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Title: PRESSURIZATION OF PRERIGOR BEEF MUSCLE AND ITS EFFECT ON THE SOLUBILIZATION OF MYOFIBRILLAR PROTEINS

Abstract approved: Dr. Allen F. Anglemier

Investigations were conducted to evaluate some of the changes occuring in the myofibrillar proteins, particularly solubility properties, resulting from the hydrostatic pressurization (15,000 psi, 2 - 3 min, 40°C) of prerigor semitendinosus and longissimus dorsi beef muscles.

Both pressurized muscles showed a sharp decline in pH (~ 0.9 unit) as the result of pressurization. Pressurized samples had an average pH of 5.9 as compared to 6.8 for the controls shortly after completion of the pressure treatment. Immediately after pressurization, the pressurized samples had higher Hunter Color "a" and "L" values than the controls indicating an increase in redness and brightness respectively.

The water-holding capacity (WHC) of both muscles was influenced by pressurization. Myofibrils isolated from pressurized muscles showed an increase in WHC with increasing molar concentrations of

KCl in which they were suspended. In 0M KCl, the WHC increased 29% while an increase of 46% was noted on pressurizing myofibrils suspended in 1.0M KCl.

The dye-binding ability of myofibrillar proteins was affected by pressurization. Approximately 25% less protein was detected in the pressurized samples by the dye-binding method than by the biuret procedure.

Extractibility of total myofibrillar proteins was reduced by pressurization. Pressurized semitendinosus and longissimus dorsi muscles yielded 39.6 and 34.4% less myofibrils respectively than their controls. When myofibrils isolated from non-pressurized muscles were pressurized and subsequently centrifuged, 35.7 and 36.8% more protein was solubilized in the supernatants of the semitendinosus and longissimus dorsi muscles respectively.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and densitometric tracings of isolated reconstituted myofibrillar pellets from pressurized muscles showed myosin (heavy chain) to be 16.6% less than its control while larger losses of about 30% were observed for M-protein, C-protein, actin, troponin-T, and tropomyosin. Electrophoresis and densitometry of supernatants from isolated pressurized myofibrils indicated actin was most affected by pressurization at low ionic strength ($\mu<0.1$) while myosin (heavy chain) was most affected at high ionic strength ($\mu\geq0.5$). Pressure-induced solubilization of actin at low ionic strength was about 48 milligrams percent while that of myosin was about 1 milligram percent. At high ionic strength, solubilization of myosin increased to 42 milligrams percent while actin was reduced to 23 milligrams percent.

Pressurization of Prerigor Beef Muscle and its Effect on the Solubilization of Myofibrillar Proteins

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INTRODUCTION

Tenderness is probably the most important criterion for assessing meat quality as consumer acceptability of fresh beef is greatly influenced by its degree of tenderness. As a result, beef tenderness has received considerable attention in recent years. Due to the increasing demand for grain in the world market for human consumption, the trend of finishing market beef cattle on more forage and less grain is continually on the increase. These animals when ready for slaughter are bound to be older and the resultant carcasses are expected to be less tender causing a marked reduction in their eating quality (Smith et al., 1977; Williams et al., 1979; Bowling et al., 1977). Consequently, meat from such carcasses may require some tenderizing techniques to render it more acceptable to the consumer.

It is not surprising, therefore, that a reasonable amount of research has been completed in various attempts to understand the causes for and variations in meat tenderness and how tenderness can be improved. Goll et al. (1974) pointed out that meat toughness or variation in meat tenderness is a function of differences in muscle proteins.

Marsh (1972, 1977) and Locker (1960) indicated that meat

tenderness could be grouped into two components which they termed "background toughness" and "actomyosin toughness." Background toughness was described as toughness attributed to stroma proteins while actomyosin toughness refers to toughness due to the contractile or myofibrillar proteins. According to Marsh (1977), the collagen of stroma proteins has long been recognized as an important influence on tenderness variation while the contractile apparatus was first established as a potent contributor to toughness only in the early 1960's. In its contribution to background toughness, the quality of collagen appears to be more significant than quantity considering the ease with which its intermolecular crosslinks are split during cooking, postmortem treatment, or acidification. On the other hand, Goll et al. (1974) described several lines of evidence demonstrating that myofibrillar proteins could have enormous influence on meat tenderness. In furtherance of this, Marsh (1977) showed that toughness due to the contractile protein complex is more amenable to control and modification than that attributed to the stroma proteins. Goll et al. (1974) indicated that the contractile protein complex can undergo two changes that affect meat tenderness. The first is the formation of actin-myosin cross-linkages which cause increased toughness due to the inextensibility and rigidity of the resultant muscle. The second change is the disintegration of the Z-disc which results in increased tenderness due to the fragmentation of the myofibrils and loss of tensile strength.

Certain tenderization techniques, more especially prerigor hydrostatic pressurization, have been shown to affect the contractile proteins thereby markedly improving tenderness (Macfarlane, 1973,

1974; Macfarlane and Mckenzie, 1976; Bouton et al., 1977, 1980). Their findings indicated that pressurization improved the yield of salt-soluble proteins about three-fold. Solubilization of the myofibrillar proteins as induced by pressure was found to be influenced by both the duration of pressurization and the temperature during pressurization. The principle behind this increased solubilization of myofibrillar proteins can be linked, in part, to events occurring during protein hydration. Macfarlane (1974) pointed out that the hydration of proteins in an aqueous system would result in a decrease in volume. This occurs because the effective volume occupied by water molecules tends to decrease as these molecules become more tightly bound to the protein. As portrayed by Le Chatelier's principle, reactions that result in a decrease in the volume of a system are enhanced by an increase in pressure while those resulting in volume increase are retarded by the same increase in pressure. It is thus conceivable that protein hydration and consequently the water-holding capacity (WHC) of such proteins would be promoted through an increase in pressure. Another aspect of pressurization as it affects tenderness has been suggested by Johnson et al. (1954), Brown (1957), and Johnson and Eyring (1970). Development of tension in muscle was reported to be influenced by prerigor hydrostatic pressurization to the point that this severe pressure-induced contraction resulted in myofibrillar damage. The degree of contraction was found to vary with the muscle temperature during pressurization and the animal species from which the muscle was obtained. It was also reported that pressurization influenced the chemical processes occuring in the muscle. Such processes according to Macfarlane (1973), Johnson

et al. (1954) , and Ikkai and Ooi (1966) include the acceleration of postmortem glycolysis, induction of zwitterion formation, F-to G-actin transformation, and dissociation of actomyosin systems.

From the above studies, it is apparent that the pressurization of prerigor muscle can influence the contraction state of the muscle and subsequent biochemical reactions thereby affecting the eating quality and ultimately, consumer acceptability of meat. The present work was undertaken to gain basic knowledge about the events taking place when prerigor beef muscle is subjected to hydrostatic pressurization especially as it affects the so-called actomyosin toughness. In addition, the nature and extent of influence of pressurization on the individual proteins of the myofibril were also investigated.

REVIEW OF LITERATURE

No biological organism can thrive and virtually no biological process can take place without the involvement of protein. Even the word "protein" connotes the importance of these molecules for it is derived from the Greek word "proteios" meaning "primary" or "holding first place" (Whitaker and Tannenbaun, 1977). Thus proteins are of vital importance in the growth and maturation of foodstuffs as well as in the growth, nutrition, and well-being of the ultimate consumer. Descriptively, proteins are high molecular weight structural biopolymers consisting of large numbers of repeating units of α-amino acid residues linked covalently by peptide bonds (Deatherage, 1975; Witkop, 1968). In addition to covalent linkages, the intricate structure of proteins is stablized by several types of bonds or forces (Whitaker, 1977).

Proteins have been grouped as fibrous or globular according to their shape and physical properties rather than by their chemical structure. Fox and Cameron (1972) described the fibrous proteins as simpler than globular and consisting of elastic and inelastic types. They are for the most part insoluble, form extended polypeptide chains held together by cross-links, and show a tendency to form gels on the application of heat. The globular proteins on the other hand show more complex structure with extensive folding of the polypeptide chains to form molecules with irregular but bulky shape.

Although fibrous or globular classification or proteins somehow denotes structural conformation, this grouping is not adequate to elucidate in appropriate terms such complex macromolecules as proteins. In keeping with this concept, the classification of proteins has been channeled towards the area of structural organization in terms of primary, secondary, tertiary, and quaternary structures as described by Anglemier and Montgomery (1976) and Jones (1964).

Skeletal Muscle Structure

Detailed discussion of skeletal muscle structure and its relationship to the myofibrillar proteins has been presented by Lawrie (1974) and Bendall (1964). They indicated that the muscle as a whole is surrounded by a sheath of connective tissue called the epimysium. Penetrating the muscle are septa of connective tissue which branch from the inner surface of the epimysium and separate the muscle fibers into bundles. Lawrie (1974) pointed out that these septa of connective tissue make up the perimysium and carry larger blood vessels and nerves which innervate the muscle. He further stated that the endomysium, a fine connective tissue framework, runs from the epimysium, to surround each muscle fiber.

According to Lawrie (1974), the muscle fiber is the vital structural unit of all muscles. He further described the muscle fiber as a long, narrow, multinucleated cell which could extend from one end of the muscle to another and attain a diameter of 10 - 100μ and a length up to 34cm. At the end of the muscle, fibers blend in with the epimysium, perimysium, and endomysium and finally converge to yield tendons. Robertson (1957) stated that each fiber

is surrounded beneath the endomysium by a double membrane, 50 - 60A° apart, called the sarcolemma.

Existing within the sarcolemma are the myofibrils which are bathed by the sarcoplasm consisting of a fluid phase containing complex internal membranes, mitochondria, soluble substances, and nuclei (Bennett, 1960; Bloom and Fawcett, 1968). According to Huxley (1960), the myofibrils are the contractile apparatus of skeletal muscle. Myofibrils measure 1 - 2μ across and run parallel to the long axis of the fiber. The cross striations are due to the presence of myofilaments which consist of the major myofibrillar proteins (myosin, actin, and tropomyosin).

Lawrie (1974) and Bloom and Fawcett (1968) described the main myofibrillar bands as the A-band, I-band, Z-line, and M-line. The A-band contains the thick or myosin filaments and when viewed under the polarizing microscope appears anisotropic and stains dark with iron hematoxylin. The I-band on the other hand contains the thin filaments consisting mainly of actin and some tropomyosin. The I-band has a central dark zone (Z-line) and extends about 1μ from each side of the Z-line. When viewed under the polarizing microscope, the I-band appears isotropic and is not stained by iron hematoxylin. The Z-line contains tropomyosin and bisects the I-band while running across the myofibril. It appears dark with various stains when viewed under phase contrast microscopy. The functional unit of a myofibril is represented by the distance between two successive Z-lines and is most frequently referred to as the sarcomere. The M-line runs through the middle of A-band and is thought to keep the myosin filaments centrally aligned in the sarcomere.

Carving out a cross section of the myofibril in the region of overlap between the thick and thin filaments, Huxley (1960, 1965) showed each myosin filament to be surrounded by six actin filaments in hexagonal arrangement whereas each actin filament is shared by three neighboring myosin filaments. Each thick filament possesses short cross-bridges which extend outward to interact with the thin filaments in actomyosin formation during muscle contraction.

Skeletal Muscle Proteins

The skeletal muscle by weight contains approximately 75% water, 19% protein, 2.5% lipid, and 3.5% soluble non-protein substances (Lawrie, 1974, 1975). Muscle proteins are often categorized as sarcoplasmic (glycolytic enzymes and pigments), stromal (connective tissue), and myofibrillar (contractile) protein fractions. Lawrie (1974) divided muscle proteins into those soluble in water and dilute salt solutions (sarcoplasmic proteins), those soluble in concentrated salt solutions (myofibrillar proteins), and those insoluble in water or salt solutions at low temperature (stroma proteins). The spatial distribution of these proteins, their concentration in the muscle, together with other data on chemical composition of a typical mammalian muscle, after rigor but prior to degradative changes, are presented in Table 1.

Sarcoplasmic Proteins

These proteins occupy all the spaces of the muscle cell not taken up by formed elements and the contractile system (Briskey and

Fukazawa, 1971). Sarcoplasmic proteins are the most soluble of all muscle proteins. Proteins found in the cytoplasm and the glycolytic enzymes are included in this category. Goll et al. (1977) indicated that the sarcoplasmic protein fraction contains 100 to 200 different proteins which constitute 30 to 35% of the total muscle protein content. They are soluble at ionic strengths of 0.1 or less at neutral pH. Generally, sarcoplasmic proteins include the albumins, globulins, myoglobin, and haemoglobin. The glycolytic enzymes form an integral portion of the albumins and globulins which constitute a large share of soluble muscle proteins. It has been reported that one enzyme alone, glyceraldehyde phosphate dehydrogenase, accounts for over 20% of the sarcoplasmic protein fraction and makes up to 25% of total glycolytic and associated enzymes (Czok and Buchner, 1960; Lawrie, 1975). This together with aldolase, enolase, kinase and lactate dehydrogenase make up about one-half of the sarcoplasmic proteins. The sarcoplasmic extract also contains other soluble nonprotein nitrogenous substances (Table 1).

The sarcoplasmic protein fraction also contains small amounts of other proteins unique to muscle. According to Heywood et al. (1974), these unique proteins possess initiation factors essential for the synthesis of myosin and myoglobin. As a sarcoplasmic protein, myoglobin is responsible for the color of meat.

Stroma Proteins

These proteins consist of connective tissue proteins, lipoproteins, and mucoproteins from cell membranes. Stroma proteins make up 10 to 15% of total proteins of skeletal muscle. Collagen and

Table 1. Chemical composition (protein and non-protein substances) of typical mammalian muscle after rigor mortis but before degradative changes postmortem.*

Components		% Wet Weight
1. WATER		75.0
2. PROTEIN		19.0
(a) Myofibrillar	11.5	
myosin (H- and L- chains)	6.5	
actin	2.5	
tropomyosin	1.5	
troponins (T, I, and C)	0.4	
actinins (α and β)	0.4	
M-protein, C-protein, etc.	0.2	
(b) Sarcoplasmic	5.5	
glyceraldehyde phosphate dehydrogenase	1.2	
aldolase	0.6	
creatine kinase	0.5	
other glycolytic enzymes	2.2	
myoglobin	0.2	
haemoglobin	0.4	
other unspecific proteins	0.4	
(c) Connective tissue/organelle	2.0	
collagen	1.0	
elastin	0.05	
mitochondrial, etc.	0.95	
3. LIPID		2.5
4. CARBOHYDRATE		1.2
5. MISCELLANEOUS SOLUBLE NONPROTEIN SUBSTANCES		2.3
(a) Nitrogenous	1.65	
(b) Inorganic	0.65	
6. VITAMINS, TRACE MINERALS, GLYCOLYTIC INTERMEDIATES Quantitatively minute amounts		

*Adapted and abridged from Lawrie (1974; 1975).

elastin are the chief connective tissue proteins with collagen constituting 40 to 60% of total stroma protein while elastin contributes 10 to 20% of its make-up. Stroma proteins are often referred to as the insoluble proteins of skeletal muscle because they are retained in the residue after exhaustive extraction of soluble muscle proteins with salt solutions of various ionic strengths (Goll et al., 1977). They proceeded to mention the deleterious effects these proteins have on meat quality which include lowering of meat tenderness, decreasing meat emulsifying capacity, lowering of water-holding capacity, and decreasing the nutritive value of the meat tissue.

Myofibrillar Proteins

These are the contractile proteins that constitute the myofibril. Myofibrillar proteins make up 52 to 56% of total skeletal muscle protein and are generally grouped as those proteins soluble in salt solutions of high ionic strength (μ>0.3) though most are water-soluble once extracted (Goll et al., 1977). Szent-Gyorgyi (1960) contended it is better to refer to these proteins as those responsible for the filamentous organization of the muscle and function directly in the contraction and relaxation process. On extraction, the high viscosity of the myofibrillar extract is attributed to the fibrous nature of the proteins brought into solution. Apart from being responsible for the contraction and relaxation process, the myofibrillar proteins account for about 97% of the muscle water-holding capacity and about 90% of its total emulsifying capacity. According to Goll et al. (1974), myofibrillar proteins appear to be responsible for anywhere from 50 to nearly 100% of the variation in meat tenderness

depending on the connective tissue content and the state of the muscle being tested. In muscles with low connective tissue content or where myofibrillar proteins have been altered by adverse postmortem treatments, tenderness is mainly a function of the myofibrillar proteins. It is also estimated that myofibrillar proteins contribute more than 70% of the protein nutrition derived from meat considering that these proteins constitute over 50% of total muscle protein while at the same time containing relatively high proportions of nutritionally essential amino acids (Briskey and Fukazawa, 1971).

The myofibrillar proteins are responsible for the complex duty of transforming the chemical energy of ATP into movement (Goll et al., 1974). In pursuance of a thorough understanding of their biochemical properties, myofibrillar proteins have been studied intensively. Intact myofibrils have been isolated and the myofibrillar proteins separated on the basis of molecular size and charge using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Goll et al., 1977; Hay et al., 1973; Dutson, 1977).

Protein Components of the Myofibril

The skeletal muscle myofibril is made up of approximately eight major proteins which include myosin, actin, tropomyosin, C-protein, M-protein, α-actinin, β-actinin, and troponins.

Myosin

Myosin is the most abundant of the myofibrillar proteins constituting 50 to 58% by weight of the myofibril (Goll et al., 1977).

Upon tryptic digestion (Briskey and Fukazawa, 1971), the myosin molecule was shown to yield two major fragments, the light (LMM) and heavy mereomyosins (HMM). Light mereomyosin is not water-soluble but soluble at higher ionic strengths (μ>0.3) and has neither actin-binding ability nor ATPase activity. Heavy mereomyosin on the other hand is soluble in water and possesses both actin-binding ability and ATPase activity. Szent-Gyorgyi (1960) reported the molecular weight of myosin to be about 500,000 with HMM and LMM being 350,000 and 150,000 respectively. More recently, Goll et al. (1977) listed the subunit polypeptide mass and composition of myosin as two 200,000 dalton components, one 21,000, two 19,000, and one 16,500 dalton components.

Myosin is the chief protein of the thick filaments constituting 94 to 96% of the protein in these filaments (Offer et al., 1973). The myosin molecule is a large, fairly rigid rod running approximately 1,600A° in length and 20A° in diameter with a thickened bulbous end of variable diameter. The bulbous end contains two globular heads and the entire molecule consists of six subunit polypeptide chains. Harrington and Burke (1972) indicated that myosin molecules by themselves apparently possess most of the information required for aggregation into thick filaments. They showed that myosin molecules form dimers spontaneously in solution as parallel aggregates joined by tail-to-tail assembly. It appears, therefore, that the thick filaments of the muscle are formed by successive addition of these parallel dimers rather than myosin monomers.

The myosin molecule or the molecular architecture of the thick filaments plays a vital role in muscle contraction. Cohen (1975)

pointed out that a muscle initiates contraction when the projecting heads of the myosin molecules extend to make contact with the actin molecules. This results in pulling together the opposed sets of thin filaments and thus the fibril shortens. However, release of calcium ions from membrane-bound storage sites in the sarcoplasmic reticulum is a required signal to trigger the event. The chemical events as elaborated by Murray and Weber (1974) involve the combination of myosin head with one molecule of adenosine triphosphate (ATP) to form myosin-ATP complex. This complex is elevated to a charged intermediate that binds to an actin molecule of the thin filaments to form the "active complex" which undergoes hydrolysis to split ATP into adenosine diphosphate (ADP) and inorganic phosphate with a release of energy. This energy powers muscle contraction.

Myosin is extractable from a muscle mince with strong salt solution (e.g., 0.5M potassium chloride) and precipitated free of water-soluble proteins by reduction in ionic strength of the solutions (Briskey and Fukazawa, 1971). Numerous procedures have been reported for the extraction of myosin. Huxley (1960) stated that two solutions have been commonly used for myosin extraction. These include the Guba-Straub solution which comprises 0.3M KCl and 0.15M phosphate buffer, pH 6.5, and Hasselbach-Schneider solution which consists of 0.47M KCl, 0.1M phosphate buffer, and 0.01M sodium pyrophosphate, pH 6.5. The first solution tends to extract some actin contaminants along with myosin but its effectiveness is improved by adding 5×10^{-4}M ATP. Hasselbach-Schneider solution extracts myosin with almost no actin contaminant though its effectiveness can be improved by the addition of 10^{-3}M $MgCl_2$. According to Bendall (1964) and Huxley (1960), myosin thus extracted remains soluble even when the ionic

strength is lowered below 0.3. This permits the removal of contaminating actin by precipitating any available actomyosin through ionic strength adjustment to 0.3. Similarly, myosin can be precipitated by adjusting the ionic strength to 0.05 thus leaving the more soluble proteins in solution (Huxley, 1960). Myosin preparations, however, have been reported to be plagued by the presence of myokinase, AMP deaminase, nucleic acids, myosin-nucleic acid complexes, and unidentified proteins (Richards et al., 1967) Attempts to extract myosin with high purity have yielded myosin with reduced ATPase activity. Due to its high content of glutamic and aspartic acids, and of dibasic amino acids, the myosin molecule is highly charged and has a strong affinity for calcium and magnesium ions. According to Bendall (1964), the isoelectric point of myosin in KCl solutions is pH 5.4 but on addition of Mg^{2+} or Ca^{2+}, this increases to about 9.3 due to its affinity for divalent ions. The contractile activity of myosin is activated by Ca^{2+} but inhibited by Mg^{2+}.

Electrophoretically, the heavy chain of myosin is the slowest migrating band on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Its protein band shows as a 200,000 dalton component and comprises 42 to 45% of the total myofibrillar protein loaded on the gel (Goll et al., 1977). The light chain of myosin is divided into alkali-1 light chain, DTNB light chain, and alkali-2 light chain which on SDS-PAGE shows as 21,000, 19,000, and 16,500 dalton subunits respectively.

Actin

Actin is the other major myofibrillar protein constituting

15 to 20% by weight of the myofibril (Goll et al., 1977). Actin can exist in two forms, the globular or G-actin and the fibrous or F-actin. G-actin consists of relatively small globular units while F-actin is a double chain formed as a result of end to end aggregation of the globular units (Lawrie, 1974). According to Huxley (1960), actin is believed to exist in vivo as F-actin. G-actin however polymerizes into F-actin in the presence of salts and small amounts of ATP. The molecular weight of G-actin monomer is understandably variable due to the inherent difficulty in obtaining purified actin and its tendency to polymerize into F-actin. Bendall (1964), Lawrie (1974), and Goll et al. (1977) give molecular weight values of G-actin as 56,000 to 70,000, 70,000, and 42,000 respectively.

Actin is the major protein of the thin filaments. It is estimated that each thin filament contains 340 to 380 actin molecules (Goll et al., 1977), though Briskey and Fukazawa (1971) indicate there can be as much as 500 to 600 actin monomers per filament. The actin molecules are small roughly spherical particles arrayed in thin filaments to form something like a twisted double strand of beads (Murray and Weber, 1974). Actin molecules are asymmetrical and are assembeld into the filaments in a "front to back" manner with the front sides of all actins facing one direction and the back sides facing the other. This directionality has been found to be essential for muscle contraction. Briskey and Fukazawa (1971) described the G-actin monomer as measuring approximately 55A° in diameter. They stated that F-actin is a double helical polymer of G-actin monomers, makes a complete turn every 700A° with 13 to

15 globular subunits per turn of the helix, and measures 80A° in diameter.

Functionally, actin is involved in muscle contraction along with myosin of the thick filaments. The two most important properties of actin appear to be G- to F-transformation and interaction with myosin in actomyosin formation (Poglazov, 1966). In reviewing G- to F-transformation, he stated that G-actin is converted to F-actin when the ionic strength of the medium is raised from 0.01 to 0.15. This transformation was found to be enhanced by Mg^{2+} and Ca^{2+} (Goll et al., 1977). In muscle contraction, it is F-actin which combines with myosin to form the contractile actomyosin of active or prerigor muscle and the inextensible actomyosin of muscle in rigor mortis (Lawrie, 1974). In resting muscle, the beads comprising the actin filaments are prevented from combining with the corresponding projections on myosin by the magnesium complex of adenosine triphosphate (Mg-ATP) which acts as a plasticizer.

An isolation procedure to obtain desirable preparation of pure actin revolves around careful manipulation of such processes like fine comminution of muscle, removal of lipid-like material, and maintenance of pH near neutrality (Seraydarian et al., 1967). They pointed out that a more satisfying actin extraction procedure involves the use of 2mM ATP, 2mM ascorbate, and 3.3M potassium chloride. ATP is needed to depolymerize F-actin in order to remove it as G-actin. The ascorbate provides protection for the sulfhydryl (-SH) groups of actin while the potassium chloride precipitation step is expected to rid the extract of α-actinin. Tropomyosin, however, appears to be the major contaminant of actin. Aldestein

et al. (1963) noted that crude actin preparations made at room temperature contained about 25% tropomyosin and when done at 0°C, about 20% of this protein.

On SDS-PAGE, actin migrates down the gel as a 42,000 dalton component. Since its subunit polypeptide composition shows it as one 42,000 body (Goll et al., 1977), actin is seen on gels as only one entity. The actin molecule, therefore, contains only one polypeptide chain and is much simpler than myosin.

M-Protein

The M-protein is a relatively new addition to the group of myofibrillar proteins. Although it was earlier referred to as "M-substance" by Masaki et al. (1968), its discovery was not documented until 1974 (Goll et al., 1977). This protein has been found to constitute 3 to 5% of the myofibril by weight. The molecular weight of M-protein appears to vary with investigators. Goll et al. (1977) gave its molecular weight as 160,000 while Trinick and Lowey (1976) reported a molecular weight of 170,000. The latter also noted the existence of a second 170,000 dalton component which purifies with M-protein through fractionations in 32% saturated ammonium sulfate and 10mM potassium phosphate. Nonetheless, several reports have indicated that a second M-protein with a 42,000 dalton subunit exists in addition to the M-protein described above (Goll et al., 1977).

M-Protein is one of the minor components of the thick filaments. According to Trinick and Lowey (1976), M-protein antibodies strongly stained the middle of the A-band thus giving credence to the belief that M-protein originates from M-line which

runs through the middle of A-band. Earlier, Masaki et al. (1968) had reported that "M-substance" (M-protein) promoted aggregation of myosin in a random manner as determined by electron microscopy. On the addition of 0.1M KCl to M-protein and myosin extracts, M-protein slowly aggregated and this aggregate markedly accelerated the lateral association of myosin aggregate in 0.1M KCl solution. This is in keeping with the concept that M-line, hence the M-protein, keeps the myosin filaments centrally aligned in the sacomere (Lawrie, 1974).

Isolation of M-protein is accomplished in essentially the same fashion as in the extraction of myosin using the Hasselbach-Schneider solution described earlier (Trinick and Lowey, 1976). Separation of pure M-protein is achieved by ion exchange chromatography on DEAE cellulose (50mM Tris-HCl, pH 8.0). M-Proteins are thus distinguishable by their sedimentation coefficients as they sediment at 5.1S while the contaminating M-line components sediment at 7.1S.

On SDS-PAGE, M-protein migrates between myosin and C-protein as a 160,000 or 170,000 dalton component as reported by Goll et al. (1977) and Trinick and Lowey (1976) respectively. Due to its high content of aspartic and glutamic acids, M-protein appears to be highly charged and has a strong tendency to bind myosin. According to Trinick and Lowey (1976), the circular dichroism spectra of the protein shows it to be virtually non-helical.

C-Protein

C-Protein has been found to be a minor component of the myofibrillar proteins constituting 2 to 3% by weight of the myofibril (Offer et al., 1973). The protein consists of a single polypeptide

chain with a molecular weight of 140,000 (Goll et al., 1977). According to Offer et al. (1973), the protein's intrinsic viscosity of 13.7ml/g suggests that the molecule is neither completely elongated nor globular. Its α-helical content is very low and the proline content is much higher than that of other myofibrillar proteins. Low ionic strength has also been found to favor the association of C-protein molecules.

C-Protein is presumed to be a component of the thick filaments since it strongly binds to myosin at low ionic strength. According to Offer et al. (1973), each thick filament contains about forty molecules of C-protein located in nine stripes spaced about 429A° apart in each half of the A-band.

C-Protein has no ATPase activity and does not affect the ATPase of pure myosin (Offer et al., 1973). However, the protein reduces the activity of the actin-activated myosin ATPase by about half, the inhibition being independent of Ca^{2+} as C-protein does not bind Ca^{2+} in the presence of Mg^{2+}. C-Protein has been suggested to function as a core protein. Offer et al. (1973) indicated that the existence of the core protein (C-protein) clarifies how the tails of myosin molecules pack together to form the backbone of the thick filament. The amount of C-protein in the myofibril could be enough to produce a cylindrical core sufficient to stretch from one end of the filament to the other. Such a core would possibly enhance the tail-to-tail aggregation of the myosin molecules. They further postulated that C-protein, in addition, could function to modify or control the movement of the cross-bridges and perhaps cross-link the thick filaments together to preserve the integrity of the A-band or

to stabilize the filament against the disruptive effect of tension in the cross-bridges.

C-Protein is extracted using Guba-Straub solution in the presence of Mg^{2+} and ATP (Offer et al., 1973). This solution consists of 0.3M KCl, 0.1M KH_2PO_4, 0.05M K_2HPO_4, pH 6.5, 0.1mM $MgCl_2$, and 1mM ATP. This extraction is followed by ammonium sulfate fractionation to remove actin which appears to be the major contaminant. Finally the protein is purified by DEAE column chromatography in which the C-protein elutes as an unretarded peak.

Electrophoretically, C-protein migrates on SDS-polyacrylamide gels as a single 140,000 dalton component (Goll et al., 1977) between the M-protein and α-actinin.

α-Actinin

α-Actinin was orignally discovered by Ebashi and Ebashi (1965) and has been found to constitute 2 to 3% of the myofibril by weight (Goll et al., 1977). As the amino acid composition of α-actinin resembles that of actin, Ebashi and Kodama (1966) suspected it to be denatured actin. α-Actinin has been found to show a molecular weight of 100,000 to 105,000 in all animal species studied (Goll et al., 1977). Preparations of α-actinin were reported to contain three components with sedimentation coefficients of 6S, 10S, and 25S (Ebashi and Kodama, 1966). According to them, all three components have the same physiological activity. It was however suggested that the 25S component might be an artifact of preparation found in low temperature extracted α-actinin (Briskey and Fukazawa, 1971).

According to Masaki et al. (1967), Goll et al. (1967), and

Briskey and Fukazawa (1971), α-actinin is located in the Z-band. By immunochemical demonstration, Masaki et al. (1967) showed localization of the α-actinin 6S component at the Z-band. Goll et al. (1977) pointed out that the transverse structure called the Z-disc is composed of α-actinin and tropomyosin.

Functionally, α-actinin is thought to be involved in the cross-linking of actin (Briskey and Fukazawa, 1971). This is because α-actinin has associative effects, having the ability to bind to F-actin and also possessing the potential to tighten the suspension during superprecipitation. The basis of this theory was given by Ebashi and Kodama (1966) who pointed out that α-actinin enhances the superprecipitation of actomyosin. They also noted that the addition of α-actinin to F-actin suspensions, under proper conditions, resulted in gel formation. This, no doubt, relates to the cross-linking phenomenon of α-actinin as described by Briskey and Fukazawa (1971).

Purified extracts of α-actinin were made by Seraydarian et al. (1967) employing the method of Ebashi and Ebashi (1965) along with their own modifications. The crude α-actinin obtained was purified by ammonium sulfate fractionation and again precipitated with 3.3M KCl.

Electrophoretic migration on SDS-polyacrylamide gels showed it to be a 100,000 dalton component as reported by Suzuki et al. (1967) or 103,000 dalton component as indicated by Goll et al. (1977). On the gel, α-actinin band is seen just below that of C-protein.

β-Actinin

β-Actinin was discovered by Maruyama (1965) who reported that this protein inhibits network formation in F-actin and restricted the

fiber length to 1-2μ. β-Actinin has an amino acid composition similar to that of actin. Its molecular weight was given by Goll et al. (1977) as 70,000 and it was found to constitute less than 1% by weight of the myofibril.

β-Actinin is one of the minor proteins of the thin filaments (Goll et al., 1977). Its regulative activity on F-actin led Ebashi and Kodama (1966) to suggest that the protein may function in muscle development rather than in muscle contraction.

Extraction of β-actinin by Maruyama (1965) involved the preparation of myofibrils followed by the removal of myosin with a solution of KCl and potassium phosphate. Actin was extracted with a solution of KI and β-actinin was separated from actin by ammonium sulfate fractionation. Column chromatography was employed to obtain relatively pure extracts of β-actinin.

Electrophoretically, β-actinin migrates as an 80,000 dalton component on SDS-polyacrylamide gels (Hay et al., 1973).

Tropomyosin

Tropomyosin was initially discovered by Bailey (1946, 1948) and when prepared according to his procedure, the extract is referred to as Bailey tropomyosin or tropomyosin B to differentiate it from tropomyosin A (paramyosin) found in invertebrate muscles capable of prolonged tetanic contraction (Poglazov, 1966). According to Goll et al. (1977), tropomyosin has a molecular weight of 70,000 and constitutes 4 to 6% of the myofibril by weight. The protein is similar to myosin in its solubility properties, amino acid composition, and an isoelectric point of pH 5.1 (Briskey and Fukazawa, 1971). It

is also almost 100% α-helical. Tropomyosin is characterized by the formation of highly polymerized (end to end polymerization) viscous solutions at neutral pH in the absence of salts. Poglazov (1966) summarized the properties of tropomyosin as soluble in water and dilute salt solutions at pH levels outside the isoelectric zone (pH 4.5 to 6.5) and resisting denaturation by acid and oragnic solvents. Tropomyosin has no ATPase activity and has been shown to decrease in viscosity with increasing salt concentration (Bailey, 1948).

Tropomyosin is one of the minor components of the thin filaments. There have been contradictory views as to the location of tropomyosin. Huxley (1963) suggested the presence of tropomyosin at the Z-line. Such a location would explain the inherent difficulty in extracting tropomyosin-free actin although later reports indicated that tropomyosin is not a Z-line component (Stromer et al., 1969; Casper et al., 1969). However, an undisputed report by Pepe (1966) shows that this protein anchors the thin filaments to the Z-line. He also reported the presence of the protein in the I-band. Briskey and Fukazawa (1971) pointed out that tropomyosin is unique among fibrous proteins in forming true three-dimensional crystals in the I-band.

Functionally, tropomyosin along with troponin have been found to possess regulatory influence on calcium ions during muscle contraction and relaxation (Murray and Weber, 1974). They stated that when actin filaments were prepared without the presence of these two proteins, contraction was insensitive to Ca^{2+} and ATP hydrolysis progressed in an uncontrollable fashion until all of the ATP was depleted. Tropomyosin plays a vital role in muscle contraction as described by Cohen (1975). In the "on" position, tropomyosin slides

off the groove on actin thus exposing the binding site for myosin which enables myosin to bind with actin to yield actomyosin or "active rigor" formation. This is done under the influence of Ca^{2+}, mediated by troponin. However, in the "off" position or relaxation state, tropomyosin slides back to block the specific site on the actin monomer leaving no room for myosin binding thus the muscle relaxes.

Isolation of tropomyosin (Bailey, 1948) involves muscle extraction with distilled water, treatment with organic solvents (ethanol followed by ether) to remove lipids and to denature unwanted components, extraction with 1M KCl, purification of tropomyosin by repeated isoelectric precipitation and ammonium sulfate fractionation.

On SDS-polyacrylamide gel electrophoresis, tropomyosin migrates as two subunits, one at 35,000 daltons and the other at 33,000 daltons (Goll et al., 1977). However, Hay et al., (1973) reported the two tropomyosin subunits to migrate as 55,000 and 52,000 dalton components when urea was used in the sample buffer and at 36,000 daltons without urea.

Troponin

Troponin has been found to possess a molecular weight of about 72,000 and constitutes approximately 4 to 6% of the myofibril by weight (Goll et al., 1977). However, Greaser and Gergley (1971) showed that troponin has a molecular weight of 69,213 and also demonstrated that the protein contains three dissimilar subunits, each with a particular physiological role. These three subunits have been listed as troponin-T (TN-T), troponin-I (TN-I), and troponin-C (TN-C). TN-T, the largest of the three subunits, possesses

a large number of basic amino acids and has an isoelectric point of pH 8.7. TN-I has a very high isoelectric point of pH 9.3 while TN-C shows a low isoelectric point in the pH range of 4.1 to 4.4 (Goll et al., 1977).

According to Greaser and Gergley (1971), TN-T is a subunit containing the principal binding site that attaches the troponin complex to tropomyosin. TN-I, however, binds to both TN-T and TN-C and also weakly to actin (Hitchcock, 1975; Cohen, 1975). It was also reported by Hitchcock (1975) that TN-I inhibits actomyosin ATPase activity in the presence of absence of Ca^{2+}. TN-C on the other hand binds Ca^{2+} and also binds to both TN-T and TN-I in the troponin complex. As a minor component of the thin filaments the troponin complex plays a vital role in muscle contraction. In vitro biochemical experiments reported by Margossian and Cohen (1973) indicate that in the absence of Ca^{2+} (about 10^{-8}M or less so that TN-C has no Ca^{2+}), TN-T strongly binds to tropomyosin, TN-C loosely binds to TN-T and TN-I while TN-I firmly binds to actin but loosely to TN-T. This situation exists in the resting muscle where the thin filament is on "off" position that prevents myosin from interacting with actin. In the presence of Ca^{2+} (10^{-6}M or enough so that TN-C has Ca^{2+}), TN-T still binds strongly to tropomyosin, TN-C now strongly binds to TN-T and TN-I, and TN-I binds to TN-T but dissociates from actin. This situation occurs when the thin filament is on "on" position that permits actin and myosin interaction due to the exposure of the myosin binding sites on actin.

Troponin has been described as a globular protein found in the I-band (Pepe, 1966). Extraction of troponin is accomplished using

Guba-Straub solution and employing the method outlined by Briskey and Fukazawa (1971). The crude extract is purified by ammonium sulfate fractionation, dialyzed and centrifuged to remove flocculated materials.

Electrophoretically, the three subunits of troponin migrate on SDS-polyacrylamide gels as described by Goll et al. (1977) and Hay et al. (1973) as follows: TN-T migrates as a 30,500 dalton component, TN-I as 20,800 while the band of TN-C shows as a 17,800 dalton component.

Pressurization of Meat

The application of hydrostatic pressure and its influence on biological systems have been studied since the later part of the nineteenth century. Early laboratory experiments on the biological effects of high hydrostatic pressures were triggered by the dredging expedition of the "Talisman" (1882-1883) which was credited with the discovery of organisms that lived in depths of the sea at 6000 meters below the surface where pressure amounted to approximately 8,820 psi (Johnson et al., 1954: Johnson and Eyring, 1970). At this point, it may be necessary to explain that hydrostatic pressure is uniformly exerted in all directions in sharp contrast to pressures of deformation. According to Johnson and Eyring (1970), pressures of 1 to 600 atmospheres for only a few minutes either retarded or accelerated certain biological processes but further increases in pressure with prolonged holding times caused death of the organisms. This is indicative of the response of living organisms to the effects of moderate hydrostatic pressure, some of the changes in activity

being reversible while others are not. Thus it was no surprise that Bridgeman (1914) reported pressures in the order of 5,000 to 15,000 atmospheres coagulated egg albumin. As a followup to these studies, technical advances since 1900 have permitted the application of hydrostatic pressures up to 100,000 atmospheres (Johnson et al., 1954)

In hydrostatic pressurization experiments, Johnson et al. (1954) always detected a change in volume. According to Le Chatelier's principle, application of pressure favors reactions that result in a volume decrease of a system but retards those that result in volume increase. Changes in the degree of ionization, hydrogen and hydrophobic bonding are frequently associated with a change in volume (Johnson et al., 1954). According to Johnson and Eyring (1970), the ionization of water is greater under pressure and some change in water structure also occurs when pressure is applied.

Changes in pH of buffered solutions due to application of pressure were discussed by Johnson et al. (1954). They reported that a phosphate buffer of pH 7.0 would become about 0.4 pH unit more acid when subjected to a pressure of 10,000 psi. It is known that a change in pH affects both the amount of zwitterion formation and the strength of hydrogen bonds. Since hydrogen bonds function to keep native proteins folded in specific configuration, changes in pH will influence the process of unfolding.

The mechanisms of change in volume through which pressurization could affect the stability of proteins were summarized by Johnson et al. (1954). They include the effect of pressurization on ionization or zwitterion formation, the process of protein unfolding following the weakening of hydrogen bonds, the solvent structure,

and the degree of ionization of the buffer system.

In meat research, several investigators have employed hydrostatic pressures of 103.5 meganewtons/M^2 (approximately 1000 atmospheres or 15,000 psi) for 1 or more minutes to prerigor beef and lamb muscles and their homogenates (Macfarlane, 1973; Macfarlane and Mckenzie, 1976; Bouton et al., 1980; Kennick et al., 1980; Elgasim, 1980). Several changes were noted to take place when pressure was applied to meat or its homogenate. The parameters involved in these changes encompass pH, water-holding capacity (WHC), protein solubilization, contraction state, rate of glycolysis, and meat color. According to Macfarlane (1973), Macfarlane and Mckenzie (1976), and Kennick et al., (1980), hydrostatic pressurization resulted in a sharp pH decrease in muscle tissues. The pH change was dependent on the amount of pressure, temperature, and duration of pressurization (Macfarlane, 1973). Since postmortem glycolysis was accelerated in muscles exposed to pressurization, Macfarlane (1973) stated that when prerigor muscle was pressurized at 15000 psi for 2 to 4 min at 25 to 35°C, glycolysis was virtually complete shortly after pressurization. This is consistent with the results of Kennick et al. (1980) who found little difference in pH between a pressurized muscle at 1 hr of storage and the same at 24 hr of storage. Myofilament damage was also reported by Macfarlane (1973) as resulting from hydrostatic pressurization. Electron microscopy of pressurized muscle sections revealed extensive disruption of myofilaments.

Macfarlane (1973) reported that the contraction state of muscle was definitely altered by pressurization. Agreeing with Ikkai and Ooi (1966), he remarked that pressurization resulted in

severe contraction of muscle leading to the myosin filaments being forced into Z-discs thus culminating in the breakdown of the myofibrillar structure.

In respect to the solubilization of proteins, pressurization was found to increase the yield of myofibrillar proteins in the supernatant three-fold over that obtained from non-pressurized samples of the same ionic strength (Macfarlane, 1974; Macfarlane and Mckenzie, 1976). Protein solubilization as influenced by pressure appears to be pH dependent. According to Macfarlane and Mckenzie (1976), pressure resulted in an increase in protein solubility as the pH increased above 5.8 although a plateau occured in the protein solubility curve from pH 6.5 to 9.0. This, they thought, was a function of the imidazole group of histidine residues. If imidazole groups are involved in the pressure-induced solubilization of myofibrillar proteins, they reasoned, then the bonds associated with histidine moieties would aid in maintaining myofibril structure at pH levels directly above the isoelectric region.

The WHC of meat proteins is influenced by pressurization. Bouton et al. (1971) reported a linear increase in WHC with increasing pH when pressurized meat was cooked at 60°C. Macfarlane (1974) showed that pressurization to 15,000 psi increased the WHC of meat homogenate but further increases in pressure resulted in WHC decrease.

Color has been shown to be affected by pressurization. The action of pressure on the behavior of chromatophores has been elucidated by Marshland (1944) and Johnson et al. (1954). They stated that the effect of pressure on chromatophores is analagous to the process of expansion and contraction as seen in amoeba under

pressure. Marshland (1944) however indicated that the expansion of pigment granules occurs on application of hydrostatic pressure and seems to reach an equilibrium value within roughly a minute of pressure application. However, he stated that pressure above 5000 psi with more than 20 min compression time did not yield samples with the same color equilibrium as those with lower holding times.

Depolymerization or dissociation of protein molecules by hydrostatic pressurization has been reported by certain investigators. Turbidity experiments on actomyosin systems reported by Ikkai and Ooi (1969) showed that in the absence of ATP and on the application of pressure, turbidity of the solutions decreased markedly. This was indicative of change in the state of molecules under pressure, most likely the dissociation of actomyosin to actin and myosin plus conformational changes in each of these molecules. Pressure has also been found to transform F-actin to G-actin. Ikkai and Ooi (1966) inferred that pressure of about 10,000 psi for few minutes caused modification of F-actin structure, depolymerized it to G-actin, and would possibly denature it if higher pressures were applied. F-actin generally has a larger volume than G-actin and on depolymerization, the molar volume of actin decreases. This is in keeping with Le Chatelier's principle which assumes that any change in state caused by pressure occurs in the direction where molar volume is decreased.

The duration of pressurization, temperature, and nature of salt and its concentration also influenced the solubilization of myofibrillar proteins. According to Macfarlane and McKenzie (1976), when the duration of pressurization was short (about 3 min) more protein was solubilized in samples pressurized at 30°C than those at

0°C. When the duration of pressurization was increased (about 15 min), there was a marked increase in solubilization from the samples at 0°C.

With respect to salts (potassium chloride, potassium iodide, sodium chloride, and sodium acetate), pressurization was found to promote protein solubilization using each of the salts but the characteristics of the pressure-solubilization effects varied (Macfarlane and Mckenzie, 1976). As a result of pressurization, sodium acetate solubilized additional myofibrillar proteins but it remained virtually constant over the wide range of salt concentrations (0.0 to 0.5M) studied. Using potassium chloride and sodium chloride, they obtained identical solubility patterns. Small additions of these salts ($<$0.1M) showed an initial increase in protein solubilization in both pressurized and nonpressurized samples. Above 0.2M concentration of these salts, a marked increase in protein solubilized in the pressurized samples was apparent. An identical situation was obtained with the use of potassium iodide where a dramatic increase in protein solubilization occured in pressurized samples even with very small ($\leq$0.05M) additions of the salt (Macfarlane and Mckenzie, 1976).

EXPERIMENTAL PROCEDURE

The research conducted in this study was carried out in three phases. These phases including sample preparation and isolation methods are described in detail below.

A comprehensive outline of the experimental procedure is presented in Figure 1. Unless otherwise stated, all media were constituted using distilled-deionized water and reagent grade chemicals. Buffers and pH adjustments were made at room temperature and the buffers stored at 4°C.

Phase 1

This phase dealt with the pressurization of small sections of beef semitendinosus and longissimus dorsi muscles prior to the extraction of myofibrils. These muscles were obtained from beef animals slaughtered at Oregon State University Meat Science Laboratory within 1 hr of exsanguination.

Meat pressurization was accomplished using the method described by Elgasim (1980). Approximately 1 lb sections of muscles were vacuum-sealed in Cry-O-Vac bags and transferred to a pressure chamber about 10.2 cm in diameter and 30.5 cm long. The chamber was filled with warm water (about 40°C) then securely closed and a pressure of 103.5 MNm^{-2} (15,000 psi) was applied for 2 min at about 40°C simulating the body temperature of the live animal. The control

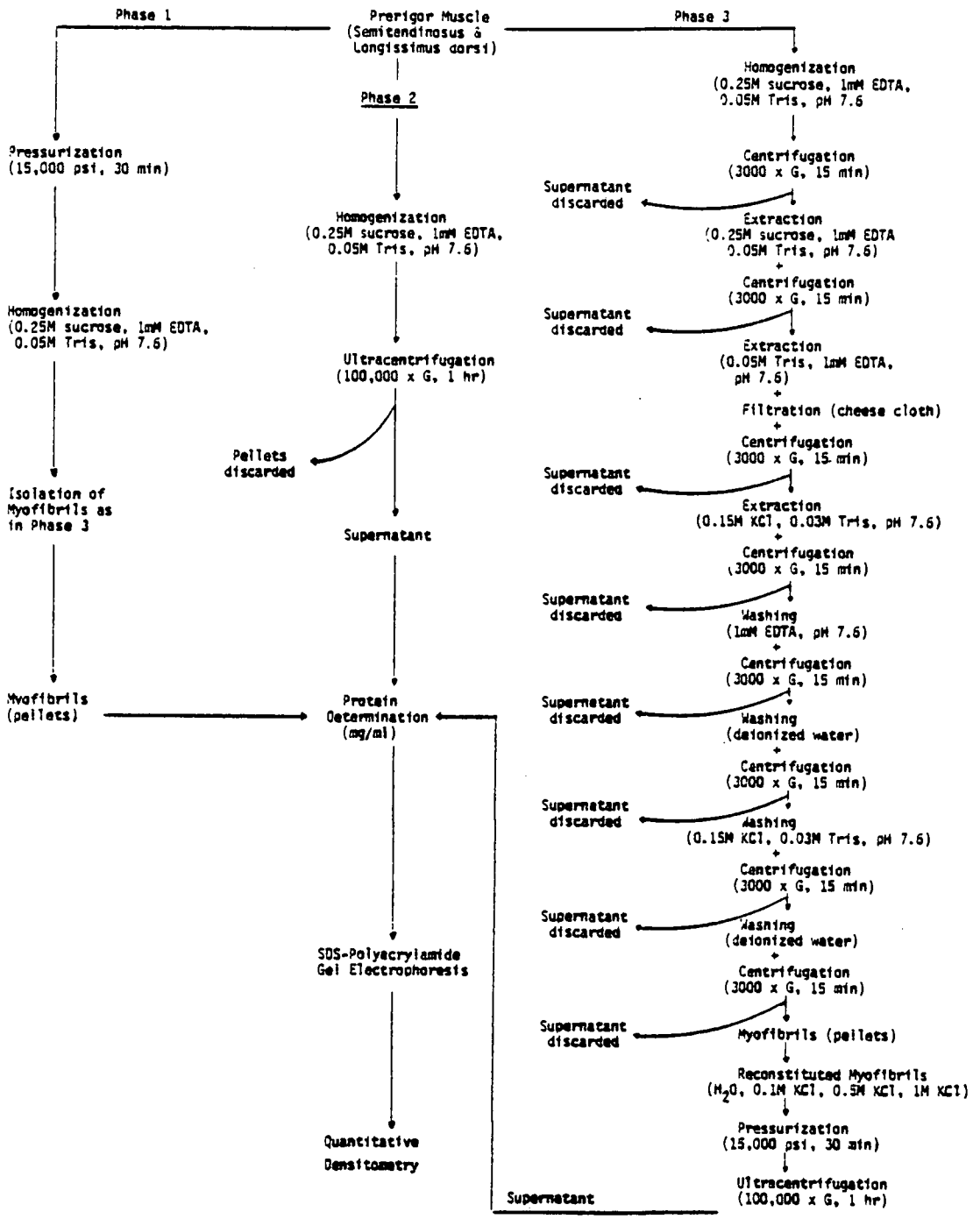

Figure 1. Outline of experimental procedure

portions were also vacuum-sealed without pressurization. Pressurized muscles and their respective controls were then stored at 4°C until the intact myofibrils were isolated from these samples. Method of myofibril isolation is described in detail in phase 3.

Phase 2

This phase covers the pressurization of muscle homogenates. Approximately 5 g portions of prerigor beef semitendinosus and longissimus dorsi muscles were introduced into large test tubes containing 3 volumes of the extracting solution (0.25M sucrose, 1mM EDTA, 0.05M Tris, pH 7.6).

Homogenization was carried out using a Tissumizer blender (Tekmar). Sample suspension was accomplished by applying three separate bursts each of 20-sec duration with a 40-sec interval between bursts. Two additional volumes of the extracting solution were used to wash down the rod of the Tissumizer and the sides of the test tubes to yield a total of 5 volumes. Homogenates were then thoroughly mixed and let stand 30 min at 4°C prior to pressurization. Equal volumes of the homogenates were set aside as controls. Fractions to be pressurized were transferred to 35 ml Monoject hypodermic disposable syringes bearing sealed end caps. Before pressurization, care was taken to eliminate air pockets from the syringes.

Pressurization of the homogenates was carried out in accordance with the method discussed by Morita (1970). The pressure cylinder employed was machined to fit a two-way through valve (Aminco Super-pressure Catalog 466A, No. 44 - 13106). The stoppered syringes bearing the homogenates were then placed in pressure cylinders to which

water at room temperature had been added. Additional water was added to fill the cylinders completely. The cylinder tops were then carefully secured by hand. The pressure line was connected to the pump assembly and the right hand valve closed. Homogenates were then pressurized by delivering a pressure of 15,000 psi at ambient temperature and the two-way through pressure valve was securely closed. The two-way through valve assembly of the pressure cylinders permitted the pressure to be maintained after the application of pressure to the system. The pressure cylinders were disconnected from the system and allowed to stand 30 min after which the pressure was released and samples taken for investigative work.

Phase 3

The third phase of study involved the isolation of intact myofibrils from prerigor beef semitendinosus and longissimus dorsi muscles followed by pressurization of the isolated myofibrils.

Myofibrils were isolated from prerigor muscles using the method described by Goll and Robson (1967) with slight modification. The 1 hr standing period in the first and second washing steps was substituted by equilibration of the samples at 4°C for 30 min during the first wash and 15 min at the same termperature during each of the 6 subsequent washings. Sodium azide (1mM), a microbial inhibitor, was added to the final wash solution to retard myofibrillar proteolysis. Sedimentation was carried out at 3000 x G for 15 min at 2°C rather than at 2500 x G for 10 min in order to obtain pellets that would adhere more firmly to bottoms of the centrifuge tubes to permit decantation of supernatant without losing portions of the pellets.

Homogenization of 5 g portions of scissor-minced prerigor muscle was accomplished as described in phase 2. The homogenates were allowed to stand 30 min at 4°C prior to sedimentation. Centrifugation was carried out at 3000 x G for 15 min at 2°C after which the supernatant was decanted and the pellets resuspended in the first extracting solution (0.2M sucrose, 1mM EDTA, 0.05M Tris, pH 7.6) and allowed to stand 15 min at 4°C. The samples were again centrifuged at 3000 x G for 15 min and the supernatant again discarded. The pellets were then suspended in the second extracting solution (1mM EDTA, 0.05M Tris, pH 7.6) and again allowed to equilibrate at 4°C for 15 min. The suspension was passed through two layers of cheese cloth to remove connective tissue. The centrifuge tubes and cheese cloth were rinsed with additional volumes of the extracting solution to recover as much of the myofibrils as possible. The ultimate amount of the extracting solution used however was 5 volumes. Sedimentation was again carried out at 3000 x G for 15 min at 2°C and the supernatant discarded. These pellets were suspended in 5 volumes of the third extracting solution (0.15M KCl, 0.03M Tris, pH 7.6) and left to equilibrate for 15 min at 4°C. The samples were again centrifuged at 3000 x G for 15 min at 2°C and the supernatant discarded. The crude myofibrils were finally washed successively with (1) 1mM EDTA, pH 7.6, (2) distilled-deionized water, and (3) 0.15M KCl, 0.03M Tris, pH 7.6. For each step, 5 volumes of solution was used in suspending the pellets and the preparation allowed to stand 15 min at 4°C prior to centrifugation. Sedimentation was carried out at 3000 x G for 15 min at 2°C in each step. Following the final washing with 0.15M KCl-0.03M Tris, the

myofibrils were washed with 5 volumes of deionized water to remove KCl. The resultant pellets were finally suspended in 5 volumes of deionized water. After thorough mixing, the myofibrils were divided into two equal fractions, one to serve as control and the other for pressurization treatment. Both fractions were then sedimented at 3000 x G for 15 min at 2°C and the supernatants discarded. The recovered pellets were stored at 4°C for use in subsequent pressurization work.

Fractions to be pressurized were resuspended in each of the following solutions: deionized water, 0.1M KCl, 0.5M KCl, and 1.0M KCl. The suspensions were carefully transferred to the 35 ml disposable syringes and pressurized at 15,000 psi for 30 min at ambient temperature as described in phase 2.

Analytical Procedures

Determination of pH

The measurement of pH of the samples was performed potentiometrically immediately after pressure treatment and at 24, 48, 72, and 168 hr postmortem using a Corning model 125 pH meter equipped with a probe-type combined electrode. Deep incissions were made in the muscle samples and the electronic voltmeter probe inserted in the grooves. Each muscle sample was firmly pressed around the probe until equilibration of the meter reading was attained. Measurements of pH were also taken for the control samples in the same manner as that just described for the pressurized samples.

Colorimetry

Color measurements of the muscle samples were conducted using a Hunter Model D25 P-2 Color Difference meter. The colorimeter was standardized against a white tile (No. DC 122, L = +94.02, a = -0.9, b = +1.2) and Hunter "L", "a", "b" color parameters taken in reflectance mode. The readings would take the form of ±a, ±b, and L where +a designates the degree of redness, -a for greeness, +b for yellowness, -b for blueness, and L for lightness or darkness. Color measurements were performed on the muscle samples immediately after pressurization and at 24, 48, 72, and 168 hr postmortem in order to monitor possible degradative changes. All samples were stored at 4°C.

Protein Determination

Protein contents of various extracts and preparations were estimated by the biuret method of Gornall *et al*. (1949). The reference calibration curve was produced using known amounts of bovine serum albumin with the biuret procedure. Absorbance was determined at 540 nm in a Perkin-Elmer Model 550 double beam spectrophotometer.

In addition to the biuret procedure, protein determinations were conducted with the Bio-Rad protein assay as outlined in the Bio-Rad Manual (1979). Absorbance was measured at 595 nm in the Perkin-Elmer spectrophotometer described above. Using myofibrillar pellets extracted from pressurized and unpressurized muscles, comparison was made between the two methods of protein determination to elucidate possible changes in protein properties due to pressurization.

Water-Holding Capacity

A model system similar to that described by Bouton et al. (1971) was used to determine the water-holding capacity (WHC) of myofibrils under study. Slight modifications were made by suspending the myofibrils in KCl solutions of different ionic strengths. Intact myofibrils were extracted as shown in phase 3 of Figure 1 using 40 g samples each of semitendinosus and longissimus dorsi beef muscles. The pellets so obtained were resuspended in 5 volumes of distilled-deionized water, divided into eight equal portions, and introduced into eight pre-weighed centrifuge tubes. Centrifugation was accomplished at 15,000 x G for 30 min at 2°C to spin off as much water as possible. Potassium chloride solutions, 25 ml each, of different ionic strengths (0M, 0.1M, 0.5M, and 1.0M) in duplicate were used to resuspend the pellets. They were weighed, then held in the cold room (4°C) for 1 hr after which they were stirred with a glass rod and one sample from each ionic strength was transferred to a 35 ml hypodermic syringe for pressurization. The remaining samples which served as controls received no pressure treatment. Following pressurization (15,000 psi, 30 min), the samples were returned to their respective pre-weighed centrifuge tubes and both the controls and the treated samples were spun at 15,000 x G for 1 hr at 2°C. The supernatants were decanted and weighed. The centrifuge tubes containing the pellets were also weighed and the total water (moisture) content and expressed juice computed. Water-holding capacity, defined as the fraction of the total moisture content remaining after deducting the total juice loss (Bouton et al., 1971), was then calculated using the equation below:

$$WHC = 1 - EJ/TW$$

where EJ stands for the expressed juice and TW designates the total moisture content. The fractions were then converted to percentages and differences between the controls and pressurized myofibrils calculated.

Polyacrylamide Gel Electrophoresis

Subjective and quantitative estimation of myofibrillar proteins in the three phases of study were carried out using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The sampling procedure and methodology applied were similar to that described by Laemmli (1970) with slight modifications. Extracts of phase 1 (Figure 1) were subjected to 24 hr dialysis against 10mM of ammonium bicarbonate prior to electrophoresis. The homogenates of phase 2 and the myofibrils of phase 3 (Figure 1) were centrifuged at 100,000 x G at 2°C for 1 hr and the resulting supernatants electrophoresed. The slab gel electrophoretic technique was used and the stock solutions prepared included acrylamide: N, N'-bis-methylene acrylamide (30:0.8); 1.5M Tris-HCl, pH 8.8; 0.5M Tris-HCl, pH 6.8; and 10% sodium dodecyl sulfate (SDS).

The acrylamide:bis solution was prepared by dissolving 30 g of acrylamide with 0.8 g of N,N'-bis-methylene acrylamide in deionized water to achieve a total volume of 100 ml. The stock solution was then filtered through Whatman No. 1 filter paper and stored in a dark bottle at 4°C. To make the 1.5M Tris-HCl buffer, 18.16 g of Tris (hydroxymethyl) aminomethane was dissolved in 80 ml of deionized water. The pH was adjusted to 8.8 with 1N HCl and the

final volume made to 100 ml with deionized water. The 0.5M Tris-HCl was prepared by dissolving 6 g of Tris (hydroxymethyl) aminiomethane in deionized water, adjusting the pH to 6.8 and adding deionized water to a final volume of 100 ml.

Different percentages of separating and stacking gels were prepared using stock solutions. High percent separating gels (15%) were used to study low molecular proteins, low percent gels (7.5%) were employed to separate high molecular weight proteins while intermediate percent gels (10%, 12.5%) were used to elucidate the entire myofibrillar protein spectrum. A 30 ml volume of 10% separating gel was made by mixing 10 ml acrylamide:bis, 7.5 ml of 1.5M Tris-HCl, pH 8.8, 12 ml deionized water, 0.3 ml of 10% SDS, 150 µl of fresh ammonium persulfate, and 12 µl of N,N,N'-tetra-methylethylenediamine (TEMED). The amounts of reagents used for the 15 and 12.5% separating gels were essentially the same as in 10% except for the amounts of acrylamide:bis and deionized water. The 15% gels required 15.0 ml of acrylamide:bis and 7.0 ml of deionized water while 12.5% gels had 12.5 ml of acrylamide:bis and 9.5 ml of deionized water. Stacking gels in the order of 5%, 4%, and 3% were made to go with the separating gels. A 15% separating gel required a 5 or 4% stacking gel, 12.5% gel went with 4% stacking gel while the 10 and 7.5% separating gels were run with 3% stacking gels. A 10 ml solution of 3% stacking gel was prepared by mixing 1.0 ml acrylamide:bis, 2.5 ml of 0.5M Tris-HCl, pH 6.8, 6.3 ml of deionized water, 150 µl fresh ammonium persulfate, and 8 µl of TEMED. The 4 and 5% stacking gels were made using the same amounts of media except for deionized water and acrylamide:bis. The 4% gels required

1.33 ml of acrylamide:bis and 5.9 ml of deionized water while 5% gels had 1.67 ml acrylamide:bis and 5.58 ml of deionized water.

The sample buffer was used to solubilize the samples prior to electrophoresis. An 8 ml volume of sample buffer was made by mixing 1.0 ml of 0.5M Tris-HCl, pH 6.8, 1.0 ml glycerol, 1.0 ml 10%(w/v) SDS, 0.1 ml β-mercaptoethanol, and 0.2 ml of 0.05% (w/v) bromophenol blue. Urea was added to achieve an 8M concentration. About 2.0 ml deionized water was added to bring the final volume to 8 ml.

Electrode buffer, pH 8.3, was prepared using 6 g of Tris base, 28.8 g of glycine and 10 ml of 10% SDS. The reagents were dissolved in large volume of water, pH adjusted to 8.3 and the solution brought up to 1 liter using deionized water.

The mini-slab gel electrophoretic equipment used in this study was the type described by Ide (1980). Glass plates measuring 15.3 cm long, 10.4 cm wide and 0.15 cm thick were used in preparing sandwiches to receive the running and stacking gels. Spacers of 0.8 mm or 1.0 mm thickness were carefully indented approximately 3 mm from the edge of the sides and bottom of the plates to restrict the gel solutions within the confines of the slab. The glass plates were then clipped together to hold the plates and spacers in proper position. A 2% molten agarose solution prepared with the electrode buffer, was poured around the outside of the three sides of the plates containing the spacers with a Pasteur pipet. The agarose was allowed to set to seal the sandwich for pouring of the gel.

After deaeration, the appropriate running gel (7.5%, 10%,

12.5%, or 15%) was prepared as described earlier and poured to about 5/6th capacity of the sandwich. The top was layered with about 2 ml of butanol and the gel polymerized in about 30 min. Following polymerization, the butanol layer was removed, the top of the gel rinsed with deionized water and dried with tissue or filter paper. The appropriate comb (1.0 mm comb for 1.0 mm spacers) was installed and the stacking gel poured. On polymerization of the stacking gel, the clips were removed along with the agarose and the spacer at the bottom of the plates. The sandwich was transferred to the slot of the upper reservoir of the mini-slab unit and the slot sealed with 2% agarose solution. The electrode buffer was poured into both the lower and upper reservoir making sure that both the top and bottom of the gels were submerged in buffer. The comb was then removed to expose the wells in the stacking gel where samples would be introduced. Samples and molecular weight standards were then injected into the wells using µl syringes. Samples were applied so that each well did not contain more than 30 µg protein as determined earlier by the biuret procedure. Electrophoresis was carried out at 125 to 200 constant voltage using a DC power supply.

After completion of the run, the gel was carefully removed from the plates. Staining of the gel was accomplished by immersing it in a Coomassie Brilliant Blue G-250 solution for 1 hr over a platform shaker. This staining solution was prepared by dissolving 2.5 g of Coomassie G-250 in 450 ml absolute methanol, 90 ml glacial acetic acid, and 460 ml deionized water. The staining solution was filtered through Whatman No. 1 filter paper prior to use. The gel was then washed with deionized water and transferred to a destaining

solution consisting of 7.5% acetic acid, 5.0% methanol, and 87.5% water. Destaining was carried out for 48 hr over a platform shaker which provided continuous agitation. The destaining solution was changed periodically during the destaining process. When destaining was complete, the gel was dried under the hood on a drying stand overnight using a dialysis sheet soaked in the destaining solution.

Molecular Weight Determination and Identification of Fractions

The electrophoretic procedure previously described was used to determine the molecular weights of the myofibrillar proteins under study and to identify those fractions affected by pressurization. A calibration curve of relative mobility versus log-molecular weight was prepared after each run using high and low molecular weight protein standards obtained from Bio-Rad Laboratories (Richmond, CA). The high molecular standards contained myosin (200,000), β-galactosidase (140,000), phosphorylase B (110,000), bovine serum albumin (75,000), and ovalbumin (45,000). The low molecular weight protein standards contained phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase (30,000), soy trypsin inhibitor (20,000), and lysozyme (15,000).

These protein standards were run under exactly the same electrophoretic conditions as were the unknown myofibrillar proteins. The total volume of the standards applied to each slab was in the order of 10 to 15 µl of 1:20 dilution while the myofibrillar protein preparation was applied at a level of 18 to 30 µg of protein per column. Relative mobility was then calculted as the ratio of the distance of migration of a given protein from the origin to that of

the known protein from the same origin. Using the high molecular weight protein standards, relative mobilities were calculated on the basis of bovine serum albumin while ovalbumin served as the base for the low molecular weight standards. The relative mobilities of the unknown myofibrillar proteins were then interpolated on the standard curve to gain their approximate molecular weights. Comparison with literature values (Goll et al., 1977) was made to tentatively identify the proteins.

Quantitative Densitometry

The dried slab gels were scanned with a Quick Scan Densitometer Model 1020 (Helena Laboratories, Beaumont, TX) equipped with an integrator as described in the Quick Scan Operators Manual (1973). Gels were enclosed in two glass plates measuring 15.3 cm long and 10.4 cm wide and placed on the carrier of the densitometer so that the sample migration distance would be at least 7.5 mm less than the scanning distance. The sample was also positioned to allow at least 4 mm background material at each end of the separation and at the ends of the sample holder. Magnets were used to hold the samples in place. Scanning was accomplished using a green filter at 570 nm wavelength and an absorbance light path of setting of 1. The speed of the recorder was adjusted to slow in order to get smooth and well defined curves.

Quantitative estimations of different amounts of proteins in the electropherograms produced by the densitometer were carried out by counting the lines of the integrator graph under the curves and expressing them as a fraction of the total proteins in the

electropherogram. Each complete up and down movement of the integrator pen represents 10 integration units. Thus the number of integration units under a curve divided by the total number of integration units for the whole spectrum multiplied by 100 gave the relative percent value of the protein fraction under the curve. The protein fractions were then expressed as milligrams percent of the total protein by multiplying the relative percent values by the total protein concentration in milligrams percent as determined by the biuret protein assay.

RESULTS AND DISCUSSION

During the early part of this research, investigations were carried out on prerigor beef semitendinosus and longissimus dorsi muscles alone. Known sections of these muscles were subjected to hydrostatic pressurization prior to the extraction of myofibrils. This was phase 1.

Data gained in phase 1 showed that less amounts of myofibrils were recovered from the pressure-treated muscle sections than from their controls. When the extracted myofibrils were electrophoresed using SDS-PAGE, there appeared to be no difference in protein concentrations within the electrophoretic patterns of the controls and pressure-treated samples. However, when the electrophoretograms were densitometrically traced, protein concentration was found to be less in the pressurized samples than in their controls. These findings were indicative of the fact that pressure induced the solubilization of some of the myofibrillar proteins resulting in the loss of this portion with the sarcoplasmic fraction when the supernatants were discarded in the extraction process. This invariably resulted in depletion of the amount of myofibrils recovered as pellets following an exhaustive extraction process.

Since phase 1 gave only an index of myofibrillar loss without typifying the proteins involved, pressurization of meat homogenates was undertaken in the second phase of study. It however, had its flaws. In this case, electrophoretic patterns were

contaminated with proteins of the sarcoplasmic fraction and it was virtually impossible to characterize the effect of pressure on the myofibrillar components.

To elucidate the effect of pressure on the myofibrillar fraction, it was justifiable to employ the third phase of study. Intact myofibrils, isolated from prerigor beef muscles, were subjected to pressurization, and their supernatants were analyzed electrophoretically in an attempt to detect changes in the major myofibrillar protein components.

Rate of pH Decline

Changes in muscle pH following pressurization were found to occur in both muscles studied. These effects are illustrated in Figure 2 for beef semitendinosus and longissimus dorsi muscles. Values in the figure reflect the average of 10 repeated experiments while employing a pressure of 15,000 psi.

As illustrated in Figure 2, semitendinosus muscle dropped from a pH of 6.68 to 5.84 immediately after pressurization. This was a pressure-induced decline of 0.84 pH unit. A similar decline is shown in Figure 2 for longissimus dorsi muscle which dropped from pH 6.83 to 5.86 soon after pressurization indicating a pressure-induced reduction of 0.97 pH unit. These data are similar to those of Macfarlane (1973) who reported pressure-induced pH reductions of 0.93 and 0.71 units for semitendinosus and longissimus dorsi beef muscles respectively. Kennick *et al.* (1980) also showed a pressure-induced pH drop for beef semitendinsus muscle to be 0.73 while that of longissimus dorsi muscle was 0.81

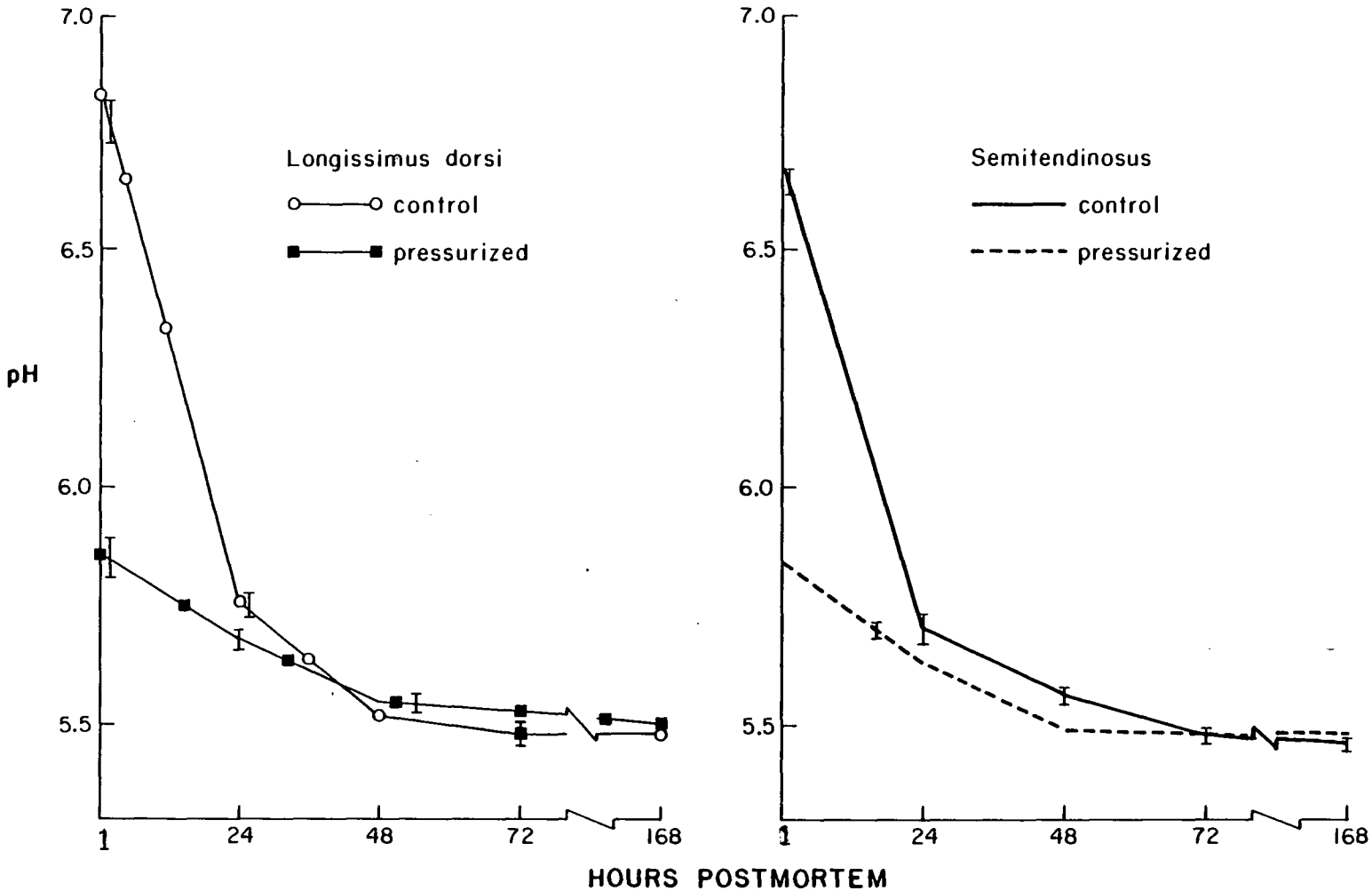

Figure 2. Effect of pressurization on pH of beef semitendinosus and longissimus dorsi muscles stored at 4°C. (Standard deviation shown by vertical lines.)

On postmortem storage at 4°C, pH of the controls dropped sharply during the first 24 hr while the pressurized muscle showed a gradual pattern of decline. By the end of 24 hr, the non-pressurized semitendinosus and longissimus dorsi muscles had dropped 0.98 and 1.08 pH units respectively. Pressurized semitendinosus muscle dropped only 0.21 pH unit and pressure-treated longissimus dorsi muscle dropped 0.18 pH unit within this same time frame. At 24 hr postmortem, the difference in pH between the control and pressurized samples was 0.07 pH unit for both semitendinosus and longissimus dorsi muscles. Similar results were obtained by Macfarlane (1973) where the difference in pH between the pressurized and non-pressurized muscle samples was 0.05 for semitendinosus and 0.17 for longissimus dorsi muscle. The trend is identical with the results of Kennick et al. (1980) who gave the difference in pH for semitendinosus muscle as 0.1 and longissimus dorsi muscle as 0.01 after 24 hr postmortem storage following pressurization.

On prolonged storage at 4°C, there were no marked differences in the pH of both the pressurized and unpressurized muscles. However, storage of up to 168 hr (7 days) showed the pressurized muscles reaching their ultimate pH in 48 hr while the non-pressurized muscles attained the lowest pH after 72 hr with the result that the non-pressurized muscles had a slightly lower ultimate pH than the pressurized. For the semitendinosus muscle, Figure 2, the point of intersection where both pressurized and non-pressurized muscles showed virtually the same pH is around 72 hr postmortem. With the longissimus dorsi muscle, this period is shorter resulting in the pressurized and non-pressurized samples attaining the same pH about

40 hr postmortem. Nonetheless, the ultimate pH for the pressurized semitendinosus muscle was 5.48 while that of the unpressurized was 5.46, an ultimate pH difference of 0.02 unit. Longissimus dorsi muscle showed an ultimate pH of 5.50 for the pressurized muscle and 5.48 for the non-pressurized, also an ultimate pH difference of 0.02 unit. Both muscles maintained the same ultimate pH up till the ultimate postmortem storage time of 168 hr.

Since the pH of postmortem muscle is an index of the lactic acid content of the muscle, variation in pH is a relative measure of the rate of postmortem glycolysis in muscle. The sharp drop in pH immediately after pressurization is indicative of enhancement of postmortem glycolysis by hydrostatic pressurization. This is consistent with the inferences made by Macfarlane (1973) that pressurization accelerates postmortem glycolysis. The failure of pressurized muscles to show appreciable differences in pH on postmortem storage tends to give credibility to his belief that glycolysis is virtually complete shortly after pressurization.

The mechanism with which pressurization achieves this massive pH decline is obviously still unresolved. However, the theory advanced by Johnson et al. (1954) on the effect of pressure on buffered solutions appears to offer some help. Working with phosphate buffer, they reported a decline in pH of 0.4 unit on application of a pressure of 10,000 psi. According to them, zwitterion formation and the strength of hydrogen bonding were both affected by pressure. It may be possible, therefore, that hydrostatic pressurization affects the buffer system of muscle tissue through charge modification and bond weakening thereby facilitating the process

of glycogenolysis while at the same time creating optimum activity conditions for the glycolytic enzymes. According to Ashmore et al. (1972), during abnormal preslaughter conditions there is activation of glycogen phosphorylase resulting in rapid depletion of muscle glycogen. In prerigor muscle, activation of this enzyme by hydrostatic pressurization would result in rapid turnover of glucose-1-phosphate moieties from muscle glycogen. Through pressure-induced hyperactivity of the enzymes of the glycolytic sequence, lactic acid would be rapidly produced resulting in sharp drop in pH.

Colorimetric Changes

Two distinct color patterns were evident between the pressurized and non-pressurized muscle samples. The color attained by the pressurized muscle was bright red much analogous to the desirable nitrosomyoglobin color of cured meat while the non-pressurized muscle possessed a dull red color. Figures 3 and 4 show the obvious differences in color patterns between pressurized and non-pressurized semitendinosus and longissimus dorsi muscles as manifested by their Hunter "L" and "a" values respectively. Hunter "L" value denotes the level of brightness of the meat while Hunter "a" value is indicative of the amount of meat redness.

Prior to pressurization, the Hunter "L" color values for semitendinosus and longissimus dorsi muscles were +20.4 and +20.0 respectively. As shown in Figure 3, these values increased to +28.8 and +27.0 for the semitendinosus and longissimus dorsi muscles respectively. Thus pressurization enhances meat brightness with a

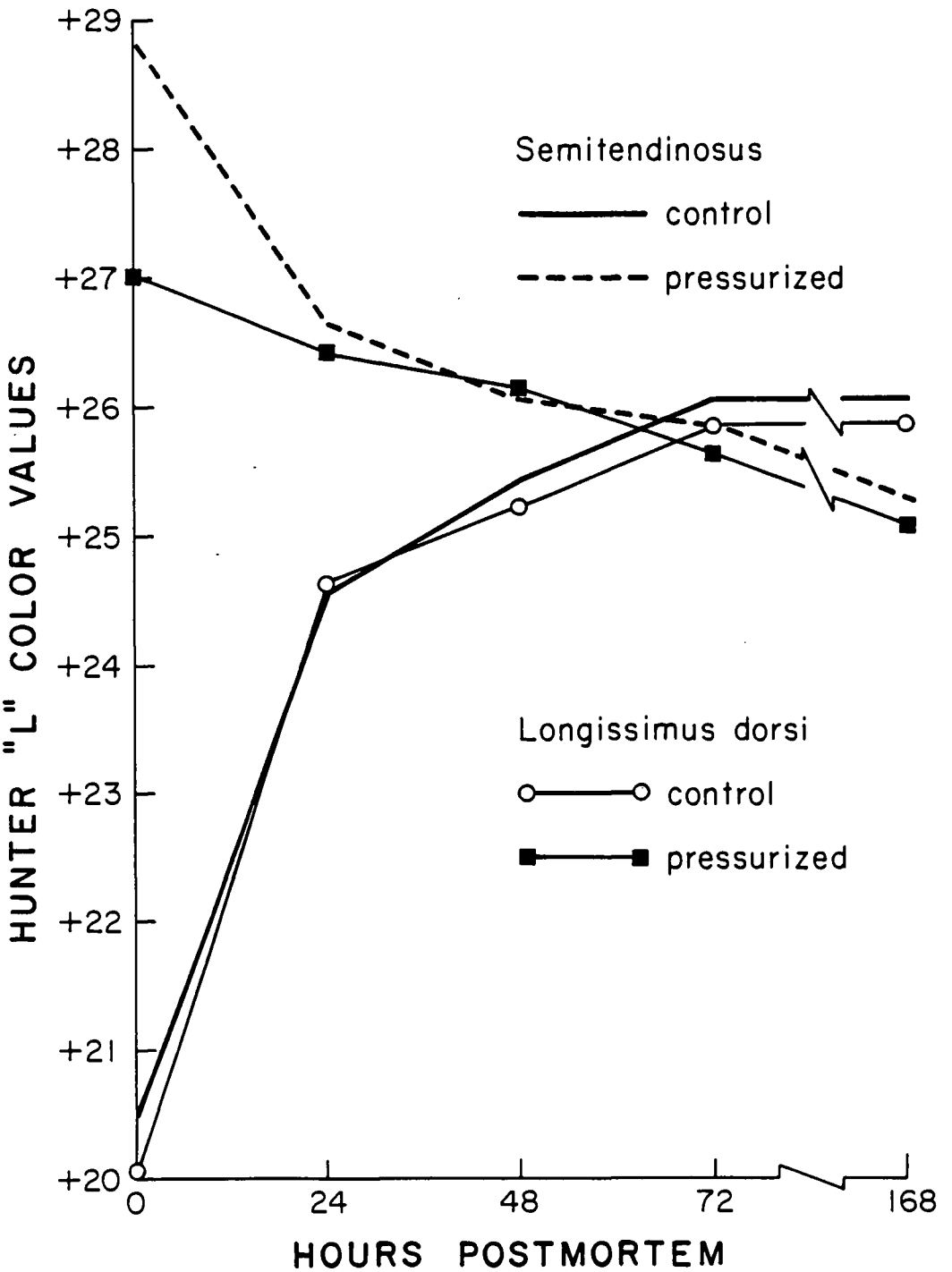

Figure 3. Effect of pressurization on Hunter "L" Color values of beef semitendinosus and longissimus dorsi muscles stored for 7 days at 4°C.

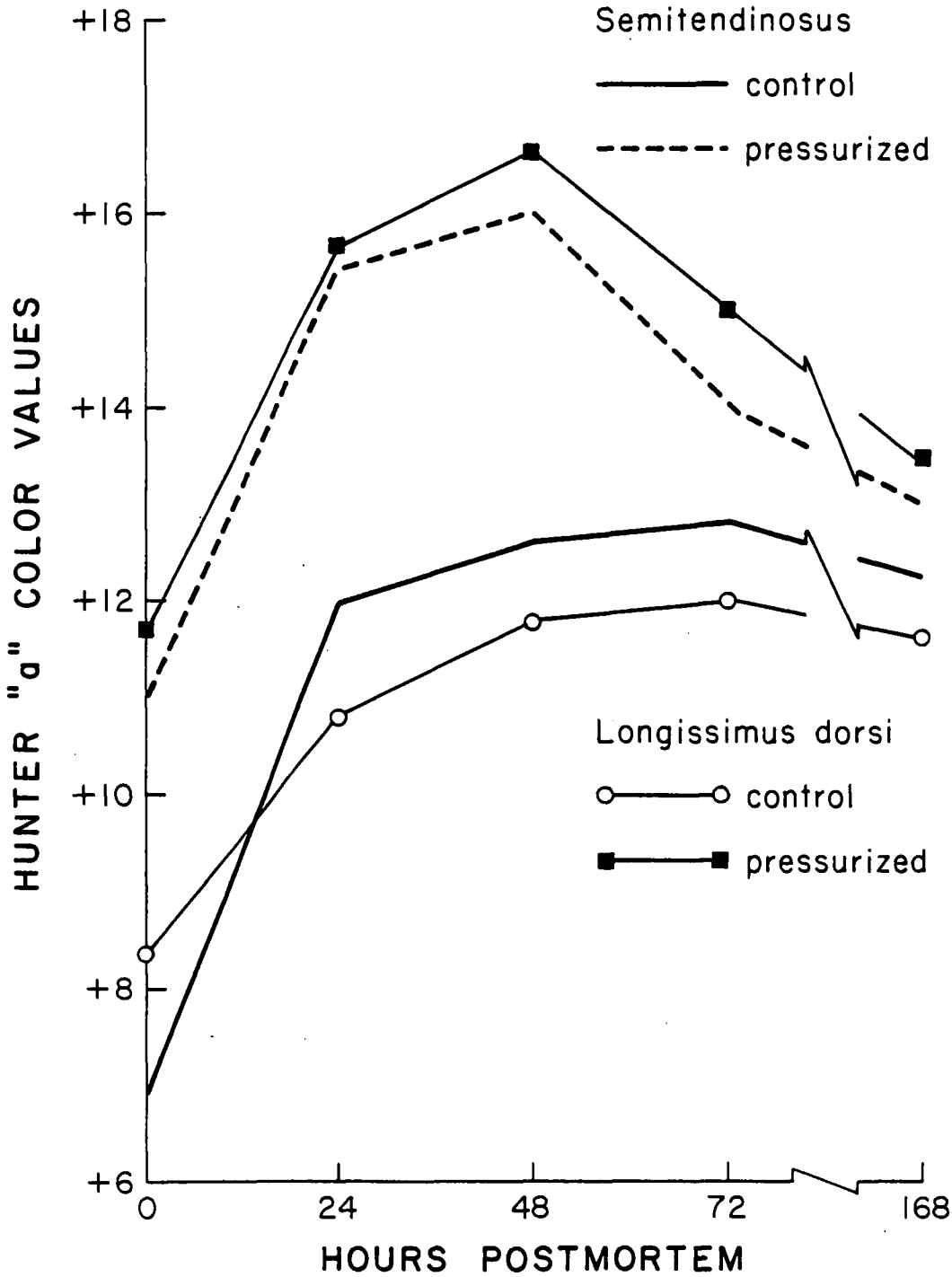

Figure 4. Effect of pressurization on Hunter "a" Color values of beef semitendinosus and longissimus dorsi muscles stored for 7 days at 4°C.

net increase of 8.4 Hunter "L" value units for semitendinosus muscle and 7.0 for longissimus dorsi muscle. On storage at 4°C, however, the brightness ("L" value) of the pressurized muscles showed a sharp drop occurring in the first 24 hr, followed by a gradual decrease for the rest of the storage period. A reverse pattern was obtained for the non-pressurized muscles which showed a sharp increase in "L" value during the first 24 hr followed by a gradual increase up to 72 hr and then remaining constant for the rest of the storage period. Though the brightness patterns of the muscles (pressurized and control) varied, they showed a similar level of brightness ("L" value) at approximately 68 hr postmortem (Figure 3). However, after 168 hr storage, the pressurized muscle samples continued to show a gradual decrease in "L" value while the non-pressurized samples maintained the same "L" value attained at 72 hr postmortem storage. At 68 hr postmortem storage, the trend slightly reversed itself with the controls showing slightly more brightness than the pressurized samples. Pressurized semitendinosus muscle was 0.85 "L" value unit less than its control while pressurized longissimus dorsi muscle showed 0.80 "L" value unit less than its control.

Changes in the amount of redness ("a" value) of pressurized and non-pressurized semitendinosus and longissimus dorsi muscles are presented in Figure 4. Immediately after pressurization, the Hunter "a" value for the semitendinosus muscle increased 4.2 units while the longissimus dorsi muscle rose by 3.4 "a" value units. On storage both muscles (pressurized and unpressurized) showed a sharp increase in "a" value in the first 24 hr followed by a more gradual increase up to 48 hr. Blooming or maximum amount of redness was

attained at 48 hr storage time for the pressurized and at 72 hr for the controls. After 48 hr storage, the pressurized muscles started to show a sharp decline in "a" value while the control samples showed gradual decline only after 72 hr storage. At the final storage time of 168 hr, the pressurized muscles still had substantially higher degree of redness ("a" value) than the control samples. At this time, pressure-treated semitendinosus muscle was 0.8 "a" value unit higher than its control while pressurized longissimus dorsi was 1.4 units more than the non-pressurized muscle.

The increase in the amount of brightness (Hunter "L" value) and redness (Hunter "a" value) of the muscle samples due to pressurization appears to be in keeping with the reports of Marshland (1944) and Johnson et al. (1954) on the effect of pressure on chromatophores. This, however, is a bit of digression as there are no available literature reports on the influence of pressure on meat pigments. On visual perception, pigment granules (chromatophores) have been found to expand and reach a certain equilibrium brightness on the application of hydrostatic pressure (Marshland, 1944; Johnson et al., 1954). When fresh meat is initially cut, the muscle pigment (myoglobin) is purplish-red. On exposure to air, myoglobin is oxygenated to form oxymyoglobin which has a bright red color (Ockerman, 1975). From the observations in Figures 3 and 4, it is obvious that pressurization enhanced the bright red color formation in these muscles. Pressurization may expand the muscle pigments thereby increasing the perceptibility of the oxymyoglobin bright red color of the muscle both visually and objectively.

The effect of pressurization on meat color may also be

analogous to the influence of ionizing radiation on muscle heme pigments as reported by Giddings and Markakis (1972). Pressurization of vacuum packaged beef muscle could result in reduction of surface ferrimyoglobin by affecting the heme prosthetic chromophore. On cutting or exposure of the meat to air, the pressure-reduced pigment could rapidly oxygenate to attain a bright red color. On the other hand, change in meat color due to pressurization may be related to the sharp drop in pH. The development of bright red color in conjunction with a sharp drop in pH following pressurization appears to be in keeping with the report of Adams (1976). He indicated that any condition that would cause the pH to drop to approximately 5.6 would cause heme dissociation, unfolding of apoglobin, and protonation of bound oxygen leading to the development of oxymoglobin (bright red color). The mechanism by which this assumption can be attained however still remains unclear.

The decrease in brightness ("L" value) of the pressurized muscles below that of the controls after 72 hr postmortem storage at 4°C gives credence to Macfarlane's (1973) report that pressurization causes tissue damage. If the myofilaments are damaged, optimum conditions would have been created for degradative psychrotrophs which on rapid proteolysis would create color taints that would reduce the brightness and redness of the meat.

Water-Holding Capacity

Data concerning the influence of hydrostatic pressure on the WHC of semitendinosus and longissimus dorsi myofibrils solubilized in different ionic strengths are presented on Table 2. Pressurization

Table 2. Effect of pressurization on water-holding capacity (WHC) of beef semitendinosus and longissimus dorsi muscle myofibrils suspended in KCl solutions of different ionic strengths.

Sample	H_2O		0.1M KCl		0.5M KCl		1.0M KCl	
	WHC[a] (%)	Diff. (%)	WHC[a] (%)	Diff. (%)	WHC[a] (%)	Diff. (%)	WHC[a] (%)	Diff. (%)
Semitendinosus								
Control	41.4 ± 1.8		27.0 ± 1.6		42.5 ± 2.1		42.2 ± 2.5	
		28.6		12.5		39.6		45.6
Pressurized	70.0 ± 3.9		39.5 ± 2.0		82.1 ± 4.3		90.8 ± 4.6	
Longissimus dorsi								
Control	42.7 ± 1.5		28.6 ± 1.4		43.2 ± 1.8		45.1 ± 2.0	
		29.2		12.8		39.8		47.3
Pressurized	71.9 ± 2.8		41.4 ± 2.6		83.0 ± 3.7		92.4 ± 3.9	

[a] Mean ± standard deviation ; n = 7.

increased the WHC of the myofibrils at all molar concentrations of KCl studied. As defined by Hamm (1960), WHC is the ability of meat to retain its own or added water during the application of external force or treatment.

Table 2 shows that pressurized semitendinosus and longissimus dorsi muscle samples suspended in deionized water (0.0 M KCl) had 28.6% and 29.2% differences in WHC respectively. At 0.1M KCl, these differences dropped to 12.5% for semitendinosus muscle and 12.8% for longissimus dorsi muscle. Suspending the myofibrils in 0.5M KCl, the differences increased 39.6% for the semitendinosus muscle and 39.8% for the longissimus dorsi muscle. Pressurized semitendinosus and longissimus dorsi muscles suspended in 1.0M KCl showed WHC differences of 45.6% and 47.3% respectively. These data show that the WHC of myofibrils increased with increasing ionic strength or molar concentration of KCl beyond 0.1. This is consistent with the findings of Macfarlane (1974) who reported a marked increase in the WHC of meat homogenates suspended in 0.5M NaCl.

Since there are no data published on the effect of pressurization on the WHC of myofibrils suspended in solutions of different ionic strengths, it is not possible to compare these results with those of other investigators. Because of the importance of protein charge on the hydration of muscle proteins, pressure-induced zwitterion formation of proteins as described by Johnson et al. (1954) would help to elucidate the differences in WHC. If pressurization initiates zwitterion formation, the charged protein moieties would have greater affinity for water thereby resulting in increased

hydration. An increase in protein hydration invariably results in less volume of the supernatant fluid which is indicative of an increase in the WHC of the pellets. However, since the WHC difference was high at 0M KCl, dropped at 0.1M KCl, then increased at 0.5M KCl and 1.0M KCl, it appears that pH, not ionic strength alone, is involved. According to Bouton et al. (1971), on either side of the isoelectric region a linear relationship exists between the WHC and pH. Their results showed increased WHC with increasing pH. This is in agreement with the report of Hamm (1960) who stated that above the isoelectric point of meat proteins, salt caused an increase in the WHC of meat as a result of loosening of a protein structure leading to increased hydration. This is because chloride ion is considerably bound by the positively charged groups of protein while sodium ion is weakly bound to the negative charges resulting in virtual annihilation of the charges (Wismer-Pedersen, 1971). This activity serves to displace the isoelectric point toward a lower pH and increases the space between the filaments to be subsequently occupied by water molecules in the process of hydration.

Dye-Binding

Pressurization seemingly interferes with the dye-binding ability of the myofibrillar proteins. Using the biuret and the Bio-Rad protein assay techniques, marked differences in dye-binding were found between the pressurized and non-pressurized muscle samples. The biuret reaction involves the reaction of alkaline cupric ions with the peptide linkages of the proteins being assayed (Gornall et al., 1949). Bio-Rad protein assay on the other hand is

a dye-binding technique based on the ability of a protein to form a complex with Coomassie Brilliant Blue G-250.

Results of three runs in triplicate using both the biuret method and the Bio-Rad (dye-binding) technique are presented in Table 3. With the biuret procedure, myofibrillar pellets isolated from pressurized semitendinosus muscle showed a protein content of approximately 27% less than its control while the pellets from pressurized longissimus dorsi muscles decreased in protein content by about 26%. This difference was markedly greater when the dye-binding method was used. A decrease in protein content of 50% was seen for the pressurized semitendinosus muscle and 48% for the pressurized longissimus dorsi muscle using the dye-binding method. This translates into approximately a net decrease in protein detectability of 25% using the Bio-Rad dye-binding method.

Certain proteins have been found to yield exceptionally low color response to the Bio-Rad dye reagent (Bio-Rad Manual, 1979). Gelatin has been found to be one such protein. From the data in Table 3, the results of the dye-binding method for the non-pressurized muscle samples were similar to those obtained with the biuret method. Since the same samples were used in both techniques, it is apparent that pressurization interfered with or reduced the dye-binding ability of the myofibrillar proteins. Because the principle of protein dye-binding is based on complexing of acid dye with proteins through linkage with the positive charges on the proteins, it is possible that pressurization modifies the charge on proteins to give less protein-dye complexes. Macfarlane and Mckenzie (1976) indicated that pressurization affected the

Table 3. Effect of pressurization on dye-binding of myofibrillar proteins obtained from beef semitendinosus and longissimus dorsi muscles.

Sample	Biuret[a] (mg protein/ml)	Diff. (%)	Bio-Rad[a] (mg protein/ml)	Diff. (%)
Semitendinosus				
Control	14.80 ± 1.2		14.01 ± 0.8	
		27.0		50.0
Pressurized	10.81 ± 0.9		7.02 ± 0.5	
Longissimus dorsi				
Control	15.0 ± 1.0		14.53 ± 0.7	
		25.8		48.0
Pressurized	11.28 ± 0.7		7.61 ± 0.4	

[a] Mean ± standard deviation ; n = 3.

imidazole ring of histidine. If this occurs, it might account for lower dye-binding ability of myofibrils extracted from pressurized meat. Since the side chain of histidine carries a positive charge, if the ring is destroyed during pressurization, there would be less positive charges to bind with the negative charges of the acidic dye, thus lower dye-binding results would be attained.

Extractibility of Total Myofibrils

Pressurization has a tremendous effect on the yield of total myofibrils of muscle tissue pressurized prior to the extraction of myofibrils. In a three-way comparison presented in Table 4, myofibrils extracted from pressurized and unpressurized semitendinosus and longissimus dorsi muscles were subjected to oven drying, centrifugation, and pellet resuspension techniques.

When myofibrillar extracts were oven-dried to constant weight, extracts from pressurized semitendinosus muscle showed a total yield of 39.6 % less than their controls. With the pressurized longissimus dorsi muscle, the difference in yield was about 34.4% lower than the control. Using the centrifugation technique, reconstituted myofibrillar pellets from pressurized semitendinosus muscle had a yield of 28.7% less than the control. Reconstituted myofibrillar pellets from pressurized longissimus dorsi muscle showed 25.1% less myofibrils than their controls. When intact myofibrils were subjected to pressurization and their supernatants analyzed after centrifugation, the trend was reversed. The supernatants from pressurized semitendinosus myofibrils had 35.7% more protein while those from pressurized longissimus dorsi myofibrils contained

Table 4. Effect of pressurization on the yield of total myofibrils.

Sample	Oven-dried Extract[a]		Pellets[b]		Supernatant[c]	
	Dry Wt.[d] (mg)	Diff. (%)	Conc.[d] (mg/ml)	Diff. (%)	Conc.[d] (mg/ml)	Diff. (%)
Semitendinosus						
Control	200.75 ± 5.18		17.03 ± 1.21		2.37 ± 0.05	
		-39.6		-28.7		+35.7
Pressurized	121.25 ± 3.26		12.14 ± 0.92		3.22 ± 0.12	
Longissimus dorsi						
Control	212.61 ± 5.46		16.35 ± 1.03		2.85 ± 0.08	
		-34.4		-25.1		+36.8
Pressurized	139.52 ± 4.14		12.25 ± 0.84		3.92 ± 0.16	

[a] Myofibrillar extract from pressurized meat oven-dried (100°C) to constant weight.

[b] Myofibrillar extract from pressurized meat centrifuged (15,000 x G, 30 min, 2°C) and pellets reconstituted with deionized water.

[c] Aqueous supernatant from pressurized myofibrils (15,000 psi, 10 min) centrifuged (15,000 x G, 30 min, 2°C).

[d] Mean ± standard deviation ; n = 5.

36.8% more protein than their controls.

These results indicate that on the average about 30% of the myofibrillar proteins are lost in the discarded sarcoplasmic fraction when hydrostatically pressurized muscle undergoes myofibrillar extraction. The slightly lower difference % values obtained with the myofibril-pellet centrifugation technique may be due to increase in hydration by the pressurized samples (Macfarlane, 1974). In effect, pressurization increases the solubilization of myofibrillar proteins. The entire trend shown above agrees with the findings of Macfarlane and Mckenzie (1976) and Macfarlane (1974). Their reports contend that pressurization results in increased yield of salt-soluble (myofibrillar) proteins. Though Macfarlane (1974) indicated that pressurization accounted for a three-fold increase in the yield of these proteins, data gained in this study consistently showed just over 30% increase. However, since the myofibrils pressurized in this study were suspended in deionized water as opposed to his which were suspended in saline solution, the reason for the staggering difference may be very obvious. There have been several documented evidences that salt enhances the extractability of contractile proteins of the skeletal muscle (Goll et al., 1977).

The major myofibrillar proteins studied include myosin (heavy chain), M-protein, C-protein, α-actinin, β-actinin, actin, troponin-T, tropomyosin, and myosin (alk-1 light chain). It appears that pressurization affects each of these proteins at different rates as will be discussed later in the section concerning the quantitative characterization of major myofibrillar proteins. Pressure has been found to influence the solubilization of these

proteins resulting in less recoverable myofibrillar pellets. According to Macfarlane and Mckenzie (1976), this solubilization is dependent on the duration of pressurization, temperature and pH of the medium. Since the temperature, pH and duration of pressurization were held constant between the pressurized and control samples in this study, the differences in results are clearly due to the effect of pressure and pressure alone.

Molecular Weight Estimation by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) is a valuable diagnostic tool for evaluating the compositional differences existing between the various proteins of the muscle tissue. Dutson (1977) used this procedure to characterize myofibrillar proteins isolated from beef muscle. The procedure was also used by Hay et al. (1973) to evaluate the proteins of the chicken muscle.

According to Shapiro et al. (1967) and Weber and Osborn (1969), protein separation by PAGE in the presence of SDS, an anionic detergent, is dependent on the molecular weights of their polypeptide chains and charge. They reported that the relative electrophoretic migration distances on gels have an inverse linear relationship with the logarithm of the molecular weights of the protein subunits bound by SDS. Thus SDS-PAGE offers a simple and convenient way of estimating the molecular weights of proteins (Weber and Osborn, 1969). Determining the molecular weights of the component proteins is, therefore, an indispensable way of identifying and characterizing

those proteins. According to Reynolds and Tanford (1970), identification of proteins through the use of SDS-PAGE is based on the principle that SDS binds to protein on gram to gram ratio thus the charge to mass ratio (e/m) of the SDS-protein complex is identical.

In the present study, pressurized muscle homogenates, myofibrillar pellets from pressurized muscle, and supernatants from pressurized myofibrils and their respective controls were electrophoresed to determine the effect of the pressurization treatment on the component proteins. Many electrophoretic runs were carried out but the electrophoretograms shown are representative of the runs. Figure 5 shows the electrophoretogram of the protein standards and the supernatant of the pressurized muscle homogenate.

The standard curve for the estimation of the molecular weights of the proteins under study is shown in Figure 6 using high molecular weight protein standards. This curve is a plot of the bands in the first column (No. 1) in the electrophoretogram presented in Figure 5. The uppermost band with the lowest mobility is myosin (heavy chain) having a molecular weight of about 200,000, followed by β-galactosidase (140,000), phosphorylase B (110,000), bovine serum albumin (75,000), and ovalbumin (45,000). This is a relatively linear curve consistent with the reports of Weber and Osborn (1969) and Hay et al. (1973). However, it disagrees with the findings of Neville (1971) who reported the standard curve to be a shallow sigmoidal type showing linearity only in the molecular weight region of 17,000 to 70,000.

Figure 5. Electrophoretogram of the supernatant from pooled pressurized prerigor beef semitendinosus and longissimus dorsi muscle homogenate.

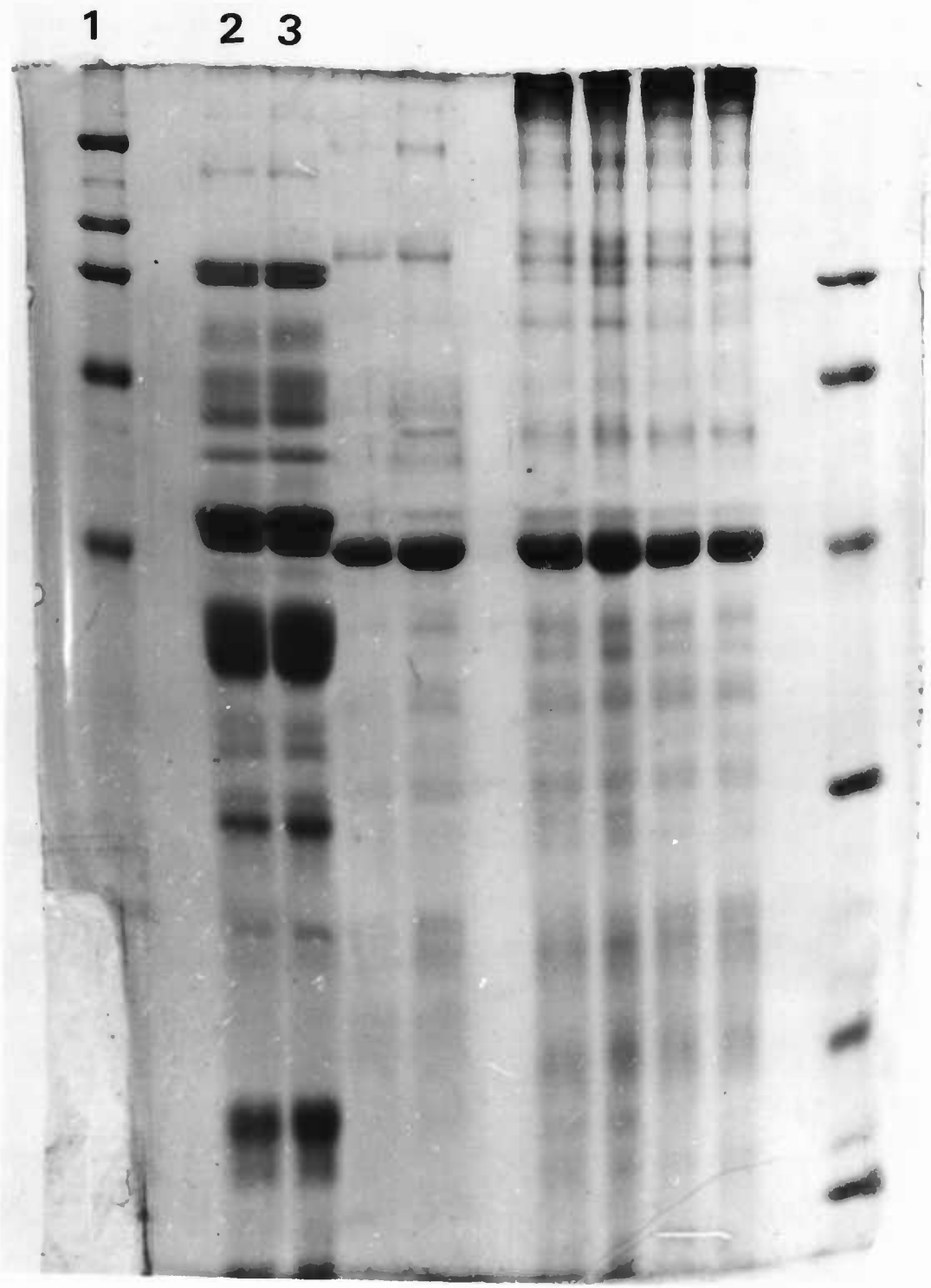

Figure 5.

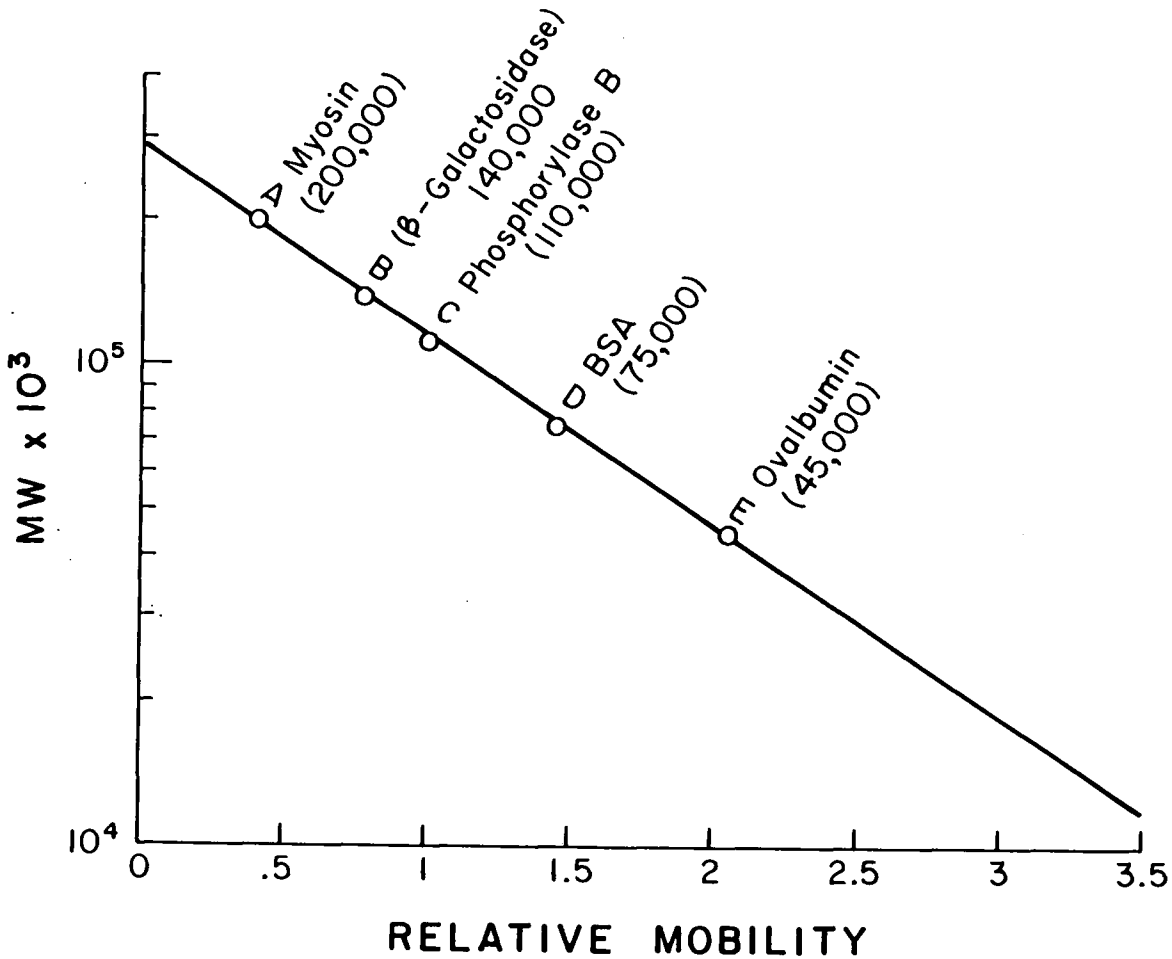

Figure 6. Plot of logarithm of molecular weights of standard proteins versus their relative mobilities (R_m) on SDS-polyacrylamide gels. Gel concentration: 10% running gel, 3% stacking gel.

To estimate the molecular weights of the unknown proteins, their relative mobilities (based on one of the standards) were interpolated on the standard curve. Table 5 shows the estimated molecular weights of proteins from supernatant of pressurized muscle homogenate as shown in columns 2 (control) and 3 (pressurized) of the electrophoretogram in Figure 5. No attempt was made to identify the bands but to illustrate the effect of pressurization on meat homogenates. From Table 5, quantitative interpretation of the effect of pressure on the homogenate using milligrams percent values (Quick Scan Manual, 1973) shows that pressure accounted for approximately 2.0 to 104.6 mg% increase in total protein for those proteins whose molecular weights are shown. Pressure-induced solubilization of these proteins was greatest in the molecular weight region of 16,000 to 55,000. It appears that this difference may have been contributed by the myofibrillar fraction which pressurization had induced to solubilize at rates higher than their controls.

The molecular weights of the major myofibrillar proteins were determined using isolated, reconstituted myofibrillar pellets from pressurized and non-pressurized semitendinosus and longissimus dorsi muscles. Figure 7 shows the electrophoretogram of these myofibrils. Column 1 contains the protein standards while the remaining columns contain the controls and pressurized samples in alternate arrangement. As presented in Table 6, myofibrillar proteins migrated according to their molecular sizes. Myosin (heavy chain) which showed a molecular weight of 210,000 had the lowest relative mobility (Rm) of 0.4. On the other hand, the alkaline-1 light chain of myosin which had the highest Rm of 2.53

Table 5. Effect of pressurization of muscle homogenates on the solubilization of muscle proteins as determined by densitometric scanning of electrophoretogram in Figure 5.

Band/peak No.[a]		Mol. Wt.	Relative Value (%)	Relative Diff. (%)	Diff. (mg% of total protein[b])
1	C	200,000	0.2		
1	P	"	0.4	0.2	2.7
2	C	170,000	0.3		
2	P	"	0.5	0.2	2.0
3	C	140,000	4.2		
3	P	"	6.5	2.3	31.1
4	C	98,000	0.6		
4	P	"	1.6	1.0	13.5
5	C	75,000	1.5		
5	P	"	2.4	0.9	12.2
6	C	63,000	1.1		
6	P	"	2.1	1.0	13.5
7	C	55,000	8.0		
7	P	"	11.4	3.4	45.9
8	C	41,000	25.9		
8	P	"	33.6	7.7	104.6
9	C	32,000	3.7		
9	P	"	7.7	4.0	54.0
10	C	27,000	13.8		
10	P	"	19.3	5.5	74.3
11	C	23,000	0.8		
11	P	"	2.6	1.8	24.3
12	C	16,000	5.6		
12	P	"	10.8	5.8	78.3

[a] C = Control; P = Pressurized.

[b] Protein content of homogenate = 1350 mg%.

Figure 7. Electrophoretogram of pellets from pooled pressurized semitendinosus and longissimus dorsi muscles. Gel concentration: 10% running gel, 3% stacking gel.

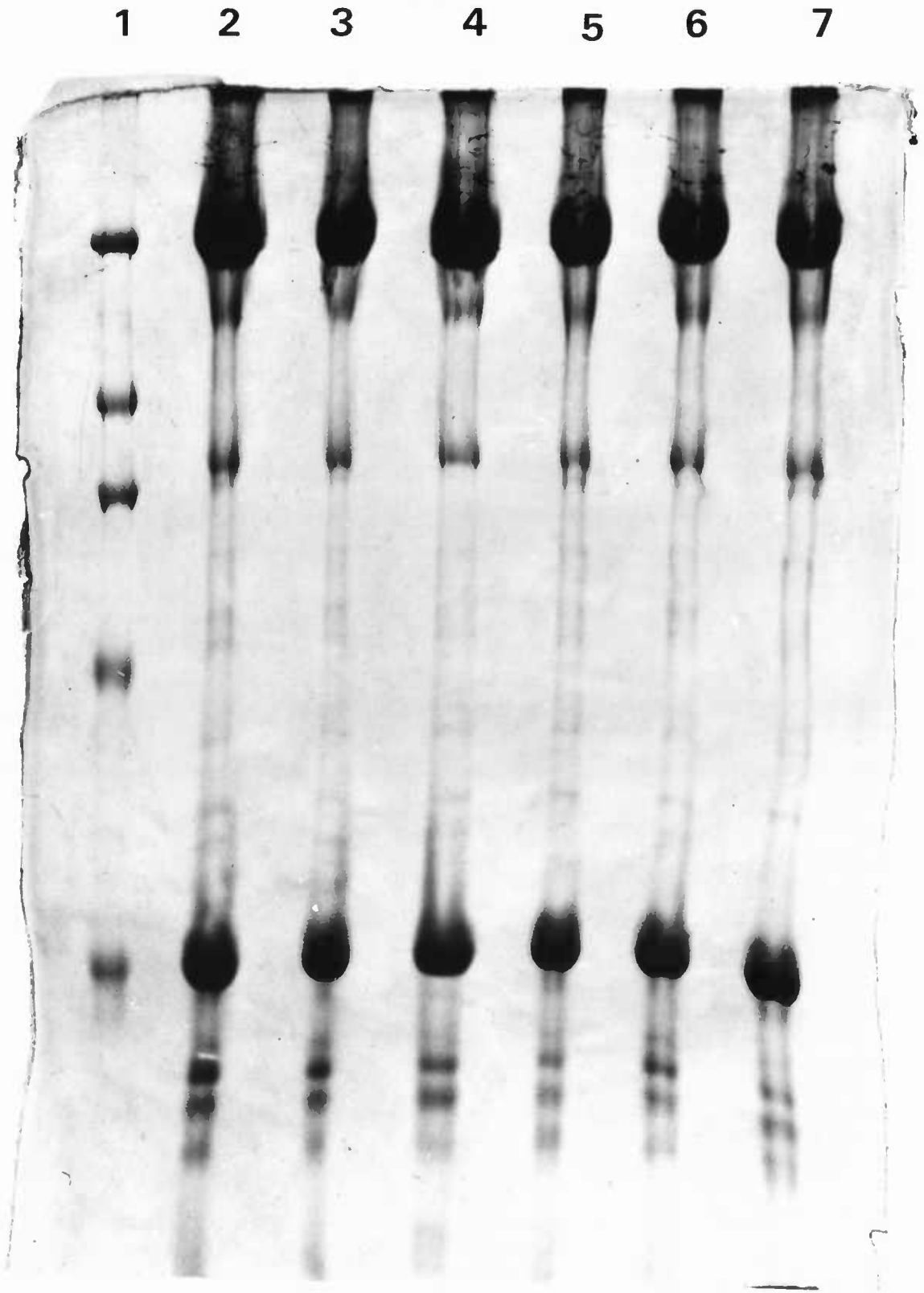

Figure 7.

Table 6. Relative mobilities (R_m) and estimated molecular weights of major myofibrillar proteins from pellets suspended in deionized water as determined by SDS-PAGE shown in Figure 7.

Protein	Band Intensity[a]	R_m	Mol. Wt.
Myosin (heavy chain)	+++	0.40	210,000
M-Protein	++	0.56	173,000
C-Protein	++	0.93	124,000
α-Actinin	+	0.95	108,000
β-Actinin	+	1.60	66,000
Actin	+++	2.18	43,000
Troponin-T	++	2.30	40,000
Tropomyosin	++	2.42	38,000
Myosin (alk-1 light chain)	+	2.53	28,500

[a] (+++) highest intensity
(++) moderate intensity
(+) low intensity

showed an estimated molecular weight of 28,500. These molecular weights are consistent with literature reports though they do vary in some cases. The molecular weight of myosin (heavy chain) of 210,000 is in complete agreement with the reports of Dutson (1977) and Hay et al. (1973). It however differs from estimations reported by Goll et al. (1977) and Weber and Osborn (1969) who gave its molecular weight as 200,000 and 220,000 respectively. The 170,000 molecular weight for M-protein is more than the 160,000 given by Goll et al. (1977). This report also gave the molecular weight of C-protein as 140,000 while it was found to be 124,000 in this study. The rest of the proteins, α-actinin, β-actinin, actin, troponin-T, tropomyosin, and myosin (alk-1 light chain) had molecular weights on SDS-PAGE of 108,000, 66,000, 43,000, 40,000, 38,000, and 28,500 compared to literature values of 103-115,000, 70,000, 42-49,000, 37,000, 36,000, and 21-27,000 respectively (Goll et al., 1977; Dutson, 1977; Hay et al., 1973; Weber and Osborn, 1969).

As presented in Table 6, the intensity of the dyed protein bands is denoted by (+) signs. The electrophoresis of reconstituted pellets showed mysoin and actin bands to have the highest intensity on visual perception. These were followed by M-protein, C-protein tropomyosin, and troponin-T. The faintest bands were α-actinin, β-actinin, and alk-1 light chain of myosin. The intensity of these bands is an index of their relative protein concentrations as would be seen in their characterization for various amounts using quantitative denistometry.

Quantitative Myofibrillar Protein Estimation by Densitometry

The densitometric tracing technique was used to quantify the electrophoretic band patterns of the myofibrillar proteins in order to elucidate the effect of hydrostatic pressurization on the major proteins of the myofibril. The principle of quantitative estimation of proteins by electrophoresis coupled with densitometric scanning has been employed by investigators since the early 1960's. Fazekas de St Groth et al. (1963) used a similar method to estimate various proteins on paper electrophoretic strips. Recently, Tregear and Squire (1973) and Potter (1974) successfully used this method to evaluate the levels of myofibrillar proteins in various tissues ranging from insect muscle to rabbit muscle.

Quantification of the myofibrillar proteins was achieved by scanning the electrophoretogram in a densitometer equipped with an integrator. As mentioned earlier, the electrophoretogram in Figure 7 shows the results obtained on SDS-PAGE of myofibrillar pellets from pressurized and unpressurized muscles. Column 2 contains the control while column 3 has the pressurized sample in alternate sequence. Based on the molecular weights determined earlier (Table 6; Figure 6), it is clear from Figure 7 that the heavy chain of myosin (M.W.210,000) shows the densest band constituting a greater percentage of the total myofibrillar proteins electrophoresed. Actin (M.W. 43,000) comes next with an appreciable percentage of the total proteins. This is in agreement with the report of Goll et al. (1977) who indicated that myosin constitutes 50 to 58% while actin comprizes 15 to 20% of the total myofibrillar proteins.

It is apparent that pressurization affects the yield or

extractibility of myofibrillar proteins, some being more affected than others. Figure 8 shows the densitometric tracing of the electrophoretogram in Figure 7. The sensitivity of the densitometer was limited to seven bands in the electrophoretogram since the remaining myofibrillar bands were too faint to be detected by the scanning instrument. The upper electropherogram (densitometric tracing) represents the unpressurized or control sample while the lower portion is the tracing of the pressurized sample. Starting from the left side, the peaks are labelled "a" to "g". Peak "a" corresponds with the band for myosin (heavy chain), "b" for M-protein, "c" for C-protein, "d" for actin, "e" for troponin-T, "f" for tropomyosin, and "g" for myosin (alk-1 light chain).

Percentage weight differences between the pressurized samples and their controls for these seven myofibrillar proteins are presented in Table 7. Calculations of these percentage weight differences were based on and reported in accordance with the instructions and illustrations outlined in the "Quick Scan" Operators Manual (1973). Using the electropherogram in Figure 8, the integrator lines under the peaks were counted and recorded as integrator units. The total integrator units under the entire tracing was used to divide the units under each curve to give the relative percent value (RV%) for each peak or protein. Since values for various protein fractions are expressed as grams percent (gm%) of the total protein, differences in RV% between the control and pressurized samples were converted accordingly. Through knowledge of the original protein concentration of the myofibrillar pellets by the biuret method, the gm% of the respective proteins was gained

Table 7. Effect of meat pressurization on yield of major myofibrillar proteins from pooled semitendinosus and longissimus dorsi myofibrillar pellets as determined by densitometric scanning of the electrophoretogram in Figure 7.

Band/Peak No.[a]		Mol. Wt.	Protein	Relative Value (%)	Actual[b] Diff. (%)
1	C	210,000	Myosin (heavy chain)	47.5	
1	P	"	"	39.6	-16.6
2	C	173,000	M-Protein	6.6	
2	P	"	"	4.0	-39.4
3	C	124,000	C-Protein	6.1	
3	P	"	"	4.0	-34.4
4	C	43,000	Actin	26.4	
4	P	"	"	18.5	-30.0
5	C	40,000	Troponin-T	7.9	
5	P	"	"	5.3	-32.9
6	C	38,000	Tropomyosin	4.0	
6	P	"	"	2.8	-30.0
7	C	28,500	Myosin (alk-1 light chain)	1.6	
7	P	"	"	1.1	-31.0

[a] C = Control; P = Pressurized.

[b] Difference in relative % values divided by control.

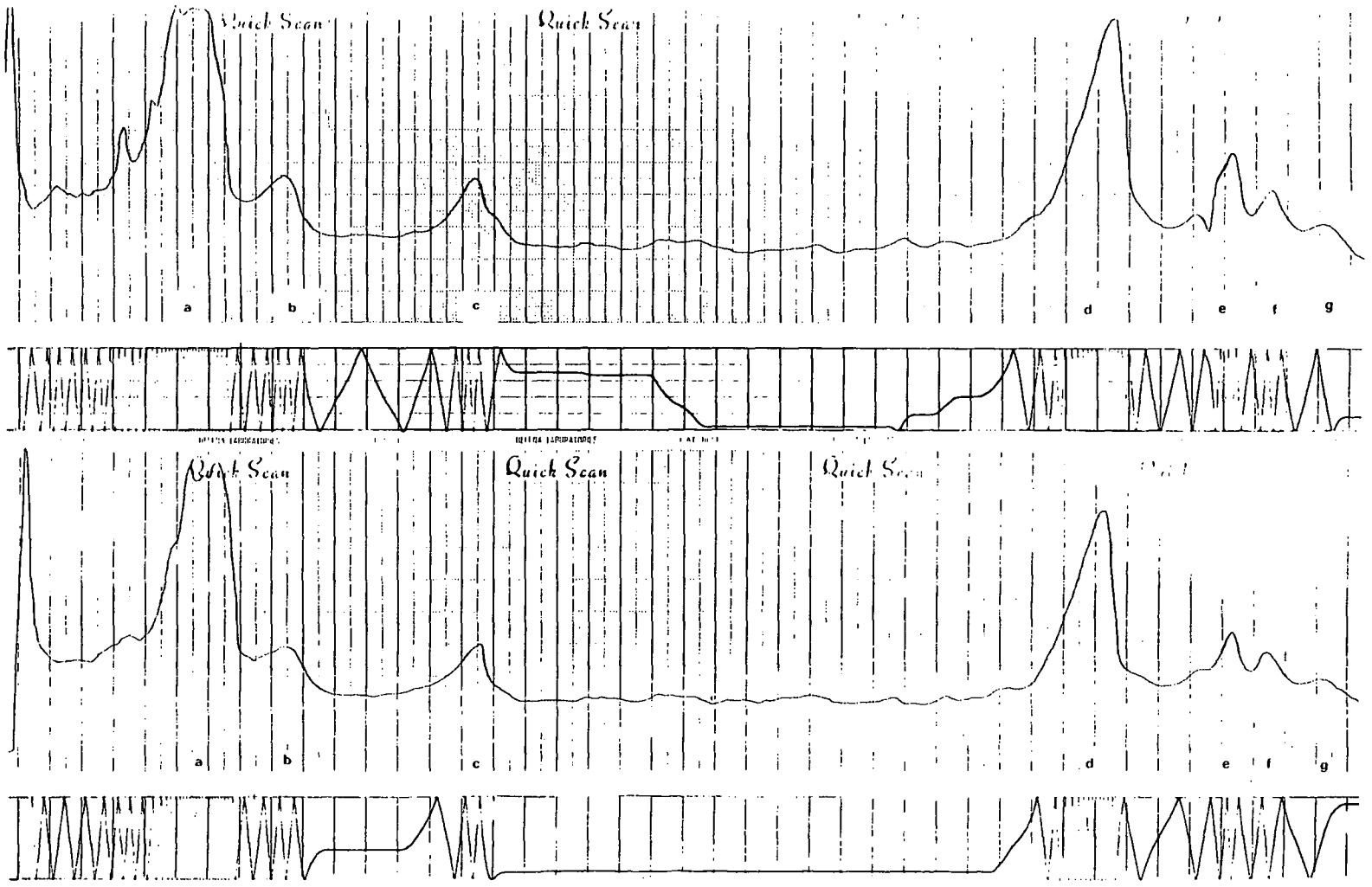

Figure 8. Densitometric tracing of the electrophoretogram in Figure 7 of pellets from pressurized muscles.

by multiplying the gm% of this value by the decimal proportions of the RV% of the respective proteins. As noted in subsequent results, these data were again converted to milligrams % since the original protein concentrations were relatively low.

Judging from the relative percent values alone, as listed in Table 7, myosin (heavy chain) appears to be least affected by meat pressurization prior to myofibril extraction. Myofibrils extracted from pressurized semitendinosus and longissimus dorsi muscles contained about 16.6% less myosin than those from the unpressurized muscles. This indicates that during the whole myofibril extraction process, about 16.6% of the myosin was lost with the sarcoplasmic fraction as a result of pressurization. Apart from myosin, the other myofibrillar proteins showed similar losses. M-protein showed about 39% reduction, C-protein was 34%, actin decreased by 30%, while troponin-T, tropomyosin, and alk-1 light chain of myosin, had losses of about 33%, 30%, and 31% respectively.

Since the extraction processes employed a maximum KCl strength of 0.15M, it may be safe to infer that at low ionic strength ($\mu \leq 0.15$), pressurization induced the disruption of about one-third of the myofibrillar proteins other than myosin. If this inference were made to encompass the entire myofibril, it would be an added dimension of enlightenment as to why prerigor meat pressurization results in high degree of meat tenderness. This is particularly interesting as we realize that the ionic strength of prerigor beef muscle lies between 0.16 and 0.18 (Goll et al., 1977) which is close to the maximum ionic strength of 0.15 used in the myofibril extraction process.

Solubilization of Total Myofibrils at Varying Ionic Strengths

Potassium chloride concentrations of 0 to 1 molarities were used to suspend the intact myofibrils extracted from beef semitendinosus and longissimus dorsi muscles. Following pressurization (15,000 psi, 30 min) at ambient temperature, the myofibril suspensions were centrifuged (100,000 x G, 1 hr) at 2°C and their supernatants assayed for protein content using the biuret procedure.

Figure 9 shows the effect of pressurization on the solubilization of these myofibrils. When the myofibrils were suspended in deionized water (0M KCl), about 74% more myofibrillar proteins were solubilized as the result of pressure. This differs from the results in Table 4 which indicated that about 36% more protein was solubilized due to pressure. However, the duration of pressurization for the Table 4 experiment was 10 min whereas a 30 min period was used in this study. This is consistent with the reports of Macfarlane and Mckenzie (1976) that the solubilization of myofibrillar proteins is also affected by the duration of pressurization.

Though protein solubilization was reduced markedly when the myofibrils were suspended in 0.1M KCl, about 100% more myofibrillar proteins were found in the supernatant as the result of pressure-induced solubilization. Suspension in 0.1M KCl showed the lowest amount of protein in the supernatant for the control and pressurized samples over the whole spectrum of molar concentrations under study. The reason for the sharp drop in protein solubilization at this molarity is unclear. Macfarlane and Mckenzie (1976), using different salts, encountered a similar trend but offered no explanations,

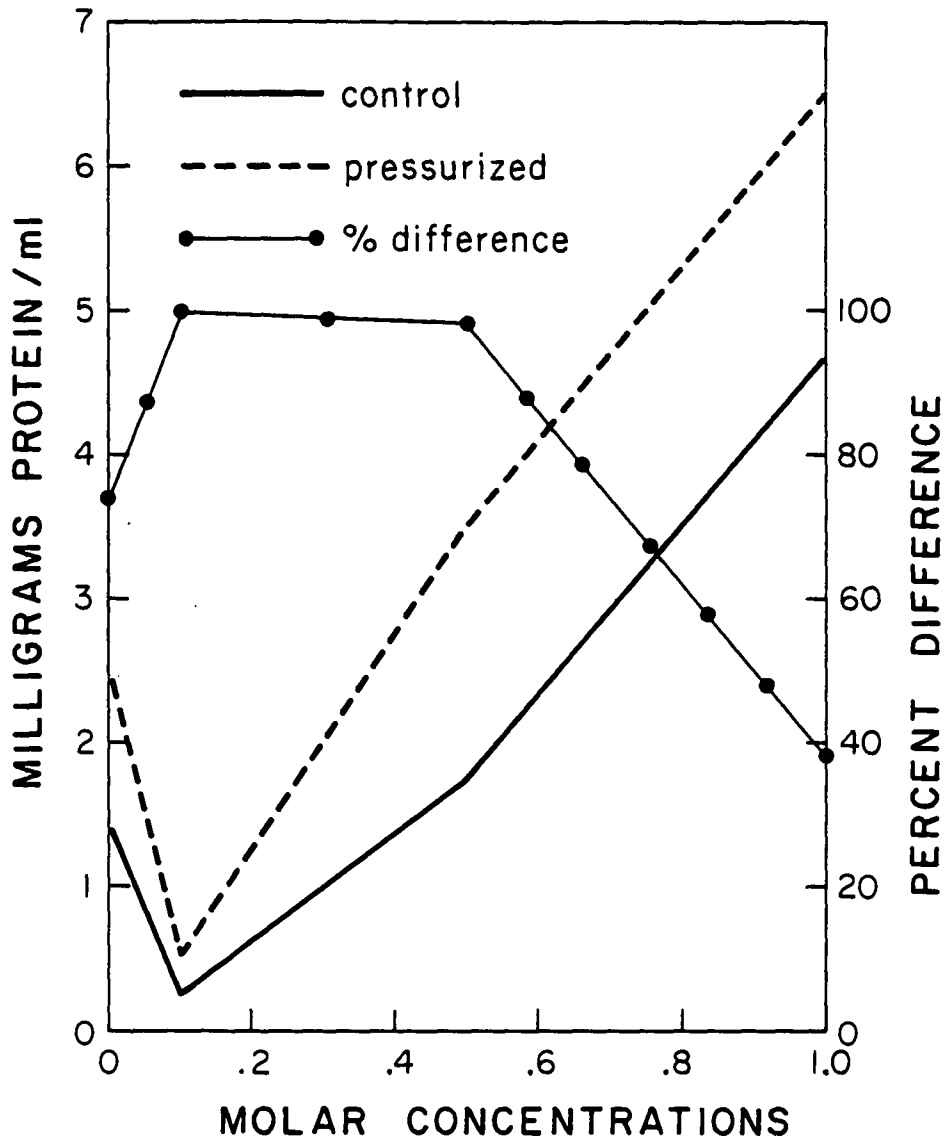

Figure 9. Effect of pressurization on the solubilization of myofibrils of pooled semitendinosus and longissimus dorsi muscles in KCl solutions of different ionic strengths.

though their lowest "expressed soluble protein" was around 0.15 molarity.

On suspension of the myofibrils in 0.5M KCl, protein recovered in the supernatant again increased. The pressurized samples contained about 98% more solubilized protein than their controls. This trend of increase continued when the myofibrils were suspended in 1.0M KCl. Both the pressurized and the control samples showed sharp increases in protein solubilization but the pressurized samples contained only 38% more protein solubilized than the controls. The drop in percentage difference in solubilization at this ionic strength (1.0M KCl) may in part be due to the immense increase in WHC at this molarity. At this point, over 75% of the myofibril suspension was reclaimed as pellets after the centrifugation of both the pressurized and control samples.

Thus the molar concentrations of KCl had identical influence on both the pressurized and unpressurized samples. At 0M KCl, both fractions had relatively high solubility, at 0.1M KCl, both sharply dropped, at 0.5M KCl, both sharply increased, and at 1.0M KCl, the sharp increase continued though in each case, the increases in the pressurized samples were considerably higher than those of their controls.

From the data gained in this study, it is evident that hydrostatic pressurization enhances the solublization of these proteins in salt solutions. The data are comparable to the findings of Macfarlane and Mckenzie (1976).

Ionic Strength Dependent Solubilization of Major Myofibrillar Proteins

Table 8 shows the effect of pressurization on the major myofibrillar proteins when isolated intact myofibrils were suspended in deionized water prior to pressurization. As can be seen from these data concerning the RV% difference and mg% difference of total protein, actin appears to be the protein most affected by pressure at this ionic strength (OM KCl). This was followed by troponin-T, β-actinin, tropomyosin, and alk-1 light chain of myosin. Myosin (heavy chain) was least affected by pressure at this low ionic strength.

The influence of pressure on the myofibrils suspended in 0.1M KCl is presented in Table 9. At this molarity, protein solubilization was extremely low for both the pressurized and control myofibrils. However, actin was most affected by pressure at this ionic strength. Due to the low concentrations of proteins in the supernatant resulting in extreme faintness of protein bands on the electrophoretogram (Figure 10), the electropherogram or the densitometric tracing failed to yield appropriate peaks for quantification.

Table 10 shows the effect of pressurization on the major myofibrillar proteins when intact myofibrils were solubilized in 0.5M KCl. It is evident that myosin was most affected by pressure at this ionic strength because the pressurized sample contained 46 mg% more protein than the control. The next to be affected was actin which showed about 27 mg% more actin in the pressurized sample. There were substantial increases in the solubilization of other

Figure 10. Electrophoretogram of pooled pressurized semitendinosus and longissimus dorsi myofibrils solubilized at different molar concentrations of KCl. Gel concentration: (a) 12.5% running gel, 4% stacking gel. (b) 10% running gel, 3% stacking gel.

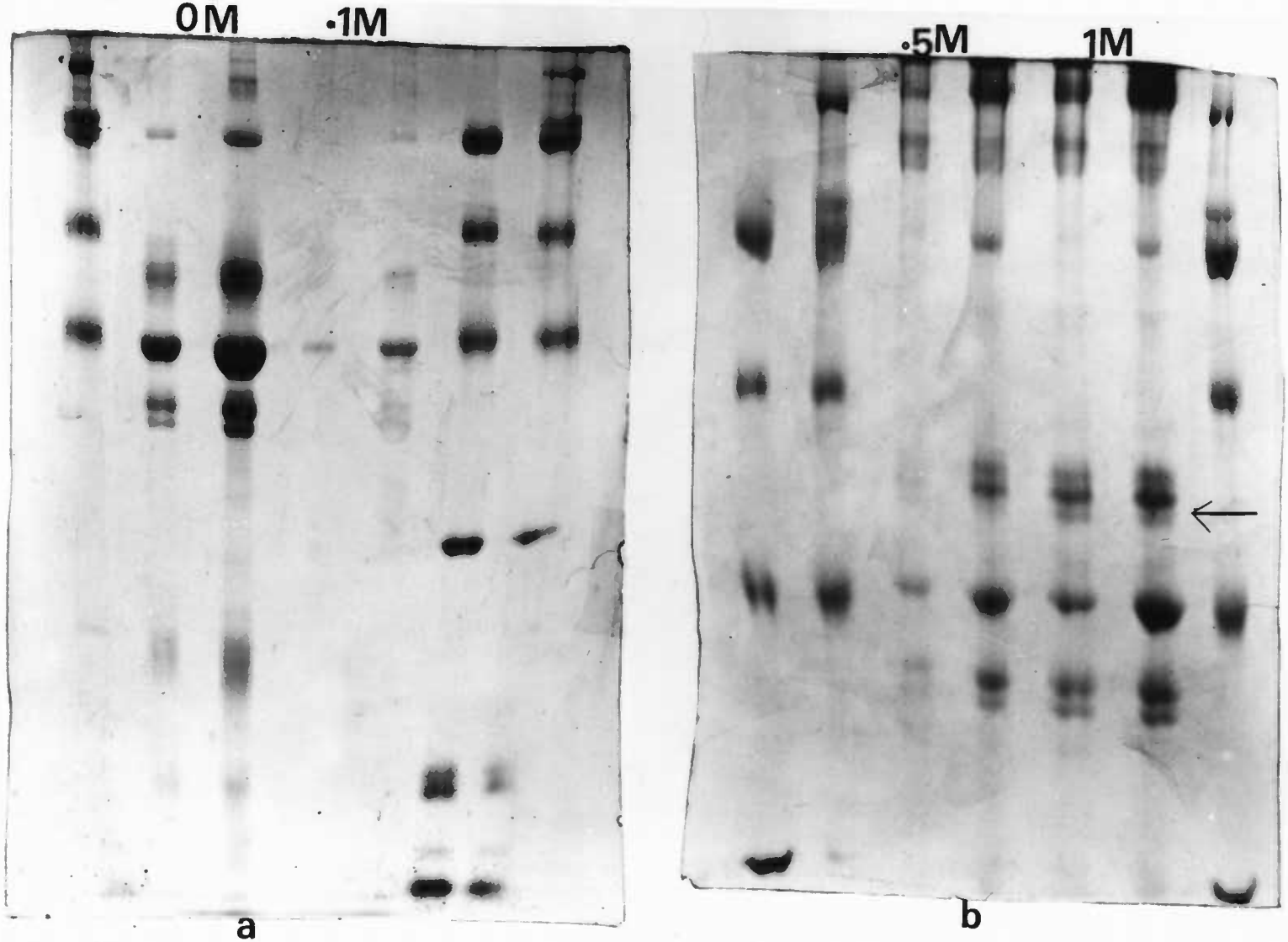

Figure 10.

Table 8. Effect of pressurization on solubilization of myofibrils suspended in deionized water as determined by densitometric tracing of the electrophoretogram in Figure 10.

Band/Peak No.[a]		Mol. Wt.	Protein	Relative Value (%)	Relative Diff. (%)	Difference (mg% of total protein)[b]
1	C	210,000	Myosin (heavy chain)	0.34		
1	P	"		0.95	0.61	0.85
2	C	172.000	M-Protein	0.27		
2	P	"	"	1.22	0.95	1.33
3	C	142,000	C-Protein	0.23		
3	P	"	"	1.39	1.16	1.62
4	C	104,000	α-Actinin	0.98		
4	P	"	"	2.47	1.49	2.09
5	C	69,000	β-Actinin	1.78		
5	P	"	"	11.23	9.45	13.23
6	C	43,000	Actin	14.45		
6	P	"	"	49.33	34.88	48.83
7	C	38,000	Troponin-T	1.62		
7	P	"	"	16.20	14.57	20.40
8	c	33,000	Tropomyosin	0.93		
8	P	"	"	6.67	5.74	8.04
9	C	24,500	Myosin (alk-1)	0.53		
9	P	"		3.52	2.99	4.19
10	C	18,000	Myosin (alk-2)	0.53		
10	P	"				
10	P	"	"	8.3	7.77	10.78

[a] C = Control; P = Pressurized

[b] Protein content supernatant = 140 mg%

Table 9. Effect of pressurization on solubilization of myofibrils suspended in 0.1M KCl as determined by densitometric tracing of the electrophoreogram in Figure 10.

Band/Peak[a] No.		Mol. Wt.	Protein	Relative Value (%)	Relative Diff. (%)	Difference (mg% of total protein)[b]
1	C	210,000	Myosin (heavy chain)	2.9		
1	P	"	"	7.03	5.74	0.12
2	C	172,000	M-Protein	1.29		
2	P	"	"	7.03	5.74	0.12
3	C	110,000	α-Actinin	1.29		
3	P	"	"	7.03	5.74	0.12
4	C	69,000	β-Actinin	1.20		
4	P	"	"	7.03	5.74	0.12
5	C	43,000	Actin	1.29		
5	P	"	"	55.0	53.71	1.07
6	C	38,000	Troponin-T	1.29		
6	P	"	"	8.50	7.21	0.14
7	c	32,000	Tropomyosin	1.29		
7	P	"	"	8.50	7.21	0.14

[a] C = Control; P = Pressurized

[b] Protein content supernatant = 2 mg%

Table 10. Effect of pressurization on solubilization of myofibrils suspended in 0.5M KCl as determined by densitometric tracing of the electrophoretogram in Figure 10.

Band/Peak[a] No.		Mol. Wt.	Protein	Relative Value (%)	Relative Diff. (%)	Difference (mg% of total protein)[b]
1	C	210,000	Myosin (heavy chain)	21.1		
1	P	"	"	35.6	14.5	45.40
2	C	172,000	M-Protein	1.2		
2	P	"	"	5.1	3.9	12.48
3	C	145,000	C-Protein	0.7		
3	P	"	"	3.7	3.0	9.60
4	C	110,000	α-Actinin	1.0		
4	P	"	"	3.6	2.6	8.32
5	C	69,000	β-Actinin	1.7		
5	P	"	"	5.3	3.6	11.52
6	C	55,000	I-Protein (?)	1.7		
6	P	"	"	4.7	3.0	9.60
7	C	42,000	Actin	19.1		
7	P	"	"	27.5	8.4	26.88
8	C	37,000	Troponin-T	6.7		
8	P	"	"	11.7	5.0	16.00
9	C	30,000	Tropomyosin	1.2		
9	P	"	"	3.6	2.4	7.68

[a] C = Control; P = Pressurized

[b] Protein content supernatant = 320 mg%

myofibrillar proteins at this ionic strength as shown in Table 10.

Data in Table 11 show the solubility pattern of the myofibrillar proteins as influenced by pressurization of the myofibrils suspended in 1.0M KCl. The trend is analagous to that seen for pressurization of myofibrils in 0.5M KCl. In this study, myosin was also most affected by pressure at this high ionic strength.

When myofibrils were suspended in 0.5M KCl and 1.0M KCl, a new myofibrillar protein (I-protein) appeared just before actin (Figure 10). This protein did not appear when myofibrils were suspended in solutions of low ionic strength (0M and 0.1M KCl). Since this band repeatedly appeared in subsequent SDS-PAGE runs, it deserves mention. For purposes of differentiation, this protein has been referred to as I-protein, with a molecular weight of 55,000. The yield of this protein was enhanced by pressurization by a difference of about 10 mg% in 0.5M KCl and 17 mg% in 1.0M KCl solutions.

The relative effect of pressurization on the solubilization of major myofibrillar proteins when the myofibrils were solubilzed in solutions of low ionic strength ($\mu \leq 0.1$) is presented in Figure 11. The pressure effect on these proteins when the myofibrils were solubilized in high ionic strength solutions ($\mu \geq 0.5$) is summarized in Figure 12. Monitoring the myofibrillar proteins individually, data indicated that at low ionic strength, pressurization affected actin the most by solubilizing about 40 mg% more actin in the pressurized sample than in its control. Troponin-T and β-actinin followed with about 20 and 13 mg% differences respectively. Myosin (light chains), tropomyosin, α-actinin, M-protein, C-protein, and myosin (heavy chain) were then affected in that order. At high

Table 11. Effect of pressurization on solubilization of myofibrils suspended in 1.0M KCl as determined by densitometric tracing of the electrophoretogram in Figure 10.

Band/Peak No.[a]		Mol. Wt.	Protein	Relative Value (%)	Relative Diff. (%)	Difference (mg% of total protein)[b]
1	C	210,000	Myosin (heavy chain)	13.0		
1	P	"		29.0	16.0	36.80
2	C	172.000	M-Protein	4.4		
2	P	"	"	9.6	5.2	11.96
3	C	145,000	C-Protein	0.27		
3	P	"	"	3.6	3.3	7.66
4	C	110,000	α-Actinin	0.46		
4	P	"	"	3.7	3.2	7.45
5	C	69,000	β-Actinin	5.6		
5	P	"	"	12.0	6.4	14.72
6	C	55,000	I-Protein (?)	2.2		
6	P	"	"	9.4	7.2	16.56
7	C	42,000	Actin	8.2		
7	P	"	"	16.3	8.1	18.63
8	C	37,000	Troponin-T	7.1		
8	P	"	"	10.6	3.5	8.05
9	C	30,000	Tropomyosin	3.0		
9	P	"	"	6.1	3.1	7.13

[a] C = Control; P = Pressurized

[b] Protein content supernatant = 230 mg%

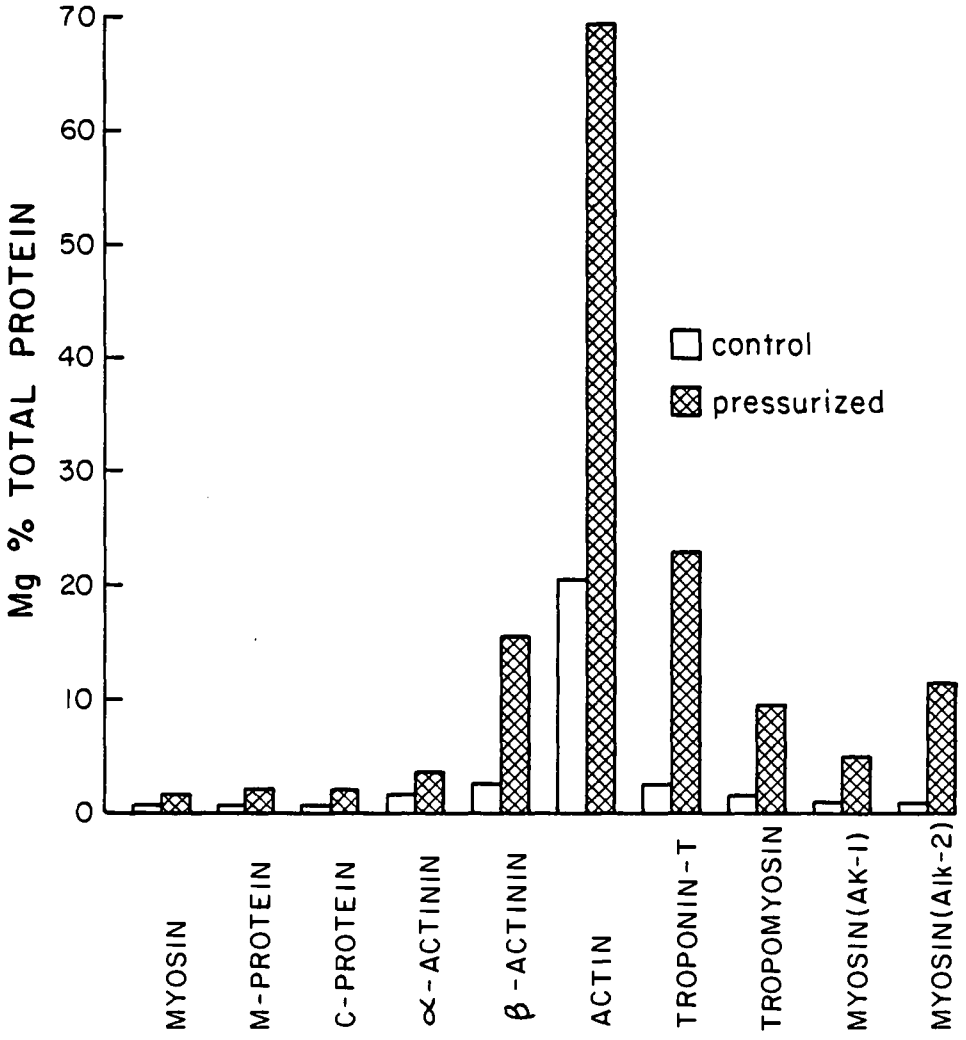

Figure 11. Relative effect of pressurization on the solubilization of major myofibrillar proteins of pooled semitendinosus and longissimus dorsi muscles at low ionic strength (<0.1M KCl)

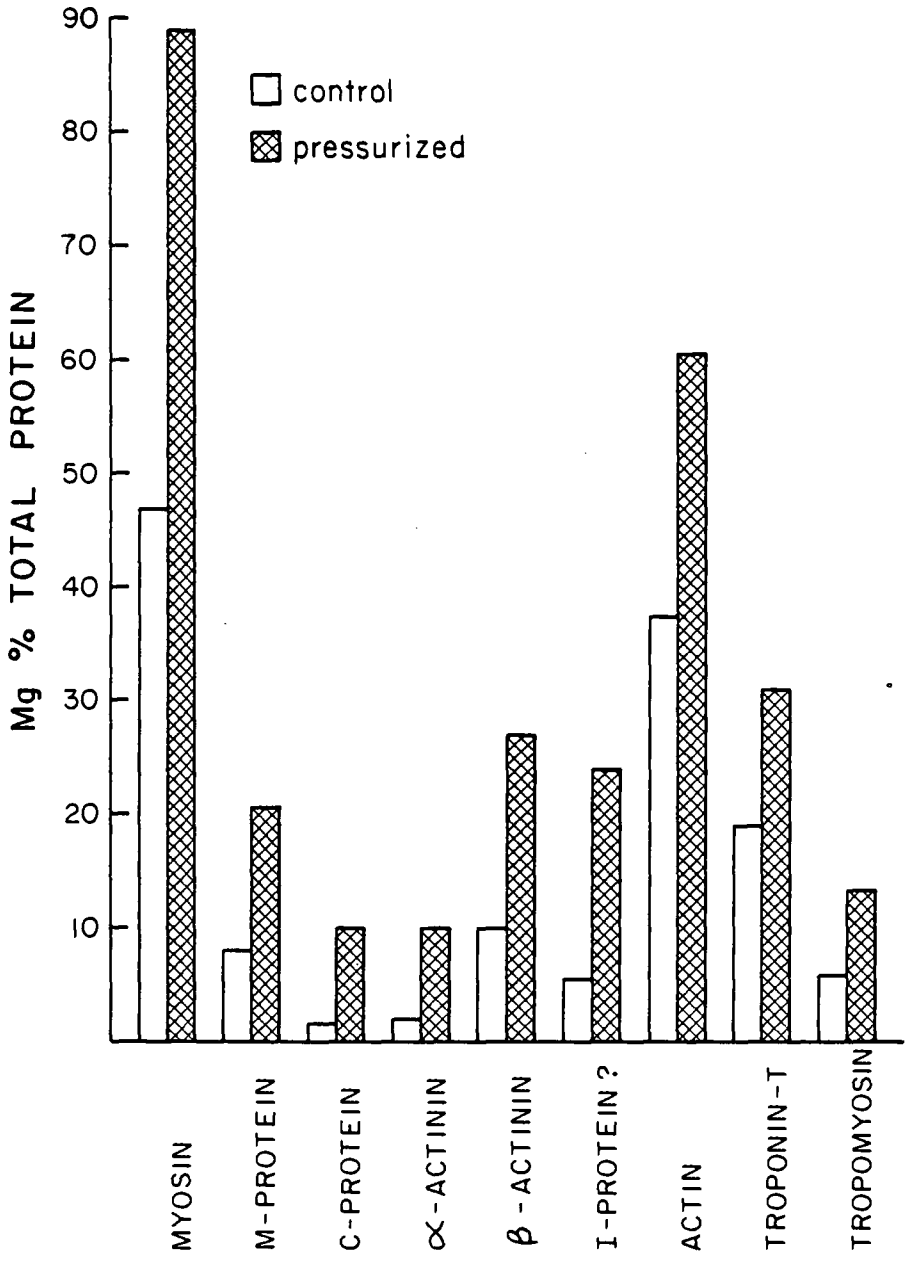

Figure 12. Relative effect of pressurization on the solubilization of major myofibrillar proteins of pooled semitendinosus and longissimus dorsi muscles at high ionic strength (0.5 - 1.0M KCl).

ionic strength, however, myosin (heavy chain) showed the greatest difference, about 42 mg% more myosin was solubilized in the pressurized samples than in the unpressurized. On the average, pressure-induced solubilization of the proteins at high ionic strength also accounted for about 23 mg% more actin, 17 mg% more β-actinin, 13 mg% more M-protein, 12 mg% more I-protein, and about 11 mg% more for each of the following: troponin-T, tropomyosin, α-actinin, and C-protein.

From the data gained in this study, it seems appropriate to infer that actin and lower molecular weight myofibrillar proteins appear to be more affected by hydrostatic pressurization when it is accomplished in solutions of low ionic strength. When pressurization is carried out in solutions of high ionic strength, myosin and to some extent actin are more affected.

It is clear, therefore, that pressurization has the most pronounced effect on only two of the major proteins of the myofibril depending on the ionic strength of the medium. These proteins are actin and myosin. Since the two proteins together constitute 65 to 78% by weight of the total proteins of the myofibril, any changes in actin and myosin would ultimately affect the entire myofibrils which constitute the contractile apparatus of the skeletal muscle. Few postulations have been advanced on the mode of influence of pressurization on actin and myosin systems. The major ones involve the transformation of fibrous to globular (F-G) actin and disaggregation of actomyosin to actin and myosin. Ikkai and Ooi (1966) inferred that pressurization caused structural modification of F-actin which was followed by depolymerization to G-actin. Large volume change was

evident in this transformation as monitored by dilatometry. In respect to actomyosin systems, pressure effects have been accounted for in terms of dissociation of the systems into actin and myosin.

From the data presented in this study, it is apparently clear that the myofibrillar proteins are greatly affected when prerigor meat is subjected to hydrostatic pressure. Since the myofibrils make up 52 to 56% of the skeletal muscle by weight, factors affecting this muscle fraction will no doubt affect meat quality as a whole.

In the light of data gained in this study, the mode with which pressurization influences meat tenderness is still not fully understood. Further investigations are needed to elucidate this phenomenon. Suggested areas of study include the influence of pressurization on the resolution of the so-called "background toughness" as contributed by stroma proteins. Such an investigation should involve quantitative analysis of the stroma proteins. Since myofilament damage has been reported by many investigators as a result of pressurization and since pressure increased the solubilization of myofibrillar proteins in this study, an investigation on rate-effect influence of pressurization on the individual myofibrillar proteins is suggested. To achieve this, each of the major myofibrillar proteins should be isolated separately, subjected to pressure, and their supernatants and pellets should be analyzed electrophoretically to determine the pressure-induced changes. Finally, a follow-up study of pressurized prerigor beef muscles is advocated to determine the rate of degradative changes occuring during storage. This study should encompass colorimetric, flavor, and microbiological changes.

SUMMARY AND CONCLUSIONS

The major objective of this study was to gain basic knowledge about the effect of hydrostatic pressurization (15,000 psi, 2 min, 40°C) on prerigor beef skeletal muscles. Using two beef muscles, longissimus dorsi and semitendinosus, pH determinations and colorimetric studies were conducted during 7 days storage at 4°C on both the control and pressurized muscles. The effect of pressurization on muscle homogenate was also studied. Intact myofibrils were also isolated from these muscles and water-holding capacity and dye-binding studies carried out. Finally, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate proteins of the intact myofibrils isolated from both pressurized and control muscle samples. Quantitative densitometry was employed to elucidate concentration differences between component myofibrillar proteins when the isolated myofibrils were suspended in solutions of different ionic strengths prior to pressurization.

The pH data indicated that pressurization induced a sharp drop in pH of prerigor beef muscles. Pressurization resulted in a rapid decline in pH of about 0.9 pH unit upon completion of the pressure treatment. This property appears to be associated with the acceleration of postmortem glycolysis due to pressure. Only slight differences in pH were noted thereafter between pressurized and control samples during 7 days postmortem storage.

Colorimetrically, the pressurized muscles differed markedly

from the unpressurized muscles. Using the Hunter "L" and "a" color values for the amount of lightness and redness respectively, marked increases were seen in these values soon after pressurization. Pressurized muscles maximized the desirable bright red color after 48 hr of postmortem storage and the amount of redness was consistently higher than that of the controls for the entire 7 day storage period. It appears that pressurization acts to expand or affect the heme prosthetic chromophore so that on cutting and exposure to oxygen, the meat attains a bright red color which could not be matched by the non-pressurized muscles.

The water-holding capacity (WHC) of the isolated myofibrils was markedly influenced by the pressure treatment though it was also ionic strength dependent. The WHC of the myofibrils was higher for the pressure-treated extracts in all concentrations of KCl (0 to 1.0M) studied. At 0M KCl, the WHC increased, decreased at 0.1M and increased again at 0.5M and 1.0M concentrations of KCl. Solubilization of myofibrils in different concentrations of KCl followed the same pattern as the WHC. It appears, therefore, that there is a relationship between the extractibility of myofibrillar proteins and WHC. Increase in hydration as induced by pressure implicates an alteration of the myofibrillar proteins thereby resulting in decreased extractibility of these proteins.

The dye-binding ability of the myofibrillar proteins was altered by pressurization. Protein levels as determined by the dye-binding method were about 25% less for the pressurized samples than those obtained on the same sample material with the biuret procedure.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed compositional differences between the major myofibrillar proteins when intact myofibrils were subjected to pressurization. The extent of these differences were obtained quantitatively using densitometric techniques. These proteins were identified using the molecular weight estimation technique employing known protein standards.

Densitometric tracings of the electrophoretograms showed that all of the myofibrillar proteins became more soluble on pressurization, though some were more soluble than the others. At ionic strengths less than 0.1M KCl, actin was most affected by pressure. Pressure-induced solubilization of actin at this ionic strength increased about 3-fold. β-Actinin, troponin-T, tropomyosin, and myosin (alk-1 light chain) were also greatly affected as actin though their concentrations were relatively low. At high ionic strength (0.5 to 1.0M KCl), pressurization induced a 2-fold increase in solubilization of myosin (heavy chain) and about 1.5-fold increase in solubilization of actin. Other myofibrillar proteins were also affected at this ionic strength though their initial concentrations were substantially less than actin or myosin.

The findings in this study relative to myofibrillar proteins as affected by hydrostatic pressurization offer some information concerning meat tenderness. The disruptive effect of pressurization on the architectural backbones of the thick and thin filaments, myosin and actin respectively, indicate that these proteins are directly involved in meat tenderness. By inducing disruption and increased solubilization of the major and minor proteins of the

myofibrils, hydrostatic pressurization would tend to reduce or minimize the so-called "actomyosin toughness" thereby rendering the meat more tender.

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