CONNECTIVE TISSUE PROTEINS

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Introduction

All mammals have an internal connective tissue framework based on the extracellular proteins, collagen (1) and elastin (2). This connective tissue framework is employed in a number of different ways; it exists to contain other tissues, e.g. skin and muscle facia; to connect one tissue to another e.g. tendons and ligaments; or to act as a support to other tissues e.g. bone. The role of animal connective tissue is therefore largely but not wholly structural and mechanical (3).

The fibrous proteins of connective tissue collagen and elastin are embedded in an amorphous ground substance of mucopolysaccharides or hydroxy apatite. Collagen fibres are highly ordered, have a high tensile strength and are inelastic. In complete contrast elastin fibres are, as the name suggests, highly elastic. The optimal functioning of the various types of animal connective tissue is dependent on the relative proportion of all these components to each other in the particular tissue.

(i) Ligaments join bone to bone and require that the fibres be highly elastic and therefore contain 60-70% elastin. The elastin fibres are laid down roughly in parallel along the length of the ligament.

(ii) Aorta similarly requires perfect elastic recovery after deformation under load, and therefore contains about 50% elastin. The fibres in this tissue are laid down in concentric lamellae. A certain rigidity is imposed by the presence of about 20% collagen.

(iii) Tendons attach muscle to bone and since they need to have a high tensile strength contain a high proportion of collagen (80-90%). The collagen fibres are aligned in parallel to give maximum strength along their length since during muscle contraction they have to transmit tensile forces with virtually no stretch.

(iv) Skin requires a little more flexibility and contains about 40% collagen and a higher proportion of mucopolysaccharides. In this tissue the fibres are randomly maintained to form a compact feltwork. This random distribution allows flexibility in all directions for animals with appendages such as arms and legs. In contrast, sharks have a laminated skin structure which gives a tough but less flexible skin. In this respect it is interesting to note that tadpole tails possess a similar laminated pattern but during metamorphosis the tail resorbs and the skin of the frog now attains the more flexible typical cross-weave pattern to permit movement of the legs.

(v) Bone has to be strong but also requires high rigidity. The collagen (20%) imparts strength whilst the incorporation of the Ca^{++} salts (70%) confers rigidity.

(vi) Cartilage contains little collagen (5-10%) and consists mainly of mucopolysaccharides stabilized by collagen fibres.

Although collagen and elastin are basic to the functioning of all the organs of the body, our interest in these proteins at the Meat Research Institute is based primarily on their effect on the texture of meat, a very important factor in its eating quality.

The structure of muscle and the distribution of the connective tissue proteins within the muscle must be considered in a study of the role played by the connective tissue proteins in the texture of meat. The collagen fibres of the tendon divide throughout the muscle to form a fine network. Each myofibril is surrounded by a collagenous sheath called the endomysium. Each

bundle of myofibrils is surrounded by another collagenous sheath termed the perimysium and finally the whole muscle is encased in a collagen tissue known as the epimysium (Fig. 1). The myofibrils of actomyosin have a low tensile strength and the contribution of the sarcoplasmic proteins must be negligible, therefore the amount of connective tissue must be an important although not the sole factor in the strength of the muscles and consequently the texture of meat.

Elastin

Elastin occurs in greatest concentration in tissues where there is a need for elastic extension and complete recovery e.g. in ligaments and aorta. A small amount, less than 1% is present in muscles and is mainly confined to the capillaries.

Elastin has a completely amorphous structure showing absolutely no crystallinity on analysis by X-ray diffraction - even when fully stretched. The fibres are extremely rubber-like and it is therefore generally regarded as being composed of randomly coiled polypeptide chains kinetically free for most of their length, but to account for its rubber-like elasticity and insolubility the chains must be linked at intervals by strong covalent bonds (2).

The nature of these bonds was discovered some six or seven years ago by Partridge and Thomas (4). Essentially they digested elastin with a whole series of enzymes and fractionated the peptides obtained. Some peptides were found to be rich in a new amino acid which, when characterized, was found to consist of two isomers termed desmosine and isodesmosine. The desmosines arise biosynthetically from four molecules of lysine. Oxidative deamination of the ϵNH_2 groups of peptide bound lysine occurs extracellularly through the presence of an amine oxidase. The resulting lysine-derived aldehydes condense together with a fourth ϵNH_2 of lysine to give the stable cross-links desmosine and isodesmosine (5) (Fig. 2). A second important cross-link is the aldol condensation product derived from the reaction of two lysine aldehydes (6). Although a precursor of desmosine it exists in the fibre in sufficient quantities to serve as a cross-link in its own right.

If the oxidation of the lysines is inhibited one can obtain the soluble precursor tropoelastin - analogous to tropocollagen. Isolation of this material has been achieved by a group of workers at the University of Utah who extracted tropoelastin from the aorta of pigs kept on a copper deficient diet (7). Copper and pyridoxal are essential cofactors for the amine oxidase. Tropoelastin contains 40 residues of lysine/1000 residues in contrast to insoluble elastin which contains 3-5 lysine/1000 residues indicating a high degree of crosslinking takes place after enzymic oxidation of the peptide bound lysines. The elastin therefore arises from a soluble precursor protein synthesised in the cell. The molecules, probably about 35,000 molecular weight (8), diffuse to the site of fibre formation and there polymerizes to produce a crosslinked three-dimensional network. Desmosines account for four of the lysine residues and the aldol condensation product for about four residues converted during the biosynthesis of the crosslinks, leaving approximately eight unaccounted for.

Since tropoelastin is considered to be globular it is difficult at present to see how we can determine how four lysines are brought into correct register for crosslinking to give desmosine and isodesmosine. Partridge has proposed a model based on globular sub-units containing hydrophobic centres. Little sequence work has been carried out on elastin (8) as yet but its amino acid composition reveals as high a content of the non-polar amino acids, glycine, proline and alanine as collagen. However, in collagen these residues are in definite sequence Gly Pro X resulting in a polyproline triple helix whereas in elastin these residues must be distributed at random so that proline acts as α-helix breaker rather than a poly proline helix initiator. This is supported by the finding that (Gly, Pro, Pro) synthetic polypeptides have a helix whereas (Gly Pro) polypeptides in which the Gly and Pro are randomly distributed do not contain a helix. The actual sequence should be confirmed in the near future now that tropoelastin can be obtained from copper deficient porcine aorta.

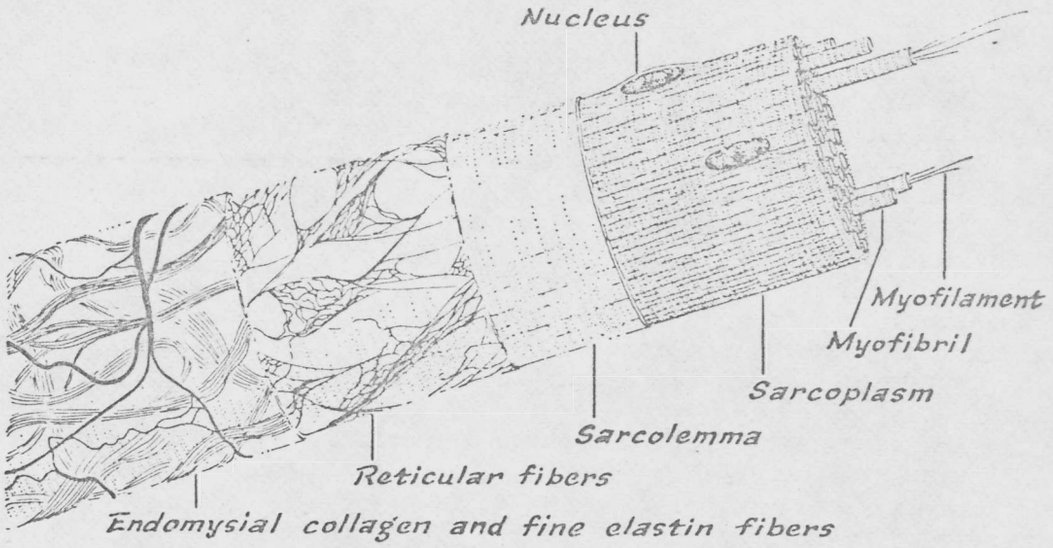

Fig.1. Diagrammatic representation of the structural components of muscle depicting the distribution of the structural components.

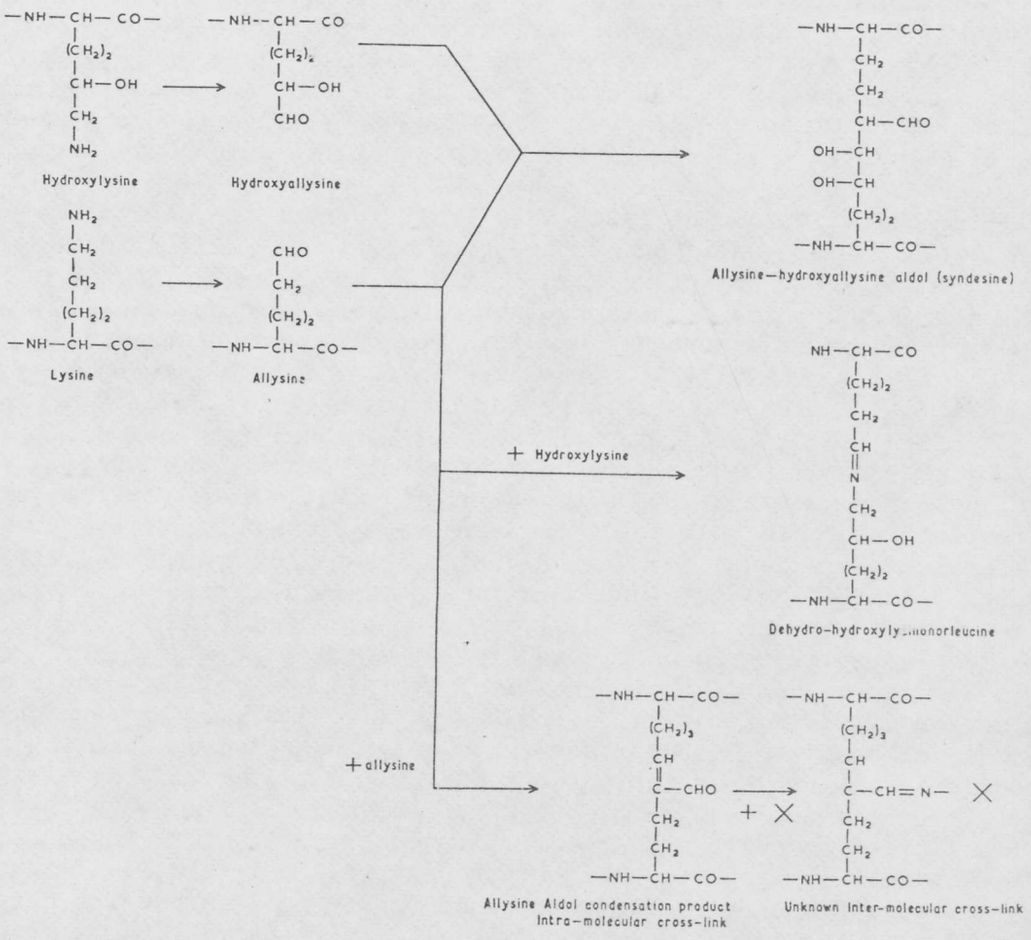

Fig.2. Biosynethetic route of the cross-links isolated from elastin fibres.

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Collagen

Numerous studies over the past few years have centred around the problem of the means by which the collagen fibres are stabilized rendering them almost completely inextensible (1,9). Before discussing the chemistry of these crosslinks I should like to briefly review the current concepts on the structure of the collagen fibre and molecule.

High resolution electron microscopy of the individual fibrils reveal the well known axial periodicity of collagen. Within each fibril one can discern that the molecules are aligned in parallel and to account for the difference in the length of molecules (3800 Å) and the axial periodicity of 700 Å Gross and his colleagues (10) proposed that the molecules were aligned in a quarter-stagger packing, rather like building bricks.

The molecules are long and thin, 2800 x 15 Å, and although a certain entanglement could occur it is difficult to account for the high tensile strength of the fibre and theoretically the molecules should be capable of slipping past each other under load. It is now generally accepted that the increase in strength is achieved by the introduction of covalent crosslinks between the molecules making up the fibre (Fig. 3). The absence of cysteine from collagen precludes the involvement of the usual covalent crosslink present in other proteins, and it is the chemistry of the crosslinks unique to collagen and elastin that have occupied our interest for the past few years.

Although the crosslinks between the molecules account for the strength and insolubility of collagen, we will firstly consider the structure of the tropocollagen molecule itself as this has an important bearing on the final mode of crosslinking.

A small proportion of collagen may be extracted by dilute acetic acid and this has been shown to exist in solution as tropocollagen monomers. Each molecule is made up of three polypeptide chains, each in a polyproline helix and all three chains wound round together into a super helix.

On heating to 40° the triple helix collapses and analysis on the Ultracentrifuge reveals two or three peaks of different molecular weight instead of a single species. Determination of the molecular weight revealed that they were single α chains, double α chains (β), or triple α chains (γ) indicating covalent crosslinking within the molecule. Further analysis revealed that the crosslinking could be α_1-α_1 (β_{11}) or α_1-α_2 (β_{12}) (11). The nature and biosynthesis of these crosslinks was elucidated by Piez and his colleagues (12). Analysis of the N-terminal peptides revealed that the crosslink was located in this region and they proposed that it was an aldol condensation product derived from the condensation of two lysine-derived aldehydes. The ϵNH_2 of the two peptide bound lysines undergo enzymic oxidative-deamination to aldehydes as the first stage in the biosynthesis of the crosslink.

In view of the ease of obtaining highly purified soluble collagen the emphasis of the crosslinking studies centred around tropocollagen. However, it must be emphasized that this crosslink exists within the molecule itself and therefore is unlikely to contribute significantly to the stability of the fibril. It is the intermolecular bonds that are the most important from a structural point of view. Unfortunately, as soon as the intermolecular crosslinks form, the collagen rapidly becomes insoluble making the analyses of these crosslinks extremely difficult.

Confirmation of the existence of intermolecular crosslinks was obtained by Veis and his co-workers (13). They extracted insoluble collagen with denaturing agents such as guanidine hydrochloride and separated the components on carboxymethyl cellulose columns. The identification of components such as β_{22} and γ_{222} could only have arisen by intermolecular crosslinking and this finding was further supported by the identification of § components i.e. polymers of molecular weight higher than γ component.

The importance of these intermolecular crosslinks for the stabilization of the fibre can be demonstrated very dramatically in experimental lathyrism. This condition can be induced by the injection of β-amino propionitrile which results in extremely fragile connective tissue (14). When injected in fertile eggs the resultant chicks, although completely formed, have no strength and on

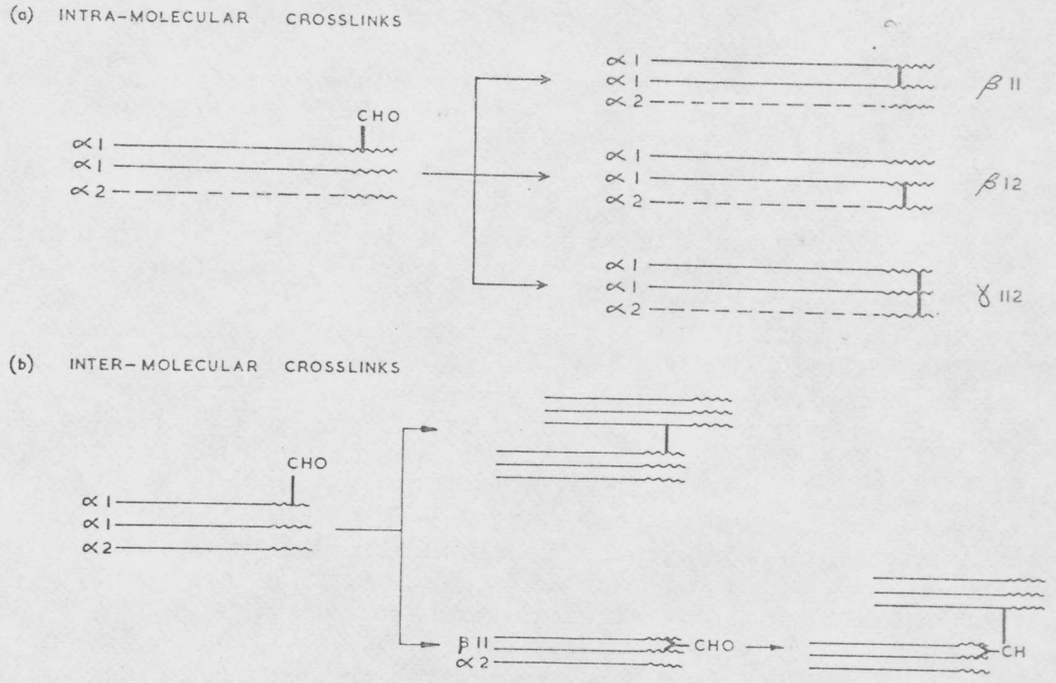

Fig.3. Diagrammatic representation of the possible location of a) the intra-molecular cross-links, and b) the inter-molecular cross-links.

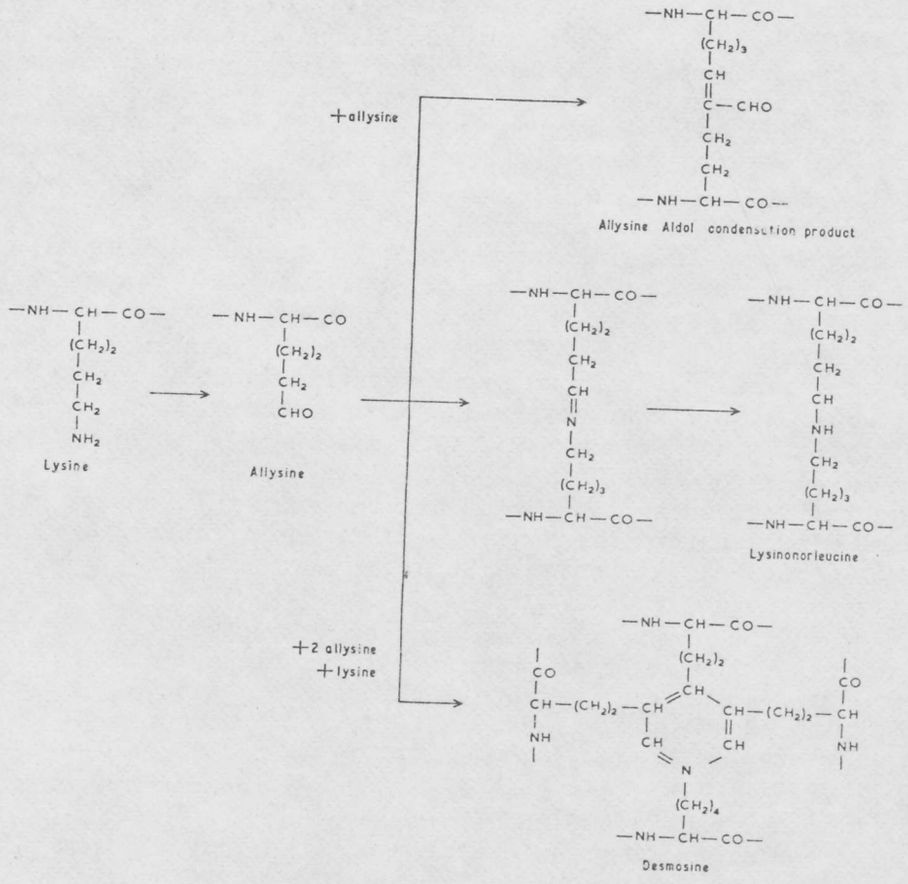

Fig.4. Biosynthesis of the intra- and inter-molecular cross links currently believed to exist in native collagen fibres. These components were isolated and characterised as the stable reduced forms after reduction of the collagen with tritiated sodium borohydride.

opening the egg the weight of the chick is sometimes sufficient to separate the body from the head. The fragility of the collagen suggests that the formation of the intermolecular crosslinks is inhibited. Examination of the collagen reveals that it is extremely soluble, and on denaturation no cross-links are detectable, i.e. the solution contains tropocollagen consisting only of complete α-chains indicating inhibition of the formation of both intra and intermolecular crosslinks. βAPN has now been shown to function by inhibiting the enzymic oxidative deamination of the ϵNH_2 of the lysine residue, thus preventing the formation of crosslinks (11).

The inhibition of both intra and intermolecular crosslinks in lathyrism indicated that both types of crosslinks are mediated through the same type of mechanism. This suggested a means of tackling the problem of intermolecular crosslinks. It was decided to stabilize the lysine-aldehyde by mild reduction and in view of the involvement of so few residues tritiated potassium borohydride was employed (15). The aldehyde precursors, and any reducible crosslinks resulting from the reaction of these aldehydes, would be stabilized by reduction thus permitting their identification after acid hydrolysis and amino acid analysis. A number of radioactive peaks were observed in positions remote from the standard amino acids indicating new amino acids. Some of these components have been isolated and identified by mass spectrometry, their structure confirming their suggested role as crosslinks (Fig. 4).

In the soft tissue collagens, e.g. rat skin and rat tail tendon the major reducible crosslinks are labile aldimine bonds one of which has been identified as dehydro-hydroxylysinonorleucine. A second more complex Schiff base crosslink is present in these tissues but its structure has not yet been elucidated. Partial characterization indicates that it is derived from the further condensation of the aldehyde function of the intra molecular aldol crosslink to a reactive group on an adjacent molecule. The ease with which these bonds are ruptured by dilute acids readily accounts for the high solubility of these tissues.

The less soluble tissues such as tendon contain an additional crosslink which we have designated syndesine (16). The bond is relatively more stable which suggests it may be related to the solubility of the tissue. This is indirectly supported by the finding that with insoluble bone and cartilage collagen syndesine is the only major reducible crosslink.

The location of these crosslinks within the collagen fibre has not yet been elucidated. However, one end of the crosslink must involve the lysine residue almost at the NH_2-terminal end of the tropocollagen molecule. This region is non-helical and thus permits enzymic oxidation of the ϵNH_2 group of the lysine or hydroxylysine residues in this region to the corresponding aldehyde. The non-helical region is also susceptible to proteolytic enzyme and can readily be cleaved from the triple helical body of the molecule as a peptide containing the intra molecular crosslink or a single peptide containing the lysine-aldehyde precursor. The location of the second residue is currently under investigation, but if the quarter-stagger alignment of the molecules in the fibre is assumed to be correct the second residue must reside along the triple helical body of the molecule.

Collagen rapidly becomes less soluble with increase in age of the tissue. Reduction of the tissue revealed that the proportion of reducible crosslinks decreased with increase in age suggesting that they must have stabilized themselves as a non-reducible form. The mode of stabilization is at present being studied.

To summarize then, we have proposed that in collagen the fibre is stabilized by a system of intermolecular crosslinks based on reactions of the lysine and hydroxylysine derived aldehydes. Two types of bonds have been shown to be present, labile aldimine bonds and stable aldols. The greater the proportion of hydroxylysine present in the non-helical terminal region converted to aldehyde the greater the proportion of the syndesine crosslink and the less soluble the collagen. The extent of hydroxylation of the telopeptide lysine may well be a controlling factor in the type of crosslink required in a particular tissue. With increase in age these reducible crosslinks appear to be stabilized resulting in a less soluble collagen. Comparison of tissues reveals that skin contains mainly the labile aldimine bonds, whilst tendons contain the additional syndesine bond, and bone and cartilage collagen contain predominantly the syndesine crosslink.

On consideration of these findings with respect to the quality control of the texture of meat we are probably at a stage where more questions can be posed than answered. However, many experimental facts can now be seen on a more rational basis. It is obvious from these studies that the quality of the collagen i.e. the type of crosslink, is more important than the quantity. Thus estimations of the quantity of collagen in various muscles can be misleading if applied to the assessment of texture.

A second consideration is the finding that there are qualitative and quantitative differences in the nature of the crosslinks between tissues of the same animal. We are now pursuing this question in relation to changes in the intramuscular connective tissue (i.e. the endo and perimysium) between various muscles. The more active leg muscles are known to contain more collagen (17) but may also contain a greater proportion of stable crosslinks than the less active muscles such as the psoas major. The coarseness of the musculature may be important, e.g. Aberdeen Angus has a fine grained muscle with a thin perimysium whilst the high growth rate animals often have a coarse grained muscle with a thick perimysium. Even with a single animal the grain varies i.e. the semimembranosus is coarse whilst the semitendinosus has a fine structure. A further consideration must be that of age of the tissue e.g. veal contains a higher proportion of collagen than older beef yet the crosslinks are thermally labile resulting in a readily soluble collagen which is exuded from the meat and often leads to a dry friable texture in the meat. Older collagen contains stable crosslinks and on thermal denaturation is not lost from the meat, and if too highly crosslinked contracts further on heating causing an excessive shrinkage of the meat on cooking, and results in tough meat. In addition, it is possible that the rate of ageing may be different in different muscles.

It is clear therefore that collagen is not something one does not want in meat but is an essential component of fresh meat in providing an acceptable texture. However, unlike most other muscle components collagen varies in its properties, primarily the extent of crosslinking, both with age and type of tissue.

Control of the rate of crosslinking could lead to meat with the optimum number of crosslinks. This may not be possible chemically but it may be possible to control the rate of growth, or, during 'finishing' of the animal it may put on new weakly crosslinked collagen (and probably fat) thus diluting out the effect of the collagen already present. Alternatively, once the nature of the crosslinks is known it may be possible to rupture these bonds specifically prior to cooking. A less selective means might be to cleave the non-helical crosslinking region from the molecule with specific proteolytic enzymes. The present processes utliizing proteolytic enzymes only act on the thermally denatured collagen and the attack is therefore non-selective and generally proceeds too far.

The above considerations apply to the role of collagen in meat texture, and other things being equal it must be a major contributor. It is unlikely that elastin makes a significant contribution to the texture of meat since it is present in extremely small amount, and is generally confined to the capillaries. However, post-slaughter conditions can also affect texture e.g. by contraction of the muscle fibres known as cold shortening (18) and these factors must be taken into account in any overall assessment of meat tenderness.

Collagen and elastin are two unique proteins perfectly tailored to match the needs of their particular function. However, it is clear that their relationship to the quality of meat requires an understanding of the biosynthesis, fibrogenesis and crosslinking of collagen and elastin at a fundamental level.

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