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Structure, Function and Ageing of the Collagens of the Eye

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I. Introduction

The collagenous tissues of the eye have received considerable attention since, apart from the importance of the organ itself, the eye is composed of several highly specialised tissues which possess distinct collagenous structures. It is, in fact, a vivid example of the biological diversity of collagenous tissues. This diversity has recently been shown to be due to the existence of a whole family of collagen molecules that are capable of aggregating in different ways to produce a variety of collagenous structures.[1] At the present time there are about twelve genetically distinct types of collagen in mammalian tissues. They have all been characterised biochemically and their precise location in complex tissues has been determined by immunohistochemical techniques. The collagens identified to date have been classified by several criteria, length of molecule, molecular weight, flexibility of the molecule, and by ultimate supramolecular structure. Employing the latter classification one can consider three groups: the fibrous collagens, the non-fibrous collagens and the filamentous collagens. The variations in structure of these aggregates is shown in Figure 1.

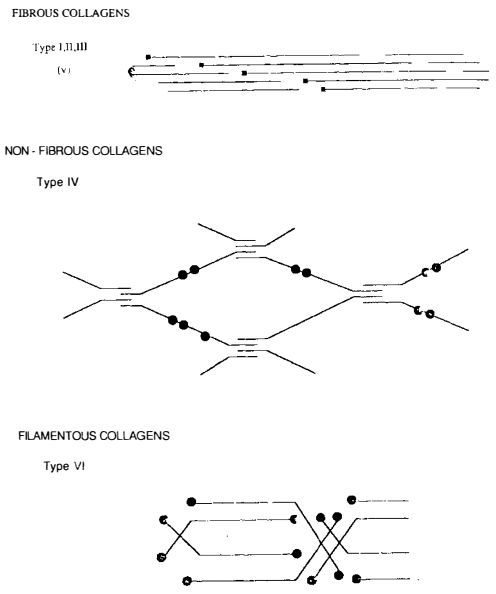

Fig. 1. *Classification of collagen types into fibrous, non-fibrous and filamentous collagen, and the corresponding supramolecular structures.*

Fibrous collagens. These collagens are revealed in the electron microscope as thick fibres with a characteristic axial repeat pattern of 67 nm. The diameter of the fibres varies considerably, from 200 nm in skin and tendon to about 25 nm in the cornea. The size distribution within a particular tissue may be uniform, for example the fibres of the cornea vary from 25 to 30 nm, or may be highly variable as in the skin, where they can vary from 20 to 200 nm.

This group of collagens is comprised of the genetic Types I, II and III collagens. Type I is the major collagen of skin, tendon and bone, and is the most abundant of all the collagens. Type II is the major collagen of cartilage, and Type III of fetal and vascular tissue.

Non-fibrous collagens. In contrast to the fibrous collagens, this group forms the non-fibrous basement membranes, which even in the electron microscope appear homogeneous. They are thin membranes separating

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the fibrous stromal tissue from the cells, and vary in thickness from 25 nm in capillaries and glomeruli to 200 nm in the lens capsule. At the present time the only collagen in this group is Type IV collagen and has been identified in all basement membranes so far examined.

Filamentous collagens. The collagens of this group form loosely aggregated fibres with little or no periodicity. They can be subdivided into pericellular and matrix collagens. This group includes Types VI, VII, IX and X.

Collagenous structures of the eye

The connective tissues of the eye can be divided into specific types for ease of description of their respective collagens:

(1) cornea—is a transparent tissue containing fine collagen fibres of uniform diameter with a high degree of spatial organisation
(2) sclera—is an opaque tissue containing thick interwoven collagen fibres
(3) vitreous body—is a polysaccharide gel containing small amounts of fine collagen fibres
(4) lens capsule—has an apparently amorphous basement membrane structure
(5) retina—the retinal pigmented epithelium is a typical thin basement membrane
(6) choroid—highly vascularised tissue of the iris.

II. Fibrous Collagens of the Cornea, Sclera and Vitreous

(a) *Cornea*

The human cornea contains 90 per cent collagen by dry weight yet, unlike other collagenous tissues, it is transparent. This feature is primarily due to the precise packing of collagen fibres of uniform diameter at a fixed distance apart. The fibres are arranged in layers of parallel sheets with each layer at right angles to the adjacent layer.

It is generally agreed that the major molecular species of collagen in cornea is Type I, although there is some disagreement on the presence of other collagens (Table I). Types II, III and V have been reported. Type III has variably been reported as totally absent, less than 1 per cent, and as much as 20 per cent.[2] Some of the differences could be due to species and age of the tissue examined, whilst others could be due to the technique employed in the analysis. On balance, Type III is probably not present and Type V is between 5–10 per cent. Type II is only present at the embryonic stage. Biochemical studies on Type I collagen show that it possesses a higher level of glycosylation of the hydroxylysine residues than Type I in other tissues. It has been suggested that the extent of glycosylation may control the size of the fibres in the cornea, but this is probably too simplistic an explanation.

Age-related studies have shown that the amount of Type V increases during maturation from 5 to 10 per cent whilst Type III decreases from 2 per cent in the embryonic eye to zero in the adult.[3] This shift in collagen types was determined from pepsin digests. More accurate data could have been obtained by analysis of CNBr cleaved peptides.

Using monoclonal antibodies to follow the developmental changes in location of Type V, Linsenmayer *et al.*[4] found the fibres to be present in a masked form, treatment with acetic acid to swell the fibres being necessary to reveal the antibody sites. Examining 4 day embryo to 1 day post-hatching the Type V in the cornea appears after the sixth day of development when the primary stroma swells and is invaded by mesenchymally derived fibroblasts. Fluo-

Table I *Major collagen types of the eye*

	Fibrous	*Non-fibrous*	*Filamentous*
Sclera	Type I, Type III (~10%)		
Cornea	Type I, Type V (~10%)		Type VI
Vitreous	Type II		Type IX
Lens capsule		Type IV	
Descemet's membrane		Type IV	Type VIII
Bowman's membrane		Type IV	
Retinal pigment epithelium		Type IV	

rescence appears throughout the cornea and it has been suggested that hybrid fibrils of Type I and Type V are formed. In this way Type V may exert an influence on the precise fibril diameter of the corneal collagen. Alternatively, the absence of Type III, which normally co-distributes with Type I, may contribute to the latter's ability to form uniform narrow fibres.

(b) *Sclera*

The sclera protects the intraocular contents from injury and the function of the collagen in the sclera is obviously structural. The strength and resilience of the sclera is imparted by close interlacing of the collagen fibres which account for 80 per cent of the dry weight. In contrast to the cornea, the fibres are like tendon and skin and vary in diameter from 30 μm to 300 μm within a single fibre bundle. The elasticity of the sclera is increased by the presence of a small proportion of elastin fibres.

Sclera contains predominantly Type I collagen (~90 per cent), and a small amount of Type III (~10 per cent). In the avian eye Type II is also present in the cartilaginous scleral support ring.[5]

The biological function of Type III is unknown although it is widely distributed in other tissues of the body. It has been suggested that increases in the proportion of Type III impart greater plasticity to the tissue, for example in fetal tissues and the vascular system. Certainly, when the ratio is disturbed in heritable disorders in favour of Type III, the tissues such as skin and aorta become more flexible and the sclera becomes translucent.[6]

Changes in the proportions of the collagen types occur with ageing and may result in structural changes which could play a role in myopia and glaucoma.

(c) *Vitreous humour*

The central part of the vitreous shows a three-dimensional network of fine collagen fibres of about 7–13 nm in diameter. The collagen is embedded in a hyaluronate gel and contributes to the maintenance of the intraocular pressure, acting as a shock absorber mitigating against the effect of body movements in the eye. The fine collagen fibres have the functions of stabilising the shape of the gel and reducing the compressibility of the hyaluronate gel when exposed to external pressure.

In the electron microscope the characteristic striations of the collagen fibre are difficult to observe in detail unless the fibres are pre-treated with typsin. Biochemical analysis has shown that the vitreous collagen is Type II, although minor modifications have been reported.[7]

The origin of the Type II of the vitreous lies in the early embryonic stages when it is derived from the neural retina which secretes Type II, and some Type V, into the vitreous body. It is not clear which cells synthesise vitreous collagen, indeed it is thought that different cells synthesise the collagen at different stages of development.

Structure and stabilisation of fibrous collagens

The biosynthesis of collagen has been extensively reviewed[8,9] and will not be dealt with here, but the subsequent extracellular aggregation of the molecules is relevant to a discussion of the different connective tissues of the eye and the effect of age on these tissues.

The fibrous collagens are initially synthesised as procollagens possessing large N- and C-terminal globular peptides. These globular domains are enzymatically removed during secretion and fibrillogenesis, thereby allowing lateral association of the long triple helical molecules to form precisely banded fibrils. The assembly is directed by acidic and basic groups of the amino acids, and stabilised by the hydrophobic groups present along the molecule, to form an end-overlap and quarter-stagger alignment of the molecules.[10]

Further stabilisation of the fibres to impart tensile properties to the tissue occurs through the formation of intermolecular covalent bonds. Specific lysines in the residual N- and C-terminal non-helical regions are enzymatically oxidised through the ε-amino groups to reactive aldehydes. The precise alignment of the molecules in the fibre ensure this reactive aldehyde is opposite the ε-amino group of a hydroxylysine in an adjacent molecule. The resulting reaction produces stable covalent aldimine or keto-imine bonds throughout the fibre (Fig. 2). The difference in the two cross-links probably arises from the different levels of lysyl hydroxylase in the

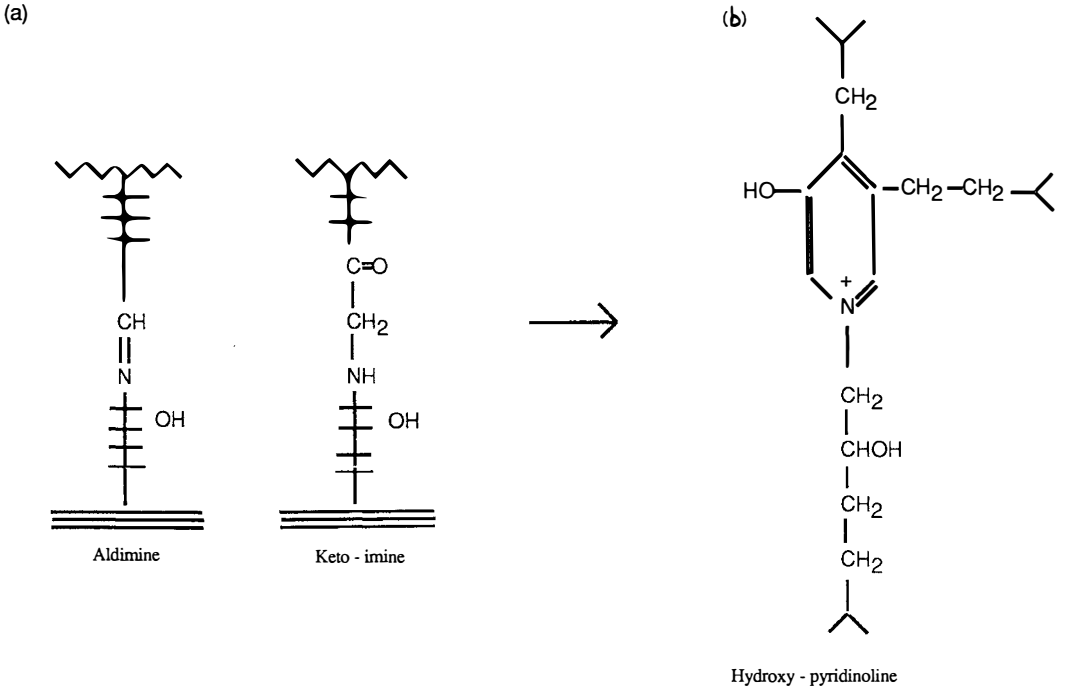

Fig. 2. *Chemistry of the collagen cross-links:*
(a) cross-links present in immature collagen, the aldimine and keto-imine divalent cross-links
(b) trivalent hydroxypyridinoline cross-link present in mature tissue

tissue such that the N- and C-terminal lysyl residue can be lysine or hydroxylysine. The physiological significance of the two different types of cross-link has not been established. This polymerisation of the molecules within the fibre imparts the mechanical properties necessary for collagen to act as a supporting tissue.[11]

The relative proportion of the keto-imine and aldimine cross-links varies with the age and nature of the collagenous tissue. Variation with age is illustrated by the high proportion of the keto-imine in embryonic corneal collagen and a changeover to only the aldimine in the young animal (Fig. 2). Tissue specificity across many species is demonstrated by the predominance of the aldimine in dermal collagen, the equal proportion of the two in Achilles tendon, and exclusively the keto-imine in cartilage collagen. Within these tissues a number of different types of collagen may be present but they will all possess the same type of cross-link. Cross-linking therefore appears to be tissue specific rather than collagen type specific.

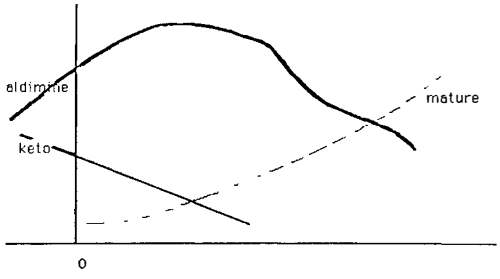

Fig. 2*(c) variation in the proportion of the different cross-links with the age of the corneal collagen.*

The divalent cross-links are crucial to the production of high tensile properties of the immature fibre. However, the mechanical stability of the fibre continues to increase during maturation, resulting in a fibre less susceptible to swelling and enzyme degradation (Table II). At the same time the proportion of the divalent cross-linking decreases. These aldimine and keto-imine divalent cross-links have now been shown to undergo further reaction to form stable multivalent cross-links

Table II *Effects of ageing on collagen fibres*

Increase in fibre size
Increase in tensile strength
Increase in thermally or chemically induced isometric tension
Decrease in solubility
Decreased susceptibility to proteolytic degradation
Decreased ability to swell
Decrease in reducible and increase in mature cross-links

thereby providing further stability to the fibre. For this type of interaction to occur the molecules must be in register rather than quarter-staggered. We suggested therefore that the polymerisation of collagen occurs in two stages. The first stage involves end-overlap head-to-tail cross-linking to form a long polymeric fibril, and the second stage the transverse cross-linking through interaction of the reducible cross-links of molecules in register (Fig. 3). The formation of these stable multivalent cross-links would result in providing a network throughout the fibre and would account for the increased stability of mature collagen.[12]

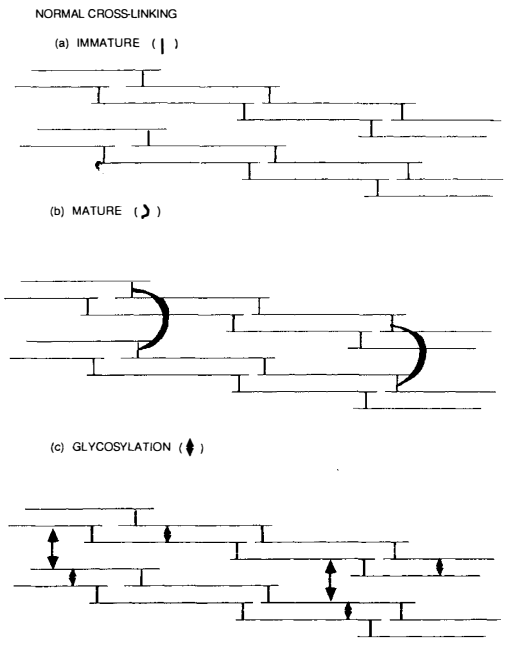

Fig. 3. *Location of the collagen cross-links:*
(a) head-to-tail cross-linking in immature tissues
(b) additional transverse cross-linking formed from the divalent cross-links to produce interfilament multivalent cross-links
(c) random cross-links within and between filaments formed through further reaction of the glucosyl lysines.

The nature of the mature cross-link has not yet been established. Several proposals have been made, and 3-hydroxypyridinoline has received considerable attention. This cross-link is formed from hydroxylysine aldehydes and is found in cartilage and bone, but is not present when the cross-link precursor is lysine aldehyde in tissue such as cornea and skin. The proposal that pyridinoline is the major cross-link in some tissues means that two different mechanisms must be operating in the maturation of collagen since other tissues do not possess pyridinoline.[13] On the other hand, Barnard *et al.*[14] have isolated a complex putative cross-link (Compound M) derived from lysine which is present in both mature bone and mature skin. The characterisation of this cross-link has not been completed, but it is suggested that the presence of this compound in all mature tissues indicates a common mechanism of maturation.

Both the corneal and scleral collagens are initially stabilised in embryonic tissue by the keto and aldimine cross-links, and subsequently only the aldimine bond in the postnatal tissue. In the mature tissue the reducible cross-links disappear and the major cross-link appears to be compound M (Fig. 2). No pyridinoline could be identified in these tissues.

On the other hand, the collagen fibres of the vitreous body are stabilised by the keto-imine bond and 3-hydroxypyridinoline in the mature eye, similar to previous studies on the stabilisation of Type II collagen in young and mature cartilage collagen. However, the proportion of cross-links is much smaller and the extent of maturation apparently slower in the vitreous compared to cartilage.[15]

It is unlikely that the maturation and slight increase in the proportion of the collagen fibres with age dramatically affects the properties of the vitreous gel, liquefaction more likely being due to a modification in the synthesis of the polysaccharide gel.

III. Non-Fibrous Basement Membrane Collagens of the Eye

(a) *The lens capsule*

The lens is surrounded by a collagenous base-

ment membrane, which because of its accessibility has been extensively studied as an example of basement membrane collagen. It is an elastic membrane and appears to be completely homogeneous. Early studies on the amino acid composition, X-ray diffraction and the use of antibodies indicated the presence of collagen in the lens capsule. Despite the transparency and apparently amorphous nature of the membrane in the electron microscope, intact collagen molecules have been isolated and this collagen is referred to as Type IV.

(b) *Descemet's membrane*

This membrane, located under the corneal stroma, is usually referred to as a basement membrane based on light microscope studies, but the electron microscope reveals a more highly organised structure. The structure appears to consist of rigidly orientated fibrils in hexagonal array about 100 nm long with nodes of 27 nm in diameter at either end. X-ray diffraction meridional spacing of 2.86 Å confirmed the presence of triple helical collagen and the 11.5 Å equatorial spacing indicates lateral association of the molecules.

Biochemical analysis indicated the presence of Type IV collagen[16] and about 10 per cent Type V. On the other hand, Schittny *et al.*[17] failed to detect Type IV using immunofluorescent techniques. Davison[3] also reported significant amounts of Type I. More recently the presence of a high proportion of Type VIII collagen has been reported.[18,19] In the light of these results a more detailed analysis of the relative proportion of the various collagen types needs to be carried out.

(c) *Retina*

The delicate light-sensitive retina is supported by a structure known as Bruch's membrane. This is in effect two basement membranes, the pigment epithelial membrane on the retinal side and the endothelial membrane on the choroidal side, with loose connective tissue in between.

Cloned colonies of retinal pigment epithelial cells from chick embryos have been shown to synthesise Type IV collagen in culture, although the conditions appear to be critical in respect of the products synthesised.[20] For example, these cells synthesise Type I and III collagen under conditions favouring fibroblastic morphology of the cells. This change may be important in defects of the retina, particularly fibrosis.

Turkeson *et al.*[21] have recently demonstrated by immunofluorescent techniques that Type IV and the other normal components of basement membrane, laminin, fibronectin, nidogen, heparan sulphate and proteoglycans are indeed components of the intact retinal pigment epithelium and established this membrane as a typical basement membrane.

Structure and stabilisation of Type IV collagen

The non-fibrillar nature of basement membrane clearly indicates a different structural assembly of the Type IV collagen molecules. The molecule itself is longer (400 nm) than the fibrous collagens and retains its large globular C-terminal peptide after secretion. Again, unlike the fibrous collagens, the crucial triple helix sequence of Gly-X-Y- is interrupted at a number of sites along the molecule. This results in a much more flexible triple helical molecule. Furthermore, the charge profile along the molecule is completely different from the fibrous collagens. Because of these unique properties Type IV molecules self-assemble to form a tetramer by anti-parallel end-overlap of the N-terminal domains.[22] It has been further proposed that these tetramers aggregate through the C-terminal globular regions to form a non-fibrillar open structure (Fig. 1), which has been referred to as a 'chicken wire net'.[23]

The single molecule framework may not be correct, indeed its obvious fragility would suggest that the molecules are associated in some form of lateral organisation. Yurchenco and Furthmayr[24] have proposed models involving lateral association but without the necessary experimental evidence. More recently Barnard *et al.*[25] have obtained evidence of loose lateral association of about four molecules by X-ray diffraction of stretched lens capsules.

Despite the novel network structure, analysis of the cross-links revealed the same keto-imine bonds in intact lens capsule and following *in vitro* culture[26] to those stabilising the fibrous collagens. The cross-links are located in the anti-parallel aligned N-terminal regions.

Further analysis indicated that the globular regions may be similarly cross-linked, and the presence of cross-links along the molecule suggested the possibility of lateral association of the molecules in the structure.[27]

The age-related changes occur rapidly, the keto-imine being barely detectable in the lens capsules from a 2–3 week old calf, suggesting maturation of this structure occurs as in the fibrous collagens. Analysis for the mature cross-link, pyridinoline, failed to reveal its presence despite its precursor keto-imine cross-link. The mature cross-link, compound M, was however detected in significant quantities. To achieve this second stage reaction of the reducible cross-links would require the sheets in the chicken wire conformation to be in precise register, such that the chains cross-linked by the keto-imine overlap similar regions of chains in the next layer.[27] This second stage polymerisation would greatly stabilise the basement membrane.

Descemet's membrane, like lens capsule, contains the keto-imine cross-link but not the pyridinoline cross-link. The mature cross-link (compound M) was also found to be present. Heathcote *et al.*[28] reported a surprising finding that Descemet's membrane contained desmosine and isodesmosine, normally confined to elastin, as the major cross-linking compounds. It remains to be established which of the different collagen types present in the membrane possess these cross-links.

These age-related changes could have a significant effect on the physico-chemical properties of these membranes.[29] In the case of the lens capsule this could contribute to presbyopia and cataracts. Research on cataracts generally concentrates on the crystallins, but a change in the diffusion of metabolites through the lens could trigger changes in the crystallins.

IV. Filamentous Collagen of the Eye

Few studies have been carried out on the presence of the filamentous collagen of the eye, but it is almost certain that a number of these new collagens will be found associated with the other major collagen types in the eye.

Type VI. Recent studies by Zimmermann *et al.*[30] have identified Type VI collagen as a major component of the extracellular matrix of the human cornea. Indirect immunofluorescence demonstrated that this collagen was distributed throughout the corneal stroma. Whether the Type VI is intimately associated with the Type I fibre or is in the extracellular matrix between these fibres is as yet unknown. In the aorta the Type VI fibrils appear to be located between the striated collagen Type I fibres. It is possible that these Type VI fibrils could act as spacers for the precisely aligned Type I fibres of the cornea.

Type VI forms loosely packed filaments with a repeat period of about 100 nm[31] produced by a unique macromolecular organisation (Fig. 1) of the Type VI molecules.[32]

The cross-linking of Type VI fibres appears to be different from other collagens. Analysis for the known reducible or mature cross-links failed to reveal any significant amounts. Further, the ready extraction of Type VI from various tissues suggests that the fibres are not cross-linked other than through disulphide bonds.

Type VIII. This collagen has only been identified *in vivo* in Descemet's membrane where it has been reported to be a major constituent and may therefore play a role in the unique structure of this basement membrane.

More recently, Type VIII has been reported to be synthesised by corneal endothelial cells.[33] Although the structure has not yet been established completely it is believed to consist of three different α-chains of 61 K with non-helical domains at each end.

The identification of polymers of the α-chains indicated the presence of cross-links but their nature has not yet been determined. Initial studies indicate that they are different from the reducible cross-links of the fibrous collagens.

Type IX. This collagen was initially reported by Duance *et al.*[34] to be closely associated with the pericellular area of chondrocytes of cartilage, but has now been reported to be associated with Type II collagen in other tissues. The vitreous contains about three times more Type IX than cartilage.[35] The cross-linking of Type IX occurs through the same mechanism as the fibrous collagen, at least in cartilage, the

changes in vitreous having not yet been reported.

V. Non-Enzymic Glycosylation

In diabetic subjects the collagen has been reported to undergo 'accelerated ageing' as evidenced by its increase in mechanical stability and resistance to enzymes. It has been suggested that this could occur by enhancement of normal cross-linking. However, we have shown this mechanism does not occur, and a proposed alternative is through the accumulation of glucose moieties.

It has been known for some time that glucose reacts with the ε-NH_2 groups of lysine in proteins, and that this reaction is particularly important in collagen because of its long biological half-life. The initial reaction results in the formation of glycosyl-lysine through an aldimine bond but this is subsequently stabilised by the Amadori rearrangement.[36,37] The presence of a keto group following this rearrangement provides the potential for reaction with further lysine residues, and thereby form an intermolecular cross-link. *In vitro* studies have demonstrated that new covalent cross-links derived from lysine and glucose are indeed formed when collagen is incubated with glucose over long periods of time.[38] These cross-links would be formed as interhelical bonds and therefore have a considerable stabilising effect on the fibril even if only a few such bonds formed. The effect of 'accelerated ageing' is dramatic in diabetics, but similar reactions are occurring slowly in normal collagenous tissues, and may therefore be important in determining age-related changes. The effect may be of little consequence to the thick fibrous tissues such as the sclera, but may be important in changing the charge profile of the fibres in precisely organised tissues such as the cornea. The effect could be even more dramatic in the more metabolically sensitive tissues such as basement membrane of the eye, particularly the capillaries. However, until the nature and extent of these possible cross-links has been elucidated the effect of non-enzymatic glycosylation ageing can only be guessed at.

VI. Conclusions

It is clear that, despite the difference in structure of the collagenous tissue of the eye, at the molecular level, at the supramolecular structure, and at the morphological level, the age-related changes in the properties of the collagen proceed by a similar mechanism. This mechanism involves the enzymic oxidation of specific lysines and the subsequent non-enzymic interaction of these groups due to the highly organised nature of the molecular aggregates. The secondary reaction of non-enzymatic glycosylation, although slow in normal subjects, may also play a role in stabilising the collagen fibre in older tissues. The formation of all these different multivalent cross-links stabilises the collagen, increasing its mechanical strength and resistance to swelling, and enzymic attack. These age-related changes in the properties of collagenous tissues, initially essential for optimal function, could if continued cease to be beneficial.

Excessive cross-linking could result in deleterious effects on the properties of the fibres, possibly causing inflexibility of the fibre, a change in its interaction with other components of the extracellular matrix, and resistance to the enzymes involved in the normal metabolism of the tissue. These changes could be particularly important in affecting the filtration properties of basement membranes. For example, they could be important in cataract formation by affecting the diffusion of specific metabolites through the lens capsule resulting in a change in the properties of the lens components, particularly the crystallins. Increasing stiffness of the lens due to cross-linking of the collagen could also be a component in presbyopia. Similar changes are occurring in the retinal basement membranes and could also lead to retinal disorders. We have learned a lot about the detailed mechanism involved but now need to relate these changes to specific property changes in the ageing collagenous tissues. Disorders of the vision are dramatic and a greater knowledge of the normal ageing process is essential to help understand and perhaps alleviate some of the aberrations.

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