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A comparative biochemical and ultrastructural study of proteoglycan–collagen interactions in corneal stroma

Functional and metabolic implications

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1. Corneas of mouse, rat, guinea pig, rabbit, sheep, cat, dog, pig and cow were quantitatively analysed for water, hydroxyproline, nucleic acid, total sulphated polyanion, chondroitin sulphate/dermatan sulphate and keratan sulphate, several samples or pools of tissue from each species being used. Ferret cornea was similarly analysed for water and hydroxyproline on one pool of eight corneas. Pooled frog (38) and ferret (eight) corneas and a single sample of human cornea were qualitatively examined for keratan sulphate and chondroitin sulphate/dermatan sulphate by electrophoresis on cellulose acetate membranes. Nine species (mouse, frog, rat, guinea pig, rabbit, sheep, cat, pig and cow) were examined by light microscopy and six (mouse, frog, rat, guinea pig, rabbit and cow) by electron microscopy, with the use of Alcian Blue or Cupromeronic Blue in critical-electrolyte-concentration (CEC) methods to stain proteoglycans. 2. Water (% of wet weight), hydroxyproline (mg/g dry wt.) and chondroitin sulphate (mg/g of hydroxyproline) contents were approximately constant across the species, except for mouse. 3. Keratan sulphate contents (mg/g of hydroxyproline) increased with corneal thickness, whereas dermatan sulphate contents decreased. The oversulphated domain of keratan sulphate was absent from mouse and frog corneas, increasing as percentage of total keratan sulphate with increasing corneal thickness. Sulphation of dermatan sulphate was essentially complete (i.e. one sulphate group per disaccharide unit). 4. Chondroitin sulphate/dermatan sulphate proteoglycans were present at the d bands of the collagen fibrils of all species examined, orthogonally arrayed, with high frequency, and occasionally at the e bands. Keratan sulphate proteoglycans were present at the a and c bands of all species examined, but with far higher frequency in the thicker corneas, where keratan sulphate contents were high. 5. Alcian Blue CEC staining showed much higher sulphation of keratan sulphate in thick corneas, e.g. that of cow, than in thin corneas, e.g. that of mouse, in keeping with biochemical analyses. 6. It is suggested that the constancy of interfibrillar volumes is regulated via the swelling and osmotic pressure of the interfibrillar polyanions, by adjustment of the extent of sulphation in two independent proteoglycan populations, to achieve an 'average sulphation' of the total polyanion similar to that of fully sulphated chondroitin sulphate/dermatan sulphate. 7. The balance of synthesis of the two kinds of proteoglycans may be determined by the O_2 supply to the avascular cornea. O_2 supply may also determine the conversion of chondroitin sulphate into dermatan sulphate.

INTRODUCTION

Connective tissues take many forms and serve many functions, but all are related by a core definition: they are 'systems of insoluble fibrils and soluble polymers which have evolved to take the stresses of movement and the maintenance of shape' (Scott, 1975). One might then ask if the means are as constant, i.e. do particular tissues and functions always use the same chemical structures? Are the interactions between them always the same?

Comparative studies dissect out the evolutionarily permanent from the species-specific. Fundamental solutions to the stress and shape problems persist across the spectrum of species, and responses to the environment appear against a constant background. Since chemical compositions of many connective tissues change dramatically with age, comparisons are subject to definitions of maturity.

The choice of tissue, as a model for connective tissue in general, is thus crucial. We chose corneal stroma, since it (1) contains mainly type 1 collagen, the most widespread and quantitatively abundant collagen, (2) has a precisely defined function, in contrast with many other connective tissues, (3) does not change its appearance and general properties for a large part of its active life, and (4) demonstrates a greater variety of interactions between fibrils and proteoglycans than have been seen in any other type-1-collagen-rich tissue (Scott, 1988), thus permitting a more complete overview of the evolutionary and physiological significance of such interactions. Previous limited studies showed differences between corneas of rat, rabbit and cow (Scott & Haigh, 1988*a*,*b*).

The present investigation therefore dealt with the interaction of chondroitin sulphate/dermatan sulphate proteoglycans (CS/DS PGs) and keratan sulphate proteoglycans (KS PGs) with the corneal fibrillar collagen matrix, as observed by electron microscopy, with the use of Cupromeronic Blue in a critical electrolyte concentration (CEC) mode to localize PGs (Haigh & Scott, 1986). Biochemical analyses of the glycosaminoglycan quantified the relationships, providing a chemically specific assessment of the cross-species variability. Histochemical staining of PGs for light microscopy with the use of Alcian Blue in CEC techniques (Scott & Dorling, 1965) confirmed and extended these

Abbreviations used: CS, chondroitin sulphate; DS, dermatan sulphate; KS, keratan sulphate; PG, proteoglycan; CEC, critical electrolyte concentration.

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results. Some of these findings have been preliminarily communicated (Scott, 1989; Scott & Bosworth, 1989).

EXPERIMENTAL

Tissues

Corneas were dissected out of freshly killed mature animals, namely cow, sheep, pig, dog, cat, rabbit, ferret, guinea pig, rat, mouse and frog, and weighed immediately. The tissue directly above the iris and the pupil was taken. Human cornea, from an eye removed at surgery for retro-retinal tumour, was similarly treated. Tissues were processed immediately for light microscopy and electron microscopy. Biochemical analyses were performed on at least 15 mg of freeze-dried tissue, or four corneas from larger animals.

Corneal stromal thickness

This was measured on fresh frozen sections of mouse, frog, rat, guinea pig and rabbit corneas in a dual-comparison microscope, with a 400-mesh electron-microscope grid as graticule (Scott & Haigh, 1988*a*).

Electron histochemistry

Corneal stromal PGs were stained with Cupromeronic Blue, in thick (10–15 μm) fresh frozen sections, at 0.3 M-$MgCl_2$ (Scott & Orford, 1981; Scott, 1985). Cupromeronic Blue was made by the method of Scott (1972). The stained sections were dehydrated, embedded in Taab resin, and ultrathin sections were cut for examination in a Philips 300 electron microscope (Haigh & Scott, 1986). Collagen fibril banding patterns (a–e) were revealed by staining with uranyl acetate. Occupancy of collagen bands by PGs was assessed by counting (Scott & Haigh, 1988*b*).

Light microscopy

Fresh frozen sections (6–8 μm thick) were stained with Alcian Blue (0.05%, w/v) in 25 mM-sodium acetate buffer, pH 5.8, containing 2.5% (v/v) glutaraldehyde and concentrations of $MgCl_2$ up to 1.4 M, in the CEC method (Scott, 1985). Alcian Blue was from ICI Dyestuffs, Blackley, Manchester, U.K.

Biochemistry

Freeze-dried corneas were digested with crystalline papain in 0.1 M-sodium acetate buffer, pH 6.0, containing 0.3 M-NaCl, 5 mM-disodium EDTA and 5 mM-cysteine hydrochloride at 65 °C for 16 h (Scott, 1960). The digest was filtered through a pad of Hyflo Supercel, which was then washed with digestion buffer. Filtrate and washings were combined, and used for subsequent analyses. Samples (2 μl) of the digest were taken for duplicate determinations of total polyanion by the Alcian Blue method (see below). Polyanions were recovered by cetylpyridinium precipitation, after dilution with water, at 50 mM-NaCl. The cetylpyridinium complexes were dissolved in propan-1-ol/water (2:1, v/v), and the polyanions were recovered as sodium salts by precipitation with saturated ethanolic sodium acetate solution.

Hydroxyproline

After hydrolysis in 5.5 M-HCl at 105 °C overnight the hydrolysates were neutralized (phenolphthalein) with NaOH. The final concentration of NaCl in the assay sample was adjusted by adding water to be less than 0.15 M. Hydroxyproline was measured by the method of Woessner (1961).

Nucleic acids

These were quantified spectrophotometrically at 260 nm, on the basis of $A_{260}^{1cm} = 6000$ per mol of nucleic acid phosphate/ml.

Electrophoresis

Electrotrophoresis was in 0.1 M-HCl (Wessler, 1970) on cellulose triacetate strips (Shandon Electrafor TRI-CAM) for 8–9 h at about 2 V/cm. Polyanions were stained as in the polyanion assay (see below). They were quantified by cutting out the stained bands, dissolving the membrane in dimethyl sulphoxide and spectrophotometrically determining Alcian Blue in the polyanion–stain complex (Newton *et al.*, 1974).

Polyanions

Polyanions were quantified by the use of Alcian Blue in a slightly modified method of Newton *et al.* (1974). Samples (2 μl) of polyanion solution were adsorbed on cellulose triacetate strips previously soaked in aq. 50 mM-sodium acetate and blotted to remove excess buffer. The strips were stained in Alcian Blue solution [0.2% (w/v) in ethanol/water (1:1, v/v) containing 25 mM-sodium acetate and 30 mM-$MgCl_2$] for 30 min and then washed in ethanol/water (1:1, v/v) containing 25 mM-sodium acetate and 30 mM-$MgCl_2$ until the background was colourless (about 1 h). The stained areas were cut out and dissolved in dimethyl sulphoxide containing 25 mM-sodium acetate and 25 mM-$MgCl_2$ (Newton *et al.*, 1974). Bovine nasal-septum CS was used as a standard polyanion, against which polyanion amounts were expressed. Two DNA preparations (gifts from the late K. S. Kirby), from rat spleen and liver, gave Alcian Blue binding equivalent to 0.045 μg of CS per A_{260}^{1cm} unit/ml. This value was used to correct total tissue polyanion values for nucleic acid content. Recovery of polyanion during isolation from papain and other enzyme digests was checked by using the above assay method.

Chondroitin sulphate/dermatan sulphate

CS and DS were measured by using the periodate/thiobarbiturate reaction on chondroitinase AC (chondroitin AC lyase, EC 4.2.2.5) or chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4) digests as described by Hascall *et al.* (1972) modified in the last stage by adding dimethyl sulphoxide to 50% (v/v) (Scott & Haigh, 1985*a*), in which the colour is more easily and sensitively measured.

Enzyme (from Sigma Chemical Co.) was dissolved at 5 units/ml in buffer (0.25 M-Tris/0.275 M-sodium acetate/0.6 M-NaCl/0.05% BSA adjusted to pH 8.0 with 2 M-acetic acid). Then 1 vol. buffered enzyme solution was added to 1 vol. of polyanion solution containing up to 2.5 mg of total polyanion/ml. Digestion was at 37 °C for 4 h. Digests were treated with cetylpyridinium chloride (5%, w/v) at room temperature to precipitate undigested polyanion and cooled to 4 °C before the combined precipitate of polyanion and cetylpyridinium chloride was recovered. Enzyme-treated standard CS gave no precipitate with cetylpyridinium, demonstrating complete digestion. The supernatants were used in the thiobarbiturate colour reaction. Precipitated glycosaminoglycans were recovered via solution in propan-1-ol/water (2:1, v/v) and subsequent precipitation as sodium salts on addition of 2 vol. of saturated ethanolic sodium acetate (about 0.7 M-sodium acetate). The sodium salts were washed in ethanol and diethyl ether.

Keratan sulphate

KS was measured by several methods, as follows. In the first the decrease in Alcian Blue units in the polyanion assay (see above) after digestion with keratanase I (keratan sulphate 4-galactopyranosylglycan hydrolase, from Sigma Chemical Co.) was measured. Enzyme was dissolved at 10 units/ml in buffer (0.1 M-Tris/0.125 M-sodium acetate/0.2 M-NaCl/0.02% BSA adjusted to pH 8 with 2 M-acetic acid). Then 1 vol. of the buffered enzyme was added to 1 vol. of polyanion solution containing up

to 2.5 mg of total polyanion/ml. Digestion was for 4 h at 37 °C. Samples (2 μl) of control (no enzyme) solution or enzyme digest were applied directly, in duplicate, to the cellulose triacetate strip.

The second method involved measurement of total Alcian Blue-stainable material migrating faster than hyaluronan on electrophoresis in 0.1 M-HCl, after digestion with chondroitinase ABC, and recovery via the cetylpyridinium salt.

In the third method highly sulphated KS, i.e. Alcian Blue-staining material running faster in 0.1 M-HCl than standard CS, after combined digestion with keratanase I and chondroitinase ABC, was quantified by means of the polyanion assay (see above).

Heparan sulphate

Heparan sulphate or other HNO_2-sensitive polyanions were determined by means of the Alcian Blue polyanion assay (see above). Polyanions were incubated as described by Scott (1979) in aq. 5% (w/v) $NaNO_2$ (1 vol.) plus 10% (v/v) acetic acid (1 vol.) for 90 min at room temperature, and then aq. 12.5% (w/v) ammonium sulphamate (1 vol.) was added to convert the HNO_2 into N_2 and water. Controls, in which all the reactants were mixed and incubated for 10 min before addition to the polyanion, were performed.

RESULTS

Corneal thickness

Corneal thickness increased with increasing body size. The thinnest were mouse (approx. 80 μm) and frog (approx. 45 μm). Other thicknesses were as follows: for cow, approx. 800 μm, for rabbit, approx. 450 μm, for rat, approx. 220 μm, and for guinea pig, approx. 220 μm. Human cornea is about 550 μm thick (Fatt, 1978). Cat cornea (approx. 755 μm; Carrington & Woodward, 1986) is considerably thicker than expected for the size of the animal. Data for corneas from sheep (500 μm), dog (730–950 μm) and pig (slightly less than 1000 μm) are from Prince *et al.* (1960).

Water content

Water content measured as loss in weight on freeze-drying, and expressed as a percentage of wet weight, was very similar in all species, at 80±2% (Fig. 1). The value for mouse is probably slightly underestimated, because the small corneas may have lost water before completion of the wet weighing.

Light microscopy

Alcian Blue staining of corneal stroma persisted to higher concentrations of $MgCl_2$ (i.e. higher CECs) with increasing stromal thickness. Thus CECs (concentrations of $MgCl_2$) were: for mouse, $\leqslant$ 0.5 M; for rat, $\leqslant$ 0.7 M; for guinea pig, $\leqslant$ 0.7 M; for frog, $\leqslant$ 0.7 M; for rabbit, $\leqslant$ 1.4 M; for sheep, $\geqslant$ 1.1 M; for pig, $\geqslant$ 1.0 M; for cow, $\geqslant$ 1.1 M. The value for cat was > 1.0 M (S. D. Carrington, personal communication). These CECs parallel the occurrence of increasingly highly sulphated KS (see below).

Electron microscopy

PGs were localized at a, c, d and e collagen fibril bands in cow, rabbit, guinea pig, rat, frog and mouse. Whereas the d band was frequently occupied by PG in all species, the a and c bands were almost devoid of PGs in the mouse. Frog and the guinea pig also showed few a-band- and c-band-associated PGs. Relative percentage occupancies have been previously reported for all four sites in cow, rabbit and rat (Scott & Haigh, 1988*a*,*b*). Because of the small number of observations, a-band/c-band relative occupancies in guinea pig, frog and mouse could not be determined accurately. The d-band/e-band relative occupancy was about 5:1 in mouse, and similarly high in guinea pig and frog. Digestion of mouse cornea with chondroitin AC lyase or sheep testicular hyaluronanase (EC 3.2.1.35, from Sigma Chemical Co.), both used as described by Scott & Haigh (1986), decreased, but did not remove, the d-band- and e-band-associated PGs. The remaining PG filaments were slightly less electron-dense, and perhaps also shorter, than in control tissues, but the d and e bands were still very frequently occupied by PGs.

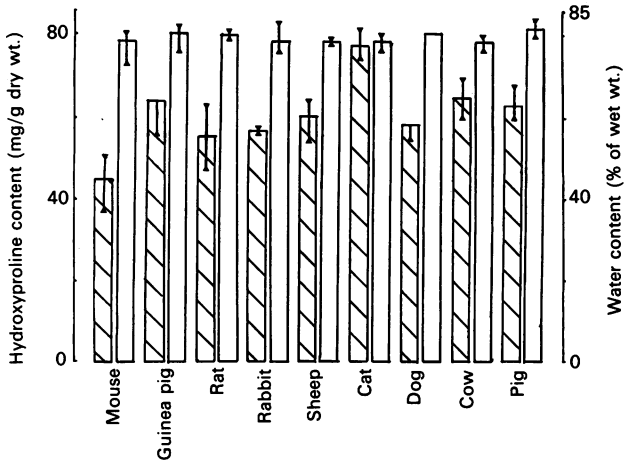

Fig. 1. Histograms of water content (□) and hydroxyproline content (▨) in corneas of nine mammals, in order of increasing corneal thickness from left to right

Water contents (loss of weight on freeze-drying overnight) were constant at 80±2%. The bars show the ranges of values. A complete bar with two triangles is based on three or more assays, half a bar with one triangle on two assays, and no bar on one assay. The mouse data were from more than 150 corneas, rat data from more than 50 and guinea-pig data from more than 40. In addition to those species listed on the Figure, a single pool of eight ferret corneas contained 79% water and 55.3 mg of hydroxyproline/g dry wt. Hydroxyproline contents in thicker corneas were relatively constant at 57.5±5 mg/g dry wt., except for cat, which was higher (in three assays), mouse and rat, the low values for which partly reflect the lower proportion of the corneal weight due to stroma and the higher contribution of epithelium and endothelium compared with thicker corneas (see the text for discussion).

Biochemistry

Results are given on recovered polyanions in those cases where recovery of sodium salts, based on the papain digest, determined by the polyanion assay, exceeded 75%. In most cases recoveries were 90±10%. Values were scaled up to a recovery of 100%, based on the polyanion assay.

Tissue nucleic acid

Tissue nucleic acid, per g dry wt., defined as the papain-resistant polyanionic material precipitated by cetylpyridinium, with a characteristic nucleic acid spectrum, decreased from 4.7 mg in mouse to 2.0 mg in rabbit and 1.6 mg in pig, parallelling the relative contribution of cells to the total bulk of the cornea, as seen in the light microscope.

Hydroxyproline

Hydroxyproline, as percentage of dry weight, increased from mouse to guinea pig, and then (with the exception of cat) remained approximately constant to pig (Fig. 1). The low value of 44.4±7 mg/g in mouse is at least partly due to the much larger contribution of the epithelium and endothelium to the corneal weight compared with those of the larger animals,

Table 1. Total sulphated polyanion in corneal stroma

Method I gives the chondroitinase ABC-resistant electrophoretically mobile material (HCl) plus chondroitinase ABC-released thiobarbiturate chromogen. Method II gives the electrophoretically mobile (HCl) material. Method III gives the total polyanion (Alcian Blue-staining) minus nucleic acid. For details see the text. Values for individual species are expressed as means ± range, with numbers of assays performed being given in parentheses. Abbreviation: N.D., not done.

Species	Content of total sulphated polyanion (mg of CS equivalent/mg dry wt.)		
	Method I	Method II	Method III
Mouse	16.2 (1)	9.21 (1)	14.1 ± 6.1 (3)
Guinea pig	22.5 ± 0.03 (2)	20.9 ± 0.12 (2)	34.3 (1)
Rat	34.9 ± 10.8 (2)	28.3 ± 15.3 (2)	25.03 ± 2.0 (2)
Rabbit	32.1 ± 7.18 (3)	33.7 ± 13.3 (3)	41.5 (1)
Sheep	41.8 ± 11.4 (2)	42.9 ± 11.9 (2)	42.8 ± 2.9 (3)
Cat	30.74 ± 8.6 (5)	27.98 ± 11.8 (5)	24.96 ± 3.1 (3)
Dog	N.D.	N.D.	24.1 (1)
Cow	34.5 ± 5.23 (3)	34.3 ± 7.1 (2)	39.6 ± 1.8 (3)
Pig	27.12 ± 3.2 (3)	31.03 ± 16 (4)	34.34 ± 7 (4)
Average	30.93 ($n = 21$)	29.76 ($n = 21$)	31.16 ($n = 21$)

Population mean (excluding mouse) 32.01 ± 9.42 (mean ± S.D.)

parallelling the nucleic acid content (above). Cat cornea had significantly more hydroxyproline than the other mammals. It is also much larger in area than the others, comprising about 30% of the entire globe (Prince *et al.*, 1960).

Excluding mouse and cat, the mean of values for seven animals (including ferret) is 57.4 ± 5.5 mg/g dry wt., and four are within 5% of this mean.

Total stromal polyanion

Total stromal polyanion, expressed as CS equivalents, was defined in a number of ways, namely (*a*) total Alcian Blue-stainable material in the polyanion assay, minus nucleic acid, (*b*) total polyanion migrating in 0.1 M-HCl, or (*c*) thiobarbiturate chromogen after chondroitinase ABC digestion, plus chondroitinase ABC-resistant Alcian Blue-staining polyanion migrating in 0.1 M-HCl.

The average of the means for the species was very similar for all three methods (29.76, 30.93 and 31.16 mg/g dry wt.; Table 1), strongly implying that the same thing was being measured by each method, but there was considerable variability, with an S.D. of 10.4 about the group mean. Reproducibility of values for duplicate samples of the same tissue (cat and pig) taken though the procedure from papain digestion to assay was better than ± 10%. The average for mouse was significantly lower ($P = 0.1$) than the average for the larger animals.

Chondroitin sulphate

CS contents (mg/g of hydroxyproline) clustered about a mean for seven species (excluding mouse) of 200 (Fig. 2). All were within 13%, four species were within 5% of the mean, with the remainder symmetrically placed about the mean.

The electrophoretic mobility of the CS in 0.1 M-HCl was much lower in all species than standard CS, presumably reflecting a low degree of sulphation.

Dermatan sulphate

By contrast, DS, defined as the difference chondroitinase ABC-released thiobarbiturate chromogen minus chondroitinase AC-released chromogen, decreased very markedly from mouse to sheep and cow. More than 60% of the CS/DS in mouse was DS, compared with 20% in the larger animals (Fig. 2). The electrophoretic mobility in 0.1 M-HCl of DS from all species was close to that of standard CS.

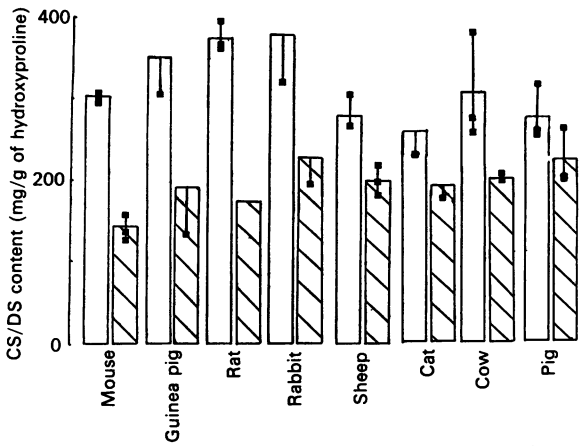

Fig. 2. Histograms of CS/DS contents of a series of mammalian corneas of increasing thickness from left to right

□, Chromogen from chondroitinase ABC digestion; ▨, chromogen from chondroitinase AC digestion. The bars indicate ranges of values. Where more than one black rectangle is shown, they indicate actual data points. No bar indicates a single determination. Assays were performed in parallel on samples taken from the same glycosaminoglycan preparation. Corneas of mouse, rat and rabbit were pooled for each set of assays. Variation between samples of the same tissue (pig) put through the procedure from papain digestion to final assay was ± 8%. The chondroitinase AC chromogen reflects CS content; the difference chondroitinase ABC-released chromogen minus chondroitinase AC-released chromogen equates with iduronate-containing galactosaminoglycan ('DS'). CS content was relatively constant throughout the series at 200 ± 25 mg/g of hydroxyproline, except for mouse, which was lower. 'DS' was highest, as percentage of total CS/DS, in mouse and rat, declining from 50–60% to 20–30% in thicker corneas.

Hyaluronan

Hyaluronan defined according to its position on electrophoresis in 0.1 M-HCl, before and after chondroitinase ABC digestion, was present in small amounts (< 5% of total glycosaminoglycan).

Heparan sulphate

Heparan sulphate, defined as material susceptible to HNO_2 degradation, moving faster than hyaluronan but not as quickly

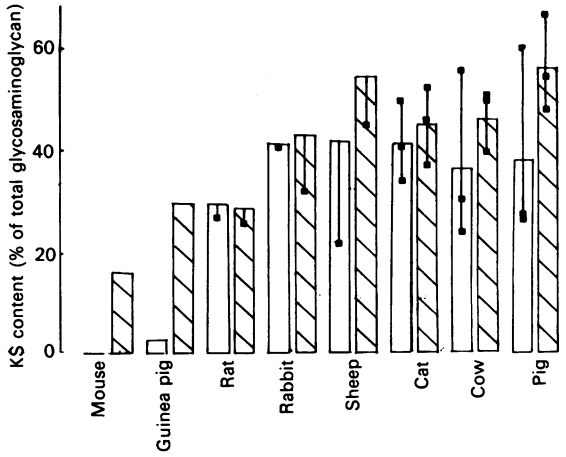

Fig. 3. Histograms of KS (▨, chondroitinase ABC-resistant Alcian Blue-stained material migrating electrophoretically in 0.1 M-HCl) as percentage of total polyanion, and oversulphated KS (□, OS KS) as percentage of total KS

Oversulphated KS was quantified by eluting the Alcian Blue-stained rapidly migrating keratanase I digestion product from the electrophoretic strip (see Fig. 4 and the text for details). Bars show ranges of values. No bar indicates a single determination. Where more than one black rectangle is shown, they indicate actual data points. KS increased markedly from mouse through rat and guinea pig to a plateau at about 50% of total glycosaminoglycans in thicker corneas. All samples except mouse were recovered from chondroitinase ABC digestions at 0.3 M-NaCl by cetylpyridinium precipitation. Further KS could be recovered at 50 mM-NaCl (about 10% more from cow glycosaminoglycan). No KS was recovered from mouse corneal glycosaminoglycan at 0.3 M-NaCl. The result was obtained after recovery at 50 mM-NaCl. In this respect mouse contains no KS of the kind present in, for example, cow (see the text for discussion). Oversulphated KS (keratanase I-resistant non-reducing terminal domain of the KS chain; Oeben *et al.*, 1987) increased from 0% in the mouse to about 40% of total KS in thicker corneas.

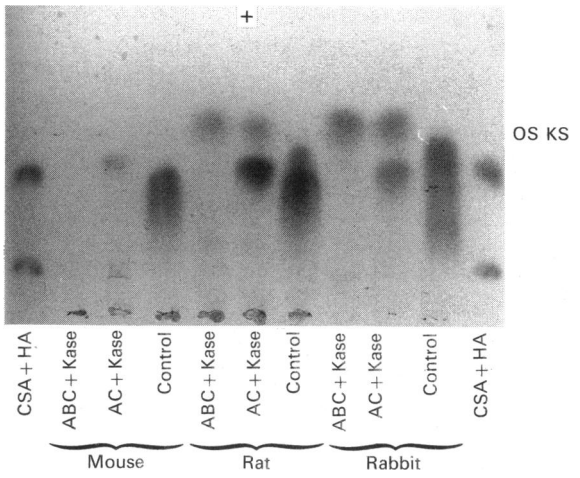

Fig. 4. Electrophoretic strip, run in 0.1 M-HCl on cellulose triacetate membrane, showing corneal glycosaminoglycans from mouse, rat and rabbit, stained with Alcian Blue

Standard CS and hyaluronan (HA) are at right and left of the strip. ABC, AC and Kase respectively indicate products of chondroitinase ABC, chondroitinase AC and keratanase digestion. ABC+Kase etc. indicate simultaneous digestion by both enzymes. No migrating polyanion was present in mouse glycosaminoglycans after digestion with chondroitinase ABC plus keratanase I, in contrast with the deeply staining spot (oversulphated KS, OS KS) in rabbit and rat. Iduronate-rich glycosaminoglycan from the three species ran at the speed of standard CS. The ratio of the amounts of this material to that of oversulphated KS was greater in rat than in rabbit. Loading of chondroitinase AC+keratanase-digested glycosaminoglycan from mouse was less than that of the corresponding control and chondroitinase ABC+keratanase-digested glycosaminoglycan. The non-migrating Alcian Blue-stained material in mouse and rat polyanion was probably nucleic acid.

as CS on electrophoresis in 0.1 M-HCl, was present in appreciable amounts (10–15% of total glycosaminoglycan) only in mouse cornea. This probably reflects the greater relative contribution of cells and basement membrane to the total cornea.

Keratan sulphate

KS, defined as Alcian Blue-staining chondroitinase ABC-resistant polyanion migrating in 0.1 M-HCl, increased dramatically as a percentage of total sulphated polyanion from mouse, through the values for rat and rabbit to an approximate plateau at > 50% in the cases of pig, sheep, cow (Fig. 3) and human. These values were obtained on glycosaminoglycan recovered from chondroitinase digestions at the ambient salt concentration (approx. 0.3 M-NaCl) by precipitation with cetylpyridinium chloride. By precipitation at 50 mM-NaCl more KS was recovered. The extra material was undersulphated, according to its electrophoretic mobility in 0.1 M-HCl, amounting in the case of cow to another 10% of total KS. The KS in mouse cornea was undersulphated, electrophoresing considerably more slowly than standard CS. This was recovered at the low NaCl concentration. The value shown in Fig. 3 refers to this material. Almost no KS was measurable in glycosaminoglycan recovered at 0.3 M-NaCl. Comparable results were obtained with the polyanion assay, comparing before and after keratanase I digestion (results not shown). KS of all other species contained material electrophoresing faster than standard CS.

'Highly sulphated KS' left after keratanase digestion (Oeben *et al.*, 1987) was not measurable in mouse cornea, but increased dramatically from rat to cow (Fig. 4). It comprised 38% of total KS in the human cornea.

DISCUSSION

Corneal thickness and chemical composition

Three biochemical parameters were approximately constant, as a function of corneal thickness: water content (% of wet weight), collagen content (mg of hydroxyproline/g dry wt.) except for mouse and cat, and CS as chondroitinase AC-released chromogen. Against this background two clear trends stood out: (1) an increase in KS from almost zero to about 50% of total glycosaminoglycan, and (2) a decrease in DS from over 60% of total CS+DS to about 20%, both with increasing corneal thickness. The most dramatic change occurred in the amounts of the oversulphated domain of KS (Figs. 3 and 4). This was not measurable in mouse cornea, and was present in smaller amounts in rat and guinea-pig corneas compared with cow, sheep, human etc. KS had previously not been detected in mouse cornea (Scott & Haigh, 1988*a*), on the basis of the absence of highly sulphated polyanion after chondroitinase ABC digestion. The Alcian Blue polyanion assay (see the Experimental section) showed that mouse cornea contained keratanase-sensitive polyanion, and, with the use of large amounts of chondroitinase ABC-treated mouse corneal glycosaminoglycan, this 'KS' electrophoresed in 0.1 M-HCl in the position of undersulphated CS. It was heterogeneous, spread over a considerable area of the electrophoretogram, present in small amount, and consequently difficult to see. This chemical demonstration of a species of KS resolves a part of the discrepancy between the results reported by Scott & Haigh

(1988*a*) and those obtained by Venn & Mason (1984), who found KS epitopes in mouse cornea and keratanase-sensitive [^{35}S]sulphate-labelled polyanion in glycosaminoglycan extracts. Both methods are extremely sensitive, and do not directly reflect the actual tissue contents, which are evidently low.

There remains the paradox that antibodies to KS can be raised in the mouse although KS epitopes are present in the cornea (Venn & Mason, 1984). There are at least two possible explanations: (*a*) that the corneal proteoglycan is not available to circulating antibodies or to its own immune system, or (*b*) that the antibodies were primarily directed to parts of the KS molecule present in bovine cartilage KS, against which the antibodies were raised, but not in mouse cornea. Many KS antibodies react with the oversulphated domain, which was not chemically measurable in mouse corneal KS (see the Results section). This material constituted up to 50% of the total KS in the corneas of larger animals.

The keratanase-sensitive and keratanase-resistant parts of KS followed similar trends through the species, as expected, since both are parts of the same chain (Oeben *et al.*, 1987). Our results suggest that KS chains in mouse are not completed by oversulphation, in contrast with the other mammals. The oversulphated domain was also absent from frog KS.

The electrophoretic mobility of KS in 0.2 M-HCl increased a little as the corneal thickness increased, presumably owing to the changes in sulphation discussed above.

Ultrastructural localization of PGs

Light microscopy. The striking increase in CEC of Alcian Blue staining with increase in thickness of corneal stroma, from < 0.5 M- to > 1.3 M-$MgCl_2$, implies an increase in sulphation of the sulphated proteoglycans, since CECs of > 0.5 M are characteristic of oversulphated CS (i.e. more than 1.0 sulphate group/disaccharide unit) or KS (Scott, 1973, 1985). Conversely, the lower CEC of the mouse material is characteristic of normally sulphated CS/DS (Scott, 1985). Both conclusions are borne out by the chemical analyses, which show no oversulphated glycosaminoglycan and very little KS in mouse cornea, and an increase in KS oversulphation through the other, larger, species. The Alcian Blue CEC procedure used at high $MgCl_2$ molarity therefore provides a relatively quick and simple means of demonstrating the presence of oversulphated domains of KS in mammalian corneas.

The KS PGs in rat and guinea-pig corneas are not mixtures of oversulphated species, of the kind found in cow or rabbit, with an undersulphated species, of the kind present in mouse. The former would stain to a high CEC, and there is no staining in rat and guinea-pig corneas above 0.7–0.8 M-$MgCl_2$. Rather, most KS PGs in rat or guinea pig must have an average sulphation lower than in cow or rabbit KS, but above that of mouse KS, giving the CEC observed, between the two extremes. This average sulphation could be due to the oversulphated domain being shorter or less sulphated, and/or to the undersulphated domain being longer or less sulphated, in rat or guinea pig than in cow or rabbit.

Electron microscopy. Four binding sites along the collagen fibril specifically associate with two KS PGs (A and B at fibril bands c and a) or two DS PGs (A and B at fibril bands d and e) (Scott & Haigh, 1985*b*; Scott, 1988). All four binding sites bound PGs in all the species examined, with the occupancy of bands a and c increasing in absolute and relative frequency with increasing thickness of corneal stroma (see the Results section), in keeping with the conclusion that these sites are reserved to KS PGs (Scott, 1988), and with the finding (Fig. 3) that KS increases with corneal thickness.

The occupancy by KS PG of the a band relative to the c band increased from rat to cow (Scott & Haigh, 1988*b*). Although accurate measurements were not possible for mouse and guinea pig, because of the small number of observable band occupancies, there were not more KS PGs at the a band than the c band. The conclusion (Scott & Haigh, 1988*b*) that the increase in total tissue KS from rat to cow was predominantly in the KS PG at the a band therefore may be generalized, namely that the a band in thicker corneas is more frequently occupied relative to the c band than in thin (mammalian) corneas, parallelling the increase in tissue KS. Since much of this increase involves the oversulphated domain (Fig. 3 and 4), the a-band KS PG may contain more highly sulphated material than the c-band KS PG, compatible with the finding (Scott & Haigh, 1988*b*) that the CEC of the a-band KS PG was higher than that of the c-band KS PG.

DS PGs were very frequently present at the d bands in all species. Thin corneas (mouse, guinea pig and frog) were similar to thicker corneas, with d-band occupancy greatly exceeding that of the e band. The contrast between the very variable KS PG band occupancies (above) and the more constant DS PG interaction (Scott & Haigh, 1988*b*) is thus confirmed and extended.

The constancy in d-band/e-band occupancy contrasts with the variable composition of the CS/DS PGs in terms of iduronate content (Fig. 2). The d-band and e-band PGs were suggested (Scott & Haigh, 1988*b*) to be the DS PG A and DS PG B described by Gregory *et al.* (1982), and DS PG B was richer in iduronate. Our results discount the possibility that iduronate contents direct the PGs to their binding sites on the collagen fibril. Most of the iduronate must go into the d-band PG in all cases, because of the great preponderance of d-band PG over e-band PG. The difference in iduronate content between d-band and e-band PGs may persist in different species, but the actual amounts must vary with the tissue iduronate concentration. Neither d-band nor e-band glycosaminoglycans in the mouse were completely digested by either chondroitinase AC or testicular hyaluronase (see the Results section), suggesting that both d-band and e-band PGs contained considerable amounts of iduronate.

This variation between species but in the same tissue parallels that between tissues in the same species, e.g. between skin or sclera, in which the DS is rich in iduronate, and annulus fibrosus CS, in which there is no iduronate (Scott & Haigh, 1986). In each case the PG associates with the d or e band, implying that the protein core, and not the glycosaminoglycan chains, determine the specific binding (Scott, 1988).

Physiological function of corneal proteoglycans

Stromal CS and KS exert independent swelling pressures (Hedbys, 1961), implying their presence on two different PGs, a result not biochemically proven for 20 years (Scott, 1989). The swelling pressure separates and maintains the collagen fibrils in a regular array, this being required for transparency of the stroma (Maurice, 1957). PGs were regularly attached to the collagen fibrils (Scott & Haigh, 1985*b*), giving order to the postulated structure.

Collagen fibrils in stromas of many species had very similar diameters (Craig & Parry, 1981) and interfibrillar separations (Gyi *et al.*, 1988). Thus the volumes between the fibrils should also be the same, this being compatible with our finding that the percentage loss on drying is constant throughout the species, assuming that most loosely bound water is interfibrillar. Hydroxyproline contents were also similar, as expected from this

simple model, except for mouse and cat (which were not examined in the works cited above).

It is therefore remarkable that the PGs are so variable. The explanation probably lies in the interchangeability of KS and CS/DS in swelling the matrix. The CS/DS percentage of total glycosaminoglycan declines as KS increases (Figs. 3 and 4). The total sulphated polyanion content per mg of hydroxyproline thus tends to an approximately constant level, as does therefore the polyanionic change per unit volume. Measurements of total stromal sulphated polyanion per g dry wt. by three methods demonstrated considerable variability (Table 1). The 25 species means of the ratio of total stromal sulphated polyanion to hydroxyproline computed from Table 1 and Fig. 1, distributed about an average of 503 ± 130 mg/g (mean $\pm$ S.D.). No species mean differed from this average at 5% significance, and there was no consistent trend with corneal thickness. To this limited extent the results support the idea that total stromal sulphated polyanion concentrations are similar in the larger species examined.

The polyanionic charge per unit volume contributes to the approximately constant water content per mg of collagen, through osmotic and swelling properties, and hence to the constancy of the interfibrillar volume.

This proposal accounts for the reciprocities in relationships between KS and CS/DS (Scott, 1989). Thus, as the content of KS increases, and the CS/DS content decreases, the average sulphation or the KS increases as that of the CS/DS decreases. There is an average extent of sulphation, taken over the whole tissue polyanion, approximating that in fully sulphated DS, which produces the required swelling pressure. By adjusting sulphation in the independent glycosaminoglycan populations, that average value is achieved.

Control of glycosaminoglycan biosynthesis in corneal stroma

The cornea is avascular and relatively anoxic, receiving O_2 and glucose by diffusion from outside. No O_2 is consumed in synthesizing KS from precursor glucose, whereas CS production requires O_2 in making glucuronate (Stockwell & Scott, 1965). It was suggested (Scott & Haigh, 1988*a*) that thick corneas, in which O_2 diffuses over long distances, would find it easier to make KS rather than CS/DS, and conversely that CS production in thin small corneas ought to proceed without difficulty, since O_2 would be easily available along very short diffusion paths. Our results confirm and extend the previous data in support of this hypothesis. The ratio of KS to CS produced in organ cultures of bovine cornea increased markedly as ambient O_2 partial pressures decreased (S. Rindi, R. Salvini, C. Balduini, G. De Luca & J. E. Scott, unpublished work).

DS formation is the final stage, after polymerization. Our results suggest that this step, probably involving the abstraction of the C-5 hydrogen atom from CS glucuronate (Malmström, 1981), may also be sensitive to ambient O_2 partial pressure, since the DS/CS ratio decreases markedly with corneal thickness.

Another key metabolite may be SO_4^{2-}, low concentrations of which were shown (Silbert *et al.*, 1986; Humphries *et al.*, 1988) to limit the extent of sulphation of chondroitin and the epimerization of chondroitin to dermatan in cell cultures. The proportion of fully sulphated iduronate-rich DS increased with SO_4^{2-} concentration. This picture resembles that in Fig. 2, where undersulphated CS was found in thick corneas and fully sulphated DS in thin corneas, with the implication that SO_4^{2-} availability may be more restricted in the former. However, it is also implied that sulphation of KS continues in these circumstances.

Rates of turnover of the CS/DS PG and KS PG must play a part in determining their tissue content, but there is no knowledge of these rates in corneas.

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