Title: Gel Electrophoretic Analysis of the Protein Changes in Ground Beef Stored at 2°C

A study was completed to determine the extent of the protein changes occurring in ground beef stored at 2°C for 10 days.

Sections of semitendinosus muscle were obtained immediately after the slaughter of three beef animals. Each section was divided into two equal portions, one of which was ground and the other remained intact (control). All samples were handled and stored under aseptic conditions.

Grinding markedly accelerated glycolysis as manifested by the rapid pH decline in the ground samples during the initial 24 hours of postmortem storage. After this storage interval, however, there was little difference in pH values between the ground and intact samples.
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was employed to monitor changes in myofibrillar and sarcoplasmic proteins extracted from at-death muscles and samples stored for 1, 3, 6, and 10 days at 2°C. The gels were examined visually and scanned densitometrically to detect protein changes.

The principal electrophoretic changes in myofibrillar proteins of the ground samples were the gradual disappearance of nebulin and desmin components and the gradual appearance of 110,000-, 95,000-, and 30,000-dalton polypeptides. In addition, there was a progressive increase in the content of a protein around 55,000 daltons and myosin light chain-3. Intact muscles showed similar changes to those of the ground samples except that the latter had a faster initial rate in some of the changes, notably the disappearance of nebulin and the appearance of the 30,000-dalton polypeptide. It seems probable that grinding caused an early release of Ca\textsuperscript{++} from the sarcoplasmic reticulum, which activated the Ca\textsuperscript{++} -activated proteinase (CAF).

Electrophoretic changes in sarcoplasmic proteins of the ground samples closely resembled those of the intact muscles. The major alterations in both muscle treatments included the gradual appearance of a 100,000-dalton polypeptide and three proteins having molecular weights
(M.W.) between 500,000 and 1,000,000 daltons, and the progressive disappearance of 300,000- and 24,000-dalton proteins. The appearance of a 100,000-dalton polypeptide and the three large M.W. proteins presumably originated from myofibrils since they appeared to be related to the changes in myofibrillar proteins.

Results of microbial testing indicated very little, if any, sample contamination by psychrotrophic microorganisms. Thus, microbial proteolysis was not a factor in this study.

It was concluded that grinding had no pronounced effect on the protein changes of beef muscle other than changes in pH.
Gel Electrophoretic Analysis of the Protein Changes in Ground Beef Stored at 2°C

by

Youling Xiong

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Densitometer tracings of electrophoretograms of sarcoplasmic proteins extracted from ground (A) and intact (B) bovine semitendinosus muscle stored at 2°C for 0, 1, 3, 6, and 10 days postmortem. Letters a, b, c, d, e, f, g, and h correspond to the changes indicated in electrophoretograms.
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GEL ELECTROPHORETIC ANALYSIS OF THE PROTEIN CHANGES IN GROUND BEEF STORED AT 2°C

INTRODUCTION

Ground beef is a popular, widely consumed meat product. Current consumption of this item is about 18.2kg per capita and the demand is increasing yearly (Raccach and Henrickson, 1978). This level of ground beef consumption is highly significant because the average person in the United States consumes approximately 36kg of beef per year.

Ground beef prepared from hot-boned beef muscle is superior to that prepared from chilled muscle in palatability and cooking properties including tenderness and juiciness (Cross et al., 1979). However, it has been found to develop characteristic off-flavors during long term storage. This defect may be associated with muscle protein degradation postmortem.

It is generally accepted that both sarcoplasmic and myofibrillar proteins are quite vulnerable to muscle endogenous proteolytic enzymes whereas proteins from the connective tissue are very stable during postmortem storage or aging (Lawrie, 1974; Penny, 1980; Goll et al., 1983). Although exhaustive studies on muscle proteins have been made, it remains controversial at the present
time which proteins undergo proteolysis and which enzymes are the causal agents for the changes occurring during the postmortem storage of meat.

Sarcoplasmic proteins of muscle contain up to one-third of total muscle proteins (Goll et al., 1970). They are the most labile of the muscle proteins. Postmortem alterations of sarcoplasmic proteins have been documented (Neelin and Ecobichon, 1966; Parrish et al., 1969; Hay et al., 1973b; Harrington and Henahan, 1982). Some of the proteolytic changes are caused by acid cathepsins (Drabikowski et al., 1977).

The myofibrillar protein fraction constitutes over 50% of the total proteins in muscle (Goll et al., 1970). Postmortem degradation of myofibrils is evidenced by limited but not extensive proteolysis in the intact muscle. The major alterations occurring postmortem are the disruption of the Z-disks (Goll et al., 1970; Penny, 1980) and the gradual appearance of a new polypeptide of 30,000 daltons (Olson et al., 1977; Penny, 1980; Parrish et al., 1981). These changes, along with several other minor changes within the myofibrils, are thought to be responsible for the increased meat tenderness during aging (MacBride and Parrish, 1977; Penny, 1980; Parrish et al., 1981). Although Ca^{++}-activated proteinase (CAF) seems to be a strong participant in these changes (Dayton et al., 1975; Olson et al., 1977; Penny, 1980),
Evidence has been accumulated that the lysosomal cathepsins are also important (Schwartz and Bird, 1977; Robbins et al., 1979; Matsukura et al., 1981; Etherington, 1981). In addition, high activities of neutral and alkaline proteinases against myofibrillar proteins have been reported (Murakami and Uchida, 1979; Goll et al., 1983).

While postmortem protein alterations in bovine muscle have been studied extensively, there is a lack of knowledge about protein changes in ground beef. Ostensibly, the grinding can rupture the cell wall and damage the myofibrils. It may also disrupt the sarcoplasmic reticulum, resulting in a release of Ca^{++} which stimulates muscle ATP-ase to accelerate glycolysis (Hamm, 1977), and rupture lysosomes to release catheptic enzymes. In addition, proteins of ground muscle become more susceptible to proteolytic bacteria due to the more exposed surface area and destruction of the cell wall (Tarrant et al., 1971; Dainty et al., 1975; Yada and Skura, 1981).

Although Harrington and Henahan (1982) found some sarcoplastic protein changes in frozen-stored ground beef, it is not known whether the grinding would influence the proteins of the bovine muscle which is ground at-death and subsequently stored at low temperatures.
The current study was therefore initiated to investigate protein changes occurring in ground beef (made at-death) during postmortem storage at 2°C by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Postmortem glycolysis and microbial contamination were also monitored.
LITERATURE REVIEW

Muscle Structure

The fiber is the essential structural unit of muscle. Fibers are long, narrow, multinucleated cells stretching from a few to as long as 34 cm with a diameter of 10-100 um (Walls, 1960). Fibers are arranged in parallel fashion to form bundles, and groups of bundles make up a muscle. Each of the above units is surrounded by a specific sheath of connective tissue: fiber by the endomysium through sarcolemma, fiber bundle by the perimysium, and finally muscle by epimysium (Figure 1).

Myofibrils are the basic structural unit of the muscle fiber. They are composed of light bands (I-bands), dark bands (A-bands) and transverse Z-disks that divide myofibrils into regular units called sarcomeres (Figure 1). The light band consists of thin filaments while the dark band contains thick filaments each surrounded by six thin filaments which can be seen through the electron microscope of cross-sections of the myofibrils (Huxley, 1972). The interdigitating thick and thin filament array has enormous implications for all phases of meat science, and a strong case can be made for the proposal that discovery of this structure is the single most important finding ever made in meat science (Goll et al., 1977).
Figure 1. Structure of skeletal muscle at several different levels of organization ranging from an entire muscle (top of fig.) to the molecular architecture of myofilaments (bottom of fig.). (From Anthony and Thibodeau, 1983)
As indicated in Figure 1 muscle cells are cross-striated and contain sarcoplasmic reticulum, T-tubules and sarcolemma. Other cell organelles such as mitochondria, lysosomes, nuclei, ribosomes and glycogen granules are also present in muscle cells (Goll et al., 1974).

Muscle Proteins

In general, muscle proteins refer to all proteins found in muscle cells (Goll et al., 1977). This includes proteins capable of rapid contraction and relaxation which are surrounded by a connective tissue network to bind them into place, and a complex system of enzymes which supply the muscle elements with energy (Bendall, 1964).

Muscle proteins can be broadly divided into those which are soluble in water or diluted salt solutions (sarcoplasmic proteins), those which are soluble in concentrated salt solutions (myofibrillar proteins), and those which are insoluble (stroma proteins or proteins of connective tissue and other formed structures) (Lawrie, 1974; Goll et al., 1977). Distribution and relative abundance of these skeletal muscle proteins are listed in Table 1.
Table 1. Major skeletal muscle proteins and their locations

<table>
<thead>
<tr>
<th>protein</th>
<th>%</th>
<th>location</th>
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<tbody>
<tr>
<td>I. myofibrillar</td>
<td>(60)</td>
<td></td>
</tr>
<tr>
<td>myosin</td>
<td>50</td>
<td>thick filaments</td>
</tr>
<tr>
<td>actin</td>
<td>20</td>
<td>thin filaments</td>
</tr>
<tr>
<td>connectin(titin)</td>
<td>5.5</td>
<td>gap filaments</td>
</tr>
<tr>
<td>tropomyosin</td>
<td>5</td>
<td>thin filaments and Z-disks</td>
</tr>
<tr>
<td>troponin T, I &amp; C</td>
<td>5</td>
<td>thin filaments</td>
</tr>
<tr>
<td>nebulin</td>
<td>4.5</td>
<td>N2-lines</td>
</tr>
<tr>
<td>M-protein</td>
<td>4</td>
<td>M-lines</td>
</tr>
<tr>
<td>C-protein</td>
<td>2</td>
<td>thick filaments</td>
</tr>
<tr>
<td>α-actinin</td>
<td>2</td>
<td>Z-disks</td>
</tr>
<tr>
<td>β-actinin</td>
<td>1</td>
<td>thin filaments</td>
</tr>
<tr>
<td>desmin</td>
<td>0.35</td>
<td>intermediate filaments(Z-disk)</td>
</tr>
<tr>
<td>others</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>II. sarcoplasmatic</td>
<td>(30)</td>
<td></td>
</tr>
<tr>
<td>glyceraldehyde phos.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dehydrogenase</td>
<td>22</td>
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<tr>
<td>aldolase</td>
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<tr>
<td>creatine kinase</td>
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<td>sarcoplasm</td>
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<tr>
<td>lactate dehydrogenase</td>
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<td>pyruvate kinase</td>
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<tr>
<td>phosphorylase b</td>
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</tr>
<tr>
<td>myoglobin</td>
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<td>extracellular</td>
<td>8</td>
<td>outside muscle cell</td>
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<tr>
<td>others</td>
<td>23</td>
<td>sarcoplasm</td>
</tr>
<tr>
<td>III. stroma</td>
<td>(10)</td>
<td></td>
</tr>
<tr>
<td>collagen</td>
<td>47</td>
<td>connective tissue,</td>
</tr>
<tr>
<td>elastin</td>
<td>2.5</td>
<td>sarcoplasmic reticula,</td>
</tr>
<tr>
<td>reticulin</td>
<td></td>
<td>sarcolemma, etc</td>
</tr>
<tr>
<td>mitochondrial</td>
<td>25</td>
<td>mitochondria</td>
</tr>
<tr>
<td>other particulate</td>
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*a* Sources are from Scopes (1970); Lawrie (1974); Dayton et al. (1975); Wang and Ramirez-Mitchell (1983); and Stanley (1983).

*b* Percentage is approximation. Data in parenthesis represent the percent of the total muscle proteins; data without parenthesis indicate the percentage of each component within the group.
Sarcoplasmic Proteins

The sarcoplasmic proteins are those proteins found in cytoplasm of the muscle and are soluble at ionic strength of 0.1 or less at neutral pH. The entire sarcoplasmic protein fraction has been estimated to contain at least 100-200 different proteins (Goll et al., 1977). Exact protein composition of the sarcoplasmic protein fraction is influenced considerably by conditions used during extraction and may vary depending on speed and extent of homogenization of the tissue before extraction, pH of the extraction, nature of extraction solvent, and centrifugal force used to separate the soluble sarcoplasmic protein fraction from unsolubilized proteins and subcellular organelles (Goll et al., 1970). A number of different procedures have been employed to extract sarcoplasmic proteins, including the use of pure water (Scopes, 1964; Harrington and Henahan, 1982), 3% iso-osmotic glycerol solution (Scopes, 1966), 0.25M sucrose solution (Hay et al., 1973a, b), and 0.03M potassium phosphate (Goll et al., 1964) as the extracting solvents. However, under most extraction conditions used, the sarcoplasmic protein fraction contains all the enzymes associated with glycolysis and most of the enzymes involved with carbohydrate and protein synthesis because these processes occur largely in the cytoplasm of
muscle cells. These enzymes, in addition to being soluble at low ionic strength, are free and readily solubilized when the muscle cell is ruptured. At least 80% of the sarcoplasmic protein extract consists of glycolytic enzymes. Of the several hundred enzymes thought to be present in the sarcoplasm, the six most abundant enzymes listed in Table 1 make up more than one-half of the sarcoplasmic protein fraction (Scopes, 1970). Most sarcoplasmic protein fractions contain some lysosomal enzymes because lysosomes are labile to muscle homogenization (Reville et al., 1976). Other important sarcoplasmic proteins are myoglobin which contributes to meat color and the calcium activated factor (CAF), a proteinase which attacks myofibrils.

Although sarcoplasmic proteins contribute little to the texture of muscle, they do contribute significantly to the development of the characteristic meat flavor. Moreover, sarcoplasmic proteins are of significant nutritive value since they account for 30% or more of the total protein in skeletal muscle (Goll et al., 1970).

Myofibrillar Proteins

Myofibrillar proteins are frequently defined as those muscle proteins that are not extractable at low ionic strength (u < 0.2), but are solubilized at high
ionic strength ($u > 0.4$) (Goll et al., 1970). Since the myofibrils must be completely disrupted, high ionic strength is usually required to destroy the intimate associations and interactions among protein molecules within the myofilaments. However, some of the recently discovered myofibrillar proteins can be extracted at very low ionic strength ($u < 0.01$) upon disruption or maceration of the muscle tissue. Once extracted, most of the myofibrillar proteins are soluble at ionic strengths from 0 to 1.5 (Goll et al., 1970). Therefore, it has been suggested that myofibrillar proteins be defined as those proteins that constitute the myofibrils (Goll et al., 1970; Goll et al., 1977).

The structure of the myofibril has been studied extensively. Unlike the sarcoplasmic protein fraction which contains many different proteins, the myofibril is composed of only about a dozen different proteins including several discovered in recent years (Table 1). Myosin and actin are the main components of thick and thin filaments of the myofibril. Both are necessary for an in vitro contraction response (Goll et al., 1977). Most of the other myofibrillar proteins function either to regulate or control the actin-myosin interaction (tropomyosin, troponin, and possibly α-actinin) or to assist in assembly of actin and myosin into the highly organized three-dimensional structure in myofibrils.
(C-protein, α-actinin, β-actinin, M-protein, and paramyosin) (Goll et al., 1977). The latter may also include several newly discovered proteins (titin, nebulin, and desmin) which have been observed in the myofibrils in the form of fine filaments (Wang and Ramirez-Mitchell, 1983).

Myosin is the major protein found in myofibrils, constituting about 50% of the total myofibrillar protein fraction. It is composed of two types of subunits, light and heavy meromyosins. The light meromyosin is water insoluble and has no ATP-ase activity or actin-binding ability while heavy meromyosin is water soluble and has both ATP-ase activity and actin-binding ability (Lowey et al., 1969).

Actin is the second most abundant myofibrillar protein accounting for some 20% of the myofibril by weight. It contains only one polypeptide chain and can exist in two forms, G-actin (globular) and F-actin (fibrillar). G-actin will polymerize into F-actin in which the globular units are aggregated end-to-end to form a double-stranded filament (Murray and Weber, 1974). F-actin combines with myosin to form the contractile actomyosin of active or pre-rigor muscle and the inextensible actomyosin of muscle in rigor mortis.

Other contractile proteins are present in relatively small quantity. Tropomyosin molecules are aggregated
end-to-end near the groove of the double-stranded actin filaments. In vertebrate muscle tropomyosin is capable of contributing mechanical stability to the muscle filaments whereas paramyosin which is tropomyosin found in certain invertebrate muscles is capable of tetanic contraction (Poglazov, 1966).

Troponin is another regulatory protein. Greaser and Gergely (1971) first demonstrated that the troponin molecule contains three dissimilar subunits which are intimately concerned in muscle contraction. The largest subunit is called troponin T (T for tropomyosin) since it binds strongly to tropomyosin. The smaller subunit, troponin I (I for inhibition), binds to actin and can inhibit the ATP-ase activity of actomyosin, thus preventing the actin-myosin interaction. Troponin C (C for calcium) is the smallest subunit which binds calcium. Troponin C is associated with both troponin T and troponin I to form troponin complex, which has very important implication in muscle contraction and relaxation (Margossian and Cohen, 1973). Cohen (1975) has illustrated and described in detail the interactions among the three troponin subunits and with other contractile components.

α-Actinin and β-actinin are structural proteins. α-Actinin is located in the Z-disks (Goll et al., 1967; Masaki et al., 1967). It promotes the lateral association
of F-actin. β-Actinin is found in thin filaments. It appears to inhibit network formation of F-actin and maintain the physical state of I-filaments in vivo (Maruyama, 1965).

The three-dimensional structure of the muscle myofibril is also maintained by C-protein and M-protein (line). However, the significance of their presence to the integrity of the myofibril remains obscure. C-protein exists in bands which completely encircle the thick filament. It has been suggested that these rings may act like staves around a barrel to hold the thick filament together during tension development (Offer et al., 1973). M-protein appears to link myosin filaments to their six nearest neighbours in order to keep the myosin filaments centrally aligned in the sarcomere.

Recently, two extremely large skeletal muscle myofibrillar proteins have been discovered and named titin and nebulin (Wang, 1981). Titin is a pair of uncommonly high molecular weight (> 700,000 daltons) myofibrillar proteins (Wang and Ramirez-Mitchell, 1979). Initially, titin was confused with connectin discovered by Maruyama et al. (1977a, b). However, recent studies suggest that a high molecular weight doublet (> 800,000 daltons) of connectin is identical to titin (Maruyama et al., 1981a). It has been suggested that titin may be the major cytoskeletal protein constituting a set of very
thin, longitudinal running elastic filaments, called gap filaments, in the sarcomere (Wang and Ramirez-Mitchell, 1979, 1983). The gap filaments are parallel to the fiber axis and provide intracellular tensile strength.

Nebulin has a molecular weight of about 500,000 daltons. It is the major component of the N\textsubscript{2}-line that is a transverse structure located in the I-band region (Wang, 1981). Wang (1981) has suggested that the N\textsubscript{2}-line may be involved in a dynamic mechanism which "steers" actin filaments to a more favorable alignment to optimize interaction with myosin thick filaments.

In the Z-disk area there exists another type of filament that functions to connect adjacent Z-disks and promote lateral registration. These filaments are composed of a smaller contractile protein called desmin (Stanley, 1983). It has been proposed that these filaments may interact with gap filaments in the region of the Z-disk to form a three-dimensional network that holds myofibrils in place and thus provides an ordering of the contractile mechanism (Stanley, 1983). The presence of the network has been documented through transmission and scanning electron microscopic studies by Wang and Ramirez-Mitchell (1983). In view of above findings, it is clear that these newly discovered thin filaments have a very significant implication to meat toughness.

It is possible that other yet unidentified proteins
may also exist within the contractile apparatus and may be discovered with advances in analytical techniques.

Stroma Proteins

The stroma proteins are usually measured as the insoluble proteins remaining after exhaustive extraction of all salt-soluble muscle proteins. They include lipoproteins and mucoproteins from cell membranes and surfaces (e.g. sarcolemmal, sarcoplasmic reticular and mitochondrial membranes) in addition to the proteins that constitute the epimysial, perimysial and endomysial connective tissues surrounding the muscle cell. Collagen alone makes up about half of the stroma protein fraction (Table 1). Stroma proteins have a significant impact on meat quality because they tend to reduce the water-holding capacity of meat (Goll et al., 1977). They also contribute to meat toughness. Besides, due to the postmortem alterations in the sarcoplasmic reticular, mitochondrial and sarcolemmal membranes, Ca$^{++}$ may be released into cytoplasm to accelerate glycolysis (Brostrom et al., 1971; Hamm, 1977) or activate the intracellular proteinases (Dayton et al., 1976a, b; Reville et al., 1976). Thus, membrane proteins have far-reaching effects on meat quality.
Postmortem Changes

Postmortem Glycolysis

Postmortem glycolysis reflects the basic metabolism of muscle. The sequence of chemical steps by which glycolysis is converted to lactic acid is essentially the same postmortem as in vivo when the oxygen supply becomes temporarily inadequate for the provision of energy in the muscle; but it proceeds further (Lawrie, 1974).

Glycolysis is coupled with ATP turnover. When the animal is slaughtered ATP is no longer produced via oxidative phosphorylation. Consequently, at the point of death (pH 6.9 - 7.2) the muscle ATP level is maintained by conversion of ADP to ATP at the expense of creatine phosphate, but when the latter is exhausted the ATP level falls. The depletion of ATP triggers the anaerobic conversion of glycogen to lactic acid. This conversion will continue until a pH is reached where the enzymes affecting the breakdown are inactivated (Lawrie, 1974). Since the production of lactic acid is dependent on the supply of glycogen, the level of this component determines the muscle ultimate pH. But the initial glycogen content is not the sole factor determining the final pH because approximately 10% of the total drop in
pH postmortem is due to protons liberated by the hydrolysis of ATP present in the tissue at death (Hamm, 1977).

In typical postmortem mammalian muscle the ultimate pH is about 5.4 - 5.5. However, both the rate and the extent of the postmortem pH fall are influenced by intrinsic factors such as species, the type of muscle and variability between animals, and by extrinsic factors such as administration of drugs pre-slaughter and the environmental temperature (Lawrie, 1974). For example, a faster muscle postmortem pH decline has been observed when the muscle is subjected to increased environmental temperature (Marsh, 1954), electric stimulation (Sonaiya et al., 1982), pressurization (Elgasim et al., 1983), and grinding (Hamm, 1977).

Postmortem Protein Degradation

Proteolysis is an important postmortem change in muscle. Postmortem protein breakdown affects meat quality in various aspects, including tenderness, functionality, and the characteristic flavor and color of meat. Although numerous studies have been conducted, it remains controversial at the present time which muscle proteins undergo proteolysis and which enzymes are the causal factors for the changes occurring during the meat aging
Generally, few proteins of connective tissue, myofibrils and sarcoplasm of muscle are subjected to extensive degradation during the aging period at temperatures above the freezing point. Many early studies have shown that no large changes were detected by various chemical measurements of proteolysis during postmortem storage (Locker, 1960; Davey and Gilbert, 1966; Parrish et al., 1969). Connective tissue proteins are generally resistant to proteolytic enzymes in muscle. Proteolysis is absent in collagen and elastin of fresh sterile meat, even after storage of 1 year at 37°C (Lawrie, 1974). Clearly, very little degradation of collagen occurs postmortem (Goll et al., 1970; Etherington, 1981).

Sarcoplasmic proteins may disintegrate and dissolve in a very concentrated solution in vivo. They are the most labile of the muscle proteins. Slight proteolytic degradation of sarcoplasmic proteins may occur during postmortem storage. Sharp (1963) demonstrated that during storage at 37°C of sterile beef muscle the total soluble protein nitrogen declined from 28% of the initial nitrogen to 13% after 20 days. The lowered concentration of sarcoplasmic proteins was due to their proteolysis to amino acids and not to precipitation (Lawrie, 1974). Parrish et al. (1969) have also shown that the sarcoplasmic protein fraction yields an increase in free
amino acids and non-protein nitrogen in postmortem bovine muscle. Although Whitaker et al. (1970) found little difference in the sarcoplasmic proteins between pre-rigor and post-rigor muscle of the rabbit and pig, other workers have shown a significant degradation of sarcoplasmic proteins in chicken (Neelin and Ecobichon, 1966; Hay et al., 1973b) and bovine (Harrington and Henahan, 1982) muscles during postmortem storage.

Because of their obvious importance in muscle contraction and because it has long been suspected that they are primarily responsible for many of the physical changes observed in postmortem muscle, the myofibrillar proteins have been the subject of many careful studies, largely by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Hay et al., 1973b; Penny, 1974, 1980; Olson et al., 1977; Bechtel and Parrish, 1983; Koohmaraie et al., 1984). The proteolytic degradative changes occurring in myofibrils during postmortem storage are appreciable. The major ultrastructural alterations in muscle observed during aging are a slow loss of the M-line, broadening and loss of fibrillar structure in the Z-disks, and the fracture seen between Z-disk and I-band (Fukazawa et al., 1969; Goll et al., 1970; Hay et al., 1973a; Penny, 1980). Paralleling the breakdown of troponin T, the most consistent and noticeable change that occurs in
myofibrils during postmortem storage is the gradual appearance of three closely spaced protein bands migrating at rates corresponding to approximately 34,000, 30,000 and 27,000 daltons in SDS-PAGE (MacBride and Parrish, 1977; Olson et al., 1977; Penny, 1980; Parrish et al., 1981). It seems highly possible that the 30,000-dalton component arises from the degradation of troponin T (Olson et al., 1977; Penny, 1980; Goll et al., 1983). Recently, Lusby et al. (1983) reported that two newly discovered myofibrillar proteins, titin and nebulin, are gradually degraded during the postmortem storage of bovine muscle.

Nevertheless, no evidence thus far has indicated any pronounced postmortem change in the major contractile proteins, myosin and actin, especially during the normal aging period. Most other contractile components also seem unaltered during the aging process.

Proteolytic Enzymes and Their Actions

Numerous studies have been made in attempt to find evidence of proteolytic activity responsible for muscle protein degradation during postmortem storage. However, the involvement of the exact enzymes remains enigmatic. A number of endogenous proteinases have been discovered from skeletal muscles. These include lysosomal
proteinases (cathepsins), neutral and alkaline proteinases, and Ca\textsuperscript{2+}-activated proteinase (CAP). Several excellent reviews about these enzymes are available (Etherington, 1981; Goll et al., 1983; Dahlmann et al., 1984).

Lysosomal Proteinases

Lysosomal proteolytic enzymes consist largely of cathepsins. Most of the cathepsins have optimum activity in the acidic pH range and are associated with lysosomes of muscle cells. Of the thirteen reported lysosomal peptide hydrolases, however, only seven, cathepsins A, B, C, D, H, L and lysosomal carboxypeptidase B, have been shown to exist inside skeletal muscle cells (Goll et al., 1983). Among these enzymes only the endopeptidases, cathepsins B, D, H and L are the most important. All four of these enzymes possess activity against myosin and actin (Goll et al., 1983). Schwartz and Bird (1977) have shown that, at pH 5.2, cathepsin B degrades myosin into major fragment of 150,000 daltons. At pH 4.0 cathepsin D degrades myosin more rapidly and more extensively than cathepsin B, forming two major fragments of 110,000 and 107,000 daltons and several smaller fragments. However, at pH values above 6.0, neither cathepsin B nor D has any appreciable effect on myosin (Schwartz and Bird, 1977).
These researchers also found that both cathepsin B and D degrade actin. Tropomyosin, the other major component of the thin filament, is degraded by cathepsin B (Dahlmann et al., 1984). Further, Robbins et al. (1979) reported that the Z-disks were substantially degraded by cathepsin D at pH 5.2 to 5.3. Cathepsin L resembles cathepsin B, so its activity may not have been separated from cathepsin B in many of the earlier studies (Okitani et al., 1980). Cathepsin L degrades actin and both heavy and light chains of myosin very quickly at pH 5.0 (Matsukura et al., 1981). Cathepsin H also has a high affinity for myosin. Goll et al. (1983) suggested that cathepsins L and H each have far greater myosin-degrading ability than cathepsins B and D combined. Dahlmann et al. (1984) have indicated that cathepsins B, D and L degrade troponin T and troponin I in vitro.

Some of the lysosomal enzymes possess collagenolytic activity, particularly cathepsins B, L, N (Etherington, 1981) and G (Starkey, 1977). But neither cathepsin G nor cathepsin N has been detected in skeletal muscle.

At the present time, data are lacking about the activity of the catheptic enzymes against sarcoplasmic proteins although there was a report that sarcoplasmic proteins can be degraded by acid cathepsins (Drabikowski et al., 1977). It is possible that this activity could be present during the postmortem storage of muscle.
Neutral and Alkaline Proteinases

Enzymes in this group have proteolytic activity in the neutral and alkaline pH range. Many of them have been detected in skeletal muscle tissue. Eight of the nine isolated neutral or alkaline proteinases are localized in mast cells rather than in skeletal muscle cells, and some are yet of unknown origin (Goll et al., 1983). Initially, identification of these enzymes was confusing but with recent immunohistochemical tests some of the problems have been resolved. Thus, it seems likely that the alkaline proteinase, the myofibrillar proteinase, the alkaline myofibrillar proteinase and the myosin-cleaving proteinase all originate from the same enzyme (Goll et al., 1983). Libby and Goldberg (1980) have suggested that most, if not all, proteolytic activity having an alkaline pH optimum in crude muscle homogenates originates from mast cells in the original tissue and not from striated muscle cells per se.

The neutral and alkaline proteinases may be important for muscle protein turnover because they have specificity against certain myofibrillar components. For example, neutral serine proteinase can cleave actin, myosin, α-actinin, tropomyosin, troponin T and I, and can rupture the Z-disks (Goll et al., 1983) while myosin-cleaving proteinase is capable of degrading
C-protein, myosin, M-protein, troponin T, I, and C, tropomyosin and Z-disks (Murakami and Uchida, 1979). However, it is difficult to conceive that these enzymes could act on myofibrils and other intracellular muscle proteins directly since they are not located inside muscle cells. The role and the significance of these proteinases to muscle postmortem proteolysis need further investigation.

The Ca$^{++}$-activated proteinase

The presence of Ca$^{++}$-activated proteinase (CAF) was discovered by Busch (1972) during the study of rabbit muscle structure by electron microscopy. It was later purified by Dayton et al (1976a). Although CAF has optimum activity between pH 7.0 and 7.5 (Penny, 1980), it is usually not classified into the neutral and alkaline proteinases group because it is localized inside skeletal muscle cells at the level of the Z-disk and adjacent to the cytoplasmic face of the plasma membrane (Dayton and Schollmeyer, 1981). Since it has a unique proteolytic characteristic, the properties of CAF has been studied extensively in the past several years.

CAF has a very unusual and limited specificity. Goll et al. (1983) have indicated that CAF has very little effect on the sarcoplasmic protein fraction from skeletal
muscle cells but it degrades the contractile proteins in these cells in a specific but limited way. Ultrastructurally, CAF selectively removes the Z-disks from myofibrils (Busch et al., 1972; Olson et al., 1977) and degrades the M-line (Dayton et al., 1976b). The ability of CAF to break down certain other myofibrillar proteins has been thoroughly investigated (Dayton et al., 1975). This includes the degradation of tropomyosin, troponin T, troponin I and C-protein, but myosin, actin, α-actinin and troponin C were not affected. However, Pemrick et al. (1980) have found that CAF may degrade myosin heavy chain if the LC₂ light chain is unphosphorylated. That CAF can degrade nebulin was also evidenced (Maruyama et al., 1981b). More recently, Zeece et al. (1983) reported that CAF can break down the newly discovered myofibrillar protein, titin.

There are two forms of CAF, high-Ca⁺⁺ CAF and low-Ca⁺⁺ CAF (Goll et al., 1983). The high-Ca⁺⁺ CAF is maximally active at pH 7.0 to 8.0 as long as the free Ca⁺⁺ concentrations range between 1 to 5 mM (Dayton et al., 1976b). The low-Ca⁺⁺ CAF, however, requires much lower Ca⁺⁺ concentration. It is completely active at 70 uM of Ca⁺⁺ and is active even at 10 uM (Dayton et al., 1981). The latter suggested that low-Ca⁺⁺ CAF is active over a slightly broader range of pH values than high-Ca⁺⁺ CAF and it may retain
significant proteolytic activity at pH values as low as 5.5.

Since high-Ca\textsuperscript{++} CAF requires Ca\textsuperscript{++} concentration of 1 to 5 mM for optimum activity while intracellular free Ca\textsuperscript{++} level rarely rises above 10 uM, Goll et al. (1983) suggested that only the low-Ca\textsuperscript{++} form of CAF is active in living muscle cells and it may be responsible for most of the postmortem proteolytic degradation of myofibrillar proteins inside muscle cells. They also suggested that high-Ca\textsuperscript{++} CAF may be a storage form of the enzyme in vivo which can be converted to the more active low-Ca\textsuperscript{++} CAF under the regulation of CAF inhibitor. Alternatively, it is assumed that high-Ca\textsuperscript{++} CAF is possibly located at the plasma membrane or in the connective tissue matrix (Barth and Elce, 1981), where it could be activated by millimolar levels of Ca\textsuperscript{++} that exist extracellularly. Low-Ca\textsuperscript{++} CAF may be localized at the Z-disk (Goll et al., 1983).

Indeed, the precise role of CAF to muscle protein degradation leading to meat tenderness and other meat quality changes remains unknown. Clearly, more studies are necessary before a thorough understanding can be achieved.
Microbial Proteinases

Certain bacteria, when growing on meat, can elaborate extracellular proteolytic enzymes that may digest muscle proteins. If microorganisms are involved, *Pseudomonas* bacteria normally predominate on meat at chill temperatures, and these organisms are unaffected by pH in the range that normally occurs in meat (Gill and Newton, 1982). It is now recognized that some *Pseudomonas* species are able to excrete proteolytic enzymes; *P. fragi* (Borton et al., 1970a, b; Tarrant et al., 1971), *P. perolens* (Buckley et al., 1974) and *P. fluorescens* (Gill and Penney, 1977) are examples of these microorganisms.

Observations pertinent to the importance of bacterial proteolysis have been contradictory. Some researchers (Jay, 1967; Ockerman et al., 1969; Jay and Shelef, 1976) believe that bacterial proteolysis plays an insignificant role in muscle protein breakdown while others (Borton et al., 1970a, b; Tarrant et al. 1971; Dainty et al., 1975; Yada and Skura, 1981) have shown significant protein degradation. Borton et al. (1970a) reported that when *P. fragi* was present during the storage of pork at 2-10°C some proteolysis of myofibrillar proteins occurred. Tarrant et al. (1971) observed considerable salt-soluble protein degradation in
pork muscle by *P. fragi* in 20 days at 10°C. *P. fragi* was also shown to alter sarcoplasmic proteins extensively (Hasegawa et al., 1970).

Gill and Penney (1977) have found that bacteria may penetrate meat upon proteolysis although it will not happen until the bacteria have reached their maximum cell density.

The effect of bacterial proteininases on muscle proteins depends on the extent of contamination. It is generally accepted that no significant degradation of meat proteins takes place until spoilage has become evident. Clearly, protein breakdown can be detected only at a very advanced stage of spoilage. Yada and Skura (1981) studied *P. fragi* and found that a count of approximately $10^{10}/\text{cm}^2$ is the minimum level at which the breakdown of sarcoplasmic, myofibrillar and connective tissue proteins by *P. fragi* became detectable by SDS-PAGE. It has been suggested that a critical number of bacteria may be required before proteolysis can be detected (Dainty et al., 1975; Jay and Shelef, 1976; Yada and Skura, 1981).

Glycolysis and Proteolysis in Ground Muscle

Grinding causes two large structural changes of the muscle, i.e., muscle surface increase and muscle tissue
damage. The more exposed surface area results in an enhanced substrate availability for the enzymes. On the other hand muscle tissue particles can be ruptured when there has been extensive tissue damage.

In ground muscle postmortem metabolism is faster than in intact muscle. It has been observed that grinding pre-rigor bovine muscle increases the rate of ATP turnover manifested by a faster depletion in the levels of ATP and glycogen and an accelerated drop of pH (Newbold and Scopes, 1971; Hamm, 1977). Hamm (1977) suggested that the acceleration of postmortem metabolism manifested by a faster depletion in the levels of ATP and glycogen and an accelerated drop of pH (Newbold and Scopes, 1971; Hamm, 1977). Hamm (1977) suggested that the acceleration of postmortem metabolism due to grinding results from damage to the sarcoplasmic reticulum of muscle cells, causing a release of Ca\textsuperscript{++} which activates myosin ATP-ase activity. The lowered ATP content triggers the activation of glycolytic enzymes so that the formation of lactic acid and pH decline are accelerated (Hamm, 1977). Two enzymes, phosphofructokinase (PFK) and, to a lesser extent, phosphorylase (PP) play a dominant role in the control of metabolic levels of glycolysis in ground muscle (Dalrymple and Hamm, 1975). Hamm (1977) reported that there was no major difference between intact and ground
tissue except a difference in the rate of postmortem changes such as glycolysis. He concluded that the destruction of the membranes by grinding can not influence the pattern but only the rate of postmortem metabolism. Recently, Etherington (1981) mentioned that rate of glycolysis can be markedly increased when the muscle temperature falls below 12°C. This lends support to the observation of faster pH decrease in ground muscle because it cools more rapidly than intact muscle.

There is very little information about the effect of grinding on muscle protein degradation postmortem. Several chemical and physical factors, e.g. pH, freezing and thawing, thermal activation and detergent treatment, have been implicated in lysosomal disruption (Sawant et al., 1964). Indeed, lysosomal membranes are very fragile and can be ruptured during electrical stimulation (Dutson et al., 1980) and pressurization (Elgasim et al., 1983) of muscle. It is highly likely that grinding may exert similar effects, especially when muscle pH has dropped to a low value (Lawrie, 1974). It is possible that a faster muscle proteolysis could take place due to the early release of catheptic enzymes from lysosomes by grinding. This possible acceleration may be triggered also by the release of Ca²⁺, a co-factor of CAF, from the sarcoplasmic reticulum.
During the grinding process myofibrils are damaged structurally and may become more vulnerable to proteolytic enzymes. While there has been no evidence that ground muscle has an accelerated proteolysis of myofibrils, Harrington and Henahan (1982) have found some differences between ground beef and intact beef in the postmortem degradation of sarcoplastic proteins.
MATERIALS AND METHODS

Muscle Sample Preparation

Semitendinosus muscles used throughout this study were obtained from three good quality heifers weighing 448 - 497 kg. The animals were slaughtered and dressed according to normal slaughter practices at the Clark Meat Science Laboratory, Oregon State University. At about 25 minutes postmortem, a two-pound muscle section was removed from each carcass and was cut immediately into two equal fractions. One fraction was subjected to grinding and the other was held intact. All equipment, including the grinder and cutting board, was sanitized with 80% alcohol. The knife was flame sterilized. Alcohol-treated and dried rubber gloves were always used during sample preparation. Muscle was ground in a Moulinex food preparation machine (Model 308) after the muscle had been trimmed free of external fat and connective tissues and minced. The ground muscle was immediately wrapped in paper toweling soaked in 0.1M NaN₃ and then wrapped in plastic film and enclosed in Ziploc plastic storage bag according to the recommendations of Bechtel and Parrish (1983). The sample bags were stored at 2°C. Each sample contained approximately 30 grams of ground muscle. The intact
muscle (3 X 7 X 15 cm) was treated similarly and stored at 2°C.

Measurement of pH

Measurements of pH were made according to the procedure of Petersen (1982) after homogenization of muscle sample in a Waring blender for 30 seconds in a 1:10 ratio of 5mM iodoacetate solution which had been adjusted to pH 7.0. Samples were taken from the cores or inner parts of the wrapped meat trimmed free of exposed surfaces. The pH measurements of ground and intact muscle samples were obtained simultaneously at the hours of 1, 2, 4, 8, 12, 16, 20 and 24, and the days of 0 (at death), 1, 3, 6 and 10 postmortem. The instrument used was a Corning Digital pH Meter (Model 125) fitted with Orion glass electrodes. The meter was standardized against pH 4.0 and 7.0 buffers and was calibrated for ambient temperature at periodic intervals. The glass electrodes were rinsed extensively with distilled water after each measurement. Each muscle sample was triplicately weighed, homogenized and measured for pH.

Preparation of Myofibrillar and Sarcoplasmic Proteins

Four grams of ground or intact muscle were taken
from the core of the wrapped muscle sample stored at 2°C. Protein extractions were prepared from both ground and intact at-death muscles and muscles after 1, 3, 6 and 10 days of postmortem storage.

Myofibrils were isolated essentially by the procedure described by Goll et al. (1974) at 2-4°C using an extracting buffer of 100mM KCl, 50mM Tris-HCl (pH 7.6), 1mM NaN₃ and 5mM EDTA as suggested by Busch et al. (1972) (Table 2). Other modifications made were the substitutions of a glass rod for polyethylene rod during vigorous stirring to suspend the pellets (steps VII to XI in Table 2), and suspension of purified myofibrils in 0.1M KCl by vigorous stirring rather than homogenization in a Waring blender (step XIV). The modifications in steps VII to XI and XIV minimized the loss of myofibrils. The supernatant from the first extraction was centrifuged at 35,000 X g for 45 minutes according to Scopes (1964). Supernatant from this centrifugation was used as the source of sarcoplasmic protein (Table 2).

Protein concentration was measured by the biuret method (Gornall et al., 1949).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Extracts of myofibrillar and sarcoplasmic proteins
Table 2. Preparation of purified myofibrillar and sarcoplasmic proteins using differential centrifugation (modified Goll's procedure)

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>Ground or minced muscle (4 grams)</td>
</tr>
<tr>
<td>II.</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>III.</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>IV.</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>V.</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>VI.</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>VII.</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>VIII to XI.</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>XII.</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>XIII.</td>
<td>Supernatant (discard)</td>
</tr>
<tr>
<td>XIV.</td>
<td>Supernatant (discard)</td>
</tr>
</tbody>
</table>

Purified Myofibrils free of membranes
were analyzed by the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure described by Porzio and Pearson (1977) using 10% acrylamide tube gels (5 X 130 mm). The major steps of the procedure included: (1) mixing all composite gel solutions except TEMED and APS (2%) at room temperature followed by degassing for 10-15 min; (2) adding TEMED and APS, mixing and degassing for 1 more min; (3) immediately transferring the gel solution into gel tubes with a Pasteur pipet; (4) mixing myofibril suspensions (about 10mg/ml) and sarcoplasmic protein extract (about 3mg/ml) in 3 and 1 volumes of sample buffer respectively (sample buffer contained 8M urea, 2.5% SDS, 5mM EDTA, 5mM 2-mercaptoethanol, and 100mM Tris / glycine (pH 8.8)); (5) solubilizing proteins in sample buffer by boiling for 3 min and then centrifuging in a Beckman Microfuge for 1 min to precipitate possible traces of undissolved materials (supernatant was utilized for electrophoretic analysis); (6) loading 30ug of protein of each sample onto the gel and running electrophoresis; (7) fixing the gels by soaking in a solution of 25% isopropanol / 10% acetic acid for 3 hours and then staining in 0.01% Coomassie Blue (R250) for 12 hours; and (8) destaining the gel with 10% acetic acid / 5% methanol till gel background became clear.

The marker proteins used were the standard mixture
of known molecular weight proteins obtained from Bio-Rad Laboratory (Richmond, CA) containing the following proteins: myosin, β-galactosidase, phosphorylase B, bovine serum albumin, carbonic anhydrase, soybean trypsin inhibitor and lysozyme.

The electrophoresis was run in gel electrophoresis cell (Bio-Rad, Model 155) with a constant voltage power supply (Buchler, Model 3-1500).

Interpretation of Gels

Myofibrillar components were identified according to the procedure of Porzio and Pearson (1977). Protein molecular weight (M.W.) was estimated against the protein standards whose logarithm M.W. were plotted against their migrating rates in the gels.

Densitometric Tracings of Gels

Gels were scanned at 580nm in a Beckman spectrophotometer (Model ACTA CIII) in conjunction with a Beckman Gel Scanner. The gel was laid in the scanning cell containing destaining solution. The size and the shape of the peak depended upon the intensity and the width of the corresponding band.
Psychrotrophic Bacteria Inspection

Microbial growth was measured at the beginning (0 day) and in the end (10 days) of postmortem storage. Samples were obtained from cores of wrapped meat stored at 2°C. The core sample was mixed with a sterilized knife and then blended in a sterile, pre-chilled Waring blender for exactly 2 minutes using 10 volumes of 0.1% sterilized peptone solution. The muscle suspension was subjected to appropriate dilutions with 0.1% peptone solution prior to inoculation.

The incubation procedure was done according to Martley et al. (1970) and Lee (1976) using Standard Methods Caseinate Agar (SMCA) medium. The inoculated media were incubated at 7°C for 10 days, which allows the detection of proteolytic organisms in total psychrotrophic bacterial counts.

Statistical Analysis

Data were analyzed according to the methods described by Snedecor and Cochran (1982). The difference in pH between ground muscles and intact muscles (from a group of animals) was analyzed using paired t-test (ground muscle and intact muscle from the same animal were paired). In an individual animal (animal No.3) this
difference was interpreted by the mean test of two samples.
RESULTS AND DISCUSSION

Postmortem Muscle pH Changes

pH Changes in Ground and Intact Muscles

Table 3 shows the changes in pH of the ground and intact bovine muscles during the postmortem storage. The pH measurements of both ground and intact muscle samples from each animal were determined in triplicate at each sampling period. The triplicate measurements were averaged. The averages obtained at the same time postmortem from all three animals were then analyzed statistically using paired t-test (Snedecor and Cochran, 1982). This allowed the detection of the effect of grinding on bovine muscle pH (Table 3). The data were depicted graphically in Figure 2.

It is clearly shown that a significantly ($P < 0.05$) faster pH fall occurred in the ground muscle than in the intact muscle within the initial 24 hours postmortem. This is in agreement with other workers' observations (Newbold and Scopes, 1971; Hamm, 1977). The rapid pH decline suggests that the grinding process accelerates the rate of glycolysis (Dalrymple and Hamm, 1975; Hamm, 1977). Hamm (1977) has hypothesized that the enhanced ATP turnover observed in the ground muscle is due to a faster
Table 3. Postmortem pH changes in ground and intact semitendinosus of bovine muscles stored at 2°C

<table>
<thead>
<tr>
<th>Time after slaughter</th>
<th>Ground (mean ± S.D.)</th>
<th>Intact (mean ± S.D.)</th>
<th>Difference between ground &amp; intact</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.50 ± 0.04</td>
<td>6.65 ± 0.05</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>2</td>
<td>6.17 ± 0.03</td>
<td>6.55 ± 0.06</td>
<td>P &lt; 0.025</td>
</tr>
<tr>
<td>4</td>
<td>5.97 ± 0.07</td>
<td>6.45 ± 0.13</td>
<td>P &lt; 0.025</td>
</tr>
<tr>
<td>8</td>
<td>5.87 ± 0.03</td>
<td>6.27 ± 0.11</td>
<td>P &lt; 0.025</td>
</tr>
<tr>
<td>12</td>
<td>5.72 ± 0.04</td>
<td>6.00 ± 0.06</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>16</td>
<td>5.69 ± 0.06</td>
<td>5.88 ± 0.08</td>
<td>P &lt; 0.025</td>
</tr>
<tr>
<td>20</td>
<td>5.64 ± 0.08</td>
<td>5.76 ± 0.08</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>24</td>
<td>5.61 ± 0.08</td>
<td>5.69 ± 0.07</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>1</td>
<td>5.61 ± 0.08</td>
<td>5.69 ± 0.07</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>3</td>
<td>5.55 ± 0.04</td>
<td>5.50 ± 0.09</td>
<td>N.S.</td>
</tr>
<tr>
<td>6</td>
<td>5.59 ± 0.05</td>
<td>5.56 ± 0.12</td>
<td>N.S.</td>
</tr>
<tr>
<td>10</td>
<td>5.45 ± 0.03</td>
<td>5.50 ± 0.10</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

a mean = average of the mean values from three animals.
b S.D. = standard deviation.
c P = probability level from the paired t-test;
N.S. = non-significant (P > 0.05).
Figure 2. Effect of grinding and postmortem storage at 2°C on pH change in the bovine semitendinosus muscles (from three animals). (A) within 24 hours; (B) within 10 days.
release of Ca\textsuperscript{++} ions from the damaged sarcoplasmic reticulum. Since glycolysis is coupled with ATP turnover it is possible that this faster release of Ca\textsuperscript{++} or faster depletion of ATP triggered glycogen breakdown to enhance the formation of lactic acid. Mitochondria may be another possible source of Ca\textsuperscript{++}. Elgasim and Kennick (1982) have provided evidence that pre-rigor pressurization of muscle causes the release of Ca\textsuperscript{++} from the damaged mitochondria. It is likely that grinding would have a similar effect since grinding and pressurization both seem to have the ability to rupture the cell organelles.

Postmortem glycolysis is also temperature sensitive. The cold-induced contraction could start earlier in the ground muscle than in the intact muscle because the former released heat more rapidly than the latter. Thus, a faster ATP breakdown can be expected to occur in the ground muscle, which accelerated the glycolysis. Etherington (1981) has reported that when the temperature of the pre-rigor muscle drops to below 12\textdegree C glycolysis is markedly hastened due to muscle contraction. Honikel et al. (1981) observed a drastic initial pH drop in the pre-rigor muscles stored at 0.5 \textdegree C in contrast to those stored at higher temperatures.

Another possible cause for the rapid pH fall could
arise from the increased substrate availability for the glycolytic enzymes. Grinding could possibly damage or rupture the glycogen granules in the muscle cells and make them more vulnerable to the enzymes.

The difference in the initial pH values between the ground and intact muscles was significant (P < 0.05) (Table 3). However, this difference was not significant (P > 0.05) after 3 days of postmortem storage. Figure 2 (A) shows that the pH dropped drastically within the first 2 hours postmortem in the ground muscle but the rate of this change decreased thereafter, probably because the initial muscle temperature remained relatively high so that the enzyme activity was maintained at a high level. Elevated substrate concentrations such as high levels of glycogen and ADP would certainly also be the contributors to this change.

The patterns of pH changes between the two muscle treatments were slightly different (Figure 2, A). In the ground beef muscle the pH declined at a gradually decreased rate (concave shaped) while in the intact muscle the pH value dropped the most rapidly within the first 2 hours postmortem, and then reduced slowly during the period of 2 to 8 hours postmortem. But after that the pH drop was accelerated again and was retarded after 12 hours of storage (convex shaped). One possible explanation for this phenomenon could be that the
conditions such as the availability of the substrates and co-enzymes favored glycolytic enzyme activity in the ground beef but not in intact beef. Initially glycogen granules in the intact muscle were tightly formed, but when the muscle became acidic the structure of these granules could be loosened and disordered, which enhanced substrate availability.

After 12 hours of storage the pH changes in both treatments leveled off because of the retarded glycolysis resulted from the limiting content of glycogen, adenosine nucleotides, and inactivation of phosphofructokinase and phosphorylase (Hamm, 1977). The change in pH values continued after 24 hours of postmortem storage, but statistical analysis indicates that the pH difference between the two muscles was no longer significant (P > 0.05) after 3 days of storage (Table 3). This observation was consistent to that reported by Hamm (1977). Similarly, during prolonged storage the pH values of both muscles fluctuated slightly (Figure 2, B), but essentially there was no significant difference (P > 0.05) between them (Table 3).

Nevertheless, one should be aware that this observation may not represent all the individual cases because both the rate and the extent of the postmortem pH fall can be influenced by intrinsic factors such as the variability between animals, and by extrinsic factors
such as exhausting exercise immediately pre-slaughter (Lawrie, 1974). Therefore, it is possible that the postmortem pH alteration in a specific animal could vary from this pH change pattern.

pH Change in Animal No.3 Muscle

Because muscle endogenous proteolytic enzymes vary in pH optimum for activity and because each specific animal could have a unique postmortem pH decline in both rate and extent, it is necessary to monitor the pH change while the muscle proteins are degrading. Table 4 and Figure 3 were made from data obtained from the same muscle (animal No.3) that was used for determining proteolysis presented in the following section of the thesis. Each pH mean value was obtained from triplicate measurements.

Basically, the pH changes in both ground and intact muscles of animal No.3 followed the pattern obtained from the group of animals (Figure 2) although there were some specific variations in the former. During the first day of postmortem storage, pH of the ground muscle was significantly lower (P < 0.005) than that of the intact muscle. But at 3 days postmortem the difference was insignificant (P > 0.05), both having a pH around 5.55 (Table 4). With prolonged storage at 2°C, however,
Table 4. Postmortem pH changes in ground and intact animal No.3 semitendinosus muscle stored at 2°C

<table>
<thead>
<tr>
<th>Time after slaughter</th>
<th>Ground (mean ± S.D.)</th>
<th>Intact (mean ± S.D.)</th>
<th>Difference between ground &amp; intact</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.47 ± 0.02</td>
<td>6.66 ± 0.02</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>2</td>
<td>6.19 ± 0.01</td>
<td>6.54 ± 0.02</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>4</td>
<td>6.10 ± 0.02</td>
<td>6.49 ± 0.03</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>8</td>
<td>5.91 ± 0.03</td>
<td>6.30 ± 0.01</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>12</td>
<td>5.75 ± 0.02</td>
<td>6.00 ± 0.02</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>16</td>
<td>5.72 ± 0.02</td>
<td>5.88 ± 0.01</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>20</td>
<td>5.68 ± 0.02</td>
<td>5.81 ± 0.01</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>24</td>
<td>5.64 ± 0.02</td>
<td>5.72 ± 0.01</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>1</td>
<td>5.64 ± 0.02</td>
<td>5.72 ± 0.01</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td>3</td>
<td>5.57 ± 0.01</td>
<td>5.54 ± 0.02</td>
<td>N.S.</td>
</tr>
<tr>
<td>6</td>
<td>5.59 ± 0.01</td>
<td>5.63 ± 0.01</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>10</td>
<td>5.48 ± 0.03</td>
<td>5.54 ± 0.02</td>
<td>P &lt; 0.05</td>
</tr>
</tbody>
</table>

a mean = mean of the triplicate measurements.
b S.D. = standard deviation.
c P = probability level from two sample mean test; N.S. = non-significant (P > 0.05).
Figure 3. Effect of grinding and postmortem storage at 2°C on pH change in the bovine semitendinosus muscle (from animal No.3). (A) within 24 hours; (B) within 10 days.
these two muscle samples, again, varied in pH value. The pH of the intact muscle was significantly higher than in the ground muscle at 6 days ($P < 0.01$) and 10 days ($P < 0.05$) postmortem (Table 4). The ground muscle exhibited a further, significant pH reduction ($P < 0.02$) at 10 days (PH 5.48) in comparison with the pH value (5.57) determined at 3 days postmortem. In contrast, during the period of day 3 to day 6 there was a significant pH increment ($P < 0.01$) in the intact muscle, but this pH value fell back to the same level of 3 days postmortem (pH 5.54). The reason is not clear but it seems possible that muscle glycogen or some residual sugars inside the cells may not have been completely metabolized within a few days postmortem. Although glycolysis may last for a prolonged period of time postmortem, it will occur at a much slower rate in the later stages than it did initially. The increased pH value in the intact muscle may be a concurrent event in which some small peptides and amino acids, or nucleotides were decarboxylated by certain enzymes present in the muscle cells. Hamm (1977) observed that glycogen breakdown and the formation of lactic acid continued in ground and intact bovine muscles even after 3 days of postmortem storage at 4°C.
Postmortem Muscle Protein Changes

Postmortem changes in beef muscle proteins were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of both ground and intact muscle samples from each of three beef animals stored at 2°C for 10 days. Since the SDS-PAGE patterns between animals were very similar per sampling period per muscle protein category, only data from animal No.3 are presented. These data are highly representative of those obtained during this investigation.

Myofibrillar Proteins

Gel Electrophoresis

Figure 4 shows the results of SDS-PAGE analysis of myofibrils isolated from the ground and intact muscle samples stored at 2°C for 10 days. Comparison of the electrophoretograms indicated that both treatments had similar protein change patterns except for a few variations. Essentially, the following major changes occurred in both muscle treatments: a gradual decrease in the intensity of a ~500,000-dalton protein band (arrow a), desmin (arrow e) and troponin T (arrow f); a gradual increase in the content of myosin light chain-3
Figure 4. SDS-polyacrylamide gel electrophoresis of myofibrils isolated from ground (A) and intact (B) semitendinosus of bovine muscle stored at 2°C. Gels 0, 1, 3, 6, and 10 represent myofibrils isolated from the muscle after 0, 1, 3, 6, and 10 days of postmortem storage. Gel S is the protein standards (note: number X 1000 = molecular weight). Arrows a, b, c, d, e, f, g, and h indicate the major changes.
Figure 4
(MLC$_3$) (arrow h) and two other polypeptides of about 110,000 and 55,000 daltons (arrow b and d); and the gradual appearance of 95,000- and 30,000-dalton components (arrow c and g). However, the ground muscle differed from the intact muscle in the rates of some of these changes. In the ground muscle the ~500,000-dalton band (arrow a) became faint after 1 day of postmortem storage, was slightly detectable after 3 days and almost disappeared after 6 days of storage (Figure 4, A). This protein is probably nebulin which was resolved from other myofibrillar components by Porzio and Pearson (1977) and identified by Wang (1981). At 10 days postmortem, a new band arose slightly beneath the original nebulin band (arrow a). Lusby et al. (1983) have suggested that this polypeptide may be the breakdown product of the titin doublet (the two bands above nebulin) or of nebulin. In contrast, nebulin degraded at a slower rate in the intact muscle (arrow a in Figure 4, B). Nevertheless, the gradually decreased intensity and the progressively broadening of the band ostensibly indicate the postmortem degradation of nebulin. The changes in both muscle treatments support the findings by Lusby et al. (1983) who demonstrated that nebulin can be degraded significantly in bovine muscle after 1 day postmortem storage at 2°C.

The gradual appearance of a 95,000-dalton component,
which was another obvious change with postmortem storage of muscle, can be seen in Figure 4 (arrow c). Although this protein band appeared in the gels of both muscle treatments at 3 days postmortem, it appeared more intense in the ground sample. The formation of this protein and the difference in the intensity between the two muscle treatments seem to parallel the changes in nebulin. These changes also seem to correlate with the subtle alteration in α-actinin (the dark band between bands b and c) which can be detected in the electrophoretograms of Figure 4. Koohmaraie et al. (1984) initially reported the appearance of the 95,000-dalton protein in the intact bovine muscle during postmortem storage. They suggested that α-actinin could be the possible source of origin. However, other workers (Olson et al. 1977; Penny, 1980) have concluded that postmortem aging has no effect on α-actinin. Obviously, no conclusive argument can be made about this change without further evidence.

The most obvious change shown in Figure 4 is the progressive appearance of a 30,000-dalton polypeptide (arrow g). It can be seen by careful examination that this polypeptide appeared as a faint band in the ground muscle at 1 day postmortem (Figure 4, A), but it was not detectable in the intact muscle (Figure 4, B). After 3 days postmortem storage the band became apparent in both muscle samples. Interestingly, however, this band
appeared slightly more intense in the intact muscle than in the muscle of ground treatment during prolonged storage. Olson et al. (1977) have pointed out that the 30,000-dalton component is most likely a degradation product of troponin T and that CAF is the causal factor for this degradation product in bovine muscle. Indeed, troponin T seemed to be degraded at 3, 6 and 10 days postmortem, which can be seen from the reduced band intensity (arrow f). This change was appreciable in the intact muscle but not in ground muscle. Likely, CAF was the causal enzyme because during day 6 and day 10 postmortem when troponin T was altering in the intact muscle, the pH value was significantly higher in the intact muscle than in ground muscle (Table 4 and Figure 3). However, other workers (Matsukura et al., 1981) believe that cathepsin L is the causal agent for the breakdown of troponin T to the 30,000-dalton component.

The observation of the gradual appearance of the 30,000-dalton polypeptide with progressive postmortem storage is consistent with the findings of numerous researchers (Hay et al., 1973b; Olson et al. 1977; Penny, 1980; Parrish et al., 1981; Koohmaraie et al., 1984).

Figure 4 shows that desmin (arrow e) diminished progressively during postmortem storage and the band almost faded completely at 6 days postmortem in both ground and intact muscle samples. The breakdown of desmin
may be significant to meat tenderness because desmin is an integral part of the Z-disks, functioning as the cross-linking transverse filaments (Wang and Ramirez-Mitchell, 1983).

Other principal changes in the myofibrils were the gradual increase in the intensity of three proteins as indicated by arrows b, d and h (Figure 4). They had molecular weights (M.W.) around 110,000, 55,000 and 15,000 daltons, respectively. From the electrophoretogram myosin heavy chain (200,000 daltons) and C-proteins (140,000 daltons) seemed without any detectable change throughout 10 days postmortem storage at 2°C whereas there were some alterations within the region of the extremely high M.W. proteins (> 300,000 daltons). It is possible, then, that the increased intensity of 110,000- and 55,000-dalton proteins could have originated from nebulin. It is also possible that any one of the myofibrillar proteins, including myosin and C-protein, which have subunit M.W. larger than 110,000 or 55,000 daltons, could be the precursors. Similarly, an original band in the area of myosin light chain-3 (MLC₃) (arrow h) was gradually intensified with extended storage time. Dayton et al. (1975) have shown that CAF can degrade tropomyosin, yielding fragments having M.W. in the range of 13,000 to 18,000 daltons. Like troponin T, tropomyosin (the unseparated band from troponin T) seemed
to undergo a subtle change during postmortem storage which was more apparent in intact muscle (Figure 4, B). Therefore, the gradually increased intensity of the band in the area of MLC₃ (arrow h) could result from the tropomyosin degradation, but this is still uncertain.

Changes in protein solubility could be another possibility which might explain the intensified bands. Initially, these native proteins (110,000, 55,000 and 15,000 daltons) were probably fairly soluble in the diluted salt solution which was used to purify the myofibrils (Table 2). Hence, significant amounts of these proteins may have been washed away. Later during postmortem storage, the proteins became less soluble possibly due to denaturation so that high yields were isolated by centrifugation (Table 2).

Densitometric Tracings

In an attempt to gain more information about the postmortem changes involved in the myofibrillar proteins, the gels were subjected to densitometric scanning technique (Figure 5). The labeled peaks correspond to the changes indicated in the electrophoretograms shown in Figure 4 using the same letters of identification. Basically, the scanning data show the major changes that were observed in the gel patterns. Figure 5 clearly
Figure 5. Densitometer tracings of electrophoretograms of myofibrils isolated from ground (A) and intact (B) bovine semitendinosus muscle stored at 2°C for 0, 1, 3, 6, and 10 days postmortem. Letters a, b, c, d, e, f, g, and h correspond to the changes indicated in electrophoretograms.
Figure 5
reveals a more rapid breakdown of nebulin in the ground muscle sample than in the intact muscle by showing a faster reduction in peak size (peak a) in the former. The 95,000-dalton component (peak c) was detected in the ground muscle at 1 day postmortem, which was barely visible in the electrophoretogram, but was undetectable in the intact muscle. Similarly, in the area of 30,000 daltons, peak g appeared at 1 day postmortem in the ground sample but not in the intact muscle. With prolonged storage, however, this peak increased in both treatments although the intact muscle showed a more pronounced change. Simultaneously, troponin T (peak f) was attenuating in both treatments but more rapidly in the intact muscle. Another marked change was the increased peak around the area of MLC₃ (peak h). Originally, peak h was not appreciable but after 10 days postmortem storage it became a major peak on the scanning profile. Other changes noted include the gradually augmented sizes of peaks b and d corresponding to the 110,000- and 55,000-dalton components. The progressive diminution of desmin (peak e) is also evident.

Causal Factors

The similarity between the patterns of protein changes of the ground and intact samples suggests that
possibly similar proteinases were involved during the postmortem storage of both treatments. Nevertheless, the small difference in the rates of these changes indicates that grinding only slightly altered the activities of certain muscle endogenous proteolytic enzymes. Structurally and enzymatically, grinding can effect muscle cells in two ways. Obviously, it can disrupt muscle cell walls and severely damage the myofibrils so that they will become more labile to proteinases. It is highly possible that grinding can rupture or impair some cell organelles, resulting in an early release of some proteinases and coenzymes. In fact, due to the fracture of cell walls, myofibrils would become more susceptible to extracellular proteinases in addition to the intracellular enzymes. In the former case, neutral and alkaline proteinases existing in mast cells which could also have been disrupted during the grinding process may attack the myofibrils. One such possible enzyme is neutral serine proteinase that has the capability of cleaving actin, myosin, α-actinin, tropomyosin, troponin T and I (Goll et al., 1983). Thus, these enzymic activities, if any, should only affect the myofibrils of ground muscle while the cell membranes of the intact muscle remained intact and protected the myofibrils from extracellular enzymes during postmortem storage. However, it is unlikely that these extracellular proteinases were
involved since the electrophoretic patterns (Figure 4) of myofibrillar proteins of both the ground and intact muscles were very similar. In addition, muscles soon became acidic after death (Table 4), which would tend to inactivate or inhibit these neutral and alkaline pH proteinases should they be found around the myofibrils.

The Ca\(^{++}\)-activated factor (CAF), however, may be activated by grinding. It has been well documented that CAF has a unique specificity against certain myofibril components (Dayton et al., 1975; Olson et al., 1977; Ishiura et al., 1979). In vivo, this activity is contributed by low-Ca\(^{++}\) CAF which is completely active at 70uM of Ca\(^{++}\) (Goll et al., 1983). Two muscle cell structures, the sarcoplasmic reticulum and mitochondria, regulate the sarcoplasmic level of Ca\(^{++}\) (Etherington, 1981). In ground muscle, the sarcoplasmic reticulum could have been ruptured, resulting in the early release of Ca\(^{++}\) (Hamm, 1977). The grinding-induced rapid glycosis can itself markedly hinder the sarcoplasmic reticulum to sequester Ca\(^{++}\) (Cassens, 1977). It is probable that grinding could also have disrupted mitochondria. Whiting (1980) has found that mitochondria are more labile than the sarcoplasmic reticulum, and under normal aging and cold-shortening conditions could be the initial agents of calcium release. An elevated sarcoplasmic Ca\(^{++}\) level tends to
promote the activity of the low-Ca\textsuperscript{++} CAF. Moreover, high-Ca\textsuperscript{++} CAF could be partially activated by the released Ca\textsuperscript{++}. Hence, a high CAF activity, and consequently, a faster protein breakdown would occur in the ground muscle. However, this likelihood is challenged by the low pH observed in the muscle postmortem since CAF has optimum pH around 7 while the pH values in both ground and intact muscle samples were below 6 during the time most of the proteolytic alterations occurred (Table 4). Hence, other proteinases may have been involved.

Lysosomal enzymes are other possible causal agents. Similarly, the initial apparently faster proteolysis of ground muscle could partially be caused by the early disruption of the muscle lysosomes. The sudden fall in pH due to grinding would promote this destruction. In the intact muscle, the disruption may also take place but at a slower rate due to the mild pH decline (Figure 3). At least four catheptical activities isolated from lysosomes of skeletal muscle cells have been shown to be active against myofibrils (Goll et al., 1983; Dahlmann and Reinauer, 1984). Bovine skeletal muscle contains cathepsins B and D (Swartz and Bird, 1977; Robbins et al., 1979). Presumably, after their release from lysosomes these enzymes could diffuse readily into the myofibrillar structure, especially when the myofibrils were damaged during the grinding process. Figure 4 shows
that the more pronounced proteolytic changes took place at the later stage of postmortem storage, during the time when the pH was maintained at about 5.5 in the muscles with both treatments (Table 4). It seems likely that cathepsins released from the lysosomes would be active during this time because they have acidic pH optima. It is not clear, however, which of the lysosomal proteinases could be the more important or more effective. The contribution of cathepsin D would seem to be small as this enzyme generally requires a pH below 4.5 for activity (Etherington, 1981). Even though cathepsins B, H and L are active within the pH range of 5.0–6.0 (Etherington, 1981) it remains uncertain whether they were the principal causal factors since they could also degrade myosin and actin in addition to other myofibrillar proteins (Dahlmann and Reinauer, 1984), which can hardly be seen from the electrophoretogram (Figure 4). However, the failure to detect changes in myosin and actin does not mean a complete absence of lysosomal activity. Most of the studies showing the activity of cathepsins against myosin and actin were conducted at ambient or higher temperatures, and used purified myosin and actin or purified myofibrils (Schwartz and Bird, 1977; Robbins et al., 1979; Matsukura et al., 1981). Evidently, these conditions may not represent the conditions in vivo, or those present in
this study, notably the integrity of myofibrils and the storage temperature (2°C). Moreover, Okitani et al. (1980) have suggested that the activity of cathepsins may vary from species to species. Etherington (1980) reported that the cysteine cathepsins (e.g., cathepsins B and L) of the muscle lysosomes were the more effective enzymes when pH was below 6.0 during the main meat aging period while CAF was the most effective enzyme above pH 6.0. Thus, it is likely that these participating cathepsins may have a substrate preference. Myosin and actin may not be significantly affected before certain other myofibrillar proteins have been markedly degraded by these lysosomal proteinases under in vivo conditions.

While the true mechanism involved in the observed myofibrillar protein alterations is still obscure, there is some evidence which tends to implicate that CAF was a possible causal agent. As shown in the gel electrophoretogram (Figure 4), nebulin (arrow a) was significantly degraded within the first 24 hours postmortem in the ground muscle but not in the intact muscle. This would suggest that the CAF was activated initially by the grinding-induced early release of Ca^{++} when the muscle pH remained high. Maruyama et al. (1981b) found that CAF can degrade nebulin. Catheptic enzyme activity could be excluded because of the high pH condition during this time. Similarly, the 30,000-dalton
component that appeared electrophoretically in the ground muscle at 1 day postmortem but was completely undetectable in the intact muscle would also be an indication that the early liberated Ca$^{++}$ did activate CAF since the 30,000-dalton polypeptide has been shown repeatedly to be derived from troponin T degraded by CAF (Olson et al., 1977; Penny, 1980). Other data suggesting the involvement of CAF are: the more pronounced decrease in the intensity of troponin T band and concurrently, the more appreciable increase in the intensity of the 30,000-dalton component in the intact muscle than in ground muscle during the main period of postmortem storage (Figure 4, 5). Interestingly, during the same time (6 days and 10 days postmortem), the pH value of the intact muscle was significantly higher ($P < 0.05$) than that of the ground muscle (Table 4). Similar results were obtained from the electrophoretic analysis of the myofibrillar proteins from other animals in this study. In one case when the pH value was significantly higher in the intact muscle than in the ground sample during the main storage time, the gradual appearance of the 30,000-dalton polypeptide seemed to be more pronounced in the former. The opposite pattern was observed in another animal when the pH value was higher in the ground muscle. Therefore, it is concluded that CAF was indeed involved in the postmortem myofibril breakdown in both ground and
intact muscles.

The existence of CAF activity in low pH muscle is not surprising. Dayton et al. (1981) have shown that low-Ca \(^{++}\) CAF is active over a slightly broader range of pH values than high-Ca \(^{++}\) CAF, and it may retain significant proteolytic activity at pH 5.5. Between pH 5.5 and 5.9, 15-25\% of the maximum activity of CAF is retained (Penny, 1980). Goll et al. (1983) suggested that 90\% or more of the postmortem proteolytic degradation of myofibrillar proteins inside muscle cells could be contributed by CAF, however, this was not obtained from actual measurement.

In view of these facts, it is possible that CAF was responsible for most of the proteolytic activities against the myofibrils in the initial postmortem storage period when the pH was still relatively high. The probable function of CAF during this stage was to disorganize the myofibrils by digesting some of the structure-maintaining proteins such as the M-line and desmin proteins in the Z-disks. This action was facilitated by grinding. When the pH value dropped to about 5.5 as observed at 3, 6 and 10 days postmortem, lysosomal enzymes became active and preferentially hydrolyzed the myofibrillar proteins. During the later period of storage, when the muscles had achieved their ultimate pH (about 5.5), CAF still retained a certain
degree of its maximum activity. It remains uncertain, however, which enzyme(s) actually played a major role in both ground and intact muscles during the postmortem storage.

Sarcoplasmic Proteins

The sarcoplasmic proteins of the ground and intact muscles after 0, 1, 3, 6 and 10 days of postmortem storage at 2°C were also analyzed by SDS-PAGE (Figure 6) and gel densitometric scanning (Figure 7). The electrophoretic patterns of both muscle treatments were very similar except that (1) three high M.W. proteins (arrows a, b, c) appeared early in the ground muscle while the 1,000,000-dalton component (arrow a) became more intense in the intact muscle during the aging; (2) two closely spaced polypeptides gradually appeared in the area around 138,000 daltons in the ground muscle but only one occurred in the intact muscle (arrow e). Other significant proteolytic changes included the disappearance of 300,000- and 24,000-dalton proteins (arrows d, h) and the gradual appearance of a 100,000-dalton polypeptide (arrow f) in both muscle treatments. Alterations in these proteins were also exhibited from the gel scanning data (Figure 7). In addition, scanning showed the diminution of a
Figure 6. SDS-polyacrylamide gel electrophoresis of sarcoplasmic proteins extracted from ground (A) and intact (B) bovine semitendinosus muscle stored at 2°C. Gels 0, 1, 3, 6, and 10 represent sarcoplasmic proteins extracted from the muscle after 0, 1, 3, 6, and 10 days of postmortem storage. Gel S is the protein standards (number X 1000 = molecular weight). Arrows a, b, c, d, e, f, g, and h show the major changes.
Figure 6
Figure 7. Densitometer tracings of electrophoretograms of sarcoplasmic proteins extracted from ground (A) and intact (B) bovine semitendinosus muscle stored at 2°C for 0, 1, 3, 6, and 10 days postmortem. Letters a, b, c, d, e, f, g, and h correspond to the changes indicated in electrophoretograms.
Figure 7
26,000-dalton protein (peak g) at 10 days postmortem (Figure 7).

Changes in the intensity in specific bands suggest a complex progression of proteolytic events. Although CAF is present in sarcoplasm it has very little effect on the sarcoplasmic proteins (Goll et al., 1983). Therefore, lysosomal proteinases may contribute to some of these changes. It is likely that contamination with non-sarcoplasmic proteins such as membrane and mitochondrial proteins or fragments of the sarcoplasmic reticulum was involved. Goll et al. (1974) suggested that centrifugation at 25,000-100,000 X g for 120-180 minutes is required to sediment microsomes, polysomes, ribosomes and fragments of sarcolemma. In our extraction, however, 35,000 X g for 45 minutes was employed (Table 2).

Contamination with myofibrillar proteins was also highly possible. Goll et al. (1970) suggested that if myofibrils are degraded the appearance of new protein components in the sarcoplasmic protein fraction should be expected. Careful comparisons of the electrophoretograms of the myofibrils (Figure 4) with those of the sarcopasmic proteins (Figure 6) reveal some interesting correlations between the two electrophoretic patterns. It can be seen that titin had a subtle alteration during the postmortem storage (Figure 4). There was a more appreciable loss of titin in the intact muscle because
the band became thinner at 6 and 10 days postmortem. Concomitantly, a new protein of the same size as titin (\(\sim 1,000,000\) daltons) gradually appeared in the sarcoplasmic extraction of both ground and intact muscles with a more intense band present in the intact muscle extraction (Figure 6, arrow a; Figure 7, peak a). This concurrent event suggests that the \(\sim 1,000,000\)-dalton component arising in the sarcoplasmic fraction probably originated from titin of the myofibrils. Goll et al. (1977) have reported that the myofibrillar proteins are soluble in water once they have been extracted, and some of them are extractable with water. Low ionic strength buffer (e.g. 5mM Tris-HCl, pH8.0) can extract most of the myofibrillar proteins except myosin (Penny, 1980). Therefore, it seems reasonable that to extract and solubilize the proteins from the myofibrils, the most important factor is to disrupt the intimate associations and interactions among the protein molecules within the myofibrils. Although Wang and Ramirez-Mitchell (1983) found that titin was salt-insoluble, their study was carried out with the fresh muscle in which titin molecules are strongly cross-linked with each other. It is probable that during the aging process the tight associations among titin molecules and the interactions of titin molecules with the myofibrils became weakened by some unknown mechanism involving certain endogenous
proteinases, and titin molecules were liberated into the sarcoplasmic protein extracting solvent during homogenization. It remains uncertain, however, whether the two proteins are identical.

Perhaps by a similar mechanism another high M.W. component indicated by arrow b (Figure 6) was removed from the lower titin doublet (the second band from the top of the gel, Figure 4). The ~600,000-dalton polypeptide of the sarcoplasmic fraction (Figure 6, arrow c) may be an extractable degradative product of titin from the myofibrils.

Similarly, the intensity of α-actinin seemed to have a slight change after 3 days of postmortem storage (Figure 4), and this change paralleled the gradual appearance of a sarcoplasmic protein of the same size about 100,000 daltons (Figure 6, arrow f). Reddy et al. (1975) have carefully studied α-actinin. They found that it was very resistant to CAF but was readily released from the Z-disk of the myofibril when CAF is present. Penny (1980) has been able to show that the binding of α-actinin to the Z-disk structure is weakened during aging and the amount of α-actinin extracted in low ionic strength solution increases with aging. In view of these findings it is likely that the 100,000-dalton protein which gradually arose in the sarcoplasmic protein fraction was the released α-actinin from the myofibrils.
by CAF during the storage period. Again, this hypothesis should be treated with caution since postmortem alterations in other cell organelles could also contribute to such a change.

In Figure 6 and Figure 7, the disappearance of the 300,000-dalton protein (arrow d and peak d) and some minor changes in 105,000 to 300,000 daltons region in both ground and intact muscles may reflect some changes in solubility and/or denaturation of the proteins in this region. Of course, the possibility of proteolysis can not be excluded. Drabikowski et al. (1977) showed that sarcoplasmic proteins can be degraded by acid cathepsins. Changes in this region have been reported by other workers (Hay, 1973b; Harrington and Henahan, 1982).

In the area of 138,000 daltons two polypeptides appeared in the ground muscle but only one in the intact muscle after 6 days of postmortem storage. The cause is not clear but this indicates that grinding can slightly influence the muscle protein alterations postmortem.

Psychrotrophic Bacteria Inspection

No growth of psychrotrophic bacteria was observed during the incubation period of 10 days at 7°C in either fresh (0 day) or stored (10 days) ground and intact muscle samples. The same results were obtained
from the preliminary tests using incubation temperature of 2°C. This is not surprising since very strict aseptic practices were employed, including use of the sterilized equipment and NaN₃ which is a microbial inhibitor. Therefore, the possibility of microbial proteolytic activity was totally excluded.

In the parallel incubations at 21°C and at 31°C, some mesophilic bacterial growth was noticed. However, no attempt was made to interpret this effect on the muscle proteins since mesophilic bacteria will not grow at 2°C which was the postmortem storage temperature used in this study.
Evidence gained throughout this study indicates that there were no extensive protein changes in ground beef muscle during the postmortem storage at 2°C for 10 days. The close resemblance between the electrophoretic patterns of ground and intact muscle sarcoplasmic proteins suggests that the grinding process had no significant influence on the sarcoplasmic protein alterations postmortem. Similarly, grinding did not render a pronounced effect upon the breakdown of the myofibrils. However, the grinding process slightly accelerated the degradation of certain contractile components particularly nebulin, but this effect diminished during the prolonged storage.

It is suggested, on the evidence presented, that some contractile proteins could be released from the myofibrils during postmortem storage and they could be extracted with diluted salt solution.

Evidence was also presented which indicates the possible participation of CAF in the alterations of myofibrillar proteins and that CAF was activated by the grinding process. The latter could result from an early release of Ca** from the damaged sarcoplasmic reticulum as manifested by the hastened glycolysis in ground muscle. It was unlikely that neutral and alkaline
proteinases were involved. In addition, there was no strong evidence which would suggest a significant activity of lysosomal enzymes in this study. Whether or not CAF acted individually or in co-operation with other enzymes (e.g. cathepsins) to produce the protein changes observed in this study remains a question.

On the other hand, grinding accelerated significantly the glycolysis reflected by the drastic pH decline in ground muscle, probably due to the activation of ATP-ase by the Ca\(^{++}\) released from disrupted sarcoplasmic reticulum and mitochondria. After 3 days postmortem storage there was no significant difference in pH between the ground and intact muscles (\(P > 0.05\)), but this does not cover all the individual cases.

Aseptic sample preparation and the use of NaN followed in this study were very effective in preventing contamination of psychrotrophic bacteria. Therefore, it is unlikely that the development of certain off-flavors in hot-boned ground beef during storage at 2\(^{\circ}\)C for up to 10 days would result from the protein changes induced by grinding or the alteration of muscle endogenous enzyme activity per se. Other mechanisms such as microbial contamination may be involved.
BIBLIOGRAPHY


