PHYSICAL AND CHEMICAL CHARACTERIZATION OF EPIMYSIAL CONNECTIVE TISSUE

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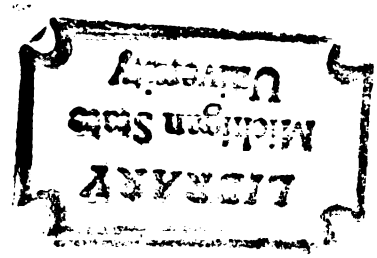

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ABSTRACT

PHYSICAL AND CHEMICAL CHARACTERIZATION

OF EPIMYSIAL CONNECTIVE TISSUE

by Philip E. McClain

Connective tissues, and collagen in particular, are thought to be closely related to the aging process in man and animals, and are involved in numerous vascular and muscular diseases. However, the chemical and physical nature of the connective tissues associated with the muscle is relatively unknown.

The first objective of this investigation was to physically and chemically characterize epimysial connective tissue, which is closely associated with muscle, utilizing histochemical, chemical and physical methods. Histochemical studies indicated that the predominant mucopolysaccharide present in porcine epimysium was hyaluronic acid. Porcine epimysium contained 3.20% of salt soluble and 0.31% of acid soluble tropocollagen. The purified acid soluble collagen fraction comprised 38.46% of the α- and 61.54% of the β-components, indicating that this fraction was 92.27% intramolecularly crosslinked.

Porcine epimysial connective tissues contained 15.02% of heat labile collagen. As determined on the melting point apparatus, the epimysium had a thermal shrinkage temperature range of 59.0 to 61.8°C. However, differential thermal analysis (DTA) data indicated that the thermal shrinkage phenomena occurred over a range of 31.53°C. The onset of shrinkage occurred at 49.44, the extrapolated onset at 60.12, the peak at 66.30 and recovery at 80.97°C.

Porcine epimysium was found to undergo osmotic swelling in neutral solutions. The epimysium had an initial dry matter content of 39.84%,

which decreased to 33.35% upon swelling. The 6.49% decrease in dry matter was thought to be due to loss of ground substance. The water uptake during swelling was 8.25 gm/gm of connective tissue.

Hydrothermally denatured epimysial connective tissues followed the theory of ideal rubber elasticity as determined by the stress-strain method. Utilizing this technique, it was found that porcine epimysium has a molecular weight between crosslinks (Mc) of 46,700, and a crosslink density of 7.73/molecule.

In the second phase of this study, emphasis was placed on a chemical and physical comparison of the epimysium from normal and pale, soft and exudative (PSE) muscles.

Markedly higher turbidity values for the PSE muscles indicated that most of the sarcoplasmic proteins had been precipitated. The ultimate pH values were similar in both normal and PSE muscles, which suggests that ultimate pH is probably not responsible for the occurrence of the PSE condition. Metachromasia and swelling studies revealed that the epimysium from PSE muscles had a lower or altered hyaluronic acid content in comparison to normal tissues.

Although no distinctive histological differences in fiber type and appearance were evident between normal and PSE connective tissues, the fibrous proteins were also altered as indicated by changes in acid and salt solubility. The PSE epimysium had a higher percentage of salt soluble collagen, indicating that it contained fewer intramolecular crosslinks. The higher percentage of heat labile collagen in the epimysium from PSE pigs also indicated either a lower number or a decreased strength of

crosslinks. The DTA data revealed that PSE epimysium was intermediate in maturity to that of 7 week and normal 6 month old pigs, and contained crosslinks susceptible to cleavage at lower temperatures than for the normal. The greater swelling in water for the PSE epimysium also indicated either a decrease in molecular ordering or less crosslinking.

Lower Mc and crosslinking values, although only significant at the 10% level, probably indicated a lowered covalent crosslinking for the epimysium from PSE pigs. No differences were detected in amino acid composition of the salt soluble collagen or in the plasma hydroxyproline levels between normal and PSE pigs.

In the third portion of this study, investigations were conducted on the alterations occurring in the connective tissues in relation to source, biological age, species and nutritional status. Special emphasis was placed on the differential thermal analysis (DTA) technique in order to evaluate its usefulness in studying connective tissues.

The DTA studies showed an increase in high melting components with increasing biological age. Stress-strain studies also revealed a corresponding increase in the amount of covalent crosslinking associated with increasing age.

Bovine epimysium had a narrower melting point range as determined by the DTA method. A much greater percentage of the melting point range occurred at higher temperatures than was found for porcine epimysium. Stress-strain studies also showed that bovine epimysium had a greater number of covalent crosslinks than that from the pig.

Corium from the 7 week old pig had a broader melting point range and a greater percentage of higher melting components than epimysium. The DTA data also indicated that connective tissues from zinc deficient pigs had a narrower melting point range and a larger percentage of higher melting components than the pigs on a normal diet. These results showed that the DTA method can be utilized for the detection of alterations occurring in connective tissues.

PHYSICAL AND CHEMICAL CHARACTERIZATION OF EPIMYSIAL CONNECTIVE TISSUE

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INTRODUCTION

Connective tissue plays an important role in muscle architecture. It is the medium supporting and surrounding the individual muscle fibers and the other tissues of the body. Consequently, connective tissues form a layer through which all substances entering and leaving the muscle fibers must pass.

Collagen is an integral part of the connective tissues. It is the most widely occurring protein in the animal body, and may constitute up to one-third of the total body protein. Collagen is the major fibrous constituent of skin, tendon, ligaments and the connective tissues supporting the muscles and vital organs. Sjoerdma (1965) stated that collagen confers the ultimate in 'togetherness', and is responsible for making one an 'intact' individual.

The connective tissues, and collagen in particular, have been implicated in numerous vascular diseases, and are known to be closely related to the aging process in man and animals. Collagen is the first, and as yet the only known protein of the body that shows continous advancing molecular changes during life, in other words, it ages (Verzar, 1964). More importantly from a food science point of view, collagen has been implicated as contributing to the tenderness and other quality attributes of meat.

A voluminous amount of information pertaining to the study of connective tissues is available in the literature. However, the primary

sources of connective tissue utilized for basic studies of collagen have been skin and tendon. The chemical and physical nature of the connective tissues associated with muscle is, therefore, relatively unknown.

Several workers (Briskey, 1963; Sayre et al., 1963) have stated that muscles in the pig carcass separate from the bone, and apparently lose their attachment to the connective tissues in severe cases of the pale, soft and exudative (PSE) condition. The literature is well documented on the biochemistry, physiology and histology of this condition in relation to the muscle mass. However, very little is known about the interrelationship of the PSE condition and the connective tissues.

The present investigation was undertaken to physically and chemically characterize the epimysial connective tissues, which are closely associated with muscle. In addition, special emphasis was placed upon comparing the connective tissues from PSE and normal porcine muscles.

REVIEW OF LITERATURE

Characteristics of Connective Tissues

Copenhaver and Johnson (1958) indicated that the fibrous elements and cellular inclusions of the connective tissues are embedded in a homogeneous material called ground substance. They further stated that ground substance is believed to be derived from the connective tissue cells and is made up of a complex mixture of proteins, glycoproteins, carbohydrates, lipids and water. The mucopolysaccharides, hyaluronic acid and the chondroitin sulfates, give the ground substance its viscosity, gelatinous nature, the capacity to bind water and other physiological properties (Copenhaver and Johnson, 1958). The ground substance also takes part in the diffusion of nutrients, effects capillary permeability, and regulates the growth of cartilage and bone (Copenhaver and Johnson, 1958).

Among the cell types found in the connective tissues are the fibroblasts, which are believed to secret tropocollagen, and the mast cells characterized mainly by their globular cytoplasmic granules (Bloom and Fawcett, 1962). These granules contain acid mucopolysaccharides, and may be the site for synthesis of hyaluronic acid, heparin and histamine (Asboe-Hansen, 1957). In addition, connective tissues contain an interlacing network of the fibrous proteins, i.e., collagen, reticulin and elastin. These proteins are embedded in the ground substance matrix (Bloom and Fawcett, 1962).

Endocrine Control of the Connective Tissues. Asboe-Hansen (1963) made an intensive review of the endocrine control of the connective tissues, which is summarized herein. Glucocorticoids and thyroid hormone generally decrease the size of the mast cells, and the quantity of acid- and sulfate-containing mucopolysaccharides. In addition, glucocorticoids inhibit fibroblast growth and production of collagen fibers. Somatotrophin, thyrotropin and mineralocorticoids exert affects opposite to those of the thyroid hormone and glucocorticoids. They stimulate mast cell activity, increase the quantity of mucopolysaccharides, bring about proliferation of fibroblasts and increase collagen production. Testosterone decreases mast cell count, increases hyaluronate content, increases collagen formation and the tensile strength of the skin. On the other hand, estrogen increases mast cell activity and water content, and reduces the permeability of the small blood vessels (Asboe-Hansen, 1963).

Physical and Chemical Characteristics of Collagen

According to Harkness (1961), collagen is the most widely occurring protein in the animal body, and may constitute up to 30% of the total body protein. Gross (1961) reported that the properties of collagen are widely diverse. For example, in tendon it has a tensile strength equal to light steel wire, but in the cornea is as transparent as water. Collagen accounts for the toughness of leather, the tenacity of glue and the viscosity of gelatin (Gross, 1961). In addition, collagen has been

implicated in arthritus, atherosclerosis and other vascular diseases, and is closely related to the aging process in man and animals (Verzar, 1964).

Amino Acid Composition. The amino acids of mammalian collagen appear to be similar in most species (Wood, 1964; Ramachandran, 1963; Piez et al., 1960). Glycine forms nearly one-third of the total number of residues. Proline and hydroxyproline account for about 22% of the total, with hydroxyproline making up 10% and proline 12% of the residues (Piez et al., 1960). Tyrosine, histidine and the sulfur-containing amino acids are present in concentrations of less than 1%. It has been suggested that the tyrosine associated with collagen may be an impurity, since the concentration is lower in the derived gelatins (Eastoe and Leach, 1958). In addition, hydroxylysine is found in very low concentrations (less than 1%). It is now believed that the presence of both hydroxylysine and hydroxyproline are a requirement for admission of a protein to the collagen class (Harrington and von Hipple, 1961). Alanine forms approximately 11% of the total residues. The polar residues including amides constitute 22%, and the rest (approximately 11%) are composed of neutral residues (Ramachandran, 1963).

The fish collagens (icthyocol) exhibit an appreciably wider range in composition than those from the mammalian species (Piez et al., 1960). The total amount of imino acids in fish collagen is significantly lower than that from the mammalian collagens (Harrington and von Hipple, 1961).

The hydroxyamino acids (serine, threonine and sometimes hydroxylysine) are enhanced, leaving the level of hydroxyl groups about the same in both fish and mammalian collagens (Ramachandran, 1963). The methionine content of fish collagen is increased over that of mammalian collagen, while tyrosine and histidine comprise less than 1% of the total (Harrington and von Hipple, 1961). Although significant variations in composition are found among the fish collagens, the glycine content remains essentially constant for all species, composing about one-third of the total amino acid residues (Piez and Gross, 1960).

Amino Acid Sequence. Electron microscope studies have revealed that collagen fibrils show a regular axial repeating periodicity of approximately 640 Å, and a distinct intraperiod fine structure of alternating dark and light bands (Gustavson, 1956). The suggestion that the intraperiod fine structure is in close correlation with the discontinuity of the primary structure has received strong support by the work of Randall (1954). More recently, Kühn (1960) was able to prove convincingly that the dark portions of the cross striations were due to the accumulation of basic and acidic amino acids, while the light areas were due to regions formed mainly by the non-polar amino acids.

Utilizing enzymatic and chemical degradation methods, Hannig and Nordwig (1965) were able to demonstrate the presence of polar and apolar regions in the collagen molecule. They showed that the apolar regions were rich in the imino acids, while the polar regionswere poor in imino acids, but rich in polar groups.

Seifter et al. (1965) reported a discernible recurrence, if not a formal periodicity, of certain similar sequences of amino acids. This arises from the almost regular occurrence of glycine as every third residue of the polypeptide chain, and the frequent occurrence of a proline or hydroxyproline residue following glycine. These authors further stated that the amino acid sequence expressed as the general tripeptide, Gly-P-X (P is either proline or hydroxyproline), is located in the crystalline or non-polar regions of the molecule. These regions appear as the dark interbands in electron micrographs. Further, a group of these tripeptides alternate almost regularly with a long polypeptide series, the character of which reflects the presence of the polar side chains of residues of glutamic acid, aspartic acid and lysine (Seifter et al., 1965). These polar regions seem to be the amorphous areas of the molecule and appear as the light bands in electron micrographs. Grassman et al. (1961) reported that residues of arginine may occur as X in the Gly-P-X triad, and also as a less frequent component of the polar polypeptide region. This finding led these workers to postulate that the triad Gly-P-Arg may act as the link between the apolar and polar regions. Hannig and Norwig (1965) have isolated good yields of the fragments, Gly-P-Hypro.

Seifter et al. (1965) have proposed a model for the polypeptide chains of collagen as follows:

$$-(\text{Gly-P-X})_n\text{-polar sequence-}(\text{Gly-P-X-})_n\text{-polar sequence-}$$

They indicated that in this model, 50 to 60% of the molecule consisted of non-polar sequences [-(Gly-P-X-)$_n$-] alternating with polar sequences. The value of n for the non-polar regions may vary in different parts of the polypeptide chain. An average value of n = 5 to 6 accounts for the regular amino acid composition of collagen. This model does not exclude the occurrence of small amounts of aspartic or glutamic acid in the non-polar sequences, nor small amounts of proline and hydroxyproline in the polar regions (Seifter et al., 1965).

Ramachandran (1963) was unable to detect any N-terminal residues in native collagen, which he attributed to masking of the terminal residues by some other group. He was, however, able to detect small amounts of three C-terminal amino acids (serine, valine and leucine), which he believed to confirm the triple helix structure.

Secondary Structure. The triple-helix arrangement originally proposed by Ramachandran and Kartha (1955) and Cowan and McGavin (1955) is generally accepted as the best model for the conformation of the polypeptide chains of collagen. The currently accepted form, which best fits the wide-angle X-ray diffraction data, is fully discussed by Rich and Crick (1961) as follows:

1. The basic molecule of collagen consists of three polypeptide chains, each of which conforms to a left-handed helix.
2. Each complete turn of an individual helix requires three residues, and an 8.4 Å rise along the fiber axis.

3. Each three residue repeating element is hydrogen bonded to each of the other chains by one hydrogen bond.
4. The group of three chains is twisted so that they coil around one another in a gradual right-handed helix.
5. One complete turn of the super-helix contains thirty residues per chain.

Structure of the Collagen Fibril. Lloyd (1938) demonstrated the dissolution of tendon in dilute alkaline solutions. The solubilization of collagen in dilute acetic acid was reported by Nageotte and Guyon (1930). Orekhovitch and Shpikiter (1957) observed a protein from the skins of young animals, which was soluble in citric acid. This protein was precipitated on addition of suitable quantities of salt to form needles, which exhibited the typical cross banding of collagen fibers. The amount of citrate soluble material diminished with the age of the animal. It was theorized that the citrate soluble material was the metabolic precursor of insoluble collagen. These workers designated this protein as "procollagen".

Harkness et al. (1953) using labeled glycine, demonstrated a collagen fraction, which was soluble in alkaline phosphate and had a very high turnover rate. On the other hand, the acid soluble fraction obtained after extraction with phosphate buffer had a low turnover rate. These workers theorized that the alkaline soluble fraction was the true collagen precursor, and that acid soluble collagen was an intermediate in the synthesis of mature insoluble collagen.

Gross (1957) demonstrated the presence of a salt soluble fraction of collagen. On warming to body temperature, it polymerized spontaneously and formed typical cross striated fibrils showing the native collagen periodicity of 640 Å. If the gel was quickly cooled, most of the fibrils redissolved. If the gel was allowed to stand at body temperature for 24 hours, however, it no longer dissolved upon cooling. Upon aging at body temperature for two weeks, it became completely insoluble in dilute acid as well. The salt soluble fraction increased directly with the growth rate of the animal from which it was derived. If growth ceased as a result of starvation for a period as short as two days, this collagen fraction disappeared from the tissues.

Lowther (1963) using tracer techniques concluded that the tropocollagen extracted with citrate represented the earliest form of collagen. At the time of extraction, the citrate soluble fraction was probably diluted by the various pools of soluble collagen and was aggregated into fibers. This author further postulated that the tropocollagen, which was extracted with sodium chloride at low ionic strength at neutral pH, represented the more recently formed tropocollagen, and therefore had the highest specific activity. Over 90% of the radioactivity was present in the tropocollagen extracted at pH 7.0. The remaining 10% was largely localized in the acid soluble fraction, while the activity in the insoluble fraction was barely significant. Thus, Lowther (1963) concluded that the transition of salt soluble tropocollagen to acid soluble and insoluble collagen was slow by comparison with its rate of synthesis.

Numerous workers (Orekhovitch and Shpikiter, 1958; Houck et al., 1961; Kao and Bouceck, 1958; Gross, 1961) have reported that the amount of extractable tropocollagen decreases with the age of animal, and that more extractable tropocollagen can be obtained from rapidly growing animals. Bowes and Raistrick (1966) found that the percent of acid soluble collagen in sheep hide was not particularly high at birth, increased during the first period of rapid growth and then decreased with age. Salt soluble collagen followed a similar trend. These workers also found that the solubility of collagen in hides from sheep and cattle was directly related to growth rate. They concluded that the collagen content of skin increases with age and gradually becomes more stable. The hides from intensively fed animals, which developed quickly, were likely to be immature compared with traditional hides of the same weight. The immature hides had more soluble collagen, swelled more during liming, and after tanning the leather tended to be softer.

Collagen extractability is also altered in lathyritic animals. Levene and Gross (1959) studied the effect of certain simple organic compounds, such as beta-aminopropionitrile and aminoacetonitrile, on the connective tissues of growing animals. Administration of these compounds resulted in weakening of the connective tissues and a decided increase in the salt solubility of the collagen. These workers attributed this condition to an inhibition of the cross-linking process.

Levene (1962) demonstrated that certain carbonyl compounds can reverse the action of lathyrogens, and concluded that carbonyl groups

are necessary for normal maturation of the collagen molecule. Piez et al. (1966) have confirmed the presence of an aldehydic component in tropocollagen, resulting from a lysyl residue. They concluded that the inhibition of cross linking in lathyritic collagen was due to blocking of the lysine-aldehyde conversion.

Schmitt et al. (1953) found that manipulation of the solvent environment would result in reprecipitation of soluble collagen in at least five different fibrous forms. By adding increasing quantities of salt to a dilute acetic acid solution of soluble collagen, they produced reconstituted collagens showing the following characteristics:

1. In 1% salt solution, they observed the normal 640 Å periodicity.
2. In 12% salt solution, they obtained a 210 Å banding pattern.
3. In 15% salt solution, there was no periodicity.
4. If they added small amounts of polyanions, such as chondroitin sulfate, sulfated dextran, gum arabic, acid glycoprotein or thrombin instead of salt, the collagen precipitated in the fibrous long spacing (FLS) form with a non-polarized or asymmetrical repeating periodicity of about 2600 Å.
5. If they added small amounts of inosine or adenosine triphosphate, the collagen precipitated as the shorter or symmetrical segments having a total length of about 2600 Å. These are known as the segment long spacing (SLS) form. Both types of fibrous precipitates can be redissolved in dilute acetic acid to give normal tropocollagen solutions. These workers interpreted their results as indicating that collagen existed as a long-filamentous-polarized molecule.

Schmitt et al. (1953) postulated that the collagen molecule produces different patterns, either by parallel alignment in a quarter staggered arrangement to form the native collagen period, or by antiparallel alignment with the ends in register to form the FLS pattern. If the alignment is parallel with the ends in register, the SLS segments are produced. These workers further assumed that the specificity for these discrete interactions was built into the molecules by specific polarized arrangements of amino acids along the polypeptide chains. This assumption has been shown to be correct by Hodge and Schmitt (1958). These workers demonstrated that both basic and carboxyl groups are located at similar positions on the intact tropocollagen molecule, and that these polar groupings increase in intensity towards the ends of the molecule.

In 1961 Gross proposed the now well accepted theory for the synthesis of collagen. According to this theory, the fibroblasts synthesize complete collagen molecules and extrude them into the ground substance outside the cells, where they polymerize to form the fibrils. These rod-like molecules are approximately 2,800 Å in length and 14 Å in diameter. They have a molecular weight of approximately 300,000. The tropocollagen molecules probably overlap by one-quarter of their length, accounting for the 640 Å periodicity found in the fibrils. The charge distribution and three-dimensional configuration of adjacent sections are complementary, and therefore, attract each other. The decreased solubility with time is probably due to the increased formation of hydrogen bonds between aggregates of tropocollagen molecules. This may be due

to thermal agitation or the formation of intramolecular cross links in the tropocollagen molecule (Gross, 1961).

Work by Piez et al. (1961), on the fractionation of denatured collagen by CMC (carboxymethyl cellulose) and characterization of the isolated components, has shown that the collagen molecule is composed of three chains. The single α_2 chain was demonstrably different in amino acid composition and chromatographic behavior from the two α_1 chains. These workers further reported that as a function of time in vivo, crosslinks are formed between chains to produce double chain components. These β-components can be of two types. When the crosslinks are intramolecular, they are designated as $\beta_{1,1}$ (α_1 and α_1) or $\beta_{1,2}$ (α_1 and α_2). Lewis and Piez (1964a) reported that dogfish shark skin contained large amounts of a γ-component. This component chromatographed as a single peak in the position predicted for the trimer, $\alpha_1 - \alpha_1 - \alpha_2$. The amino acid composition was also consistent with this structure. Therefore, this component was appropriately designated as $\gamma_{1,1,2}$. This triple chained component arises from the denaturation of collagen, in which all three chains are covalently crosslinked. These studies led Lewis and Piez (1964a) to conclude that the β - and γ - components in soluble collagen arise as a result of an interchain, intramolecular process, which produces covalent crosslinks. However, later work by Bornstein et al. (1964) revealed the presence of a new β-component, which was identified as the dimer of $\alpha_2 - \alpha_2$ ($\beta_{2,2}$), by its amino acid analysis and sedimentation properties. Since each collagen molecule contains only one α_2 chain, $\beta_{2,2}$ must

originate from an intermolecular crosslink. These studies provided direct evidence indicating that both inter- and intramolecular crosslinking result from the same continuous process which produces aggregation.

Piez et al. (1965) reported that salt soluble collagen was composed predominantly of α_1 and α_2 chains, accounting of 80-90% of the total. In acid extracted collagen, there were larger amounts of $\beta_{1,1}$ and $\beta_{1,2}$, the combined total making up about 50-60%. Consequently, these workers theorized that acid extracted collagen was biologically older, and therefore, was more highly crosslinked than salt extracted collagen. It was further reported that the ratio of α_1 to α_2 was always 2.0, indicating that the collagen molecule had the formula $(\alpha_1)_2\alpha_2$.

Amino acid analysis of the collagen components (Rice et al., 1964; Piez et al., 1963) has shown that α_1 and α_2 differ significantly in their contents of 3- and 4-hydroxyproline, glutamic acid, proline, valine, isoleucine, leucine, hydroxylysine, lysine and histidine. Analysis of $\beta_{1,2}$ revealed that its composition was equivalent to the average for α_1 and α_2. On the other hand, analysis of $\beta_{1,1}$ showed it to be identical to α_1, and therefore a dimer of α_1 (Rice et al., 1964). The composition of unfractionated collagen was found to be the same as would be predicted from the structure of $(\alpha_1)_2\,\alpha_2$. The molecular weight studies of Lewis and Piez (1964b) have substantiated the above findings. The α_1 and α_2 subunits were found to have identical molecular weights, about 98,000. The molecular weight of $\beta_{1,2}$ was twice that of α_1 or α_2, with a value of

196,000. Based on these findings and assuming three α chains per molecule, native collagen should have a molecular weight of approximately 294,000. This value is in good agreement with the molecular weight of 310,000 reported by Rice et al. (1964).

Recent evidence (Piez et al., 1965) indicated that each of the three chains may be different. These workers found three α chains in cod fish collagen, which were present in equal amounts but differed significantly in their chromatographic behavior and amino acid composition.

Covalent Crosslinks. Franzblau et al. (1963) reported finding γ -glutamyl peptide linkages in native and soluble collagen. Their results indicated that 30% of the glutamic acid in ichthyocol collagen and 13% of the glutamic acid in soluble calf skin collagen occurred in the γ -peptide linkage. Gallop and Seifter (1962) also reported finding γ -glutamyl linkages in gelatin. Harding (1965) indicated that all of the natural occurring γ -glutamyl peptides have free α-carboxyl groups, and therefore, do not constitute branching points or crosslinks. However, Franzblau et al. (1963) pointed out that γ -glutamyl linkages introduce two methylene groups into the peptide chain. These may be of great importance in the configuration of the chains. Franzblau et al. (1963) further suggested that such linkages, presumably occurring in the polar regions of the collagen molecule, may be responsible for the disordered arrangement.

Ester linkages have been shown to occur and participate in the bonding of gelatin, and of insoluble and soluble collagen (Harding, 1965).

Blumenfeld and Gallop (1962) found that gelatin binds about 5 moles of hydroxamate per 100,000 gm. Sedimentation studies showed a decrease in the molecular weight to 20,000. This suggests that there are 4 to 5 polypeptide units in collagen, which are held together by ester-like linkages (Gallop et al., 1965). These workers also suggested that the terminal carboxyls of tropocollagen are involved in ester linkages. They also demonstrated that the α- and β-carboxy groups of aspartic acid participate in the hydroxylamine sensitive linkages.

Harding (1965) indicated that tropocollagen may contain carbohydrate as an integral part of the protein. All of the evidence indicates that the hexosamine found in collagen can be readily removed, whereas, about 50% of the original hexose is firmly bound to the peptide chains and can not be removed without being destroyed (Harding, 1965). Furthermore, the bound hexoses occur in the more unavailable regions of the collagen molecule, suggesting that they may take part in the crosslinks. Blumenfeld et al. (1963) have shown that mature collagen can be dissolved completely by periodate, and thus concluded that the oxidizable cross-links must involve hexoses.

Harding (1965) presented evidence showing that intramolecular cross-linking takes place by transferring the ester groups on to hexose residues. The origin of the alcohol donor of the ester linkage is not known. An ester to hexose ratio of 2 to 1 appears to be wide spread in tropocollagen, indicating two ester linkages per hexose unit (Harding, 1965). Mature collagen has approximately the same hexose content as tropocollagen,

but has an ester to hexose ratio of close to 3 to 1. This suggests that one hexose unit may be involved in three ester linkages, two of which are intramolecular, and one of which is intermolecular (Harding, 1965). Blumenfeld and Gallop (1962) suggested that the crosslinks occur in pairs every 640 to 700 Å in the collagen molecule. This indicates that the intermolecular linkages occur at the ends of the tropocollagen molecule, and that the intramolecular linkages arise from the same region. The side chain carboxyl groups of both aspartic and glutamic acid are thought to be involved in these linkages (Blumenfeld and Gallop, 1962).

Rubin et al. (1965) have shown that proteolytic enzymes will dissolve soluble collagen. This led them to conclude that the crosslinks are either a part of a separate peptide chain, or that the collagen molecule adjacent to the crosslink contains pepsin or trypsin sensitive bonds.

Recent studies by Rojkind et al. (1966), Bornstein et al. (1966a,b) and Bornstein and Piez (1966) demonstrated the presence of an aldehydic component in tropocollagen, and in cyanogen bromide-cleaved peptides from α_1 and α_2 chains of rat skin collagen. These workers concluded that a lysyl residue in each chain is converted to the δ-semialdehyde of α-aminoadipic acid. They further suggested that this step is preliminary to the formation of an intramolecular crosslink by aldol condensation. This postulation has been supported by Piez et al. (1966), who showed that inhibition of crosslinking is a result of blocking the lysine-to-aldehyde conversion.

Boedtker and Doty (1956) postulated that the collagen molecule is arranged so that the chains are staggered. A dangling peptide protrudes

beyond the rigid three-stranded portion at either end of the molecule. Hodge and Schmitt (1958) proposed that these dangling peptides are necessary for aggregation of native tropocollagen. Gallop (1955) reported that the molecular length of native collagen was not affected by tryptic attack. However, the native type aggregates were not obtainable after trypsin digestion, which suggests that trypsin attacks the end regions of the molecule.

Hodge et al. (1960) subsequently isolated an acidic tyrosine-containing peptide from tryptic digests. This led them to suggest that the proteolytic activity was confined to the end regions of the molecule. However, Hodge and Petruska (1963) demonstrated that tropocollagen monomers overlap instead of aggregating end-to-end. They implied that if peptide appendages are needed for polymerization, they are not necessarily restricted to the end regions of the tropocollagen molecule. Numerous workers (Drake et al., 1966; Rubin et al., 1965; Kühn et al., 1955; Schmitt, 1964) have postulated that the tropocollagen molecule is composed of non-helical portions at the ends with other peptides protruding from the sides. This would allow both side-to-side and end-to-end aggregation.

Swelling of Collagen. Collagen undergoes characteristic swelling and dimensional changes, when subjected to various solvents. The swelling characteristics of collagen have been extensively reviewed by Gustavson (1956). He has classified collagen swelling as being due to either osmotic or lyotrophic swelling. Osmotic swelling occurs in solutions of either

acids or bases, and causes the fibers to decrease in length and increase in diameter. Osmotically swollen fibers have a glassy appearance and are pressed together. The plumping effect of osmotic swelling can be depressed or reversed by the addition of neutral salts to the solution.

Gustavson (1956) reported that osmotic swelling is due to the freeing of ionic groups by acids or bases, which leads to the establishment of a Donnan membrane potential inside the fibers. This in turn brings about an inflow of water to equalize the concentration of ions between the substrate and the external solution. These changes distort the structure by twisting the fibers, shortening their length, and expanding their diameter. The depressing effect of neutral salts on osmotic swelling apparently is the result of a reduction in anion concentration differences between the two phases.

According to Gustavson (1956), lyotrophic swelling is due to the interaction of ions and molecules with nonionic protein bonds, probably hydrogen bonds. In lyotrophic swelling, only the width of the fiber increases. This is a result of lessened cohesion and the separation of the component fibrils, while they remain flaccid and opaque. This type of swelling is only partially reversed by removal of the lyotrophic agent, and more or less permanent swelling is produced.

Gustavson (1956) further pointed out that the swelling of collagen in dilute solutions of weak acids, for instance a 3 M solution of acetic acid at pH 2.0, apparently involves both the lyotrophic or Hofmeister effect, and the electrostatic or osmotic effect. This is not a specific

ion effect but a specific molecule effect, since the un-ionized acid is the swelling agent. The molecule probably competes with the peptide groups involved in the intermolecular linkages of the protein chains. This results in a partial rupture of the hydrogen bonds and an association of the acetic acid molecules with the exposed groups. Gustavson (1956) has attributed the different swelling characteristics in acids and bases to the efficient crosslinking of the collagen chains.

On the basis of X-ray diffraction and density studies, Fels (1966) reported that changes in the swelling characteristics of collagen were due not only to crosslinking, but also to the degree of crystallinity or molecular ordering. Gustavson (1956) also reported that the amount and arrangement of the collagen fibers had a marked affect on swelling. He further stated that isolated fiber bundles of oxhide would take up about 20 times their weight in water, whereas, the whole hide took up only 3-4 times its weight. Similar results were found by Kwon et al. (1964). These workers reported that removal of the restrictive membrane from tendon increased the amount of swelling in distilled water, and reduced the thermal shrinkage temperature.

Thermal Shrinkage. According to Gustavson (1956), the hydrothermal shrinkage of connective tissue is one of the outstanding characteristics of the principal constituent of connective tissue, i.e., collagen. Mammalian collagen fibers in water at 60 to 70°C contract from about one-quarter to one-third of their initial length. The shrunken specimen feels like glue and shows a rubber-like elasticity. The tensile strength of

the fiber is greatly lowered, and the original resistance of native collagen to trypsin is destroyed (Gustavson, 1956).

The shrinkage temperature of collagen has been reported to be markedly affected by interaction with acids, bases and tanning agents (Harrington and von Hipple, 1961); by biological age (Verzar, 1964); by species differences (Gustavson, 1956); and by ionizing radiation, pregnancy, nutritional status and disease (Verzar, 1964).

According to Rigby (1964), collagen exhibits a number of thermal transitions on heating. The most widely known change takes place at the shrinkage temperature (T_s), which is defined as the temperature at which an unconstrained sample in 0.9% sodium chloride undergoes an abrupt dimensional change. Another thermal transition occurs at the equilibrium melting temperature (T_m), which is defined as the temperature of equilibrium between the crystalline and amorphous phases in a partially melted sample. Unconstrained mammalian collagen in 0.9% saline has a T_s of approximately 59-60°C, and a T_m of about 49-50°C (Rigby, 1964).

A further transition takes place in unextended collagen at temperatures well below the T_s and T_m (Rigby, 1964). This transition has some of the characteristics of a glass transition, and is denoted as T_g. It occurs between 40 and 50°C in native collagen. The melting or uncoiling of the three polypeptide chains of the tropocollagen molecule in solution is referred to as T_d, and for mammalian samples occurs at 35-40°C (Rigby, 1964).

Flory and Garrett (1958) have hypothesized that the shrinkage temperature (T_s) of native collagen represents fusion of the crystalline-

oriented polypeptide chains. These authors further stated that the difference between T_s and T_m is due to superheating, with the T_s measurements being as much as 10°C above the true melting point (T_m).

Mason and Rigby (1963) reported that the abrupt changes in viscosity and optical rotation, which occur in dilute solutions of native collagen upon heating to 35°C (T_d), are related to the T_s of macroscopic fibers. They are actually manifestations of the same configurational transformations, except for the difference in concentration. Thus, T_s, T_m, T_d and T_g are apparently a part of the same phenomenon (Mason and Rigby, 1963).

Scheraga (1960) has further clarified the thermal changes occurring in collagen. According to him, both intrahelical backbone hydrogen bonds and interhelical side chain hydrogen bonds are disrupted by the temperature of thermal contraction. This results in the disappearance of the crystalline structure. The stability of the helix is provided by hydrogen bonds between the polar side chain groups. The destruction of these bonds results in the randomly coiled form.

Scheraga (1960) further postulated an intermediary reaction, in which only the side chain hydrogen bonds are destroyed, while the backbone hydrogen bonds still exist. This would result in liberation of collagen components having no intermolecular covalent linkages. Upon further heating, the backbone hydrogen bonds are also destroyed and the fiber goes from the crystalline to the randomly coiled stage (Scheraga, 1960). At this point, however, heat-resistant covalent crosslinks remain. Thus, the fiber will be rubbery and elastic, and will remain in this

state if cooled immediately. If the temperature is raised or the heating period prolonged, however, then the covalent bonds will also be destroyed. Complete relaxation will follow, elasticity will disappear, and no thermal contraction will be possible (Scheraga, 1960).

Piez and Gross (1960) reported on the relationship between the amino acid composition of numerous collagens and their T_s values. They found statistically significant correlations between T_s and proline, hydroxyproline and total imino acid content. The best correlations were found to exist between T_s and the total imino acid residues. von Hipple and Harrington (1959) reported that there was a restricted rotation of bonds adjacent to the pyrrolidine rings in the collagen structure. This led the above workers to postulate that the stereochemical properties of pyrrolidine ring-containing residues in the polypeptide chain environment might be the key factor in stabilizing the collagen structure, rather than interchain hydrogen bonding. However, Doty and Nishihara (1958) theorized that in the solid state, both the stabilization provided by the energy of crystallization and the intramolecular forces stabilizing the molecules themselves must be overcome. They found that in all classes of collagen, the temperature differences (T_s-T_d) were essentially constant at 27° C. On this basis, they concluded that differences in thermal stability of the various collagens depend on intramolecular processes rather than intermolecular interactions.

Harding (1965) reported that the shrinkage temperature of collagen isolated from the skin of young children was lower than that of adult

human collagen. Verzar (1964) found that the shrinkage temperature of collagen increased with the age of the individual, but decreased in certain pathological conditions.

In addition to the physical changes observed in collagen during thermal shrinkage, Verzar (1964) found that chemical changes also occur. He found that a certain amount of collagen was released from the fiber and went into solution during thermal contraction. The dissolved collagen was referred to as labile collagen and was found to decrease as the animal became older. This suggests the formation of a larger number of cross-links with age. Verzar (1964) also postulated that hydrogen bonds are destroyed by heating to the T_s, thus liberating some hydroxyproline containing complexes. He further reported that a slow and diminished release of labile collagen was characteristic of old age, supporting the theory that aging of collagen is caused by increased crosslinking.

Wiederhorn *et al.* (1953) postulated that crosslinking explains the stress-strain properties of heat-shrunk collagen. These authors suggested that heat-shrunk collagen obeys the kinetic theory of rubber elasticity. They stated that an ideal crosslinked rubber is a network of flexible chains tied together at various points along their length by linkages, which have a total stability approximating that of the bonds comprising the chains. They further indicated that there must be no attractive or repulsive forces between the different chains or between various portions of the same chain.

Wiederhorn and Reardon (1952) further clarified the application of the kinetic theory of rubber elasticity to collagen. They stated that

the tensile stress required to deform a solid, which does not flow, may be given as the sum of two terms:

$$f = \left(\frac{\partial E}{\partial L}\right)_T - T \left(\frac{\partial S}{\partial L}\right)_T \qquad (1a)$$

where:

f = the retractive force per unit area.

$(\partial E/ \partial L)_T$ = change in internal energy with length at constant temperature.

$(\partial S/ \partial L)_T$ = change in entrophy with length at constant temperature.

T = absolute temperature.

Equation 1a is a general one and applies to all materials (Wiederhorn and Reardon, 1952). In the case of rubbery polymeric materials, which do not exhibit flow, the energy term is small. For ideal rubbers it is assumed to have no influence. For such materials the following equation developed by Wiederhorn and Reardon (1952) applies:

$$f = - T \left(\frac{\partial S}{\partial L}\right)_T \qquad (1b)$$

Wiederhorn and Reardon (1952) further stated that for a material consisting of a large network, whose basic element is a flexible chain extending between junction points, the following equation applies:

$$f = - T \left(\frac{\partial S}{\partial L}\right)_T = \frac{RT\theta \, v_2^{1/3}}{Mc} \left(\alpha - \frac{1}{\alpha^2}\right) \qquad (2)$$

where:

Mc = average molecular weight of the chain between junction points.

α = extension of the material, i.e., the stretched length divided by the initial length.

ρ = density of the dry elastomer (1.33).

v_2 = volume fraction of the elastomer being stretched.

R = gas constant.

T = absolute temperature.

According to Wiederhorn and Reardon (1952) Equation 2 predicts that the elastic behavior of a rubber-like solid is determined by only one molecular constant, Mc, which is the molecular weight between junction points. Equation 2 is applicable to any chain-like molecule, irrespective of its structure or chemical composition (Wiederhorn and Reardon, 1952).

Utilizing this technique on kangaroo tail tendon, Wiederhorn and Reardon (1952) reported a value of 55,000 as the molecular weight between crosslinks. They calculated a unit cell of 566 Å, which corresponds to that weight. The authors pointed out that this value is in close agreement with the 640 Å periodicity exhibited by dry collagen. Utilizing the same material after formaldehyde treatment, the molecular weight between crosslinks fell to approximately 15,000, indicating the introduction of new crosslinks (Wiederhorn et al., 1953).

Kulonen et al. (1963) reported a decrease in molecular weight between crosslinks in rat skin collagen, which was associated with biological age. Their results indicated that the crosslink density in rat skin collagen increases with age.

Gustavson (1956) pointed out that, inasmuch as the collagen structure varies directly with chain spacing and spatial orientation, the crosslinks probably possess different energies. In addition, some regions apparently contain fewer crossbridges than others. The author further indicated that small regions of the collagen lattice, which contain a limited number of crosslinks of low energy, should offer ideal conditions for initiating shrinkage, i.e., a shrinkage nucleus. As the temperature is raised, more energy would be supplied and an additional number of crosslinks would be ruptured, increasing the thermal movement of the chains. Finally a point will be reached, at which the structure is weakened to the extent that chain folding will occur. Gustavson (1956) further stated that the process of shrinkage is a rate phenomenon and that a true shrinkage temperature does not exist from the thermodynamic point of view.

Witnauer and Wisnewski (1964) described a method for the absolute measurement of the shrinkage temperature of leather utilizing differential thermal analysis (DTA). This method involves continuous comparison of the temperatures of the sample and an inert reference material (usually glass beads), while both are heated at a constant rate. The temperature differential is plotted as a function of sample temperature. An endothermic phase change, such as that observed during collagen shrinkage, results in a negative deviation from the base line. This results in a peak at the point of maximum transition. The temperature at which shrinkage begins and the general shape of the curve yields valuable additional information about the hydrothermal stability of the sample.

Witnauer and Wisnewski (1964) noted that the transition for leather specimens took place over a 10°C temperature range. In addition, the temperature from the onset to the peak, which ranged from 8 to 20°C, appeared to depend on the type of leather. No attempt was made to correlate this range with type of tanning agent, source of skin or any other variable. The same authors also observed that the shrinkage temperatures, as determined by conventional measurements (change in length versus temperature measurements), fell somewhere between the extrapolated onset and the peak DTA temperatures. They concluded that the peak in the DTA thermogram was a manifestation of the shrinkage of leather.

Dickson (1966) also utilized DTA to study different areas of the human intervertebral disk. His results indicated that the peak temperature of both nuclear and annular areas of the disk was higher in specimens from young individuals than in those from older ones.

Pale, Soft and Exudative (PSE) Pork

Ludvigsen (1953) first described a condition in swine, which he called "muscle degeneration disease". The muscle is pale, soft and watery in appearance and has been called "white muscle disease" (Kjølberg, 1963), or more recently has been abbreviated from "pale, soft and exudative" to "PSE" (Briskey and Wismer-Pedersen, 1961). This condition has been encountered in swine throughout the world and has been estimated to vary from an incidence of 18% of all hogs slaughtered in the United States (Forest et al., 1963) to 35-40% in Denmark (Clausen et al., 1960).

Characteristics of Pale, Soft and Exudative (PSE) Tissue. It is now generally accepted that the PSE condition is associated with an accelerated post-mortem glycolysis and a rapid onset of rigor-mortis (Briskey and Wismer-Pedersen, 1961; Briskey et al., 1962; Kastenschmitt et al., 1964). Briskey (1963) reported that when post-mortem changes take place at a rapid rate under acid conditions and high temperature, the meat develops the undesirable characteristics associated with PSE muscle.

Bendall and Wismer-Pedersen (1962) reported that muscles allowed to go into rigor-mortis at a temperature of 37°C always become watery and pale in appearance. However, this was not consistently observed with intact carcasses by Sayre et al. (1963b), or in muscle strips by Briskey et al. (1962). Briskey (1964) postulated that the enzyme systems in the muscles of certain pigs were highly sensitive to temperature.

From the findings of McLoughlin (1963) and Sayre and Briskey (1963), it seems certain that the sarcoplasmic proteins are severely denatured or reduced in solubility during rapid glycolysis. Cassens et al. (1963) reported that the sarcoplasmic proteins appear to be denatured in severe PSE muscle. In addition, the ultrastructure of the myofibrillar proteins also appears to be altered. Numerous workers (Wismer-Pedersen, 1959; Bendall and Wismer-Pedersen, 1962; Scopes, 1964) have reported decreased myofibrillar protein solubility in muscles which undergo an accelerated drop in pH while at high temperatures. Bendall and Wismer-Pedersen (1962) concluded that the decrease in solubility was due to denaturation of the sarcoplasmic proteins, which were deposited upon and firmly bound to the myofibrillar proteins.

Connective Tissues in PSE Muscle. The literature on the biochemistry, physiology, and histology of this condition is well documented in relation to the muscle mass. However, very little has been reported on the relationship of the PSE condition to the connective tissues.

Briskey (1963) postulated that a relationship existed between the development of this condition and the loss of intermuscular binding in PSE hams. Sayre et al. (1963b) demonstrated that in severe cases, PSE muscles lose their connective tissue attachments and can be easily pulled from the carcass. These investigators attributed this to a combination of high temperature and low pH, which apparently resulted in thermal shrinkage or hydrolysis of the connective tissue attachments to the bone. However, Briskey (1963) in reviewing some unpublished work by Sayre indicated that there was no difference in the hydroxyproline content of muscle extracts from normal and PSE tissue.

MATERIALS AND METHODS

Selection and Treatment of Samples

The epimysial connective tissues from the l. dorsi muscles of 12 market weight pigs and 2 commercial grade cows were used for physical and chemical characterization. In addition, epimysium and corium samples from 2 normal seven-week old pigs, and 2 zinc deficient seven-week old pigs were also utilized.

The epimysium was removed from the anterior portion of the loin in the region of the 3rd and 4th ribs at 48 hr post-mortem, and dissected free of adhering fat and muscle. Corium samples were removed from the same general area and treated in a similar manner.

A portion of the sample was used for histochemical and histological studies. The remaining portion of each sample was frozen in liquid nitrogen and stored at -20°C for subsequent physical and chemical studies.

Turbidity and Hydrogen Ion Determinations

Turbidity Values. The turbidity method of Hart (1962a) was used in order to determine the severity or relative freedom from the PSE condition in the market weight pigs. A 10 gm aliquot of the sample was placed in a 50 ml centrifuge tube along with 30 ml of distilled water. The muscle-water solution was mixed thoroughly and extracted for 18 hr at 2°C. The mixture was then centrifuged for 30 min at 1,000 rpm, and the supernatant was filtered through Whatman #1 filter paper. Exactly 5 ml

of disodium phosphate-citric acid buffer at pH 4.6 was mixed with 1 ml of the muscle extract. The mixture was allowed to stand for 30 min at 20° C. A blank was also prepared by mixing 1 ml of the muscle extract with 5 ml of distilled water. The degree of turbidity was measured in a spectronic 20 spectrophotometer at a wavelength of 600 mμ, after setting the blank to read zero. Turbidity was always measured within 5 min after removing the sample from the 20° C water bath.

Hydrogen Ion Concentration. The pH of the muscle samples was determined at the same time as the turbidity. Equal volumes of muscle tissue and distilled water were mixed in a Waring blender, and pH readings were made with a Beckman Zeromatic expanded scale pH meter to the nearest 0.01 pH unit.

Histological and Histochemical

Fixing, Embedding and Sectioning. Samples for histological and histochemical studies were fixed for 24 hr in a formalin-TCA-picric acid fixing solution (Appendix 1). After fixing, the samples were washed for 2 hr in 35% ethanol followed by a 2 hr wash in 50% ethanol. They were then stored in 70% ethanol until used. Prior to staining, the fixed samples were dehydrated, cleared and infiltrated by passing them up through the alcohol series to xylene and paraffin (15 min/step). The samples were then embedded in paraffin and sectioned 10 μ in thickness. The paraffin was removed from the sections by placing them in xylene for

15 min. They were then rehydrated by going down through the alcohol series to water, allowing 1 min in each solution.

The frozen samples were mounted on chucks and sectioned 8 μ in thickness using an International Cryostat.

Verhoeff's Elastin Stain. The fixed sections were stained with Verhoeff's elastin stain according to the method of Pearse (1960). Sections were brought down through the alcohol series to tap water, stained in Verhoeff's stain until black (15 min), and rinsed with water. The samples were treated with 2% ferric chloride until the elastic fibers and nuclei were properly differentiated (30-45 sec). Sections were then rinsed in distilled water, dehydrated by taking them up through the alcohol series, and mounted with Permount. Staining by this method rendered the elastin fibers black, the nuclei gray, the collagen fibers red and the background structures and cells yellow.

Hematoxylin-Eosin. Similar sections of all fixed samples were also stained with hematoxylin-eosin by a modification of the methods of Davenport (1961) and Lillie (1954). The sections were brought down through the alcohol series to water, stained for 15 min in Ehrlich's hematoxylin, washed in tap water and differentiated for 30 sec in 0.5% HCL in 95% ethanol. They were then rinsed with two changes of distilled water, and stained for 3 min in 0.1% eosin-Y in 50% ethanol. They were rinsed with 70% ethanol and dehydrated for 1 min in both 95% and absolute ethanol. The sections were then cleared in xylene for 10 min, and mounted in Permount. Staining by this method resulted in blue nuclei. The calcium and

cartilage deposits were dark blue, with the cytoplasm, muscle fibers and other structures being stained various shades of blue.

Mallory's Anilin Blue Stain. Comparable fixed sections were stained with Mallory's stain according to the method of Davenport (1961). Sections were brought to 70% ethanol, and stained for 10 min in 0.5% aqueous acid fuchsin. Without washing, the samples were then passed into anilin blue-orange G stain and stained for 20 min. Samples were then rinsed in 3 changes of 95% ethanol, dehydrated in absolute alcohol for 1 min, cleared in xylene for 10 min and mounted in Permount. Staining by this procedure rendered collagen blue, elastin fibers pale pink or yellow, the ground substance blue and the nuclei red.

Toluidine Blue Stain. Both fixed and frozen samples were stained with toluidine blue by the methods of Van Alten and Fennell (1957) and Pearse (1960). The fixed samples were taken down through the alcohol series to water, placed in buffer solutions at either pH 2.2, 5.6, or 8.0 for 3 hr and stained for 30 min in 0.1% toluidine blue buffered to the same pH. The samples were then rinsed in the same buffer and mounted in Permount.

The frozen sections were handled in the same manner as the fixed samples after the latter had been rehydrated by going down through the alcohol series.

Two independent series of samples were prepared for each animal at each pH and fixing condition. The light transmitted through the preparations was measured in arbitrary units with a Leitz Dialux microscope

equipped with a Vickers Instruments photo-multiplier and an automatic integrating exposure unit.

Extraction and Purification of Salt and Acid Soluble Collagen

Extraction Procedure. The salt and acid extraction procedure was a modification of the methods of Gross (1957) and Harkness et al. (1953). Samples of the epimysium to be extracted were comminuted in a Waring blender with dry ice. Aliquots weighing 6 gm were placed into 100 ml polyethylene centrifuge tubes. A total of 60 ml of 0.45 M sodium chloride was added, and the samples were extracted for 18 hr at 2°C. They were then centrifuged at 2,500 rpm for 60 min, and filtered through coarse- and medium-sintered glass filters. The samples were washed with water (30 ml) and recentrifuged for 30 min at 2,500 rpm. The rinse water was filtered and added to the extract. The combined filtrates comprised the salt-soluble fraction.

A total of 60 ml of 0.15 M citrate buffer at pH 3.7 was then added to the residue. Extraction was carried out for an additional 18 hr and the samples were centrifuged and filtered as before. This extract composed the acid soluble fraction. Aliquots of the salt and acid extracts were removed for hydrolysis and hydroxyproline determinations.

Purification Procedure. The remaining acid extracts were purified according to a modification of the method of Rubin et al. (1965). Solid potassium chloride was added to the acid filtrates to bring the concentration to 0.6 M. Dibasic potassium phosphate was added to adjust the

pH to 5.8, and the solution was allowed to stand 48 hr at 2°C. It was then centrifuged at 2,500 rpm for 60 min, and the supernatant was decanted. The precipitate was redissolved in 0.2 M acetic acid overnight. The solution was then centrifuged at 25,000 g for 60 min. The supernatant was decanted and dialyzed against 1% sodium chloride for 48 hr. The solution remaining in the dialysis tubing was centrifuged at 2,500 rpm for 60 min. The precipitate was then dissolved in 0.2 M acetic acid, and dialyzed against 0.2 M acetic acid for 24 hr. The contents of the dialysis bag were then lyophilized, and the dry, salt-free collagen was stored at -20°C.

Disc Gel Electrophoresis

Cyanogum (E. C. Apparatus Co.) was used for making the gels instead of the usual acrylamide. The working solutions utilized for disc gel electrophoresis are shown in Appendix 2. The ammonium persulfate solution was mixed with equal volumes of either lower or upper gel immediately before use. TEMED (N, N, N', N'-tetramethylethylenediamine) was added to the combined ammonium persulfate-gel solution at the rate of 0.6 ml/100 ml. The disc gel tubes were immediately filled to within 2 cm of the top, and carefully overlaid with distilled water. The tubes were allowed to polymerize at room temperature for 15-20 min. After polymerization, the water and unreacted monomer were poured from the tubes, and the gel was rinsed with upper gel solution. A 1.5 cm column of upper gel solution was added to the tubes, overlaid with water and allowed to polymerize for 15-20 min.

The tubes were then placed in the disc gel apparatus, and buffer (Nagai et al., 1964) was added to the buffer tanks.

The lyophilized protein was dissolved in 0.05% acetic acid by stirring overnight at 3°C. Prior to applying the samples to the disc gel tubes, sucrose was added to a final concentration of 5%. The protein solution was then heat denatured by warming to 45°C for 30 min. The sucrose increased the density of the protein solution and permitted layering of the sample directly on the spacer gel. A current of 5 ma/tube was applied for a period of 1 hr.

After electrophoresis, the gels were removed from the tubes and stained for 30 min. The dye solution contained 2 gm of Amido Black 10-B dissolved in a mixture of 250 ml of water, 250 ml of methanol and 50 ml of acetic acid. The gels were destained by soaking overnight in 7% acetic acid.

The α to β ratios were determined on the destained gels utilizing a Canalco, Model F, Electrophoresis Microdensitometer with an electronic integrator.

Thermal Labile Collagen

Determination of the heat labile collagen was according to a modification of the procedure of Verzar (1964). A total of 10 ml of a 0.9% sodium chloride solution was placed in a test tube along with 0.5 gm of connective tissue. The samples were heated in a water bath for 10 min at 65°C, and then cooled in an ice bath. Centrifugation was carried out at 1,000 rpm for 15 min, and the supernatant was filtered into test tubes

through Whatman #1 filter paper. Both the supernatant and the residue were brought to 6 N by adding concentrated HCL. The test tubes containing both the supernatant and the residue were heat sealed and hydrolyzed for 15 hr at 120°C. The hydrolyzates were then neutralized, made to volume and aliquots were taken for subsequent hydroxyproline determination.

Hydroxyproline Determinations

Hydroxyproline was determined according to the procedure of Woessner (1961) as modified by McClain et al. (1965). The hydrolyzed samples were diluted to contain 0.5-2.5 μg/ml of hydroxyproline. A 2 ml aliquot was placed in 16 by 150 mm test tubes. Hydroxyproline oxidation was initiated by adding 1 ml of chloramine T solution to each tube. The contents were mixed and allowed to stand for 5 min. One ml of p-dimethylaminobenzaldehyde solution was added, the solution was thoroughly mixed, and the tubes were placed in a 60°C water bath for 5 min. The absorbancy of the developed color was measured at 557 mμ in a Beckman DU spectrophotometer. Hydroxyproline was determined directly from the standard curve, which was prepared prior to each run. A conversion factor of 7.14 was used to convert hydroxyproline to collagen.

Thermal Shrinkage Temperatures

Melting Points. Melting points were determined on the epimysial collagen by a method similar to that of Gross (1964). Samples were floated in 0.9% sodium chloride on the heating stage of a Fisher-Johns

melting point apparatus. A cover slip was placed over the heating stage, and the samples were subjected to heat at the rate of 3°C/min. The temperature was noted and recorded at the beginning of shrinkage, at the end of shrinkage and at the temperature of complete dissolution.

Differential Thermal Analysis. Differential thermal analysis (DTA) was employed for studying the thermal behavior of the epimysial connective tissue. The instrument used for this investigation was the DuPont 900 Differential Thermal Analyzer. Approximately 30 mg of sample along with 60 mg of 0.9% saline solution was placed in a glass tube 25 mm in length and 4 mm in diameter. Glass beads were placed in a similar tube to a depth of 7 mm, and served as the reference standard. Thermocouples were placed into the center of the tubes, and the samples were inserted into position. The T scale was set at 10°C/in, the Δ T scale at 0.2°C/in and the base line slope at -0.2. The starting temperature was adjusted to approximately 25°C by means of the cooling unit. The heating rate was 10°C/min under an air atmosphere.

Stress-Strain Studies

Apparatus and Sample Preparation. Before conducting stress-strain determinations, samples approximately 2 cm in length and 1 cm in width were soaked for 2 hr in 0.9% sodium chloride solution.

The procedure employed for these measurements consisted basically of mounting the sample described above between two clamps. The upper

clamp was attached to a Statham, Model UL5, Microscale Assessory and Universal Transducing Cell. The universal cell was attached to a Statham Precision Readout (Model UR4). The lower clamp was mounted on a rigid rod, which could be moved vertically, thus extending the sample. The distance between the upper and lower clamp could be recorded accurately by means of a dial micrometer.

The apparatus was arranged so that a constant temperature bath could be raised to the height of the two clamps, thus immersing the sample in water at 85°C.

Measurements and Calculations. Samples were held at 85°C for 30 min before making the length and force measurements. Samples were subsequently cut from the clamps and their mass determined. The dry weight of the sample was determined by drying at 95°C for 24 hr. The cross sectional area was calculated from the dry weight and the length after thermal shrinkage, assuming a density of 1.33 gm/cc. The volume fraction of the protein in the sample was calculated from the dry and wet weight. The molecular weight between crosslinks was calculated according to a modification of the formula of Wiederhorn et al. (1953) as shown in Appendix 3.

Swelling Studies. Swelling characteristics of the epimysial connective tissues were evaluated by the change in weight observed after soaking in various solvents. Dimensional changes were also measured for the swollen samples.

Amino Acid Analysis and Plasma Hydroxyproline Levels

Amino Acid Analysis. Amino acid analysis was performed on 24 hr acid hydrolyzates of the acid soluble collagen using a Beckman Amino Acid Analyzer, Model 120B (Moore et al., 1957). About 3 mg of dried protein was weighed directly into a 10 ml hydrolysis tube fitted with a ground glass stopcock. The contents of the tubes were frozen in a dry-ice-ethanol bath, and the tubes were evacuated under high vacuum. The samples were allowed to melt slowly while under vacuum in order to remove any gases present. After evacuation the tubes were sealed, and the protein solution was hydrolyzed for 24 hr.

The hydrolyzate was transferred to an evaporating flask, and evaporated to dryness on a rotary evaporator. The residue was taken up in a small amount of distilled water and again evaporated to dryness to remove the HCL. The sample was then taken up in citrate-HCL buffer (pH 2.2). An aliquot was removed for analysis on the amino acid analyzer. Standard amino acid mixtures were also run.

Plasma Hydroxyproline. Blood samples were taken at the time of exsanguination. Samples were collected in heparinized centrifuge tubes, and centrifuged for 40 min at 2,000 rpm. The plasma was frozen and stored at -20°C for subsequent hydroxyproline determinations.

Statistical Analysis

The statistical methods employed in this study were outlined by Snedecor (1956). Analysis of variance and standard deviations were calculated for the various treatment groups.

RESULTS AND DISCUSSION

Turbidity and pH Values

Table 1 lists the turbidity and ultimate (48 hr) pH values for the *L. dorsi* muscles from the pigs used in this study. The mean turbidity value for the PSE muscles was 92.00 as compared to 26.67 for the normal muscles. The markedly lower turbidity for the PSE samples indicated that much of the protein in the PSE muscles had been precipitated.

Analysis of variance revealed a highly significant ($P < 0.01$) difference between the turbidity values of the normal and PSE muscles (Appendix 4). These results agree with those of McLoughlin (1963) and Sayre and Briskey (1963), who reported that the sarcoplasmic proteins are severely denatured and lose much of their solubility as a result of rapid glycolysis at high temperatures.

Results of the turbidometric measurements were also in good agreement with subjective evaluations. In general, there was very little turbidity in the sarcoplasmic extracts from PSE muscle. Certain muscles were observed to be soft and exudative, but appeared to be normal in color. On the other hand, others were pale but not soft and exudative. It is suggested that perhaps these types represent intermediate stages of the PSE condition. A study of the sarcoplasmic protein solubility and pH changes in these muscle types would be desirable.

The mean ultimate pH values for the normal and PSE muscles are listed in Table 1. The average pH value for the PSE muscle was 5.49, and that of the normal was 5.50. Data from analysis of variance are shown in

Appendix 4. The difference was not statistically significant, as would be expected in view of the similar values for the two types of muscle.

Table 1. Turbidity values and ultimate pH of normal and PSE 1. dorsi muscle.

PSE			Normal		
Animal No.	Turbidity[1] value	Ultimate pH	Animal No.	Turbidity[1] value	Ultimate pH
1	99.00	5.52	2	52.00	5.50
4	95.50	5.60	3	33.00	5.55
6	93.00	5.43	5	35.00	5.40
8	98.50	5.40	7	18.00	5.52
10	98.50	5.50	11	12.00	5.45
14	97.50	5.52	13	10.00	5.60
Mean	92.00	5.49		26.67	5.50
Std. dev.	11.72	0.06		16.24	0.06

[1] % transmittance

These results do not agree with those of Briskey et al. (1959a,b), who reported that muscles developing the PSE condition had significantly lower pH values at 24 hr post-mortem than normal muscles. Extremely low ultimate pH values have also been observed by Hart(1962b) and Lawrie et al. (1958). Briskey and Wismer-Pedersen (1961) reported that PSE muscles reached an ultimate pH of as low as 5.1, but frequently increased to between 5.3 and 5.6 after cooling.

Results of the present study are substantiated by Bendall and Wismer-Pedersen (1962), who refuted the suggestion that the depression and elevation of ultimate pH was a factor in the development of PSE muscle. Wismer-Pedersen (1959) also found that PSE muscles from Danish Landrace pigs usually have normal ultimate pH values.

Based on the above reports and the results of the present study, it appears that the temperature at which the pH value falls below 5.5 is probably more critical than the ultimate pH in determining the occurrence of the PSE condition.

Histological and Histochemical Studies

PSE Musculature. Plate 1 shows typical examples of PSE and normal *l. dorsi* muscles. The characteristic pale grayish color was readily apparent in the PSE samples, while the normal muscles maintained a desirable red color and dry surface. The phenomenon of special interest was the apparent loosening of the attachment between the muscle and the epimysial connective tissue (Plate 2).

Beta Metachromasia. Plate 3a illustrates the typical beta metachromasia encountered in the epimysial connective tissues. According to Pearse (1960), toluidine blue metachromasia results from the presence of free electronegative surface charges of certain minimum density. Increasing charge density increases the amount of complexing of the dye. The alpha form of the dye is the monomer and results in the blue color. Beta metachromasia is the result of dimer formation and yields a violet or

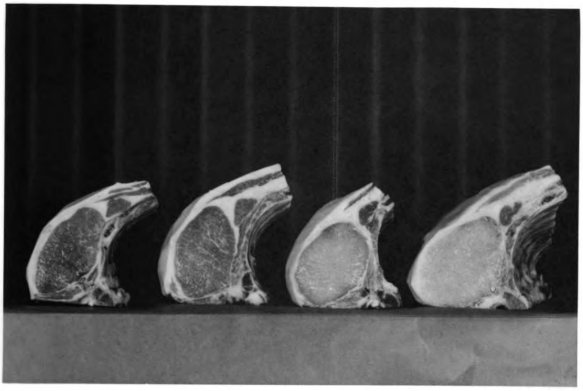


Plate 1. Comparison of normal and PSE musculature. The two samples on the left are normal, the two on the right are PSE.

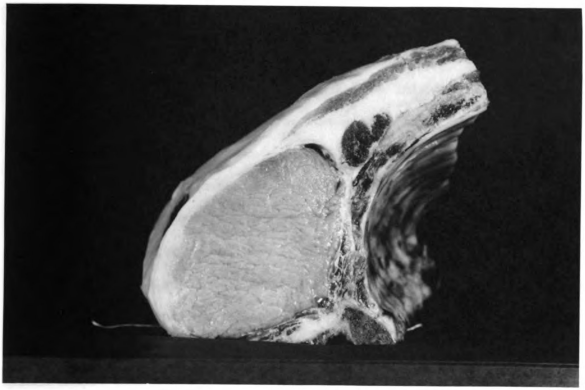


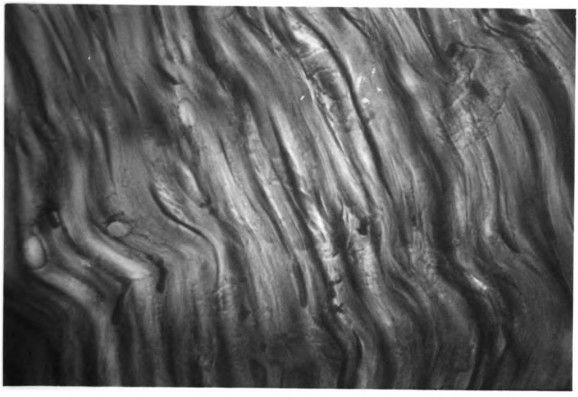
Plate 2. Illustration of the loosening of the attachment of the muscle to the epimysial connective tissue.

purple color, while the formation of gamma polymers results in a red color (Pearse, 1960).

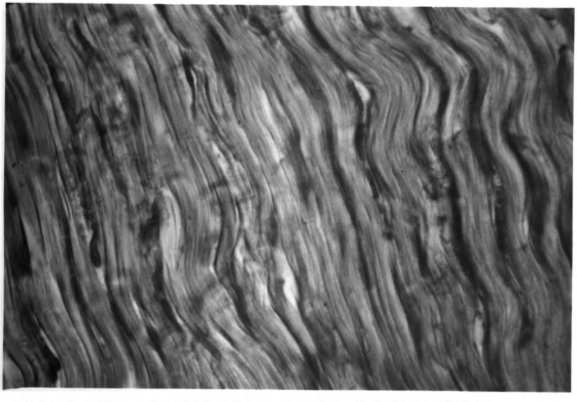
Stain Intensity. Plate 3 illustrates the metachromasia observed in normal and PSE samples stained at pH 8.0. The intensity of the stain was much less in the epimysium from PSE samples. A lower stain intensity in PSE connective tissue was also observed in samples stained at pH 5.6 (Plate 4). The stain intensity in both normal and PSE samples at pH 2.2 was so slight that photographs were unattainable.

Light intensity values in arbitrary units for normal and PSE tissues stained at pH 8.0 and 5.6 in both fixed and unfixed samples are shown in Table 2. Two independent series of samples were run for each animal at each pH and fixing condition, and three observations were obtained for each sample within a series. Therefore, each value given in the table represents the average of 6 observations.

Appendix 5 shows analysis of variance of the light intensity values for the samples studied. The light intensity was greater at pH 5.6 ($P < 0.01$) than at pH 8.0 for both PSE and normal samples, thus indicating less dye uptake at the lower pH. In addition, a fixing effect was evident ($P < 0.05$), as well as an interaction between pH of staining and the method of fixation ($P < 0.05$). The differences in light intensity between the fixed and unfixed samples stained at pH 8.0 were small. However, at pH 5.6 the light intensity was greater in the fixed samples.



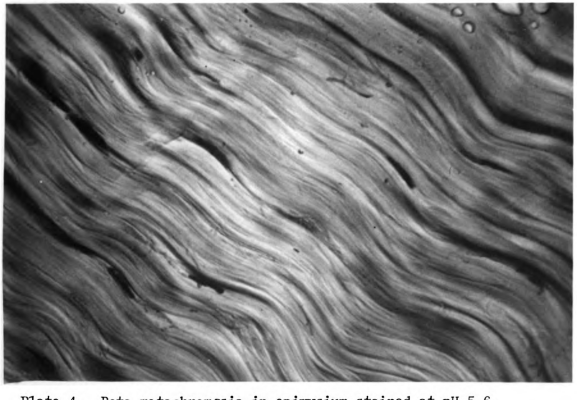
A



B

Plate 3. Beta metachromasia in epimysium stained at pH 8.0.
A = normal
B = PSE

A



B

Plate 4. Beta metachromasia in epimysium stained at pH 5.6.
A = normal
B = PSE

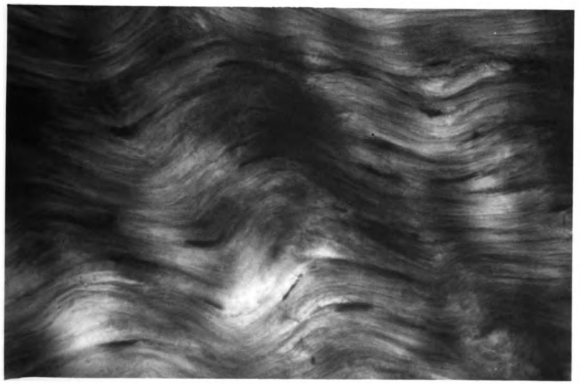
Table 2. Light intensity values for toluidine blue stained epimysial connective tissues.

	pH 8.0		pH 5.6	
Animal No.	Fixed	Unfixed	Fixed	Unfixed
PSE				
1	14.50	14.25	14.50	15.75
4	15.50	13.00	19.25	16.00
6	13.00	16.50	18.50	15.50
Mean	14.33	14.58	17.41	16.08
NORMAL				
2	11.00	10.50	15.50	13.75
3	12.00	11.25	16.25	12.00
5	11.00	12.50	16.90	14.50
Mean	11.33	11.41	16.21	13.41

Regardless of the pH or fixing condition, there was a significant ($P < 0.05$) difference in the light intensity of normal and PSE connective tissues, with the PSE samples having higher values.

Loss of Beta Metachromasia. Plate 5b shows the same sample as shown in Plate 5a after an alcohol rinse. The loss in beta metachromasia was readily apparent, with the purple color completely reverting to the blue alpha color. The loss in beta metachromasia has been clarified by Pearse (1960). He theorized that the bond angle between carboxyl groups, such

A



B

Plate 5. Illustration of the loss in beta metachromasia after an alcohol rinse.
A = beta metachromasia in epimysium from normal tissue stained at pH 8.0.
B = loss in beta metachromasia after an alcohol rinse.

as are found in hyaluronic acid, results in a weakly stable metachromasia that can be reversed by washing in alcohol. Conversely, sulfate ester groups, such as are found in the chondroitin sulfates, results in a stable, strong, irreversible metachromasia.

Therefore, the beta metachromasia observed in porcine epimysium in the present study is apparently due to hyaluronic acid. This conclusion is further substantiated by the lack of dye binding at pH 2.2. The lack of dye binding can be explained on the basis of the work of Szirmai (1963). He theorized that hyaluronic acid, which contains only one carboxyl group per disaccharide unit, is unable to bind dye below pH 3.0. He further stated that chondroitin sulfate, which contains both a carboxyl and a sulfate group, can bind dye at pH values below 3.0 because the sulfate group is still nearly completely dissociated at this pH. Therefore, he concluded that any dye binding observed at pH values below 3.0 is due almost entirely to the sulfate groups. This conclusion is complicated by the fact that Szirmai (1963) also found that the proteins present in cartilage resulted in a competitive lowering of the dye binding capacity of mucopolysaccharides at pH 2.0. Van Alten and Fennell (1957) also reported a protein-dye competition in chick embryo connective tissue mucopolysaccharides at pH 2.5 to 3.5. However, the beta metachromasia observed in these tissues was alcohol stable. In other words, the dye binding exhibited by proteins at pH 2.0 could lead to the erroneous conclusion that sulfate groups are present. However, lack of dye binding at this pH is a good indication that the beta metachromasia observed at higher pH values is due to carboxyl groups.

Based on the alcohol reversible metachromasia and the almost non-existent dye binding observed at pH 2.2 in the present study, it can be assumed that the predominent mucopolysaccharide present in porcine epimysial connective tissue is hyaluronic acid. In addition, differences in light intensity values, indicated that the hyaluronic acid content of the epimysium from PSE tissues is less or is in some way altered in comparison to that of normal epimysium.

Connective Tissue Fibers. Plate 6 shows connective tissues stained with Verhoeff's elastic stain. The black elastin fibers and nuclei were readily apparent. Plate 7 shows connective tissues stained with Verhoeff's-Van Giesson stain. The wavy collagen fibers were rendered pink, the elastin fibers and nuclei black. These two staining techniques were found to be the most desirable in that greater contrast and detail were attainable.

No distinctive histological differences were evident in either fiber type or appearance between PSE and normal connective tissues. Certain samples appeared to have a higher intracellular activity than others as evidenced by a greater number of nuclei, however, no definite trend was established.

Acid and Salt Soluble Collagen

Solubility. In order to further characterize and differentiate between the epimysial connective tissues from PSE and normal pigs, both salt and acid soluble collagen were extracted. Normal epimysial connective

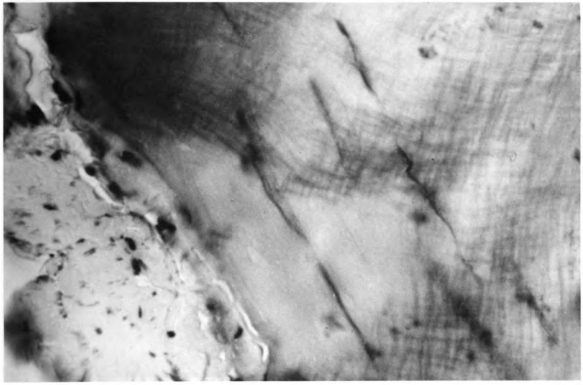


Plate 6. Epimysial connective tissue stained with Verhoeff's elastin stain.

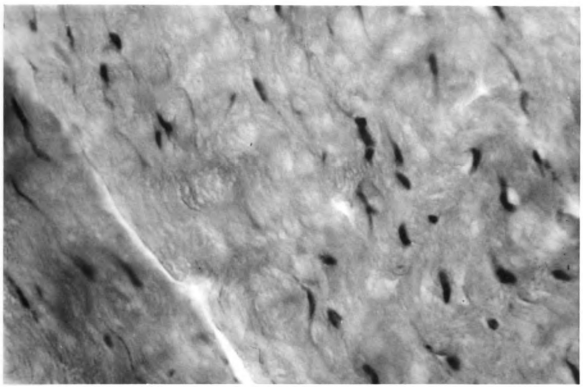


Plate 7. Epimysial connective tissue stained with Verhoeff-Van Giesson stain.

tissue was found to contain an average of 3.20% of salt soluble and 0.31% acid soluble collagen, while the epimysium from PSE tissues had 4.29% and 0.24%, respectively (Table 3).

Table 3. Percentage of acid and salt soluble collagen in epimysial connective tissues from normal and PSE *l. dorsi* muscles.

PSE			Normal		
Animal No.	Salt soluble[1]	Acid soluble[1]	Animal No.	Salt soluble[1]	Acid soluble[1]
1	4.16	0.22	2	2.63	0.21
4	3.93	0.25	3	3.41	0.28
6	4.66	0.25	5	4.52	0.51
8	4.10	0.24	7	3.30	0.36
10	5.37	0.31	11	2.62	0.27
14	3.57	0.21	13	2.75	0.27
Mean	4.29	0.24		3.20	0.31
Std. dev.	0.63	0.03		0.72	0.10

[1]% of total collagen

The small quantities of acid soluble collagen in comparison to the salt soluble fraction is in contrast to the results reported by Harkness *et al.* (1953). These workers found 11.0% of acid soluble and 21.5% salt soluble collagen in rabbit skin. In addition, the total soluble collagen content of porcine epimysium was lower than the values of 10 to 20% for extractable tropocollagen as reported by Lowther (1963). However, he reported that the quantity of extractable collagen was greatly affected

by the age and growth rate of the animal. The results of Nimni (1965), who reported values of 3.43% salt soluble and 1.29% acid soluble collagen, are in reasonably good agreement with the results of the present study.

There was significantly ($P < 0.05$) more salt soluble collagen in the PSE tissues than in the normal. The difference in acid soluble collagen was small and not significant (Appendix 6). Although histological studies revealed no discernible alterations in the fibrous proteins of either normal or PSE epimysium, the solubility data indicated that not only the ground substances are altered in PSE tissues, but also the fibrous proteins. Results also suggest that acid and salt solubility is more sensitive than the histological techniques for detecting changes in the fibrous proteins of connective tissues. This conclusion is substantiated by the work of Houck and Patel (1964), who reported no remarkable changes in the staining characteristics of dermal collagen in vitamin A and papain treated rats, even though there was a 50% decrease in the total collagen content of the skin and a 60% decrease in the salt soluble fraction.

It is extremely difficult to pinpoint the cause of increased tropocollagen solubility in the PSE connective tissues. The fact that only the salt soluble or newly synthesized tropocollagen was high in PSE connective tissues could indicate increased collagen anabolism. In light of Bowes and Raistrich's (1966) finding of an immature type collagen in the hides of rapidly growing sheep and beef, it is possible that the connective tissues of animals developing the PSE condition are also more immature than that of normal animals. In any case, the epimysium from PSE pigs contains collagen which has fewer intramolecular crosslinks than that of the normal.

Disc Gel Electrophoresis. In an effort to characterize the α- and β- subunit composition in epimysial connective tissue, and to attempt to separate the α_1- α_2 and $\beta_{1,1}$-$\beta_{1,2}$ subunits, several variables pertaining to disc gel electrophoresis were investigated.

Cyanogum (E. C. Apparatus Co.) was found to be satisfactory for disc gel electrophoresis of acid soluble tropocollagen. Cyanogum had a number of advantages over acrylamide in that preparation of working solutions was greatly simplified, allowing fresh solutions to be made up daily. In addition, polymerization time was greatly reduced, and the same working solutions could be used for both flat-bed and disc gel electrophoresis.

Concentration of sample gel or spacer gel was not a critical factor in achieving good separation or resolution. In fact, separation could be achieved without spacer or sample gel, by layering the sample in sucrose on the lower gel. However, failure to use a spacer gel resulted in a significant decrease in the sharpness of the bands.

The application of the sample in 5% sucrose (Plate 8) was definitely superior to the use of sample gel. This was probably due, in part, to the fact that the sample gel would not polymerize in the presence of the sample.

Runs of 1 to 1.5 hr resulted in the sharpest α- and β- bands. As the duration of electrophoresis was increased, sample separation also appeared to increase. In the case of runs of up to 3 hr, the α-subunits began to separate. After this period, however, the bands were located

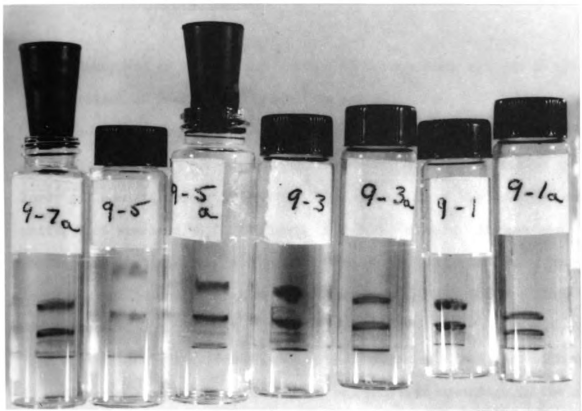


Plate 8. Effect of gel concentration and method of sample application on disc gel patterns of acid soluble collagen.

Tube No.	Lower gel concentration	Method of sample application
9-1	10.0%	Sample gel
9-1a	10.0%	5% sucrose
9-3	7.5%	Sample gel
9-3a	7.5%	5% sucrose
9-5	5.0%	Sample gel
9-5a	5.0%	5% sucrose
9-7a	7.5%	5% sucrose

at the lower end of the gel and further electrophoresis was not possible. No separation of β-units was evident in any run.

Good separation of the α- and β-bands could be achieved with various gel concentrations. However, the sharpness of the bands decreased at lower gel concentrations (Plate 8). For separation of the collagen subunits, 7.5% appeared to be the ideal gel concentration. It had been anticipated that at lower gel concentrations, the pore size would be increased sufficiently to allow the γ-polymer to move. However, this did not occur.

Urea in the gel system did not greatly enhance separation, although it did increase mobility. Thus, there was no great advantage in the use of urea in this system.

α - β Subunit Composition of Acid Soluble Collagen. The α-and β-subunit composition was determined by disc gel electrophoresis on purified acid soluble extracts of the epimysial connective tissue from normal and PSE muscle. A typical densitometer tracing of the α-and β-subunit patterns obtained from the disc gels is shown in Figure 1.

The acid soluble collagen from normal epimysium contained 38.46% of the α and 61.54% of β-subunits. The corresponding values for PSE epimysium were 37.92% for the α-and 62.08% for the β-subunits (Table 4). These results can be compared with those of Piez *et al.* (1963), who found that salt soluble collagen from rat skin contained 50% of the β-components. Without prior salt extraction, the acid soluble collagen from rat

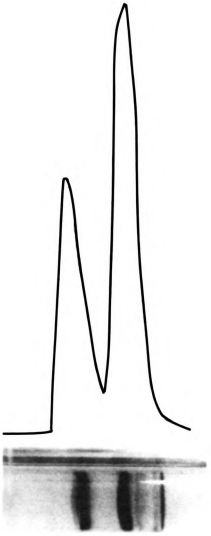


Figure 1. Typical densitometer tracing of α and β subunit patterns from disc gel electrophoresis.

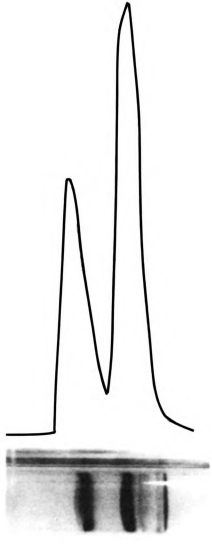


Figure 1. Typical densitometer tracing of α and β subunit patterns from disc gel electrophoresis.

tail tendon contained 60% of β-subunits, that from carp swim bladder 27% and from dogfish skin 67% (Piez et al., 1963). These authors theorized that the collagen with a higher percentage of β-subunits probably had a greater proportion of intramolecular crosslinks.

Table 4. Percentage of α-and β-subunits in acid soluble collagen of epimysial connective tissues from normal and PSE *l. dorsi* muscles.

PSE			Normal		
Animal No.	% α	% β	Animal No.	% α	% β
1	40.00	60.00	2	39.00	61.00
4	36.50	63.50	3	38.25	61.75
6	40.00	60.00	5	40.00	60.00
8	35.50	64.50	7	39.00	61.00
10	38.00	62.00	11	36.00	64.00
14	37.50	62.50	13	38.50	61.50
Mean	37.92	62.08		38.46	61.54
Std. dev.	1.66	1.66		1.22	1.22

From the α-and β-subunit composition it was calculated that the acid soluble collagen from normal epimysium was 92.27% crosslinked. Similar calculations showed that the acid soluble fraction from PSE epimysium was 93.02% crosslinked. It is difficult to compare these values with those reported in the literature due to differences in extraction methods. Some values are reported as acid extracted collagen

with no previous salt extraction, while others received a prior salt extraction. Piez et al. (1963) reported that salt extractable rat skin tropocollagen was 28% crosslinked, while the acid extractable collagen was 76% crosslinked. Without prior salt extraction, acid soluble rat tail tendon was 91% crosslinked, carp swim bladder 41 and dogfish collagen 99% crosslinked. Thus, it appears that the porcine epimysial connective tissues used in the present study were intermediate in crosslinking density between rat skin and rat tail tendon.

Analysis of variance (Appendix 7) releaved that the difference between normal and PSE connective tissues in α- and β-subunit composition was not statistically significant. However, it must be remembered that there was a significantly greater amount of salt extractable collagen in the PSE connective tissues. Salt soluble collagen is composed mainly of non-crosslinked α-components (Piez et al., 1963). Therefore, if the sample had not received a prior salt extraction, the α- and β-subunit composition of the acid soluble fraction from PSE epimysium would have had a lower percentage of β-components. Thus, indicating decreased intramolecular crosslinking.

Similar results have been reported by Martin et al. (1961), who extracted acid soluble collagen without prior salt extraction. The effluent pattern from CMC chromatograms of the acid soluble collagen from rats with chronic lathyrism was similar to the pattern observed for salt extracted collagen from normal animals. Compared to acid soluble collagen from normal animals, there was an increase in the weight ratio of single

chains (α_1 and α_2) to chain pairs ($\beta_{1,1}$ and $\beta_{1,2}$) from 1.0 to 2.8. These authors suggested that there is a defect at the molecular level in the maturation of collagen in lathyritic animals. The failure to produce chain pairs by intramolecular crosslinking results in a higher proportion of collagen in acid extracts. It is similar to newly formed collagen (salt extractable) in normal animals. This apparently results in the gross symptoms characteristic of lathyrism, i.e., a general weakening of tendons and ligaments, and an increased tissue fragility (Martin et al., 1961).

It is possible that a similar maturation defect might be responsible for the altered solubility characteristics and subunit distribution of the PSE connective tissues observed in the present study.

Heat Labile Collagen

The results of heat solubility on the epimysium from normal and PSE tissues is shown in Table 5. These data revealed that porcine epimysial connective tissue contains approximately 25% total collagen. It is also apparent that 22.98% of the collagen from PSE tissue is released upon heating as compared to 15.02% from the normal tissues.

Results agree very well with the work of Verzar (1964), who reported 25% heat labile collagen in tendons from 3 yr old rats. They are also in essential agreement with Hill (1966), who found 22% heat labile collagen in the residues isolated from the loose connective tissue network of 5 month old pigs.

Table 5. Heat solubility parameters of epimysial connective tissues from normal and PSE *l. dorsi* muscles.

PSE					Normal				
Animal No.	Total collagen[1]	Heat soluble collagen[1]	Residual collagen[1]	% heat soluble collagen	Animal No.	Total collagen[1]	Heat soluble collagen[1]	Residual collagen[1]	% heat soluble collagen
1	251.62	55.81	195.74	22.23	2	266.84	31.50	235.36	11.20
4	262.63	64.76	197.88	24.62	3	268.69	42.62	226.07	15.89
6	256.18	72.46	183.72	28.29	5	247.01	37.75	209.26	15.25
8	262.17	54.73	207.43	20.88	7	232.93	38.24	194.68	16.58
10	224.93	39.83	185.09	17.70	11	249.84	42.75	207.09	17.10
14	284.72	69.00	215.71	24.19	13	257.65	36.42	221.23	14.13
Mean	257.04	59.43	197.60	22.98		253.82	38.21	215.62	15.02
Std. dev.	19.42	11.90	12.47	3.61		13.44	4.21	14.71	2.14

[1] mg/gm connective tissue

There are, however, apparent differences in the amount of heat labile collagen due to source, species and animal age. Verzar (1964) found 75-80% of heat labile collagen in the tendons of young rats, while the corium from the same animals had 7.4-11.8%. At 2 yr of age, the heat labile collagen in the corium had decreased to 2-3%. Goll et al. (1964) also found differences in heat labile collagen from collagenous residues isolated from the loose connective tissues of calves and old cows. They reported that the amount of heat labile collagen decreased from 42% for calves to 2% for 10 yr old cows. Hill (1966) using a similar procedure isolated 22% of heat labile collagen from 5 month old hogs, which compared to 3.9% for old sows.

The exact nature of the heat labile collagen is not clear. Verzar (1964) reported that during thermal contraction, a certain amount of collagen is released or dissolved and goes into solution. He described this fraction as labile collagen, as differentiated from tropocollagen. According to Gustavson (1957) and Verzar (1964) the release of collagen by heating is caused by a rupture of the interchain crosslinkages, which are made up primarily of hydrogen bonds.

Results of the present study also indicated that heat labile collagen is of a different type than that liberated by neutral salts and dilute acids (tropocollagen). This is substantiated by the fact that the combined salt and acid soluble collagen accounted for only 3.51 to 4.53% of the total, while heat labile collagen composed 15.02 to 22.98% of the total. On the basis of these results, it is suggested that factors other than

intermolecular hydrogen bonds are involved in the release of heat labile collagen. It is possible that certain heat labile covalent bonds are also broken.

Analysis of variance (Appendix 8) revealed that the differences in the amount of total collagen between normal and PSE connective tissues were not statistically significant. However, there was a highly significant difference ($P < 0.01$) between the percentage of heat labile collagen in the normal and PSE epimysium. These results again indicated that the epimysium from normal tissue has greater numbers or an increased strength of crosslinks as compared to the PSE connective tissue. This conclusion is in agreement with numerous workers (Verzar, 1964; Goll et al., 1964; Hill, 1966), who have postulated that the decrease in heat labile collagen as a result of increasing animal age is due to greater numbers and increased strength of crosslinks.

Hydrothermal Shrinkage Characteristics

Thermal Shrinkage. The hydrothermal shrinkage of collagen is a unique characteristic of connective tissues, and is affected by numerous factors, such as biological age (Verzar, 1964), species (Gustavson, 1956) and nutritional state and disease (Versar, 1964). Therefore, a study of the thermal shrinkage temperature (Ts) of epimysium from PSE and normal tissues was initiated.

Temperatures at the onset of shrinkage, completion of shrinkage and denaturation were very similar in the epimysium from both PSE and normal

tissues (Table 6). The differences between the various temperature parameters were not statistically significant (Appendix 9).

The temperature range at which the epimysial collagen underwent thermal contraction agrees closely with other values reported in the literature. Gustavson (1956) has summarized the Ts values for skins of different species. His values of 58-62°C for the sheep, and 60-62°C for the deer, cat, dog, moose and reindeer agree quite closely with those for porcine epimysial collagen as found in the present study. Somewhat higher Ts ranges have been reported for collagen isolated from the canine, bovine or human aortas (68-70°C) by Melch (1965), and from canine ligaments and tendons (69-71°C) according to Peacock (1966). The values of 63-65°C for the calf, 65-67°C for the cow and 64-66°C for the goat as reported by Gustavson (1956) are slightly higher than the values found for porcine epimysium in the present study. Gross (1964) also found considerable variation in the thermal shrinkage temperature of rat tail tendon. His values varied from 54-59°C.

It is readily apparent, therefore, that collagen from different sources exhibits differences in thermal shrinkage temperatures. It is interesting to note that the porcine epimysial collagen used in the present study had a Ts of 3-7°C lower than that of bovine hide collagen as reported by Gustavson (1956). It is tempting to speculate that similar variations in Ts might have a direct effect on meat quality factors, such as tenderness.

The fact that normal and PSE tissues did not differ in their thermal shrinkage temperature was surprising in light of the differences in acid

Table 6. Thermal shrinkage temperature (Ts, °C) of epimysial connective tissues from normal and PSE *l. dorsi* muscles.

PSE				Normal			
Animal No.	Onset of shrinkage[1]	Completion of shrinkage[2]	Denaturation[3]	Animal No.	Onset of shrinkage[1]	Completion of shrinkage[2]	Denaturation[3]
1	60.00	61.00	64.00	2	59.00	60.00	62.00
4	58.50	59.50	61.00	3	59.00	60.00	62.00
6	59.00	60.00	61.00	5	59.00	60.00	61.50
Mean	59.16	60.16	62.00		59.00	60.00	61.83

[1]Temperature at beginning of shrinkage.
[2]Temperature at end of shrinkage.
[3]Temperature at complete loss of opacity.

and salt solubility and the heat labile collagen. These results indicated a decrease in the amount of crosslinking, or a less stable type collagen in the PSE tissues. Thus, it was reasonable to expect a lower Ts in the epimysium from PSE tissues.

Differential Thermal Analysis. Gustavson (1956) stated that the process of thermal shrinkage is a rate phenomenon, and that a true shrinkage temperature does not exist from a thermodynamic point of view. With the advent of differential thermal analysis (DTA), the absolute measurement of shrinkage temperature has been made possible. However, this technique had not previously been used in investigations with native collagen. Therefore, a study was initiated utilizing differential thermal analysis to investigate the thermal shrinkage phenomena, and to further characterize various connective tissues, including that from normal and PSE pigs.

Since differential thermal analysis had not previously been applied to native collagen, it was necessary to determine the conditions for conducting such a study. Benzoic acid was used to standardize the apparatus daily. Figure 2 illustrates a typical DTA thermogram of benzoic acid, and defines the location of significant transition temperatures as outlined in the Dupont 900 DTA instruction manual. According to the manual the following temperatures are often of value:

a). Onset temperature = the temperature at which the thermogram starts to depart from the base line. This is the first indication of a physical change.

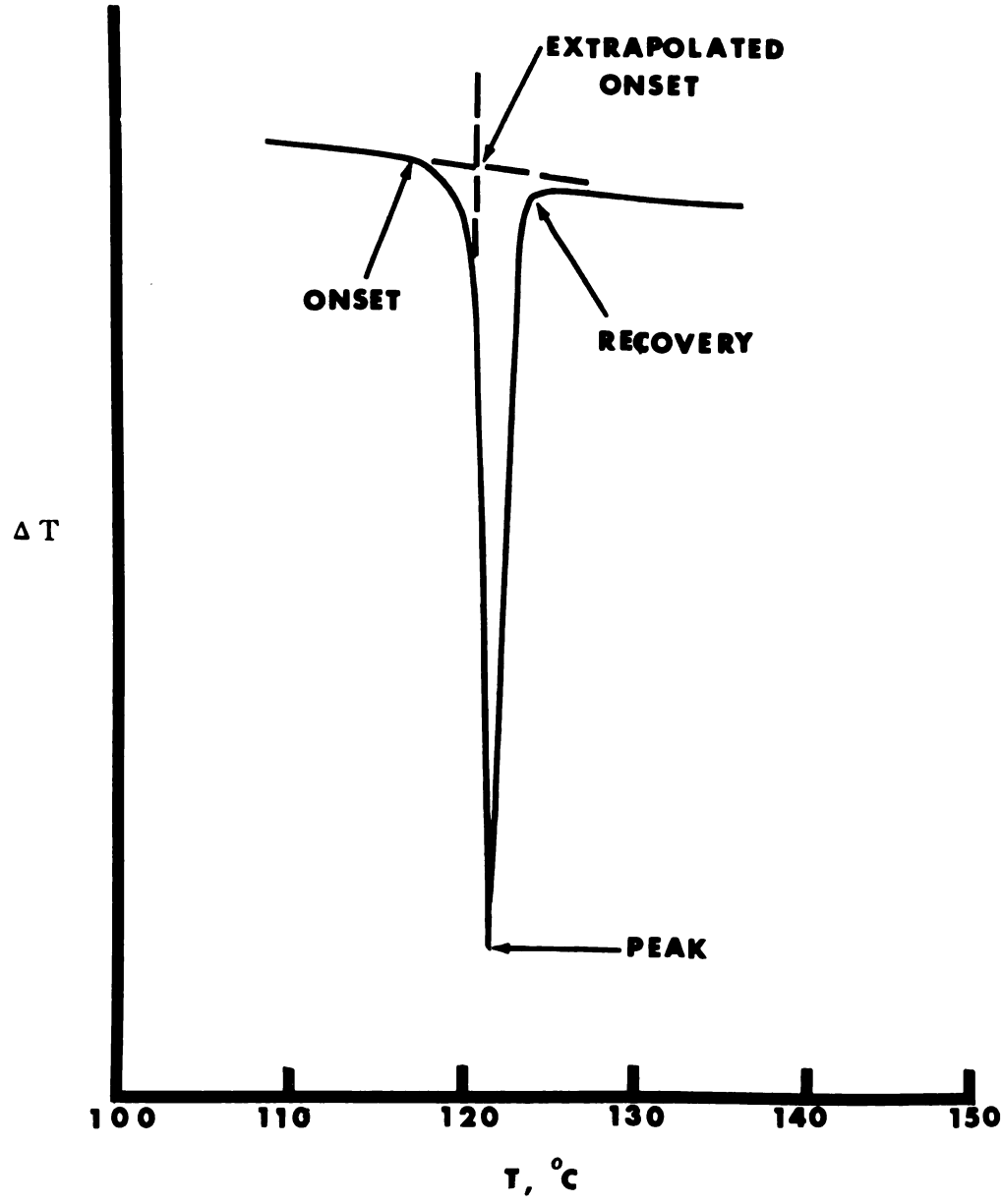


Figure 2. DTA thermogram of benzoic acid.

T scale = 10°C/in; ΔT scale = 2°C/in; heating rate = 20°C/in.

Code: ΔT = temperature differential between sample and reference; T,°C = sample temperature.

b). Extrapolated onset = the temperature corresponding to the intersection of extrapolations of the base line and the longest straight line section on the low temperature side of the peak. This point represents the starting temperature of the major portion of the transformation.

c). Peak = the temperature of reversal, or the temperature at which the differential between sample and reference is greatest. The transformation taking place in the sample is almost complete at this temperature, and for compounds undergoing fusion, it is reported as the melting point.

d). Recovery = the temperature at which the thermogram returns to either the same or a different base line.

The thermogram of benzoic acid (Figure 2) shows maximum absorption of endothermic heat by the sample in a temperature range of 117-123°C. The peak temperature of the melting endotherm is very reproducible and should be within ±2° of the corrected temperature of 121°C. The peak temperature corresponds to the melting point of benzoic acid as determined by the capillary tube method.

A conflict was encountered when heating rates were investigated. According to Gustavson (1956), an increase in the Ts of native collagen occurs when the heating rate is increased. For example, the Ts of calf skin heated at 2.5°/min was found to be 64.5°C, while samples heated at 4°/min had a Ts of 67.5°C. He attributed this to a failure to attain thermal equilibrium on rapid heating. According to the Dupont manual,

faster programming rates will give greater sensitivity in the detection of thermal changes, but may result in broadening or coalescing of the peaks associated with separate thermal phenomena. Conversely, slower programming rates give better resolution at the expense of sensitivity.

Thermograms of porcine epimysial connective tissue run at heating rates of 1, 2, 5 or 10°C/min are shown in Figure 3. The thermal shrinkage temperatures were found to be 62.0, 63.0, 64.6 and 65.8°C, respectively, as the heating rate was increased from 1 to 10°C. Sensitivity was greatly improved as the heating rate was increased, as evidenced by the depth and sharpness of the thermograms. However, resolution was greater at lower heating rates, as indicated by deviations from the base line. The deviations observed at the lower heating rates probably reflect expansion and settling of the sample, or perhaps background noise of the apparatus. The manufacturer recommends heating rates of 15 to 20°C in order to overcome such problems. Due to the greater sensitivity and reproducibility of the thermograms, 10°C/min was chosen as the heating rate for subsequent studies.

Flory (1958) and Mason and Rigby (1963) reported that the difference between Ts and Tm is due to a superheating phenomenon. These workers found that after establishment of a fresh crystalline amorphous interface by partially melting and cooling to room temperature, the value of Ts was reduced towards the equilibrium melting point (Tm). Remelting studies in the present investigation (Figure 4) tend to confirm the superheating phenomenon. The normal thermogram of porcine epimysium revealed a Ts of

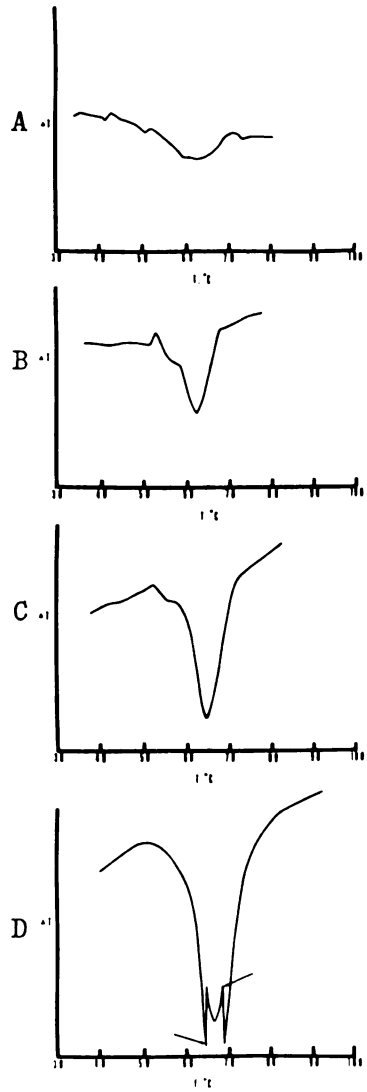


Figure 3. Effect of heating rate on DTA thermograms of native collagen.
T scale = 10°C/in; ΔT scale = 0.2°C/in.
Code: ΔT = temperature differential between sample and reference; T°,C = sample temperature.

A = heating rate of 1°C/in
B = heating rate of 2°C/in
C = heating rate of 5°C/in
D = heating rate of 10°C/in

Arrows indicate a shift in baseline to accommodate the peak on scale.

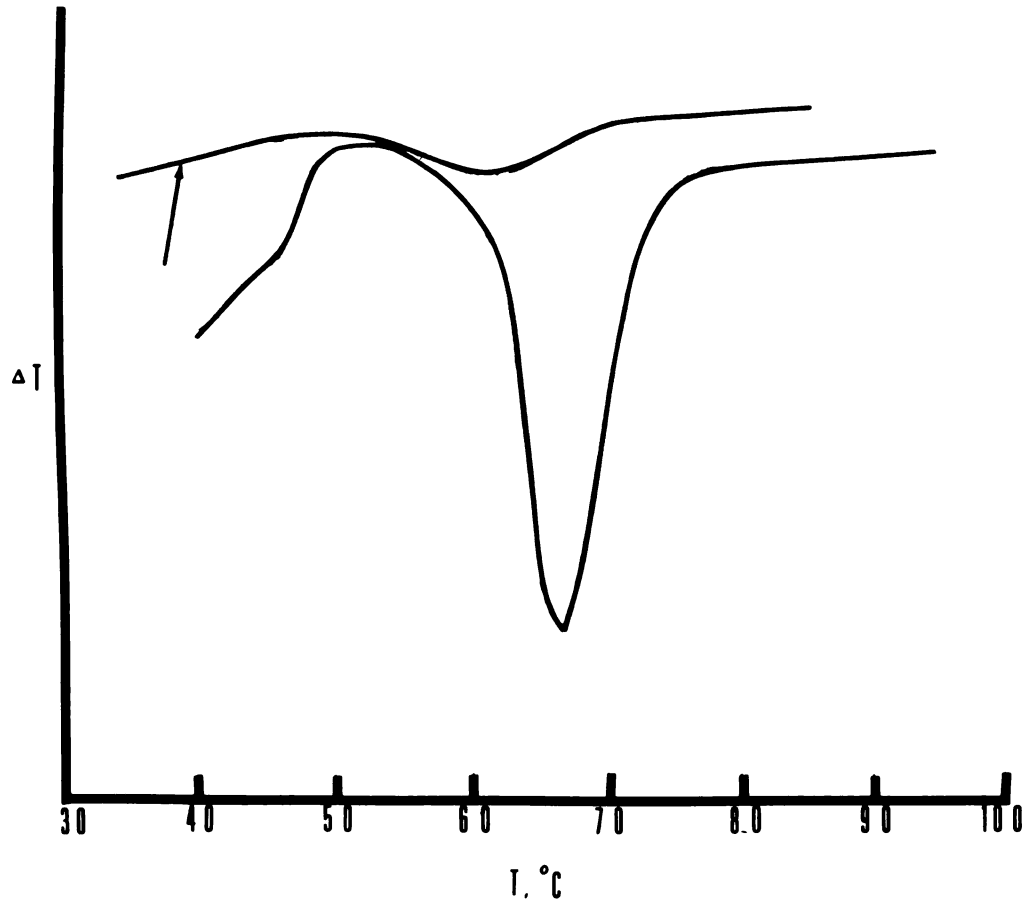


Figure 4. DTA thermogram showing remelting of native collagen. T scale = 10°C/in; ΔT scale = 0.2°C/in; heating rate = 10°C/in.
Code: ΔT = temperature differential between sample and reference; T,°C = sample temperature; arrow indicates remelt curve.

65.8°C. After cooling to room temperature, the sample was reheated and resulted in a Ts value of 62.4°C. The sensitivity in remelted samples was very low. Attempts to remelt for the second or third time resulted in complete loss of the transition maxima.

The results of a study to determine proper sample size for DTA is shown in Figure 5. The main observable effect of increased sample size was an increase in the endothermic response. The extrapolated onset and peak temperatures were very similar, regardless of sample size. Some variation in onset and recovery temperatures was noted.

Initial DTA thermograms revealed that the base line tended to slope upward, indicating that the reference material was heating at a faster rate than the samples. The Dupont manual states that differences in thermal conductivity between the reference material and the sample may be encountered. Silicone oil may be added to the glass bead reference to overcome this problem. Figure 6 shows thermograms utilizing various reference materials. The Ts values obtained were the same in every case. The only reference material influence noted was the change in base line slope to the endothermic side in the case of the water-glass beads reference (Figure 6c). Since no particular advantage was noted for any reference material, glass beads were used throughout the study. The exothermic base line slope was corrected by setting a -0.2 base line slope correction on the apparatus.

In order to evaluate the reproducibility of the 4 temperature parameters measured by DTA, 4 separate determinations were run on various sample sources. From the data (Table 7), it is evident that the most

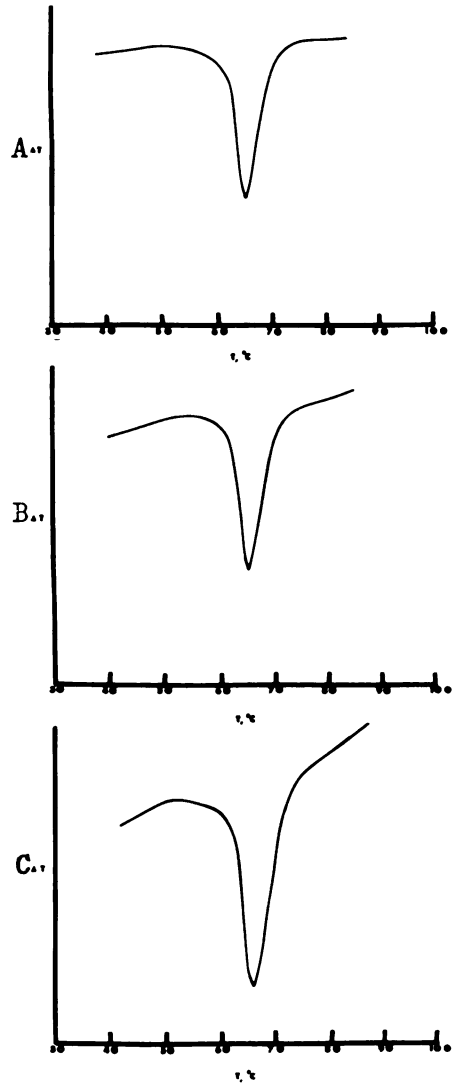


Figure 5. Effect of sample size on DTA thermograms of native collagen. T scale = 10°C/in; ΔT scale = 0.2°C/in; heating rate = 10°C/in.
Code: ΔT = temperature differential between sample temperature and reference; T,°C = sample temperature.

A = 18.5 mg
B = 31.0 mg
C = 50.0 mg

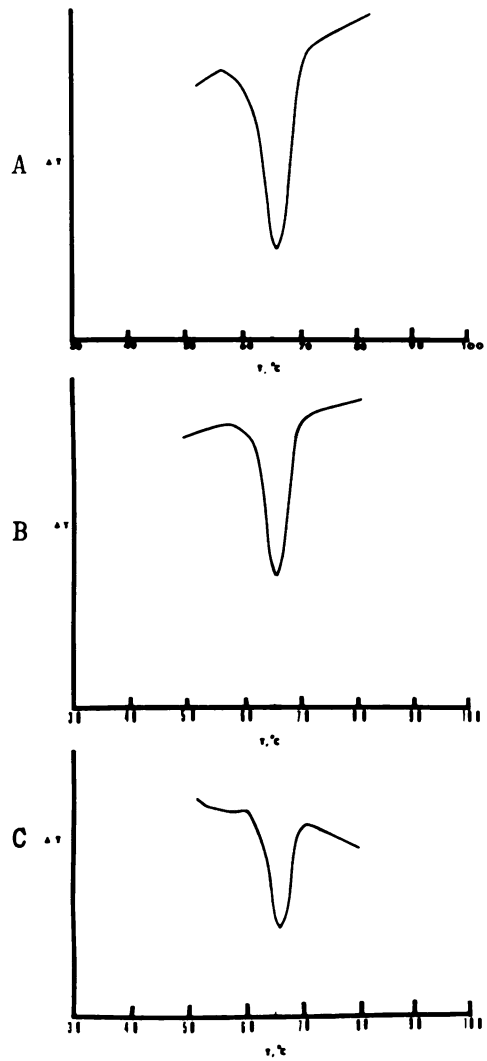


Figure 6. Effect of reference material on DTA thermograms of native collagen.
T scale = 10°C/in; ΔT scale = 0.2°C/in; heating rate = 10°C/in.
Code: ΔT = temperature differential between sample and reference; T,°C = sample temperature.

A = glass beads
B = silica oil
C = water-glass beads

Table 7. Multiple DTA determinations on native collagen from various sources.

Source	Determination No.	Onset	Extrapolated onset	Peak	Recovery
	1	45.63	60.74	66.65	77.75
Normal	2	45.00	61.50	66.19	80.37
7 wk old pig	3	48.12	61.50	66.50	78.37
epimysium	4	45.63	61.50	66.82	79.00
	Mean	46.10	61.31	66.54	78.87
	Std. dev.	1.38	0.38	0.27	1.12
	1	48.37	58.06	65.25	75.87
Zinc deficient	2	47.50	60.25	65.56	75.25
7 wk old pig	3	45.50	59.94	65.87	75.87
epimysium	4	48.12	59.13	65.87	75.25
	Mean	47.37	59.35	65.64	75.56
	Std. dev.	1.30	0.98	0.30	0.36
	1	45.00	60.87	67.12	82.25
Normal	2	45.01	59.94	67.44	83.17
7 wk old pig	3	45.90	60.94	67.44	84.25
corium	4	45.21	60.50	67.30	83.00
	Mean	45.28	60.56	67.53	83.17
	Std. dev.	0.42	0.46	0.15	0.82
	1	54.63	62.12	65.25	81.50
Zinc deficient	2	55.25	62.44	65.87	83.00
7 wk old pig	3	57.75	62.44	65.42	81.50
corium	4	53.62	61.66	65.87	81.62
	Mean	55.31	62.16	65.60	81.91
	Std. dev.	1.76	0.37	0.32	0.73
	1	52.50	60.56	66.81	82.25
6 month old	2	48.75	60.25	66.50	80.25
market pig	3	48.75	60.25	66.80	81.94
epimysium	4	49.00	60.30	66.60	81.42
	Mean	49.75	60.34	66.68	81.47
	Std. dev.	1.84	0.15	0.15	0.88
	1	59.63	62.28	66.18	84.88
Commercial grade	2	58.28	62.22	66.66	87.37
beef	3	60.87	62.12	65.25	84.87
	4	61.50	62.12	65.25	89.88
	Mean	60.07	62.19	65.84	86.75
	Std. dev.	1.42	0.08	0.70	2.39

reproducible temperature parameters were the extrapolated onset and the peak temperature. The standard deviations for these two parameters were extremely small. The standard deviations for the onset and recovery temperatures were larger, but were also quite reproducible. In most cases, the onset temperature exhibited the greatest variation.

Difficulty was encountered in trying to interpret differences due to age, species or source of tissue in the DTA parameters (Table 7). In order to overcome the difficulty in interpretation, the DTA data were retabulated as shown in Table 8. The data are presented in terms of total melting range, temperature range from onset to peak, temperature range from peak to recovery and the percentage of the total range represented by the latter two parameters. On presenting the data in this manner, distinct differences were noted between connective tissue sources.

The total melting range for the samples varied from 26.60 to 37.89°C. These results substantiate Gustavson's (1956) assertion that a true shrinkage temperature does not exist for collagen. Indeed, the present data show that the shrinkage phenomena occurs over a wide and variable temperature range. It is theorized that the total melting range is a good indicator of the heterogeneity of the components. It seems plausible that a sample melting over a range of 26°C would be more homogeneous than one melting over a range of 37°C. This conclusion is substantiated by Gustavson (1956), who outlined the phenomena which occurs during thermal shrinkage. He stated that at a certain temperature, the tendon forms tiny nodules at various points. Their appearance marks the onset of

shrinkage. These nodules grow into lumps and form gelatinous nodes. As the nodules continue to grow, they coalesce and shrinkage takes place. Gustavson (1956) further theorized that the collagen structure probably varies as to chain spacings and spatial orientation. It is likely that the crosslinks possess different energies, and that some regions of the molecule contain fewer crosslinks than others. He thus concluded that a small region of the collagen lattice, containing few crosslinks of low energy, should offer the right conditions for initial shrinkage. As the temperature is raised, an additional number of crosslinks are ruptured. Finally a point will be reached at which the structure is so weakened that it will take on a more stable configuration by chain folding (Gustavson, 1956).

Therefore, it is suggested that the DTA temperature range from onset to peak represents the breaking of low energy crosslinks, while the range from peak to recovery indicates the rupturing of high energy crosslinks. If this interpretation is placed on the data in Table 8, some interesting conclusions can be drawn. Examination of the total melting point range in epimysial connective tissue reveals that the 7 week old pigs and the 6 month old market weight pigs were very similar. However, the temperature range from onset to peak as a percentage of the total melting range was 62.4% for the 7 week old pigs and 53.5% for the 6 month old pigs. Conversely, the temperature range from peak to recovery as a percent of the total melting range was 37.6% and 46.5%, respectively. Apparently, there is a decrease in the components melting at low temperatures and an increase in the higher melting fraction with an increase in age. It is

Table 8. DTA determination of melting ranges on native collagen from various sources.

Source	Total melting range °C	Onset-peak °C	Onset-peak as a percentage of total melting range	Peak-recovery °C	Peak-recovery as a percentage of total melting range
Normal 7 wk old pig epimysium	32.77	20.44	62.40	12.33	37.60
Zinc deficient 7 wk old pig epimysium	28.19	18.27	64.80	9.92	35.20
Normal 7 wk old pig corium	37.89	22.05	58.20	15.84	41.80
Zinc deficient 7 wk old pig corium	26.60	10.29	38.70	16.31	61.30
Normal 6 month old market pig epimysium	31.53	16.86	53.50	14.67	46.50
PSE 6 month old market pig epimysium	30.53	18.88	61.80	11.65	38.20
Commercial grade beef epimysium	26.68	5.77	21.60	20.19	75.70

interesting to note that commercial beef had a narrower melting point range, with the onset and recovery occurring at much higher temperatures than that of either the 7 week or 6 month old pigs (Table 8). In the epimysium from commercial beef, only 21.6% of the total temperature range occurred between the onset and peak, while 75.7% occurred between the peak and recovery. These differences may well reflect alterations in salt and acid solubility and in thermal labile collagen as a consequence of changes in the age of the animal.

Another interesting comparison was found between epimysium and corium (Table 8). Normal corium from 7 week old pigs had a broader melting point range, higher recovery temperature and a greater percentage of higher melting components than that from epimysium.

Alterations in connective tissues from zinc deficient pigs were also detectable with DTA (Table 8). The epimysium from the zinc deficient pigs had a much narrower melting point range than that from the normal pigs, but only slight differences occurred in the amount of high and low melting components. In the corium, however, the differences were more dramatic. The corium from zinc deficient pigs had a much narrower melting point range, a higher onset temperature and a much larger proportion of the components melted at a higher temperature.

Results of the DTA study on the epimysium from normal and PSE tissues is shown in Table 9. Although differences between the epimysium from normal and PSE pigs in all 4 DTA parameters appeared to be small, analysis of variance (Appendix 10) revealed them to be highly significant ($P < 0.01$). This again points out the extreme sensitivity and reproducibility

Table 9. DTA determinations on epimysial connective tissues from normal and PSE 1. dorsi muscles.

Animal No.	Onset °C	Extrapolated onset °C	Peak °C	Recovery °C
PSE				
1	46.41	58.88	65.87	78.99
4	46.98	59.47	65.97	78.99
6	46.25	59.00	65.64	76.03
10	49.69	58.62	65.25	74.94
8	46.04	58.58	65.41	76.60
14	45.42	57.96	65.87	78.37
Mean	46.79	58.75	65.67	77.32
Std. dev.	1.50	0.50	0.29	1.70
Normal				
2	49.38	60.93	66.81	81.25
3	48.75	59.63	66.03	80.98
5	49.37	59.15	66.03	77.96
7	50.00	60.35	66.70	81.48
11	49.74	60.18	66.03	81.19
13	49.37	60.45	66.19	82.93
Mean	49.44	60.12	66.30	80.97
Std. dev.	0.42	0.63	0.36	1.63

of the DTA method. From the data in Table 8, it is apparent that the epimysium from PSE pigs had a total melting point range similar to that of the normal. However, onset and recovery occurred at lower temperatures. The PSE tissues also contained a higher percentage of components or cross-links that melted at a low temperature. This probably explains the increased amount of salt soluble collagen and the greater release of heat labile collagen, which was found for the PSE tissues.

It is interesting to note that the DTA parameters for the epimysium of the PSE pigs are intermediate to those for the 7 week and 6 month old pigs. The percentage of the total melting point range from onset to peak

and from peak to recovery, respectively, are 62.40 and 37.60% for the 7 week old pigs, 61.80 and 38.20% for the PSE pigs and 53.50 and 46.50% for the normal pigs. It is suggested, therefore, that the PSE epimysial collagen is more immature than that from normal animals.

The results of this study may also explain why no differences in Ts values were observed between normal and PSE epimysium in the thermal shrinkage studies conducted with the melting point apparatus. It is readily apparent that thermal shrinkage does not occur at a given temperature, but rather over a wide and variable range. The Ts observed at the point of visual contraction appears to conform more nearly to the extrapolated onset temperature observed with the DTA apparatus. This is probably the case, since by definition the extrapolated onset temperature is that temperature representing the start of the major part of the transition. In addition, DTA data revealed that the peak temperatures or melting points were very similar, although statistically significant. This suggests that small differences in melting or thermal shrinkage temperatures may be physiologically significant. Due to the extreme sensitivity of DTA, these small differences can be measured, although they are not normally detected by visual procedures.

Differential thermal analysis is limited by superheating, which undoubtedly results in peak temperatures higher than those occurring at slower heating rates. However, this technique has the advantages of excellent reproducibility and extreme sensitivity. This method also more nearly explains the nature of the thermal shrinkage transition by depicting the total melting range and the relative portions of the melting

range, which occur at high or low temperatures. It is suggested, therefore, that the use of differential thermal analysis will be a very useful tool in determining changes in the connective tissues as a result of variables, such as species, biological age and disease.

Stress-Strain Studies

Swelling Properties. At the beginning of the stress-strain studies, samples were placed in distilled water until the run could be made. These samples were held in distilled water for periods varying from 15 min to 2 hr. It was noted that the molecular weight between crosslinks from the same sample became greater as the length of time in distilled water was increased. This indicated a loss or breaking of the crosslinks (Table 10). It was also noted that swelling occurred and dimensional changes increased as the time in distilled water became longer.

Table 10. Increase in molecular weight between crosslinks with increased soaking time.

Soaking time[1]	Mc x 10^4[2]	Crosslinks/molecule[3]
0.5	1.20	24.99
1.0	1.31	22.88
1.5	1.49	20.04
2.0	1.76	16.99
2.5	2.25	13.36

[1]Hours in distilled water.
[2]Mc = molecular weight between crosslinks.
[3]Assuming a molecular weight of 300,000 for the collagen molecule.

This swelling was not expected since Gustavson (1956) stated that there was a wide pH range around the isoelectric point (pH 5-7), in which pH variation had little influence on the extent of swelling. He further indicated that in this pH range, collagen contains water of only two types, i.e., the water of hydration and the water mechanically held in the interstices of the molecule.

Previous work pertaining to the swelling of collagen, while fundamentally important to the understanding of collagen and its interaction with aqueous solutions, has generally neglected swelling in physiological pH ranges. This is probably due to the fact that the maximum swelling effects are obtained with pH values outside the physiological range. Recent work, however, has shown that the neutral swelling of collagen may also have important physiological implications (Fels, 1966; Harkness and Harkness, 1965; Milch, 1965; Verzar, 1964).

The effects of various solutions on the swelling of collagen from porcine epimysial connective tissue are shown in Table 11. The samples soaked in 0.9% saline were opaque, underwent little or no dimensional change, and imbibed only a very small amount of water. Samples placed in acetic acid developed the characteristic appearance attributed to osmotic swelling (Gustavson, 1956). The samples imbibed large amounts of water and developed a clear or glassy appearance. The fibers clumped together and became plump in appearance, decreasing in length and increasing in width. The samples placed in distilled water imbibed large quantities of water, decreased in length and increased in width. Some of the samples

soaked in water had an appearance more like the samples soaked in saline, i.e., they remained opaque and showed only small dimensional changes. Others, however, appeared more like those placed in acetic acid, especially if the fiber network was disperse. Still other samples appeared intermediate in appearance. The alterations in appearance and structure were reversed in all cases by placing the samples in 0.9% saline, indicating that swelling in water was of the osmotic type.

Table 11. Neutral swelling characteristics of porcine epimysial connective tissue.

Animal No.	Treatment	Water uptake[1]	Length after soaking[2]	Width after soaking[2]
11	Distilled water 2 hr	6.27	92.67	128.67
3	Distilled water 2 hr	9.29	93.17	132.00
4	Distilled water 2 hr	10.00	82.00	255.67
4	0.9% saline 2 hr	0.06	97.00	110.67
4	3 M acetic acid 2 hr	16.00	86.33	372.00

[1] Grams of water/gm tissue.
[2] Percent of initial length or width.

The results of a swelling study conducted on epimysial connective tissues from normal and PSE muscles are shown in Table 12. Analysis of variance (Appendix 11) revealed a significantly ($P < 0.05$) lower initial dry matter content and a higher initial moisture content in the epimysium

Table 12. Neutral swelling characteristics of epimysial connective tissues from normal and PSE 1. dorsi muscles.

Animal No.	Initial moisture[1]	Initial dry matter[1]	Dry matter after soaking[2]	Dry matter lost[2]	Water uptake[2]
PSE					
1	63.25	36.75	29.28	7.47	10.35
4	62.74	37.26	33.76	3.50	11.00
6	61.90	38.10	36.75	1.32	14.78
8	63.78	36.22	28.92	7.30	10.47
10	63.01	36.99	31.88	5.11	13.84
14	63.94	36.06	31.88	4.68	16.58
Mean	62.96	36.90	32.08	4.90	12.84
Std. dev.	0.62	0.74	2.92	2.33	2.61
Normal					
2	59.84	40.16	34.46	5.70	5.41
3	62.14	37.86	35.78	2.08	10.29
5	58.96	41.04	35.65	5.39	12.85
7	56.50	43.50	29.69	13.81	9.03
11	61.33	38.67	29.49	9.18	7.27
13	62.17	37.83	35.05	2.78	4.63
Mean	60.16	39.84	33.35	6.49	8.25
Std. dev.	2.20	2.20	2.95	4.38	3.10

[1]Percent of initial wet weight of tissue.
[2]Grams of water/gm tissue.

from PSE tissues. These differences were approaching significance at the 1% level. There was also a significantly greater ($P < 0.05$) amount of water imbibed by the epimysium from PSE pigs. No significant differences were evident between the two tissue types in the dry matter content after soaking. The epimysium from PSE tissues lost an average of 4.90% dry matter during soaking, while the normal tissue lost 6.49%. Although differences in dry matter losses were not statistically significant, they are thought to be real and will be further discussed later.

Table 13 shows the results of a study to determine the nature of the material lost during imbibing of water. Holding epimysial connective tissues in 1 M sodium chloride for 15 hr resulted in a loss of 3.58% of the total dry matter. Lowther et al. (1967) reported that 86% of the total tissue hexosamine from heart valves was removed by extraction in 1 M sodium chloride. These workers also reported that 70% of the total tissue hexosamine was removed by distilled water extraction. The acid mucopolysaccharides had been almost completely extracted. Thus, the loss in dry matter in the present study can probably be explained by the extraction of ground substance from the connective tissues.

Table 13. Effect of various soaking and heating treatments on the dry matter content of porcine epimysial connective tissues.

Treatment	% Dry matter	% Dry matter loss	Mc x 10^4[1]
1) Native epimysium	39.50	--	--
2) 15 hr in 1M NaCl	35.92	3.58	--
3) 2 hr in distilled water	35.78	3.72	--
4) 15 hr in 1M NaCl and 2 hr in distilled water	34.91	4.09	--
5) 1 hr in 85°C distilled water	20.26	19.24	1.34
6) 0.5 hr in 85°C distilled water	20.14	19.36	1.39
7) 2 hr in distilled water then 0.5 hr in 85°C distilled water	14.90	24.60	4.68
8) 24 hrs in formaldehyde	33.00	6.50	--

[1]Mc = molecular weight between crosslinks.

Several hypothesis can now be put forth to explain some of the previous results observed on the epimysium from PSE tissues. The lower dry matter content of PSE epimysium (Table 12) could reflect a lower ground substance content. This may also be reflected by the lower losses of dry matter from the PSE tissues during soaking in water. This conclusion is substantiated by the histochemical study, which also revealed a lower content of mucopolysaccharide material in the epimysium from PSE pigs.

The increased water uptake in PSE epimysium is not completely compatible with our knowledge on water binding by connective tissues. Ground substance is noted for its water holding capacity. Thus, the low content of ground substance in the PSE epimysium should limit the water imbibing capacity of the tissues. On the other hand, if the swelling characteristics of collagen are reflections of looser molecular ordering and less crosslinking (Gustavson, 1956; Fels, 1966), then the epimysium from PSE tissues would be expected to imbibe larger amounts of water. Results of acid soluble, salt soluble, heat labile collagen and DTA studies have all indicated that the collagen matrix in PSE epimysium is looser or less highly crosslinked, and thus should imbibe larger amounts of water.

It is suggested that the alterations in the ground substances and fibrous proteins observed in the PSE epimysium may indicate changes in the water permeability characteristics of this tissue. If this is the case, the increased moisture content of PSE epimysium could be due to the unrestricted movement of moisture from the PSE muscles into and through the surrounding connective tissues. This, along with the lowered water-

holding capacity of the PSE muscle proteins, could account for the exudative condition observed in PSE muscles.

Stress-Strain Determinations. Very little work has been reported on the stress-strain approach of measuring the heat stable convalent bonds in native collagen (Wiederhorn et al., 1953). However, it is widely held that the changes in solubility, thermal shrinkage temperature and swelling characteristics, all of which are encountered with advancing biological age and with certain diseases, are actually manifestations of the extent of crosslinking. In addition, several workers (Goll et al., 1964; Hill, 1966) have postulated that stronger or more extensive crosslinking in collagen occurs with increasing biological age, and may explain the toughness of meat. Therefore, a study was initiated to determine the crosslinking characteristics of epimysial connective tissue in relation to biological age, species and the PSE condition in swine.

As indicated previously (Table 10), initial stress-strain determinations revealed an increase in molecular weight between crosslinks (Mc), which was associated with an increase in the length of the soaking period in distilled water. Data in Table 13 revealed that several changes occurred in the tissue during the course of the stress-strain determinations. The initial soaking period in distilled water apparently removed ground substance (3.58% of the dry matter) from the epimysium. Heating in distilled water without an initial soaking period resulted in a loss of approximately 19% dry matter. The amount lost was essentially the same regardless of whether the sample was heated for 0.5 or 1 hr. The heat labile collagen

studies reported previously showed that 14 to 24% of the total collagen was released by heating to 70°C for 10 min (Table 5). Therefore, it seems likely that the dry matter lost on heating during the stress-strain studies is identical to the heat labile fraction reported earlier herein.

When samples were subjected to soaking for 2 hr in distilled water and then heated for 0.5 hr in 85°C distilled water, a loss of 24.60% of the dry matter occurred (Table 13). This represents an increased loss in dry matter of 5.24% in comparison to those samples which were not soaked in water. Therefore, in the course of stress-strain measurements, two moieties appeared to be lost from the native collagen, i.e., the water soluble ground substance and the heat labile collagen.

The soaking treatment prior to heat shrinkage also affected the molecular weight (Mc) between crosslinks (Table 13). Heat treatment with no prior soaking in water resulted in Mc values of 1.34 to 1.39 x 10^4. Samples receiving a prior water treatment had corresponding values of 4.68 x 10^4. At first glance, it would appear that the loss of ground substance resulting from soaking in water decreased the number of crosslinks. However, the data shown in Table 14, indicated that this was probably not the case. As indicated by the percentage of dry matter, soaking samples in 0.9% saline undoubtedly removed the ground substances, and yet the Mc determinations were relatively constant, even at soaking periods of up to 3.5 hr. It is suggested, therefore, that it was the swelling *per se* and not the loss of ground substance which resulted in a decrease in the crosslink density.

Table 14. Changes in porcine epimysial connective tissue characteristics with increased soaking time.

Soaking time (hrs)[1]	Dry matter[2]	Mc x 10^4[3]	Crosslinks/molecule[4]
0.5	18.16	2.43	12.22
1.5	16.48	2.66	11.26
2.5	16.04	2.75	10.91
3.5	17.85	2.99	10.00

[1]Samples soaked in 0.9% saline solution for period indicated, before subjection to 0.5 hr in 85°C distilled water.
[2]Percent on a wet weight basis.
[3]Mc = molecular weight between crosslinks.
[4]Assuming a molecular weight of 300,000 for the collagen molecule.

The reason for the decreased crosslinking observed in the water swollen epimysium is not clear. However, similar phenomena have been observed by other workers. Harkness and Harkness (1965) found that incubation of rat skin in weak buffer solutions at pH 7.0 resulted in a decrease in tensile strength. The decrease was not observed in stronger buffers. These authors suggested that this might be the result of an internal pH change produced by cellular activity in a weak external buffer. This conclusion was substantiated by Milch (1965), who reported that a sample of goatskin incubated in distilled water had a Ts of 64-65°C, while samples incubated in phosphate buffer had a Ts of 59-60°C. However, Verzar (1964) indicated that other factors are responsible for changes in connective tissues held in distilled water. He reported that immersion of collagen fibers from rat tail tendon for 10 minutes in distilled water increased the

thermal shrinkage temperature by 4°C. He found that the fibers lost all of their sodium and potassium in distilled water during periods as short as 10 min. The influence of distilled water was found to be completely reversed by immersing the fibers in 0.9% sodium chloride. Thus, Verzar (1964) concluded that lowering of the thermal shrinkage temperature, as a result of immersion in water, was due to the removal of electrolytes.

An increase in molecular weight between crosslinks (Mc) was also found to occur with increasing time in 85°C distilled water (Table 15). Mc values increased from 2.04×10^4 at 0.5 hr to a 3.69×10^4 after 2 hr. Thus, there was a decrease in crosslinks from 14.74 to 8.12/molecule. It was at first thought that the samples continued to shrink during this period, however, this was obviously not the case (Table 15). The data in Table 13 also indicated that the time in the 85°C water bath did not effect the release of heat soluble components, and therefore, should not effect the molecular weight calculations. The only parameter, which varied with time, was the length the sample attained at a given retractive force (Table 15). The implication being that covalent crosslinks were broken with increased periods of heating.

In the present investigation, heat labile collagen studies and DTA data also indicated that collagen contains bonds which vary in their sensitivity to heat. This conclusion is also substantiated by the work of Verzar (1964). He reported, that if collagen samples are held at or above the shrinkage temperature, the fibers will undergo spontaneous relaxation. As a result of relaxation, the original fiber length will eventually be

reached. He attributed the relaxation to the destruction of the strong covalent crosslinkages.

Table 15. Changes in porcine epimysial connective tissue characteristics with increased time in 85°C distilled water.

Hours in 85°C water bath	Contracted length[1]	Length to attain 5.43 gm of force[1]	Mc x 10^4[2]	Crosslink/ molecule[3]	Crosslinks broken
0.25	.156	.190	2.04	14.74	0
0.50	.160	.197	2.43	12.33	2.41
0.75	.163	.204	2.81	10.69	1.31
1.00	.163	.207	2.96	10.12	0.57
1.25	.160	.210	3.11	9.63	0.49
1.50	.160	.214	3.31	9.06	0.57
1.75	.160	.218	3.51	8.55	0.51
2.00	.160	.222	3.69	8.12	0.43

[1]Length in inches.
[2]Mc = molecular weight between crosslinks.
[3]Assuming a molecular weight of 300,000 for the collagen molecule.

The number of crosslinks broken per unit time apparently reached a minimum after 1 hr at 85°C (Table 15). Therefore, the time was standardized to 1 hr in all subsequent studies.

A graph of extension force (F) against $\alpha - \frac{1}{\alpha^2}$ (α = the relative elongation of the sample is given in Figure 7. Provided hydrothermally denatured collagen follows the theory of ideal rubber elasticity, which defines the force-extension relationship for a swollen elastomer, such a

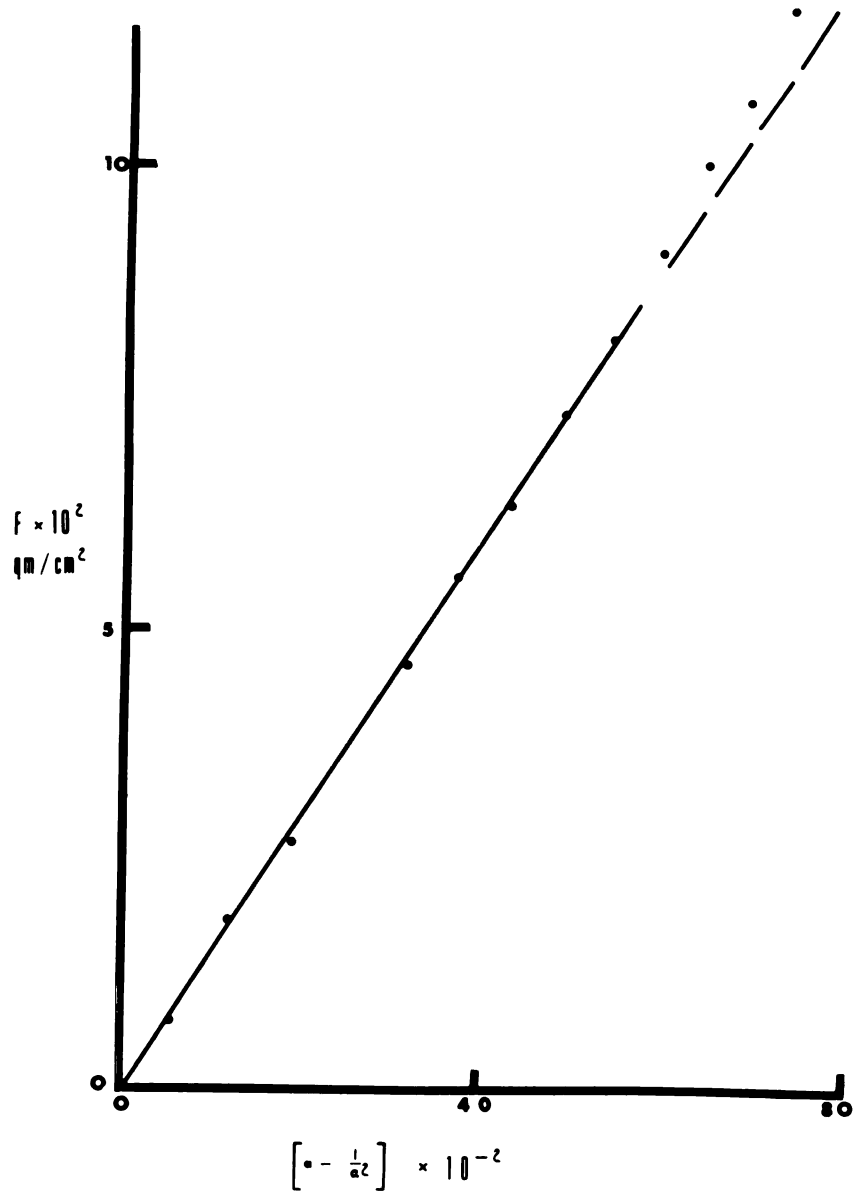


Figure 7. Plot of extension force (F)/unit cross sectional area against $[\alpha - \frac{1}{\alpha}2]$

where α = relative elongation of the sample.

graph should be a straight line passing through the origin (Cater, 1963). Figure 7 shows that the response for epimysial connective tissues was linear up to an $\alpha - \frac{1}{\alpha^2}$ value of approximately 55 x 10^{-2}, indicating that the behavior of epimysium over this range was identical to an ideal rubber. At larger deformations, a non-linear response occurred. However, determination of the molecular weight between crosslinks can be calculated from the region of linear response. Data shown in Table 16 illustrate the type of replication which can be attained with stress-strain techniques.

Table 16. Multiple determinations of molecular weight between crosslinks for porcine epimysial connective tissue.

Determination	Mc x 10^4[1]	Crosslinks/molecule[2]
1	9.108	3.29
2	8.686	3.45
3	9.515	3.15
Mean	9.103	3.30

[1]Mc = molecular weight between crosslinks.
[2]Assuming a molecular weight of 300,000 for the collagen molecule.

The results of a study on the effects of biological aging on the covalent crosslinking in epimysial connective tissues are shown in Table 17. These results clearly showed a decrease in the Mc value and a corresponding increase in the number of covalent crosslinks with advancing biological age. The Mc value declined from 8.00 to 4.67 x 10^4, with the corresponding number of crosslinks increasing from 4.09 to 7.73/molecule as age increased from 1 wk to 6 mo. The small Mc value and resultant large number of crosslinks observed in commercial grade beef may partially

reflect species differences. However, it is probably due largely to a manifestation of the aging process.

Table 17. Effect of biological aging and species on the molecular weight between crosslinks in epimysial connective tissues.

Source	Number of animals	Mc x 10^4[1]	Crosslinks/molecule[2]
1 week old pig	2	8.00	4.09
7 week old pig	3	6.58	4.81
6 month old pig	6	4.67	7.73
Commercial grade beef	2	1.42	21.31

[1]Mc = molecular weight between crosslinks.
[2]Assuming a molecular weight of 300,000 for the collagen molecule.

A comparison with values reported in the literature is difficult, due mainly to the limited amount of work reported and the variety of sources. Wiederhorn and Reardon (1952) reported an Mc value of 5.5 x 10^4 for kangaroo tail tendon. Cater (1963), however, found a value of 19.9 x 10^4 for freshly isolated kangaroo tail tendon, as compared to a value of 5.5 x 10^4 after preparation for surgical sutures. Cater (1963) also reported a value of 6.4 x 10^4 for wallaby tail tendon that had been acetone dehydrated and stored in the air dry state for a number of years.

The only study found in the literature pertaining to stress-strain studies in relation to the age of the animal was reported by Kulonen et al. (1963). They found that the Mc value for rat skin was 15.0 x 10^4 at 3 mo of age, 9.5 x 10^4 at 6 mo, 4.5 x 10^4 at 12 mo and 3.7 x 10^4 at 24 mo of age. These same workers found a value of 5.0 to 6.5 x 10^4 in rat tail tendon, regardless of age.

The results of the present study, showed that epimysium from commercial grade beef had a crosslink density of approximately $7.0/10^5$ gm of collagen (Table 17). This is in good agreement with the work of Blumenfeld and Gallop (1962), who reported that gelatin had 5 esterlinks/10^5 gm of gelatin. Harding (1965) also found 6 esterlinks/10^5 gm for calf skin collagen, and 6.4 esterlinks/10^5 gm of soluble hide collagen from mature cattle. The above values do not, however, agree with those obtained for porcine epimysium in the present study. The number of crosslinks for the porcine epimysium varied from 1 to $2/10^5$ gm of collagen, depending upon the age of the animal.

The changes in molecular weight between crosslinks due to formaldehyde treatment of epimysial connective tissues are shown in Table 18. Formaldehyde treatment decreased the Mc values from 4.67×10^4 to approximately 8.0×10^3. Consequently, the number of crosslinks increased from 7.73 to 36.01/molecule. These values are lower than those reported by Wiederhorn and Reardon (1952), who found an Mc value of 1.5×10^4 for formaldehyde treated kangaroo tail tendon. Cater (1963), however, found a value of 8.9×10^3 for kangaroo tail tendon, and a value of 2.68×10^4 for calf tendon. Apparently, the increase in crosslinks due to formaldehyde are effected by age and species.

It is also interesting to note that formaldehyde treatment reduced the loss in dry matter in comparison to untreated samples (Table 13). Apparently formaldehyde was effective in crosslinking the collagen fraction, which is normally lost during heating of the untreated material.

Table 18. Effect of formaldehyde tanning on the molecular weight between crosslinks in porcine epimysial connective tissue.

Incubation time in 0.1% formaldehyde	Mc x 10^3[1]	Crosslinks/molecule[2]
4 hrs	7.87	38.14
24 hrs	8.27	36.27
48 hrs	8.33	36.01

[1]Mc = molecular weight between crosslinks.
[2]Assuming a molecular weight of 300,000 for the collagen molecule.

The results of stress-strain studies on epimysial connective tissues from normal and PSE muscles are shown in Table 19. Analysis of variance (Appendix 12) revealed that the differences in crosslinks/molecule were only significant at the 10% level. However, based on data from acid and salt solubility, heat labile collagen and DTA studies, it seems probable that the differences in covalent crosslinkages between normal and PSE tissues are real.

Table 19. Stress-strain measurements on epimysial connective tissues from normal and PSE 1. dorsi muscles.

PSE			Normal		
Animal No.	Mc x 10^4[1]	Crosslinks/ molecule[2]	Animal No.	Mc x 10^4[1]	Crosslinks/ molecule[2]
14	4.34	6.90	7	2.97	10.09
4	10.83	2.76	2	9.10	3.30
1	7.65	3.91	13	5.91	5.08
6	4.90	6.11	3	2.51	11.95
8	4.50	6.68	11	3.60	8.33
10	5.94	5.04	5	3.92	7.64
Mean	6.37	5.23	>	4.67	7.73
Std. dev.	2.51	1.65		2.46	3.17

[1]Mc = molecular weight between crosslinks
[2]Assuming a molecular weight of 300,000 for the collagen molecule

Although the results of stress-strain studies comparing normal and PSE epimysium are not clear cut, nevertheless, it is suggested that this technique provides a useful tool for measuring variation in the cross-linking characteristics of connective tissues.

Amino Acid Analysis and Plasma Hydroxyproline Levels

It was anticipated that the amino acid composition might be helpful in explaining the differences in solubility, DTA and stress-strain values observed between normal and PSE epimysium. Several workers have reported a relationship between amino acid composition and the thermal shrinkage temperature (Ts) of collagen. Gustavson (1956) found that the Ts increased with the amount of hydroxyproline. He interpreted this as being due to the hydrogen bonding capacity of the hydroxyl group of hydroxyproline. Piez and Gross (1960) found that Ts correlated equally well with the proline content, and that the sum of the two imino acids gave the best correlation. They attributed the correlation to the steric hindrance of rotation by the imino acid residues, when they are located adjacent to each other.

Therefore, a study of the amino acid composition of pooled samples of purified acid soluble collagen extracted from the epimysium of normal and PSE muscles was initiated.

The results of this analysis were limited by the fact that standard runs revealed that hydroxyproline came off the column at the same time as aspartic acid. Although hydroxyproline exhibited only minimum absorption at 570 mμ, where the aspartic acid-ninhydrin complex is measured, the

value for aspartic acid is undoubtedly high. In addition, it was not possible to quantitatively determine the hydroxyproline content in this analysis. However, the percent hydroxyproline was determined utilizing the colorimetric method of Woessner (1961). Using this method, values of 13.10 and 13.25% hydroxyproline were found for normal and PSE acid soluble collagen, respectively.

The partial amino acid composition of the acid soluble collagen from normal and PSE epimysium was very similar (Table 20). Thus, variation in the primary structure of the collagen molecule was probably not responsible for the differences between PSE and normal epimysium in the acid and salt solubility, thermal lability or DTA values.

Bates et al. (1962) postulated that the level of plasma hydroxyproline could be used as an indicator of the breakdown of collagen. In addition, LeRoy et al. (1964) concluded that the relative and absolute amounts of free, peptide bound, and protein bound hydroxyproline in the blood plasma was a reflection of collagen biosynthesis and metabolism. Therefore, a study of plasma hydroxyproline levels was initiated in order to determine if there were differences between normal and PSE tissues in the rate of collagen synthesis or breakdown.

It was noted that most of the heparinized plasma samples contained a precipitate after freezing and thawing. LeRoy et al. (1964) also reported the formation of a precipitate in frozen and thawed heparinized plasma. They found that removal of the precipitate reduced the protein bound hydroxyproline level by 30%. These authors found no losses on

Table 20. Partial amino acid composition of acid soluble collagen from the epimysium of normal and PSE muscles.

Amino acids	Normal[1]	PSE[1]
Lysine	3.81	3.68
Histidine	0.99	0.94
Arginine	5.13	5.03
Hydroxylysine	0.65	0.67
*Aspartic acid	6.15	6.00
Threonine	2.62	2.57
Serine	5.09	4.89
Glutamic	8.56	8.48
Proline	15.04	15.97
Glycine	29.51	30.19
Alanine	10.30	10.61
Valine	3.02	2.91
Methionine	0.59	0.64
Isoleucine	1.71	1.61
Leucine	3.98	3.75
Tyrosine	0.97	0.93
Phenylalanine	1.89	1.77

[1]Molecule percent amino acid, average of two determinations.
*Value for aspartic acid is high due to interference from hydroxyproline.

freezing or storage when citrate was used as the anticoagulant. Although hydroxyproline levels in the present study agree very well with those reported by LeRoy et al. (1964), it is entirely possible that some protein bound hydroxyproline was lost due to precipitation.

The results of this study (Table 21) revealed a slightly higher plasma hydroxyproline level in the normal animals. Analysis of variance showed that this difference was not statistically significant (Appendix 13). It is suggested, however, that further study on the amounts and types of plasma, and perhaps urinary, hydroxyproline might yield valuable information into the nature of the PSE condition.

Table 21. Plasma hydroxyproline levels in normal and PSE pigs.

PSE		Normal	
Sample No.	Plasma hydroxyproline[1]	Sample No.	Plasma hydroxyproline[1]
11	7.30	3	11.80
2	9.65	4	9.10
17	7.80	6	9.10
18	14.15	9	10.80
19	8.15	13	9.90
Mean	9.41		10.14
Std. dev.	2.79		1.16

[1]Values are expressed in μg of hydroxyproline per ml of plasma.

Some Implications On Possible Causes of the PSE Condition

A number of possible explanations can be put forth to explain the alterations in the PSE epimysial connective tissue. The rapid pH drop at high temperature could conceivably alter the ground substance and fibrous proteins. The immediate formation of lactic acid (1%) in high temperature and low pH muscle (Briskey, 1963) may bring about an alteration in the collagen fibers similar to that described by Gustavson (1956). He reported that lactic acid and certain other solvents (phenol, m-cresol, formamide and formic acid) break the collagen crosslinks and cause dissolution of the collagen fibers.

In addition, it must be recognized that some of the PSE musculature may be pathological or dystrophic in nature. Briskey et al. (1959b) have

reported that all porcine muscles are dark and firm at the time of death. Several workers (Bendall and Wismer-Pedersen, 1962; Cassens et al., 1963) have also shown that there is no histologically visible evidence of degeneration at the time of death in muscle which ultimately becomes PSE. However, Briskey (1964) in a review of unpublished work by Norman and Sayre, reported that structural abnormalities have been found in PSE, as well as in dark, firm and dry muscles. Bodwell et al. (1965) also reported the presence of acid and alkaline phosphatase activity in the muscles of seven pork carcasses, and suggested that a degenerative condition existed.

The work of Woessner (1965) may also be pertinent to the problem of PSE musculature. He found that vitamin A administration to Xenopus larvae caused changes in the mucopolysaccharides of the connective tissues. In the rabbit, collapse of the ear cartilage and damage to the knee cartilage was induced by excess Vitamin A. Similar studies on the rat showed that mucopolysaccharides were also lost from the skin. Woessner (1965) concluded that the vitamin A excess in vivo caused liberation of hydrolases from the lysosomes. In further studies, Woessner (1965) found that rudiments of limb explants lost protein, hexosamine and metachromasia most rapidly at pH 4 to 5. He thus concluded that the low pH brought about a release of hydrolases into the extracellular matrix. The proteases then attacked mucopolysaccharide-protein complexes, liberating both components from the matrix. Muir (1964) also reported that vitamin A caused release of liver lysosomal enzymes. These enzymes reduced metachromasia and caused the release of chondroitin sulfate. A similar mechanism could conceivably

be functioning in PSE muscle. The release of lysosomal enzymes by muscle or connective tissue would account for the decreased metachromasia and increased salt soluble and heat labile fraction of the PSE epimysium in the present study.

Tappel et al. (1963) demonstrated the presence of a large number of lysosomal enzymes in the leg muscles of rabbits and mice, and in the breast muscle of chickens. They also found that acid pH and high temperatures favored lysosomal activity in the breakdown of the mitochondrial structure. These workers further stated that production of metabolic acidity may be the most important factor for physiological autolysis. Lysosomal preparations have the capacity to cleave cytochromes from the mitochondria. This cleavage destroys the energy yielding capacity of the mitochondria and leads to disorders in several energy dependent processes of the cell. The metabolic acidity, thereby produced, could be sufficient to activate the cathepsins, and thus hydrolyze the subcellular constituents at a faster rate (Tappel et al., 1963).

The implication of these findings to the PSE condition are obvious. The rapid pH drop observed in PSE muscle could be a result of lysosomal cleavage of the cytochromes from the mitochondria, thereby producing rapid metabolic acidity. The lysosomal action could be mediated by cortisone. Muir (1964) reported that cortisone prevents the vitamin A effect, apparently by increasing lysosomal stability. Thus, cortisone prevents release of cathepsins into the circulation, which otherwise follows traumatic shock (Muir, 1964). Whether or not lysosomes actually are the cause of

the rapid pH drop observed in PSE tissues remains to be determined. At any rate, the low pH and high temperature conditions found in PSE muscle are ideal for the release and activation of lysosomal enzymes. The hydrolytic effect of these enzymes under low pH and high temperatures could be responsible for the development of the pale, soft and exudative appearance.

Another interesting and highly pertinent finding has been reported by Houck and Patel (1965). They found abrupt losses of insoluble dermal collagen in response to necrotic injury, and to the administration of various hormones such as cortisol, thyroxin or prolactin. Concurrently, a non-lysosomal protease was released into the extracellular compartment. Along with the release of this protease, the amount of activated collagenase also increased. The activated collagenase caused the dissolution of insoluble collagen into two products, one diffusing away from the tissue and the other being soluble in dilute acid.

In light of the results of the present investigation, it is suggested that further study on the influence of lysosomes and naturally occurring collagenases on problems such as aging, tenderness and pale, soft and exudative pork may be of benefit.

SUMMARY AND CONCLUSIONS

The first part of this investigation was conducted to physically and chemically characterize porcine epimysial connective tissues. The following observations were made:

1. Alcohol reversible metachromasia and the almost nonexistent dye binding at low pH indicated that the predominant mucopolysaccharide in porcine epimysial connective tissues was hyaluronic acid.

2. Solubility studies showed that normal porcine epimysium contained 3.20% of salt soluble and 0.31% of acid soluble tropocollagen.

3. Densitometer tracings of disc gels revealed that purified acid soluble collagen from the epimysium contained 38.46% of α- and 61.54% of β-components. These results indicated that the acid soluble collagen was 92.27% intramolecularly crosslinked.

4. Normal porcine epimysial connective tissue contained 15.02% of heat labile collagen.

5. As determined with the melting point apparatus, the thermal shrinkage temperature of porcine epimysium was 59.0 to 61.8°C. However, DTA data indicated that collagen does not have a true thermal shrinkage temperature, but rather the thermal shrinkage phenomena occurs over a wide and variable range.

6. Porcine epimysial connective tissue was found to undergo osmotic swelling in neutral solutions. The water uptake was 8.15 gm/gm of tissue. Dry matter decreased during swelling, and appeared to be due to a loss of ground substance.

7. Stress-strain studies showed that hydrothermally denatured epimysial connective tissue followed the theory of ideal rubber elasticity. The molecular weight between crosslinks (Mc) was found to be 46,700, and the number of crosslinks was calculated to be 7.73/molecule.

8. Formaldehyde tanning of epimysium resulted in a decrease in Mc values to approximately 8,000, and a subsequent increase in crosslinks to 36/molecule.

The second phase of this study dealt with a comparison of the epimysium from normal and from pale, soft and exudative (PSE) pigs. The results were as follows:

1. Markedly higher transmission values were found for the PSE muscles, indicating that most of the sarcoplasmic proteins had been precipitated.

2. The pH values were similar for both the normal and PSE muscle tissues, indicating that the ultimate pH is not responsible for the occurrence of the PSE condition.

3. Light intensity values showed that epimysium from PSE muscles had a lower or an altered hyaluronic acid content. No distinctive histological differences in either the fiber type or in their appearance were evident between normal and PSE connective tissues.

4. Solubility studies revealed that the epimysium from PSE tissues contained significantly ($P < 0.05$) more salt soluble tropocollagen than that from the normal tissues, while the difference in acid soluble collagen was not significant. These results showed that the fibrous proteins were also altered in the PSE epimysium, and apparently contained fewer

intramolecular crosslinks, or were of a more immature type. The percentage of α- and β-components in the acid soluble extract from PSE epimysium was not significantly different from the normal.

5. Heat labile studies revealed that both normal and PSE epimysium contained 25% collagen. In the case of the PSE epimysium, however, 22.98% was released upon heating as compared to 15.02% for the normal. These results also indicated a lower number or a decreased strength of crosslinks in the epimysium from PSE muscles.

6. Differential thermal analysis revealed that epimysial connective tissue from PSE muscles had a total melting point range similar to that of normal tissues. However, onset and recovery occurred at lower temperatures. The epimysium from PSE muscles also contained a higher percentage of low temperature melting components. The DTA data also indicated that the epimysial collagen from PSE pigs was intermediate in maturity between that of 7 week and 6 month old pigs.

7. Neutral swelling studies showed that the epimysium from PSE muscles had a significantly ($P < 0.05$) higher water uptake than that from normal pigs, indicating a lower degree of molecular ordering or crosslinking. The initial dry matter content and the dry matter losses during swelling were also lower in the PSE epimysium. These results substantiate those of the histochemical studies, which indicated a lower ground substance content in the epimysium from PSE muscles.

8. Stress-strain studies revealed an increased Mc value and a decreased number of crosslinks in the epimysium from PSE muscles. These

results showed that the PSE epimysial collagen had fewer covalent cross-links than that from normal pigs.

9. The amino acid composition of purified acid soluble extracts from normal and PSE epimysium was very similar. Therefore, variation in the primary structure of the collagen molecule was probably not responsible for differences between PSE and normal epimysium.

10. No differences were detected in blood plasma hydroxyproline levels between normal and PSE pigs.

The third phase of this investigation dealt with alterations occurring in the connective tissues in relation to source, biological age, species and nutritional status. The following observations were made on this phase of the study:

1. Biological age influenced values obtained by both the DTA and stress-strain methods. The peak DTA temperatures in epimysium from 7 week old pigs was similar to that for 6 month old pigs. However, there was a decrease in low melting components and an increase in high melting components associated with age. A decrease in Mc values and a corresponding increase in the number of covalent crosslinks were also noted for the pig as age increased from 1 week to 6 months.

2. The epimysium from commercial grade beef had a much narrower DTA melting point range, with onset and recovery occurring at much higher temperatures than that for porcine epimysium. Stress-strain studies also showed that bovine epimysium had much lower Mc values and a much greater crosslinking density than that from porcine tissues.

3. Variations due to source of connective tissue were also observed in the DTA parameters. Corium had a broader melting point range and a higher recovery temperature than epimysium.

4. The DTA data showed that zinc deficient pigs had a much narrower melting point range, a higher onset temperature and a much larger percentage of higher melting components than the pigs on a normal diet.

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APPENDIX

Appendix 1. Fixing solution

Formalin	15.0 ml
Trichloracetic acid	0.5 gm
Picric acid (2% in 50% ethanol)	85.0 ml

Appendix 2. Disc gel working solutions.

Lower gel (per 100 ml)

12.00 ml 1N KOH

5.60 gm Glacial acetic acid

15.00 gm Cyanogum

Final pH = 4.2

Upper gel (per 100 ml)

12.00 ml 1N KOH

0.78 gm Glacial acetic acid

6.00 gm Cyanogum

Ammonium persulfate solution

0.40 gm ammonium persulfate

100.00 ml distilled water

Appendix 3. Equation for calculating molecular weight between crosslinks

$$Mc = \frac{v^{1/3}}{f} R T d (\alpha - \frac{1}{\alpha^2})$$

where:

f = retractive force per unit cross-section

α = relative elongation of the sample, i.e., the ratio of the stretched length to the initial length.

d = density of the unswollen rubber

T = temperature in degrees K

R = gas constant (8.478×10^4)

Mc = average molecular weight of the chains between crosslinks

v = volume fraction of the rubber constituent of the sample

Appendix 4. Analysis of variance for turbidity values and ultimate pH.

Source of variation	Sum of squares	d.f.	Mean square	F value
Turbidity				
Treatment	12805.3333	1	12805.3333	68.8245**
Error term	2006.3333	10	200.6333	
Total	14811.6666	11		
pH				
Treatment	0.0002	1	0.0002	0.392
Error term	0.0509	10	0.0051	
Total	0.0511	11		

* $P < 0.05$

** $P < 0.01$

Appendix 5. Analysis of variance for light intensity values.

Source of variation	Sum of squares	d.f.	Mean square	F value
Rep	15.1666	2	7.5833	4.6075
Tr	59.6302	1	59.6302	36.2310*
Rep x Tr	3.2916	2	1.6458	
pH	118.7552	1	118.7552	35.2956**
Tr x pH	6.3802	1	6.3802	1.8962
Rep x pH + Rep x Tr x pH	13.4583	4	3.3645	
Fix	18.1302	1	18.1302	4.5281*
Tr x Fix	3.7968	1	3.7968	0.9482
pH x Fix	19.3802	1	19.3802	4.8403*
Tr x pH x Fix	4.3802	1	4.3802	1.0939
Er	128.1250	32	4.0039	

Rep = Replicates
Tr = treatment - PSE vs normal
pH = pH of staining
Fix = Fixing condition
Er = Error term
* $P < 0.05$
** $P < 0.01$

Appendix 6. Analysis of variance for acid and salt soluble collagen.

Source of variation	Sum of squares	d.f.	Mean square	F value
		Salt Soluble		
Treatment	3.5861	1	3.5861	7.6889*
Error term	4.6640	10	0.4664	
Total	8.2501	11		
		Acid Soluble		
Treatment	0.0147	1	0.0147	2.3532
Error term	0.0624	10	0.0062	
Total	0.0771	11		

* $P < 0.05$

** $P < 0.01$

Appendix 7. Analysis of variance for percent α- and β-subunits.

Source of variation	Sum of squares	d.f.	Mean square	F value
Treatment	0.8802	1	0.8802	0.3417
Error term	25.7604	10	2.5760	
Total	26.6406	11		

* $P < 0.05$

** $P < 0.01$

Appendix 8. Analysis of variance for percent heat labile and total collagen.

Source of variation	Sum of squares	d.f.	Mean square	F value
		Heat Soluble		
Treatment	190.0848	1	190.0848	21.5626**
Error term	88.1547	10	8.8155	
Total	278.2394	11		
		Total Collagen		
Treatment	31.0087	1	21.0087	0.1111
Error term	2788.8300	10	278.8830	
Total	2819.8387	11		

* $P < 0.05$

** $P < 0.01$

Appendix 9. Analysis of variance for thermal shrinkage temperatures (Ts)

Source of variation	Sum of squares	d.f.	Mean square	F value
		Shrinkage Onset		
Treatment	0.0417	1	0.04167	0.1429
Error term	1.1666	4	0.2917	
Total	1.2083	5		
		Shrinkage Completion		
Treatment	0.0417	1	0.0416	0.1429
Error term	1.1667	4	0.2916	
Total	1.2083	5		
		Denaturation		
Treatment	0.0417	1	0.4167	0.0270
Error term	6.1667	4	1.5417	
Total	6.2083	5		

* $P < 0.05$

** $P < 0.01$

Appendix 10. Analysis of variance for DTA determinations.

Source of variation	Sum of squares	d.f.	Mean square	F value
		Onset		
Treatment	20.8560	1	20.8560	17.0755**
Error term	12.2140	10	1.2214	
Total	33.0700	11		
		Extrapolated Onset		
Treatment	5.5760	1	5.5760	17.0519**
Error term	3.2700	10	0.3270	
Total	8.8461	11		
		Peak		
Treatment	1.1907	1	1.1907	11.1722**
Error term	1.0657	10	0.1066	
Total	2.2564	11		
		Recovery		
Treatment	39.8581	1	39.8581	14.3293**
Error term	27.8158	10	2.7816	
Total	67.6738	11		

* $P < 0.05$

** $P < 0.01$

Appendix 11. Analysis of variance for neutral swelling characteristics.

Source of variation	Sum of squares	d.f.	Mean square	F value
		Initial Moisture		
Treatment	23.6322	1	23.6322	9.0138*
Error term	26.2179	10	2.6218	
Total	49.8499	11		
		Dry Matter		
Treatment	26.0485	1	26.0485	9.6349*
Error term	27.0356	10	2.7035	
Total	53.0840	11		
		Dry Matter After Soaking		
Treatment	4.8769	1	4.8769	0.56610
Error term	86.1490	10	8.6149	
Total	91.0259	11		
		Dry Matter Lost		
Treatment	7.6161	1	7.6161	0.6187
Error term	123.0985	10	12.3099	
Total	130.7147	11		
		Water Uptake		
Treatment	63.2043	1	63.2043	7.7065*
Error term	82.0140	10	8.2014	
Total	145.2184	11		

* $P < 0.05$
** $P < 0.01$

Appendix 12. Analysis of variance for stress-strain measurements.

Source of variation	Sum of squares	d.f.	Mean square	F value
	Molecular Weight Between Crosslinks			
Treatment	8.6360	1	8.6360	1.3933
Error term	61.9813	10	6.1981	
Total	70.6174	11		
	Crosslink/Molecule			
Treatment	18.7250	1	18.7250	2.9286[1]
Error term	63.9386	10	6.3938	
Total	82.6636	11		

* $P < 0.05$

** $P < 0.01$

[1] $P < 0.10$

Appendix 13. Analysis of variance for plasma hydroxyproline levels.

Source of variation	Sum of squares	d.f.	Mean square	F value
Treatment	1.3323	1	1.3323	0.2915
Error term	36.5690	8	4.5711	
Total	37.9013	9		