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Collagen as a model system to investigate the use of aspirin as an inhibitor of protein glycation and crosslinking

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Abstract

Aspirin has been shown to be a powerful inhibitor of post-Amadori Maillard reactions, although the exact mechanism of this action remains unclear. We have used corneal and scleral collagen as a model system: (i) to assess how aspirin, either alone or in combination with sugars, affects the surface charge distribution along the collagen fibrils; (ii) to see how sugars and/or aspirin affect the swelling properties of the cornea; and (iii) to see if sugars and/or aspirin change the distribution of water within the corneal stroma as the tissue swells. Charge changes were detected by examining changes in the uptake of phosphotungstate ions as seen in the electron microscope. Swelling was measured by monitoring the uptake of water as a function of swelling time, and water distribution was determined by using synchrotron X-ray diffraction to follow changes in the interfibrillar Bragg spacing as the cornea swells. Aspirin has a marked effect on the positive staining pattern of scleral collagen. This is different to the changes in stain uptake produced by glycation. Incubation with both sugars and aspirin showed that the sugar binding takes precedence over the effects of aspirin which, in turn, suggests that protein acetylation by aspirin is unlikely under these circumstances. However, aspirin completely suppresses corneal swelling. Even when the aspirin is removed, swelling in distilled water is reduced, and this is accompanied by changes in the water distribution. The results suggest that water is more evenly distributed in aspirin-treated corneas that are subsequently swollen than in swollen glycated corneas. Fructation, glucation and ribation on their own have little effect on the uptake of water as the cornea swells. This suggests that any sugar-derived crosslinks formed at this stage do not limit swelling.

Keywords: Collagen; Cornea; Sclera; Aspirin; Glycation

1. Introduction

The development of natural cross-links in collagen is fairly well established. However, an additional, non-enzymic pathway involving sugars has been postulated. Simple sugars can interact with proteins in two ways. Enzymic glycosylation is tightly regulated and the resulting sugar attachment is site specific, involving only a few amino acid residues at specific sites along collagen chains (Reiser et al., 1991). Non-enzymic glycosylation, or glycation, involves the addition of sugar molecules to tertiary amino groups to form Schiff base adducts. Further chemical modifications can produce Amadori products (AP) or Heyns products (HPs). A number of subsequent reactions result in the formation of advanced glycosylation end products (AGEs), some of which crosslink within, and between proteins (Kent et al., 1985; Brownlee et al., 1986; Brownlee, 1994). The pathway is not regulated but can be influenced by the type and concentration of sugar, by the half-life of the protein, and by the availability of free amino groups on the protein. Glycation and subsequent crosslink formation are thought to contribute to many of the complications seen in ageing and diabetes mellitus.

Aspirin (acetylsalicylic acid) is a non-steroid, anti-inflammatory drug that has been in general use since the end of the last century. It was thought to act as an anti-glycation agent by acetylating free amino groups on proteins, and limiting the sugar-induced formation of APs and AGEs. Aspirin also inhibits the increase in the collagen molecular spacing seen with glycation (particularly fructation) (Malik and Meek, 1994, 1996). Yue et al. (1984, 1985) have shown that aspirin inhibits the formation of glycoxidation products and prevents the crosslinking of rat tail tendon in vitro, but it has no effect on the extent of glycation of proteins, suggesting that aspirin acts via free radical scavenging rather than by acetylation of lysine residues (Fu et al.,

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1994). The purpose of the present study was to use biophysical measurements to investigate the effects of sugars and aspirin on corneal and scleral collagen.

Type I collagen fibrils display a characteristic positive staining pattern when viewed in the electron microscope, which can be quantitatively correlated with the axial distribution of positively charged amino acid residues (Chapman and Hardcastle 1974; Tzaphlidou and Hardcastle, 1984). Reactions that alter the surface charge distribution along the axis of a collagen fibril lead to changes in stain uptake that have provided considerable insight into the reaction mechanisms of a range of different molecules (Tzaphlidou et al., 1982a,b; Tzaphlidou and Chapman, 1984; Meek and Chapman, 1985a,b). Recently, we used the method to demonstrate that glycation alters the distribution of surface charge along the collagen fibrils (Hadley et al., 1998). In this paper, we use the same method to investigate the effects of aspirin on the staining pattern, and hence on the surface charge distribution of collagen fibrils.

We have previously shown that glycation and subsequent AGE formation alters the molecular packing of corneal collagen, both in vivo (Malik et al., 1992) and in vitro (Malik and Meek, 1996). When immersed in a bathing medium such as water, normal corneal tissue initially swells dramatically. The hydration then plateaus off, as the collagen fibrils are no longer able to move further apart to accommodate the water. It is not known if glycation-induced crosslinks occur between fibrillar collagen and proteins in the extrafibrillar matrix, in vivo, although this has been shown to occur in vitro (Sajithlal et al, 1998). If such crosslinks do occur, they might be expected to inhibit the swelling of the tissue. We have therefore carried out swelling studies to examine if swelling of the corneal stroma is modified by incubation with sugars and/or aspirin.

The collagen interfibrillar spacing is sufficiently regular to give rise to a low angle equatorial Bragg spacing in X-ray diffraction patterns (Goodfellow et al., 1978; Sayers et al., 1982)). This spacing is known to increase with hydration; as more water enters the tissue, it moves into spaces between the fibrils, which thus move apart (Meek et al., 1991). The presence of crosslinks between fibrils or between fibrils and other matrix components might be expected to change the rate or extent to which the interfibrillar spacing changes with hydration. The effects of fructose and/or aspirin on the corneal collagen interfibrillar spacing were thus measured using synchrotron X-ray diffraction.

2. Materials and methods

Six human corneoscleral tissues (donor ages over 60 years) were taken from the United Kingdom Transplant Service Eye Bank (Bristol) and comprised of the cornea and a 5 mm scleral rim. Each specimen was sectioned to allow parts of it to be used for electron microscopy, for X-ray diffraction, and for swelling studies.

2.1. Electron microscopy

The tissues intended for electron microscopy were stored frozen at −40°C. They were later thawed and the scleral rims from each cornea were dissected into 2 × 2 mm pieces and incubated at 37°C in solutions detailed below:(A) tissue was stored frozen for 11 days; (B) tissue was incubated in 0.05 M phosphate buffer containing 25 mM aspirin for 11 days; (C) tissue was incubated in 0.5 M fructose in 0.05 M phosphate buffer with 25 mM aspirin for 11 days; and (D) tissue was incubated in 0.5 M glucose in 0.05 M phosphate buffer with 25 mM aspirin for 11 days.

All incubated samples were subsequently dialysed against distilled water (three hours at room temperature, with change of water every 30 min). In all incubations, 3 mM sodium azide was included to prevent bacterial contamination. The incubation medium also contained the protease inhibitors 5 mM EDTA, 2.5 mM benzamidine hydrochloride, and 0.01% trypsin inhibitor.

The scleral pieces were then mechanically disintegrated to disperse the collagen. A small amount of liquid nitrogen was used to aid fracture of the scleral tissue. The collagen fibrils were briefly suspended in distilled water and captured on carbon-coated electron microscope grids. They were stained for one hour at room temperature using 2% aqueous phosphotungstate acid (PTA), pH 3.2, washed with distilled water to remove unbound stain ions, and then allowed to dry at room temperature. The grids were examined using a Philips 301 transmission electron microscope, calibrated using a diffraction grating replica with 2160 lines/mm, and micrographs were taken at high magnification (72,000 ×).

The positively stained collagen fibrils were examined as described previously (Hadley et al., 1998). Briefly, this involved selecting fibrils with a clear banding pattern and making 20 densitometric tracings along fibril axes using a laser densitometer (LKB Ultrascan XL, LKB Instruments Inc., Gaithersburg, MD). The tracings were processed and averaged to obtain a characteristic banding pattern of the scleral collagen from each sample (Tzaphlidou and Hardcastle, 1984).

2.2. Swelling studies

Swelling studies were carried out on both human and bovine corneas. The bovine corneas were collected immediately after death from a local abattoir. The central regions of bovine and human corneas were dissected into 2 × 2 mm^2 pieces and each piece was weighed. Small tissue pieces were used to facilitate penetration of sugars and aspirin. The tissue samples were incubated for one week at 37°C in one of the following solutions: (A) 0.05 M phosphate buffer; (B) 0.5 M glucose in 0.05 M phosphate buffer; (C) 0.5 M fructose in 0.05 M phosphate buffer; (D) 0.5 M ribose in 0.05 M phosphate buffer; and (E) 25 mM aspirin in 0.05 M phosphate buffer. Antibacterial agents and protease inhibitors were included as above.

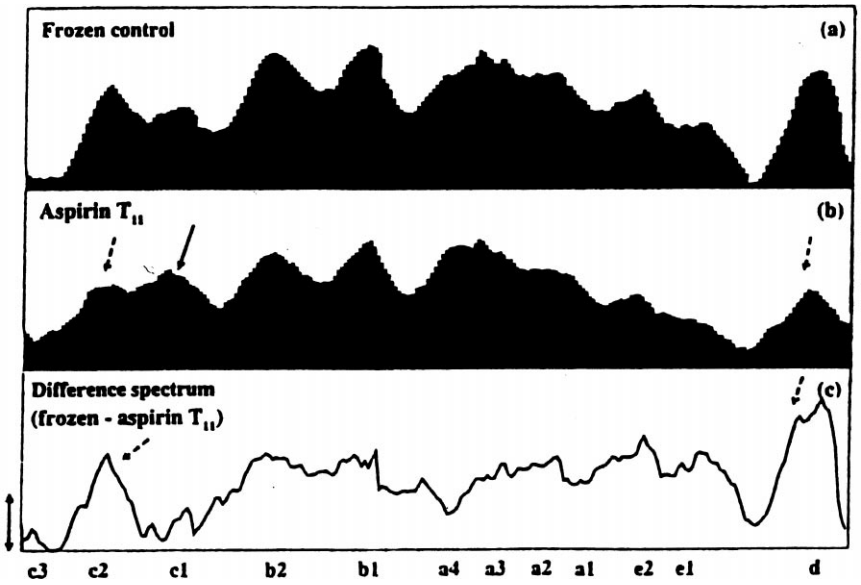

Fig. 1. The PTA stain distribution in the axial D-period along scleral collagen fibrils. (a) Average of 20 microdensitometric traces for the control tissue; (b) average of 20 microdensitometric traces for the scleral tissue incubated in aspirin for 11 days; and (c) difference spectrum, the difference between the control staining profile (shown in (a)) and the staining profile for the aspirin-incubated tissue, T_{11} (shown in (b). The dashed arrows show where aspirin has a marked effect, the full arrow shows where fructose has been shown to reduce staining (Hadley et al., 1998). The double-headed arrow shows the maximum noise level, quantified by comparing the variances of the noise in the averaged traces (the traces having been corrected for varying background intensities and scaled with respect to each other (Tzaphlidou and Hardcastle, 1984)).

The tissue samples were then swollen in distilled water and weighed at various time points over a period of eight hours. They were dried for four days in a 37°C incubator and the hydration at each measurement point was subsequently calculated using:

$$\text{Hydration}(H) = \frac{\text{wet weight} - \text{dry weight}}{\text{dry weight}}$$

The swelling rates were calculated from:

$$\text{Rate} = \Delta H/\Delta t,$$

where ΔH is the change in hydration over a time interval Δt.

2.3. X-ray scattering

In another set of experiments, bovine and human corneas were swollen in order to monitor the effects of aspirin on the change in the interfibrillar spacing. Central regions were dissected into 2×2 mm^2 pieces and incubated in the same solutions used in the swelling study described above. In addition, some bovine pieces were incubated in 0.5 M glucose/25 mM aspirin/0.05 M phosphate buffer or 0.5 M fructose/25 mM aspirin/0.05 M phosphate buffer under the same conditions as above (temperature, duration, protease inhibitors, etc.) to assess the effectiveness of aspirin as an inhibitor of glycation-induced changes in swelling behaviour. After incubation, the human tissues were dialysed against 1.5% polyethylene glycol (PEG), and the bovine issues against 2% PEG, both with 0.15 M NaCl for four days at 4°C. This equilibrates the cornea as near to physiological hydration as possible (Hadley, 1998). The corneas were then examined using Station 2.1 at the Daresbury synchrotron radiation source (CLRC, Daresbury, UK). Details of the procedures used to collect the low angle X-ray diffraction patterns have been published elsewhere (Huang et al., 1996; Rawe et al., 1997). The interfibrillar Bragg spacing (d) was obtained (time $t = 0$). The samples were subsequently swollen in distilled water, blotted, weighed and examined again. This process was repeated as the corneas were further swollen. Because the cornea swells in two dimensions only, the dependence of interfibrillar spacing on tissue hydration was plotted in the form of linear graphs of d^2 against H (Meek et al., 1991).

3. Results

3.1. Electron microscopy

We have previously shown that fructose binds at several sites along the collagen D-period, reducing the binding of stain ions. Most prominent amongst these sites is the $c1$ staining band (Hadley et al., 1998). Fig. 1(a) shows that in untreated collagen, all the positive staining bands except the four a bands can be identified (labelled $c3$–d at the bottom of the figure). PTA staining does not show four clear a bands because of the broad distribution of positively charged amino acid residues in this region (Tzaphlidou et al., 1982a). Following incubation in aspirin, the $c1$ band is

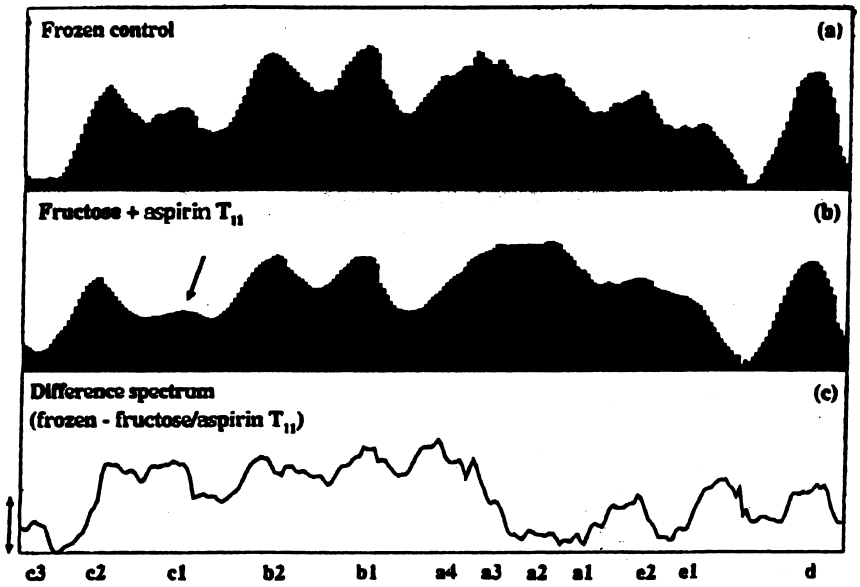

Fig. 2. The PTA stain distribution in the axial D-period along scleral collagen fibrils. (a) Average of 20 microdensitometric traces for the control tissue; (b) average of 20 microdensitometric traces for the scleral tissue incubated in fructose and aspirin for 11 days; and (c) difference spectrum, the difference between the control staining profile (shown in (a)) and the staining profile for the fructose/aspirin-incubated tissue, T_{11} (shown in (b)). The arrows are as described in the legend to Fig. 1.

relatively unchanged (Fig. 1(b), solid arrow). However, on the assumption that aspirin reduces rather than enhances stain uptake, there are a number of sites where stain uptake is markedly reduced, for example the *c*2 and *d* bands (dotted arrows). Across the rest of the D-period, binding is less specific, although there is some correlation with the positions of the other staining bands.

Fig. 2 shows the results for tissue incubated in fructose and aspirin. The difference plot (Fig. 2(c)) shows changes in stain uptake that are greater than the noise level. There is a decrease in stain uptake at a number of regions along the D-period; at *c*2, *c*1, *b*2, *b*1, and *a*4; also, smaller peaks are identifiable at *e*2, near *e*1 and at *d*. These sites of decreased PTA stain uptake coincide with those observed when

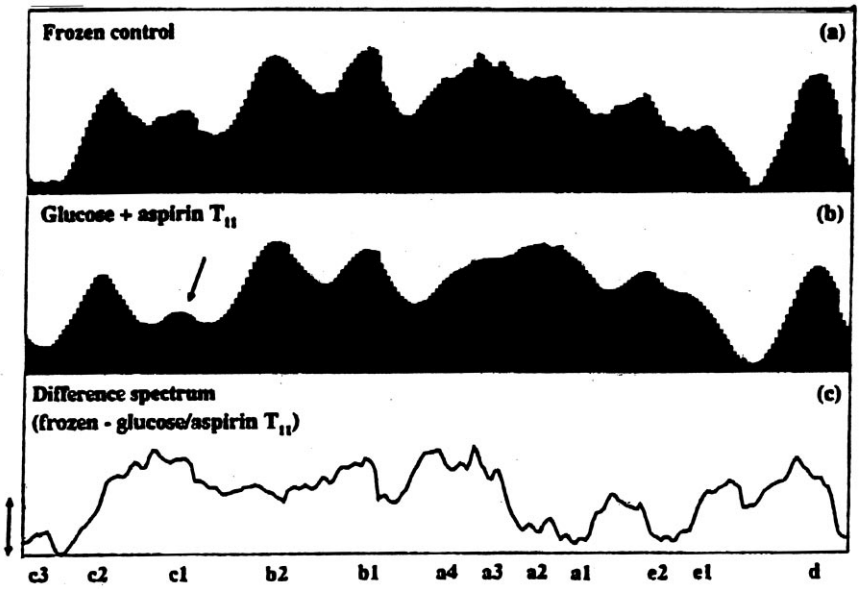

Fig. 3. The PTA stain distribution in the axial D-period along scleral collagen fibrils. (a) Average of 20 microdensitometric traces for the control tissue; (b) average of 20 microdensitometric traces for the scleral tissue incubated in glucose and aspirin for 11 days; and (c) difference spectrum, the difference between the control staining profile (shown in (a)) and the staining profile for the glucose/aspirin-incubated tissue, T_{11} (shown in (b)). The arrows are as described in the legend to Fig. 1.

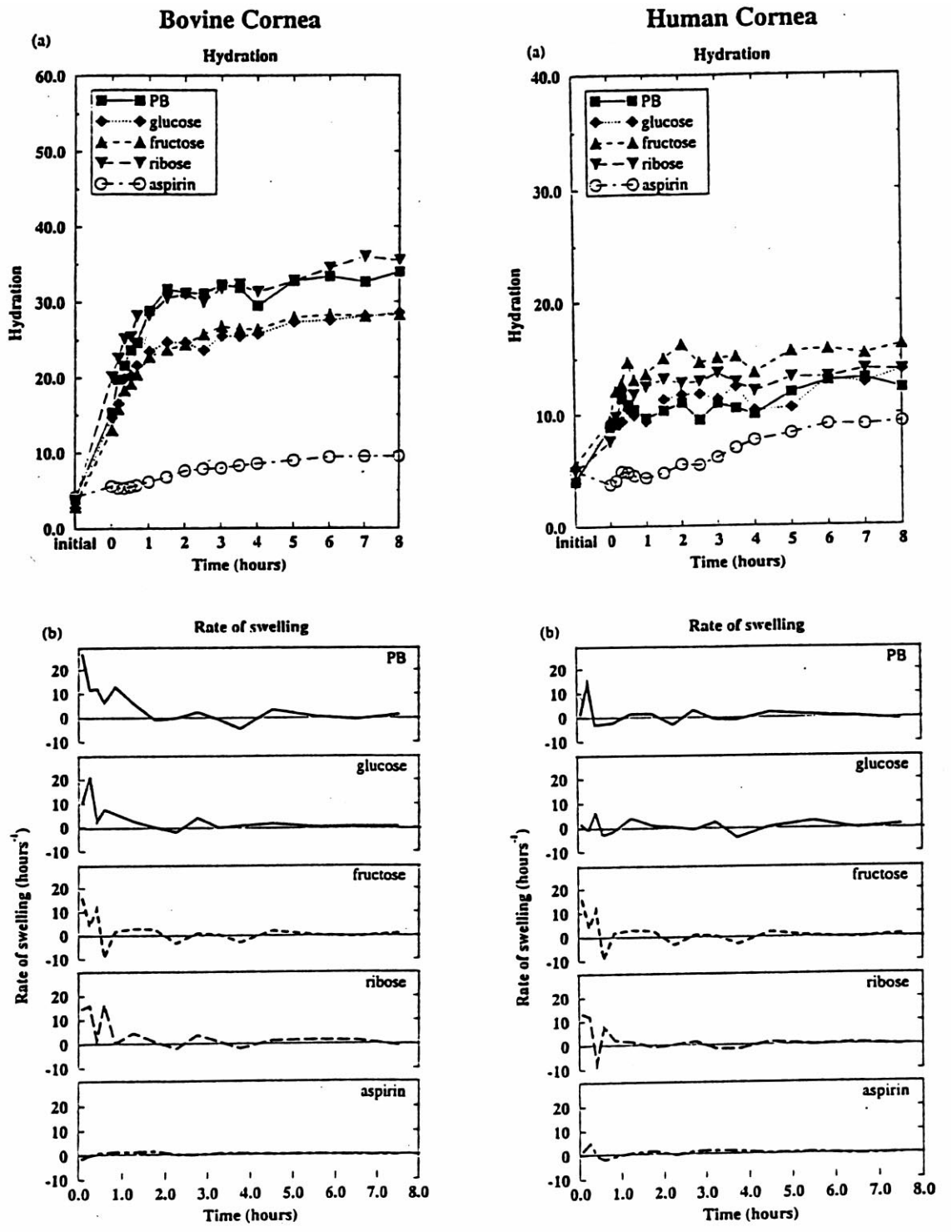

Fig. 4. Swelling of corneal samples incubated in phosphate buffer (PB), sugars and aspirin for one week. The results from bovine cornea are shown on the left, and those from human cornea on the right. (a) in both cases shows the swelling curves after various incubations; and (b) shows how the rate of swelling varied as a function of time. In general, bovine corneas swell more than human corneas.

fructose was used in the absence of aspirin (see Fig. 5 in Hadley et al., 1998), suggesting that fructose is binding. Aspirin was shown to bind to collagen after 11 days incubation (Fig. 1), but the similarity of the difference spectra using fructose and fructose/aspirin suggests that fructose is the molecule binding predominantly.

In Fig. 3, the effects of glucose are examined. Once again, there are several regions of reduced stain uptake. These are

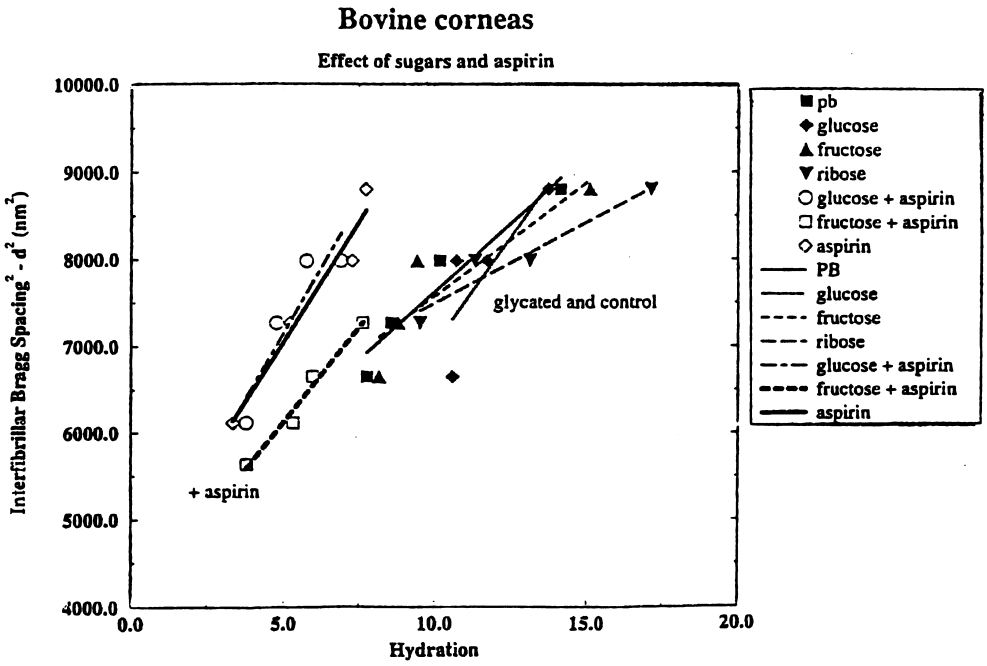

Fig. 5. The effect of glycation and aspirin on bovine corneal tissue hydration and interfibrillar Bragg spacing. The change in the interfibrillar Bragg spacing squared (determined by X-ray diffraction) is plotted as a function of tissue hydration.

similar to those in Fig. 2(c) but different from those where aspirin was used alone. (Fig. 1(c)).

3.2. Corneal swelling

The hydration of the bovine corneal stroma is plotted as a function of swelling time in Fig. 4. The initial hydrations of all the samples in the one week incubation (Fig. 4(a)) were very similar and close to the physiological value ($H = 3.2$). In this experiment, the initial weights were measured prior to incubation in the various solutions (designated $T =$ initial). This allowed an assessment of the hydration effect of incubation in buffer, sugar or aspirin at $T = 0$. After incubation, the hydration values varied widely (comparing $T =$ initial with $T = 0$). The tissue incubated in aspirin showed a much lower hydration compared to the phosphate buffer control and the glycated samples. The ribosylated sample swelled the most during incubation, and hence showed the highest $T = 0$ hydration. During the swelling proper (in distilled water), the tissue pre-incubated in phosphate buffer swelled at the fastest rate, whereas the aspirin-incubated sample hardly swelled at all (Fig. 4(b)).

Similar results were obtained with human corneas, except that the hydrations achieved were much lower than with bovine corneas (Fig. 4(a)) and that the initial swelling rates in glucose and phosphate buffer were lower (Fig. 4(b)).

3.3. X-ray diffraction

Fig. 5 shows the changes in the bovine interfibrillar Bragg spacing as a function of hydration. Linear regression plots are also shown and correlation coefficients r calculated from: $r =$ covariance/ product of variances. In each case there was a linear relationship between the Bragg spacing and the tissue hydration, with the following r-values: PB = 0.962; glucose = 0.808; fructose = 0.893; ribose = 0.963; glucose + aspirin = 0.911; fructose + aspirin = 0.988; and aspirin = 0.978.

To obtain the data in Fig. 5, both the hydration and the interfibrillar Bragg spacing were measured as a function of incubation time between zero and two hours. Thus there is a relationship between each of these parameters and the swelling time at which the measurement was made. Any differences in each parameter caused by the incubation medium can therefore be compared at each swelling time measurement point.

At the end of the two-hour swelling period, aspirin-treated samples reached lower hydrations than samples without aspirin. Aspirin therefore inhibits the extent to which corneas can swell over this time period. The hydration values of the treated samples at each time measurement point were compared with the corresponding hydration values of the PB control using t-test analysis. The analysis showed that there was no significant difference between the hydrations of the glycated samples and the control but that samples pre-incubated in aspirin had significantly lower hydrations than the control.

The d^2 values of the glycated samples at each time measurement point were not significantly different to those of the PB control whether or not aspirin was present. The exception was the fructose + aspirin samples whose d^2 values were significantly lower than the control (PB) values

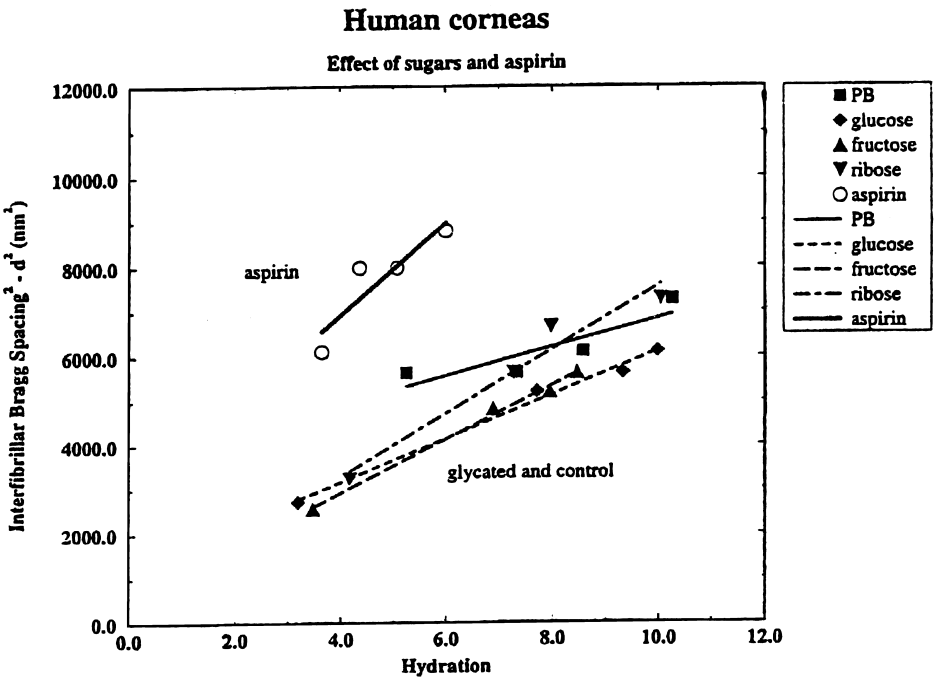

Fig. 6. The effect of glycation and aspirin on human corneal tissue hydration and interfibrillar Bragg spacing. The change in the interfibrillar Bragg spacing squared (determined by X-ray diffraction) is plotted as a function of tissue hydration.

at the $p = 0.1$ level, but this was not significant at the 0.05 level.

The results for human corneas are shown in Fig. 6. Again there was a linear relationship, with the following *r*-values: PB = 0.875; glucose = 0.995; fructose = 0.997; ribose = 0.978; and aspirin = 0.905. The aspirin-incubated samples reached higher d^2 values at lower hydrations compared to the glycated and control samples. Statistical analysis (*t*-test) showed that (i) there was no significant difference between the hydrations of the glycated samples and the control throughout the swelling period; (ii) the hydrations of the aspirin-incubated samples were significantly lower than those of the control ($p < 0.05$); (iii) there was no significant difference between the d^2 values of the glycated samples and the control; and (iv) the aspirin d^2 values were higher at the $p = 0.1$ level than the control d^2 values, although not at the 0.05 level.

4. Discussion

The positive staining pattern of collagen provides a very sensitive system to detect changes in the distribution of surface charge (Chapman, 1974; Tzaphlidou et al., 1982a,b; Chapman et al., 1990). Our results showed that after 11 days incubation, aspirin reduces the stain uptake by positive charges throughout the D-period. This could be the result of steric exclusion but it is more likely that aspirin alters the surface charge on the collagen. There is no obvious correlation between the aspirin staining pattern and the distribution of positively charged amino acids, or lysine or arginine residues on collagen separately. Hence there is no evidence for acetylation, but this mechanism cannot be ruled out since *all* positive charges may not be involved. The mechanism by which aspirin prevents stain uptake by collagen therefore remains to be elucidated.

We previously showed that fructose incubation reduced stain uptake at specific sites, particularly at the *c*1 band (Hadley et al., 1998). We have now examined the effects of co-incubation with fructose and aspirin to see if the presence of aspirin inhibits the charge changes due to fructation. After 11 days incubation in fructose/aspirin, fructose appears to be bound to collagen, and the heights of the peaks in the difference spectrum are similar to those observed for samples fructated for 11 days (Hadley et al., 1998). This suggests that aspirin does not prevent fructose binding, nor the production of reactive intermediates.

Co-incubation of glucose and aspirin was also investigated to analyse whether or not aspirin had a similar effect on glucation as seen with fructation. After 11 days incubation in glucose/aspirin, the difference spectrum produced is similar to that seen after 11 days incubation in glucose alone (result not shown), suggesting that, like fructose, glucose too binds in preference to aspirin.

Aspirin was originally thought to inhibit glycation by acetylating the free lysine residues (Day et al., 1979; Li et al., 1984; Rao et al., 1985; Rendell et al., 1986; Huby and Harding, 1988; Rao and Cotlier, 1988; Abraham et al., 1989). However, Yue et al (1984, 1985) showed that aspirin had no effect on glycation of rat tail tendon collagen, so

lysine residues were unaffected at this stage of the Maillard reaction and the mechanism of inhibition was apparently not due to acetylation. Aspirin is also thought to act as a free radical scavenger and/or chelator that inhibits the oxidative pathways of the Maillard reaction (Fu et al., 1992, 1994). So it could be speculated that by using longer incubation periods with aspirin/fructose or aspirin/glucose, inhibition of the later Maillard reaction would become apparent. After 11 days in the presence of 0.5 M fructose or 0.5 M glucose however, no such inhibition of intermediate formation is discernible.

The presence of aspirin had a marked effect on the capacity of the bovine cornea to swell. In the presence of 25 mM aspirin, neither human nor bovine cornea swelled (compare $T =$ initial with $T = 0$ in Fig. 4). When swollen in distilled water following one week's incubation with aspirin, the bovine corneas failed to swell to a hydration above $H \approx 7$ (see Fig. 4). This was also the case when fructose or glucose was included with the aspirin, but not when sugars alone were used. Corneal swelling is driven by Donnan potentials controlled by the charged proteoglycans and a chloride binding ligand (Hodson et al., 1992). Although the exact modes of action of aspirin are as yet unclear, it could be postulated from the impairment of swelling seen here, and from the effects of aspirin on the charge distribution on the collagen, that aspirin alters the p*K* and hence the charge on proteoglycans and/or chloride-binding ligands, thereby reducing the swelling pressure.

The presence of glycation-induced crosslinks *between* fibrils (either directly or mediated by other proteins) may not per se be observable by a reduction in the swelling of the tissue. This is because, in crosslinked tissue, water could be prevented from moving into spaces between the fibrils but an equivalent amount of water could enter other spaces between domains of fibrils, or between lamellae, producing the same measured hydration. To investigate if this is happening, X-ray diffraction was used to monitor the interfibrillar separation as a function of tissue water content. The results indicated that aspirin-treated tissues reached the same or greater interfibrillar spacings at lower hydrations than tissues incubated in sugars or buffer alone. There were no differences in this second group, suggesting that glycation does not affect the swelling of the cornea in any detectable way. The effect of aspirin, however, is puzzling. The results must be interpreted to mean that aspirin allows a more uniform tissue distribution of water than occurs when tissue is swollen under the same conditions in its absence. Collagen fibril spacing is thought to be governed by the constituents of the interfibrillar matrix, particularly proteoglycans and type VI collagen. When corneas are mechanically disrupted and interfibrillar components removed, corneal collagen fibrils have a strong tendency to clump and even fuse together (Meek and Holmes, 1983). It now seems likely that aspirin changes the electrostatic charge on some or all of these constituents (by altering the local pH or by acetylation), and this may weaken the attractive forces between fibrils or somehow increase the swelling pressure provided by the interfibrillar matrix.

Although aspirin is thought to inhibit the production of adverse end-products by inhibiting the oxidative pathways of the Maillard reaction, we have shown that it can alter the charge distribution at several sites along the D-period before AGEs have been formed. The possibility that, under the conditions used here, acetylation is taking place cannot, therefore, be excluded. Co-incubation of sugar with aspirin showed that sugars were still able to bind to collagen. The close similarity of the co-incubation difference spectra (Figs. 1(c) and 2(c)) with the difference spectra for sugars alone (Hadley et al., 1998) suggest that aspirin does not inhibit the binding of sugars to collagen. This provides further evidence that the inhibitory functions of aspirin are more likely to be post-Amadori, which strengthens the theory that aspirin behaves as a free radical scavenger. The current work also demonstrates the ability of aspirin to control, in vitro, corneal oedema, illustrating yet another potentially useful property of this low-cost analgesic.

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