Age and Ageing 1996;25:279–284

Vitamins and Analgesics in the Prevention of Collagen Ageing

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Summary

The ageing of connective tissues involves modifications of collagen, which are currently generating much interest amongst protein researchers. Protein glycation, a non-enzymic reaction involving sugar, appears to play a role in the evolution of age-related physical changes and diabetic complications—retinopathy, neuropathy, renal failure and atherosclerosis. Our studies show that the glycation of human corneal and scleral collagen produces increases in the collagen intermolecular spacing—these increases are similar to those we previously reported on the ageing of collagen in these tissues. The present investigation employs X-ray diffraction to look at the structural effects of various substances that are believed in inhibit protein glycation. Aspirin-like compounds and certain vitamins successfully prevented the sugar-induced molecular changes from occurring in corneal and scleral collagen, suggesting that such compounds could have a useful role in this aspect of ageing.

Key words: Ageing, Collagen, Cornea, Sclera, Aspirin, Glycation.

Introduction

Glycation is initiated by the reaction of a free amino group, typically on a long-lived protein such as collagen, with the carbonyl group of a reducing sugar. It forms the initial stage of the Maillard reaction, which was first described in 1912 by Louise-Camille Maillard, and is widely recognized in the food industry for the spoilage of food during storage. Glycation represents a post-translational modification of a protein by the covalent attachment of a sugar residue. The amino-carbonyl condensation results in the reversible formation of a Schiff's base. The latter undergoes a rearrangement to produce a more stable Amadori product (AP). It is this AP which, after a series of complex reactions, results in the irreversible production of advanced glycation end-products (AGEs), some of which are fluorescent [1]. A proportion of these fluorescent products can form cross-links between protein molecules. These advanced products of glycation appear to play a role in the evolution of age-related and diabetic complications and, since they are slowly-formed, glucose-derived compounds and are chemically irreversible, they can accumulate with time as a function of age [2] or glycaemic control [3]. Although natural collagen cross-links exist within tissues, the nonreducible 'mature' ones tend to reach a plateau with age so the physical changes exhibited by collagen-containing tissues on ageing and in diabetes could be accounted for by the accumulation of non-enzymically derived cross-links, i.e. the sugar-induced ones.

We have shown that glycation of human corneal and scleral collagen increases with age [4]. By using synchrotron X-ray diffraction, we have been able to show that elevated tissue glycation is accompanied by increases in collagen cross-linking and intermolecular spacing [4]. These ageing effects have also been reproduced *in vitro* [5]. We have also shown that, when different sugars are used to incubate the protein *in vitro*, the rate of increase of collagen intermolecular spacing and fluorophore production is dependent upon the type of sugar used [5]. In the present study we have used fructose as a glycating agent, since this sugar reacts with collagen at a faster rate than glucose. Fructose also commonly occurs in fruits and, interestingly, is often recommended in diabetic diets as a substitute for sucrose.

Frequently, rat tail tendon is employed as a model tissue for much *in vitro* work in studies of ageing of collagen. We have used human corneal and scleral tissue as models for the ageing of collagen—when young corneal and scleral collagen was incubated with glucose, the increasing glycation of the protein was accompanied by increases in the intermolecular spacing similar to those that occur with age [5]. We have also shown that the use of aspirin inhibits this process [6]. In this paper we report the effects of several inhibiting compounds on collagen glycation and ageing.

Aspirin has previously been used in clinical trials in the prevention of cataract in humans [7]. Since glycation is a possible cause of cataract formation, aspirin was one of the inhibitors tested in the present work. Ibuprofen and paracetamol have also been shown to protect against cataract in diabetic rats [8] and aminoguanidine (AG) has been demonstrated to inhibit AGE formation in diabetic rat proteins [9]. Since one

route to AGE formation may involve autoxidation reactions catalysed by metal ions [10], the metal ion chelator diethylenetriaminepentaacetic acid (DETAPAC) was chosen as an alternative inhibitor. The vitamins C and E were used as inhibitors of glycation since they have both been previously employed in the prevention of sugar-induced cataract [11].

Part of this work has been published in abstract form [12].

Materials and Methods

X-ray diffraction of corneal and scleral tissue: Corneal and scleral tissue was sectioned and incubated for different periods in buffer solutions containing 0.5 *M* fructose with or without an inhibitor [12]. The inhibitors used were aminoguanidine, acetylsalicylic acid (aspirin), diethylenetriaminepentaacetic acid, α-methyl-4-[2-methyl-propyl]benzeneacetic acid (ibuprofen), acetaminophen (paracetamol), L-ascorbic acid (vitamin C) and α-tocopherol (vitamin E) all at a concentration of 25 mmol/l. One section of each cornea used was incubated in 0.05 *M* phosphate buffer (pH 7.4) for 24 days at 37°C in the absence of fructose. The remaining corneal sections were incubated for varying periods, together with one of the seven inhibitors, in the presence of fructose. Control corneas and scleras were also used.

X-ray diffraction of collagen was performed as described elsewhere [6]. Changes in the intermolecular Bragg spacing reflect corresponding changes in the cross-sectional area associated with each molecule within a collagen fibril. The parameter of interest, therefore, is the Bragg spacing squared. Significance of changes in the intermolecular spacing was assessed using Student's *t* tests.

Fluorescence of corneal and scleral tissue: Collagen-associated fluorescence was measured as follows. Once the glycated corneas and scleras had been observed via X-ray diffraction, they were digested overnight with papain (1 mg/ml citrate buffer pH6) in a water bath at 45°C. The digests were centrifuged at 10 000 r/min for 10 min. The fluorescence of each supernatant was then measured using an AMINCO-BOWMAN spectrophotofluorimeter (Model no. J4 8960). Emission was recorded at 440 nm upon excitation at 370 nm since studies on the nature of the fluorophores which accumulate in ageing human collagen have revealed the presence of a major fluorophore with excitation-emission maxima at 360/440 nm [13]. Current research on collagen commonly employs excitation-emission wavelengths of 370/440 nm.

Significance of changes in fluorescence was assessed using Student's *t* tests.

Results

X-ray diffraction: Figure 1(a) shows that all seven compounds significantly inhibited the expansion of the corneal collagen molecular network associated with fructation ($p < 0.038$). In the absence of an inhibiting

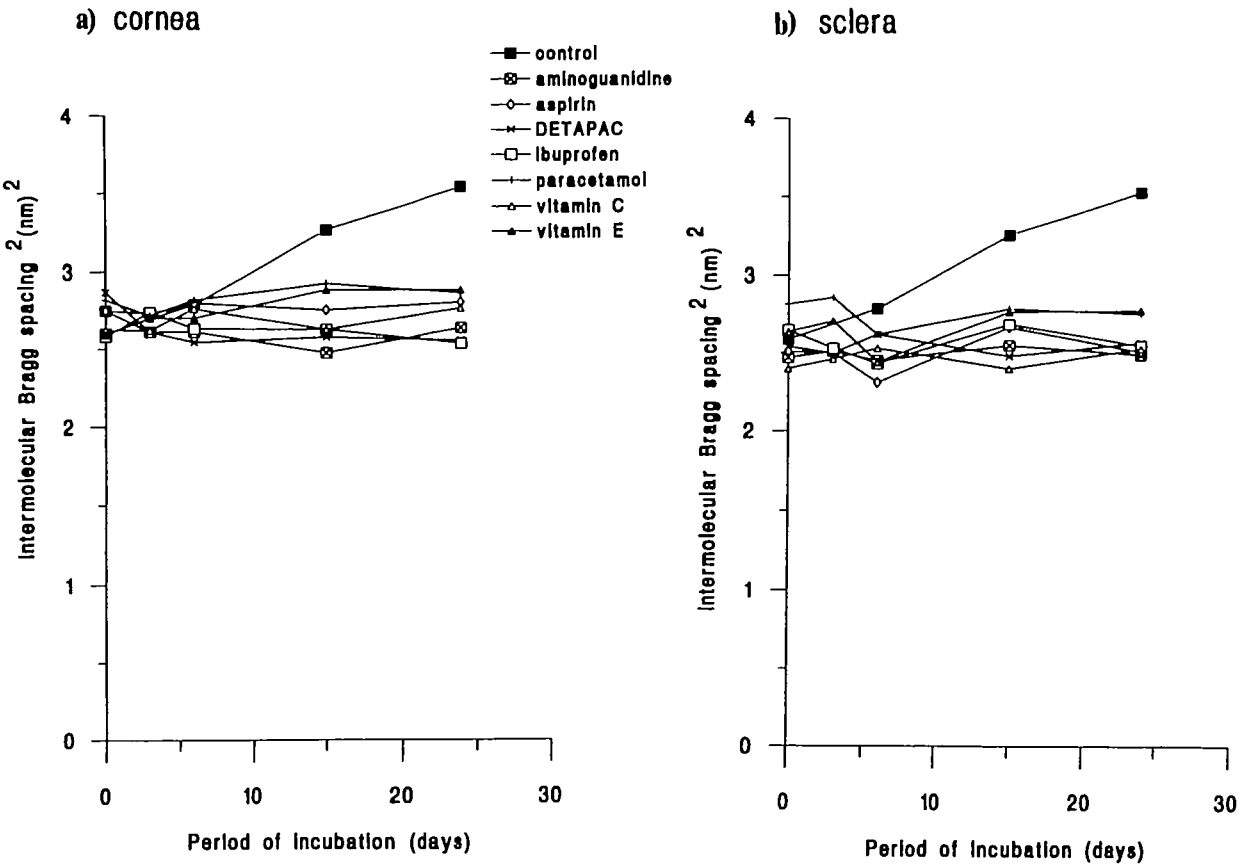

Figure 1. X-ray diffraction of corneal and scleral tissue. Changes in the intermolecular spacing of human corneal (a) and scleral (b) collagen occur in the absence of an inhibiting compound but are hindered in the presence of the various inhibitors.

compound, the corneal collagen molecular network expanded by 49% after 24 days, compared with 2%, 2%, 5% and 11% when aspirin, paracetamol, vitamin C and vitamin E respectively were included. In observing the individual graphs, small fluctuations are visible. However, with the exception of DETAPAC which significantly reduced the molecular spacing ($p < 0.0005$), none of these fluctuations is significant.

Figure 1(b) illustrates that in the sclera, as with the cornea, the inhibitors prevented the expansion of the molecular network ($p < 0.045$). In the absence of an inhibiting compound, the scleral molecular network expanded by 36% after 24 days, compared with 1%, 2% and 4% when aspirin, paracetamol and ibuprofen respectively were included and, 3% and 5% when vitamins C and E were included. DETAPAC and aminoguanidine both produced only a 1% decrease in the collagen molecular network. Once again, there are apparent fluctuations in the molecular spacing in the presence of the aspirin-like analgesics and both vitamins. However these were not statistically significant ($p > 0.2$).

The effect of the inhibitors *alone*, i.e. in the absence of sugar, was also investigated. After 24 days incubation, DETAPAC and aminoguanidine had caused a reduction in the corneal molecular spacing by 10%. Aspirin caused an initial reduction in the corneal molecular spacing by 6% ($p < 0.001$) after 3 days, but the spacing then remained constant throughout the remainder of the 24-day incubation period. In scleral collagen, aspirin had no effect in the absence of sugar, i.e. the molecular spacing remained unchanged. Vitamin C, on the other hand, produced a small (3%) but significant increase in this parameter ($p < 0.15$).

Collagen-associated fluorescence: Figure 2(a) shows that the fructose-induced fluorescence of corneal collagen is reduced in the presence of all the inhibitors tested ($p < 0.006$). However, there were clear differences in the effects of the individual inhibitors at different times during the incubation period. During the early stages of incubation (0–6 days), fructose produced a significant increase in the fluorescence of corneal collagen at the specified wavelengths in the presence of vitamin C or vitamin E ($p < 0.03$ and $p < 0.01$ respectively) and a smaller increase in the presence of paracetamol or ibuprofen ($p < 0.05$ and $p < 0.15$). After the total incubation period (24 days), the fluorescence of corneal collagen had increased only slightly in the presence of aspirin, aminoguanidine and DETAPAC but somewhat more when paracetamol, ibuprofen or vitamin E were used and to an even greater extent with vitamin C.

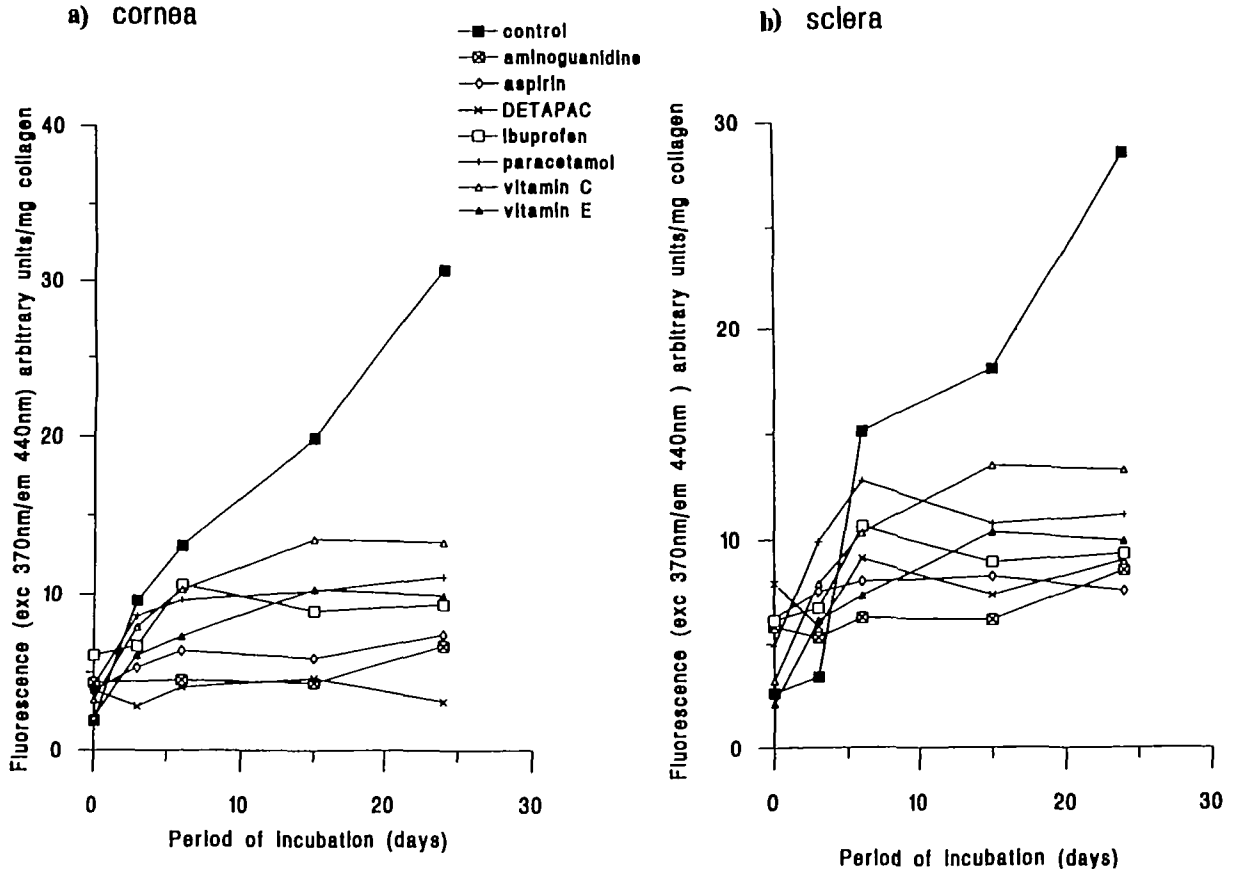

Figure 2. Fluorescence of corneal and scleral tissue. Changes in the level of collagen-associated fluorescence of human cornea (a) and sclera (b) occur in the absence of an inhibiting compound but are diminished in the presence of the various inhibitors.

Figure 2(b) shows the changes in the level of scleral collagen fluorescence in the presence of the seven compounds used. As with corneal collagen, in the absence of an inhibitor the fluorescence of scleral collagen increased dramatically and this was clearly prevented to different extents by different inhibitors. The results, as with the scleral intermolecular alterations, were rather more unpredictable during the early incubation stages where, in the presence of ibuprofen, paracetamol and vitamin C, fructose produced significant increases between 0 and 6 days ($p < 0.15$, $p < 0.03$ and $p < 0.017$ respectively). With the exception of paracetamol and vitamin C, all the other inhibitors prevented fructose from producing a significant overall increase in the level of scleral collagen-associated fluorescence ($p < 0.006$).

Discussion

Using synchroton X-ray diffraction, the need to process the tissues before analysis is avoided. Any changes in tissue hydration are essentially eliminated by sealing the sample in an air-tight cell and using short exposure times. The technique also allows structural measurements of protein molecules to be made *in situ* within the tissue.

The sugar fructose was chosen for the experiments in order to speed up the rate of glycation *in vitro*. Normally, the glucose concentration of the human corneal epithelium is approximately 5.6 μmol/g dry weight and that of the corneal stroma 8.8 μmol/g [14]. With ageing, individuals experience an increased glucose intolerance [15]; hence one would expect the corneal sugar levels to vary. It is also known that the sorbitol pathway operates in the corneal epithelium [16]. This produces sorbitol and fructose from glucose and, in instances where excess glucose exists, an accumulation of sorbitol can result in osmotic cell damage. The role of such damage in ageing and diabetic corneal epithelial abnormalities is not clear and it is possible that fructation within the cornea plays a role.

Expansion of the collagen molecular network has been attributed to the rigid sugar-derived cross-links, which are capable of pushing adjacent molecules apart in the closely packed overlap regions of the fibrils [17]. The use of inhibiting compounds leads to relatively little change in the intermolecular spacings and this implies that the production of new cross-links is somehow being hindered. In this paper we have shown how both vitamins and analgesics prevent sugar-induced structural change. The results suggest that, overall, the analgesics are better able to restrict collagen molecular changes that occur in the presence of sugar.

The acetylation of free amino groups on the collagen by aspirin may reduce the amount of Amadori product (i.e. the level of glycation) and in turn the level of AGE production. However, aspirin has been shown to have no effect on the glycation of rat tail tendon collagen in diabetic rats but it was found to inhibit cross-linking [18]. Since salicylate has been found to have a similar effect, i.e. the inhibition of cross-linking without an effect on glycation both *in vivo* and *in vitro*, the implication is that aspirin may not be acting by acetylation in inhibiting advanced glycation reactions. An alternative mechanism suggests that aspirin, paracetamol and salicylate possess oxygen radical scavenging activity and possible chelating properties [19].

The aspirin-like analgesic, ibuprofen, has been shown to reduce the rate of binding of fructose to lens proteins [20]. However this compound, unlike aspirin, will not be able to acetylate since it lacks an acetyl group. It may bind to the collagen resulting in inhibition of nonenzymatic modification or it may act indirectly by attacking the sugar. Since ibuprofen has been shown to reduce fasting blood glucose and to improve glucose tolerance in diabetic and non-diabetic patients [20], this compound may have a role in the inhibition of glycation. Although ibuprofen appears to prevent the molecular alterations in collagen that normally occur in the presence of sugar alone, the role of this drug in the inhibition of glycation and AGE formation is still uncertain, particularly since our results showed that it was not as effective in scleral tissue as in corneal tissue.

Vitamins C and E have previously been employed in the prevention of sugar-induced cataract [11, 21]. Vitamin C is structurally very similar to glucose, hence one would predict an *increase* in the level of glycation and subsequent AGEs. It has been shown that ascorbic acid produces rapid protein cross-linking *in vitro* [22]. However, we have shown that the collagen molecular spacing in both cornea and sclera remains fairly constant either when vitamin C is used alone or when it is used in the presence of sugar. Under these circumstances, it appears that the antioxidant activity of this compound is predominating and its potential to create changes in collagen-associated fluorescence is being hindered.

The oxidation of vitamin C, however, can result in collagen glycation as a result of the formation of 'glycators' such as L-threose and 2,3-diketogluconic acid [22]. It is therefore possible that vitamin C and fructose compete for sites of glycation. Our experiments do suggest that vitamin C was the least effective of all the inhibitors in terms of minimizing changes in collagen-associated fluorescence in both cornea and sclera even though it seemed to prevent structural changes associated with glycation. The observed change in fluorescence was most likely a result of those fluorophores that do not produce a change in collagen molecular structure. The role of vitamin C in any given system is likely to depend on the tissues and sugars used and also the incubating conditions, such as the concentration and type of buffer used. Ortwerth and Olesen [22] used equimolar concentrations of ascorbic acid and glucose (20 mM) in 0.1 M phosphate buffer at pH 7 whereas our experimental set-up used 25 mM ascorbic acid and 0.5 M fructose in 0.05 M phosphate buffer at pH 7.4. The extent of oxidation of ascorbic

acid is also dependent on the amount of oxygen prevailing in the incubation set-up.

Vitamins C and E are capable of scavenging free radicals and in this way may prevent advanced glycation reactions from taking place *in vitro*. When combined, the antioxidant activity of the vitamins is enhanced and the combination has been demonstrated to prevent low-density lipoprotein oxidation which plays an important role in the development of atherosclerosis [23].

DETAPAC and AG inhibit the conversion of the Amadori product to an AGE. DETAPAC is a metal ion chelator which can prevent oxidative reactions from occurring. It has been suggested that AG does not act on the protein but is capable of reducing the concentration of the active aldehyde form of the sugar [24]. It has also been proposed that AG can block free carbonyl groups on the reactive dicarbonyl compounds derived from the Amadori product [24], resulting in the inhibition of advanced glycation. However, our results [6] have suggested that both aminoguanidine and DETAPAC might themselves lead to structural alterations in the absence of sugar.

With the exception of DETAPAC and aminoguanidine, which are relatively toxic, we have shown that the other inhibitors, aspirin, paracetamol, ibuprofen, vitamin C and vitamin E, are suitable for preventing the structural effects of glycation on collagen *in vitro* and also, probably, *in vivo*. Analgesics are commonly employed for various therapeutic purposes, for example aspirin is recommended in the treatment of heart attack patients. Vitamins C and E not only form part of our daily dietary requirements but possess antioxidant properties that have been explored, in the case if vitamin E, in the treatment of coronary heart disease [25]. Free radicals are generated by several processes within the body. They are another factor resulting in the onset and progression of the various complications that can occur in ageing and in diabetes. The scavenging activity and the consequent inhibition of glycation and age-related collagen cross-linking are likely to be additional but useful side-effects of these compounds.

The collagen intermolecular spacing is one of the factors that contribute to the transparency of the cornea [26]. The importance of this work, in relation to the tissues used, lies in minimizing alterations in the transparency of the cornea and the physical properties of the sclera that occur with age, but the use of inhibitors would also be expected to minimize the detrimental effects of glycation in other collagen-containing tissues. Furthermore, in diabetes the increased collagen fluorescence intensity and collagen insolubility are accelerated and the fluorescence level correlates well with the severity of diabetic complications such as cataract formation [27]. The use of inhibitors would be expected to reduce the rate of development of such age-related complications.

Many of the compounds used during this study are already conveniently available and have endured various clinical trials and testing procedures prior to becoming routinely available. The results of this study suggest that their therapeutic values may be more widespread than first imagined.

Acknowledgements

We are very grateful to British Telecommunications plc through Research Into Ageing for funding this work and also thank Bayer plc for financial support. We wish to thank the UK Transplant Service Eye Bank (Bristol) for their kind donation of corneoscleral discs and Anna J. Furth for her suggestions on this manuscript.

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Received in revised form 28 November 1995