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Chemical and physical properties of various fractions of connective tissue proteins of bovine muscle

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NOTE TO USERS

CHEMICAL AND PHYSICAL PROPERTIES OF VARIOUS FRACTIONS OF CONNECTIVE TISSUE PROTEINS OF BOVINE MUSCLE

by

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INTRODUCTION

Tenderness in meats is one of the most desired qualities. Tenderness must, perforce, accrue from the chemical and physical properties of the constituents of muscle tissue. The belief has long been paramount that the connective tissue in muscle is one of the primary determinants of tenderness. The morphologic complexity of muscle tissue has always challenged and, to a great degree, defied investigators who would partition it. The skeletal muscle cells are long, unbranching cells usually termed muscle fibers; the membranous covering is called the sarcolemma. The muscle cells are held together and unified into a functioning structure by a matrix of connective tissue or stroma. The multiplicity of methods that have been devised for the estimation of tissue components and the divergency of results are ample evidence of the difficulties encountered in the assessment of skeletal tissue.

An evaluation of the connective tissue must first deal with the complicated problem of separating and removing, quantitatively, the complex of proteins held within the sarcolemma from the stroma proteins. This necessitates the disruption of the sarcolemma, and desirably without altering

the proteins. Traditional solubility classifications of proteins provided generalized approaches in methodology, but fall short of precise guidance because of the diversity and multiplicity of the intracellular proteins. A considerable number of variables which affect the solubility of each of the proteins must be reckoned with. There is the concomitant requisite of making certain that none of the stroma proteins are removed during the fractionation and removal of intracellular components. The efficaciousness with which the intracellular proteins, and only those, are removed will be a peremptory determinant of the validity of assessment of the remaining connective tissue proteins. The term stroma or connective tissue is employed throughout this thesis with these limitations in mind.

The second phase of the problem is the appraisal of the not uncomplicated constituents of the connective tissue proper.

Several methods of fractionation have been developed, and the results have been both verified and controverted. It was deemed worthwhile to investigate some of the most pertinent problems dealing with the isolation and estimation of connective tissue in bovine muscle because of its importance in the tenderness of meats.

REVIEW OF LITERATURE

Architecture of Skeletal Tissue

Striated muscle is fabricated from individual elongated cells, or fibers, oriented in parallel alignment with each other and with the longitudinal axis of the muscle. The muscle fiber is an elongated, multinucleated, cylindrical cell; its covering is a thin structureless membrane, the sarcolemma. The sarcolemma, appraised in multiple, must comprise an important portion of the total muscle structure; it is considered here as a part of the stroma proteins. The basic framework of muscle structure is considered as stroma; in the work reported here the term stroma is used interchangeably with connective tissue. The intracellular milieu, within the sarcolemma, is made up of myofibrils surrounded by a sarcous medium, the sarcoplasm. The myofibrils are aligned in register and are composed of myosin(s); there are several myosins. The sarcoplasm is described as being comprised of myogen, myoalbumin, and globulin-X.

Ramifying between and around the muscle fibers, encircling them into bundles or groupings called fasciculi, and joining the fasciculi into the gross muscle, is the

connective tissue. The functional role of connective tissue in holding and uniting parenchymatous elements together is implicit in the name. An extensive vascular network ramifies throughout skeletal muscle; the rich capillary bed traverses the connective tissue septa in muscle. Three types of components make up the connective tissue; the cellular, the fibrillar, and the ground substance. The cells in connective tissue are minimal in terms of quantity, and hence are usually neglected or omitted in problems dealing with structural ratios and amounts. The predominant cell-type is that of the fibroblast. The fibrillar elements are made up of strands of collagen(s), finer fibrous structures debatedly termed reticulin by some but considered immature or procollagen by others, and elastin fibers. The fibers or strands are embedded in a homogeneous matrix described as an amorphous ground substance. This interfibrillar material, having adhesive or cementive function, is thought to be composed largely of mucopolysaccharides. The structure and composition of the complex ground substance, coupled with the causes and significance of changes therein, are only beginning to be investigated.

Connective Tissue Components

Connective tissue is made up of the cellular components, the collagen strands, and the elastin fibers held and supported in an adhesive matrix of ground substance. The relative ratios of each of the constituents, the chemical composition, and the architectural disposition are believed to show some differences between species, within an organism, and as a function of age, in addition to the differences and distortions caused by pathological conditions.

The mesenchymal cell population (potentially) differentiates into a diversity of morphological cells, dependent on physiological stimuli and responses; but the primary cell type is believed to be that of the fibroblast. The role of the fibroblast is indicated in its name. Using tissue culture explants, Parker (1933) demonstrated that fibroblasts from different parts of the body are physiologically different. It seems plausible to anticipate that their products may well exhibit differences in diverse tissues of the organism, in addition to certain phylogenetic variations.

The characteristics used to describe and categorize the components of connective tissue must be appraised in the light of the diverse methods and techniques out of which they have their inception. These methods have a dependency

on the work of the anatomist or morphologist, for other specialists train their methods of attack on those segments which the morphologist delineates as connective tissue. The chemist pursues his analysis of connective tissue, usually on tendon, or hide, or ligament, but always on those portions of tissue which the morphologist has identified as tendon, or hide, or ligament.

Collagen

The morphologist sees the collagens as groups or bundles of thread-like fibers following an undulatory path. A hierarchial description of the structural organization of collagen fibers was presented by Bear (1952) in which he summarized much of the work done in microscopy. Bear envisaged the thinnest filament embodying the basic structural configuration of collagen as a protofibril. The specific configuration of the protofibril is such that, when the protofibrils are oriented into groups to make up the fibrils, their axial juxtaposition results in a transverse matching of neighboring structural composites. Thus, the fibrils, viewed with the electron microscope, exhibit repetitive banding, or periodic striae, along their lengths. The larger fibers are made up of aggregates of fibrils. The common forms of mammalian collagen which have been

studied indicate a rather universal fibrillar period of about 640A° (Schmitt, 1951) for dry specimens. It should be noted that periodicity is only one kind of measurement, and is observed in samples which have of necessity been subjected to rather severe treatment, including complete desiccation. It has been suggested that the seemingly constant striae, at the level of electron microscopy, of collagens from different sources should be interpreted as evidencing only a similarity of band and interband components (Martin, 1953).

In tendon (rat tail) there is a great diversity of fibril diameters, whereas in skin (human) the fibrils appear to be of quite uniform caliber. The diameters of fibrils in tissue cultures are extremely fine as contrasted with those of spleen, which in turn are only a fraction as large as those of hide or tendon. Tendon fibers are several times the size of those of hide. Skin fibrils are also so tightly integrated they rarely exhibit longitudinal splitting, but tail tendon readily cleaves along the fibril (Gross and Schmitt, 1948). Thus there appear variations in the lateral forces holding the protofibrils together.

The collagen fibers have considerable strength and are slightly flexible, but in comparison with certain other fibers, are considered inextensible. Some extensibility has been indicated with the finer fibrils; this may eventually

prove to be artefact, relative to the pretreatment requisite for electron microscopy (Wyckoff, 1952).

The histological interpretations of collagen have been based on the response to certain stains and dyes. The concept is crystallizing that the affinities for many of the dyes are quite non-specific and diffuse (Bennett, 1951). The complexity of the reactions, the variability in staining reagents, coupled with the requisite subjective judgment of the operator in most procedures give rise to considerable variability in tinctorial response. The empiricism of staining methods is pointed out by many workers in the field. Lillie (1952), discussing the much-used silver method and the argyrophilic response of fibers, said, "It seems utterly capricious." There are also the inherent problems of scanning many fields and deciding what will be adjudged a representative sample.

Collagen is classified as one of the fibrous scleroproteins, made up of long and oriented polypeptide chains, and characterized by marked insolubility in neutral solvents.

Low (1953) concisely reviewed the theory of molecular configuration and structure of collagen. She emphasized the significance of hydrogen bonds and salt linkages as interchain binding mechanisms. Jordan Lloyd (1938) referred to this as internal molecular scaffolding. Attention was directed to the preponderance of proline and hydroxyproline residues in collagen and the restrictions their bulkiness imposed on the polypeptide chains. A unique model of the structural

configuration of collagen, consisting of three polypeptide chains coiled into a helix, three such helixes having a common axis, has been proposed by Pauling and Corey (1951).

The chemical reference is not much more satisfying nor unequivocal than are the other avenues of investigation. Many tables have been compiled listing amino acid content, but these cannot be taken without reservation. Determinations of amino acids by direct chemical analysis have been dependent on the vagaries of isolation techniques. The instability of certain amino acids, for example tryptophane, upon subjection to hydrolytic conditions, calls into question the reliability of some tabulated analyses. Another deterrent to progress has been the lack of definite criteria of homogeneity. The earlier work was fraught with inadequate and relatively non-specific methods. In many cases the workers unknowingly used mixtures, and in most instances their analyses were restricted to specific tissue which could rather readily be excised, such as tendon or hide. This latter factor, the use of dense, macroscopic aggregates, is always cited as one of the weaknesses of the Roentgen-ray technique, yet it is no less true for the chemical analyses. Ox hide collagen was used by Bowes and Kenten (1949) as a reference for the comparison of other collagens. They were careful to tabulate amino acid values from native collagen or ox hide which had not received the alkaline or enzyme treatment. However, hide collagen has been shown to be considerably more refractory than some of the other

collagens from the same animal.

There are marked similarities, though not identities, in the amino acid content of the mammalian collagens that have been analyzed. The collagens are unique among proteins in their minimal amounts of aromatic amino acids, in their high content of non-polar amino acids, glycine and alanine making up about one-third of the residues, and in their unusually high percentage of proline and hydroxyproline; the latter constitute another one-third of the total residues. Neuman and Logan (1950a) stated the range of hydroxyproline content for most collagens as determined from the amino acid composition of gelatin to be from 13 to 14 per cent. Their hydroxyproline figure for gelatin from bovine tendon was 13.4 per cent.

It is accepted that the collagens swell in acids and alkalies. More than two decades ago, Knaggs (1929) discussed the changes collagen undergoes when treated with cold solutions of NaOH or HCl. He made note of the internal structural changes induced and the difference in the hydrolytic products in relation to the nature of the precursive agent. The caution was advanced that the chemical constitution of collagens varies with different tissues. Nageotte and Guyon (1930) demonstrated how differently homologous tissues in the same animal may respond to solubilization. They dissolved tendon from rat-tail in very dilute acetic acid (0.0006 N); the tendons of rat-paws required a preliminary treatment with N NaOH before the acetic

acid effected their solution. Rabbit and dog tendon necessitated treatment similar to that for rat-paws. Bovine tendons were more resistant than any of the other collagens.

The swelling of collagens in alkali with resultant weakening of structure through the breaking of linkages was pointed out by Jordan Lloyd (1938). She demonstrated the swelling and tearing of rat-tail tendon in very dilute NaOH. In 0.002 N NaOH, pH 11.4, the tail tendon was dissolved.

Careful studies have been made by Bowes (1951) on the composition of skin collagen and the effect of alkalies on collagen. She reported the hydrolysis of some peptide links; the extent of hydrolysis of amide groups increased with pH and with time of treatment. There were increases in water uptake at all pH levels used. This uptake of water with subsequent swelling of the collagen was interpreted as meaning decreased cohesion of collagen due to rupture of intermolecular bondings. The possibility of the particular involvement, in the hydrolysis of peptide links, of the imino groups of proline and hydroxyproline was pointed out. Speculative though this was, the inference of a hypersusceptibility of the imino-amide links to the action of alkali would have considerable significance for collagen and elastin with their high pyrollidine content. Bowes showed that 5 per cent of the collagen was dissolved by the alkali. This measurable chemical solubility is probably not the most important result of the investigation. The

postulated changes in the breaking of the intermolecular bonds, the loosening up of the fibers, with induced alterations in the subsequent behavior of that collagen would appear to be of considerable import. Credit should be accorded Bowes that she stressed the fact that tendon and cartilage collagen are more readily dispersed in alkali than is hide collagen, and warned that findings on skin collagen are not necessarily applicable to other collagens.

The possibility of hydroxyacids in protein combination exhibiting a special lability toward alkali was shown by Nicolet and Shinn (1941). The applicability of this concept to hydroxyproline, and the question of the lability of this acid in the denaturation of connective tissue by alkali, are, however, conjectural.

Partridge (1948) presented the theory that chondroitin sulfate cements the collagen molecules together to form macromolecules and these in turn are cemented to form fiber bundles. If this is the manner of organization, then the ready degradation of the acid polysaccharides in strong alkali would have marked effects on the decreased cohesion of collagen and its subsequent response to treatment.

The earliest characterization of collagen was the conversion, upon adequate heating in an aqueous medium, to the water-soluble protein, gelatin. This dissolution of native collagen has served as the separatory technique in analytical methods.

Because the tyrosine content of collagen is consistently reported as low or nil, some investigators have used negative tests of tyrosine in gelatin dispersions as the criterion of purity. This assumption rests on somewhat shaky premises. It has been demonstrated that the tyrosine content of gelatin is dependent on the method of preparation. Loofbourow et al. (1949) carried out studies of the absorption spectra of collagens, using collagens from different sources. For comparative purposes they used "purified" rat-tail collagen as the standard of pure collagen. They were unable to get beef tendon to dissolve in acetic acid as rat tail did. In their tests with collagens from different sources and with diverse treatments for purification, they reported small absorption maxima at 270 and 280 millimicrons. This could be construed as presumptive evidence of the presence of aromatic amino acids in gelatin.

Ultraviolet spectroscopy was used by Sizer (1952) in estimating the purity of collagen. Collagen samples from several sources were used; samples were hydrolyzed at 100°C. in 6 N NaOH. Tryptophane was given as low or lacking and phenylalanine made a relatively small contribution. Assuming the absence of other absorbing substances, the tyrosine content was estimated from the absorption at 275 millimicrons. By this method the tyrosine content was several fold the value by chemical measurement. Sizer resolved the anomaly by discussing the possibility of absorbing impurities; the justification for this appears dubious.

When collagen fibers are subjected to temperatures of 60°C., they exhibit pronounced contraction to about one-fourth the initial length (Bear, 1952). As the fiber cools, it lengthens spontaneously, but its original length is not recovered. Winegarden et al. (1952) in work with dense sections of connective tissue indicated the thermal changes more nearly at 65°C.

The response of the collagens to enzymic degradation is in need of cautious repetition of much of the early work with critical appraisals of the results. The statement appears often in the literature that the native collagens are resistant to digestion by proteolytic enzymes such as trypsin, chymotrypsin, and papain. In analytical work on excised tissues, or in determinations on muscles, or meats, the procedure invariably involves a segmentation of the tissue, often to a very fine state of subdivision. The crux of the problem involves, not how does a gross portion of intact tendon respond to enzyme action, but rather, what is the reactivity of finely divided collagen to enzymic treatment. Even more important is the question of the surface area of the connective tissue which is presented to the enzyme in use. Variations in surface area would influence the degree of lability to the action of the enzyme.

Trypsin has had a long and varied applicability in the degradation of proteins and particularly in the fractionation

work involving connective tissue. The intent of the application of trypsin has been the removal of all and only non-collagen protein (Bowes, 1951; Loofbourow et al., 1949). It was demonstrated by Sizer (1949) that native tendon in large pieces was refractory to trypsin action and other proteases, except pepsin. However, when the collagen was comminuted, it was attacked by trypsin. With increases in degree of division of the fibrils there was an increase in the rate of digestion by trypsin. In light of the known variations in the diameters of collagen fibers, in the compactness and cohesion of collagen fibers and in the gradations in macerative procedures, Sizer's findings should have profound implications both for potential enzymic changes in muscle in situ and as it is subjected to analytical procedures.

The significance of the all too prevalent disregard of aseptic techniques in the preparation of enzymes and in the assessment of their activity is becoming increasingly clear. Ashley et al. (1935) reported work in their laboratory with commercial trypsin and crystalline trypsin. They revealed the finding of both sources of trypsin contaminated with bacteria, and the former very heavily so. They carried out a procedure for freeing the crystalline trypsin of bacteria and ascertained that the subsequent activity of the enzyme was greatly decreased. When this type of extraneous causative agent is present in producing and confounding degradative changes, the

specific enzymic effects cannot be differentiated nor assessed. How extensively this condition has existed or how persistently it should be kept in view when perusing the literature is a matter of conjecture.

The alterations of collagen samples incident to preparatory processes were shown by Neuman and Tytell (1950) to markedly influence later enzymic treatment. When any step of the preliminary procedure is harsh or drastic the collagen is thereby modified and weakened, and it then exhibits increased susceptibility to digestion by proteolytic enzymes. Hence, the response of hide powder collagen (prepared by drastic procedures), gelatin, or otherwise modified and "cleaned" collagens to enzymic degradation cannot be applied in speculation to native collagen reaction. In Neuman and Tytell's work, all of the rigorously pre-treated collagens were degraded more readily by the proteases which they tested. Using what they described as mild processes, collagens from several sources and from several species showed resistance, at pH's near neutrality, to trypsin, chymotrypsin, and papain. These collagens were readily attacked by the proteolytic enzyme(s) in the filtrates of Cl. histolyticum, by pepsin at pH 2, and to a lesser degree by proteases of the Cl. perfringens.

The collagenases represent, in all likelihood, a family of enzymes; the substrate preferences required by the various collagenases from different sources have not been established.

The collagenase activity of papain and ficin has been

noted by Rosenblum and colleagues (1953) to take place in a pH range of 2.0 to 4.5, but was not detected near neutrality. Their belief was that in the indicated acidic range collagen first undergoes a physical alteration which then causes the protein to be enzymically digestible.

There are changes in collagenous tissue with age. Studies of human skin morphology, using the electron microscope, indicated that the skin of infants had much greater proportions of ground substance to fibers than was observed in mature skin (Gross and Schmitt, 1948). The amorphous material did not seem as tightly adherent to the fibers in infant skin. Andrew (1952) called attention to the very gelatinous nature of infant skin and thereby indicated a fundamental difference in the amorphous matrix in the skin of the very young as contrasted with adult skin. Gross (1950) reported increases in the width and amount of collagen fibers in rat skin aging from two days to three months.

Some histologists have emphasized the presence of fibers finer than collagen in connective tissue areas, and these have been termed reticulin. The differentiation is based primarily on size of fiber and tinctorial response, particularly the argyrophilic (the Maresch-Bielschowsky method) reaction. Reticulin fibers are always described as fine and sometimes branching. The fine fibers are often traced to a merging with larger fibers. The phenomenon of branching is never applied

to collagen fibers, but this branching may only appear because the fine fibers have not aggregated or coalesced with enough neighboring fibrils to present the bulk necessary for the waviness typical of the mature collagen fiber. Gross's work, employing electron optics in the study of rat skin, would indicate the so-called reticulin is one of the collagens, probably immature, and with aging shows transition to collagen. As the term reticulin was originally used it referred to the framework of the special parenchymatous organs such as the spleen. Robb-Smith (1952) stressed differences in the architectural fashion in which reticulin fibers are laid down, such as strap-like in the skin and a membranous type in muscle. The evidence would appear to indicate that reticulin will find its place in the category of the collagens.

It is accepted that there are a number of collagens within the same organism; these have certain common denominators and hence some distinct similarities, yet it has been established that they also possess differences with respect to certain other phases.

Elastin

A meager amount of work has been done on elastin. Out of the investigations on elastin certain properties appear quite trenchant and characterizing, yet this apparent clarity may

delude because of the paucity of research upon which it is based. It may well be that many of the concepts take their rise from inference rather than fact.

Elastin is classified as one of the scleroproteins. It is considered insoluble in water, in dilute salt solutions, and in most other solvents. The elastin fibers have great mechanical strength; their response to stress is indicated in the name. These fibers are straight, thread-like, refractive fibers with a marked tendency to anastomose. The elastic fibers in a young animal are clear and glistening; with advancing age their color characterization is yellowish.

Great differences in the caliber of elastic fibers are observed, varying with placement throughout an organism. In the ligamentum nuchea the fibers are gross and coarse as compared with the finer anastomosing elastic fibers found in loose connective tissue. In the aorta both coarse and fine fibers are laid down in rather dense plates or as lamallae (Lansing, 1952).

The differentiations in morphologic aspects are reflected as variations in staining responses. Within the skin some of the elastic fibers will show a strong affinity for certain dyes, while other dermal elastic fibers stain very lightly. The widely used hematoxylin stain is not taken up by young elastic fibers, but hematoxylin does stain old elastic fibers in varying degrees (Lansing, 1952). It can be said that with

all of the stains employed there are numerous variations in the tinctorial responses of the diversely architectured elastic fibers.

In studying compilations of the amino acid composition of elastins, it should be kept in mind that the separation of elastin from other tissue components is based on the established concept of insolubility. Classically, in analyses, elastin is the residuum after everything else has been extracted with dilute acids or alkali. Chemical analyses indicate that the elastins are rich in glycine, alanine, the leucines and valine. Their hydroxyproline content has been estimated to be approximately 2 per cent; Neuman and Logan (1950a) report 1.9 per cent hydroxyproline from bovine ligamentum nuchae and aorta. There are differences in the amino acid compositions of elastin in relation to source and also differences which are correlated with age. In the aged type of elastic fibers Lansing et al. (1951) presented data showing appreciable increases in the amounts of aspartic and glutamic acids.

When rings of canine aorta were extracted with 88 per cent formic acid for 96 hours, Ayer and co-workers (1951) reported that the elastic fibrils showed considerable extension and became readily separable. Their electron microscopy studies revealed linear branching threads having faint longitudinal striations. This latter appearance might be construed as

evidence of a composite of very fine fibrillae.

It is generally believed that elastin is slowly digested by the enzyme trypsin. However, this belief is now being questioned. From crude pancreatin Balo and Banga (1950) isolated an enzyme named elastase, which solubilized elastin fibers. It was also shown that trypsin preparations which were obtained after the removal of elastase were considerably less active. The mode of action was considered not a hydrolytic one for they could not detect released amino acids; the interpretation was that of conversion of an insoluble fibrous protein into a solubilized globular one. With this clarification it appears that earlier reported tryptic digestion of elastin has been due to the activity of elastase-contamination in trypsin.

The German workers, Schwarz and Dettmer (1953), used the electron microscope in studying human aorta. Among other approaches they treated unfixed aortic tissue with elastase. They reported a dissolution from the elastic fibers of a cementing substance with fine fibrillae left intact. Microscopic examination of these fibrillae appeared to show fibrils (elastic) identical with the conventional picture of collagen fibrils. Banga (1953) reasoned that previous failures to detect this cementing substance as an integral part of elastic fibers have been due to traditional application of severe and caustic treatments to "purify" the elastin, and hence it has always been a denatured product that has been studied. Banga

subjected collagen to certain denaturing procedures, then treated the collagen with elastase, and reported rapid solubilization of the collagen. He theorized that mucopolysaccharides, bonded by collagen's dicarboxylic acids, enveloped the collagen fibrils; and when this coverage was weakened or removed the elastase was then able to attack the macromolecule.

This points up the limitations in the use of the term elastase for an enzyme solubilizing collagen. More interesting than the morass of nomenclature is the postulation of the homology of collagen and elastin fibers. This has long been a tantalizing concept, for with the derivation of the two types of fibers believed to be from a common origin, the fibroblast, the enigma of their seemingly diverse structures begged resolution. If this work can be verified, what has thus far appeared as basic differences between the types of fibers will be attributable to artefacts imposed by manipulative procedures.

In the alterations of elastin by elastase, Lansing (1951) discussed the release and separation of a fraction having lipid-like properties. This would appear to be a very intimate if not integral part of the elastic fibers, because even after treatment with a number of defatting extractives some lipid-like content remains. The composite of protein complexed with lipid would appear to be appropriately termed proteolipid (Folch and Lees, 1951); its solubilities appear similar to

those of lipids rather than the lipoproteins.

That there are distinct changes in elastic tissue with age has been noted, but the degree of alteration differs within an organism. In general, the fibers increase in size with maturity; they fray and fragment with advancing time, and an increased mineralization is evidenced as calcium phosphate is deposited in aged elastic tissue. Some restrictive mechanism is in play *in vivo*, however, for the elastin from the pulmonary artery does not show the increases of aspartic acid and glutamic acid nor the calcific accumulations so pronounced in aged aortic tissue (Lansing *et al.*, 1951).

The Ground Substance

The ground substance of connective tissue is the milieu in which the fibrillar components are held. This extracellular and interfibrillar material varies in consistency from a jelly-like substance to one that is considerably more fluid. This variability in physiochemical behavior is evidenced in the organism when, in some states or conditions, certain connective tissue is cohesive, tough, and difficult to cut; then in altered or exaggerated situations it can be shown to be soft, very watery, and readily separable with a probe.

A complex of mucoproteins makes up this matrix termed the ground substance; several differing carbohydrate-protein

combinations have been isolated from various connective tissues (Meyer et al., 1953). The mucoproteins which have been isolated, not all from the same source, include: hyaluronic acid or a complexed substance similar to hyaluronic acid, chondroitin sulfates A, B, and C, and a sulfated mucopolysaccharide termed keratosulfate. The latter has thus far been found only in bovine cornea (Meyer et al., 1953). Undoubtedly there are other saccharide polymers and protein complexes which will, with extending investigations, be isolated and identified, but these are the best known at present. Little is known about the composition of the chondroitin sulfates except that upon hydrolysis they yield acetyl galactosamine, ester sulfate, and glucuronic acid. The mucopolysaccharides are thought to be loosely combined with proteins or may exist in the free state. Day (1949) suggested that the architectured matrix was held by linkages that were rather weak and labile. He used trypsin and reported the digestion of the interfibrillary substance; his contention was that a scaffolding of peptide linkages constituted the continuum of the ground substance. In working with hyaluronic acid Meyer (1947) referred to the weak bondages between the polysaccharide molecules and the proteins, as well as between the polysaccharide molecules as they form polymers. He pointed out that the weak linkages were readily broken during isolation procedures. Repetitious as the concept is, it is worthy of note that all of these

delineated mucoproteins have been fractionated from rather dense or specialized areas of connective tissue, such as heart valves, cartilage, skin tumors, symphysis pubis, umbilical cord, and synovial fluid.

The mucoproteins in the amorphous ground substance appear to exist in varying degrees of aggregation. Gersh and Catchpole (1949) pointed out the plausibility of considering different degrees of polymerization in an organ at different times, and in diverse organs at the same time. When the substances are highly polymerized there is a high concentration of immobile colloid which appears to exert selective effects toward ion exchanges and water (Joseph et al., 1952). With disaggregation the selective effects are lost, the components of the ground substance become depolymerized to varying extents, and there is an increase in the water-soluble fraction. In the pioneering work on chondroitin sulfates the nature and extent of polymerization was not noted because, as Partridge (1948) indicated, the extracting agents used were strong alkalies. Subsequent work has revealed that the polysaccharides were rapidly degraded by strong alkalies. This lability toward alkali would not only alter the continuum of the ground substance but would also condition secondary changes in the architecture of the fibrous elements, if the mucopolysaccharides function in cementive roles in the structuring of collagen fibers (Partridge, 1948) and in elastin fibers (Banga, 1953).

The ground substance has exhibited an evasiveness and refractoriness toward extractive procedures which parallels that of other tissue components. Though of hydrophilic nature, it cannot be leached out with water (Day, 1949). The isolation of the constituents causes considerable depolymerization. Meyer and Rapport (1952) have observed that during the isolation of hyaluronic acid it is degraded, and the viscosity of the isolated constituent is considerably less than in the original source.

In the absence of reliable extractive procedures, many of the data available have been based on histological work. It is now recognized that most of the staining techniques used in pioneering experiments did not differentiate the components of the ground substance; they have, indeed, been quite non-specific. The metachromatic staining, for example, has enjoyed considerable popularity. This was the use of a specific basic dye, such as toluidine blue, and the resultant development of the purplish-red color was considered due to the reaction of the acid mucopolysaccharides. However, it has been shown that any macromolecule with a free acid radical will exhibit metachromasia; so this falls short of specificity (Angevine, 1951).

The utilization of enzymes as cleavage tools of the ground substance has offered considerable promise. Gersh and Catchpole (1949) reported that the mucoproteins of subcutaneous connective tissue were affected by pepsin or trypsin, as well as by

the toxin of Clostridium welchii, and hyaluronidase. The use of trypsin in this capacity has been cited above. The site of attack of the proteases would be at peptide linkages. The cleavage of any portion of the protein-polysaccharide complex would appear to increase the possibility of solution of either moiety or both.

Work with the depolymerizing enzymes has been reported under the heading "mucinases", "spreading factors", and hyaluronidase. There appears to be agreement that hyaluronidase is a general term applying to a family of enzymes. Hyaluronidase is elaborated from diverse origins, including certain pathogenic bacteria, snake venom, and certain mammalian tissues. A source of high concentration of hyaluronidase, and hence available commercially, is from mature bovine testes. The appreciable discrepancies in results reported from various laboratories are probably based on differences in the origin of the enzyme, modifications in methods of preparation, as well as a diversity of methods of assessment.

Hyaluronidase of testicular origin hydrolyzes not only hyaluronic acid, but also two of the chondroitin sulfates. In contradistinction the hyaluronidases of streptococcal and pneumococcal origins do not digest the chondroitin sulfates. The marked variations in the extent of hydrolysis and in the size of the constituent cleavage products were related by Woods (1947) to the source of the enzyme. The variations in

cleavage of the polymers resulting from hydrolytic procedures are suggested by Woods as having a determinative effect on the activity of the enzyme, for with a decrease in the molecular weight of the substrate there was a decrement in the enzyme-substrate affinity. Testicular hyaluronidase has been shown to release less reducing sugar than do the bacterial hyaluronidases for a given physiochemical change (Meyer and Rapport, 1952). The presence of a rather large grouping, designated as a "carrier", was described by Malmgren (1953) in his comparison of crude testicular extracts with more highly fractionated enzymic material. Differences in the degree or extent of removal of this "carrier" in the purification process, as well as possible differences in the carrier per se, may help account for the lack of concordant results from enzymatic studies.

Hyaluronic acid is the primary substrate of hyaluronidase. Meyer and colleagues (1952) have done a preponderate amount of work on hyaluronic acid. They delineate it as a straight chain polymer of equimolar quantities of N-acetyl glucosamine and glucuronic acid, with the reducing group on the acetyl glucosamine portion. In the native state this highly polymerized mucopolysaccharide has considerable hydration capacity and is gel-like in character. The easy opening of labile bonds in hyaluronic acid, during isolation procedures, results in marked lessening of the viscosity because of the formation of

readily diffusible low-molecular-weight substances. This same pattern of physical changes occurs when degradation is brought about by hyaluronidase activity, in addition to which there is an increase in reducing power owing to the liberation of the aldehydic group as the glucosamine bond is cleaved.

In a comparison of methods of assay of hyaluronidase activity, Rapport et al. (1950) contrasted physiochemical methods with an increase in reducing sugar. When the substrate system could be relatively purified they concluded that the increment in reducing sugar was a more valid index of the progress of enzyme activity than the decrease in viscosity or the loss of turbidity. Using testicular hyaluronidase in a system buffered with 0.02 M citrate-phosphate, they established the optimal pH values for enzyme activity. Their pH activity curve showed a rather broad optimum at values 5.4 and below; with more alkaline values there was a rapid decrease in activity. They speculated from their data that the site of the enzymic attack was within the macro-chain and not that of terminal attack.

There has been no unequivocal investigation to particularize the mucopolysaccharides which constitute the ground substance of connective tissue as it ramifies throughout skeletal muscle. Meyer et al. (1952) hypothesized that connective tissue in muscle contained not hyaluronic acid but rather two of the chondroitin sulfates and only one of them was responsive to the

hydrolytic attack of testicular hyaluronidase. The formation of ground substance was discussed by Gersh (1949) as the result of fibroblastic activity, and also the dynamic equilibrating activity, in vivo, of depolymerizing enzymes, elaborated presumably by the fibroblasts.

An increase in density or an increased degree of polymerization of the carbohydrate-protein complexes in the ground substance is considered as part of the aging process (Andrew, 1952).

Methods of Assessment

Since the turn of the century there have been numerous attempts to devise methods of objectively measuring the connective tissue of muscle because workers have so firmly believed the stromal tissues play a dominant role in the tenderness of meats. The attainment of a chemical method of quantitation has lured researchers with a Meccan appeal. The chemical methods of assessment have been reviewed by Prudent (1947). Since Prudent's review, a method designed for the rapid determination of collagen has been published, in 1948, by Hartley and Hall. Neuman and Logan (1950b) proposed a method for the determination of collagen and elastin based on their colorimetric method for the estimation of hydroxyproline (1950a). This procedure for the estimation of the collagen content of

tissues is based upon the belief that hydroxyproline does not occur in tissue other than the connective tissue.

A repetitive pattern of procedural similarity threads through the evolvement of all the methods.

The beginning was always concerned with some means of comminuting the tissue. Many approaches have been tried: trituration in a mortar, slicing thin sections from a frozen block, grinding through food chopper, macerating in a ball mill, shaking with sand, and homogenizing in a Waring blendor. The two primary objectives were minimization of sampling variation and mechanical severance of cells to facilitate the release and subsequent extraction of intracellular constituents.

The presumptive bases inherent in this first step were: the tissue would be rather uniformly disrupted; neither the manipulations nor the temperatures used would cause denaturation of the various proteins and thereby alter their subsequent solubility behavior; the slurry could quantitatively be removed from the apparatus used in its maceration.

The second phase has usually been the dispersal of the macerated tissue with an extracting solution. In many cases this step has been combined with the execution of the first one. The extractants have included: distilled water at differing temperatures, alkaline solutions of strengths from 5 to 0.4 per cent NaOH, lithium chloride of 7 per cent concentration, dilute HCl, and 20 per cent urea solutions. The methods of mixing

the tissue with the solution, the temperatures employed, the time-intervals for solubilization, and the number of repetitive extractions are as varied as the sundry laboratories in which the methods have been developed.

They have assumed that the connective tissue proteins in what must have been varied states of dispersion were inert toward the extractants, and that the response of the proteins within the muscle fibers was diametric to that of the stromal proteins in relation to the extractant. It was necessary also to assume that the agitation necessary for dispersion did not cause denaturation which would alter solubilities; and that each of the types of protein, that within the sarcolemma as well as connective tissue proteins, reacted independently of the other.

Manifestly, the next phase was concerned with the separation of the dispersed proteins. An early attempt used filtration through a cotton-lined Büchner funnel. In some methods separation was mechanically effected by sieving. Then the residue was taken up in extractant, washed thoroughly, and re-filtered through the sieve. This washing and sieving was usually repeated several times, the filtrates being discarded. The meshes of sieves varied from 40 to 120. Water at temperatures of 40° to 50°C. was sometimes used because the fat then presented less of a problem. In one procedure the tissue dispersion was poured on a sieve and, using several liters of water for washing, the residue was massaged between the fingers

until it felt resilient. Later in other laboratories exhaustive extractions were made using centrifugation and decantation as the separatory scheme. In an adaptation of an earlier method to cooked tissue the filtrates from the sievings were drained gently through linen on a Büchner funnel. The finely granular material thus retained was added back to the sieved residue.

In this fractionation step, irrespective of the specific procedure, it appears that the assumptions were: all of the connective tissues were retained by the sieves, filters, or centrifugations; that stroma proteins were not lost in the manifold manipulative processes; and that the finely granular material legitimately belonged with the connective tissues.

In all the methods the retained residues were then hydrolyzed for the conversion of collagen to gelatin. Autoclaving periods varied from 1/2 hour to 6 hours, using 15 to 50 pounds pressure. The most commonly used time-interval was two hours. The residues were frequently washed several times, and the washings decanted and filtered. Early workers used hot 0.5 per cent NaOH to solubilize the collagen. In one method (Spencer et al., 1937) no attempt was made to remove the intracellular proteins. Minced muscle was dehydrated with acetone, it was finely ground, then water was added and the entire slurry was autoclaved for two hours. Hot-water washings with centrifugations removed the gelatin. The gelatin was

precipitated with 5 per cent tannic acid. The collagen content of all the filtrates, decantates, or precipitates was estimated from nitrogen determinations.

In all methods it was assumed that prior separatory processes had removed all the non-stromal proteins, and only those, or, if any intracellular proteins were retained, that they had no effect on connective tissue proteins and their measurement. Implicit also was the assumption that the autoclaving and washings, of whatever duration, selectively hydrolyzed the collagen quantitatively to gelatin. Concomitant with this was the assumption that a nitrogen determination of the solubilized hydrolysate would serve as a valid estimation of collagen. Spencer and colleagues (1937) assumed that tannic acid selectively precipitated gelatin.

It was assumed that an estimation of the gelatin constituted an accountal of the most important part of connective tissue. For an estimation of the elastin three approaches predominated. In some as soon as the collagen was washed from the residue, a nitrogen determination was made on the residue and this calculated as elastin. Others subjected the residue to trypsin digestion, filtered and washed the residue, and calculated the elastin from the nitrogen content of the terminal residue. In the Lowry, Gilligan and Katersky (1941) method the collagen-free residue was extracted with 0.1 N NaOH in boiling water bath, centrifuged and washed, then dried to a

constant weight. This terminal weight was used in estimating the percentage of elastin.

Hartley and Hall (1949) employed Waring blendor homogenization, with an acidic adjustment of pH, and centrifugation as the separatory technique. They did not attempt a measure of elastin. Difficulty in obtaining a sharp separation by centrifugation in the cooked samples, subsequent to autoclaving, was mentioned. Conspicuous in their work was the assumption that the intracellular proteins, numerous though they are, had a single or common isoelectric point. The assumption was also made that by repeated centrifugations sharp separations could be achieved.

Neuman and Logan (1950b) presented a method for the determination of collagen and elastin designed particularly for applicability to small amounts of tissue. Their signal contribution was that the quantitation of the collagen and elastin was based, not on a general nitrogen value, but on the estimation of a distinctive constituent hydroxyproline. This latter determination was made by a colorimetric method these same authors had published earlier (1950a). In their method for tissue analysis the preliminary preparation differed according to the approximate amount of connective tissue in the sample. In the assessment of tissues relatively low in connective tissue, as in muscle, the intracellular proteins were extracted by grinding in a mortar with sand and a solution

of 20 per cent urea. The muscle tissue slurry was stirred in the urea solution for an hour, centrifuged, and washed. The residue was autoclaved, washed, and decantations evaporated to dryness. The hydroxyproline content was determined according to the Neuman and Logan method (1950a). In pursuing the estimation of elastin the collagen-free residue was autoclaved and washed again to decrease the possibility of adherent non-elastin proteins. The terminal residue was then analyzed for hydroxyproline in the same manner as for collagen. The authors noted that the precision of the elastin estimation leaves much to be desired.

Neuman and Logan (1950b) assumed that none of the connective tissue was lost or solubilized by the grinding with sand and extracting with urea. However, the solubility-promoting action of urea has been demonstrated for many proteins (Schmidt, 1938). Neuman and Logan (1950b) called attention to their assumption that the collagen was quantitatively fractionated from the residual proteins. One of the vulnerable spots in this method is the essentiality of an elastin reference of known hydroxyproline content for use in the calculation of the analyzed sample. In the beef tissue analyses the value used is that in bovine ligamentum nuchae, yet the elastin in this area may differ considerably from that found intramuscularly. It appears of significance, also, that a goodly portion of the data which Neuman and Logan (1950b) presented was from

work with skins or hides of different species.

A comparison of the Hartley and Hall method with the Lowry* method (essentially an extraction of non-stromal proteins with 0.1 N NaOH) was made by Griswold and Leffler (1952). The comparative study was precipitated by the anomalous finding of more collagen in 60 per cent of their cooked samples than in the raw samples when analyzed by the Hartley and Hall method. They then attempted tests for tyrosine and tryptophane on the filtrate. Comment has been made earlier regarding these aromatic amino acids. As a reference control in comparing the two methods they ran analyses on purified steer-hide collagen. Their results on purified hide collagen alone indicated agreement within 5 per cent by the two methods. The use of purified hide collagen as a check on the two methods may have contributed more distortion to the picture than the desired sharpening of focus. In the employment of purified collagen from the corium of the hide they evaded the problem of solubilizing and removing non-stromal proteins; so they did not actually test the difficult initial riddance of the intracellular proteins. In the use of a purified collagen (prepared according to Buechner's method as reported by Jacobs, 1949) they were using a material which had already been subjected to considerable

*Throughout the remainder of this discussion, for purposes of simplification, the Lowry, Gilligan, and Katersky (1941) method will be referred to as the Lowry method.

processing and manipulation. In addition to this, the literature now contains considerable evidence, as well as speculations and implications, that the other collagens in the body may well respond differently from hide collagen.

In Griswold's study, the collagen content of raw samples, as determined by the Lowry method, was one-half or less than that determined by Hartley and Hall method. In all instances the collagen of cooked samples was, by the Lowry method, less than that found in paralleled raw samples. Using the Lowry method, the determinations on the cooked samples were from one-fourth to one-ninth the amounts resulting from the employment of the Hartley and Hall procedure. The summarizing statement made by the authors was that the Lowry method was superior to the other one. This appears a completely untenable conclusion; the data do not substantiate it. Their data showed only considerable divergency of results. Just the fact that less collagen was found, by a nitrogen determination, when using the Lowry procedure could scarcely be construed as proof of superiority. The speculation is legitimate that the Hartley and Hall data may be spuriously high; the reverse side of the same coin might suggest that the Lowry procedure yields results unduly meager. The authors have offered no evidence that measurable amounts of intramuscular collagen may not have been lost in the Lowry alkaline extraction prior to autoclaving.

Cognizance should be taken of the general use and dependency of early workers on powdered hide collagen from limed hides as their reference. In view of the harsh treatment employed in the preparation (Theis and Jacoby, 1942) and keeping in mind the known diversities among collagens and also the changes which have been demonstrated to be induced by alkalies, the use of limed hide powders as reference collagen invites considerable skepticism.

It should also be noted that there is an inexplicable range in the quantities of stroma proteins in muscle tissue reported from different laboratories. The distribution of the fractions of muscle proteins was given by Weber (1950) as: albumin 20 per cent, globulin-X 20 per cent, myosin 40 per cent, and stroma 20 per cent. The averaged collagen and elastin values, combined, reported by Prudent (1947) were roughly 4 per cent. Griswold's collagen values (1952) by the Lowry method were in the same category as Prudent's. While it may be considered that Weber's 20 per cent for stroma proteins should be viewed as maximal, the discrepancy between these values cannot be dismissed.

OBJECTIVES

The objectives of this study were to explore the possibilities of several approaches in an endeavor to achieve an effective fractionation of the intracellular from the extracellular components of skeletal tissue. Diverse methods of initial disruption of the tissue as well as the employment of differing extracting solutions and separatory mechanisms were to be investigated. The first aims diffuse inextricably into a second grouping of objectives, namely attempts to evaluate the effects of the above procedural manipulations on the isolated moiety which remained. One of the most difficult and baffling aspects of fractionation efforts is that there is rarely any independent evidence of the homogeneity of the resultant fraction. Nitrogen determinations were to be made on the various fractions, with the view that such an over-all measure would not delineatively identify nor differentiate, but might serve as a general framing within which to make comparisons. Determinations of the hydroxyproline content of some extractions and residues were to be made; these, it was anticipated, might be quite characterizing. Some preliminary probings of the possibilities of certain enzymes as cleavage agents were planned. The assessment of the latter was to be in terms of nitrogen determinations, hydroxyproline content, and reducing activity.

EXPERIMENTAL PROCEDURE

Tissue Used

Bovine skeletal muscle was the tissue used throughout this study. Minimization of the problems arising from biological variation among tissue was desired. The study was restricted to the employment of few muscles from few animals. The major portion of these investigations employed one muscle. In the initial phase some cooked and correlated raw samples were employed, but following this unit all of the tissues were uncooked.

Chemical Determinations

The nitrogen determinations were carried out on the micro-Kjeldahl scale. No one who has routinely employed Kjeldahl determinations will gainsay that they are onerous. In an attempt to lessen the tedium, the estimation of nitrogen content by Nesslerization was tried. But this method presents its own problems; and in this study where certain extractions were in the alkaline range, considerable turbidity was often imparted and a colorimetric method was then vitiated. Because of difficulties with Nesslerization, most of the nitrogen determinations were obtained by Kjeldahl procedure. Nitrogens

resulting from the use of the Kjeldahl procedure are indicated as "N by Kj." in several tables.

The hydroxyproline determinations were made according to the Neuman and Logan (1950a) method. To calculate the collagen nitrogen, the mgm. of hydroxyproline were multiplied by the factor 1.365. Neuman and Logan (1950a) list the hydroxyproline content of bovine tendon as 13.4 per cent and the nitrogen content of gelatin is listed by Block and Bolling (1951) as 18.3 per cent; thus $\frac{18.3}{13.4} = 1.365$. The elastin factor, 8.943, was computed in a similar manner. The hydroxyproline content of bovine nuchea and aorta is 1.91 per cent according to Newman and Logan (1950a), and Stein and Miller (1938) give the nitrogen content of elastin as 17.1 per cent; hence $\frac{17.1}{1.91} x = 8.943$. Thus the mgm. of hydroxyproline as indicated from the colorimetric test multiplied by the factor 8.943 yield the mgm. of elastin nitrogen estimated from the hydroxyproline content. The collagen nitrogen and elastin nitrogen calculated from hydroxyproline content are listed in the tables as "N by Ho-p". Martin and Axelrod (1953) developed a modification of the Neuman and Logan method of determination of hydroxyproline. The Martin and Axelrod modification was employed in analyzing certain samples previously assessed by the Neuman and Logan method. It could not be established that the modified method gave more consistent data. Therefore, the

original Neuman and Logan method was employed to obtain all of the hydroxyproline values of the tissues studied during these investigations.

It is notable that either procedure requires carefully standardized preparation of the reagents. A particularly troublesome task was to obtain a satisfactory p-dimethylaminobenzaldehyde. Several attempts were made to recrystallize the p-dimethylaminobenzaldehyde from several sources, such as alcohol, benzene, and other solvents; but in no case could a colorless product with a sharp melting point be obtained, even after several recrystallizations. The reagent from the source suggested by Martin and Axelrod proved to be no better than that prepared in the laboratory. Criteria used to determine whether a given preparation of p-dimethylaminobenzaldehyde was satisfactory were based upon rate and intensity of final color development and stability of the final colored complex.

The evaluation of collagen content was in all cases an estimation of the protein solubilized by autoclaving for 4 to 6 hours at 15 to 20 pounds pressure.

The final residuum is conventionally designated as elastin. In the modified Lowry method this had been subjected to 0.1 N alkali at boiling water-bath temperatures, washing and centrifugation, and then had been refluxed in 87 per cent reagent grade formic acid for 24 hours. In all other fractionation efforts, the elastin was the end residue but accrued from less severe treatment.

The Park and Johnson (1949) method for determination of glucose was used in the assessment of the extent of enzymatic activity on the ground substance. The procedure was used in the belief that the release of aldehyde groups is a measure of the extent of depolymerization. The samples were read in an Evelyn colorimeter at 660 millimicrons.

Tissue Analyses

Lowry method

The origin of this entire study was in the analysis of certain samples of beef muscle, using a modification of the Lowry method. An extensive project was in progress, in related laboratories, in which the Lowry method with certain modifications was the method of assessment of connective tissue content. The alterations in the Lowry method were in the initial masceration of tissue and in the substitution of a volumetric assessment of collagen and elastin for the original gravimetric determination, and in the final step for solubilizing the elastin. The sample of meat was comminuted by grinding through a food-chopper, mixing with crushed dry ice, and then pulverized by passing the mix through a small mill. After the CO_2 had been dissipated, weighed portions were mixed with water and Waring blended for about 45 minutes. The entire dispersion was transferred to volumetric flasks. Aliquots

from this mix constituted the analyzed portions. Reference has already been made to the plan for solubilization of elastin in formic acid. To reiterate, these modifications of the Lowry method had been planned and the modified method was in use in routine analyses in a related project. Because of certain anomalies in the data, a familiarity with the modified Lowry method and its application in some analyses was utilized as the point of departure in this study. Samples of uncooked meat and related samples of cooked meat were analyzed for collagen and elastin content. Samples 1 and 2 were from a beef carcass graded Commercial . Sample 1 was from the longissimus dorsi and sample 2 was from the semitendinosus. Samples 3 and 4 were from a beef carcass graded Choice . Sample 3 was from the longissimus dorsi and sample 4 was from the semitendinosus. The values are reported in terms of collagen nitrogen and elastin nitrogen as per cent of total nitrogen.

Initial investigation with KCl

The foremost desideratum in initiating exploratory attempts was the employment of an extractant which would be mild. Bate Smith (1934) had suggested that LiCl was an efficient extractant for muscle protein. The salts which have been used include KCl, NaCl, $CaCl_2$, Na_2CO_3, $NaHCO_3$, NaH_2PO_4, and KCNS. However, since it has been shown that KCl and NaCl have less destructive effect upon the stroma proteins than the

other salts, KCl salt extractions were employed. Extractions of muscle protein with LiCl were not as efficient as those made with extracting solutions containing KCl. Gradations of molarities of KCl, with two successive extractions, were tried. The tissue for these first investigative extractions was from the semimembranosus muscle of a carcass graded Good . It was cut into compact segments; these were frozen, foil-wrapped, and stored in the freezing compartment of a laboratory refrigerator. The tissue was shredded from the frozen segment, minced with a sharp knife, and then a 25-gram portion was Waring blended in 250 ml. ice water for 3 minutes. Aliquots of 50 ml. were taken, solutions of KCl ranging from molarities 1.0 M to 2.5 M were added, volumes adjusted to 60 ml., and the stoppered tubes shaken for 30 minutes. The tubes were centrifuged and decanted. These extractions were made at pH 9.0.

The advisability of first removing a water-soluble fraction, followed by successive KCl extractions, was pursued. The method of initial dispersion was identical with that just indicated, except that the 50-ml. aliquots were diluted to volumes of 200 ml., with the addition of KCl equivalent to 1.0 M. Subsequently, a comparison of the extent of dilution of the 50-ml. aliquot to 60 ml. contrasted with 200 ml. was made. The first two extractions were water, followed by a KCl extraction and finally extraction with $NaHCO_3$ of 0.01 M.

Freezing to disrupt the muscle fibers

Because the literature contains numerous suggestions for the freezing of tissue as a method of disrupting the cell wall of muscle fibers this technique was tried. The manipulation of tissue up to this time had been done at room temperature. The ease with which the proteins may be denatured at room temperatures may explain, in part, some of the variability between samples. In the treatment of the three following samples the comminution and shaking were carried out in a cold room (5°C.). The shaker was the end-over-end type. Facilities were such that it was still necessary to do the centrifuging and redispersion at room temperatures. The object of the treatment of the next three samples, with replicates of four each, was to determine the effect of repeated freezing and thawing 7 times on the extractability of intracellular proteins. The results are recorded in Table 5. Extractions from the frozen samples are given in the rows numbered 1. Extractions from unfrozen or control samples will be seen in the rows numbered 2. The sequence of steps in handling of the samples was: first extraction with water for approximately 24 hours; then two successive 1 M KCl extractions each of about 12 hours duration; a water wash followed; then the residue, in 25 ml. of H_2O, was autoclaved 4 hours at 15 pounds pressure. It was deemed desirable for purposes of comparison to determine the effect of 0.1 NaOH on the connective tissue residue after autoclaving.

Hence, a 24-hour extraction with 0.1 N NaOH at room temperature was made. A water wash followed, and the terminal residue was considered elastin. The content of the collagen of the autoclave-solubilized fractions was determined by three methods: from nitrogen values obtained by Nesslerization, from its hydroxyproline content, and from the gelatin estimation by the tannate procedure (Cover et al., 1944). The terminal residue was estimated by the first two methods listed above in the assessment of collagen.

Fractionation by centrifugation

Subsequent work was carried out at 0.5 to 2°C. in a cold room. The psoas major muscle was excised from a beef carcass graded Commercial which had hung in the cooler 10 days. All extraneous surface fat was carefully trimmed from the muscle, but no attempt was made to remove the epimysial covering. The entire muscle was comminuted for three minutes in a Silent Cutter, which is a revolving bowl with a rapidly spinning central knife. This was done to effect more uniform sampling. The mascerated tissue was mixed and blended by hand, then 30-gram portions were wrapped in aluminum foil. The foil-wrapped portions were frozen and held at -30°C.

Preliminary variations were made using centrifugation for separation. In initial dispersions there were no real differences between using the Waring blendor for two minutes, for

five minutes, or carefully blending by hand with a glass rod. When water was the first extractant an increase of shaking time from 7 hours to 24 hours did not increase the amount of nitrogen removed. A series of extractions using varying concentrations of KCl, all buffered with 0.03 M $NaHCO_3$, indicated: little if any difference in extraction between water extraction and 0.25 M KCl; no difference between 0.5 and 0.75 M KCl as extractants; marked increase in the gelation within the tube when molarities were increased to 1.0 M and 1.25 M. In these trials there was considerable gelling in the tube. There was often a stratification of the gelatinous material. The high viscosity of the gelatinous material precluded effective sedimentation. Therefore a more efficacious method of partitioning the fractions needed to be sought.

Glass wool entrapment

The possibility of physical entrapment of the scleroproteins on a mass of glass wool in an Erlenmeyer flask was explored. The procedure was an aliquoting of tissue-dispersions into flasks containing glass wool. The stoppered flasks could be shaken gently at the cold-room temperatures. The dispersed proteins were then to be decanted from the flask through a filter pad of glass wool. Rather thorough washing of the glass wool with the extracting solution was feasible. The stroma proteins would, theoretically, be entrapped and retained on the glass wool.

In a series of preliminary trials the effect of the following factors was investigated: dispersion of tissue by hand-mixing versus use of Waring blendor, differing grades of glass wool, lengths of glass wool strands, quantities of glass wool, varying dilutions of the 5 grams of tissue from 30 ml. to 100 ml., length of shaking times, decanting and washing at 0°C. to 2°C. versus room temperature. In the modifications imposed by varying the factors listed above, there were no marked differences in the amounts of protein extracted. Successive trials gave good agreement in percentage of total nitrogen removed; the average was approximately 85 per cent.

Because earlier work did not indicate differences in the efficiency of 0.5 M as contrasted with 0.75 M KCl it was decided to use 0.6 M KCl, plus 0.03 M $NaHCO_3$, which was very similar to the widely used Weber-Edsall extracting solution (Mommaerts, 1950). The pH of this KCl extracting solution is 8.3.

Based on the rather exhaustive trials relating to environmental factors noted above, certain conditions were established because of feasibility of manipulation; these were kept uniform throughout the remainder of the work. Erlenmeyer flasks of 300 ml. volume with tightly fitting aluminum foil-covered rubber stoppers were used. Glass wool was cut in 3/4-inch lengths and 1.25 gm. of glass wool was used in each flask.

For the first extraction the flasks were shaken 3 hours. The speed of the horizontal shaker could be adjusted (about 25 excursions per minute) so the solution moved completely through the mat of glass wool with each back-and-forth motion of the shaker, but care was taken to avoid foam formation.

Five grams of the frozen tissue was weighed into the flask; and then approximately 1 ml. of water was introduced, but care was taken that the water was not in contact with the tissue. The flasks were tightly stoppered, and the tissue thawed overnight. The following morning the tissue was manipulated into a uniformly soft mass by using a glass rod. Small portions of the extracting solution were blended with the tissue so that the slurry was kept as uniform in consistency as possible. The glass wool was added gradually in small portions in order to effect uniform dispersion of the tissue.

For the total nitrogen determinations, a 10-gm. portion was homogenized in a Waring blendor with 190 ml. of water for 3 minutes. Aliquots of 5 ml. were used in Kjeldahl determinations. In the procedural handling of the glass wool-entrapped residues, a triple washing of 100 ml. each of distilled water was routinely employed to wash the mass before another solution or another treatment was utilized.

All of the subsequent work, with a few exceptions which will be indicated, was done with tissue from the semitendinosus muscle of a beef carcass graded Choice . The muscle was

prepared exactly in the manner previously described for the psoas major muscle.

It was planned to extract the tissue with 0.6 M KCl and then subject the entrapped residue to varying treatments. With one series, extractions with 0.1 N NaOH were compared with those with 1 N NaOH; and following the extraction with alkali, the protein solubilized by autoclaving was quantitated. After the KCl extractions and decantations the residues were triple-washed; then 50 ml. of planned extractant was added and the flasks were shaken. Aliquots were removed at 4-hour intervals for a 48-hour period. Residues were again washed, 50 ml. of water was added to each flask, and the samples were autoclaved 4 hours at 15 pounds pressure. These data are recorded in Table 6.

In the subsequent series the alkaline extractions prior to autoclaving were repeated. Correlated samples were autoclaved immediately after the KCl decantations and then subjected to 0.1 N NaOH extractions.

In the next series the samples were KCl extracted and then subjected to autoclaving. Kjeldahl nitrogens and hydroxyproline estimations were made on the fraction solubilized by this hydrolysis. For the hydroxyproline determinations, the residues were washed and hydrolyzed with 25 ml. of 6 N HCl by autoclaving 8 hours at 20 pounds pressure. They were filtered while hot, neutralized, and brought to volume (Neuman and Logan, 1950a).

It seemed expedient to vary the normality of the alkaline extracting solution from 0.05 to 0.25 N. Fifty ml. of NaOH solution was in contact with the residues for 20 hours at room temperature; then the residues were washed and autoclaved for 4 hours at 15 pounds pressure. Two samples in this series were autoclaved as soon as the KCl extractant had been decanted and the residues washed; these were not treated with alkali. The values on the non-alkaline-treated samples are listed in the last two columns of Table 9. As in the prior series, the Kjeldahl nitrogen and hydroxyproline determinations were made on the gelatin fraction. After washing, the final residues were hydrolyzed with 6 N HCl preparatory for hydroxyproline determinations. The values so obtained were used to calculate the elastin content.

A repetition of the design reported in Table 9 was executed, with the addition of two samples. The additional samples were not KCl-extracted; the initial extracting solution of the added samples was 0.1 N NaOH.

Because routine analytical work on meat samples, using the modified Lowry method, was proceeding in a related laboratory, there was an opportunity to make certain comparisons. A sample of tissue (semimembranosus from a Choice beef carcass) which the technician had ground with dry ice was obtained. Five grams of this tissue was weighed into each flask and carried through the improvised glass wool procedure. Two flasks were

extracted with 0.6 M KCl and two with 0.1 N NaOH. An aliquot of autoclave-solubilized collagen from this same sample of semimembranosus tissue which the technician had prepared by the Lowry procedure was obtained. Nitrogen content by Kjeldahl procedure and by hydroxyproline determination was estimated; these could be compared with the earlier values obtained when the semimembranosus tissue had been subjected to the glass wool procedure. Determinations of nitrogen content were also made on the final residues.

The next step appeared to be that of taking the semitendinosus tissue, used in most of these exploratory problems, and analyzing it by the Lowry procedure. This was done in the related laboratory so all special equipment could be utilized. Comparative determinations were carried out on the collagen and terminal residues.

It was repeatedly observed that the color of the gelatin and elastin hydrolysates varied with the method of preparing the residues. The acid hydrolysates resulting from the glass wool procedure and by the Lowry method prepared for the hydroxyproline determinations were employed for the absorption studies. Absorption readings were made using a Beckman D U spectrophotometer over the range of 250-700 millimicrons at intervals of 20 millimicrons.

The absorption spectra of gelatin hydrolysates and the elastin hydrolysates, obtained by the glass wool procedure and

by the Lowry method, were determined using a Beckman D U spectrophotometer. The acid hydrolysates were prepared by the usual methods for the determination of hydroxyproline. The objective in view was a comparison of the color components in relation to the method of derivation of residues. No quantitative determination was intended; the aim was a comparison of the qualitative patterns of optical densities. These data are recorded graphically in figures 1 and 2.

Use of enzymes

Because there was a reference in the literature (Rosenblum et al., 1953) indicating that papain possessed collagenase activity, attempts to assess the response of the entrapped residue to papain were made. Buffered solutions of pH's 4.0, 8.5, and 2.7 were made up as described by Hurst (1953). Activation of the enzyme was accomplished by blending 1 gm. of papain with 1.3 gm. of cysteine hydrochloride and 10 ml. of water, and incubating the preparation at 37°C. for 1 hour. The pH was adjusted to 7.0 with the addition of 2.5 N NaOH and the entire dispersion brought to 100 ml. One ml. of the papain solution was used for each gram of muscle tissue. Five ml. of the papain suspension was used on the residue in each flask; then 95 ml. of the respective buffering solution was added. One ml. of toluene was added to each flask; the flasks were tightly stoppered and incubated at 37°C. for 48 hours.

In the first group, 4 samples were to be extracted with KCl and 4 with 0.1 N NaOH. Then the papain suspension and 95 ml. of water were added to each flask. The flasks were swirled occasionally during the incubation period. The washings, autoclaving, and determinations on extractions and on terminal residues were as indicated for earlier treatments. Subsequently the pH of the buffered solutions, for papain activity, was adjusted to 4.0 for some samples and 8.5 for others.

In a third series of KCl-extracted residues buffered solution of pH 2.7 and pH 4.0 were used.

Trials were made using hyaluronidase to determine if any measurable changes in the entrapped residues could be induced. Ten-gram samples of tissue were used. Two samples were extracted with KCl and two with NaOH. Half of the residues were subjected to papain for 48 hours at 37°C. prior to serving as substrate for the hyaluronidase. Commercial testicular hyaluronidase was procured. Twenty-five mgm. of hyaluronidase was dissolved in 25 ml. of 0.1 M NaCl; 5 ml. of this solution was used in each flask. To each flask was added 45 ml. of 0.1 M acetate solution adjusted to pH 5.2. A vial of toluene was placed in each flask. The flasks were tightly stoppered and incubated at 37°C. for 24 hours. The glucose equivalents per gram of tissue were estimated after the papain digestion and following the hyaluronidase treatment.

For the concluding unit, the pattern just described was

repeated with the extensions of checking the reducing activity at the end of 48 hours, and 72 hours incubation. Two additional KCl-extracted samples were carried throughout the processing; these are indicated in Table 19 as flasks 3 and 4. Flasks 3 and 4 had H_2O on the residues during the papain digestion. Hyaluronidase was omitted from flask 3, but 0.1 M sodium acetate solution was on the residue during the 72-hour incubation. Flask 4 (KCl-extracted) had hyaluronidase addition, identical with the aliquots used in flasks 1, 2, 5, and 6. Kjeldahl nitrogens and hydroxyproline determinations were made on the papain digests.

RESULTS

The tissue used throughout this study was bovine skeletal muscle.

For the sake of convenience, the nitrogen, however estimated, in the autoclave-solubilized fraction is termed collagen nitrogen, whereas the nitrogen contained in the hydrolysates of the terminal residues is designated as elastin nitrogen.

Several attempts were made to determine whether the KCl extracts contained hydroxyproline, but no evidence that they contained this amino acid was obtained.

Use of the Lowry Procedure

The employment of the modified Lowry procedure in the analysis of certain samples of raw (r) tissues and of the correlated cooked (c) samples from the paired location in the carcass gave the values recorded in Table 1. Samples 1 and 2 were from a beef carcass graded "commercial"; samples 3 and 4 were from a "choice" beef carcass.

Table 1

Collagen Nitrogen and Elastin Nitrogen

in Raw and Cooked Samples of Four Muscles

Sample	Collagen N	Elastin N
	(Per cent of Total Nitrogen)	
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
Ave. r	1.88	0.26
c	0.31	0.71

Initial Investigation with KCl

The separation of fractions by centrifugation gave persistent and considerable difficulty. The residue was often soft and gel-like and lacked a sharp line of demarcation from the supernatant. More often than not some of the strand-like particles in the supernatant would not sediment down efficiently. In the cooked samples there was such marked gelation within the centrifuge tube that decantations were highly dependent on the manipulator's judgment. The surfacing of the tubes, in the first three extractions with lipid-like material and enmeshed strands, was a constant problem.

The trial use of increasing molarities of KCl solutions from 1.0 M to 2.5 M as extractants is recorded in Table 2.

Table 2

Effect of Concentration of KCl in Extracting Solutions on Amount of Protein Extracted

(All values expressed as %N of T.N.)

Molarity of KCl:		1.0 M	1.5 M	2.0 M	2.5 M
Series	I	75.2	60.8	53.1	49.6
	II	64.8	60.1	58.6	47.4
	III	63.4	62.2	60.2	55.0

In the KCl extractions some of the supernatants were rather turbid. Surface films formed on all of the tubes; with an increase in concentration of salt there was an increase in the thickness of film on the surface. The residues in the centrifuge tubes were somewhat gelatinous in consistency.

Table 3 shows the comparison between having the first extraction that of water, as in I, versus an initial KCl extraction, as shown in II.

Table 3

Efficiencies of Initial Water and Salt Extractions of Protein

(All values expressed as %N of T.N.)

Treatment	I	II
Water	31.5	----
KCl, 1.0 M	----	53.9
KCl, 1.0 M	33.8	7.4
KCl, 1.0 M	4.0	3.7
Sum	69.3	65.0

In Table 4 are the results obtained by diluting the 50-ml. aliquot (containing 5 gm. tissue) to 60 ml. and to 200 ml.

Table 4

Effect of Volume of Extracting Solution
in Amount of Muscle Protein Solubilized
(All values expressed as %N of T.N.)

Series	Extractions	60 ml.	200 ml.
I	Water	27.4	29.3
	Water	3.4	9.3
	KCl, 1.0 M	13.7	45.4
	$NaHCO_3$, .03 M	2.7	6.5
	Sum	47.2	90.5
II	Water	27.5	28.1
	KCl, 0.5 M	10.7	19.9
	KCl, 1.0 M	9.3	27.1
	$NaHCO_3$, .01 M	2.5	8.1
	Sum	50.1	83.2

The water extractions were opalescent in appearance. Suspended particles in the salt extractions imparted a cloudiness.

Freezing

The influence of freezing and thawing the initial water extraction 7 times is shown in Table 5.

Table 5

Effect of Freezing on Solubilization of Muscle Protein by Water and by KCl (All values expressed as %N of T.N.)

Series		I		II		III	
Samples		A	B	A	B	A	B
Water	1.*	24.6	26.3	24.6	24.6	27.4	27.4
	2.	23.4	23.4	21.0	21.6	24.8	23.5
KCl, 1.0 M	1.	15.6	12.0	18.1	15.8	14.4	14.4
	2.	28.1	29.3	27.5	27.5	35.3	34.0
Collagen							
N, Ness.	1.	11.4	13.1	12.6	14.9	15.2	15.2
	2.	9.2	9.2	9.6	8.8	12.2	12.4
N, Tann.	1.	----	7.8	----	9.1	----	----
	2.	5.5	----	----	5.2	7.8	8.8
N, Ho-p	1.	2.7	4.2	3.5	3.5	3.3	3.4
	2.	2.4	2.4	2.6	2.3	2.3	2.9
Elastin							
N, Ness.	1.	9.3	9.4	18.5	17.8	8.8	8.8
	2.	----	----	8.8	8.8	15.1	14.1
N, Ho-p	1.	0.18	0.22	0.6	0.6	0.3	0.3
	2.	0.04	0.05	0.14	0.04	0.3	0.3

*1. Frozen samples
2. Unfrozen samples

In Table 5 above is the only work reported in which the nitrogens were determined by Nesslerization; they are indicated in the table as "N, Ness." The values for the water and

KCl extractions were obtained by Nesslerization. In Table 5 also are the only presentations of the assessment of the collagen fraction by the tannate procedure; these values are listed as "N, Tann."

An alkaline extraction with 0.1 N NaOH was made after the collagen fraction had been removed. The values for the alkaline extraction of Series II were, by hydroxyproline estimation, 0.18 and 0.16 for the frozen samples A and B respectively, and 0.18 and 0.16 for the correlated unfrozen samples. These values were calculated as collagen nitrogen in per cent of total nitrogen.

It should be noted that maximum absorption for solutions of hydroxyproline prepared for assay by the Neuman and Logan (1950a) procedure is at 560 millimicrons, and not the 540 millimicrons that Neuman and Logan reported. This observation has been reported by Howard (1951) and Baker et al. (1953). Figures 1 and 2 also show that maximum absorption of the color complex in the colorimetric determination for hydroxyproline is at 560 millimicrons. The major portion of the hydroxyproline assays had been completed before this discrepancy was discovered. Therefore all hydroxyproline values reported were read at 540 millimicrons. The color components of acid hydrolysates of collagen by either the glass wool procedure or the Lowry method of fractionation and of standard solutions of hydroxyproline had absorptive characteristics which gave

essentially equivalent optical density patterns.

The use of the end-over-end shaker resulted in considerable foam formation. In the water extractions there were visible particles in the supernatants. The frozen samples A and B upon final defrosting had a matted, filamentous residue in the bottom of the container with a watery upper layer. The KCl supernatants appeared somewhat cloudy. In the samples which had been frozen no surface films formed on centrifugation, but they were very difficult to sediment. It was necessary to centrifuge, chill, and recentrifuge; a conspicuous amount of suspended material was taken off in the supernatant. In the non-frozen samples the KCl extractions formed stringy surface films on centrifugation.

Glass Wool Entrapment of Connective Tissues

In the following work the muscle was from the semitendinosus of a Choice beef carcass, except one sample, which was used in a comparative manner, and its grade and source have been indicated. Also, in the subsequent work the improvised glass wool method of retaining the stromal proteins was used except in a comparative problem with the Lowry procedure.

The influence of the length of time on the amounts of protein solubilized by 0.1 N NaOH and 1.0 N NaOH are reported in Table 6. An initial KCl extraction was made; then the

residue was extracted with alkali; sample A was extracted with 0.1 N NaOH, while B was subjected to 1.0 N NaOH. The residues were autoclaved subsequent to the alkaline treatment. All values in Table 6 result from the Kjeldahl determination.

Table 6

Effect of Length of Time on Amount of Protein Extracted by 0.1 and 1.0 N NaOH (All values expressed as %N of T.N.)

	KCl 0.6M	Alkali			
		4 hr.	8 hr.	24 hr.	48 hr.
Sample A 0.1 N NaOH	87.3	4.8	5.4	6.7	6.6
Sample B 1.0 N NaOH	83.1	8.4	8.8	10.9	14.2

The collagen content of sample A in Table 6 (N by the Kjeldahl method) after 0.6 M KCl and 0.1 N NaOH extractions, was 4.2 per cent of the total nitrogen; while that of sample B, which had been treated with the more concentrated alkali, contained 0.9 per cent collagen nitrogen.

The contrasting effects of an alkaline treatment before autoclaving and subsequent to the autoclaving are reported in Table 7.

Table 7

Effect on Collagen Content of Alkaline Treatment Prior to and Following Autoclaving

(All values expressed as %N of T.N.)

Series:	I	II	III
Treatment			
KCl, 0.6 M	80.7	84.8	83.6
Alkali, prior to autoclaving	7.6*		
Collagen N	3.8	10.2	11.4
Alkali, following autoclaving		0.1*	0.9**

* 0.1 N NaOH used in series I and II

**1.0 N NaOH used in series III

Estimation of the protein solubilized by autoclaving, immediately after decantation of the salt-dispersed portion, is shown in Table 8 for 4 samples. A comparison of the collagen values as determined by two different methods, Kjeldahl nitrogen and hydroxyproline, is given in Table 8.

Table 8

Comparison of Two Methods of Assessment of Collagen Content

(All values expressed as %N of T.N.)

Extracted by 0.6 M KCl			
85.7	86.4	86.4	85.0
Autoclave-solubilized fraction			
(N by Kj.)			
12.6	14.2	12.6	13.6
(N by Ho-p.)			
4.3	4.3	4.0	4.0

Gradations in the normality of NaOH used for extraction, varying from 0.05 N to 0.25 N, were used on the residues immediately after the KCl extraction and are reported in Table 9. Samples 7 and 8 had no alkaline extraction, but were autoclaved immediately after decantation of the salt-dispersed protein.

Table 9

Fractions of Muscle Proteins Resulting
from KCl and NaOH Treatments
(All values expressed as %N of T.N.)

Extraction with 0.6 M KCl							
72.9	78.5	77.3	75.9	79.3	80.5	78.9	78.7
Normality of NaOH							
0.05	0.075	0.10	0.15	0.20	0.25		
Extraction of residue by alkali							
(N by Kj.)							
12.4	10.7	10.5	12.8	10.6	10.4		
(N by Ho-p.)							
.13	.15	.15	.19	.19	.23		
Autoclave-solubilized fraction							
(N by Kj.)							
5.3	3.8	3.9	3.9	3.6	3.6	16.5	14.9
(N by Ho-p.)							
4.1	3.7	3.8	3.8	3.7	3.8	4.3	4.3
Elastin N in terminal residue							
(N by Ho-p.)							
2.4	2.3	2.4	2.5	2.5	2.6	2.6	2.1
N not accounted for							
(N by difference)							
7.0	4.7	5.9	4.9	4.0	2.9	2.0	4.3

In the subsequent tables 10a and 10b, the pattern of extractions and methods of analyses of the fractions used in securing data in Table 9 were repeated. Two samples were added; these appear in the third and fourth columns of Table 10b. The two additional samples were not extracted with 0.6 M KCl, but had an initial extraction of 0.1 N NaOH.

Table 10a

Fractions of Muscle Proteins Resulting from KCl and NaOH Treatments

(All values expressed as %N of T.N.)

Extraction with 0.6 M KCl					
82.6	82.2	80.0	81.1	82.8	83.3
Normality of NaOH					
0.05	0.075	0.10	0.15	0.20	0.25
Extraction of residue by alkali					
(N by Kj.)					
8.8	8.7	9.3	8.4	9.1	9.8
(N by Ho-p.)					
.16	.2	.2	.2	.2	.3
Autoclave-solubilized fraction					
(N by Kj.)					
5.0	4.7	4.0	4.0	3.9	4.0
(N by Ho-p.)					
4.0	3.9	3.7	3.6	3.6	3.7
Elastin N in terminal residue					
(N by Ho-p.)					
2.2	2.4	2.3	2.2	2.4	2.4
N not accounted for					
(N by difference)					
1.4	2.0	4.4	4.3	1.8	0.5

Table 10b

Fractions of Muscle Proteins Resulting from KCl and NaOH Treatments (All values expressed as %N of T.N.)

Extraction with 0.6 M KCl			
84.9	84.3		
Extraction of residue by alkali (N by Kj.)			
		90.5	90.1
Autoclave-solubilized fraction (N by Kj.)			
12.5	13.3	4.1	4.1
(N by Ho-p.)			
4.0	4.1	3.9	4.1
Elastin N in terminal residue (N by Ho-p.)			
2.6	2.0	2.4	2.4
N not accounted for (N by difference)			
0	0.4	3.0	3.4

The pH of the KCl decantations, diluted to 250 ml., was 8.4 to 8.5. The pH of the 0.1 N NaOH extraction, diluted to 250 ml., was 12.3 to 12.5 (uncorrected).

A sample of semimembranosus from a Choice beef carcass, ground with dry ice and initially prepared for analysis by the Lowry procedure, was obtained. In 5-gram portions this tissue was analyzed using the glass wool procedure. The values are listed in Table 11.

Table 11

Fractionation of Semimembranosus Tissue
Using Glass Wool Procedure
(All values expressed as %N of T.N.)

Extractant			
0.6 M KCl		0.1 N NaOH	
82.0	81.4	94.1	95.0
Autoclave-solubilized fraction			
(N by Kj.)			
11.0	15.7	2.8	2.1
(N by Ho-p.)			
2.0	2.4	1.8	2.1
Elastin N in terminal residue			
(N by Ho-p.)			
0.8	0.8	0.8	0.8
N not accounted for			
(N by difference)			
6.2	2.1	2.3	2.1

Aliquots of an autoclave-solubilized fraction prepared by the Lowry procedure were secured. These aliquots accrued from the fractionation of the same sample of semimembranosus reported in Table 11. On these collagen aliquots the Kjeldahl nitrogens were determined and hydroxyproline determinations were also made. The elastin residues were quantitated by these two methods also. The values from the residual fractions of collagen and elastin are given in Table 12.

Table 12

Fractionation of Residual Connective Tissues
Using Lowry Procedure
(All values expressed as %N of T.N.)

Autoclave-solubilized fraction					
(N by Kj.)					
1.1	1.1	1.1	1.1	1.2	1.2
(N by Ho-p.)					
1.3	1.4	1.5	1.3	1.6	1.4
Elastin in terminal residue					
(N by Kj.)					
		0.15			0.18
(N by Ho-p.)					
0.12	0.15		0.16	0.17	

The semitendinosus tissue used in the work recorded from Table 7 through Table 10b was assessed by the modified Lowry method and is reported in Table 13. Hydroxyproline determinations of the collagen fraction and of certain elastin residues are included.

Table 13

Collagen and Elastin Content of Residues of Semitendinosus Tissue by the Lowry Method (All values expressed as %N of T.N.)

Autoclave-solubilized fraction					
(N by Kj.)					
3.7	3.7	3.4	3.4	3.5	3.6
(N by Ho-p.)					
3.9	4.1	3.8	3.8	3.9	4.2
Elastin N in terminal residue					
(N by Kj.)					
2.4		2.5		2.3	
(N by Ho-p.)					
	2.8		2.8		2.7

The hydroxyproline preparations on the elastins prepared by the Lowry procedure exhibited a color development on oxidation with the H_2O_2 which was analogous with that developed

in the collagen hydrolysates; it was a yellow-brown color with a brown precipitate sedimenting out, whereas in the terminal residues derived by glass wool entrapment, the elastins were always a distinct yellow-green at the stage of the peroxide reaction.

The terminal color developed in the acid hydrolysates of elastins prepared by the Lowry method was the same color, a purplish-red, as that in the collagens and in the standard hydroxyproline solutions. The terminal color of the acid hydrolysates of the glass wool-entrapped elastins was characteristically and observably different from that of the collagens; these elastins were of a brownish or bronzy cast.

The optical density curves of the acid hydrolysates of collagen and gelatin obtained by glass wool retention are shown in Figure 1. In Figure 2 are the patterns of optical densities as they resulted from analysis of the acid hydrolysates of collagen and elastin prepared by the Lowry procedure.

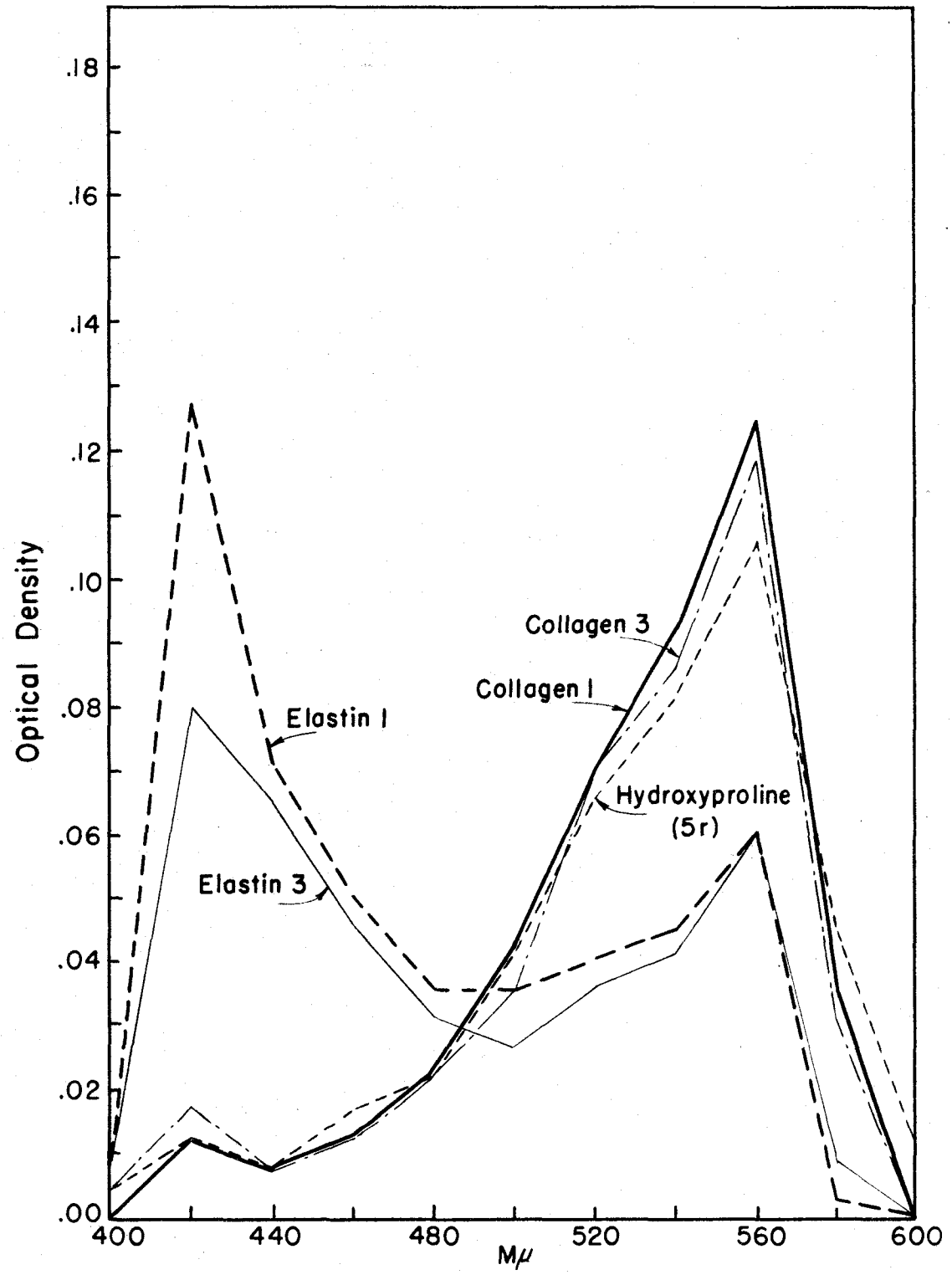

Fig. I Absorption Spectra of Acid Hydrolysates of Glass Wool Entrapped Collagen and Elastin

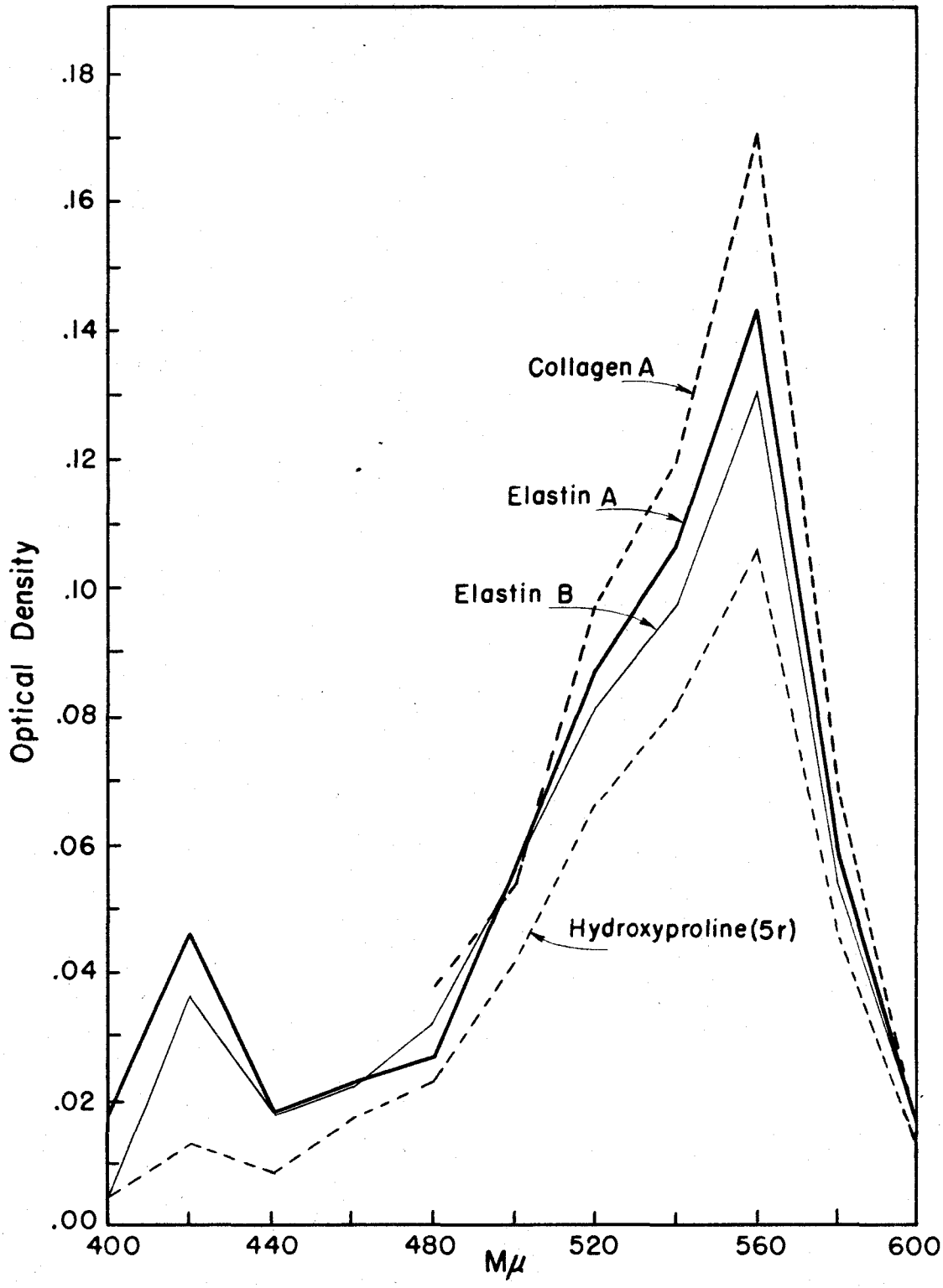

Fig. 2 Absorption Spectra of Acid Hydrolysates of Collagen and Elastin Prepared by the Lowry Method

Use of Papain and Hyaluronidase

The results of the treatment of muscle tissue residues with papain prior to autoclaving are given in Tables 14a through 17. The first extracting solution is indicated in each table. The initial pH at which each sample was buffered is also indicated. The pH's of certain papain digests and of the autoclave-solubilized fractions are given in Table 16. These pH values were determined on the fractions reported in Tables 15a and 15b.

Table 14a

Influence of Papain on KCl-extracted Residues of Muscle Tissue

(All values expressed as %N of T.N.)

Extraction with 0.6 M KCl			
77.2	75.5	75.1	74.9
Papain-solubilized fraction			
(N by Kj.)			
1.6	1.7	1.8	1.7
(N by Ho-p.)			
.02	.02	.04	.04
Autoclave-solubilized fraction			
(N by Kj.)			
11.6	12.4	11.7	12.9
(N by Ho-p.)			
4.1	4.1	4.1	4.1
Elastin N in terminal residue			
(N by Ho-p.)			
2.6	2.3	2.3	2.4
N not accounted for			
(N by difference)			
7.0	8.1	9.0	8.1

Table 14b

Influence of Papain on NaOH-extracted Residues of Muscle Tissue

(All values expressed as %N of T.N.)

Extracted with 0.1 N NaOH			
89.8	89.4	90.1	89.8
Papain-solubilized fraction			
(N by Kj.)			
1.1	1.2	1.0	1.0
(N by Ho-p.)			
.04	.03	.03	.03
Autoclave-solubilized fraction			
(N by Kj.)			
4.8	4.8	4.8	4.8
(N by Ho-p.)			
3.8	3.8	3.8	4.0
Elastin N in terminal residue			
(N by Ho-p.)			
2.1	2.1	2.1	2.4
N not accounted for			
(N by difference)			
2.2	2.5	2.0	2.0

Table 15a

Influence of Papain on Residues of Muscle Tissue

(All values expressed as %N of T.N.)

Extractant			
0.6 M KCl		0.1 N NaOH	
75.2	74.8	85.8	85.6
Initial pH 4.0			
Papain-solubilized fraction			
(N by Kj.)			
2.2	2.2	1.7	1.7
(N by Ho-p.)			
.06	.06	.08	.09
Autoclave-solubilized fraction			
(N by Kj.)			
12.5	12.5	4.7	4.7
(N by Ho-p.)			
3.8	3.4	3.1	3.1
Elastin N in terminal residue			
(N by Ho-p.)			
2.2	2.2	1.9	2.0
N not accounted for (N by difference)			
9.9	8.3	5.9	6.0

Table 15b

Influence of Papain on Residues of Muscle Tissue
(All values expressed as %N of T.N.)

Extractant			
0.6 M KCl		0.1 N NaOH	
69.5	69.2	88.2	87.8
Initial pH 8.5			
Papain-solubilized fraction			
(N by Kj.)			
6.0	3.7	1.8	2.0
(N by Ho-p.)			
.02	.02	.06	.03
Autoclave-solubilized fraction			
(N by Kj.)			
10.7	12.8	4.6	4.6
(N by Ho-p.)			
3.5	3.4	3.2	3.1
Elastin N in terminal residue			
(N by Ho-p.)			
1.9	1.8	2.1	2.1
N not accounted for (N by difference)			
11.9	12.5	3.3	3.5

Table 16

The pH of Papain Digests and Autoclave-solubilized Fractions

0.6 M KCl-extracted residues Initial pH 4.0			
Papain-solubilized fraction			
4.3	4.3	4.25	4.3
Autoclave-solubilized fraction			
9.0	9.15	8.8	9.4
0.1 N NaOH-extracted residues Initial pH 8.5			
Papain-solubilized fraction			
9.1	9.05	9.1	9.1
Autoclave-solubilized fraction			
8.8	9.0	9.0	9.0

Table 17

Influence of Papain on Residues of Muscle Tissue

(All values expressed as %N of T.N.)

Extraction with 0.6 M KCl			
78.2	75.6	76.0	72.8
Initial pH 2.7		Initial pH 4.0	
Papain-solubilized fraction			
(N by Kj.)			
4.0	5.4	3.1	3.2
(N by Ho-p.)			
0.1	0.1	0.1	0.1
Autoclave-solubilized fraction			
(N by Kj.)			
10.6	11.0	11.3	11.6
(N by Ho-p.)			
4.2	4.1	4.1	4.4
Elastin N in terminal residue			
(N by Ho-p.)			
2.4	2.4	2.3	2.2
N not accounted for			
(N by difference)			
4.8	5.6	7.3	10.2

The influence of hyaluronidase on the residues, with and without prior papain digestion, is recorded in Tables 18 and 19. The measurement of reducing sugar is in mgm. of glucose-equivalents per gram of tissue.

Table 18

Glucose-equivalents in Residues from Muscle Tissue
(Mgm./g. of tissue)

Treatment	Papain	Hyaluronidase	Total
0.6 M KCl	--	0.03	0.03
0.6 M KCl	0.84	0.07	0.91
0.1 N NaOH	--	0.03	0.03
0.1 N NaOH	0.81	0.12	0.93

Table 19

Glucose-equivalents in Residues from Muscle Tissue
(Mgm./g. of tissue)

Treatment	Papain	Hyaluronidase		Total
		48 hr.	72 hr.	
0.6 M KCl	--	0.04	0.04	0.04
0.6 M KCl	0.71	0.13	0.15	0.86
0.6 M KCl (KOH)	0.11	0.01	0.01	0.12
0.6 M KCl (KOH)	0.14	0.02	0.02	0.16
0.1 N NaOH	--	0.04	0.05	0.05
0.1 N NaOH	0.72	0.11	0.13	0.85

In Table 20 are shown the results of Kjeldahl nitrogen and hydroxyproline determinations on the papain digests and the extractions after 72 hours of hyaluronidase treatment. The samples are the same ones indicated in the left column of Table 19.

Table 20

Nitrogen and Hydroxyproline Determinations on Papain and Hyaluronidase Digests

Papain digest		Hyaluronidase digest	
Nitrogen mgm./g. tissue	Ho-proline mgm./g. tissue	Nitrogen mgm./g. tissue	Ho-proline mgm./g. tissue
Extraction with 0.6 M KCl			
----	----	0.11	0
2.58	trace	0.33	0
0.81		0	0
1.07		0	0
Extraction with 0.1 N NaOH			
----	----	0.11	0
1.79	trace	1.49	0

It can be seen that small amounts of protein were solubilized during the extractions, but hydroxyproline was not detected or was found only in traces.

DISCUSSION

The analyses by the Lowry method of certain correlated raw and cooked beef samples, tabulated in Table 1, indicate two concepts. The Kjeldahl nitrogen content of the autoclave-solubilized fraction and of the terminal residue (in the Lowry method these fractions represent collagen and elastin respectively) formed a very small proportion of the total nitrogen; in the raw samples the additive amount was slightly over 2 per cent. Secondly, the cooked samples contained a larger amount of terminal, residual nitrogen, in every instance, than did the corresponding raw sample.

Prudent (1947) used the Lowry method, with some modifications, in the analysis of beef samples from two animals; the roasts were stored at 34° to 36°F. for varying lengths of time. With the Lowry method of assessment she was not able to demonstrate any consistent relationship, or effect, of the length of storage on collagen and elastin content. Study of the summary table of Prudent's data showed the average of all collagen nitrogen in the uncooked samples was 3.5 per cent and the uncooked elastin nitrogen was 0.9 per cent of the total nitrogen. In 4 out of 10 muscles the collagen content of the cooked sample was higher than the collagen content of the corresponding uncooked sample. In 2 out of 10 of the cooked

samples the elastin nitrogen showed an increase over the amount in the uncooked sample. If the percentages of collagen and elastin nitrogen are averaged for all the muscles, one point is cogent: the cooked samples showed increases of both collagen and elastin content contrasted with the uncooked samples.

It appears unequivocal that the validity of the Lowry method of assessment must be challenged, for there can be no real increase of collagen or elastin *per se* with cookery.

Griswold and Leffler's (1951) utilization of the Lowry method in analyzing beef round resulted in values not very divergent from those in Table 1 or Prudent's data. For the raw samples their averaged value was 1.05 per cent collagen, and the cooked samples varied in per cent of collagen from 0.33 to 0.93. They did not determine the nitrogen content of the terminal residue. In their hands the Lowry procedure yielded consistent decrements in collagen from the raw samples to cooked ones. This points out one of the characteristics of the Lowry method, namely, the variability in results due to differences in individual manipulation in different laboratories.

Another procedure for effecting release of the intracellular components of the muscle cells was investigated. Haurowitz (1950) in discussing the extraction of proteins stated, "The simplest and best method for the destruction of the cellular

membranes is disintegration by repeated freezing and thawing."

Initial extractions of repeatedly frozen and thawed samples of muscle tissue were carried out by using water followed by 1.0 M KCl solution. Centrifugation was utilized as the separatory mechanism. The data in Table 5 reveal that this method of effecting the release of intracellular components was quite inefficient. It will be noted that the initial water extraction was little affected by the freezing and thawing, but the subsequent KCl extraction was markedly influenced by the freezing process. Furthermore, in the frozen samples the per cent of total nitrogen extracted was disappointingly low; only about 40 per cent was removed with the first two extractions.

In these experiments the collagen nitrogen content of the residues prepared from repeatedly frozen and thawed tissues were measured by three different methods of assessment. It is apparent that estimates of the collagen content of the autoclave-solubilized fractions by the Nesslerization and tannate procedures were significantly higher than those obtained by hydroxyproline estimations. If it can be assumed that only the connective tissues contain hydroxyproline, the data obtained by Nesslerization or by the tannate method are spuriously high.

The tannate procedure invites error and criticism because as a precipitant of gelatin tannic acid is non-specific. If it selectively precipitated gelatin the values obtained by the

tannate method would be expected to parallel the hydroxy-proline estimations.

In view of the ineffectiveness of obtaining the fractions of the nitrogen-bearing components of muscle tissue by centrifugation, other separatory techniques were investigated. The difficulties which attended the fractionation of the proteins by centrifugation dictated the need for avoiding the use of the centrifuge. It was for this reason that the possibility of entrapping connective tissue on glass wool was explored. The data shown in Tables 6 through 19 were obtained on tissue residues prepared by glass wool entrapment.

There is not an adequate base of reference concerning the composition of the stroma proteins of muscle tissue owing to the paucity of data. Weber's estimate (1950) of 20 per cent for stroma proteins may be viewed as maximal, but not necessarily an absolute value. Several questions must be raised about the validity of this estimation. This figure was obtained by subtracting the nitrogen representing the protein extracted by KCl from the total nitrogen content of the tissue. It must also be acknowledged that the order of magnitude of a 20 per cent stromal moiety is disturbing in contrast with the values which accrue following alkaline extractions of muscle tissue such as that employed in the Lowry procedure. The response of muscle tissue to fractionation procedures revealed

several marked differences dependent upon prior treatment.

The data in Tables 7 through 10b appear to indicate that:

1. Rather large amounts of protein measured by Kjeldahl nitrogen were solubilized when the residues were autoclaved immediately after the KCl extractions. Classically, it is assumed that the autoclave-solubilized protein represents the collagen content of muscle.

2. The hydroxyproline determinations on this fraction indicated about one-third of the nitrogen was collagen nitrogen. It could be presumed from this that in much of the earlier work in the literature, where not all of non-collagen protein was removed, there would be considerable doubt about the collagen content reported. This evaluation is based upon the assumption that the hydroxyproline is a valid measure of connective tissue content.

3. When 0.1 N NaOH extraction was made prior to autoclaving the KCl-insoluble residue, the Kjeldahl nitrogen determination and the hydroxyproline estimation indicated similar collagen content.

4. Hydroxyproline determinations on several of the alkaline extractions showed the solubilization of very small amounts of material containing hydroxyproline.

5. The elastins estimated by hydroxyproline determinations were approximately the same in quantity regardless of the extractions made prior to the removal of the collagen fraction.

6. The constancy of the collagen nitrogen resulting from the Kjeldahl nitrogen and hydroxyproline estimations on the autoclave-solubilized fraction was striking. An aggregate average may be used to describe a ratio: Kjeldahl nitrogen/ hydroxyproline nitrogen. The samples which had been extracted with 0.6 M KCl prior to autoclaving yielded a ratio of 3.4. The samples extracted with 0.1 N NaOH before autoclaving showed ratios of 1.2. The difference in these two ratios is attributable to the removal of protein which contained negligible amounts of hydroxyproline by the alkali treatment.

Comparisons of the results of analysis by the Lowry method and of the glass wool procedure were made in an effort to investigate further the nature of the KCl-insoluble residue. The collagen nitrogen contents of the autoclave-solubilized fractions were very similar; the elastin nitrogen contents of the terminal residues also paralleled. The nitrogen content of the residues by the Kjeldahl determinations are closely related to the method of prior treatment.

Throughout these investigations efforts were made to quantitatively account for the total nitrogen content of the muscle tissue. The following discussion attempts to encompass the inherent problems in obtaining quantitative nitrogen data for fractionated muscle tissue.

Channels through which connective tissue proteins escaped

measurement may include: mechanical losses in decantation of extracting solutions, mechanical losses in washings, solubilization in extracting solutions, the weakening of certain fibers by contact with solutions and their subsequent dissolution in washing of the residues.

The amount of strand-like substances mechanically lost appeared quite significant as determined by visual examination of the KCl supernatants. The data in Tables 9, 10a, and 10b do not indicate that this fraction contained appreciable amounts of nitrogen. The amount of nitrogen actually accounted for is completely dependent upon the method of assessment. Whether the material decanted with the KCl supernatants was an important connective tissue component remains unknown. In this connection, nitrogen data per se should be interpreted with caution.

Little data can be found on the specific effects of salt solutions on connective tissue proteins. Neutral salt solutions were employed with two views: solubilizations of the intracellular muscle proteins such as the myosins (Mommaerts, 1950), and less destructive effects on the stroma proteins than alkali would exert. It may be suggested that the use of neutral salts may have effects on the very delicate strands and finer fibers of muscle tissue. Some swelling of the fibers appeared to occur when they were in contact with solutions of KCl. This interfibrillar swelling could have sequential

effects, such as, during subsequent washing of the entrapped residues, the weakened, swollen fibers would be more susceptible to hydration and perhaps would undergo solution during the washing of the residue. The possibility that the water washings contained hydroxyproline was not pursued.

The mean of the percentage of nitrogen not accounted for in samples extracted with KCl before autoclaving was 5.7 per cent of the total nitrogen, whereas an alkaline extraction of the sample resulted in a mean of 3.2 per cent nitrogen of total nitrogen not accounted for.

The values in the tables indicating the nitrogen not accounted for were calculated by difference. The Kjeldahl nitrogen content of each fraction, and the hydroxyproline derived value for elastin nitrogen, were summed. The total of the partitioned values was subtracted from the value for total nitrogen content of the tissue. The remaining fraction is the nitrogen not accounted for. In the autoclave-solubilized fraction there was opportunity for assessment by two methods, the moiety measured by the acceptance of the hydroxyproline values accounted for only a portion of the nitrogen contained by this fraction as determined by the Kjeldahl method. In the case of the terminal residues, conventionally considered elastin, and measured only by hydroxyproline determinations, the Kjeldahl method might have also given higher nitrogen values.

A further endeaver to obtain information concerning the properties of the KCl- and alkali-insoluble protein residues involved the use of enzymatic digestion. Papain has been reported to possess collagenase activity in the pH range of 2.0 to 4.5 (Rosenblum et al., 1953). The results of the treatment of glass wool entrapped residues to papain prior to autoclaving are given in Tables 14a to 17. The nitrogen data for the papain-treated residues are listed in the tables. Opportunity for papain digestion was provided prior to autoclaving. Analysis of the papain digest yielded an unimpressive amount of nitrogen as determined by the Kjeldahl method. The amount of collagen nitrogen in the papain digests as indicated by hydroxyproline estimations was consistently so small that it can be regarded as negligible.

The subsequent solubilization of protein by autoclaving appeared not to have been influenced by previous papain treatment. Repeated trials show values very closely correlated, and also in close agreement with earlier determinations on this fraction. The elastin values determined by analysis of hydroxyproline content were uniform and are in good agreement with the data previously obtained. Thus it may also be stated that the results of these investigations, as indicated by the hydroxyproline data, show that papain does not digest collagen or elastin at acid or alkaline pH's. These findings are not in harmony with those reported by Rosenblum et al. (1953).

Attempts were made to see if changes in mucopolysaccharides could be induced and assessed by hyaluronidase treatment. The data in Tables 18 and 19 suggested that treatment of the residue with papain prior to the use of hyaluronidase was conducive to increased activity of the hyaluronidase. In each instance the reducing sugar measurements exhibited increases when the residue had first been subjected to papain. Increased activity of the hyaluronidase appeared related to some previous effect of the papain, and was not merely the result of the effect of solutions in which the enzyme incubation was carried out. This was substantiated by measurements on samples in flasks 3 and 4, in Table 19, which were held in these solutions but were not subjected to papain digestion.

Kjeldahl nitrogen and hydroxyproline determinations on the papain digests and also on the extractions following hyaluronidase activity resulted in values in agreement with the papain results reported in Tables 14a to 17. Small amounts of protein were solubilized, but they did not contain hydroxyproline. Hence they were not collagen or elastin strands if it can be assumed that these constituents contain appreciable amounts of hydroxyproline. The investigations with hyaluronidase revealed, on the other hand, that not all of the ground substance is removed by KCl, alkali, or by papain treatment. The apparent content of reducing sugar shows that at least some ground substance remains after these treatments. While

some nitrogen was present in the fractions solubilized by papain and by hyaluronidase, the presence of hydroxyproline in these fractions could not be demonstrated. Evidence was obtained that papain affected the ground substance because following papain treatment of residues the values for milligrams of glucose-equivalents per gram of tissue were considerably higher than those observed following hyaluronidase treatment. This suggestion is further substantiated by the fact that the component of the residue released by papain was not collagen or elastin since the presence of hydroxyproline could not be demonstrated in the digests. It further appeared that the effect of hyaluronidase was enhanced by prior papain treatment. The significance of these observations at present is not known except that collagen or elastin fibers *per se* were not affected by these enzyme treatments.

Depolymerization of the mucoproteins by hyaluronidase is believed to be the result of hydrolysis of some of the glucosaminidic bonds. The breaking of a relatively few bonds in the center of, or within, the polysaccharide chain (Rapport *et al.*, 1950) would result in considerable disaggregation, with the liberation of but few reducing groups. The amounts of reducing sugar estimated per gram of tissue, Tables 18 and 19, appear small. Based on their theoretical derivation, they may represent considerable depolymerization in the ground substance.

There is the possibility that the reducing groups may not be attributable to the cleavage of the polysaccharide polymers, but derived from other sources, such as contamination with glycogen. However, since alkali treatment of the sample did not lower the reducing values, it is quite unlikely that this was an involvement.

In areas from which connective tissue can be excised or teased, it has been demonstrated that there are marked variations in the sizes of fibers, in the cohesive forces that maintain the integrity of the fibers, and in the response of the varied fibers to diverse treatments. Reference has been made to the dissolution of certain tendons in extremely dilute acetic acid (Nagoette and Guyon, 1930) and to the solubilizing of certain tendons in very dilute NaOH solutions (Jordon Lloyd, 1938). Orekhovich et al., Russian investigators, extracted skin with a citrate buffer at pH 4.0 (1948). They postulated the viscous extract of solubilized protein was a precursor of collagen on the basis that this material had many properties associated with gelatin. Analogous concepts of diversity of components and of treatment-responses apply equally well to the ground substance, not withstanding the meager amount of work which has been done.

The literature concerning connective tissue is prodigious; however, relatively little is known with definitiveness of intramuscular connective tissue, and even less is known about

its composition. There must be an extensive gradation in fiber sizes relevant to the functional role in the architecture of muscle, with some fibers being extremely fine and delicate; there must also be related variations in the proportions and composition of ground substance, as a reflection of functionalism and location. In light of the demonstrated responses of diverse fibers to chemical treatments it may be expected that alkaline solutions may produce varying effects upon them. The length of time the tissue is in contact with the alkali would be expected to be an important factor. The data in Table 6 substantiate this concept. In the Lowry procedure extracting periods as long as 20 hours at room temperature were employed. The inference may be drawn that only the more gross and compactly organized fibers would be able to withstand the degradative effects of alkaline extractants. Actually (Table 10a) the hydroxyproline values for the protein extracted by alkali from tissue residue failed to confirm that alkali dissolves connective tissue fibers to any appreciable extent.

The data in Tables 9, 10a, and 10b show that there exists an appreciable fraction containing nitrogen which can be identified only as being KCl- or water-insoluble but which can be solubilized by 0.1 N NaOH and by autoclaving. It was further demonstrated that this fraction was not appreciably solubilized by papain treatment regardless of the pH at which

digestion was effected. It was also shown that the fraction which was solubilized by papain treatment did not contain significant amounts of hydroxyproline. The amounts of hydroxyproline in either the alkali or papain solubilized fractions of the residues remaining after KCl extraction and water washing were consistently negligible.

It is noteworthy that, regardless of the prior treatment of residue, similar hydroxyproline values were obtained for the autoclave-solubilized portion of the residue. The nitrogen values as determined by the Kjeldahl method did reflect differences in the earlier treatment of the autoclave-solubilized fraction. The hydroxyproline content for the hydrolysates of the terminal residues were not influenced by the different procedures used in their preparation. The only difference in the response of the terminal residues was that attributable to variations in optical properties (see figures 1 and 2, p. 77 and 78).

If it is accepted that the presence of hydroxyproline specifically identifies collagen and elastin in muscle tissue, procedures which involve the Kjeldahl nitrogen determination for estimating the collagen content of autoclave-solubilized muscle protein are in serious error in the absence of rigorous and prior alkali treatment of the residue.

If it is assumed that 0.6 M KCl extractions quantitatively remove proteins of intracellular derivation then there exists

a fraction which cannot be described as fibrillar connective tissue since it does not contain appreciable amounts of hydroxyproline. This fraction can be further characterized as being labile to autoclaving and somewhat susceptible to papain digestion. It was further demonstrated that in the absence of alkali treatment, the acid hydrolysates of the residues remaining after autoclaving possessed a characteristic color when prepared for the hydroxyproline assay. The maximum absorption of this colored complex was observed to be at 420 millimicrons. Optical density readings for similar preparations obtained by the Lowry method were characteristically consistent with that observed for solutions containing hydroxyproline only.

The KCl extraction removed approximately 80 to 85 per cent of the total nitrogen of the muscle tissue when the glass wool procedure was used. Thus there remained from 15 to 20 per cent of the nitrogen to be accounted for. This residual fraction has been designated by Weber (1950) as stroma protein. On the assumption that connective tissue fibers contain hydroxyproline, of the 15 to 20 per cent moiety approximately 5 to 6 per cent is accounted for as collagen and elastin. The remaining fraction is accounted for almost quantitatively by the nitrogen contained in the alkali-soluble portion of the residue. Thus the only way that one can account for a stroma protein content of 15 or 20 per cent for muscle tissue is to

include the alkali and autoclave labile fraction. In view of the fact that this fraction does not contain hydroxyproline in significant amounts the question whether this fraction is associated with connective tissue per se is conjectural.

It can be suggested, however, that the alkali labile fraction is indeed an integral portion of the ground substance, which is the very matrix of connective tissue. Support for this belief can also be obtained from the observations made by Partridge (1948), who states that ground substance is readily degraded by alkali.

If these postulations can be verified it means that even a sensitive measure of the collagen and elastin content would not serve as a quantitative measure of connective tissue content.

SUMMARY

Extractions of proteins from bovine skeletal tissue using KCl, NaOH at several concentrations, and at different pH's, were studied. Repeated freezing and thawing of beef muscle tissue was used in preliminary attempts to facilitate removal of the intracellular proteins, but was found to be ineffective. The KCl- and NaOH-insoluble proteins were obtained by centrifugation and by entrapment on glass wool. Nitrogen content of the KCl- and NaOH-insoluble residues, before and after autoclaving, and papain digestions were determined by the Nesslerization, tannate precipitation, Kjeldahl, and hydroxyproline procedures. Some residues following KCl, NaOH, and papain treatment were subjected to hyaluronidase digestion.

1. KCl extracting solutions (Weber-Edsall) were found to remove 80 to 85 per cent of the total nitrogen of muscle tissue when glass wool was used in shake flasks to entrap the connective tissues during the extraction period.

2. NaOH at concentrations of 0.1 N caused removal of approximately 90 to 95 per cent of beef muscle protein as determined by the Kjeldahl nitrogen procedure.

3. It was demonstrated that the KCl-insoluble fraction contained a nitrogen-bearing fraction which could not be removed by papain to any appreciable degree, nor could it be

removed by water; but it could be solubilized by autoclaving at 15 pounds pressure for 4 hours, and by extraction with 0.1 N NaOH for 3 hours at 0.5 to 2.0°C. This fraction did not contain significant amounts of hydroxyproline.

4. The collagen and elastin content of selected beef muscle tissue, based upon the results of the hydroxyproline assay, were not influenced by prior KCl or NaOH extraction or by papain digestion of the tissue.

5. Estimates of collagen and elastin content of autoclaved KCl-insoluble muscle tissue based on Kjeldahl nitrogen values are much higher than the collagen or elastin contents determined by the hydroxyproline procedures if 0.1 N NaOH is not used on the residue after the KCl extraction.

6. Most procedures for analyzing muscle tissue for collagen and elastin which have been described in the literature give results which are spuriously high when compared with results obtained by hydroxyproline determinations.

7. The acid hydrolysates of the terminal residues of the KCl-insoluble fractions prepared for hydroxyproline assay exhibited a characteristic color with maximum absorption at 420 millimicrons. Analogous preparations of the terminal residues prepared by the Lowry procedure showed colors and optical density reading which paralleled those of standard solutions containing only hydroxyproline with maximum absorption at 560 millimicrons.

8. Papain did not digest collagen or elastin from the residues, based upon hydroxyproline data. Absence of hydroxyproline in the enzyme digests demonstrated that papain had not solubilized collagen or elastin fibers *per se* at either acid or alkaline pH's.

9. Papain was more effective than hyaluronidase in releasing reducing substances from KCl- and NaOH-insoluble muscle tissue residues. Prior treatment of the residues with papain enhanced the activity of the hyaluronidase on the residues. Some ground substance remained after treatment of the residues with 0.6 M KCl, 0.1 N NaOH, or papain.

10. Results of these investigations indicate quantitative accounting of the nitrogen in bovine skeletal tissue may be effected by acceptance of the following fractions: a KCl-soluble portion, approximating 80 to 85 per cent; a collagen and elastin fibrillar fraction of 5 to 6 per cent; an alkali-labile, autoclave-labile moiety of 8 to 12 per cent. The insignificant amounts of hydroxyproline in the latter preclude its classification as collagen or elastin. It is suggested that this fraction is a component of the ground substance.

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APPENDIX

MODIFICATION OF THE LOWRY ET AL. (1941) PROCEDURE FOR THE DETERMINATION OF COLLAGEN NITROGEN AND ELASTIN NITROGEN

1. Grind muscle tissue through a meat grinder twice. Using crushed dry ice with the ground meat, pass through a pulverizing mill.
2. Weigh out two 35-g. samples and put into chilled blendor jars with trace of water.
3. Blend 10 times for 2-minute periods in Waring blendor with intermittent chilling in an ice bath. After the first 3 periods, wash down, keeping the volume of distilled water low. (An additional washing is usually necessary.)
4. Transfer blended mixture to a 500-ml. volumetric flask and bring to volume with water. Shake to mix thoroughly.

TOTAL NITROGEN

5. Pipette 5 ml. of #4 into a 50-ml. volumetric flask, bring to volume and take 2-ml. duplicates into micro-Kjeldahl flasks and add 2 ml. N-free 36 N sulfuric acid and digest. (Do in triplicate.)
6. Calculation:

$$(\text{ml. } 0.01 \text{ N HCl}) \times (\text{N.F.}) \times \frac{500}{5} \times \frac{100}{35} \times \frac{1}{1000} \times \frac{50}{2} = \frac{\text{grams}}{\%\ \text{Nitrogen per 100 g. fresh tissue}}$$

COLLAGEN

7a. Pipette 80 ml. aliquots of #4 into 100-ml. centrifuge tubes, using 50-ml., 25-ml., and 5-ml. pipettes. (Do in triplicate.)

b. Centrifuge and discard supernatant. (3 min. at 3000 r.p.m.)

8a. Add 70 ml. of water. Bring to 80 ml.

b. Add 4 ml. of 2 N NaOH.

9. Stir vigorously and occasionally for 1 hr. and let stand overnight at room temperature (about 20 hr.).

10. Stir vigorously, centrifuge at 3000 r.p.m. for 5 min. and discard supernatant liquid which may not be entirely clear.

11. Add 70 ml. of 0.1 N NaOH and stir vigorously.

12. Let stand 2 hr. with occasional stirring.

13. Centrifuge 5 min. at 3000 r.p.m. and discard supernatant liquid.

14. Add 40 ml. water and 1 drop of 0.1% phenol red.

15. Adjust to a very faint pink with 0.1 N HCl (pH 7.0) with vigorous stirring and allowing time (1 hr.) for diffusion of alkali from the suspended particles.

16. Centrifuge 5 min. and discard supernatant liquid.

17. Add 40 ml. of a 3:1 mixture of 95% alcohol and anhydrous ether.

18. Let stand 10 min., centrifuge, and discard supernatant.

19. Add 40 ml. anhydrous ether, stir, centrifuge, and discard supernatant liquid.

20. Add 20 ml. distilled water and bend a loose tin foil cap over each tube.
21. Autoclave 6 hr. at 20 lb. pressure.
22. Centrifuge and transfer supernatant liquid to a 50-ml. volumetric flask for collagen determination.
23. Wash remaining tissue with 20 ml. distilled water, centrifuge, and add wash water to #22 and bring to volume with distilled water. (Store in refrigerator in test tubes.)
24. The supernatants from #22 and #23 serve as material for collagen determination by nitrogen method. Use 5-ml aliquots in micro-Kjeldahl flasks.
25. Collagen determined and reported as collagen nitrogen.

Calculation:

$$(\text{ml. } 0.01 \text{ N HCl}) \times (\text{N.F.}) \times \frac{50}{5} \times \frac{100}{35} \times \frac{500}{80} \times \frac{1}{1000} = \frac{\text{grams \% collagen}}{\text{nitrogen}}$$

ELASTIN:

26. Tube #23. Add 40 ml. of 0.1 N NaOH
27. Place centrifuge tubes in boiling water bath for 30 min.
28. Centrifuge and discard supernatant liquid.
29. Wash with 40 ml. water, stir vigorously, centrifuge, and discard supernatant liquid.
30. Reflux for 24 hr. in 3 ml. of 87% formic acid. Use 50-ml. Erlenmeyer flasks inverted over centrifuge tubes as air condensers. (May need to replace formic acid which evaporates.)

31. Transfer digest to micro-Kjeldahl flasks with washings. Use formic acid for the first 2 washings. Last washing is 1 ml. H_2O.

32. Evaporate water from micro-Kjeldahl flasks over open flame to avoid bumping and to shorten digestion period, adding 1 to 2 drops of H_2SO_4 and water to keep volume to 1 to 2 ml. Important to free solution of all formic acid.

33. Elastin determined and reported as elastin nitrogen.

Calculation:

$$(\text{ml. } 0.01 \text{ N HCl}) \times (\text{N.F.}) \times \frac{500}{80} \times \frac{100}{35} \times \frac{1}{1000} = \begin{matrix}\text{grams \%} \\ \text{elastin} \\ \text{nitrogen}\end{matrix}$$