Chemical Responses of Connective Tissue of Bovine Skeletal Muscle

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The connective tissue in skeletal muscle has long been considered important in the tenderness of meats. Attempts to partition muscle and measure the connective tissue fraction have claimed the attention of meat analysts for more than half a century. The lack of agreement of results from diverse laboratories focuses attention on the validity of the methods, and makes manifest that gross analyses will reveal little of the fundamental causes of tenderness. Connective tissue is composed of fibrillar elements, the collagen and elastin strands, held in an adhesive matrix, the ground substance. The latter moiety has been neglected in attempts to assess tenderness and to unravel the basic causes of changes in tenderness, as in the ripening of meats. Alkali has been an extractant classically and widely used. In these studies a mild extracting solution of potassium chloride is compared with alkali. The responses of various fractions to extractions, to autoclaving, and to certain enzymatic digestions form the bases for the postulations presented.

TENDERNESS IN MEATS is one of the most desired qualities. Manifestly, tenderness accrues from the chemical and physical properties of muscle components. The belief has long been held that the connective tissue plays a distinctive role in the tenderness of meat. Numerous attempts have been made to partition muscle tissue into distinct fractions and to make quantitative measurements of the various components. Values reported in the literature for the different fractions of muscle tissue evidence a lack of concordance and cast little light on the fundamental causes of tenderness. It becomes apparent that the assessment of tenderness will not be completely revealed by determinations of the quantities of the diverse fractions of muscle tissue. There remains the need for a better understanding of the role of each component in relationship with other constituents and of the post-mortem alterations in the tissue components with their resultant effects on tenderness.

It has been established that during the ripening of beef, held at refrigerated temperatures, certain changes occur; among these is an increase in tenderness. Numerous reports (*3, 5, 6, 18*) have shown that there is a change in the degree of tenderness as assessed by organoleptic evaluations and mechanical shear force measurements. The theory subscribed to by many workers is that enzymes, presumably proteases, are responsible for alterations in the tenderness (*1*), the basic belief being that these enzymes are capable of modifying the structure of skeletal tissue. However, there appears to be little or no consistent relationship between changes in degree of tenderness and changes in nonprotein nitrogen (*6*). In Bate-Smith's 1948 review (*1*) he stated, "the tenderness changes have therefore to be explained in terms of much less drastic proteolysis than the complete breakdown to noncoagulable end products."

Others (*7, 19*) have been unable to demonstrate that increases in tenderness of beef during ripening have been associated with decrements of connective tissue as measured by rather classical methods of assessment, such as the Lowry, Gilligan, and Katersky method (*10*). Recently, Wierbicki and others (*23*) have suggested that connective tissue does not appear to be involved in tenderness increases during ripening, but that such changes are primarily related to the association of actomyosin or some similar protein changes which were revealed by changes in extractability. They further suggested that meat may be markedly modified in tenderness by changing the ionic atmosphere in muscle.

An adequate appraisal of the reports of investigation of the role of the skeletal tissue components is difficult. The multiplicity and complexity of the constituents of biological material impose inherent obstacles in fractionation and isolation procedures. The aim, succinctly, is a partitioning of the intracellular proteins from the extracellular constituents, which make up the connective tissue. The primary components of connective tissue are the collagen fibers, the elastin fibers, and the mucinous adhesive ground substance. The definition of the diverse tissue components, from an assessment viewpoint, has traditionally been in terms of the characteristics evidenced in general response to specific treatment, such as solubility, resistance to acids and alkalies, thermal treatment, and staining reactions, and as enzymatic substrates.

In the assessment of connective tissue the separatory procedure for the estimation of collagen has been by autoclaving the tissue with water for the conversion of collagen to gelatin. The use of this partitioning technique has invoked the assumptions that the collagen was not affected by the prior treatment of the tissue, that autoclaving conditions selectively hydrolyzed the collagen to gelatin, and that determination of nitrogen in the solubilized hydrolyzate constituted a valid estimate of the collagen content. The moiety designated as elastin is based on the concept of its chemical inertness, its insolubility; the final residuum after exhaustive extraction with dilute acids or alkali is conventionally defined as elastin. It has been rather widely accepted that collagen is not solubilized by 0.1*N* sodium hydroxide. However, Lloyd (*9*) demonstrated the dissolution of tendon in very dilute alkaline solutions; and the solubilization of collagen in extremely dilute acetic acid was reported by Nageotte and Guyon (*13*). The Lowry, Gilligan, and Katersky method (*10*) is essentially an exhaustive fractionation using 0.1*N* sodium hydroxide for the extractant. Work in this laboratory, using a modification of the Lowry procedure, in the analysis of certain raw (r) tissues and correlated cooked (c) samples from the paired location in the carcass yielded the values for collagen and elastin recorded in Table I.

In the cooked samples the fraction assayed as collagen showed consistent decreases in relation to the values for the corresponding raw samples. It is easy to document this phenomenon. The

Table I. Collagen Nitrogen and Elastin Nitrogen in Raw and Cooked Samples of Beef Muscle

Muscle Sample	Collagen N	Elastin N
Longissimus dorsi		
1 r	0.98	0.06
1 c	0.10	0.07
Semitendinosus		
2 r	3.50	0.32
2 c	0.48	1.17
Longissimus dorsi		
3 r	1.26	0.03
3 c	0.04	0.06
Semitendinosus		
4 r	1.80	0.66
4 c	0.60	1.56

Nitrogen values expressed as per cent nitrogen of total nitrogen. Samples 1 and 2 were from a carcass graded commercial; samples 3 and 4 were from a carcass graded choice.

values for the terminal residuum, elastin, should also be noted. In all instances, there were increases in the elastin values of the cooked sample in comparison with corresponding values for uncooked samples. This anomaly casts considerable doubt upon the validity of the method of assessment. In many investigations which have been reported only the collagen values have been determined; lacking residual values for summation and comparison, the validity of the method of analysis perhaps did not plague the analyst. Trypsin has also been used in muscle tissue fractionation, with the belief that it did not attack collagen. However, Sizer (*21*) showed that the response of collagen as substrate for trypsin activity was related to fineness of division of the fibers. In 1950 Neuman and Tytell (*15*) reported that the sensitivity of the collagens to enzymatic digestion is a reflection of the prior treatment, and that the use of hide powder collagen as a reference could not be relied upon in analytical work.

It is known that there are variations in the sizes of both the collagen and elastin strands and in the forces which bind the fibrils together. These variations have their origin in species differences, in functional diversities within an organism, and in age alterations. In addition to the diversities among the fibrillar components of connective tissue, there are also differences in the adhesive milieu, the ground substance, in which the collagen and elastin fibers are embedded (*4*). The differing combinations of the mucopolysaccharides found in the ground substance of various connective tissues and their varying labilities have been suggested by Meyer and others (*11*).

The literature which deals with or refers to connective tissue is prodigious, yet there is a dearth of unequivocal information concerning the composition of intramuscular connective tissue; and even less is known with certainty of the role of connective tissue components in meats. It was deemed of value to pursue certain exploratory studies in fractionation and to draw comparisons of certain moieties with the characterization of fractions wherever feasible.

Experimental Procedure

Bovine muscle was the tissue used throughout this study. In the interest of minimizing the problems arising from biological variation, only the semitendinosus and the semimembranosus muscles were used in these investigations. Numerous methods of maceration of muscle tissue were explored. When a meat grinder or the Waring Blendor is used, the coarse connective tissue strands become enwrapped on the worm or the blades and thence are not included, or at best only partially so, in the analysis which is aimed at their measurement. If the entangled strands are scraped from the grinder, they still are not adequately comminuted and spell failure in uniformity of sampling. The use of the silent cutter in the comminution of muscle tissue resulted in the most nearly uniform values of any of the many macerative procedures pursued.

Several extracting solutions of differing molarities were compared for efficiency of protein extraction. Because a mild extractant was desired, a solution of 0.6*M* potassium chloride and 0.03*M* sodium hydrogen carbonate was chosen. This is very similar to the widely used Weber-Edsall solution. Varying strengths of sodium hydroxide were also compared as extracting solutions. For the greater part of these investigations, however, when an alkaline extractant was employed a 0.1*N* sodium hydroxide solution was used.

The muscles were removed from the carcass at time of slaughter, trimmed of extraneous surface fat, and comminuted for 3 minutes in a silent cutter. Samples of the comminuted muscle tissue were wrapped in aluminum foil and stored at −34.4° C.

In preliminary investigations 25 grams of beef tissue was Waring-blended with the extracting solution. The separation of the insoluble proteins from the solubilized fractions was attempted by the commonly used centrifugation. These operations and subsequent manipulations, unless otherwise indicated, were carried out at 0.5° to 2° C. in a cold room. Considerable gelling of extracts occurred in the centrifuge tubes and resulted in ineffective sedimentation of the insoluble materials; hence physical entrapment of the stroma proteins on a mass of glass wool in an Erlenmeyer flask was devised as a separatory technique. The procedure for isolating the stroma proteins was conducted as follows. Five grams of frozen comminuted beef tissue was placed in 300-ml. Erlenmeyer flasks. The tissues in the tightly stoppered flasks were placed in the cold room to thaw. When the tissue had thawed, small portions of extracting solution were blended with the tissue into a soft mass by using a glass rod. Additional amounts of ice-cold extracting solution (total volume 95 ml.) were blended concurrently with small portions (1.25 grams) of medium grade glass wool cut in $^3/_4$-inch lengths. The tightly stoppered flasks were gently shaken in a horizontal shaker; foam formation was avoided by controlling the speed of the shaker. The dispersed proteins were decanted through a filter pad of glass wool, and the entrapped stroma proteins were retained on the glass wool. The entrapped residues were triple-washed with portions of dis-

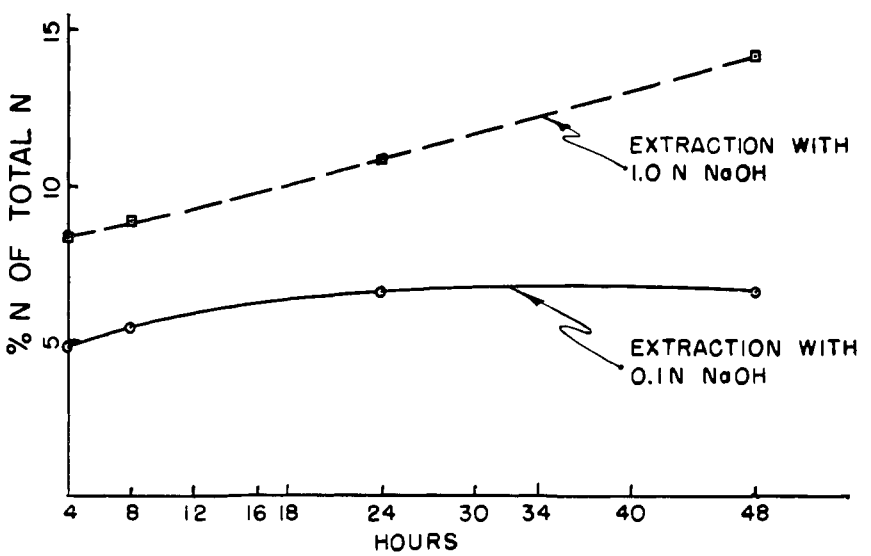

Figure 1. Percentages of protein extracted with 0.1*N* sodium hydroxide vs. 1.0*N* sodium hydroxide during 48-hour period

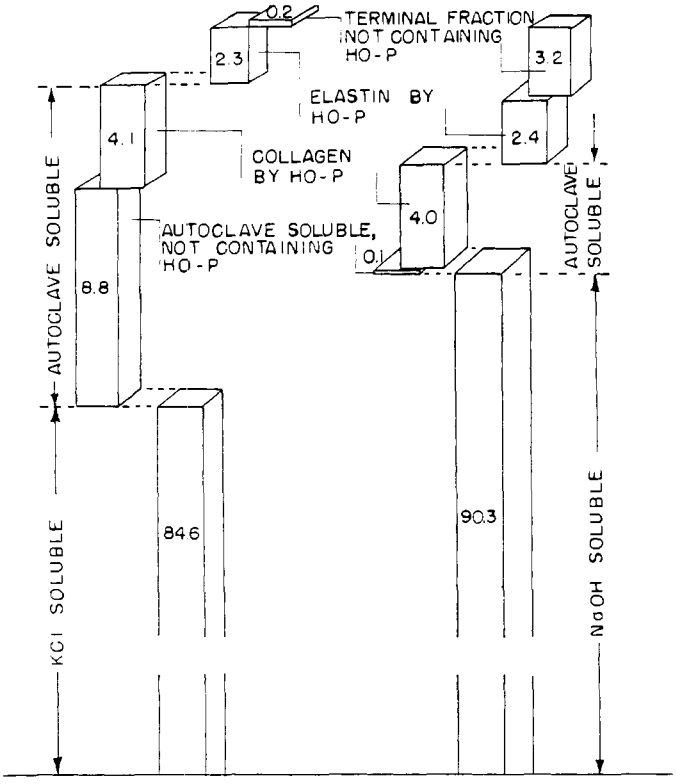

Figure 2. Fractionation of semitendinosus tissue by potassium chloride and sodium hydroxide extractions and by autoclaving. Nitrogen values expressed as per cent nitrogen of total nitrogen. Assessment of nitrogen by Kjeldahl and hydroxyproline determinations

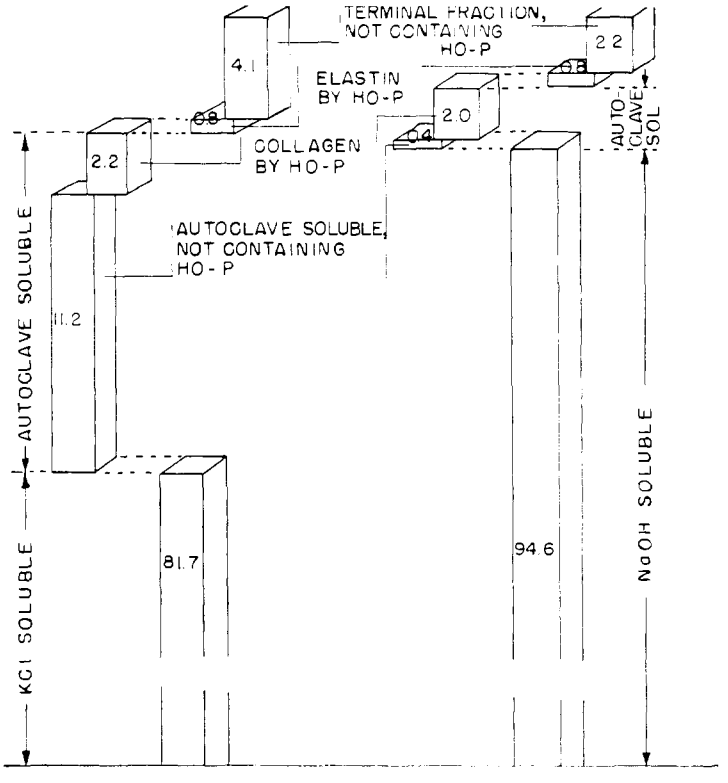

Figure 3. Fractionation of semimembranosus tissue by potassium chloride and sodium hydroxide extractions and by autoclaving. Nitrogen values expressed as per cent nitrogen of total nitrogen. Assessment of nitrogen by Kjeldahl and hydroxyproline determinations

tilled water, totaling 100 ml., before another solution or treatment was used.

Nitrogen determinations were carried out on the micro-Kjeldahl scale and the hyhroxyproline analysis was executed as described by Neuman and Logan (*14*). In the figures the nitrogen calculated from hydroxyproline content is listed as "N by Ho-p"; other nitrogen values were determined by the Kjeldahl procedure and for comparison are indicated as "N by Kj."

The papain digestion investigations were carried out as follows: Ten milligrams of papain, previously activated by incubation with cysteine hydrochloride for 1 hour at 37° C. and then neutralized to pH 7.0, was employed in the digestion of the stroma proteins isolated from 1 gram of muscle tissue. The digestions were carried out in buffered solutions of pH 4.0, 8.5, 7.0, and 2.7 for 48 hours at 37° C. in the presence of toluene.

The testicular hyaluronidase trials were effected on similar quantities of tissue, using 5 mg. of enzyme dissolved in 0.1*M* sodium chloride. These enzymatic digestions were allowed to proceed for 24, 48, or 72 hours in 45 ml. of 0.1*M* sodium acetate, adjusted to pH 5.2. The glucose equivalents per gram of tissue were estimated according to the Park and Johnson (*16*) method for determination of glucose, after the papain digestion and the hyaluronidase treatment of stroma protein which had been subjected to one or more prior treatments.

Results

A preponderance of the work reported in the literature on the fractionation of skeletal tissue with a view to the assessment of the connective tissue has been based on alkaline extractions, such as the Lowry, Gilligan, and Katersky method or some modification thereof. The Lowry method employs exhaustive 0.1*N* sodium hydroxide extraction with the measurement by nitrogen determinations of certain residual fractions. It appeared of value in these investigations to use alkaline extractions for comparison with the potassium chloride extractions.

The effectiveness of alkali as a protein extractant with successive increments of time and with increase in the normality of the alkali is indicated in Figure 1. These alkaline extractions were made on tissue residues, entrapped on glass wool, after the intracellular proteins had been removed by potassium chloride treatment.

The extraction of tissues with potassium chloride, followed by extraction with 0.05*N* to 0.25*N* sodium hydroxide, evidenced that increases in the alkalinity of the extractant resulted in consistent decreases in the nitrogen content of the residue. The hydroxyproline values of the alkali-soluble fractions were small, but showed increases paralleling the strength of the sodium hydroxide extractant. The data substantiating these concepts have been reported (*8*).

Characteristic responses of the comminuted tissue to extraction with the respective potassium chloride or sodium hydroxide solutions, with the entrapment of the connective tissue on a mat of glass wool, and the assessment of the fractions are presented in Figures 2 and 3. The initial extraction of the intracellular proteins was estimated in terms of Kjeldahl nitrogen, the portion solubilized by autoclaving the entrapped residue was assessed in terms of both Kjeldahl nitrogen and hydroxyproline content, and the terminal residue was subjected to hydroxyproline evaluation. Efforts were consistently made to account quantitatively for the total nitrogen content of the tissue; the value recorded as terminal fraction not containing hydroxyproline was a computed difference.

In the preparation of certain fractions for hydroxyproline assay there were distinctions in the colors which could be related to the method of fractionation of the connective tissue residues. Absorption readings were made with the Beckman Model DU spectrophotometer. The color components of the acid hydrolyzates of the autoclave soluble fraction, prepared by either the Lowry method or glass wool entrapment, gave absorbance patterns essentially equivalent to those of the standard solutions of hydroxyproline. These absorbance curves showed maxima at 560 mμ (Figures 4 and 5). The absorption spectrum of the acid hydrolyzates of the terminal residues prepared by the Lowry method (Figure 4) was also equivalent to the absorbance pattern of the standard solutions and evidenced maximum absorption at 560

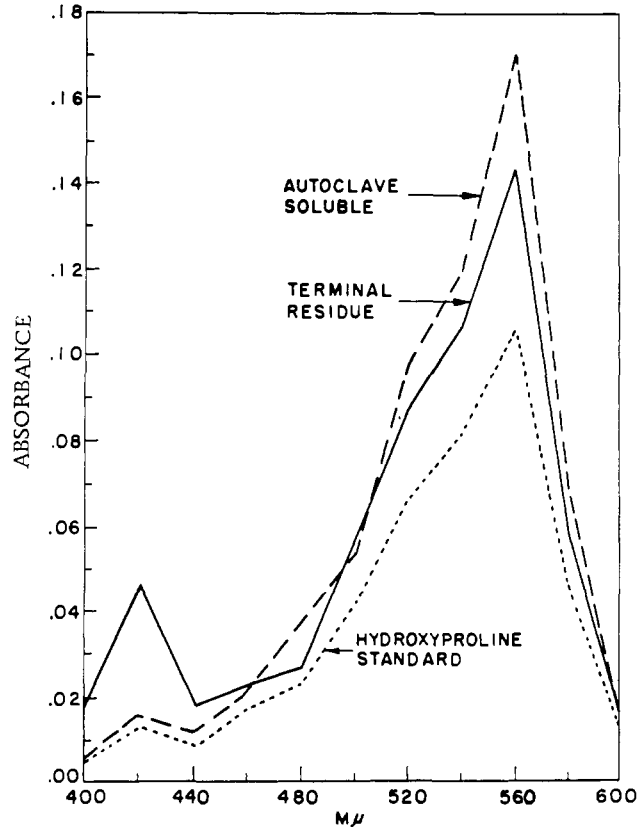

Figure 4. Representative absorption spectra of acid hydrolyzates of autoclave soluble and terminal residue fractions (by Lowry method) prepared for hydroxyproline analysis. Absorbance curve of standard hydroxyproline solution indicated in figure

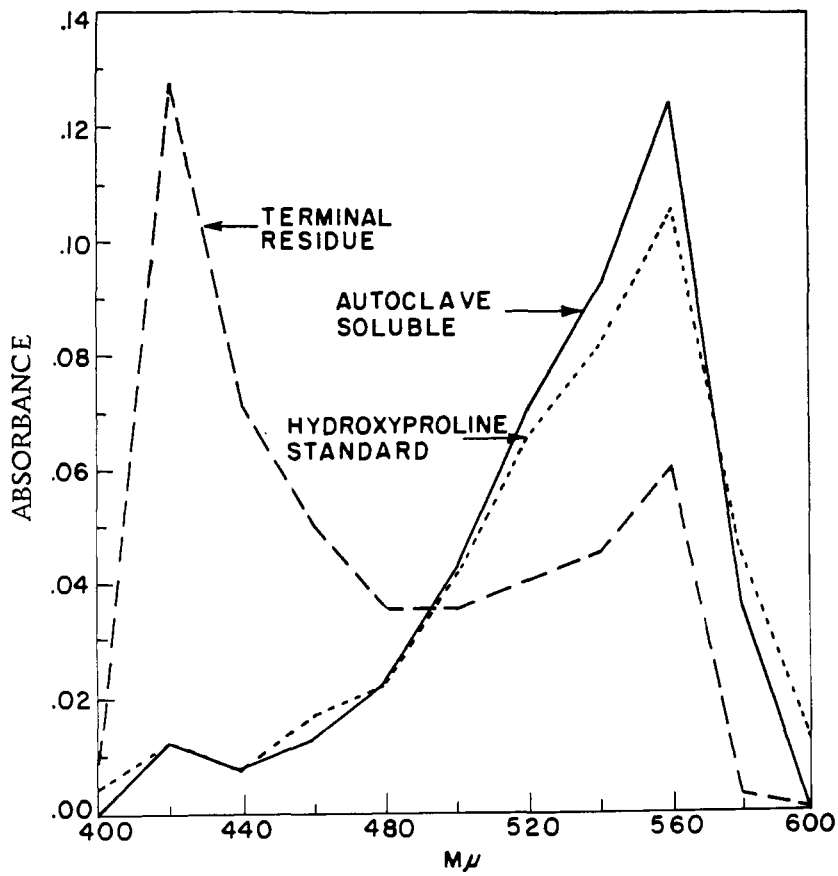

Figure 5. Representative absorption spectra of acid hydrolyzates of autoclave soluble and the terminal residue fractions (by potassium chloride extraction and by glass wool entrapment) prepared for hydroxyproline analysis. Absorbance curve of standard hydroxyproline solution indicated in figure

mμ. However, the terminal residues which had not had exhaustive alkaline treatment, as in the case of the glass wool–entrapped residues extracted with potassium chloride (Figure 5), developed a characteristically different color in the hydrolyzates when prepared for hydroxyproline assay. The maximum absorption of this latter colored complex was at 420 mμ (Figure 5).

The effect of the protease, papain, on the stroma proteins was evaluated by subjecting glass wool–entrapped tissue residues to the activity of papain in solutions buffered at acid, neutral, and alkaline pH's. The tissue samples were extracted with potassium chloride or sodium hydroxide solutions prior to incubation with the papain dispersion. The extent of papain digestion was assessed in terms of the Kjeldahl nitrogen released and hydroxyproline determinations on the same fraction. Following the opportunity for papain digestion, the residues were autoclaved and the autoclave labile portion was evaluated in terms of Kjeldahl nitrogen and hydroxyproline content. The terminal residuum was then quantitated by a hydroxyproline measurement.

The fractionation of the potassium chloride–extracted residues digested with papain is shown in Figure 6. Although some protein by Kjeldahl nitrogen measurement was solubilized by papain digestion, in no case was an appreciable amount of hydroxyproline released. The hydroxyproline content of the subsequently autoclaved portion was consistently uniform and in alignment with the values reported for similar samples which had not had enzymatic treatment (Figure 2). The hydroxyproline content of the terminal fraction, elastin, was in close agreement with the elastin content reported earlier for samples not treated with papain.

Initial extraction with sodium hydroxide (Figure 7) resulted in a lesser amount of protein exhibiting lability to papain digestion, but reaffirmed the ineffectiveness of papain in releasing hydroxyproline. Observation of the hydroxyproline values of the autoclave soluble and the terminal elastin fraction reveals harmony with those shown in Figure 6 and also with values from previous samples where papain had not been involved (Figure 2).

Table II. Glucose Equivalents in Residues from Muscle Tissue

(Mg. glucose/mg. insoluble nitrogen)

Initial Extractant	Papain	Hyaluronidase (72 Hr.)	Total
0.6*M* KCl	...	0.01	0.01
0.6*M* KCl	0.13	0.03	0.16
0.1*N* NaOH	...	0.01	0.01
0.1*N* NaOH	0.22	0.04	0.26

Preliminary attempts were initiated to induce alterations in the connective tissue by hyaluronidase digestion. The extent of depolymerization of the ground substance effected by enzymatic digestion was estimated in terms of glucose equivalents. Representative responses of the connective tissue residues to enzymatic treatments are summarized in Table II. The tissue extracted with potassium chloride had 5.3 mg. of insoluble nitrogen per gram of tissue as entrapped stroma, whereas the sodium hydroxide-extracted tissue had 3.3 mg. of insoluble nitrogen per gram of tissue. Certain tissue residues were incubated with papain prior to the hyaluronidase digestion and consistently exhibited increases in apparent reducing sugar content upon digestion with hyaluronidase, as indicated in the Table II. Kjeldahl nitrogen and hydroxyproline determinations were made on the papain digests and on the extractions after hyaluronidase incubation. Small amounts of nitrogen were solubilized during the enzymatic digestions, but hydroxyproline was found only in traces in the papain digests and could not be demonstrated in the extractions after hyaluronidase activity.

Discussion

The major portion of the fundamental work on connective tissue has been done on massive aggregates such as tendon, ligament, and hide. To what degree one can, with equity, extrapolate these findings to the fine, netlike web of connective tissue as it ramifies throughout skeletal muscle is highly speculative. Much that is believed to be known about the stroma of skeletal muscle is more inference than fact, or at best awaits validation. Traditional approaches to the partitioning of skeletal tissue for quantitative appraisal of the connective tissue moiety appear to have suffered from a restricted concept of the morphologic complexity of connective tissue. Connective tissue is comprised of the adhesive milieu, or the ground substance, in which the cellular and fibrillar constituents are held. There are marked differences in the relative concentrations of the fibrillar and amorphous components. The ground substance is an amorphous matrix of mucopolysaccharides and mucopolysaccharide-protein complexes in differing degrees of polymerization. It is not surprising that the amorphous moiety of connective tissue has been neglected, for the elucidation of the variants in ground substance has scarcely been initiated. Historically, the emphasis has been on the fibers, collagen and elastin; and with the results dependent on the methods of isolation, and, with little to characterize the fractions other than definition, there

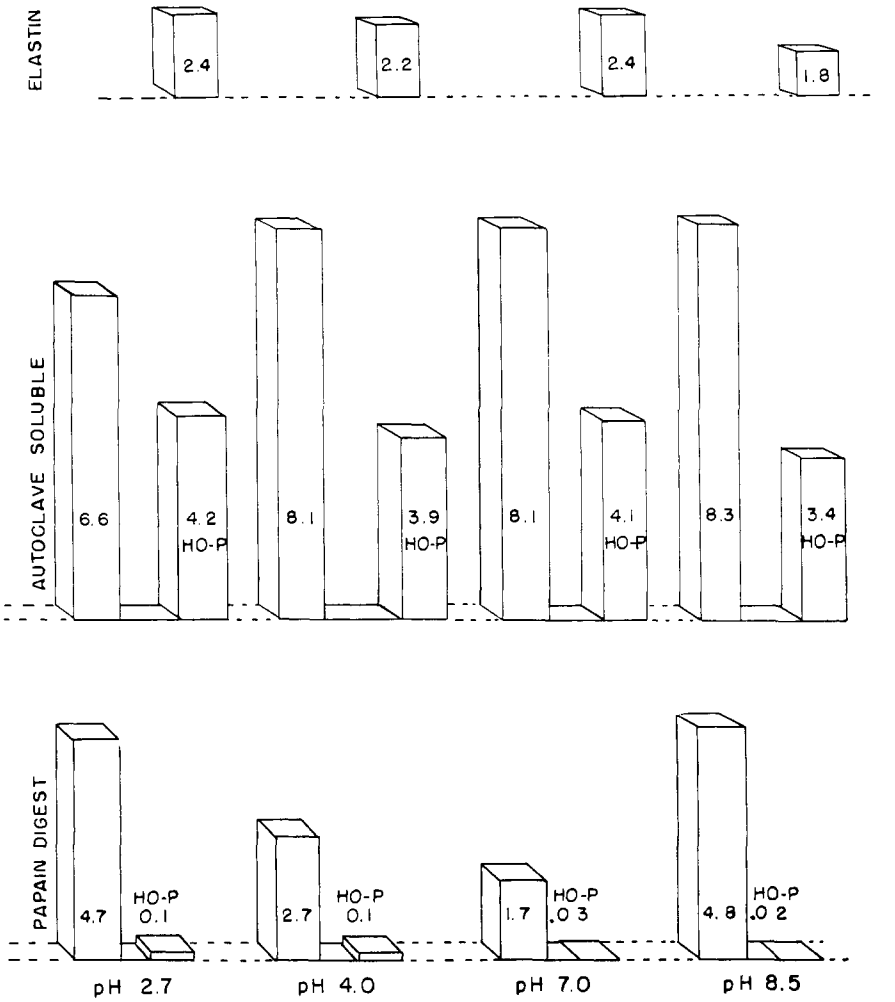

Figure 6. Fractionation of potassium chloride-extracted residues of semitendinosus tissue by papain digestion and subsequent autoclaving. Nitrogen values expressed as per cent nitrogen of total nitrogen. Assessment of nitrogen by Kjeldahl and hydroxyproline determinations

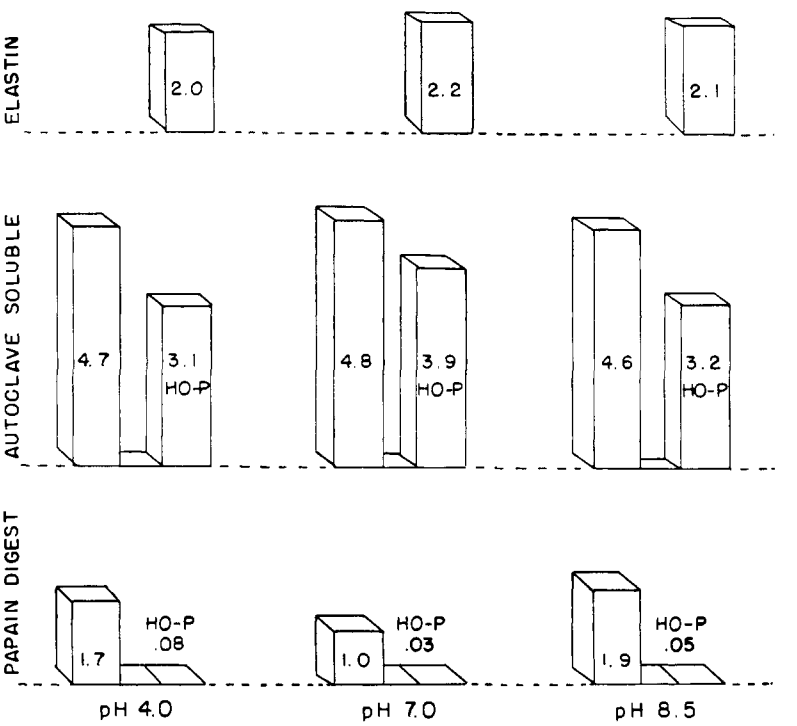

Figure 7. Fractionation of sodium hydroxide-extracted residues of semitendinosus tissue by papain digestion and subsequent autoclaving. Nitrogen values expressed as per cent nitrogen of total nitrogen. Assessment of nitrogen by Kjeldahl and hydroxyproline determinations

has been a paucity of corroboration of results. Weber (*22*) reported stroma protein content of about 20%. The average collagen and elastin content determined by Prudent (*19*) was of the order of 4%.

The investigations reported here show that residual connective tissue proteins differ markedly relevant to the initial extracting solution (Figures 2 and 3). In terms of Kjeldahl nitrogen 15 to 20% of the total nitrogen remained as insoluble in potassium chloride, whereas only 5 to 10% was not extracted by 0.1*N* sodium hydroxide. Autoclaving the entrapped stroma proteins in water at neutral pH revealed further disparities. The inveterate concept of collagen has been that it is that protein which is solubilized by autoclaving. Based on a nitrogen determination the collagen in the potassium chloride–insoluble residue would be severalfold that of collagen in the sodium hydroxide–insoluble portion. Acceptance of the belief that the hydroxyproline content specifically identifies collagen and elastin permits a more delineative criterion. From the autoclave solubilized fraction of the potassium chloride–insoluble residue, the collagen by hydroxyproline determination constituted only a portion (one fifth to one third) of the value indicated by the nitrogen determination. The collagen values by hydroxyproline determinations, whether from potassium chloride or alkali-extracted tissues, were in close agreement. The terminal residues, elastin, assessed by hydroxyproline content evidenced concordance irrespective of which initial extractant was used. Many of the procedures for the fractionation of muscle tissue described in the literature, which are in terms of a Kjeldahl nitrogen estimation of the autoclave solubilized portion for the collagen content, may well yield results spuriously high when compared with values obtained by hydroxyproline determinations.

Papain has been reported to possess collagenase activity (*20*). Measurable amounts of protein, by Kjeldahl assessment, were solubilized by papain digestion, but the absence of significant amounts of hydroxyproline in the digests demonstrated that the papain had not solubilized the collagen or elastin fibers at either acid or alkaline pH's (Figures 6 and 7). Papain was effective, however, in releasing reducing substances from the residues (Table II). The protein components of the mucopolysaccharides and the mode of linkage of the polysaccharide-protein complexes in ground substance in muscle have not been studied. Day (*2*) used certain proteases and reported the cleavage of linkages which were postulated to serve as scaffolding of the continuum of the ground substance. Some experimental evidence from the studies here reported may be adduced supporting this theory. The treatment of the residues with papain released reducing substances. Furthermore, treatment of the residues with papain prior to the employment of hyaluronidase on the residues greatly enhanced the activity of the hyaluronidase as measured by the apparent content of reducing sugar.

These data show that there is in bovine muscle tissue a nitrogen-containing fraction which can be characterized as insoluble in 0.6*M* potassium chloride solutions, can be extracted with 0.1*M* sodium hydroxide, and is labile to autoclaving. Although the papain did digest certain amounts of the nitrogen-bearing components, they were not fibrillar, for only traces of hydroxyproline could be detected in the digests.

It was demonstrated that approximately 80 to 85% of the total nitrogen of muscle tissue was extracted with the potassium chloride solution. There remained 15 to 20% of the nitrogen to be accounted for. This residual fraction has been designated by Weber (*22*) as the stroma proteins. On the basis that only the connective tissue fibers contain hydroxyproline, of the 15 to 20% moiety about 5 to 6% may be categorized as collagen and elastin. The remaining portion is accounted for by that fraction which was alkali labile. Partridge (*17*) has reported that the ground substance was readily degraded by alkali. It is conjectured that the fraction which does not contain hydroxyproline and which can be solubilized by autoclaving and by alkali may be an integral portion of the ground substance.

It seems evident that gross analyses will not reveal the presence of delicate chemical substances, nor suggest their morphologic disposition in complexly structured material. With the acceptance of the concept that connective tissue is composed of more than just the structured strands of collagen and elastin, that these fibers must indeed be held in an amorphous matrix, it follows that even a sensitive measure of collagen and elastin will not afford a quantitative measure of connective tissue, nor will it elucidate the nature and cause of changes in the tenderness of muscle tissue. The site of lability in connective tissue appears to be in the amorphous area rather than in the fibrillar components. It is speculated that post-mortem changes in the tenderness of meats may be caused by alterations in the ground substance of the connective tissue. The hypothesis is tenable that certain constituents in muscle tissue, though minimal in quantity, may well play a determining role in architectural aspects, and hence in tenderness of meats.

The unraveling of the myriad factors involved in the tenderness of meat, and in the alterations in tenderness that may be induced, is contingent on the clarification of the function of each of the multiple constituents of muscle tissue.

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