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Review

Mechanisms of maturation and ageing of collagen

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Abstract

The deleterious age-related changes in collagen that manifest in the stiffening of the joints, the vascular system and the renal and retinal capillaries are primarily due to the intermolecular cross-linking of the collagen molecules within the tissues. The formation of cross-links was elegantly demonstrated by Verzar over 40 years ago but the nature and mechanisms are only now being unravelled. Cross-linking involves two different mechanisms, one a precise enzymically controlled cross-linking during development and maturation and the other an adventitious non-enzymic mechanism following maturation of the tissue. It is this additional non-enzymic cross-linking, known as glycation, involving reaction with glucose and subsequent oxidation products of the complex, that is the major cause of dysfunction of collagenous tissues in old age. The process is accelerated in diabetic subjects due to the higher levels of glucose. The effect of glycation on cell-matrix interactions is now being studied and may be shown to be an equally important aspect of ageing of collagen. An understanding of these mechanisms is now leading to the development of inhibitors of glycation and compounds capable of cleaving the cross-links, thus alleviating the devastating effects of ageing.

Keywords: Collagen; Cross-links; Ageing; Glycation; Cell-matrix

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1. Introduction

The overall shape and function, in terms of flexibility and locomotion, of the human skeletal system depend on a basic framework of collagen fibres (Alexander, 1981). The collagen fibres are essentially inextensible and therefore provide mechanical strength and through that strength confer and maintain form whilst allowing flexibility between various organs of the body. Thus, the randomly orientated fibres of the skin permit considerable extension of the tissue until the fibres themselves are loaded. The fibres of tendons are aligned in parallel and therefore loaded instantly, permitting maximum transfer of the energy of muscle contraction to the skeleton. The fibres of bone are organised in concentric layers to maximise for torsional and compressive stresses and the rigidity is conferred on the bone by mineralisation. The surface of cartilage on the bone extremities allows efficient movement of the skeleton through lubricating joints following muscular contraction, and stability is provided by a small proportion of fine collagen fibres in a mainly polysaccharide gel. The cornea provides an example of well ordered fibres in precise layers at a defined angle to each other thereby allowing the transmission of light. In contrast to these fibrous structures the network structure of basement membranes provides a filtration system and an attachment site for cells, for example the kidney glomeruli and arterial basement membranes. This biological diversity of function of collagenous tissues is primarily due to the fact that these fibres are biopolymers of one of several genetically distinct collagens, which are to some extent tissue specific as will be discussed later.

During ageing, changes occur in the collagenous framework. These changes in the physical properties of the fibres are reflected in the well-documented increases in stiffness of skin, tendon, bone and joints in old age. The major changes are an increase in rigidity of the tissue, the fibres ultimately becoming brittle (Torp et al., 1975; Viidik, 1982; Uitto, 1986). Such changes are clearly deleterious to the optimal functioning of the locomotive system, the elastic vascular system and the filtration properties of the basement membranes. Recent studies have emphasised the importance of cell-matrix interactions, particularly during development, and preliminary studies indicate that a reduction in the efficacy of cell-collagen interactions also occurs during ageing, the effect being particularly important in the case of basement membranes (see later).

In order to understand the mechanism of these age-related changes it is essential to understand the role of collagen in determining the mechanical properties of the tissue; the changes in the cross-linking as a major cause of the observed change in mechanical properties; the role of the collagen types comprising the fibrous framework, and changes in the activity and collagen type expression of the cells associated with a particular tissue, occurring with senescence.

1.1. Age-related changes in mechanical properties

Several structural features of collagen bestow particular tissues with mechanical properties appropriate to their widely varying functions. The total collagen content

of the tissue is obviously an important determinant of mechanical strength. The ability of the tissue to sustain an applied load is also determined by the orientation of the fibres which can vary markedly between tissues, e.g. unidirectional in tendon, laminated in cornea and random in skin. The collagen fibrils possess little strength in flexion or torsion but exhibit a very high tensile strength. The tensile strength increases considerably with age. In tendon the toe region of the stress-strain curve is shortened as the fibre packing is tighter and at the same time the stiffness of the fibre increases. Little change occurs in the linear modulus during maturation but in old age there is an increase in failure stress accompanied by a decrease in failure strain (Fig. 1), indicating increased cohesion between the microfibrils thus preventing slippage (Torp et al., 1975).

The diameter of the collagen fibres also plays a significant role in determining the mechanical properties of the tissue. For example, the plastic deformation or 'creep' of tissues is directly related to the proportion of small diameter fibrils (Parry, 1988). Conversely, the ability to withstand high stress levels is related to the proportion of large diameter fibrils. As the diameter increases the flexibility of the tissue decreases and there is a decrease in the ability to resist crack propagation. The variation in diameter of collagen fibres between tissues is illustrated by tendon (200 nm), skin (approx. 100 nm), cartilage (approx. 50 nm), and cornea (20 nm) and this can be related to their relative mechanical properties. In some tissues there is a bimodal distribution of fibre diameter, the voids between the large fibres being filled by small fibres, thus allowing a high collagen content but maintaining a flexibility of the tissue (Parry et al., 1978). With maturation, fibril diameters increase and may be either unimodular or bimodally distributed, whilst with senescence, diameters may decrease and tend towards bimodality in many tissues (Parry et al., 1978; Jones, 1991).

An additional structural feature of the collagen fibre is its possession of a crimp structure, a periodic light and dark banding structure of 100 μm with an angle of 5–25° seen under polarised light, which results from the planar zigzag wave along the fibre path. This crimp is believed to act as a shock absorber system, and is

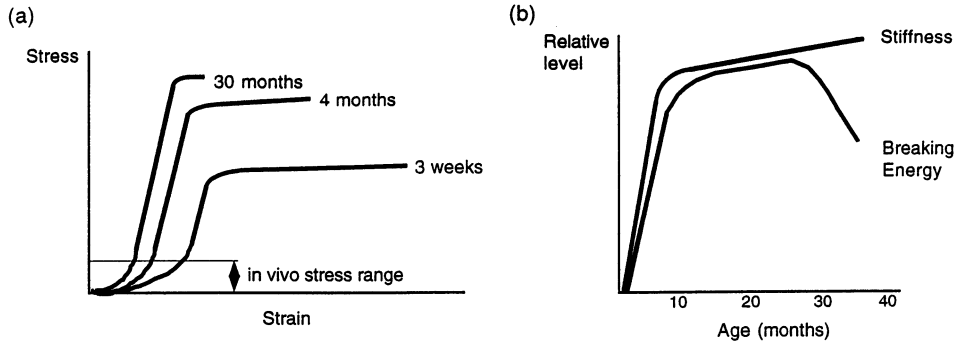


Fig. 1. (a) Typical stress-strain curves for ageing rat tail tendon. (b) Age-related biomechanical changes in rat tail tendon: note decreased energy to break as stiffness (brittleness) increases in old age.

represented within the toe-region of the stress–strain curve, that is, within physiological levels of stress (Fig. 1a). With increasing age the angle of the planar waveform increases thus decreasing the shock absorbing effect (Gathercole and Keller, 1975).

The effect of physical training is of considerable interest to the understanding of the control mechanisms. Collagenous tissues appear to have a general systemic response to exercise, skin, tendon, and bone readily showing increases in weight and strength (Viidik, 1986). It has been suggested that load resistance training is more effective than endurance training in the development of connective tissue (Stone, 1988). Under normal conditions the flexor tendons are stronger than the extensor tendons, but following training the extensor tendons can approach the strength of flexor tendons (Woo et al., 1982). The mechanisms involved are as yet unknown but could be a direct effect on the collagen synthesising cells as evidenced by elevated levels of hydroxylases, by initiation of a growth promoter, and by a change in the blood hormone levels, or a change in the number and activity of the hormone receptors. On the other hand extremes have a different effect, excessive tension on fibres leads to degradation due to the stimulation of MMPs (Bailey et al., 1994).

1.2. The collagen superfamily

The collagens exist as thick striated fibres, as non-fibrous networks in basement membranes, as non-striated filamentous structures, or fibril associated molecules. These morphologically different collagen structures are aggregates of one of more of over a dozen different collagen molecules. These molecules are, in fact, a family of closely related but genetically distinct proteins, possessing a basic structure of three polypeptide chains each with a Gly-X-Y repeat forming tightly bound triple helices which subsequently aggregate to form various types of supporting structures (for reviews see Kielty et al., 1993; Comper, 1996). Before further discussion of the mechanisms of ageing of collagen, it would be advantageous at this stage to review the nature of these different collagens (Fig. 2). At the present time there are 19 genetically distinct collagens, but the function of many of the minor collagens has yet to be elucidated.

1.3. Genetically distinct collagen types

The majority of the collagens can be classified according to the nature of their aggregated forms.

1.3.1. The fibrous collagens

These collagens are long (300 nm) rod-like molecules which self-assemble in a parallel, quarter-staggered end over-lap arrangement (Fig. 2a) to form fibres possessing a characteristic band pattern, with a periodicity of 67 nm identifiable in the electron microscope. The collagen types in this group are Types I, II, III, and the minor collagens V and XI.

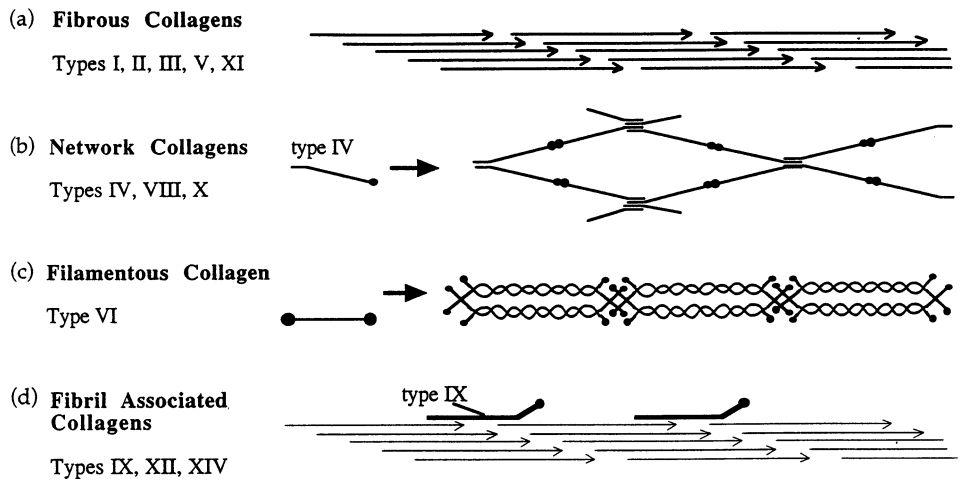


Fig. 2. Diagrammatic representation of the aggregated forms of the collagen superfamily of proteins.

1.3.2. The network collagens

The type IV molecules are very long (400 nm) and flexible due to irregularities in the Gly-X-Y sequence. They form a 'chicken-wire' network which acts as the basic framework of basement membranes of vertebrates and invertebrates (Fig. 2b). Four molecules are associated in an antiparallel fashion through the amino termini to form a 110 nm overlap known as the 7S region, whilst the carboxy termini, which are comprised of a large non-triple helical peptide (NC1), interact with the NC1 region of an adjacent molecule to build up the 'network'.

Types VIII and X collagen also form networks, and are often classed as 'short-chain' collagens. Both have been reported to form hexagonal lattices in Descemet's membrane (Benya and Padilla, 1986) and growth plate cartilage respectively, although their supramolecular structures in other tissues have not yet been elucidated.

1.3.3. Filamentous collagen

Type VI is observed as a loosely packed filamentous structure with an axial repeat of 100 nm and is formed by end-to-end alignment of tetramers (Bruns et al., 1986), Fig. 2c. These fibres occur in many tissues and it has been suggested that it may separate and align larger type I fibres (Bonaldo et al., 1990). Lateral aggregation of the tetramers has also been observed in both normal and diseased tissues.

1.3.4. The fibril associated collagens

Several collagens do not form homotypic fibres or networks but are associated with other fibre forming collagens, for example, type IX collagen decorates the surface of the type II collagen fibre (Vaughan et al., 1988) (Fig. 2d). Type IX, which consists of three collagenous domains separated by non-triple helical regions, aligns on the type II collagen fibre in an anti-parallel manner (Wu et al., 1992). Types XII

and XIV are generally associated with the surface of type I collagen fibres (Sugrue et al., 1989; Van der Rest and Dublet, 1996), although both have also been localised in foetal cartilage (Watt et al., 1992). One of the three domains of type IX extends into the extracellular space and we have shown it to be more thermally stable (Miles et al., 1998).

Not all collagens fall easily into these groups. Type VII for example forms microfibres which underlie some basement membranes acting as short anchoring fibrils between the membrane and the underlying matrix. These short fibrils are formed by lateral aggregation of anti-parallel overlapped (60 nm) dimers. Many of the remaining collagen types are known only by their DNA sequence, and as such their macromolecular structures are unknown (Comper, 1996).

1.4. Variation of collagen type in different tissues

The biological diversity of function of collagenous tissue is primarily due to the variety of aggregated forms derived from the genetically distinct collagens listed above, which are to some extent tissue specific. Bone and tendon are predominantly fibrous type I collagen, the vascular system contains both types I and III, whilst cartilage contains predominantly type II collagen. The thin basement membranes are primarily type IV collagen. Most collagenous tissues also contain other, 'minor' collagens, and in some cases the individual fibres themselves may contain small proportions of another collagen type.

At the present time there is little information on the role of these additional collagens in modifying the physical properties of the fibre. For example, the presence of type III fibres is believed to confer the greater elasticity to embryonic and vascular tissues primarily because of their small diameter, but its effect when co-polymerised with type I is unknown. On the other hand type XI in cartilage and type V in many other tissues, may be involved in the nucleation of fibrous type II and type I respectively, whilst type IX, which occurs on the surface of type II, may play a role in determining the diameter of the fibril (Wotton et al., 1988). Types X and VIII collagens, which are structurally very similar have apparently widely differing functions. Type X is restricted to the calcifying edge of the growth plate and may form both hexagonal structures and type II associated fibrous mats (Reginato and Jimenez, 1991), whilst type VIII is expressed in a wide range of tissues in addition to Descemet's membrane (Benya and Padilla, 1986), in which its macromolecular structure is not yet clear.

1.5. Variation in collagen type with age

Changes in the collagenous matrix are readily observed during development (Reichenberger and Olsen, 1996) and are often reported as age changes, mainly because the differences in old age are harder to identify. However, it is now becoming clear that there are significant quantitative and qualitative changes in the collagenous tissues in old age. The composition of tissues may vary and involve alteration in the proportion of different collagens in a particular tissue. Few studies

however have been carried out on compositional changes during ageing, although some have been studied during development. For example, embryonic dermis contains about 50% type III collagen, but this reduces to about 15% during post-natal growth (Epstein, 1971). However, one study has indicated that in the last few decades of life there is an increase in the proportion of type III collagen present in the dermis (Lovell et al., 1987). This effect could be due to a loss or reduced synthesis of type I collagen or an increase in type III collagen due to a change in the phenotypic expression of the fibroblasts in old tissue. Either way it almost certainly affects the functional properties of the skin. Similarly, the osteoblasts of bone or the chondroblasts of cartilage could alter their phenotypic expression in old age due to a changing environment. However, we have recently shown that in the case of human iliac bone no detectable changes in collagen type could be detected (Bailey et al., 1998). On the other hand we have recently observed changes in the collagen type in the subchondral bone of elderly osteoarthritic subjects (unpublished data). The effect of such changes in the collagen type on the functional properties of the fibre, primarily mechanical, has yet to be established.

The rate of collagen metabolism also varies substantially with maturation and senescence, although the overall turnover rate is comparatively slow. Collagen synthesis decreases steadily with maturation, and with subsequent ageing drops 10-fold in the majority of tissues. Collagen degradation, on the other hand, has been reported in rats to increase with maturation, and as a result the majority of newly synthesised collagen in old rats appears to be destined for degradation (Mays et al., 1991).

The functional properties of the tissue also depend on the primary structure of the collagen molecules themselves, and despite the relatively low rate of collagen turnover, changes can be significant in terms of decades of life as the matrix is slowly being renewed. In old age it is therefore possible that the composition of the fibres can be altered. Indeed, the extent of hydroxylation and glycosylation has been reported to decrease with maturation (Barnes et al., 1974; Royce and Barnes, 1985). Based on studies of the effect of increased lysyl hydroxylation, for example, in osteogenesis imperfecta type II, the fibre diameter is reduced with a consequent decrease in mechanical strength (Byers, 1993). These modifications reflect changes in the phenotypic expression of the fibroblasts, osteoblasts and chondroblasts of the various tissues with increasing age. At the same time modifications of the amino acid side-chains, whether pre- or post-translational could affect the cell/matrix interactions, which would further exacerbate the decline in functional properties of these collagenous tissues. However, little is known of the effects of these changes and they certainly warrant further research.

Isomerization and racemisation of aspartyl residues in proteins (Clarke, 1987) have been shown to increase with age in long lived proteins such as dentine, tooth enamel, and lens protein (Helfman and Bada, 1975; Masters et al., 1977). Recently, type I collagen has been shown to undergo β-isomerization of Asp-Gly (Fig. 3) within the C-telopeptide (Fledelius et al., 1997). This modification was demonstrated in bone tissue by direct bone analysis and indirectly in urine and the extent of isomerization was shown to increase with age. The identification of the β-Asp in

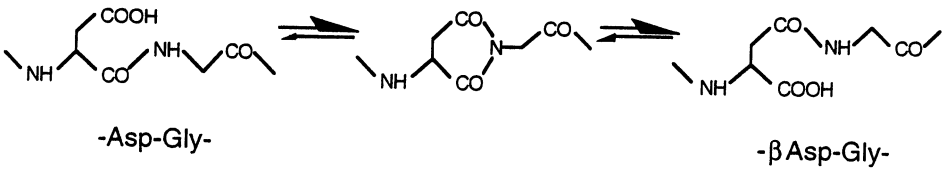


Fig. 3. β-Isomerization of the -Asp-Gly- sequence in the C-telopeptide of type I collagen.

urine as a monitor of turnover would be limited since if turnover increased, for example in Paget's disease, the amount of bone collagen would be underestimated as there would be an excess of newly synthesised collagen that had not undergone isomerization. To be an effective method for ageing or turnover, a baseline level of the rate of racemisation with age under the conditions pertaining in vivo needs to be established.

2. Enzymic intermolecular cross-linking

The most dramatic changes in collagenous tissues with age are in the physical properties and few of the changes discussed so far directly influence the strength of the tissue. Such changes in mechanical properties must involve polymerisation of the collagen molecules in the fibre through inter-molecular cross-linking.

The cross-linking theory of ageing has a long history. Bjorksten (1962) proposed an ageing theory based on the loss of function of all proteins by excessive cross-linking through the reaction with aldehyde metabolites. Verzar (1957) was the first to carry out a detailed study on the ageing of collagen, and using the increasing tension generated on heat shrinkage as a function of ageing clearly demonstrated the presence of cross-links. The most dramatic changes occur between birth and maturity but Rasmussen et al. (1964) reported an additional increase in isometric tension with senescence. The work of Verzar over several years (Verzar, 1964) opened up work on the role of cross-linking in the ageing of collagen although the nature of the cross-links was unknown. For many years suggestions ranged from exogenous aldehydes, through hexoses to ester bonds.

In fact, we now know that the change in mechanical properties with age can be related to two distinct mechanisms of cross-linking of the fibre to form a large polymeric network, one direct and specific and the other an indirect and non-specific method. The initial cross-linking of collagen molecules during development involves a precisely controlled enzymic cross-linking, the divalent products of which mature spontaneously to form stable multivalent cross-links. The reduced metabolic turnover of collagen allows the second indirect cross-linking mechanism to occur through the reaction with glucose and its oxidation products, a process referred to as glycation. The precise mechanisms of these two different processes must be fully understood if one is to monitor the changes involved in ageing and ultimately inhibit them.

2.1. Immature enzymic cross-links

The enzymic cross-linking mechanism in the fibrillar collagens is based upon aldehyde formation from the single telopeptide lysine or hydroxylysine residue. The enzyme which oxidatively deaminates these residues is lysyl oxidase, a copper dependent enzyme which acts only upon telopeptide residues when bound to a highly conserved amino acid sequence (Hyl-Gly-His-Arg) opposite the N- and C-terminals of an adjacent quarter-staggered aligned molecule. The enzyme does not, therefore, act on individual molecules (Siegel, 1979). Oxidative deamination of the ε-amino group of the telopeptide lysine residue occurs by α-proton abstraction and carbanion formation. Inhibition of this enzyme during copper deficiency and lathyrism has long been known to have profound effects on all collagenous tissues leading to extreme fragility due to the subsequent reduction in cross-linking. The activity of lysyl oxidase has been found to decrease with age although the rate depends on the tissue (Sanada et al., 1978; Quaglino et al., 1993). After this initial enzymic step, the subsequent reactions of the aldehydes are spontaneous and governed by post-translational modifications and the structural organisation of the collagen.

2.2. Aldimines

The telopeptide lysine-aldehydes condense with either lysine or hydroxylysine residues in the conserved sequence of the triple helix to form reducible Schiff bases (Fig. 4a). In tissues such as skin and rat tail tendon with a low level of telopeptide lysyl hydroxylation, aldimine cross-links predominate. The first and most common intermolecular lysine-aldehyde cross-link, dehydro-hydroxylysino-norleucine (deH-HLNL), was identified by Bailey and Peach (1968), and involves reaction of the lysyl-aldehyde with a helical hydroxylysine. Dehydro-lysinonorleucine (deH-LNL), is formed following reaction with a helical lysine residue. These aldimines are in equilibrium as Schiff bases which under physiological conditions are effective intermolecular cross-links. In vitro they are readily cleaved at acid pH or by elevated temperature, accounting for the high solubility of collagenous tissues such as young skin in organic acids and hot water. These Schiff bases are readily stabilised for analysis by mild reduction with borohydride to form hydroxylysino-norleucine (HLNL) and lysino-norleucine (LNL), respectively.

2.3. Keto-imines

The second group of immature cross-links is formed when the telopeptide lysine is hydroxylated. The hydroxylysyl-aldehyde derived from this residue reacts with the ε-amino group of a helical hydroxylysine to form the Schiff base which then spontaneously undergoes the Amadori rearrangement to form the cross-link hydroxylysino-5-ketonorleucine (HLKNL). The keto-imine is stable to acid and heat thus accounting for the insolubility of bone and cartilage collagens even at the foetal stage. This compound can be stabilised prior to acid hydrolysis by reduction with borohydride to form dihydroxy-lysinonorleucine, (DHLNL).

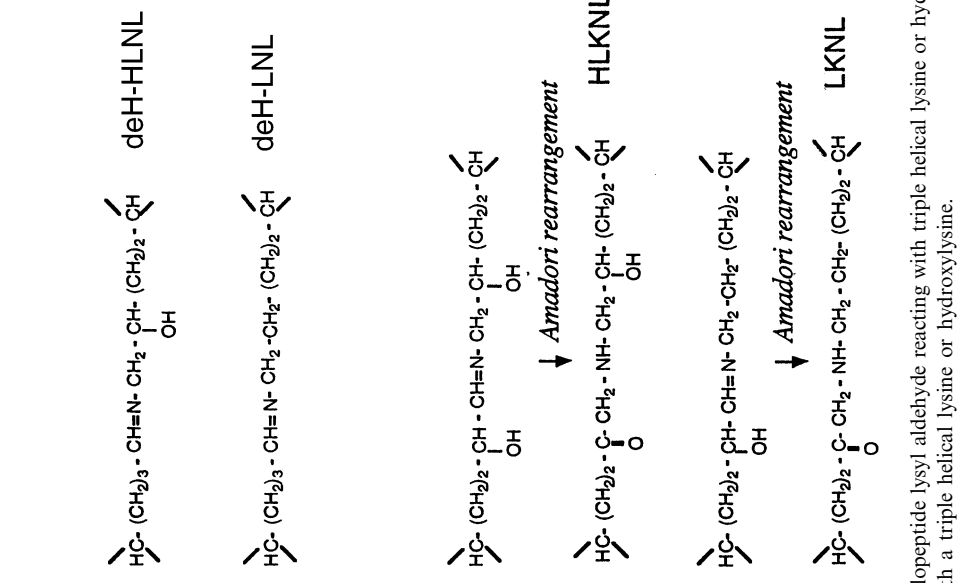


Fig. 4. Formation of the immature cross-links. (a) The aldimines formed from telopeptide lysyl aldehyde reacting with triple helical lysine or hydroxylysine. (b) The keto-imines formed from telopeptide hydroxylysyl aldehyde reacting with a triple helical lysine or hydroxylysine.

In bone and other calcifying tissues, a cross-link may also form between an hydroxylysine aldehyde and a helical lysine (Robins and Bailey, 1975), Fig. 4b. This Schiff base also undergoes an Amadori rearrangement to form the stable lysino-5-ketonorleucine (LKNL) which can be reduced to form lysino-hydroxynorleucine (LHNL). LKNL is rarely considered in the literature, partly because in most other tissues very little is formed, and also because the reduced form is a structural isomer of HLNL (see above) and co-elutes with this reduced aldimine cross-link on analysis (Robins and Bailey, 1975; Richard-Blum and Ville, 1988).

2.4. Aldol-derived cross-link

Analysis of borohydride reduced skin and tendon collagen revealed the presence of a tetravalent cross-link, histidino-hydroxymerodesmosine (HHMD) formed from reaction of the carbon-carbon double bond of the intramolecular aldol condensation product with histidine and its free aldehyde with the ε-amino group of hydroxylysine. These reactions convert the intramolecular aldol, which plays no role in the mechanical properties of the fibre, to a tetravalent intermolecular cross-link which would have a significant effect on the mechanical properties of the fibre. However, whether the non-reduced form of this cross-link, i.e. the aldimine form, exists in vivo or is formed during borohydride reduction is controversial. The cleavage of the aldimine bond by mild acid or penicillamine should reveal the presence of the histidine-aldol product since this reaction product is stable, but it could not be detected suggesting that the histidine only adds across the aldol double bond during the borohydride reaction (Robins and Bailey, 1973). Bernstein and Mechanic (1980) refute this suggestion but surprisingly test for the presence of HHMD by borohydride reduction. Further alternative techniques are clearly required to clarify the in vivo existence of this potential tetravalent cross-link.

2.5. Control of cross-link type by lysine hydroxylation

Although cross-linking occurs extracellularly it is obvious from the above that the type of cross-link clearly depends on the previous intra-cellular post-translational modifications to the collagen molecule, in particular, hydroxylation of lysine residues. The extent of their hydroxylation, both within the triple helical sequences and the terminal amino and carboxy telopeptides, varies greatly between collagen types and tissues. The specific hydroxylation of these lysines governs the nature of the cross-link formed, and as a consequence, the biomechanical properties of the tissue. In particular, the hydroxylation of the telopeptide lysine results in the formation of the keto-imine type cross-link, which is chemically stable. The importance of this post-translational modification of the collagen is exemplified in heritable disorders of collagen, for example Ehlers–Danlos syndrome (EDS) type VI where a hydroxylation deficiency results in collagen fibre fragility. Conversely, over-hydroxylation of collagen, as a consequence of retarded triple helix formation seen in several forms of osteogenesis imperfecta results in altered collagen fibril formation and compromised bone strength (Kirsch et al., 1981). Among the

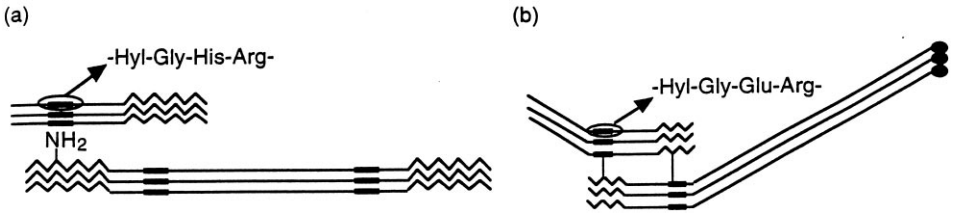


Fig. 5. Placement of collagen molecules and location of cross-links. (a) Head to tail placement and cross-linking through the telopeptide and the hydroxylysine in the conserved sequence in the triple helix of the fibrous collagens. (b) Head to tail antiparallel placement and cross-linking through the *N*-telopeptide and the conserved sequence in the triple helix of type IV collagen of basement membrane.

acquired diseases transient increases in hydroxylation occur in collagen during bone fracture repair (Glimcher et al., 1980) and osteoporosis (Bailey et al., 1992; Batge et al., 1992), and in all cases there is an increase in hydroxylated cross-links and evidence for a decrease in fibre size.

The difference in the primary sequences of the telopeptide and triple helical domains, together with reports that purified lysyl hydroxylase does not hydroxylate telopeptide lysine residues whilst the crude mixture does (Royce and Barnes, 1985), suggest that another lysyl hydroxylase enzyme exists to ensure independent control of helical and telopeptide lysines. This proposal is further supported by studies on the cross-link profile during the mineralisation of bone and tendon (Knott et al., 1995, 1997) in which there is an overall reduction in lysine hydroxylation but an increase in the hydroxylated cross-links. Similarly Gerriets et al., (1993) found that the increased lysine hydroxylation of hypertrophic tendon was confined to the telopeptide lysine. Lysyl hydroxylase has been cloned (Hautala et al., 1992) but a search for a second form of lysyl hydroxylase could prove interesting.

2.6. Head to tail placement of the immature cross-links

The location of the divalent cross-linking residues has been identified for most of the enzymic cross-links in the fibrous collagen type I (Kang, 1972), type II (Miller, 1971), and type III (Nicholls and Bailey, 1980). These chemical studies confirmed the end-overlap quarter-staggered alignment of the molecules in the fibre previously deduced from electron micrographs. There are several cross-linking sites per molecule, one in each of the telopeptides (residues 9N and 16C) and two sites in the helix (residues 87 and 930). These latter sites have the specific amino acid sequence Hyl-Gly-His-Arg-Gly. An aldehyde in each of the telopeptide sites can react with the lysine or hydroxylysine in this sequence of the helix due to the staggering of the molecules by four D-periods (Fig. 5a).

Yamauchi and co-workers (Yamauchi et al., 1989) have suggested that calcifying collagen is unusual in that it lacks cross-links at the amino-terminal and contains the free carboxy-terminal aldehyde due to the dissociation of the immature cross-links (Otsubo et al., 1992). However, others (Robins and Duncan, 1987; Mansell et

al., 1994) have located cross-links at both carboxy- and amino-terminal telopeptides in bone and dentine. The presence of the free carboxy terminal aldehyde appears to be due to cleavage of the cross-link during demineralisation of the bone with EDTA (Knott et al., 1997).

The Hyl-Gly-His-Arg sequence is a conserved feature of the fibrous collagens, however, the sequence appears to be modified by substitution of Glu for His in the type IV network structure (Fig. 5b). The sequence and the cross-links occur primarily in the 7S N-terminal region although there is some evidence of cross-links along the body of the triple helix indicating lateral alignment of the molecules (Bailey et al., 1984). The NC1 domain is linked to adjacent molecules solely by stable disulphide bonds (Reddy et al., 1993).

2.7. Mature enzymic cross-links

The presence of mature cross-links derived from the immature reducible cross-links was first suspected when the levels of reducible cross-links were found by Bailey and colleagues to decline with age in skin despite an increase in strength (Bailey and Shimokomaki, 1971). It was also suggested that these mature cross-links would be more stable and possibly tri-functional, thereby explaining the increase in both insolublity and strength of older collagenous tissues.

2.8. Histidino-hydroxylysinonorleucine

Dehydro-HLNL reacts spontaneously with histidine to form histidino-hydroxylysinonorleucine (HHL; Fig. 6a). The presence of this compound in skin (Yamauchi et al., 1987), and recently cornea (Yamauchi et al., 1996), to the exclusion of most other tissues is due in part to the predominance of lysine-aldehyde in the telopeptide. It has also been suggested that it is due to the steric tilt of the molecules in skin being greater than in skeletal tissues which results in the characteristic banding pattern of collagen in skin having a periodicity of 65 nm compared to the 67 nm in skeletal tissues. This also allows a histidine rather than a lysine residue to react with the cross-linking aldehyde. (Mechanic et al., 1987). No histidino-lysyl-norleucine has been reported indicating that the helical lysine is virtually fully hydroxylated in dermal collagen.

2.9. Pyridinolines

Hydroxylysyl-pyridinoline (HL-Pyr) was first identified by Fujimoto et al. (1978) and is a common mature cross-link found in a wide variety of tissues. This cross-link is composed of two hydroxylysyl-aldehyde residues and a helical hydroxylysine (Fig. 6b), hence its predominance in highly hydroxylated collagens such as type II collagen in cartilage. Lysyl-pyridinoline (L-Pyr) is formed from two hydroxylysines and a helical lysine residue (Ogawa et al., 1982) and is found primarily in calcified tissues (Eyre, 1981). L-Pyr is often incorrectly termed deoxy-pyridinoline, which suggests it is a condensation product, whereas in fact it is derived from lysine

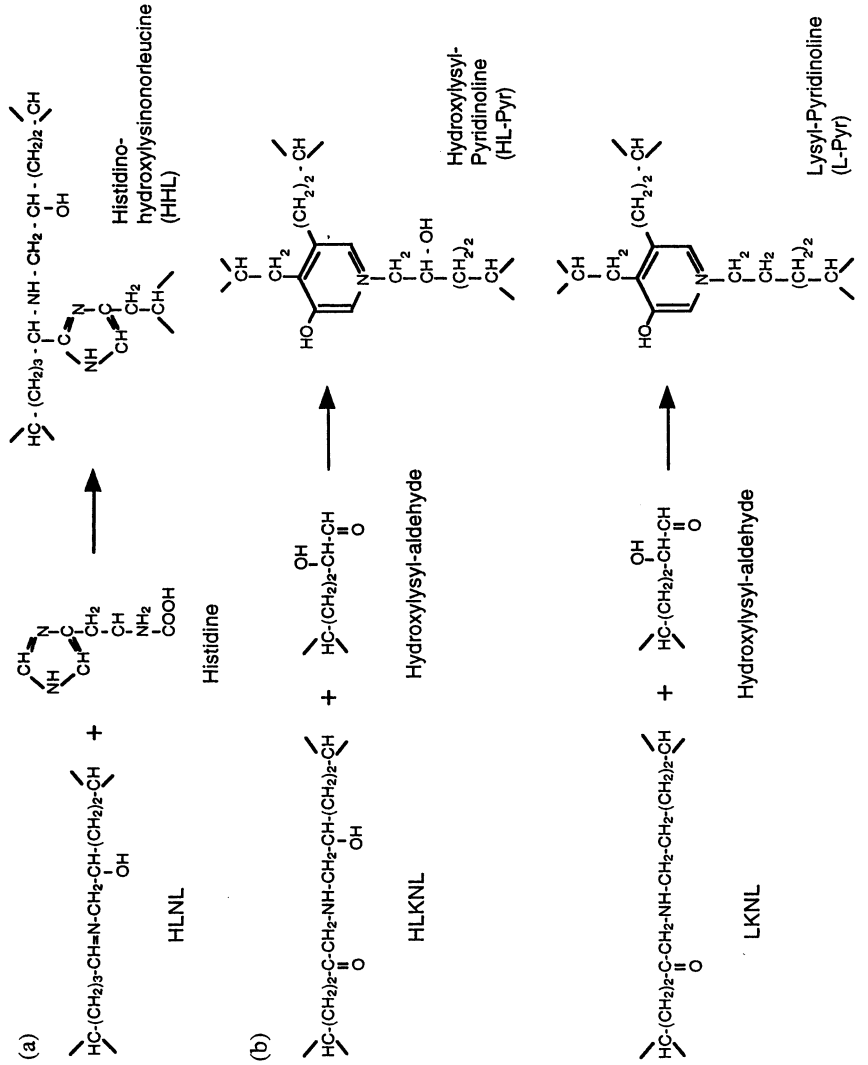


Fig. 6. Formation of the characterised mature cross-links. (a) Histidino-hydroxylysinonorleucine (HHL) derived from the divalent Schiff base HLNL and triple helical histidine. (b) The pyridinolines derived from the divalent keto-imines and the telopeptide hydroxylysyl aldehyde.

rather than hydroxylysine. A true deoxy derivative is the reducible, non-fluorescent pyridinoline tentatively localised in skin (Barber et al., 1982; Tilson et al., 1985). This pyridinium analogue lacks the hydroxyl group at ring position 3 following a condensation reaction. The precise content of this pyridinoline in skin has not been determined but it is believed to be a minor cross-link.

The pyridinium cross-links are acid stable, UV sensitive, fluorescent compounds, and although the cross-linking peptides have been isolated and sequenced (Hanson and Eyre, 1996), the exact mechanism of formation is controversial. Eyre (1981) postulated that this trifunctional cross-link is formed from two immature HLKNL cross-links based upon the loss of HLKNL being equal to double the increase in the two pyridinium cross-links during in vitro incubations. The condensation of two HLKNL cross-links would require the release of an hydroxylysine residue which would involve the cleavage of a covalent bond, an unlikely event in vivo (Yamauchi and Mechanic, 1985). An alternative, sterically more feasible, theory was proposed by Robins and Duncan (1987) involving the condensation of an HLKNL cross-link with another hydroxylysine aldehyde (Fig. 6b). This model of pyridinoline formation is less constrictive of the molecular and fibrillar organisation of the collagen. L-Pyr would then be formed through the reaction of LKNL with an hydroxylysine aldehyde (Fig. 6c). As a consequence, this pyridinium cross-link is formed in tissues such as bone, which have a relatively low lysyl hydroxylation (Robins and Duncan, 1987). Indeed, EDS type VI, characterised by a paucity of hydroxylysine, has elevated levels of L-Pyr in bone. Why L-Pyr is apparently confined to calcified tissues is not known. However, not many tissues have been analysed in detail and we have found low levels of L-Pyr in intramuscular and visceral collagen (unpublished data).

The deficiency of pyridinoline levels in bone collagen (1 per 5 collagen molecules) has been the subject of some speculation, particularly since bone collagen is very insoluble and resists acid swelling compared to other tissues such as cartilage, which possess even higher levels of the mature pyridinoline cross-links. We have proposed that the deficit in mature cross-links can be accounted for by the novel pyrrole cross-link (Knott et al., 1995).

2.10. Pyrrole cross-link

Since initial confusing reports of the almost ubiquitous presence of pyrrole in many tissues (Scott et al., 1981; Kemp and Scott, 1988), recent papers have provided further convincing evidence in support of this compound as a potential trivalent collagen cross-link (Kuypers et al., 1992). Based on the amino acid sequence of peptides containing the pyrrole, they postulated that this cross-link could form from the reaction of a telopeptidyl lysine-aldehyde and the immature cross-link HLKNL (Fig. 7a). This mechanism would be in line with the mechanism of pyridinoline formation postulated by Robins. If the telopeptides are highly hydroxylated pyridinoline would form, if not then the pyrrole would form. However, there is again an alternative hypothesis for its formation, with Eyre and his colleagues proposing that the pyrrole is formed through the interaction of HLKNL

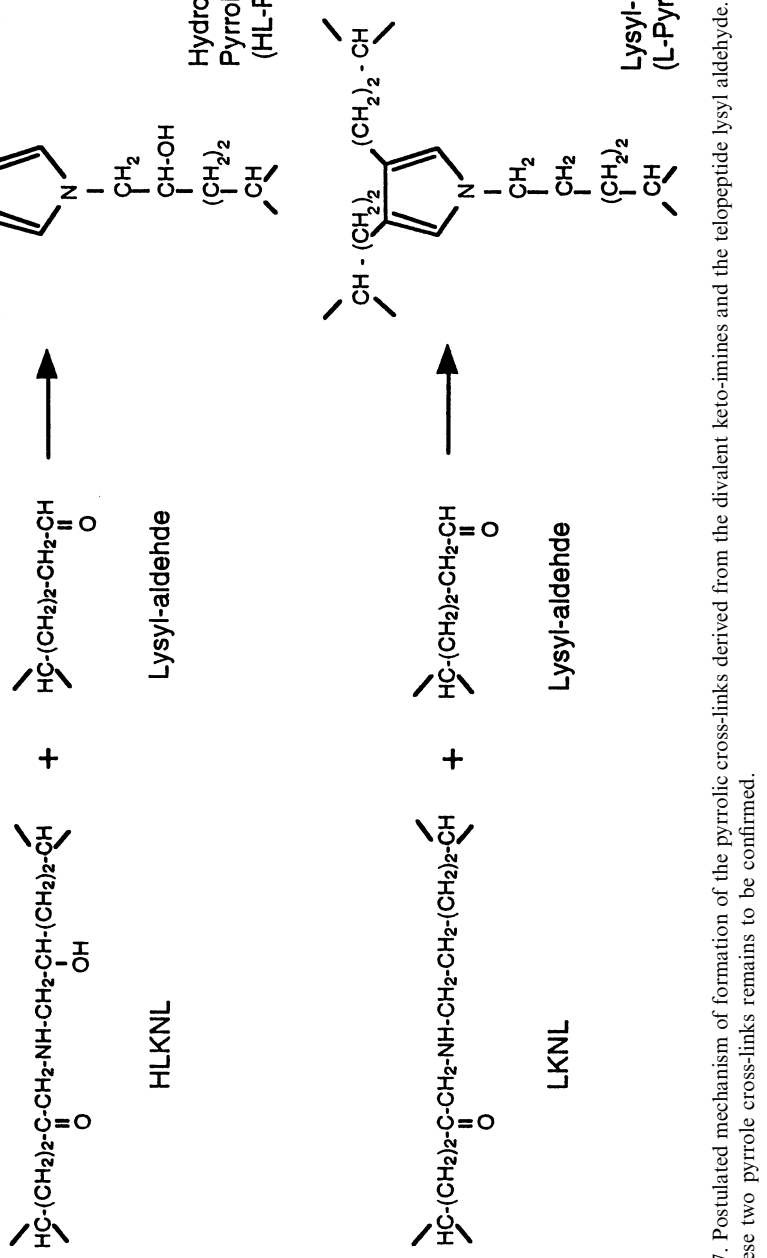


Fig. 7. Postulated mechanism of formation of the pyrrolic cross-links derived from the divalent keto-imines and the telopeptide lysyl aldehyde. The structure of these two pyrrole cross-links remains to be confirmed.

and deH-HLNL (Hanson and Eyre, 1996). Isolation of the actual cross-link itself has proved difficult due to its inherent instability during the isolation procedures. The structure suggested by Kuypers is illustrated in Fig. 7. It can be seen that, as with the pyridinolines, pyrroles should form hydroxylysyl and lysyl variants. Recently Kleter et al. (1998) reported the isolation and characterisation of a modified pyrrole, a pyrrolinenone, in acid hydrolysates of bovine dental collagen. Which of these two pyrroles is the major cross-link remains to be elucidated.

2.11. Transverse placement of the mature cross-links

The location of the pyridinoline moiety is crucial to its role in the biomechanical properties of a tissue in that it must link more than two collagen molecules if it is to confer any additional strength to the collagen matrix. At the present time investigations to demonstrate this are equivocal. Several groups have proposed that the cross-link bonds three molecules (Eyre, 1981; Henkel et al., 1987), whilst other workers (Light and Bailey, 1985; Robins and Duncan, 1987) believe that only two molecules are bound together.

The divalent immature cross-links occur at the 4D stagger of the aligned collagen molecules, hence the reaction of another lysine- or hydroxylysine-aldehyde with this cross-link can only occur through the additional collagen molecule being in parallel alignment. Bailey and coworkers proposed that this can be achieved by the cross-linking of two molecules in register each on different microfibrils (Bailey et al., 1980), Fig. 8b. Such cross-linking between microfibrils would certainly increase the stability of the fibre and account for the increase in mechanical strength with age as these transverse cross-links continue to form.

We have shown that the ratio of pyridinoline to the pyrrole in human bone is different at the N- and C- termini, and proposed a different function for each of the cross-links (Knott et al., 1995). Recently pyridinoline has been reported to be located equally at both the N- and C-termini whilst lysyl pyridinoline and the pyrrole are preferentially located at the N- terminus of the molecules (Hanson and Eyre, 1996). In view of our biochemical data correlating the pyrrole, but not

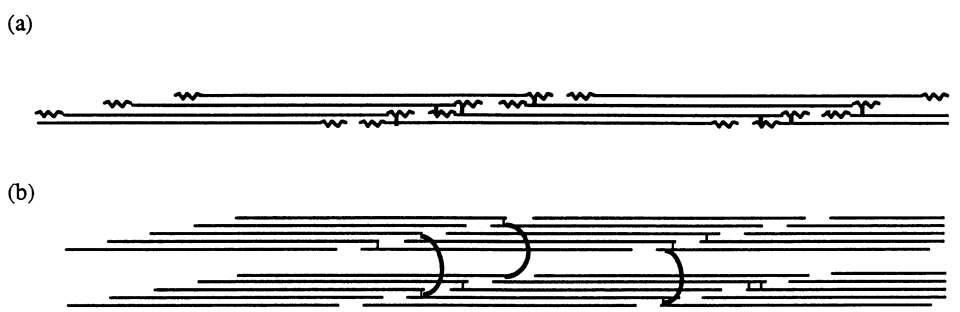


Fig. 8. Location of the divalent immature cross-links in immature collagen fibres aligned in the quarter-staggered end-over lap arrangement. (b) Location of the trivalent mature cross-links, derived from the immature cross-links and the linking of the microfibrils in register.

pyridinoline, with mechanical strength (Knott et al., 1995) we suggest that the pyrrole could act as an interfibrillar cross-link involving three different molecules whilst pyridinoline only cross-links two collagen molecules within the fibril (Knott and Bailey, 1998).

2.12. Age variation of cross-links in tissues

Virtually all the collagens identified to date, where the aggregated form has been analysed, are stabilised by the lysyl-aldehyde derived cross-links. Although type IX does not form fibrils it is cross-linked to the type II collagen by pyridinoline cross-links (Wu et al., 1992). As far as we are aware only type VI collagen appears to rely solely on disulphide bonds, no lysine derived cross-links having been detected. Some of the recently identified minor collagens have not yet been analysed.

The collagens of all species of animals examined have also been shown to possess the lysyl-aldehyde derived cross-links, from the most primitive sponges through amphibians on up to man (Eyre and Glimcher, 1971; Bailey, 1984) and basically similar age-related changes in the cross-link profile occur in the different species. Although it has been suggested that the continuous growth of fish precludes ageing (Goss, 1974) there are now reports of ageing in terms of increased tensile strength and cross-linking in fish, amphibians, and reptiles (Panigrahy and Patnaik, 1976; Cannon and Davison, 1978).

The cross-link profiles are to a certain extent tissue specific and consequently different tissues change with age by a different mechanism depending on the extent of lysyl hydroxylation.

2.13. Skin

The turnover of dermal collagen is rapid during growth and consequently stabilised by the divalent immature cross-links, but as turnover slows towards maturity and the half life of collagen increases there is an increase in the proportion of mature cross-links. Following maturation the predominant cross-links are the mature trivalent bonds which are maintained into old age (Fig. 9). A change in metabolism, for example in wound healing, results in a repeat of this change in cross-link profile. The ratio of immature to mature cross-links therefore provides a measure of the metabolism of collagen.

Both type I and type III collagens possess the same cross-links in young and old skin. In foetal skin they possess the keto-imine cross-link when lysyl-hydroxylation is high, but as this reduces post-natally the aldimine predominates in both types. This major cross-link, deH-HLNL, is converted to HHL during maturation of human and bovine skin (Fig. 9). However, it is interesting to note that old rat skin, rat tail tendon and mouse skin do not contain HHL despite the fact that deH-HLNL decreases with age (Mechanic et al., 1987). No alternative maturation product has as yet been identified, despite the extensive use of mice and rats in ageing research.

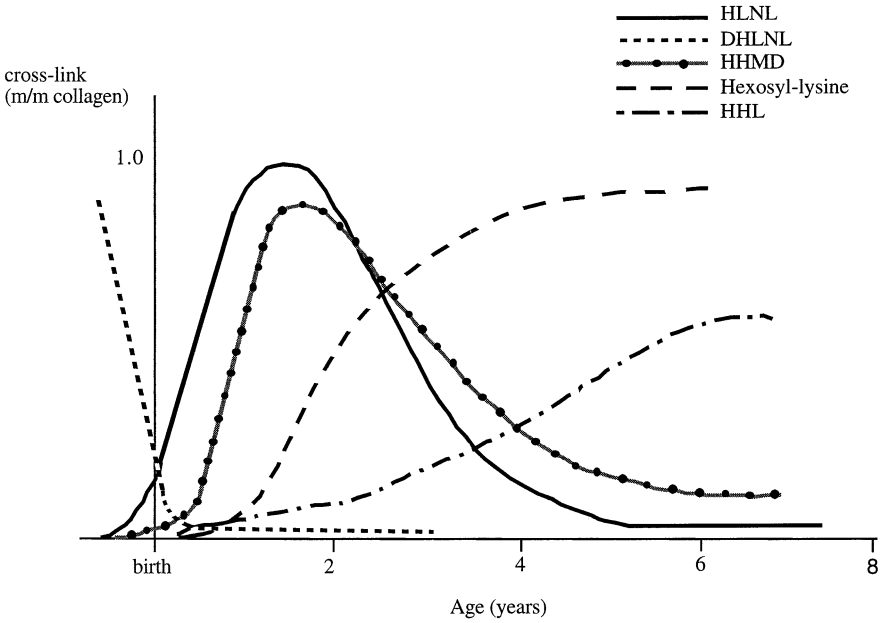


Fig. 9. Variation in the levels of the collagen cross-links in skin with increasing age. (---), DHLNL; (– –), HLNL; (–•–•–•–), HHMD; (– -), HHL; (– –) Hexosyl-lysine.

The disputed reducible cross-link, HHMD decreases slowly with age of the tissue (Bernstein and Mechanic, 1980; Yamauchi and Mechanic, 1985) but the formation of a stable product from this disputed cross-link has not as yet been reported.

The non-fluorescent pyridinoline cross-link, deoxy-pyridinoline (Barber et al., 1982), has been reported to be present in skin, but no supporting evidence has been published since the initial papers, hence its relative importance in young or aged tissue is currently unknown.

A further complication to the maturation of skin collagen is the effect of photoageing. Although HHL, the major cross-link, is stable to UV light, exposure to sun light has been reported to impede HHL formation, and as such sun-damaged skin is more soluble than unexposed skin (Sams and Smith, 1961; Yamauchi et al., 1991). Pyridinoline is susceptible to UV but is not present to any significant extent in skin. Photoageing is a specialist effect and we have not concerned ourselves with this aspect of pseudo-ageing in this review.

2.14. Bone and other calcifying tissues

Although bone is less hydroxylated than tendon, skin or cartilage, the immature cross-links are primarily derived from the telopeptide hydroxylysyl-aldehydes, the major cross-link being the keto-imine HLKNL. In view of the lower hydroxylation of the helical residues in bone collagen an additional keto-imine formed following reaction with a helical lysine, LKNL, is formed. Foetal bone contains predomi-

nantly HLKNL whilst post-natally both HLKNL and LKNL are present although the former is the major cross-link. The ratio of the two depending on the species and particular site of bone collagen.

During maturation the keto-imines form HL-Pyr and L-Pyr, the proportion of the two cross-links again varying with the species, for example 1:5 in humans, and 1:1 in mice. The immature keto-imine is present at a concentration of 1/molecule of collagen but the pyridinoline is only present at 1/5 molecules of collagen. This low level of cross-linking is inconsistent with the insolubility and resistance to swelling of bone, suggesting additional mature cross-links derived from HLKNL. We have proposed that the deficiency can be accounted for by the pyrrole cross-link (Knott et al., 1995).

Type I collagen in bone may also be co-polymerised with type V collagen (Broek et al., 1985), an association which has been suggested to regulate fibre diameter (Birk et al., 1990). Cross-links have been found both between type V molecules and between type V and type I molecules in a head to tail manner. Although these cross-links were found to be derived from telopeptide aldehydes, they were not fully identified and their maturation products were not investigated (Niyibizi and Eyre, 1994).

Type X collagen is localised at sites of endochondral ossification, in close association with type II collagen. The immature cross-links HLKNL and deH-HLNL/LKNL have been identified in fractions of type X collagen, however, the corresponding telopeptide to which the cross-links are attached is as yet unknown, although presumed to be type II collagen (Chen et al., 1992; Rucklidge et al., 1996). Only trace amounts of the pyridinolines were found, and it is postulated that an alternative maturation pathway for these immature cross-links may exist (Rucklidge et al., 1996). However, as type X collagen is a transient collagen, its mechanism of cross-link maturation is only of interest from a developmental point of view, and may not be relevant to the ageing of collagenous tissues.

2.15. Cartilage

Hyaline cartilage is comprised of a heterotypic network of collagen fibres within a highly hydrated proteoglycan matrix. The predominant collagen in cartilage is type II which is believed to form fibres around a core of type XI collagen fibrils. Type IX collagen molecules are located on the surface of these fibres. These collagens are cross-linked, both within and between collagen types, so conferring the material strength of the tissue. Type II collagen is highly hydroxylated such that the major cross-links in cartilage are the keto-imine (HLKNL) and its maturation product, HL-Pyr. This cross-link maturation occurs at an early age (10–15 years old), and the overall levels of HL-Pyr either do not change after maturity (Eyre et al., 1988; Uchiyama et al., 1991), or may even decrease (Kim et al., 1994). The age-associated increase in insolubility of cartilage could therefore be due to the formation of non-enzymatic cross-links, as opposed to the pyridinolines. Indeed, pentosidine, a Maillard reaction product (see later) increases six-fold with age in rabbit cartilage (Pokharna et al., 1995). The change in the mechanical properties of

cartilage with ageing has significant implications for the functional integrity of the tissue. The increase in collagen fibre diameter, which leads to microscar formation, may be a direct consequence of the increase in cross-linking (Mallinger and Stockinger, 1988).

The minor collagens of cartilage, types IX and XI, also contain hydroxylysine-aldehyde derived cross-links, however their cross-link maturation is unclear, although the maturation of type XI collagen is apparently impeded by fibril formation (Eyre et al., 1990; Reiser et al., 1992a).

2.16. Tendons and ligaments

The majority of tendons and ligaments contain both the keto-imine and the aldimine immature cross-links. The ratio depends upon the particular function of the tissue, in particular whether there is tension on the tissue, in which case the level of the keto-imine (the more stable immature cross-link) tends to be higher. Ligaments therefore, contain mainly keto-imine cross-links, whilst tendons have a higher aldimine content (Amiel et al., 1984). This phenomenon may also be due to the difference in turnover rate between these two tissues. Ligaments also possess a significant amount of type III collagen, and an apparent higher level of lysyl hydroxylation, both factors indicative of a high turnover rate. At the extreme, periodontal ligament has an unusually high turnover rate of about two days (Sodek, 1977), and consequently, a high immature cross-link content.

With maturation of tendons HL-Pyr is formed, but little HHL, the aldimine maturation product, is detected (Birch, 1993). The main maturation product of the aldimines in tendon has yet to be elucidated. Again, the levels of the mature cross-links are related to the function of the tissue. Even within flexor tendons of the equine foot for example, the level of pyridinoline was positively correlated to the amount of stress the tendon was subjected to. In this case, a difference in turnover rate was found not to be cause of the difference in cross-link levels (Birch, 1993).

In short lived species, such as rats and mice, the pyridinoline content continues to increase after maturity. In contrast however, pyridinoline levels in human Achilles tendon decreased after 30 years of age (Moriguchi and Fujimoto, 1978). The reason for this phenomenon is unclear, but may be due to some degenerative change with age, or less likely, an increase in tissue turnover.

2.17. Basement membrane

Type IV collagen of basement membrane is highly hydroxylated and the immature cross-link is consequently the keto-imine. Analysis of mature basement membrane surprisingly fails to reveal the normal product of the keto-imine, pyridinoline, and the mechanism of stabilisation of the keto-imine in type IV collagen remains to be elucidated (Bailey et al., 1984). The lysyl-aldehydes only cross-link the anti-parallel molecules in the N-terminus, whilst the C-terminus cross-links to other molecules through disulphide bonds (Reddy et al., 1993). Tissues such as the

anterior lens capsule become more rigid and brittle with age (Krag et al., 1997), and although the mechanism is as yet unknown, increased cross-linking, either enzymic or glycation mediated cross-links, is likely to be responsible (Bailey et al., 1993) in a similar manner to other ageing collagenous tissues.

Type VIII collagen is also found in basement and other membranes, and is similar in structure to type X collagen. In Descemet's membrane, type VIII forms an hexagonal lattice, whilst in other tissues it is found associated with other matrix molecules (Sutmuller et al., 1997), however its mode of stabilisation and structural changes with age have not yet been elucidated.

In summary the age-related mechanism of stabilisation of the collagen fibre, initially by lysyl oxidase and the spontaneous conversion of the divalent cross-links of the immature tissue to the trivalent cross-links of the mature tissue, is now fairly well established. The location of the divalent cross-links has been identified and confirms the quarter-stagger alignment of the molecules in the fibre and thus leads to the head to tail polymerisation of the molecules that accounts for the high tensile strength of the fibre. However, the nature of the further increase in strength of the fibre during maturation due to the trivalent mature cross-links is not clear. Our proposal that the trivalent cross-links form inter-fibrillar cross-links requires chemical confirmation. Other questions also remain to be answered; to confirm that the deficit of pyridinoline cross-links in bone is made up by the pyrrole cross-links, whether the pyrrole forms interfibrillar links, and to identify the nature of the mature cross-links in rat and mouse skin and in basement membranes. Continuing investigation in this fundamental area of research is required to elucidate these questions and further our understanding of ageing and disease processes.

3. Non-enzymic cross-linking (glycation)

The second mechanism of intermolecular cross-linking of collagen that increases with age is via the non-enzymic reaction with glucose, now generally referred to as glycation.The glycation of proteins is purely adventitious and is therefore likely to be more important in proteins possessing a long biological half-life, such as mature collagen. Indeed, the deleterious effects of glycation on collagen play a central role in the pathogenesis of ageing (Schnider and Kohn, 1982; Monnier, 1989; Paul and Bailey, 1996). Collagen provides the basic structural properties of the most vulnerable tissues, such as renal basement membrane, the cardiovascular system and retinal capillaries, and the most serious late complications of ageing involve the modifications of the collagen which result in the dysfunction of these tissues. The typical age-related changes are accelerated in diabetic patients due to hyperglycaemia and are the major cause of premature morbidity and mortality in these subjects (Diabetes Control and Complications Trial, 1993). Glycation can affect the properties of collagen in a number of ways, for example, its optimal biomechanical functioning in the diverse tissue forms, its ability to form precise supramolecular aggregates, the alteration of its charge profile and hence its interaction with cells, and, additionally, glycated collagen can act as an oxidising agent. The most

damaging effects are believed to be caused by the formation of glucose-mediated intermolecular cross-links. These cross-links decrease the critical flexibility and permeability of the tissues and reduce turnover.

The nature of these glycation cross-links is now being unravelled and this knowledge is crucial in any attempt to understand the mechanisms of the ageing process and ultimately inhibit these deleterious glycation reactions.

3.1. Reaction of glucose with collagen

The aldehyde of the open chain form of glucose reacts with the free ε-amino group of a peptide bound lysine to form a glucosyl-lysine. This reaction, known as the Maillard reaction after the French chemist who studied the reaction between glucose and glycine, but it was not until 1953 that Hodge laid out the basic mechanisms (Hodge, 1953). The glycation of proteins was first noted with the formation of an adduct between glucose and the α-amino end-group of the terminal valine in haemoglobin (Holmquist and Schroeder, 1966; Bookchin and Gallop, 1968), but collagen was the first protein shown to be glycated through the ε-amino groups of peptide bound lysine by Robins and Bailey (1972). The hexosyl lysine formed is then stabilised by spontaneous Amadori rearrangement to form a keto-imine (Fig. 10). This reaction has been shown to be common to other proteins investigated (for reviews see Baynes and Monnier, 1989; Labuza et al., 1994). The formation of the Amadori complex may be confirmed by isolation of its borohydride reduced product (Robins and Bailey, 1972) and by direct acid hydrolysis to furosine and pyridosine (Schleicher and Wieland, 1981). Glucose and fructose are present in significant concentrations in vivo but react slowly in contrast to the ketoaldehydes and glyoxals which react rapidly but are present at very low concentration in vivo. The rate of reaction of glucose with the lysines and arginines in a protein depends on the neighbouring groups which influence the pK_a of the side-chain.

3.2. Site-specificity of glycation

The lysine and arginine levels are generally high in proteins, but only a few residues are glycated, it is therefore of interest to know if some of these residues are preferentially glycated, or whether the reaction occurs at random along the polypeptide chain.

The specificity of the lysines initially involved has been reported for some proteins to depend on the nature of the neighbouring groups, e.g. histidine (Shilton and Walton, 1991). Baynes et al. (1989) have reviewed possible mechanisms which include decreasing pK values, proximity to carboxyl groups and the presence of phosphate. It is important to note that chelators, antioxidants and sulphydryl compounds have little effect on this initial stage of glycation. In the case of collagen the specificity is unclear and has usually been based on particular cyanogen bromide peptides, the latter produced by cleavage of the collagen α-chain into well established specific peptides. The results are equivocal, varying from attachment of

ribose to all the CB peptides (Tanaka et al., 1988b) to glucose attachment to αICB6 (Le Pape et al., 1984) to preferential glycation of particular lysine residues in the short peptide α1CB3 and α2CB3,5, (Reiser et al., 1992b) although the latter constitutes almost two thirds of the α2 chain. Wess et al., (1993) provided evidence from neutron scattering for glycation in the gap region of the fibre. This region might be more accessible to glucose than the over-lap region but again does not define a specific lysine(s). These reports indicate some preference between lysine residues, and the sites are believed to be consistently preferred whether glycation occurred in vitro or during ageing in vivo, but the precise residues involved are

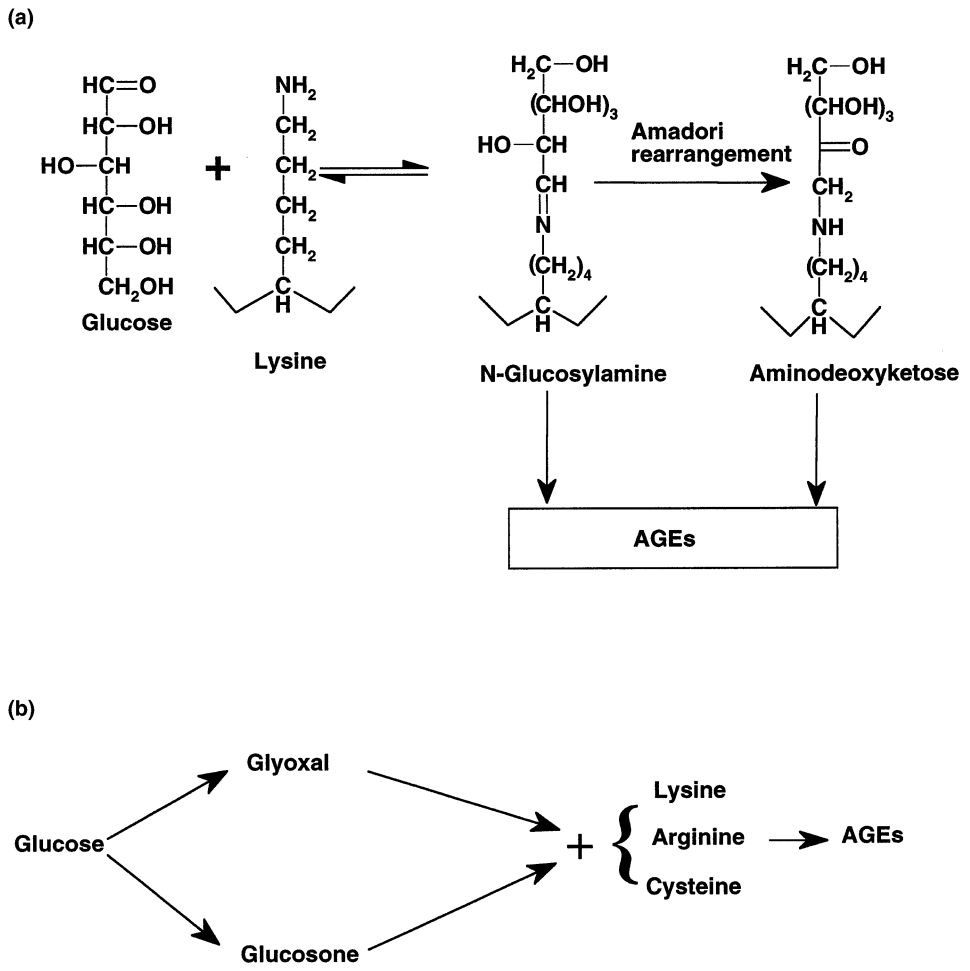


Fig. 10. (a) Reaction of glucose and peptide bound lysine to form a Schiff base which spontaneously undergoes an Amadori rearrangement to give the Amadori product, aminodeoxyketose. Both the Schiff base and the ketose are believed to react further to form AGEs. (b) Oxidative degradation of glucose to glyoxal and glucosone, which then react with protein side-chains to give AGEs.

ambiguous. It is possible that some of the variation may be due to the nature of the sugar involved and the extent of glycation employed by different workers. We have therefore recently carried out a detailed analysis of the initial reaction and found that there is indeed a preference for specific lysines in α1 CB7 of fibrous type I collagen.

3.3. Advanced glycation end-products

Both the Schiff base adduct and the keto-imine undergo further reactions with other amino acid residues, (Cerami et al., 1987; Baynes et al., 1989), or following metal-ion induced oxidative breakdown (Ahmed et al., 1986) to form AGEs. The term AGE is used to describe any protein bound moiety detected after formation of the initial Schiff base/Amadori product that appears to be the final product. For example, it may result from a one-step conversion to carboxymethyllysine (Fig. 11a), or involve a complex series of reactions to form an intermolecular cross-link such as pentosidine (Fig. 11b). In addition, oxidative breakdown can lead to the formation of more reactive sugars, such as 3-deoxyglucosone (Kato et al., 1989) and the glyoxals (Wellsknecht et al., 1995), which then also complex with other lysines to form AGEs (Fig. 12). Wolff and Dean (1987) proposed an alternative mechanism in which glucose in the presence of protein is itself oxidised to form a more reactive species, again 3-deoxyglucosone or smaller sugar aldehydes (Wolff and Dean, 1987), which then react with the ε-amino group of lysine (Fig. 10B). However, the importance of this pathway is now being questioned. There is little real evidence for this pathway and the precise nature of the oxidative process of the Amadori product is similarly a matter of some controversy. Confirmation of free radical and oxidative mechanisms have been demonstrated by addition of radical scavengers and anti-oxidants to collagen-glucose in vitro incubations, when the formation of AGEs and the resultant changes in the physical properties are inhibited, thus supporting the contention that at least some of the AGEs are responsible for the latter effects (Chace et al., 1991; Fu et al., 1994a). Baynes and his colleagues (Zyzak et al., 1994, 1995) have suggested that although the Amadori product is slowly formed and oxidised to AGEs the major reactions in vitro are the direct auto-oxidation of glucose and oxidation of the rapidly formed Schiff base adduct as proposed by (Hayashi and Namiki, 1986). However, this conclusion is based on in vitro reaction products, whilst the predominant reaction in vivo will depend on the relative concentrations of the reactants, local pH, and the presence of additives, such as metal ions and anti-oxidants, in the tissue. The latter are unknown but could vary considerably from these in vitro artificial mixtures.

The presence of AGEs in the tissues most vulnerable to age-related changes has been confirmed by AGE specific fluorescent antibodies (Makita et al., 1992) but the antigen is often an unknown mixture of AGEs, usually the total products of glycated bovine serum albumin. This is inevitable since only a few AGEs have been actually been isolated and characterised. More importantly, direct evidence that AGEs do affect these tissues has recently been neatly demonstrated by administration of AGEs prepared in vitro (again total glycation products of BSA) to normal

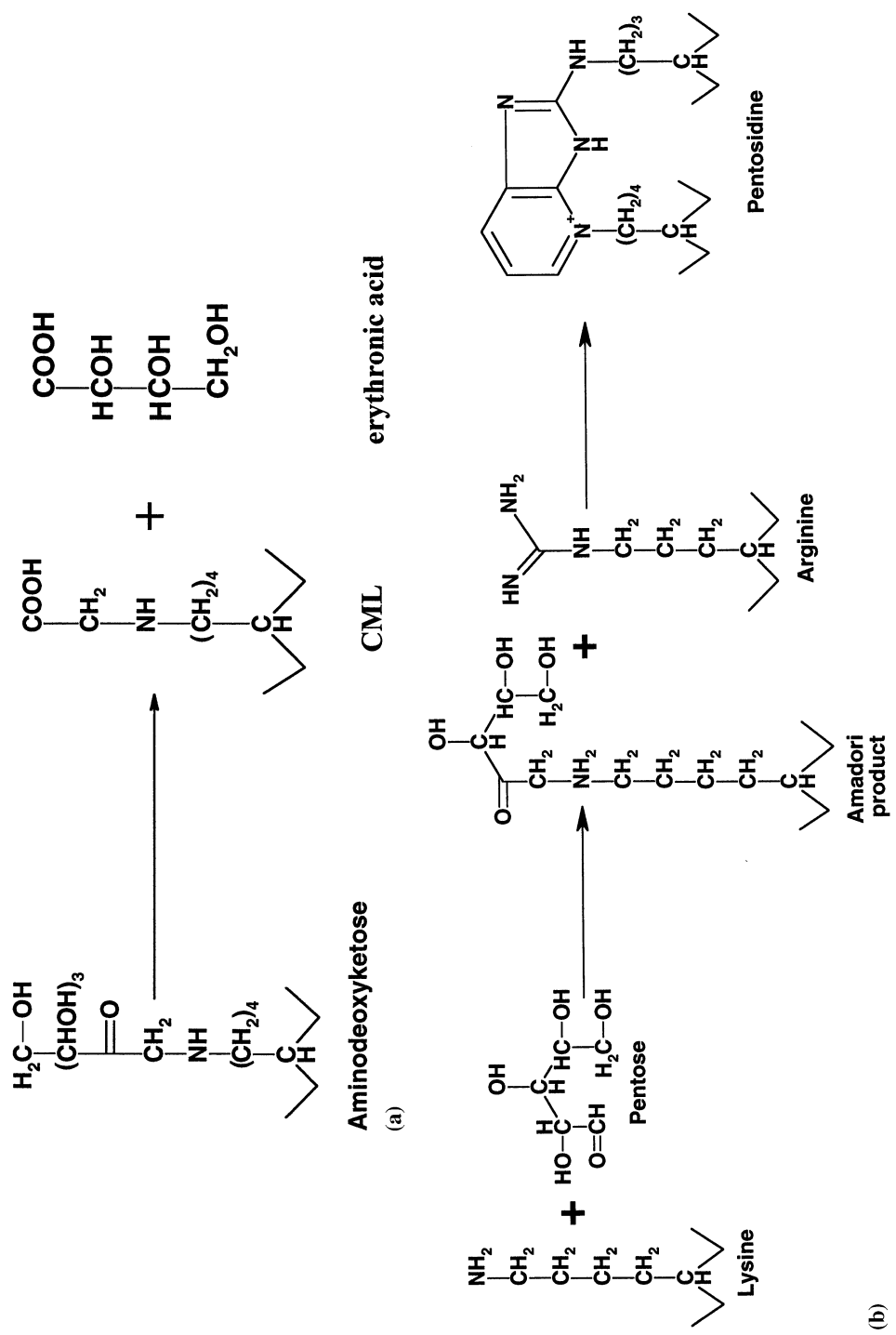
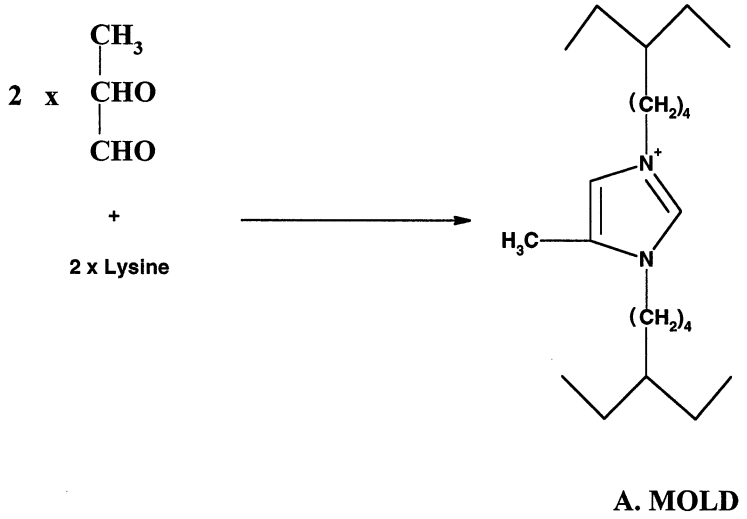
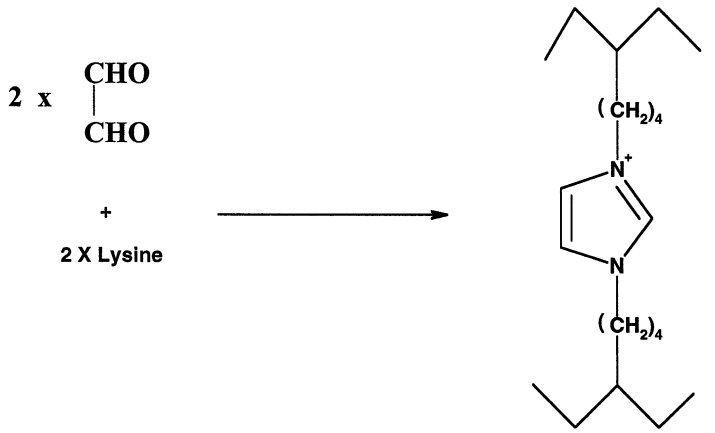


Fig. 11. (a) Oxidation of the Amadori product or Schiff base to form carboxymethyl-lysine (CML). (b) Reaction of a pentose (e.g. ribose) with peptide bound arginine and lysine on different peptide chains to form the cross-link pentosidine.



A. MOLD



B. GOLD

Fig. 12. (A) Reaction of methylglyoxal with two peptide bound lysines to form the potential cross-link MOLD. (B) Reaction of glyoxal with two peptide bound lysines to form the potential cross-link GOLD.

rats which subsequently revealed the typical age/diabetes changes, basement membrane thickening, glomerular hypertrophy and an increase in the mesangial volume in the absence of hyperglycaemia (Vlassara et al., 1994). This finding is of interest in relation to a recent report by Cerami et al., (1997) that glycation products are present in tobacco smoke that can react rapidly with proteins to form AGEs, and suggest that they may contribute to increased incidence of atherosclerosis in smokers.

We believe that the most damaging AGEs would be those forming intermolecular cross-links between the collagen molecules in the fibre. The nature of these cross-linking AGEs is therefore the subject of intense research, not least because identification of the mechanisms involved could lead to alleviation, or even inhibition, of these damaging late complications both in ageing and diabetes mellitus.

3.4. Intermolecular glycation cross-links

Long term glycation of fibrous collagen occurs during ageing and renders it less soluble, more resistant to enzymes and less flexible in vivo, and similar reactions occur following incubation with glucose in vitro (Kent et al., 1985; Monnier et al., 1986). The process is accelerated in diabetic subjects due to the higher glucose levels (Schnider and Kohn, 1982) and in experimentally diabetic animals (Andreassen et al., 1981). These effects on the properties of collagen are consistent with formation of the intermolecular cross-links between the collagen molecules within the fibre. This would involve reaction between ε-amino groups of lysine in the triple helical parts of the collagen molecule rather than the globular ends of the molecules as in the case of enzyme generated cross-links (see above), and consequently lead to more rapid stiffening and enzyme resistance of the collagen fibre. The non-fibrous basement membrane type IV collagen not only becomes stiffer due to increased cross-linking (Bailey et al., 1993) but as a consequence also results in increased permeability and reduced endothelial attachment (Haitoglou et al., 1992).

The formation of intermolecular cross-links within the collagen fibres and basement membranes are obviously consistent with the decreasing elasticity of retinal capillaries, renal glomeruli and arterial vessel walls, which are characteristic effects of ageing. These changes in physical properties induced by the cross-links must therefore be considered the most important modification of the collagen during glycation. Thus, there is considerable circumstantial evidence that some of the AGEs form intermolecular cross-links but the nature of these cross-links has proved elusive. To date the following structures have been proposed as cross-links.

3.4.1. Pentosidine

Pentosidine is an imidazo pyridinium compound (Sell and Monnier, 1989), derived from lysine, arginine and ribose (Fig. 11b). It has now been identified as the end-product of the Maillard reaction elicited by hexoses, pentoses, ascorbate, and a variety of Amadori compounds in vitro (Sell and Monnier, 1989; Dyer et al., 1991; Grandhee and Monnier, 1991). The major in vivo carbohydrate source leading to pentosidine formation is therefore not clear. The oxidative nature of the reactions prompted Baynes to coin the phrase 'glyco-oxidation product' to describe pentosidine and other glycation compounds similarly dependent on oxygen availability (Baynes, 1991).

Pentosidine has now been identified in numerous collagenous and non-collagenous tissues and shown to exhibit a linear increase with age (Sell and Monnier, 1989). However, in view of the low concentration in tissues, amounting to only one pentosidine cross-link/2–300 collagen molecules (Dyer et al., 1991; Bailey et al.,

1995) its significance as a major cross-linking component affecting the properties of collagen has been largely discounted and is now regarded mainly as a biomarker in the assessment of cumulative damage to proteins by non-enzymic glycation.

3.4.2. Non fluorescent component-1

In view of the low yields of fluorescent cross-links observed during in vitro and in vivo investigations Bailey and his colleagues (Bailey et al., 1995) turned their attentions to a detailed analysis of non-fluorescent components associated with highly cross-linked collagen and identified a non-fluorescent component, NFC-1, the presence of which could be correlated to a change in physiochemical properties consistent with increased cross-linking. The concentration of NFC-1 present in tissue (1 NFC-1/collagen molecule) and its increase with age suggested that it could be an important intermolecular cross-link. More recent evidence has shown that NFC-1 is a complex consisting of imidazolones derived from glyoxal and methyl glyoxal reacting with arginine (see below) and an as yet uncharacterised high molecular weight component, the latter is believed to be the cross-link moiety (Paul et al., 1998).

3.4.3. Imidazolium compounds

Two acid stable imidazolium compounds designated MOLD and GOLD, have recently been proposed as potential intermolecular cross-links, both of which are non-fluorescent. MOLD, a methylimidazolium compound (Fig. 12a) was originally proposed as a potential cross-link species by Brinkmann et al. (1995) following its isolation from in vitro incubations of N^{α}-hippuryllysine and methylglyoxal (MGO). The major source of MGO in vivo is from the non-oxidative degradation of triosephosphates, acetone, and ketone body metabolism and aminoacetone and threonine metabolism (Thornalley, 1996). The continual synthesis of MGO in vivo could lead to a gradual build up of imidazolium cross-linked collagen. Nagaraj et al., (1996) isolated MOLD from in vitro glycated human serum albumin and lens crystallin and showed a time dependent increase, reaching levels of 7–8/HSA molecule and 5–6/α-crystallin sub-unit. Two mechanisms have now been proposed for its formation. Both involve the reaction of two MGO molecules and two lysine residues with a subsequent elimination of an acyl group, the first (Brinkmann et al., 1995) prior to imidazolium formation and an alternative proposed by Alabed et al. (1996b) in which hydrolytic deacetylation occurs after formation of a 2-acyl-4-imidazolium intermediate. The second imidazolium compound, GOLD (Fig. 12B), has also been islolated from model in vitro systems following the incubation of N^{ε}-hippuryllysine and glyoxal a dicarbonyl formed by lipid peroxidation and degradation of glucose and glycated protein (Wellsknecht et al., 1995). However, isolation of this cross-link species from protein hydrolysates has yet to be achieved.

3.4.4. Vesperlysines

Three fluorescent cross-links, vesperlysines A, B, and C have recently been detected in the acid hydrolysates of in vitro glycated bovine serum albumin (Nakamura et al., 1997a). Incubation of protein in the presence of glucose results

in the cross-linking of two lysine residues by a mixture of pyrrolopyridinium rings (Fig. 13A). The original six carbon skeleton of glucose is not incorporated into the cross-link structure indicating that they are glyco-oxidation products derived from two molecules of glucose via dehydration, oxidative fragmentation, and condensation of the sugar moieties. In addition they can be formed from a number of shorter chain sugars e.g. ribose and glyceraldehyde. Although, peaks co-eluting with authentic vesperlysines have been observed in the hydrolysate of glycated protein they have not been isolated and their presence in vivo remains uncertain.

3.4.5. Crossline

Following in vitro reaction of glucose and N^{α}-acetyl-lysine Nakamura et al. (1992) proposed crossline (Fig. 13B) as a potential cross-link. Formed by the cyclo-condensation of two glucose and two lysine molecules, crossline is a fluorophore existing in two epimeric forms, A and B. In contrast to candidates described previously they are acid labile and have not been isolated from tissues. However, immunohistochemical studies using sera derived from N^{α}-acetyl derivatives has indicated their presence in both in vivo and in vitro glycated protein (Nakamura et al., 1992; Ienaga et al., 1996) but needs to be confirmed chemically.

There is no doubt that the presence of glucose in tissues results in the intermolecular cross-linking of collagen, which has a deleterious effect on its optimal functional properties, but the isolation and characterisation of these cross-links from tissue has proved difficult. Further investigation on the relative importance of those described above and the nature of as yet undiscovered cross-links is crucial if we are to fully understand these important mechanisms of ageing.

3.5. Site of the glycation cross-links

The location of any of the proposed cross-links in the collagen fibre has not been elucidated. The rapid decrease in susceptibility of the collagen to enzymes and the increased thermal denaturation temperature following glycation is consistent with inter-helical cross-linking, which may involve lysines and arginines at random along the length of the molecule (Fig. 14). Although there is a suggestion of preferential glucose reactive lysine(s), which one might expect would be the source of the cross-link, it is also possible that late reacting carbonyls, for example glyoxal, attach at completely different sites along the collagen molecule (e.g., arginine).

3.6. Lipid derived glycation cross-linking

The low density lipoprotein (LDL) of the cardiovascular system and its close association with collagen is of particular interest in the context of this review of ageing in view of the mechanisms involved in atherosclerosis, the main age-related cause of mortality in western society (Ross, 1993). Coronary risk factors are multiple but the increased incidence in diabetics suggests that glycation cross-linking may play an important role in ageing of non-diabetics. For example, Lee et al., (1994) reported on the binding of LDL to collagen although the nature of the cross-links were not studied.

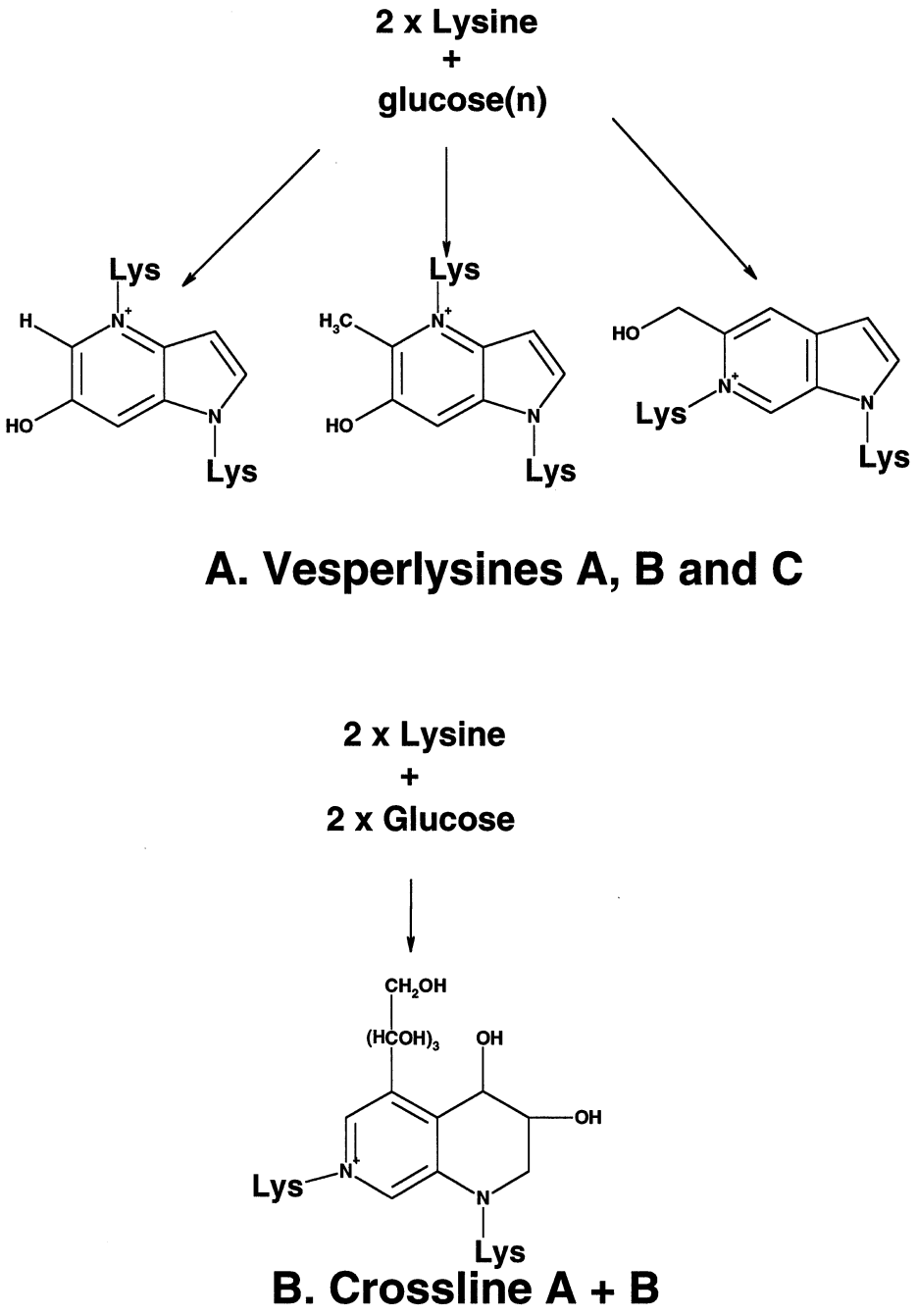


Fig. 13. Proposed alternative reaction of two peptide bound lysines with glucose to form potential cross-links. (a) Vesperlysines A, B, and C. (b) Crosslines A and B.

A possible important aspect of the glycation reactions of collagen is the surprising ability of 'glycated collagen' to accelerate the oxidation of compounds such as lipids, and produce the potential cross-linking compound, malondialdehyde (Hicks et al., 1988).

We have, therefore, recently proposed that a second but indirect glycation modification of collagen can occur through reaction with the reactive oxidation products of lipids induced by glycation, such as malondialdehyde, to form stable intermolecular cross-links (Slatter et al., 1998).

Polyunsaturated fatty acids are sensitive to peroxidation with accompanying diene conjugation and hydroperoxide formation. Following the initial oxidation reaction fatty acids breakdown readily to a variety of reactive aldehydes, the occurrence and formation of which has been extensively reviewed (Esterbauer et al., 1991). Two distinct classes of aldehyde are formed from this reaction, that is, lipid aldehydes in which the aldehyde function remains attached to the parent lipid molecule and released fragments such as n-alkenals, 2-alkenals, (Alabed et al., 1996a) and malondialdehyde (MDA), which are derived from the methyl terminus of the fatty acid chain. These aldehydes are long lived and can diffuse from their site of origin in membranes and react with intracellular or extracellular targets, such

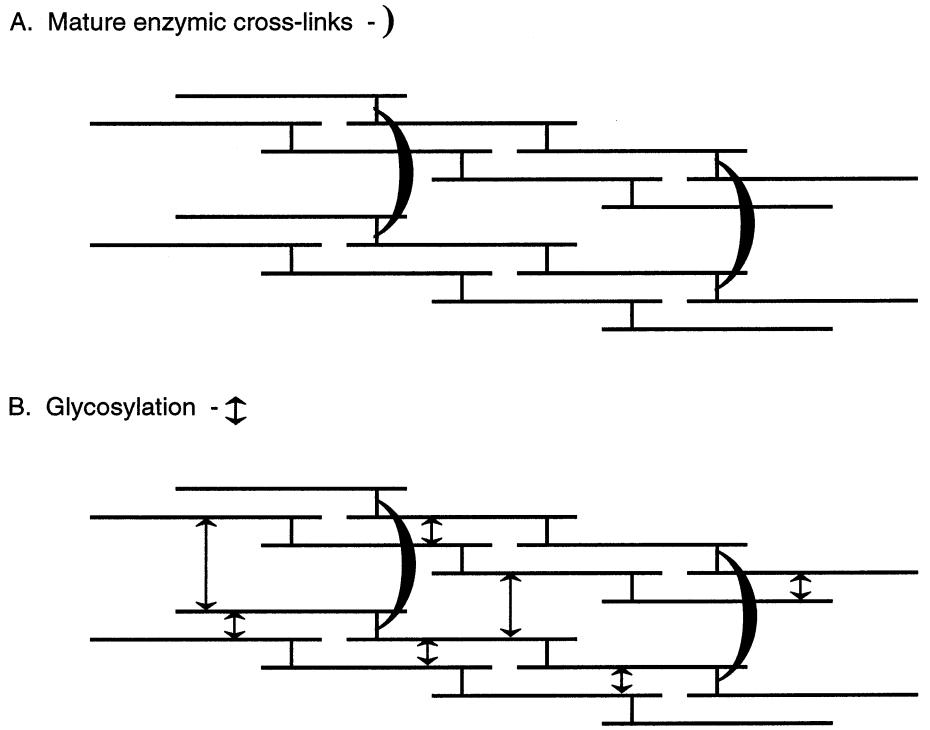


Fig. 14. Summary of the location of cross-links. Immature, (|); mature, (); and senescent, glycation, (↕) cross-links during ageing of the collagen fibre.

as protein, some distance away. Similar products have been identified by mass spectrometry from lipid-AGE reactions confirming the ability of glycation products to oxidise unsaturated fatty acids. Despite the very high lysine content of ApoB the site has been mapped to a 67 residue peptide, which results in a conformational change in the LDL thus affecting its clearance and age-related progression to an atherosclerotic plaque. Malondialdehyde is produced in both situations and it is noteworthy in that MDA not only modifies proteins under physiological conditions but can introduce non-enzymic cross-links through its dialdehyde character.

The possible cross-linking of collagen by di-aldehydes derived from lipid peroxidation is of particular importance since it will not only have a direct affect upon collagen function but may also act as a link between glycation and further lipid peroxidation. In other words MDA type cross-linking will decrease the turnover of the collagen and consequently result in an increase in its susceptibility to further glycation which can in turn initiate further oxidation of fatty acids with concomitant formation of reactive aldehydes, thereby propagating a vicious circle of protein and fatty acid damage (Fig. 15). The formation of these 'MDA-cross-links' in the fibrous collagens and non-fibrous basement membrane collagens of the cardiovascular system would certainly affect the flexibility and permeability of the aorta. Indeed, damage to the endothelium and the stiffening of the vessel walls are well established early stages of atherosclerosis, a process which is known to occur prematurely in diabetic subjects (Brownlee, 1994; Vlassara, 1996) and has been shown to be related to cross-linking (Sims et al., 1996; Winlove et al., 1996). It is interesting to note that elevated levels of MDA have been detected in the serum of diabetics (Ozdemirler et al., 1995) but whether they are raised in the aged population, or maintained at a significant level is not known.

Employing model compounds such as polylysine (Kikugawa et al., 1985), bovine serum albumen (Beppu et al., 1988) and γ-crystallin (Riley and Harding, 1993) it has been suggested that MDA forms cross-links via Schiff bases with each of the aldehyde groups to give a stable amino-imino-propen structure. In our hands the reaction of MDA with collagen results predominantly in the formation of a dihydro-pyridine derivative (Fig. 15). This is based on following the reaction of MDA with α-amino blocked lysine by NMR and mass spectrometry (Slatter et al., 1998). The pyridine derivative possesses two aldehyde groups which are capable of reacting with a further two lysine-containing molecules making it a trivalent cross-link. Such a link should be of considerable importance in establishing the mechanisms of aortic stiffening in atherosclerosis during ageing. It is possible that, as in the case of glucose, there are preferred sites for the reaction of MDA with collagen but these remain to be identified.

3.7. Non-crosslinking amino acid modifications

In addition to intermolecular cross-linking other properties of collagen may be modified by the above glycation reactions. For example, glycation modifies the amino acid side chains and consequently the charge profile of the collagen molecule or fibre. Such modifications are therefore likely to affect its interactions with cells and other matrix components and these effects will increase with age.

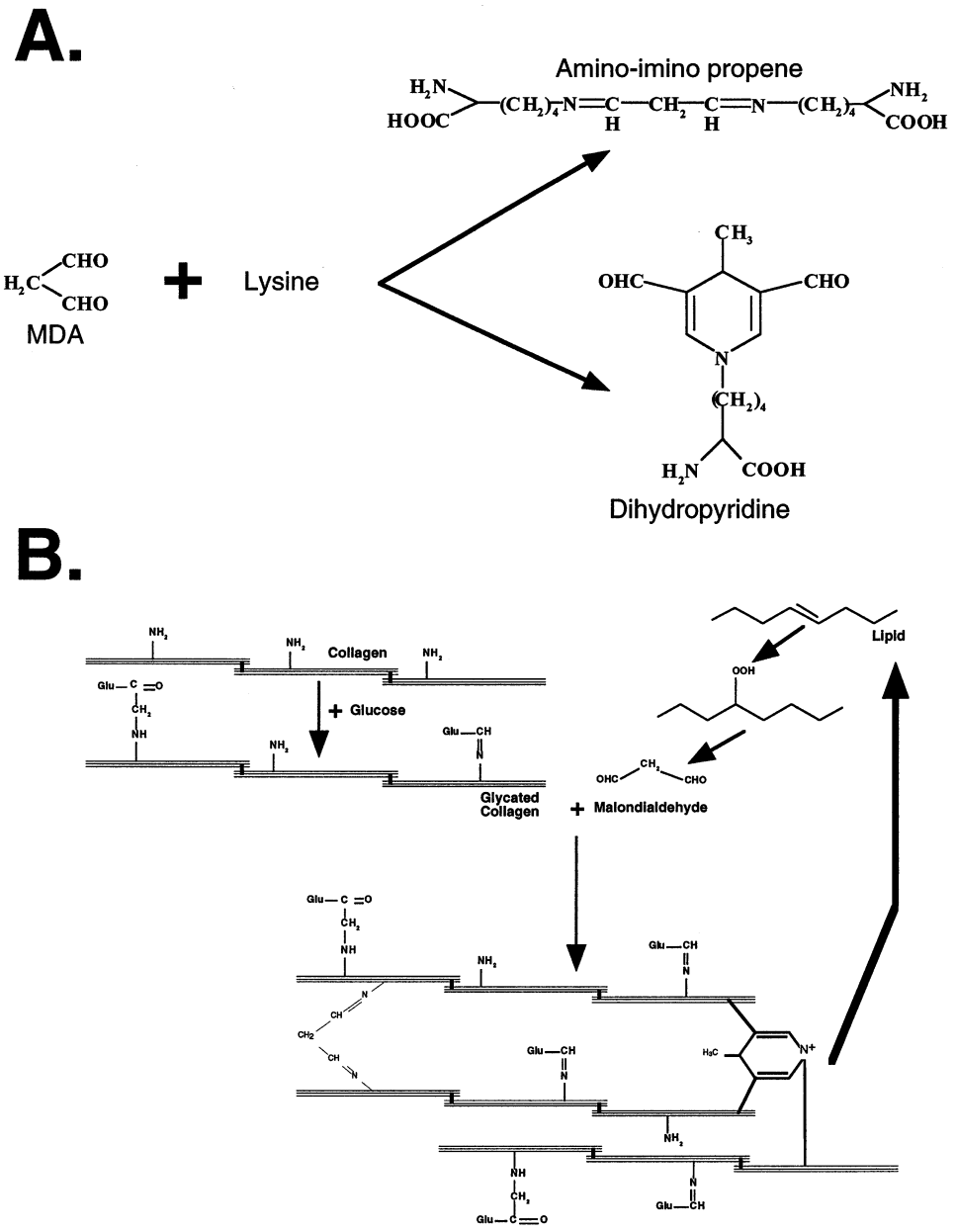


Fig. 15. (A) Reaction of malondialdehyde (MDA) and lysine to form either the divalentamino-iminopropene or the trivalent dihydropyridine. (B) Proposed cycle of glycation through lipid derived MDA and glucose, the glycated collagen stimulating degradation of LDL to MDA resulting in further cross-linking and glycation.

Glycation has in fact been shown to interfere with cell-matrix and collagen-matrix interactions. For example, glycation of basement membrane alters its binding characteristics with heparin and laminin (Sensi et al., 1986) and fibronectin (Tarsio et al., 1988) and glycation of the cell binding domain of type IV decreased endothelial adhesion and spreading (Haitoglou et al., 1992). Similarly, platelet aggregation and adhesion to collagen are known to be dependent on the quaternary structure of the collagen (Barnes et al., 1980) and involve specific domains in the molecule. Glycation of collagen increases platelet aggregating potency which could lead to increased risk of thrombotic disease in elderly subject. Nitric oxide is a key regulator of vascular tone and studies have demonstrated that glycation indirectly quenches its activity and specifically blocks the anti-proliferative effect in vascular smooth muscle cells (Hogan et al., 1992). The vasodilatory response to nitric oxide has been reported to correlate directly with the level of AGEs (Bucala et al., 1991) although the nature and location of these AGEs has not been determined. It is possible that the glycation of the basement membrane type IV collagen of the endothelium is involved in these changes. We can assume that the changes involved in all these reactions are modifications of the existing amino acid side chains, either simple additions of glucose to form the hexosyl-lysines and thus nullify the positive charge of the lysine, or oxidative modifications such as carboxymethyl-lysine which changes the side chain lysine charge to a negative carboxyl group, but other modifications probably remain to be identified.

In short, a number of side-chain modifications have been reported, and these changes, if sufficiently extensive to affect the charge profile, or possibly the cell or matrix interaction sites, may affect the remodelling of collagenous tissue and turn out to be a very important aspects of the ageing mechanism. It is therefore of interest to identify these modifications.

3.8. Lysine modifications

3.8.1. Nε-(Carboxymethyl)lysine

*N*ε-(Carboxymethyl)lysine is a non-fluorescent product (Ahmed et al., 1986) which has been shown to accumulate with age (Dunn et al., 1991; Dyer et al., 1993). Several mechanisms of formation have been reported, oxidative degradation of the Amadori product (Ahmed et al., 1986; Dunn et al., 1991), the reaction of glyoxal with lysine (Alabed and Bucala, 1995) and by oxidation of both the Schiff base and the Amadori product (Glomb and Monnier, 1995) (Fig. 16a). *N*ε-(Lacatolysine), (LL), is also formed by the oxidative degradation of the Amadori product via a 3,4-ene-diol intermediate. Formed during the degradation of synthetic fructosamine it has also been detected in proteins glycated in vitro, urine and in lens protein (Ahmed et al., 1988). *N*ε-(Carboxyethyl)lysine, (CEL), a homologue of CML has also been detected in lens protein at levels comparable to CML and has been shown to increase with age and has been proposed as a marker of oxidative stress (Ahmed et al., 1997). It can be formed in vitro by the reaction of lysine or protein with a number of carbonyl compounds, the highest yields being with methylglyoxal and triose phosphates (Ahmed et al., 1997).

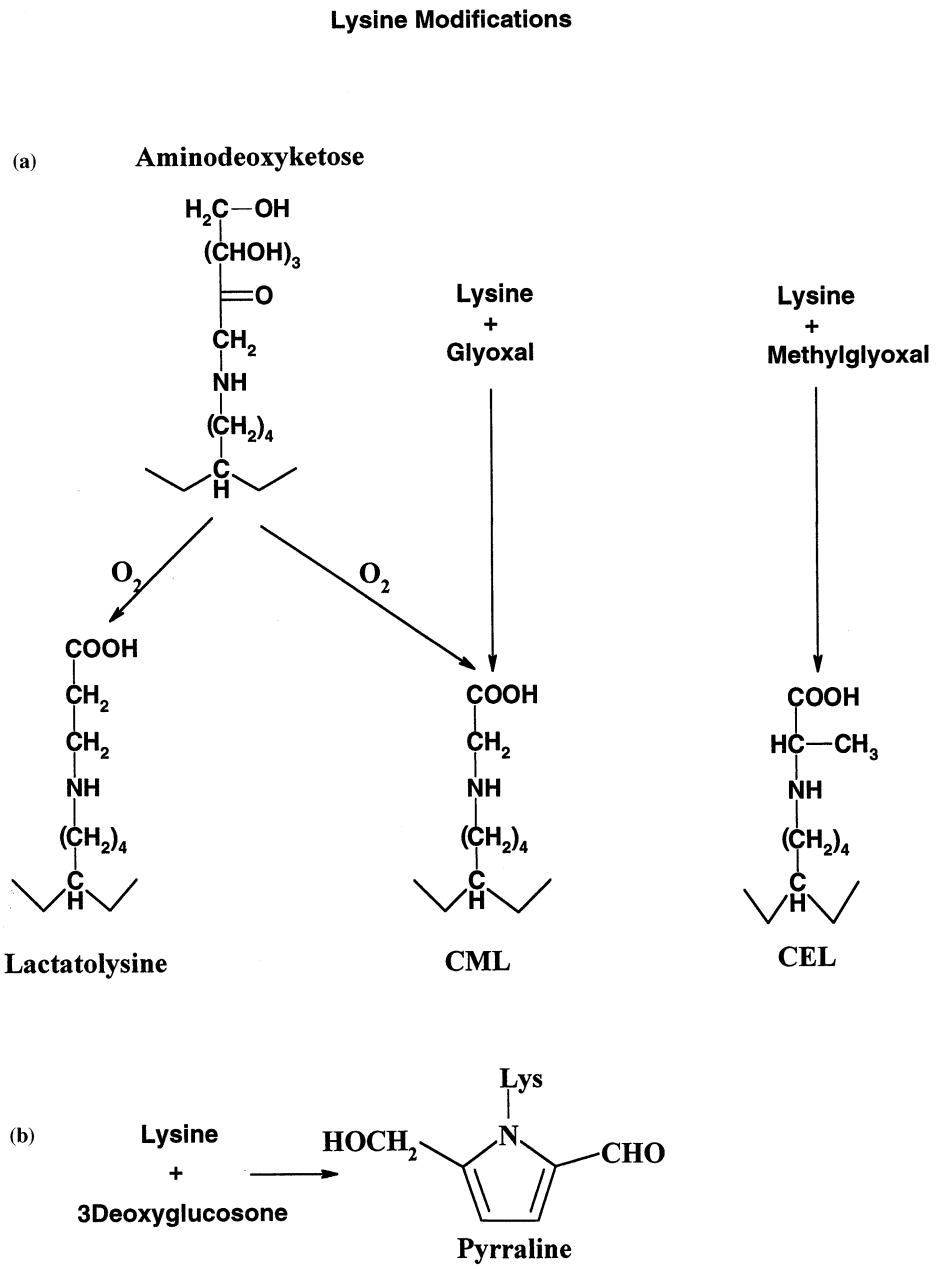


Fig. 16. Modification of lysine side-chains. (a) Oxidation of the Amadori product to carboxymethyl-lysine (CML) and lactato-lysine, or direct reaction with glyoxal and methylglyoxal to form CML and carboxyethyl-lysine respectively (CEL). (b) 3-Deoxyglucosone reacts with the lysine side-chain to form pyrraline.

3.8.2. Pyrraline

Pyrraline is another lysine derivative, has also been identified in a variety of tissues by the use of antibodies (Miyata and Monnier, 1992; Marion and Carlson, 1994; Smith et al., 1994) and by chromatographic techniques (Henle and Klostermeyer, 1993; Portero-Otin et al., 1995) (Fig. 16b). The mechanism of formation is believed to involve 3-deoxyglucosone (3-DG) as the immediate precursor and reaction with the ε-amino of lysine to form 5-hydroxymethyl-1-alkyl pyrrole-2-carbaldehyde (Hayase et al., 1989; Miyata and Monnier, 1992).

3.9. Arginine modifications

3.9.1. Imidazolones

The modification of arginine residues in collagen by carbonyl compounds results in the formation of the respective imidazolones (Paul et al., 1998), Fig. 17A. Glyoxal reacts to form either *N*ε-(4-oxo-5-dihydroimidazol-2-yl)-L-ornithine (Paul et al., 1998) or 1-(4-amino-4-carboxybutyl)-2-imino-5-oxo-imidazolidine (Schwarzenbolz et al., 1997), whereas methylglyoxal forms a 4-imidazolon-2-yl derivative existing in three tautomeric forms (Westwood et al., 1997; Paul et al., 1998). Imidazolones derived from 3-deoxyglucosone have also been synthesised in vitro (Konishi et al., 1994; Hayase et al., 1995). Antibodies specific to these imidazolones have been used to indicate their presence in situ (Niwa et al., 1997) but they have not been confirmed by isolation from collagenous tissues.

3.9.2. Argpyrimidine

A pyrimidinium compound has also been shown to be formed during the in vitro incubation of methylglyoxal and NH_2 chemically blocked arginine (Alabed et al., 1996b). This compound was later described by Shipanova et al., (1997) and named argpyrimidine (Fig. 17B). Two mechanisms for its synthesis have been proposed one via the formation of double Schiff base adducts (Alabed et al., 1996b) and the second through a 3-hydroxypentane-2,4-dione intermediate (Shipanova et al., 1997). It is acid labile and has distinctive fluorescent characteristics which resemble that of modified protein. However, it has not been isolated and definitively characterised from an in vivo glycated protein.

3.10. Modification of cell-matrix interactions

The modification of arginine raises the important question of how this would affect the crucial cell-matrix interactions involving motifs containing arginine, such as RGD. This would obviously be particularly important in the case of cell-basement membrane (type IV collagen) interactions since they support various cell types and influence their differentiation during remodelling.

Recent studies have also indicated that imidazolones can act as monocyte/macrophage AGE receptor ligands which may also have profound effects upon collagen-cell interactions (Westwood et al., 1997). Researchers have attempted to locate cell-matrix recognition sites and a number of arginine dependent sites have

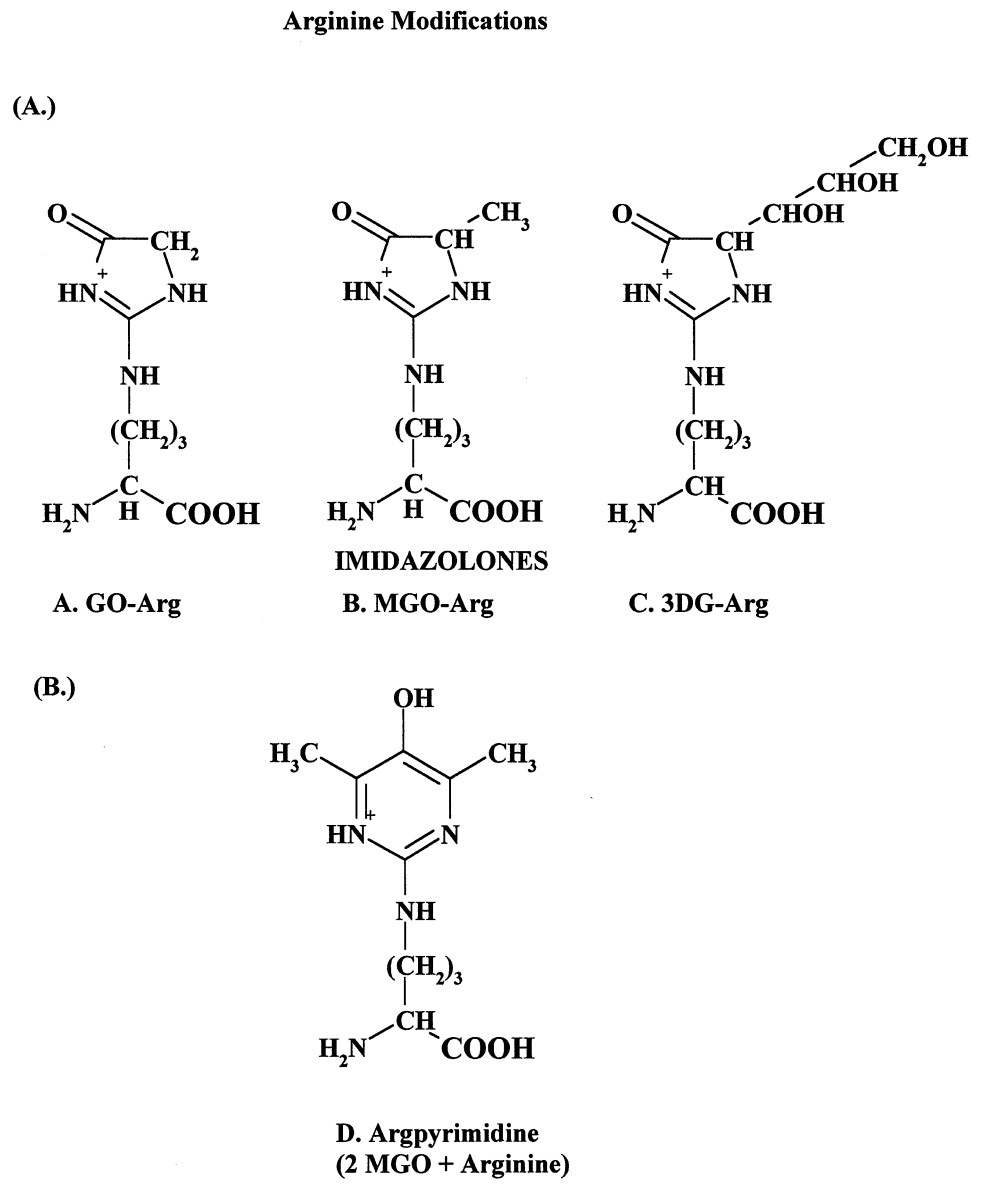


Fig. 17. Modification of arginine side-chains. (A) Imidazolones formed by reaction of glyoxal, methylglyoxal and 3-deoxyglucosone with arginine side-chains. (B) Argpyrimidine is formed from the reaction of two methylglyoxal molecules and peptide bound arginine.

been identified. Early studies (Cardarelli et al., 1992) demonstrated that certain cell lines adhere to collagen type I via $\alpha 2\beta 1$ integrins and that cellular adhesion to this matrix could be inhibited by cyclic RGD containing peptides which interact directly

with $\alpha 2\beta 1$ integrin. Similarly, Ruggiero et al., (1996) reported cyclic RGD peptides dramatically reduced adhesion of HT 1080 cells to type V collagen. Native type V collagen supports adhesion of a large variety of cell lines and normal fibroblasts with $\alpha 1\beta 1$ and $\alpha 2\beta 1$ integrins as the major receptors. Basement membrane (type IV) was also an obvious target for the demonstration of cell-collagen interactions and the role of the RGD peptide. Eble et al., (1993) found that the main recognition site for $\alpha 1\beta 1$ on type IV is 80 residues long and 100 nm from the N-terminus and that there are two sites for $\alpha 2\beta 1$ but this latter receptor is RGD independent (Kern et al., 1993). However, within the $\alpha 1\beta 1$ site there are two aspartate residues on the $\alpha 1$ (IV) chain and one arginine on the $\alpha 2$ (IV) chain which were shown to be essential for interaction with $\alpha 1\beta 1$. The spatial consideration of Asp-Arg at the same position in two different α(IV) chains only occurs once in the entire $\{\alpha 1(IV)\}2\ \alpha 2(IV)$ collagen molecule and this is found in CB3 (IV) near the N-terminus. Grab et al., (1996) have recently demonstrated the importance of a peptide containing Arg, but not in an RGD sequence, in the mechanism of degradation of collagen. They found that the peptide, which corresponded to the sequence of amino acids from 772-782 in type I collagen was almost as potent in adhesion of skin fibroblasts as native collagen itself. This 15 amino acid peptide contains Arg at residue nine. There is evidence that the peptide 772-786 mediates the proposed mechanism of collagen turnover by regulating the production of MMPs 1, 2, and 8 that cleave the triple helix of type I collagen at residue 775 (Gly-Leu). Although the precise mechanism is unknown it may be related to $\alpha 1\beta 1$ binding of this peptide of type I, in which the Arg residue at 781 may be involved in tyrosine phosphorylation and induction of MMP-1 mRNA levels (Riikonen et al., 1995). An Arg residue containing peptide Gly-Gly-Pro-Hyp-Gly-Pro-Arg which corresponds to residues 522-528 of type I collagen has been reported as the minimum recognition site for $\alpha 2\beta 1$ during the aggregation of platelets (Morton et al., 1997).

Arginine residues are clearly involved in recognition sites for the two matrix integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ (Tuckwell and Humphries, 1996) and if modified by glycation in vivo could have far reaching effects in all aspects of cell matrix communication. In preliminary experiments we have demonstrated that the interaction of both MG63 and HT1080 cells with collagen is significantly affected by prior incubation of the collagen with methylglyoxal (10 mmol, 19 h at 37°C). Both the attachment of cells and their morphology was affected. Adherence of MG63 and HT1080 cell lines to the modified matrix was inhibited by 70 and 90% respectively. In both cases the cells that did attach to the modified matrix were much rounder in appearance than cells incubated in the presence of unmodified matrix. Adherence of both cell types to normal matrix was similarly inhibited by incubation of cells in the presence of anti-$\alpha 2\beta 1$ antibodies confirming the importance of integrin mediated binding to collagen in both cell lines. Clearly the formation of imidazolones on the arginine side-chains affects collagen-cell interactions. Glycated collagen-cell interactions and the nature of the cell receptors should become a major area of investigation in the future, particularly in respect of basement membrane type IV collagen.

A number of AGE specific receptors have now been described (Table 1) although, Shaw and Crabbe (1994) have suggested that the interaction of AGEs and macrophages is predominantly non-specific.

AGE receptor interactions have also been linked to the development of pathological conditions resulting from AGE formation. Interaction with RAGE (Receptor for Advanced Glycation End-products) is thought to play a central role in the onset of vascular disease in diabetes through chemotaxis, generation of cellular oxidant stress, and associated increase in the levels of vascular adhesion molecule-1 (VCAM-1). This has the effect of increasing attachment of mononuclear macrophages to the vessel wall and results in the generation of a pro-inflammatory environment necessary to promote vascular pathology (Hori et al., 1996). A cascade of events that is thought to be linked to the activation of NF-kB sites within the promoter of VCAM-1 and is instigated by the binding of AGEs to RAGE and increased oxidative stress (Schmidt et al., 1995). The interplay between AGEs and cellular receptors is complex and elicits multiple effects which have been reviewed (Vlassara et al., 1985; Schmidt et al., 1996; Yan et al., 1997). However, a clear understanding of AGE/receptor interactions is hampered by the diverse array of AGE compounds used in the previous studies. A number of different proteins have been used, all modified to various degrees and the nature of the AGEs involved was unknown. Consequently, very little is known about which AGEs react with the varied receptors described so far. Westwood et al., (1994) have suggested that there are both competitive and non-competitive binding sites for methylglyoxal (MGO) and glucose modified proteins on murine macrophages. More recently, this same group has shown that one of the imidazolones described earlier i.e. the 4-imidazolon-2-yl derivative, could displace MGO modified protein bound to human monocytic THP-1 cells and indicates that it is the receptor recognition factor for MGO modified protein (Westwood et al., 1997). Further studies are needed to concentrate on the effect of specific glycation modifications rather than proteins with several different AGEs.

Table 1

AGE binding protein	Identity/homology	References
AGE-1	Oligosaccharyltransferase-48 complex (OST-48)	(Li et al., 1996; Stitt et al., 1997)
AGE-2	80 K-H, a protein kinase C substrate	(Li et al., 1996; Stitt et al., 1997)
RAGE	35 kDa immunoglobulin	(Neeper et al., 1992; Schmidt et al., 1992)
LFL	Lactoferrin	(Schmidt et al., 1992)
Galectin -3	Mac-2 or carbohydrate binding protein-35 (CBP-35)	(Vlassara et al., 1995)
MSR	Macrophage scavenging receptor	(Elkhoury et al., 1994)

3.11. Structural modifications

The intermolecular spacing of the collagen molecules in the fibril has been shown to be increased by glycation with ribose (Tanaka et al., 1988a). The extent of glycation per molecule is usually limited and the mechanism of the increased spacing is obscure. Since glycation changes the charge profile of the collagen it is not surprising that it has been reported to affect the precise aggregation of collagen monomers into fibres (Guitton et al., 1981). Similarly, glycation of the non-triple helical C-terminal (NC1) domain of the type IV monomer has been reported to inhibit lateral association into the typical basement membrane network structure (Tsilibary et al., 1988). The nature of these glycation side-chain modifications is unknown. However, the question arises as to whether glycation of the newly synthesised monomer can occur so rapidly after synthesis and before aggregation occurs in vivo is debatable. The observations do demonstrate a real possibility of a change in the interactions of collagen with other matrix components such as proteoglycans, vitronectin and laminin during tissue development.

3.12. Inhibition of advanced glycation endpoints formation

Current knowledge of the mechanisms involved, albeit limited, has led to attempts or inhibit the formation of AGEs and hence alleviate the effects of glycation during ageing. The most likely route would seem to be pharmaceutical intervention and could involve compounds such as aminoguanidine (Brownlee et al., 1986) a compound which has been shown to inhibit the formation of AGEs and reduce the changes characteristic of glycation damage. Indeed aminoguanidine is currently undergoing patient trials. The mechanism of action is still controversial and may involve direct reaction with the keto-imine or with the released oxidation products, such as 3-deoxyglucosone (Lewis and Harding, 1990) to form substituted triazines (Hirsch et al., 1992) (Fig. 18). On the other hand, Ou and Wolff (1994) report that aminoguanidine accelerates hydrogen peroxide production during glycation and therefore make the interesting suggestion that if aminoguanidine is indeed an effective inhibitor then the formation of AGEs cannot be due to increased tissue oxidation. Whether this in vitro finding translates to in vivo conditions remains to be seen. Clearly the mode of action of aminoguanidine to inhibit glycation damage is at present unknown and requires further investigation.

Several other agents have been shown to be effective in the inhibition of the detrimental effects of excessive protein cross-linking e.g. aspirin (Huby and Harding, 1988; Rao and Cotlier, 1988; Swamy and Abraham, 1989). Fu et al. (1994a) reported that aspirin had little effect on the initial glycation, in contrast to Huby and Harding (1988), but did inhibit glyco-oxidation and cross-linking of collagen. A variety of other compounds have also been investigated as inhibitors of AGE formation and their potential as therapeutic agents highlighted. Hipkiss et al., (1995) demonstrated that the competitive, preferential glycation of carnosine (β-alanyl-L-histidine), prevented the formation of glycation mediated cross-links in protein-glucose incubations, and proposed its use as a non-mutagenic product. The

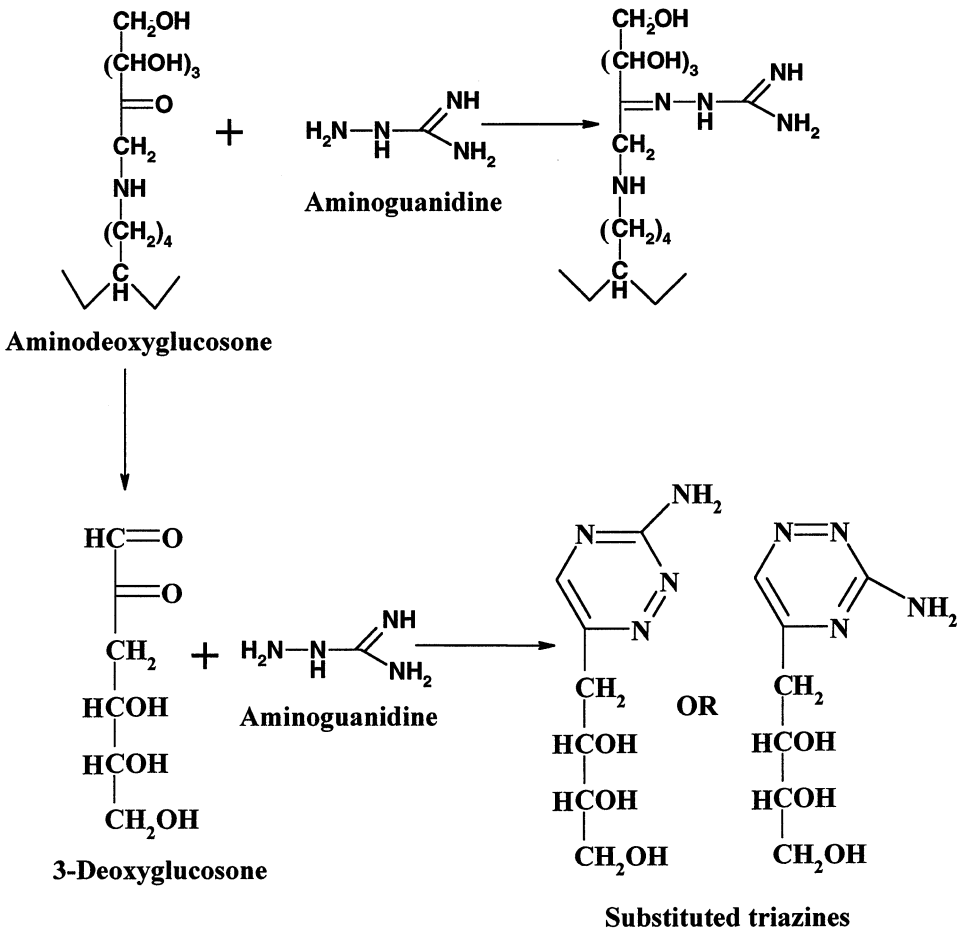


Fig. 18. Inhibition of glycation by aminoguanidine, either by (a) reaction with the Amadori product to form a complex with the keto group. Or (b) with 3-deoxyglucosone to form triazines.

anti-dementia drug Tenilsetam has also been shown to inhibit AGE mediated cross-linking (Munch et al., 1997; Shoda et al., 1997) indicating the possible significance of the Maillard reaction in the progression of Alzheimer's disease. Other candidates with possible roles in the inhibition of AGEs and associated pathologies include thiazolidines (Nakamura et al., 1997b) and organic germanium compounds (Nakamura et al., 1996). Pyruvate and α-ketoglutarate have also been shown to inhibit initial glycation and formation of protein aggregates indicative of cross-linking, the beneficial effects presumably due to competitive inhibition and the radical scavenging properties of these compounds (Varma et al., 1997). The role of other antioxidants, such as vitamin E and flavanoids in the diet (Ceriello et al., 1991; Hartog et al., 1993) do not appear to have been adequately investigated as alternative inhibitors in relation to glycation reactions.

Recently Vasan et al., (1996) reported the chemical synthesis of a compound capable of breaking glycation cross-links. The cross-link breaker, *N*-phenylacyl thiazolium bromide (PTB) reacts with and cleaves covalent AGE derived protein cross-links in vivo (Fig. 19). The carbon-carbon bond of α-diketones is selectively cleaved by thiazolium compounds. These authors reported that PTB decreased AGE cross-linking in glycated rat tail tendon when given to rats with established diabetes. The success of this type of compound assumes that diketones are the major source of collagen glycation cross-linking, although their reactivity would suggest that they are not end-products. However, this type of compound may be the forerunner of treatments to ameliorate the deleterious effects of glycation.

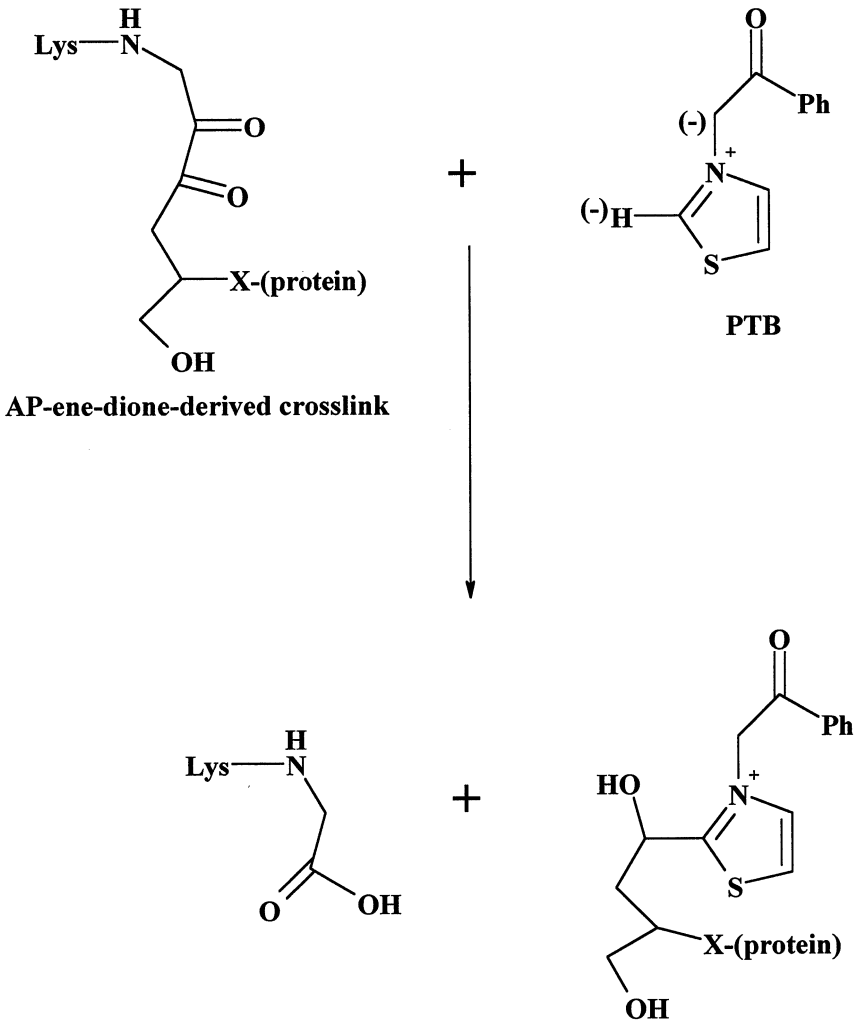


Fig. 19. Proposed cleavage of AGE cross-links possessing a dicarbonyl structure with a thiazolium compound.

An interesting concept, first proposed by Vlassara et al., (1985) is that the body itself has a mechanism for removing AGEs in that the AGE/receptor interactions may be involved in a process by which AGE modified proteins can be targeted and removed by macrophages. AGEs have been reported to be chemotactic for macrophages and induce recruitment from the circulation across the endothelium. In addition it has also been shown that the interaction of AGEs with cellular receptors induces the secretion of interleukin-1 and TNF-α suggesting a regulatory system in which macrophages selectively remove AGE modified proteins and then contribute to the repair remodelling process (Vlassara et al., 1985).

4. Summary and conclusions

The age-related changes causing a functional deficiency of collagenous tissues are clearly due primarily to increased intermolecular cross-linking. This can account for the observed increased stiffness, enzyme resistance, and permeability and decreased swelling of the tissue. Two completely different mechanisms have been identified.

The first mechanism involves the enzymically controlled lysine-aldehyde cross-links which are now fairly well established and the differences between tissues depends on the extent of hydroxylation of the collagen, particularly the telopeptide lysines, which may be different at the N and C-termini. There is a clear route from immature divalent cross-links to mature trivalent cross-links, although not all the maturation products have been characterised. The location of the divalent bonds via the end-overlap regions has been determined and correlates with the mechanical properties of the fibre. However, the subsequent mechanisms of increase in strength with age, which we have proposed involves interfibrillar cross-links, remains to be confirmed. Some other questions remain to be answered, for example, to confirm that the deficit of pyridinoline cross-links in bone is made up by the pyrrole cross-links; whether the pyrrole forms interfibrillar links; what is the nature of the mature cross-links in rat skin, mouse skin and basement membrane. The basic biochemical principles of the age-related changes in enzymic cross-linking have been established but the important relation of the mature and immature cross-links to increased mechanical properties remain to be elucidated.

The second mechanism is non-enzymic and involves the fairly random addition of glucose to the collagen as its turnover is reduced with age, the products of which ultimately react further to form intermolecular cross-links. It is clear that the cross-link AGEs of collagen accumulate with age and result in substantial dysfunction of just those collagenous tissues responsible for the morbidity and mortality in age, primarily renal, cardiovascular and retinal tissues. The glycation of other proteins involves the same mechanisms, but the long biological half life of collagen ensures that it plays an important role in ageing. The nature and relative importance of the cross-links formed in vivo as opposed to those reported to be formed in vitro with model compounds needs to be established.

The recent studies on the effect of glycation on cell-matrix interaction are of considerable interest and their relative contribution compared to cross-linking needs

to be determined. The age-related glycation changes appear to be similar to those occurring at an accelerated rate in diabetes mellitus and considerable information on the glycation mechanism has been learned from these studies involving higher glucose levels.

Our rapidly increasing understanding of the mechanism of formation of AGEs, particularly those involved in intermolecular cross-linking, will help to develop new inhibitors against the distressing effects caused by the glycation of collagenous tissues during ageing. As the life span increases in affluent societies, the need for ageing research becomes even more important and ageing of collagenous tissue will play a major role.

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