Chemistry of collagen cross-links: glucose-mediated covalent cross-linking of type-IV collagen in lens capsules

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The incubation of lens capsules with glucose *in vitro* resulted in changes in the mechanical and thermal properties of type-IV collagen consistent with increased cross-linking. Differential scanning calorimetry (d.s.c.) of fresh lens capsules showed two major peaks at melting temperatures T_m 1 and T_m 2 at approx. 54 °C and 90 °C, which can be attributed to the denaturation of the triple helix and 7S domains respectively. Glycosylation of lens capsules *in vitro* for 24 weeks caused an increase in T_m 1 from 54 °C to 61 °C, while non-glycosylated, control incubated capsules increased to a T_m 1 of 57 °C. The higher temperature required to denature the type-IV collagen after incubation *in vitro* suggested increased intermolecular cross-linking. Glycosylated lens capsules were more brittle than fresh samples, breaking at a maximum strain of 36.8 ± 1.8 % compared with 75.6 ± 6.3 % for the fresh samples. The stress at maximum strain (or 'strength') was dramatically reduced from 12.0 to 4.7 $N \cdot mm \cdot mg^{-1}$ after glycosylation *in vitro*. The increased constraints within the system leading to loss of strength and increased brittleness suggested not only the presence of more cross-links but a difference in the location of these cross-links compared with the natural lysyl-aldehyde-derived cross-links. The chemical nature of the fluorescent glucose-derived cross-link following glycosylation was determined as pentosidine, at a concentration of 1 pentosidine molecule per 600 collagen molecules after 24 weeks incubation. Pentosidine was also determined in the lens capsules obtained from uncontrolled diabetics at a level of about 1 per 100 collagen molecules. The concentration of these pentosidine cross-links is far too small to account for the observed changes in the thermal and mechanical properties following incubation *in vitro*, clearly indicating that another as yet undefined, but apparently more important cross-linking mechanism mediated by glucose is taking place.

INTRODUCTION

Non-enzymic glycosylation of collagen *in vivo* is a well-established phenomenon which has been the subject of extensive research (for reviews see Baynes and Monnier, 1989; Reiser, 1991). Our initial studies demonstrated the condensation of glucose with the ϵ-amino groups of lysine residues along the collagen backbone, and that the extent of this reaction increased with age (Robins and Bailey, 1972). The first step is the condensation of glucose with the ϵ-amino group of lysine and hydroxylysine of collagen, to form a Schiff's base or aldimine linkage. The extent of glycosylation varies considerably between these residues (Le Pape et al., 1981a), presumably depending on the relative accessibility of the ϵ-amino groups (Watkins et al., 1985; Reiser and Amigable, 1990), and the hexosyl-lysine formed is spontaneously stabilized by undergoing an Amadori rearrangement to form a keto–imine linkage. This glycosylation reaction is accelerated several fold in diabetes mellitus (Monnier et al., 1979; Cohen et al., 1980; Schnider and Kohn, 1980). The hexosyl-lysines subsequently undergo further reaction to form advanced Maillard products, some of which may be fluorescent. Attempts were therefore made to correlate the extent of fluorescence with age, and particularly with the dramatic changes occurring in diabetic patients. With increasing age the physical properties of collagen change, it becomes more soluble, more resistant to enzymes and increases in mechanical stiffness, and all these factors are accelerated in diabetes mellitus (Andreassen et al., 1981; Schnider and Kohn, 1980; Kohn et al., 1984; Yue et al., 1984). We have provided evidence for glucose-mediated cross-linking (Kent et al., 1985), while Brennan (1989) suggested that cross-linking was due to changes in the lysine–aldehyde cross-linking.

Elucidation of the chemical nature of the glucose-mediated intermolecular cross-linking of collagen has only recently been reported and is as yet incomplete. Employing a model system of reacting polylysine with glucose *in vitro*, Pongor et al. (1984) synthesized a fluorophor, 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI), as a possible cross-link, and Chang et al. (1985) used a radioimmunoassay to detect its presence *in vivo*. Despite this evidence, Monnier and his colleagues (Njoroge et al., 1989) reported that the structure identified was an artefact of the isolation procedure. However, it may be that FFI-like structures exist *in vivo*. Subsequently Sell and Monnier (1989) characterized a putative cross-link from glycosylated fibrous collagen, designated pentosidine in view of its derivation from a pentose. The formation of pentosidine would appear to be derived directly from ribose or involve the prior degradation of glucose to a pentose (Dyer et al., 1991; Grandee and Monnier, 1991). The formation of cross-links such as pentosidine could have a significant effect on sensitive tissues such as basement membrane. Indeed, one of the characteristic manifestations of long-term diabetes mellitus is the thickening of collagenous basement membranes (Vrako, 1978) resulting in diabetic microangiopathy, which is believed to lead to renal failure, blindness or arteriosclerosis (Spiro, 1976). Although biochemical analysis of the basement membrane has revealed few changes, a consistent finding is the increased glycosylation, i.e. hexosyl-lysine, of the type-IV collagen of the basement membranes of the kidney (Cohen et al., 1980) and lens capsule (Mandel et al., 1983; Trueb et al., 1984; Garlick et al., 1988). Cohen et al. (1980) suggested

Abbreviations used: d.s.c., differential scanning calorimetry; HMF, 5-hydroxymethylfurfural; T_m, denaturation (melting) temperature of lens capsules; T_D, denaturation temperature of purified type-IV collagen molecules.

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that non-enzymic glycosylation of glomerular basement membrane could interfere with the normal lysine–aldehyde cross-linking of the collagen framework, thus leading to large pores and a loss of size-selective filtration capacity. However, such glycosylation would have to occur on the soluble type-IV molecule before incorporation into the cross-linked macrostructure. Le Pape et al. (1981b) also showed a doubling of the glucose binding to glomerular basement membrane during the hyperglycaemic state in streptozotocin-induced diabetic rats and proposed a decrease in normal cross-linking. Our own studies indicated that the lysyl-aldehyde cross-links were unaffected (Andreassen et al., 1981). Tsilbury et al. (1988) reported that glycation of the NC1 region of type-IV collagen interfered with the normal assembly of the macromolecular structure. The increase in glucose binding amounts to about 1–2 residues of hexose per collagen molecule. Consequently, several authors have suggested that this is a small effect which is unlikely to be a primary cause of the late complications of diabetes (Trueb et al., 1984). On the other hand, the type-IV collagen of basement membrane would certainly be more sensitive to minor changes in the level of glycosylation, compared with fibrous type-I collagen. It is possible that 1–2 residues of glucose could attach at sensitive regions of the type-IV molecule, for example, the collagenase site, or the glycoprotein interaction sites, and thereby affect the selective filtration properties of the membrane. Indeed, Tarsio et al. (1987) reported a 3-fold reduction in affinity of type-IV collagen fibronectin and heparan sulphate when these proteins are glycosylated, and there have been reports of decreased heparan sulphate content of glomerular basement membrane in diabetes (Brown et al., 1982; Parthasarathy and Spiro, 1982; Rohrbach et al., 1982). Chemical cross-linking of glomerular basement membrane by dimethylmalonimide or glutaraldehyde has been shown to render the membrane permeable to proteins (Walton et al., 1992) analogous to the changes in its properties in diabetes. Similarly, intermolecular cross-linking mediated by non-enzymic glycosylation could not only affect the permeability, but also the unusual mechanical properties of lens capsule which are so important in accommodation, i.e. a high elasticity analogous to rubber at low stress and an elasticity modulus ten times that of rubber at high stress (Fisher and Wakeley, 1976).

The physical properties of the lens capsule could be sufficiently perturbed by glucose-mediated intermolecular cross-linking to result in the observed pathology. We have therefore undertaken a study of the effect of non-enzymic glycosylation on basement membrane. The apparent absence of lateral alignment of the type-IV molecules in the 'network' model of Timpl et al. (1981) poses the interesting question as to whether such intermolecular cross-linking could form in basement membrane. In this paper we report changes in the mechanical and thermal properties of the anterior lens capsule which are consistent with increased cross-linking of the type-IV molecules of the basement membrane framework.

MATERIALS AND METHODS

Materials

Anterior lens capsules were dissected from 300-day-old bovine eyes within 4 h of slaughter. To ensure similar orientation full-width strips (approx. 8 mm × 16 mm) were cut from the horizontal axis of capsules to be used for mechanical testing, otherwise the capsules were left intact.

Human lens capsules were obtained from the National Eye Bank, Bristol, courtesy of Professor D. L. Easty. Capsules from diabetic and normal age-matched controls were stored at −20 °C until analysed for pentosidine content. Insufficient human capsules were available for repetitive mechanical and differential scanning calorimetry (d.s.c.) analyses.

Collagenase treatment

For collagenase treatment capsules were taken up at 1 mg dry wt./ml in 0.05 M Tris/HCl, pH 7.5/0.2 M NaCl/0.002 M $CaCl_2$ and incubated with 0.1 mg of collagenase/ml (collagenase CLSPA, Cooper Biomedical) for 20 h at 35 °C. The incubation mixture was dialysed into 0.05 M acetic acid and freeze-dried, then taken up in PBS (0.15 M NaCl/0.02 M sodium phosphate, pH 7.4) and analysed in the differential scanning calorimeter. D.s.c. was also performed on purified reprecipitated type-I collagen before and after collagenase treatment to demonstrate the effect of collagenase on the d.s.c. thermogram.

Extraction of proteoglycan

(i) Lens capsules were incubated in 4 M guanidine hydrochloride/50 mM Tris/HCl (pH 7.5) at room temperature overnight, then washed extensively in PBS before analysis by d.s.c.
(ii) Lens capsules were also incubated with hyaluronidase (1 mg/ml) in PBS at room temperature for 72 h then washed in the same buffer before analysis.

Analysis for glycosaminoglycans

The extracted lens capsules were analysed for residual glycosaminoglycans by the method of Bitter and Muir (1962) using the carbazole reagent and employing glucuronolactone as the standard.

Monomeric type-IV collagen

Purified solubilized type-IV collagen and the isolated helical 'leg' domain of the type-IV collagen molecule were also examined by d.s.c. These fractions were prepared as described previously for pepsin digestion of human placenta (Bailey et al., 1984). Briefly, the intact basement membranes were homogenized and suspended in 0.5 M acetic acid and digested with pepsin (ratio 100:1) at 4 °C for 24 h. The solubilized collagens were reprecipitated, type IV remaining in solution. The presence of typical type-IV tetramers was demonstrated by electron microscopy following rotary shadowing.

To obtain the single molecule helical region a second pepsin digestion was carried out on the isolated type-IV collagen (2 mg/ml) at 20 °C for 70 h. Confirmation of the presence of helical or 'leg' regions was obtained by electrophoresis and electron microscopy.

Glycosylation *in vitro*

Lens capsules were incubated in screw-cap bottles with PBS at 35 °C for periods of up to 24 weeks. Toluene (50 μl) was added to 20 ml of PBS to prevent bacterial and fungal growth. The use of streptomycin and amphotericin was discontinued following the production of spurious fluorescent peaks after hydrolysis. Glycosylated samples were incubated *in vitro* with 133 mM glucose while controls were incubated without added glucose or in the presence of 133 mM mannitol to provide conditions of similar osmolality. After the incubation period samples were dialysed into PBS at 4 °C to remove unbound glucose.

The extent of glycosylation of lens capsules was measured by a modification of the method of Fluckiger and Winterhalter

(1976) in which 5-hydroxymethylfurfural (HMF) liberated from hexosyl-lysine or hexosyl-hydroxylysine by hydrolysis with oxalic acid was determined colorimetrically at 443 nm after reaction with thiobarbituric acid.

Mechanical properties

The mechanical properties of lens capsules were tested in a miniature tensile-testing apparatus (Nene Instruments, Wellingborough, Northants., U.K.). A rectangular support (14 cm × 30 mm) was cut from an acetate sheet, and a rectangular hole (4 mm × 8 mm) cut in the centre. Strips of lens capsule (1.5 mm × 16 mm) were fixed with cyanoacrylate to the acetate support, and the length suspended across the central hole was measured using a travelling microscope. The acetate support was then glued to the two arms of the testing device, which was immersed in PBS. The central portion of the acetate support was cut away leaving the strip of lens capsule hanging freely.

The strip was extended at a constant rate, and allowed to return to the original length nine times, then extended to breaking point. The recycling procedure was carried out to provide evidence of change at low strain, that is in the physiological range. Force and extension were measured on a chart recorder. Following rupture of the capsule the two broken ends were cut from the support, freeze-dried and weighed.

Stress/strain curves were plotted, stress being expressed as (force × length)/dry weight of sample and strain as a percentage change in length [(increase in length/original length) × 100]. Also calculated were the maximum strain at breaking point, and the stiffness (stress/strain) at 30% strain.

D.s.c.

Lens capsules were analysed in a Perkin–Elmer DSC-2C at a heating rate of 10 °C/min from 5 to 95 °C. Samples (7–10 mg wet wt) of material were used for each run, with an approximately equal quantity of PBS. The method was essentially as described by Miles et al. (1986). Lens capsules were tested in the fresh state or after incubation *in vitro* for 4, 12 or 24 weeks either without addition or in the presence of 133 mM glucose or 133 mM mannitol. Samples of lens capsules were also pretreated with collagenase or guanidine hydrochloride before analysis by d.s.c. From the energy input peaks obtained on the thermograph T_m (the denaturation temperature for intact capsules) and T_D (the denaturation temperature for purified type-IV collagen) (for molecules in solution) were determined as the temperatures at which the peak reached a maximum.

Cross-link analysis

The samples were hydrolysed in 6 M HCl under nitrogen, the HCl was removed by rotary evaporation and the residue dissolved in water. The cross-linking amino acids were separated from the standard amino acid using a CF1 cellulose column employing organic buffers, the cross-links were then eluted with water, and the eluate freeze-dried. The reducible lysine-derived cross-links and their mature products were analysed by ion-exchange chromatography using an LKB 4000 AutoAnalyser (Pharmacia) employing a ninhydrin detection system. An aliquot of the eluate from the CF1 column was applied to this system as previously described in detail (Sims and Bailey, 1992).

The total hydrolysate was analysed by reversed-phase h.p.l.c. (LKB) using a Hypercarb S 1000 mm × 4.6 mm column employing a gradient of 12–34% acetonitrile containing heptafluorobutyric acid (0.5%) at 1.1 ml/min. The coefficient of variation for retention times of pentosidine was 2.16%, and for reproducibility of response to constant loadings of pentosidine was 1.9%.

RESULTS

Thermal properties of lens capsules

(a) Fresh lens capsule

Figure 1(a) shows a typical d.s.c. thermogram of fresh bovine anterior lens capsules. There were two major peaks, the first around 53–55 °C (T_m 1), and the second at 88–90 °C (T_m 2), which indicates two separate regions of different stability within the structure of lens capsules. Treatment of lens capsules with highly purified collagenase abolished both of these characteristic peaks, giving a featureless thermogram (Figure 1b), demonstrating that the peaks in Figure 1(a) were due to the collagenous component of lens capsules. As a control, purified reprecipitated type-I collagen was treated with collagenase and a similar loss of the thermographic profile was observed (results not shown). Figure 1(c) shows a thermogram of lens capsules treated with guanidine hydrochloride to remove glycoprotein components of the basement membrane which were not covalently bound within

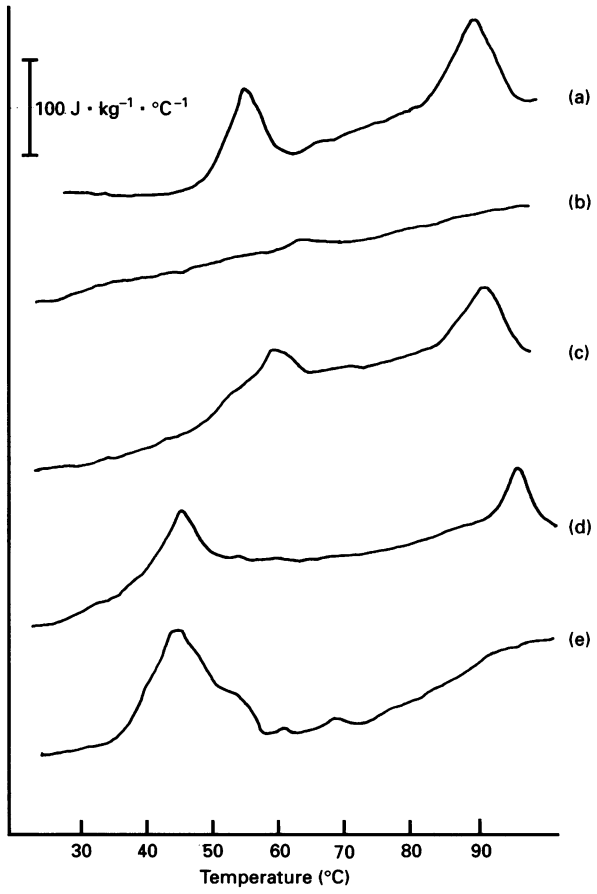

Figure 1 D.s.c. thermograms of lens capsules and purified type-IV collagen

Thermograms of fresh lens capsules (**a**), collagenase-treated fresh lens capsules (**b**), guanidine hydrochloride-treated fresh lens capsules (**c**), purified tetramers of type-IV collagen (**d**), and the isolated helical 'leg' region of type-I collagen (**e**).

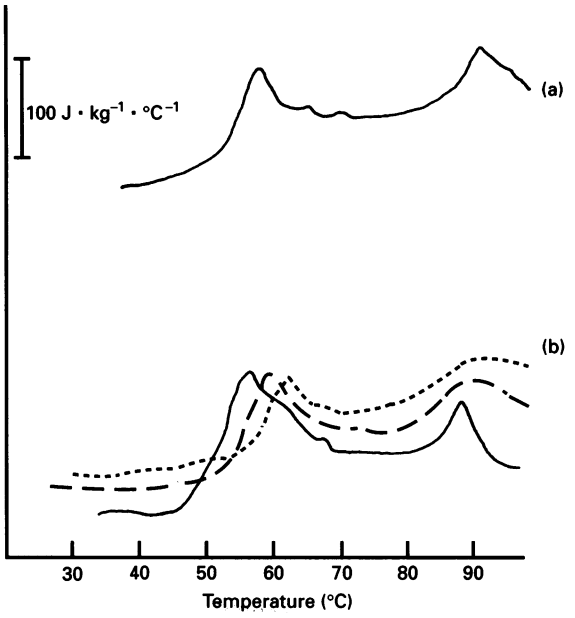

Figure 2 The effect of glycosylation *in vitro* on d.s.c. thermograms of lens capsules

(**a**) Control incubated without glucose for 24 weeks; (**b**) incubated with glucose for 4 weeks (——), 12 weeks (– – –), and 24 weeks (. . . .).

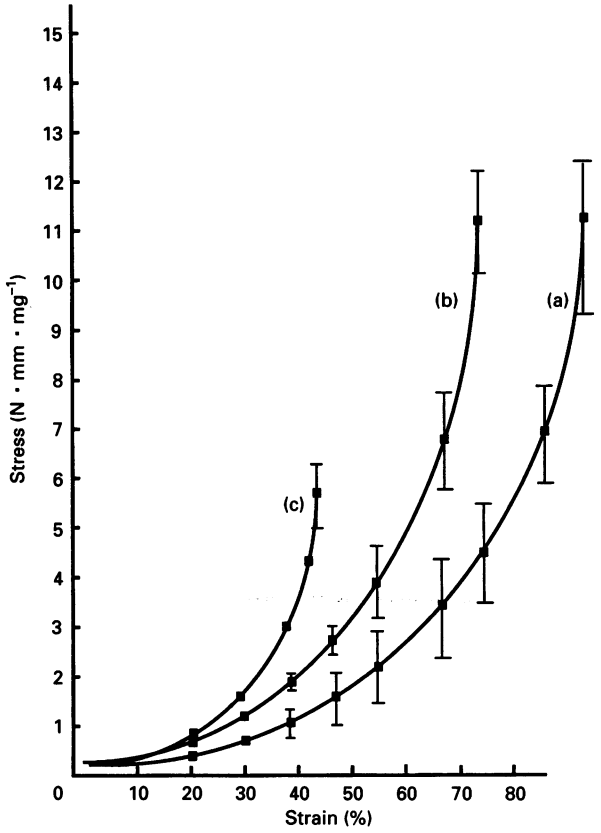

Figure 3 Stress/strain curves of anterior bovine lens capsules

Mechanical tests were performed on fresh lens capsules (**a**); capsules incubated *in vitro* without glucose (**b**); or capsules incubated *in vitro* with 133 mM glucose (**c**). *In vitro* incubations were for 12 weeks.

Table 1 Melting temperature (T_m) of bovine anterior lens capsules determined by d.s.c.

Results are expressed as means ± S.D. of (*n*) samples

Sample	T_m 1 (°C)	T_m 2 (°C)
Fresh lens capsule (0 weeks)	54.5 ± 0.5 (8)	88.8 ± 0.8 (8)
Lens capsules incubated *in vitro* in:		
PBS		
(0 weeks)	54.8 ± 0.5	88.8 ± 0.8
(4 weeks)	55.8 ± 0.5 (3)	89.3 ± 0.2 (2)
(12 weeks)	56.2 ± 0.3 (4)	88.1 ± 0.5 (4)
(24 weeks)	57.0 ± 1.1 (6)	89.8 ± 1.2 (5)
PBS + 133 mM mannitol		
(24 weeks)	56.3 ± 0.6 (3)	89.4 ± 0.2 (3)
PBS + 133 mM glucose		
(0 weeks)	54.5 ± 0.5	88.8 ± 0.8
(4 weeks)	57.5 ± 1.6 (2)	88.8 ± 1.9 (2)
(12 weeks)	58.6 ± 0.5 (3)	89.9 ± 0.7 (3)
(24 weeks)	61.2 ± 1.8 (10)	91.5 ± 1.9 (9)

the matrix. The early peak (T_m 1) was considerably reduced, while the later peak (T_m 2) was still prominent.

Guanidine hydrochloride was more effective than the hyaluronidase in reducing the glycosaminoglycan content to 9% and 24% of their original levels respectively. It is clear from the thermal denaturation thermographs that the removal of glycosaminoglycans had no significant effect on the actual temperature at which thermal denaturation of the helix occurred, i.e. T_m 1.

(b) Soluble type-IV collagen

The d.s.c. thermograms of purified type-IV tetramers, and of the isolated triple-helical 'leg' regions are shown in Figures 1(d) and 1(e). The tetramers revealed two peaks, T_D 1 at 45 °C and T_D 2 at 90 °C, while the helical 'leg' regions gave a single peak around 45 °C.

(c) Reprecipitated type-IV fibrils

Type-IV monomers were reprecipitated in 0.02 M Na_2HPO_4 to form non-striated fibrils as previously described (Barnes et al., 1980). The d.s.c. thermograph of these random fibrils reveals a T_m 1 of 51–54 °C, indicating that some lateral aggregation of these helical molecules had taken place in a sufficiently specific manner to produce a higher melting point than that of the individual molecules.

(d) Glycosylated lens capsules

Lens capsules incubated in the presence of 133 mM glucose for 12 or 24 weeks showed an elevation by several degrees of both T_m 1 and T_m 2 compared with those obtained from control incubated capsules, whereas 133 mM mannitol had no significant effect on the melting temperatures (Figure 2, Table 1). Mannitol is a sugar alcohol and therefore cannot form Schiff's bases with the ϵ-amino group of lysine or hydroxylysine. Mannitol acted as a control to demonstrate the possible effect of dehydration, as

Table 2 Mechanical properties of bovine lens capsules after incubation *in vitro*

Results are expressed as means ± S.D. ($n = 3$)

Incubation condition	Stress at 30% strain ($N \cdot mm \cdot mg^{-1}$)	Strength (maximum stress) ($N \cdot mm \cdot mg^{-1}$)	Maximum strain (%)
Without glucose			
(0 weeks)	0.37 ± 0.18	12.0 ± 1.41	75.6 ± 6.3
(8 weeks)	1.31 + 0.22	11.01 ± 0.59	58.7 ± 3.4
(12 weeks)	1.20 ± 0.12	12.14 ± 2.19	59.8 ± 2.2
With glucose			
(0 weeks)	0.37 ± 0.18	12.0 ± 1.41	75.6 ± 6.3
(8 weeks)	2.89 ± 0.75	7.24 ± 0.48	42.2 ± 2.4
(12 weeks)	2.95 ± 0.15	4.69 ± 0.56	36.8 ± 1.8

133 mM mannitol has the same osmolality as 133 mM glucose. This indicates that the elevation of the T_m value in the presence of glucose was a result of glycosylation rather than dehydration of the lens capsule.

Mechanical properties of lens capsules glycosylated *in vitro*

Stress/strain curves of lens capsules incubated *in vitro* for 12 weeks with or without glucose, and of non-incubated controls are shown in Figure 3. Glycosylated lens capsules were stiffer at a given strain (30%) than non-glycosylated capsules incubated *in vitro* and non-incubated controls. The difference in strength at maximum stress between the fresh capsules and those incubated without glucose was not significant, while the strength at maximum stress of the glycosylated capsules decreased dramatically from 12.0 to 4.7 $N \cdot mm \cdot mg^{-1}$ (Table 2).

The glycosylated capsules were also considerably more brittle, breaking at a lower maximum strain of 36.8% compared with 75.6% for the non-incubated controls. A much smaller, although significant, decrease in maximum strain at breaking point was also recorded for the capsules incubated in the absence of glucose, decreasing from 75.6% to 59.8%.

Glycosylation of lens capsules incubated *in vitro* for 8 weeks gave a similar pattern of stress/strain curves, although the changes were less marked. Results for both 8 and 12 weeks are summarized in Table 2.

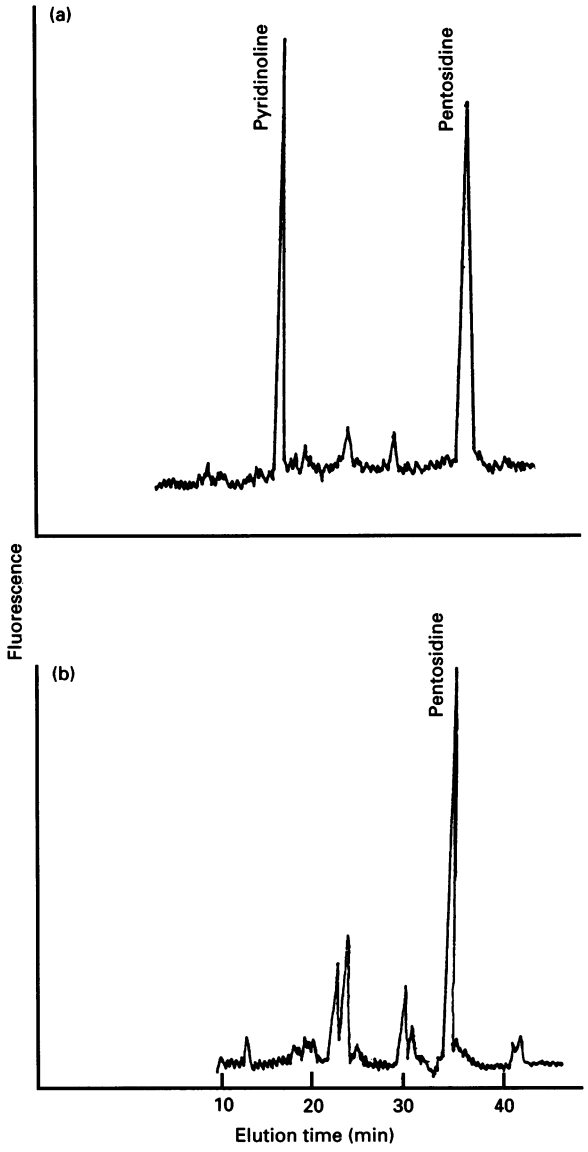

Figure 4 H.p.l.c. chromatograms of fluorescent components

(**a**) Standards of hydroxylysyl-pyridinoline and pentosidine. (**b**) Bovine lens capsule following incubation *in vitro* with glucose for 24 weeks.

Chemical analysis of glycosylated lens capsules

Extent of glycosylation

The level of glycosylation of the control lens capsules was determined as HMF and was 12.5 nM HMF per mg wet wt compared with a level of 43.2 nM HMF per mg wet wt of the lens capsules incubated with glucose for 12 weeks.

Cross-linking components

Lens capsules incubated without glucose were analysed for the reducible intermediate and mature cross-links derived from lysine–aldehyde. No reducible intermediate or the mature cross-links histidinohydroxylysinonorleucine and hydroxylysyl-pyridinoline could be detected in these samples. Previous studies had demonstrated the presence of the precursor-reducible cross-links in young lens capsules (Heathcote et al., 1980). Clearly maturation is very rapid in lens capsules and the mature cross-link remains to be identified.

Lens capsules incubated in the presence of glucose were analysed on the h.p.l.c. system (Figure 4) and revealed the presence of increasing amounts of pentosidine at longer incubation times (Table 3). The concentration increased from negligible levels of one pentosidine per 3000–4000 collagen molecules to one molecule per 600 collagen molecules after 6 months, and one per 200 collagen molecules after incubation for 12 months.

As anticipated from our original collagen incubation studies (Robins and Bailey, 1977) and in line with the results of Fu et al.

Table 3 Pentosodine content of lens capsules

Incubation conditions/source	Pentosidine content (mol collagen/mol pentosidine)
Bovine lens capsules (after incubation *in vitro*)	
Without glucose	
(0 weeks)	3500 (±800)
(8 weeks)	2500
(24 weeks)	2000
With mannitol	
(24 weeks)	3000
With glucose	
(8 weeks)	2000
(24 weeks)	600
(52 weeks)	200 (±17)
Human lens capsules	
Uncontrolled diabetic	74 (±13)
30-year-old control	1000

(1992) lens capsules incubated in the absence of oxygen revealed reduced amounts of pentosidine (results not shown).

Human lens capsules from diabetic subjects revealed the presence of pentosidine with an average content of pentosidine of approx. 1 pentosidine per 100 collagen molecules.

DISCUSSION

The results of the studies reported in this paper provide some conclusions on the mode of action of glucose in non-enzymic glycosylation of basement membrane collagen, and on the macromolecular structure of type-IV collagen in basement membrane.

Thermal analysis

The peaks observed in d.s.c. represent the temperature (T_m) of maximum power input, or heat capacity, and correspond to the temperature at which disruption of a stable structure within the material occurs at the maximum rate. The fresh lens capsules produced two major peaks when studied by d.s.c., T_m 1 and T_m 2 at 53–55 °C and 88–90 °C respectively (Figure 2). The basement membrane is a complex tissue, the major constituents of which are type-IV collagen (40 %), laminin, heparan sulphate and nidogen (Timpl and Martin, 1982). Confirmation that T_m 1 and T_m 2 were derived from the shrinkage of collagen was obtained by treatment of the lens capsule with collagenase after which both peaks were eliminated, and with guanidine hydrochloride after which T_m 1 became reduced but T_m 2 remained prominent. The d.s.c. scans of a purified solution of type-IV tetramers revealed two peaks, T_D 1 at 43–44 °C and T_D 2 at 90–91 °C, while the isolated triple-helical 'leg' regions produced the single T_D 1 peak. The T_D 1 at 43–44 °C obtained by d.s.c. is slightly higher than the value of 40.0 °C obtained by c.d. spectrometry of ovine lens capsules (Gelman et al., 1976).

A representation of the structural units of type-IV collagen in basement membrane has been proposed by Timpl et al. (1981). Four molecules associate in an anti-parallel fashion at their N-terminal ends to form the 7S region. The triple-helical 'leg' regions are bounded by the non-helical NC1 and NC2 domains. Polymerization of the type-IV tetramers occurs by association with adjacent NC2 regions to form a type of 'chicken-wire' network (Timpl et al., 1981). A more complex random organization of the type-IV molecules involving some lateral aggregation has been proposed by Yurchenco et al. (1986).

The d.s.c. thermogram of type-IV collagen may therefore be interpreted as follows; T_D 1 (43–45 °C) is due to the collapse of the triple-helical domain of the type-IV monomer. The second denaturation peak (90–91 °C) is due to the collapse of the 7S domain of the type-IV tetramer. The latter region is stabilized by both disulphide bonds and lysine-derived cross-links between the type-IV monomers and would therefore be expected to possess a high denaturation temperature. Risteli et al. (1980) have reported that the melting of isolated, reduced and alkylated 7S collagen occurs around 70 °C using c.d., but melting of highly cross-linked collagen would occur at an even higher temperature. The intact lens capsule also revealed two denaturation temperatures, T_m 1 at 53–55 °C due to the collapse of the helices in the aggregated form of type IV and T_m 2 at 90 °C due to the 7S domain. The more precise values for T_m obtained by d.s.c. agree with the range 50–55 °C reported by Linsenmeyer et al. (1984) using the indirect technique of following triple-helix denaturation by the use of conformation-dependent monoclonal antibodies.

It is interesting to note that the triple helix of the isolated type-IV monomer melts at about 44 °C while in the intact lens capsule denaturation of the helical domain occurs at about 54 °C (Figures 2a and 2e). This 10 °C difference between T_D and T_m can be compared with the difference of about 27 °C between the shrinkage of the tropocollagen molecule in solution and in the fibre, which represents lateral interaction between the molecules in the fibre. It is unlikely that the increase in denaturation temperature is due to proteoglycans, as their removal by guanidine hydrochloride does not reduce the temperature at which the helices in the intact membrane denature, and secondly because on reprecipitation of purified type-IV molecules the denaturation temperature is close to T_m 1. Thus the difference in T_D 1 and T_m 1 clearly indicates lateral aggregation of the helical regions of the type-IV molecule within the basement membrane, although certainly not to the same extent as the close packing of the fibrous type-I collagen molecules. This evidence for lateral aggregation of the type-IV molecules is consistent with the model proposed by Yurchenco et al. (1986) and our own studies of the X-ray diffraction of stretched lens capsules (Barnard et al., 1987).

Having established the origin of the major peaks in the d.s.c. thermogram we used the technique to demonstrate changes in the stability of type-IV collagen of lens capsules following non-enzymic glycosylation. Increases in the denaturation temperature T_m 1 occurred in the incubated control but a larger increase in T_m 1 occurred on incubation in the presence of glucose. Only a small elevation of T_m 2 was noted in the glycated membrane. The raised denaturation temperature (T_m 1) after glycosylation *in vitro* can therefore be accounted for by subsequent glucose-mediated intermolecular cross-linking between the helical parts of the type-IV molecules in the basement-membrane framework.

The temperature of the d.s.c. peak maximum is affected by instrument response times, but in this work is given approx. by

$$\frac{r\Delta E}{aR} = T_{max.}^{\ 2} \cdot \exp\left(\frac{-\Delta E}{RT_{max.}}\right)$$

(Sanchez-Ruiz et al., 1988; Miles, 1993) where r is the scanning rate, R is the gas constant, E is the activation energy and a is defined by the Arrhenius equation governing the effective rate constant k controlling the denaturation;

$$k = a\exp\left(\frac{-\Delta E}{RT}\right)$$

The increase in temperature $\delta T_{max.}$ caused by simultaneous changes in a and ΔE is therefore given by;

$$\delta T_{max.} = \frac{\delta(\Delta E)\left(\frac{1}{\Delta E}+\frac{1}{RT_{max.}}\right)-\frac{\delta a}{a}}{\frac{2}{T_{max.}}+\frac{\Delta E}{RT_{max.}^{2}}}$$

which may be approximated to;

$$\delta T_{max.} = T_{max.}\left(\frac{\delta\Delta E}{\Delta E}-\frac{RT_{max.}}{\Delta E}\cdot\frac{\delta a}{a}\right)$$

provided $\Delta E = 2RT_{max.}$, as is the case here. Thus an increase in δT could be caused by an increase in E or a decrease in a or some combination of both. For example, the increase in the thermal stability of the collagen by 7 °C, due to interhelical cross-linking, represents an increase of just 1 % in ΔE.

Mechanical properties

The incubation of lens capsule with glucose renders the capsules increasingly brittle, as shown by the greater stiffness at 30 % strain and shorter elongation at the breaking point (Figure 3). Such changes can only be interpreted in terms of additional constraints in the tissue compared with the controls (Viidik, 1973), clearly indicating the formation of additional intermolecular cross-links. The dramatic drop in breaking load at maximum stress from 12 to 4.7 $N \cdot mm \cdot mg^{-1}$ suggests increased brittleness, presumably due to the extensive interhelical cross-linking.

Extent and location of the cross-linking

The observed changes in the mechanical and thermal properties of the lens capsule following non-enzymic glycosylation are consistent with increased stability due to glucose-mediated cross-linking. Indeed, we have identified the presence of the trivalent cross-link pentosidine in the glycosylated capsules.

The total number of pentosidine cross-links is very low, amounting to only 1 cross-link per 600 collagen molecules after incubation for 24 weeks. To account for the changes in mechanical properties of the lens capsule these potential cross-links would have to link sheets of aggregated type-IV molecules and it is possible that few cross-links would be required. However, the increase in the T_m 1 cannot be accounted for on the basis of so few cross-links, as many interhelical links would be necessary to stabilize the helices.

The lysine–aldehyde-derived cross-links are located between very specific lysine residues in the N-terminal non-helical region, opposite a binding site for lysyl oxidase, also close to the termini of the molecule. These intermediate cross-links, typical of fibrous collagen, have been identified in basement membrane (Heathcote et al., 1980), and they decrease in amount during maturation, analogous to the fibrous collagens (Bailey et al., 1984). Cross-linking sites have been identified on the 7S region where the sequence Hyl-Gly-Glu-Arg is present (Siebold et al., 1987). This is similar to the Hyl-Gly-His-Arg of fibrous collagens (Fietzek et al., 1977) and similarly may act as the binding site for lysyl oxidase. Unfortunately the mechanism of maturation of the intermediate cross-links is currently unclear, but is likely to be confined to the N-terminal region as we have recently demonstrated the absence of lysine-derived cross-links in the C-terminal NC1 hexamer (Reddy et al., 1993).

In contrast, initial glycosylation of the lysine and hydroxylysine residues could occur at random along the whole length of the triple helix, although there is some preliminary evidence of site specificity (Reiser and Amigable, 1990). As we have shown some lateral aggregation of the type-IV molecules occurs in basement membrane the subsequent formation of the glucose-mediated cross-linking between helical parts of the molecules would readily occur. Such interhelical cross-linking would be more effective in increasing both the stiffness of the membrane and in increasing the denaturation temperature (T_m) than the lysine–aldehyde-derived cross-links which are confined to the termini of the molecules.

Increasing stability of controls

Similar, but much less marked, changes in the solubility, T_m and mechanical properties occurred during incubation in the absence of glucose (Tables 1 and 2). Such changes are analogous to previous studies on tendon collagen and purified reprecipitated type-I collagen which demonstrated age-related changes similar to *in vivo* ageing when the specimens were incubated *in vitro*. This effect has been demonstrated to be due partly to the maturation of the lysine–aldehyde-derived cross-links (Bailey et al., 1974). However, the mature cross-links known to be present in mature fibrous collagen, histidine–hydroxylysinonorleucine in skin and hydroxy–pyridinoline in bone and cartilage could not be detected in these lens capsules. The mechanism of stabilization of the precursor keto-imine known to be present in fetal lens capsule (Heathcote et al., 1980) and normally converted into hydroxylysylpyridinoline in fibrous collagens remains to be elucidated in basement membrane. At this time we also demonstrated a secondary reaction causing insolubility which involved an oxygen-dependent mechanism (Robins and Bailey, 1977). Fu et al. (1992) have reported recently the importance of oxidative reactions in the formation of pentosidine. Thus, during incubation in the presence of oxygen cross-linking could be occurring by two oxidative mechanisms, one unknown route described above and one involving the more rapid formation of glucose-mediated cross-links. The lens capsules contain glycosyllysine, the precursor of pentosidine, which could be converted into pentosidine in the control incubations. However, the levels are too low to account for the physical changes and we conclude that the maturation of lysine-derived cross-links is the major pathway for stabilization in the absence of glucose.

In conclusion, stabilization of the lens capsule by cross-linking following non-enzymic glycosylation is occurring and could lead to even greater physiological deterioration of the lens, e.g. in its accommodation (Bito et al., 1987), than that occurring during normal ageing. However, the mechanism of cross-linking is still unclear as the contribution of the Maillard reaction fluorphor, pentosidine, appears to be minimal.

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