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Keratan sulphate and the ultrastructure of cornea and cartilage: a 'stand-in' for chondroitin sulphate in conditions of oxygen lack?

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INTRODUCTION

Maurice (1957) suggested that corneal transparency depended on the maintenance of regular spacing of the collagen fibrils in the cornea, and Balazs (1965) speculated that this regularity was maintained by structure in the interfibrillar space solution, set up by glycosaminoglycans (GAGs). Hedbys (1961) had shown that the corneal GAGs exerted a swelling pressure, sufficient to inflate the collagenous matrix, and that the two GAGs, 'chondroitin sulphate' (CS) and keratan sulphate (KS) contributed independently to the swelling pressure. Using new ultrastructural methods (Scott, 1985) it was shown that corneal GAGs had specific binding sites along the collagen fibrils (Scott & Haigh, 1985*b*). Dermatan sulphate (DS) was associated at the d and e bands, and KS at the a and c bands. This specific and regular localisation seemed to meet the requirements of Balazs' hypothetical interfibrillar structures, which help to maintain corneal transparency.

Keratan sulphate, as its name implies, was, when it was christened (Meyer, Linker, Davidson & Weissman, 1953) regarded as characteristic of the cornea. Later it was found in the intervertebral disc (Gardel & Rastgeldi, 1954), and then in many cartilages (e.g. Kaplan & Meyer, 1959). Like all other sulphated glycosaminoglycans, KS is attached to a protein core, but whereas skeletal KS is ether-linked to serine or threonine, corneal KS is bound via amide to asparagine. Moreover, while in the large cartilage proteoglycan (PG), several KS chains share the same protein core with many chondroitin sulphate chains, giving a PG of very high mol. wt (2×10^6 Da), corneal KS-PG contains only 1–2 GAG chains, in a PG of mol. wt. 10^5 DA (Gregory, Cöster & Damle, 1982). The 'chondroitin sulphate' of cornea contains iduronic acid, and therefore qualifies as a dermatan sulphate (Stuhlsatz, Muthiah & Greiling, 1972). The CS-DS-PG of cornea is similar in size and number of glycan chains per molecule to that of the KS-PG (Gregory *et al.* 1982). Thus the corneal PGs are quite different from the large cartilage PG.

The KS-containing large cartilage PG does not appear to be regularly located with respect to collagen fibrils (Scott, 1985), but it serves a similar function to that of the PGs of cornea in keeping the collagen meshwork inflated, and hence endowing the tissue with rigidity and elasticity (Stockwell & Scott, 1965; Scott, 1975).

KS is not distributed uniformly in cartilage and the pattern of its distribution changes with age (Stockwell & Scott, 1965, 1967; Inerot & Heinegård, 1983). There is a reciprocal relationship between the regions of high CS content and those of high KS content. KS is present in greatest amounts in regions of cartilage furthest from

supplies of nutrients and oxygen, which are derived mainly from the synovial fluid and from the underlying bone in the young animal. In later life the route from bone is cut off, when communicating canals close, and a rise in the KS content of the cartilage ensues in the parts nearest the bone (Stockwell, 1970). Shortage of oxygen should not seriously impede biosynthesis of KS, which requires no overall oxidation from the precursor glucose whereas in the conversion of glucose to glucuronic acid in CS biosynthesis oxygen is consumed. In low oxygen tensions CS biosynthesis might be diminished.

There are analogies between cornea and cartilage. Both are relatively avascular, and metabolism depends on diffusion from fluids outside the tissue. It is a corollary of the 'oxygen-lack' hypothesis that very thin tissues (cartilage or cornea), e.g. from small animals, should not be affected, since diffusion paths therein are short. The content of KS in such tissues should be lower than in thicker and bigger tissues, obtained from larger animals. We examined mouse corneal stroma, which is about 15% of the thickness of rabbit corneal stroma, using Alcian blue staining, and found that there were indeed marked differences between rabbit and mouse, indicative of there being much less KS in the latter. Venn & Mason (1985) found evidence for KS in mouse cornea, based on ^{35}S uptake into keratanase-sensitive GAGs and staining with KS specific antibodies, but neither technique is quantitative. We have therefore investigated rabbit, rat and mouse cornea, biochemically and by light and electron microscopy, for their content of KS and the consequent impact on their ultrastructure.

Some of these results have been the subject of a preliminary communication (Haigh & Scott, Biochemical Society 621st Meeting, London, Dec. 1986, Abst. No. 280).

MATERIAL AND METHODS

Source of materials

Papain was 2 × crystallised (BDH, Poole, Dorset). Chondroitinase ABC (EC 4.2.2.4), chondroitinase AC (EC 4.2.2.5) and hyaluronidase (testicular, 350 WHO units/mg) were from Sigma, Gillingham, England. Keratanase (keratan sulphate 4-galactopyranosylglycanohydrolase purified from pseudomonas, not less than 20 units/mg) was from Seikagaku Kogyo Co Ltd, Tokyo, Japan.

Cupromeronic blue was from Aldrich Chemical Co., Milwaukee, U.S.A., and Alcian blue 8G was from I.C.I. Ltd, Blackley, Manchester.

Chondroitin-4-sulphate (whale) was from Sigma, dermatan sulphate was prepared from pig skin, and hyaluronate from mesothelioma fluid, both by the method of Scott (1960). Keratan sulphate (nos. 70 and 82, 0·77 and 1·4 mol S/mol glucosamine respectively) was from bovine cornea, a gift of Dr H. Greiling, Aachen, FRG.

Tissues

Tissues for biochemical analysis were taken from eyes of young adult Sprague–Dawley rats, rabbits and mice from the Manchester University breeding colony. Rats and mice were killed by blows to the back of the head, rabbits by injection of air into the ear vein while under nembutal anaesthesia.

Preparation of tissues

Corneas were taken from immediately over the iris, from recently killed mice, rats and rabbits. The tissues were frozen, sectioned at 10 μm, stained (with or without prior digestion by keratanase, hyaluronidase or chondroitinases) in 0·05% w/v Cupromeronic blue solutions containing either 0·3 M or 0·05 M $MgCl_2$, at pH 5·6 (0·025 M Na

acetate buffer) dehydrated in graded ethanols, embedded in Taab epoxy resin and sectioned for electron microscopy (Scott & Haigh, 1985*a*, *b*; Haigh & Scott, 1986). Other fresh-frozen sections were stained in 0·05% w/v Alcian blue solutions containing $MgCl_2$ at 0·3, 0·5 and 0·7 M at pH 5·6 (0·025 M Na acetate buffer) (Scott & Dorling, 1965).

Ultrathin sections were stained with 0·5% w/v uranyl acetate to demonstrate the collagen fibril banding pattern, a–e.

Statistics of PG localisation at the collagen bands were obtained by counting all the cases in which an orthogonally placed PG filament could be unambiguously seen to be at one of the a–e bands.

Biochemical investigations

Recovery of acid glycosaminoglycan. Corneas (20–60 pooled mice, 6–12 pooled rats, 2–6 pooled rabbits) were digested with papain, the GAG was precipitated as the cetylpryridinium salt, dissolved in propanol:water 2:1 and precipitated as the sodium salt after the addition of saturated ethanolic sodium acetate (Scott, Orford & Hughes, 1981; Scott & Haigh, 1986). *Hexuronate* and *hydroxyproline* were assayed by the methods of Bitter & Muir (1962) and Woessner (1961) respectively. *The unsaturated hexuronate chromogen* from chondroitinase ABC-digested GAGs was measured by a modification of the method of Hascall, Riolo, Hayward & Reynolds (1972), in which dimethyl sulphoxide was added before measurement of the colour (Scott & Haigh, 1986). *Electrophoresis* was on cellulose acetate, in 0·1 M HCl (Wessler, 1970*a*, *b*), 0·1 M calcium acetate or 0·1 M sodium acetate solutions, respectively. The strips were stained with Alcian blue 8G (Newton, Scott & Whiteman, 1974). Electrophoresed GAG was estimated by eluting the dye into dimethyl sulphoxide (Newton, Scott & Whiteman, 1974).

RESULTS

Light microscopy

Mouse corneal stroma stained with Alcian blue in 0·5 M $MgCl_2$, but not at all in 0·7 M $MgCl_2$, in contrast to rat and rabbit, which stained strongly in both (Fig. 1). This strongly suggests that mouse cornea is deficient in KS, but contains GAG similar in degree of sulphation to standard CS (Scott & Dorling, 1965; Stockwell & Scott, 1965). On this basis, as a result of their content of KS, rat and rabbit contain highly sulphated material. These conclusions were confirmed by electrophoresis of the stromal GAGs in 0·1 M HCl (see below).

Electron microscopy

In all, 1061 micrographs from 96 blocks of rabbit sections, 504 micrographs from 44 blocks of mouse sections, and 148 micrographs from 12 blocks of rat sections were examined. Micrographs were taken at random from the full depth of the stroma.

Rat stroma showed a generally complex picture (Fig. 2*a*), with numerous PG filaments orthogonal to the collagen fibrils, and many others which were randomly orientated, or parallel to the fibrils. Mouse stroma often showed a high degree of order (Fig. 2*b*). Some pictures of mouse stroma contained large numbers of PG filaments less ordered than those in Figure 2*b*, but no picture of rat stroma demonstrated as clear a pattern as that in Figure 2*b*. There were more PG filaments per D repeat unit of rat collagen fibril than in the case of mouse (Table 1). Rabbit corneal stroma was similar in appearance to that of rat (Scott & Haigh, 1985*b*).

Uranyl acetate staining of the rat collagen a–e bands located the PG filaments

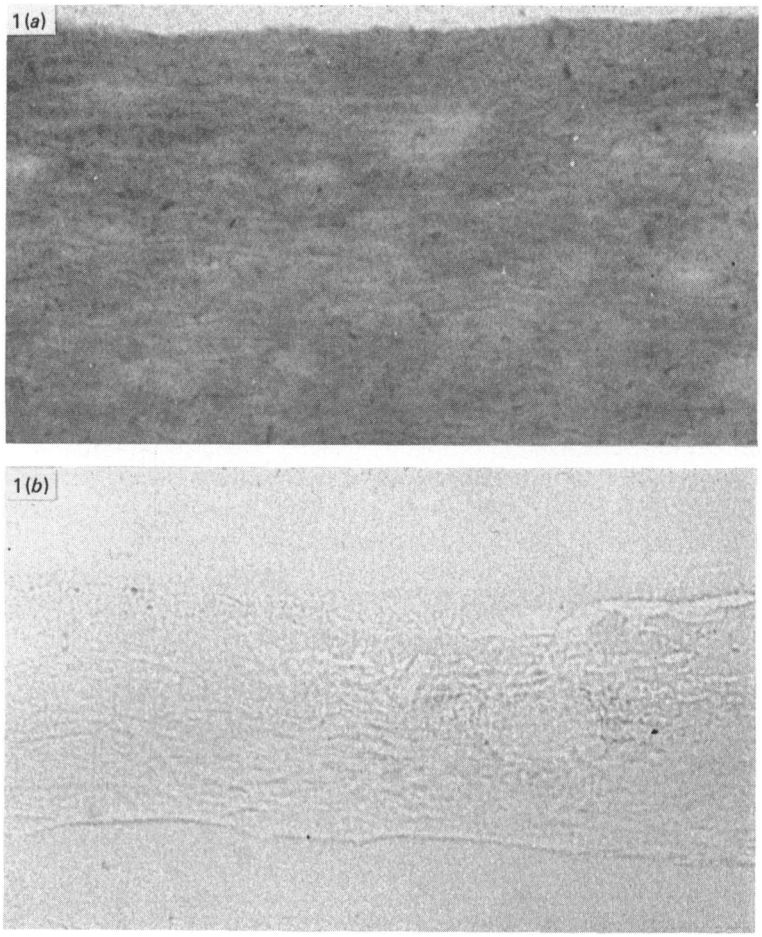
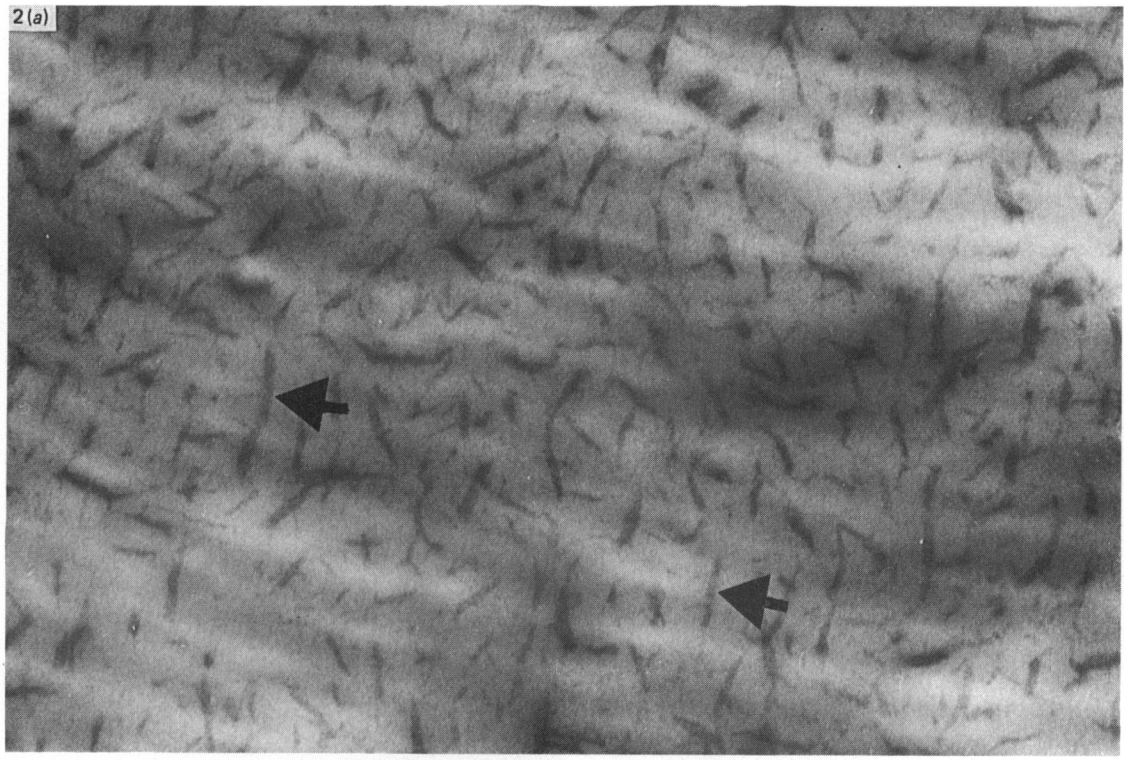


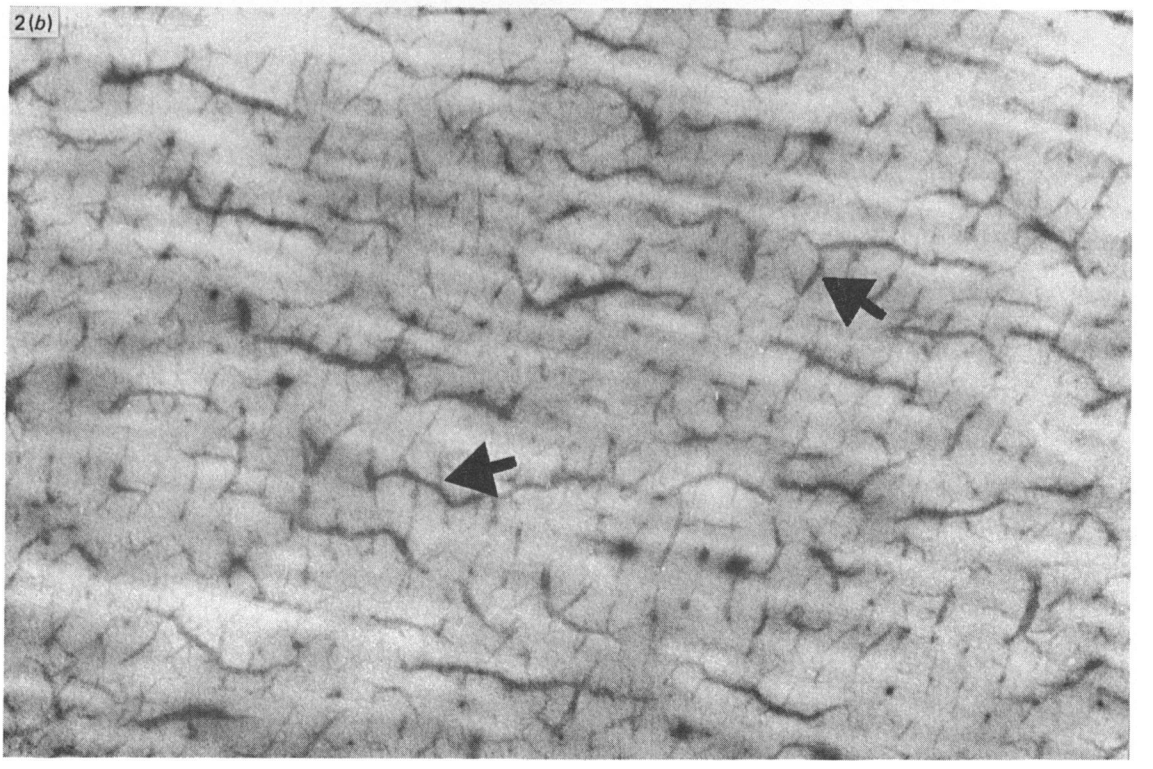
Fig. 1(*a–b*). Staining of rabbit (*a*) and mouse (*b*) cornea with Alcian blue in 0·7 M $MgCl_2$ solution (Scott & Dorling, 1965). The pictures show the full depth of mouse cornea (*b*), including endothelium and epithelium, whereas (*a*) shows less than half the depth of rabbit stroma, at the epithelial edge. The strong staining of rabbit stroma contrasts with the complete absence of staining of mouse stroma, indicating that the latter is lacking in the highly sulphated GAG present in the rabbit (Scott & Dorling, 1965). × 240.

predominantly at the c and d bands (Table 1). After digestion with chondroitinase ABC many filaments were left, those at identifiable sites being mainly at the a and c bands. The effect of chondroitinase AC was similar, in that there were many fewer PG filaments left at the d bands and the largest proportion was at the c band. There were still significant amounts of PG at the d band after digestion with chondroitinase AC, however. These results are similar to those obtained on rabbit corneal stroma (Table 1) (Scott & Haigh, 1985*b*) and suggest (i), the a and c band-associated PGs are KS-rich, and (ii), the PG at the d band is CS or DS, probably containing a significant proportion of DS, in view of its partial resistance to chondroitinase AC digestion.

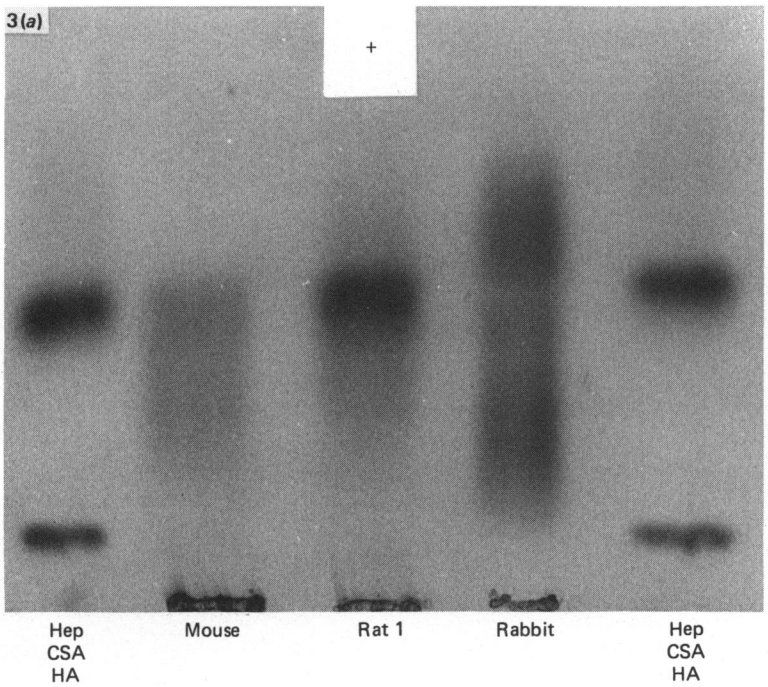
Fig. 2(*a–b*). Electron micrographs of (*a*) rat and (*b*) mouse corneal stromas stained with Cupromeronic blue in 0·3 M $MgCl_2$ (for details see text) to demonstrate PGs. These preparations were *not* stained with UO_2^{2+}, thus collagen fibrils are seen as *light* bands, travelling from left to right, and PGs (arrowed) are dark filaments arrayed mainly orthogonally to the fibrils. × 105000.



2(*a*)



2(*b*)



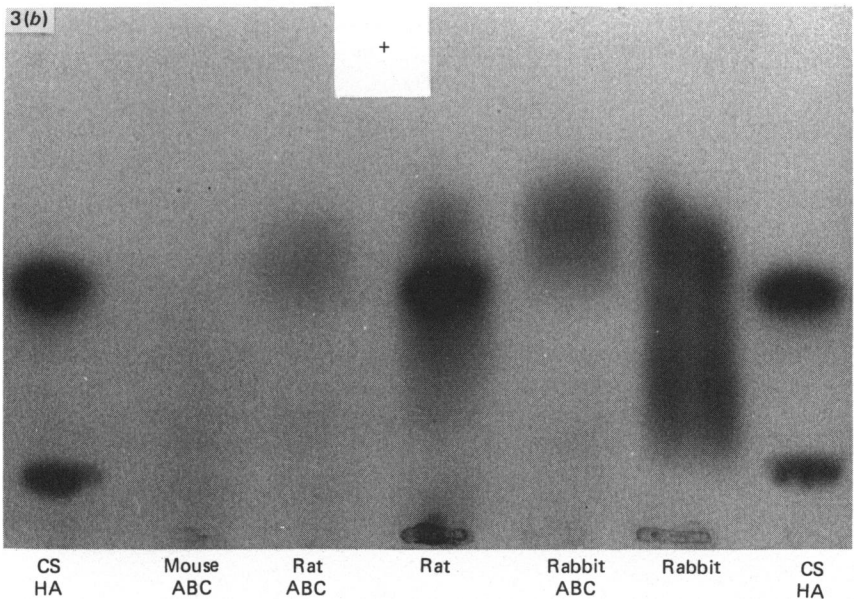


Fig. 3(*a–b*). Patterns of corneal GAGs after electrophoresis in 0·1 M HCl (Wessler, 1970*a*) on cellulose acetate membranes (*a*) before and (*b*) after digestion with chondroitinase ABC. GAGs were stained with Alcian blue (Newton, Scott & Whiteman, 1974).

The amount of GAG applied to the electrophoretic strip after chondroitinase ABC digestion was adjusted according to the amount of cetylpyridinium precipitate visible at the recovery stage. Clearly visible precipitates were present in both digested rat and rabbit samples, so the loading was at the same level as the control samples, which went through the entire procedure, minus the enzyme. There was no visible precipitate in the digested mouse sample. The loading was therefore increased to 3 times that of the undigested control.

Mobility in 0·1 M HCl depends on the content of ester sulphate (Wessler, 1970*a*, *b*). The faster

Table 1. *Percentage occupancy by proteoglycan filaments of collagen fibril bands*

Species	CEC* of staining solution ($MgCl_2$)	Enzyme treatment	Bands a	b	c	d	e	Number of PG filaments counted
Mouse	0·3		4	1	12	62	20	89
		Chondroitinase ABC	56	6	19	13	6	8
		Chondroitinase AC	8	9	8	53	22	101
		Hyaluronidase	12	10	11	43	24	120
	0·05		10	3	9	59	19	199
Rat	0·3		12·5	12·5	34·5	34·5	6	67
		Chondroitinase ABC	32	6	46	12	5	121
		Chondroitinase AC	19	9	48	19	2	97
Rabbit	0·3		21	3	39	29	8	282
		Chondroitinase ABC	41	6	48	3	1	285

* CEC, critical electrolyte concentration.

Assuming that a and c band-associated GAG contained KS, most of the KS was associated at the c band, in both rat and rabbit (Table 1).

Mouse presented a very different picture. There were very few a and c band-associated PG filaments, at least 80% of the located PG was at the d and e bands (Table 1). Almost nothing was visible after chondroitinase ABC digestion. Proteoglycans associated with the d and e bands were resistant to chondroitinase AC and hyaluronidase digestion, although the filaments which remained were apparently shorter and somewhat less electron-dense, suggesting that parts of the filaments may have been removed by the enzymes.

The picture obtained by staining at 0·05 M $MgCl_2$ did not differ significantly from that at 0·3 M $MgCl_2$. If the tissue had contained significant amounts of undersulphated low molecular weight GAGs (Scott, 1973) there would have been extra material, visible either as increased numbers of PG filaments or as a change in their distributions at the a–e bands.

Not all the band-associated PG filaments were necessarily specifically associated. In every picture there were randomly distributed filaments, sometimes numerous, and some of these must occasionally have been present at specific bands.

Biochemistry: qualitative

Electrophoresis in 0·1 M HCl demonstrated remarkable differences in GAG pattern between mouse, rat and rabbit (Fig. 3). Mobility in this system is determined by

running polyanions are heparin (faintly visible forward of CS, in the standard tracks to the left and right of Fig. 1*a*) and oversulphated KS in rat and rabbit CAG. The slowest running is hyaluronate (HA) in the standard tracks. Some 2 μg of standard CS and HA were applied, and 1 μg heparin. In parallel experiments at 2 μg loading the heparin band was more clearly visible, extending well forward of CS, as above.

The patterns are quite different in the three species. Rabbit corneal GAG contains large amounts of undersulphated (CS-DS, see text) and oversulphated (KS) material, but only a little approximately at the position of standard CS. Mouse contains no oversulphated KS, and rat shows a major band at the position of standard CS.

After chondroitinase ABC digestion (Fig. 1*b*) the major GAG is oversulphated KS, running forward of standard CS in both rat and rabbit, but there is nothing in the KS or CS positions in mouse. The CS and HA standards in Fig. 1*b* were not digested with chondroitinase.

sulphate content (Wessler, 1970*a*, *b*). KS samples '70' and '82' behaved according to Wessler's generalisation. Whereas rat and rabbit both contained very significant amounts of GAG which were more highly sulphated than the chondroitin sulphate standard, running with the same speed as oversulphated KS (1·4 S/GlcNH$_2$), mouse GAG contained no material of this kind (Fig. 3). The oversulphated GAG was unaffected by digestion with chondroitinase ABC, suggesting that it was KS.

GAG with sulphate contents similar to that of standard CS comprised much of the GAG from mouse and rat cornea, but hardly any of that from rabbit cornea (Fig. 3). Whereas a large proportion of rabbit GAG was markedly undersulphated much less of the rat and mouse GAG was so. All the undersulphated and normally sulphated GAG from rabbit, rat and mouse was digested by chondroitinase ABC.

Electrophoresis in 0·1 M calcium acetate, which separates DS from CS, showed two bands of similar intensity in mouse corneal GAG. Both ran much more slowly than standard CS, the faster in the DS position. Since practically all mouse GAG was digested with chondroitinase ABC, both bands must be DS or CS. The mobility of the slower band was similar to that of hyaluronate, and since the HCl system (above) showed no component with so low a degree of sulphation, the low mobility in calcium acetate was probably due to the presence of iduronate, as well as a degree of undersulphation. Thus the faster band is normally sulphated DS, and the slower is probably undersulphated DS.

Oversulphated KS ran slightly behind standard CS, from which it could not be clearly differentiated. Both rat and rabbit GAGs contained at least two bands, the lower of which (chondroitinase ABC digestible) ran in the same position as the DS from mouse. Fast moving bands in rat and rabbit GAGs still present after chondroitinase ABC digestion are therefore oversulphated KS (see above).

Electrophoresis in 0·05 M sodium acetate, which separates GAG according to total charge, did not resolve corneal GAG into separate bands. All GAG ran as elongated overlapping blocks, with maximum mobility equal to standard CS. After digestion with chondroitinase ABC both rat and rabbit showed broad bands (of KS) moving somewhat slower than standard CS. Keratanase digestion of rabbit GAG removed faster moving material – the remaining GAG (CS-DS) was considerably slower than standard CS. The fastest-moving material in rat GAG was chondroitinase ABC digestible.

Much of the mouse GAG moved at a speed close to that of standard CS, and the major part of this material was resistant to hyaluronidase.

In summary, mouse cornea contains two CS-DS GAGs, of differing sulphate and iduronate content, but no KS. Rat and rabbit both contain oversulphated KS, and varying amounts of undersulphated CS-DS, probably of two kinds, of varying iduronate and sulphate content.

Biochemistry: quantitative

Because of the functional significance of measurements on fully hydrated tissue, an attempt was made to express results on a wet weight basis. However, only data on rabbit were sufficiently precise, the samples of mouse and rat were very small (< 0·5 mg). Rabbit wet cornea contained 20·1 mg/g hydroxyproline (19·4–21·3, three determinations, 2·32 mg/g acid glycosaminoglycan, based on uronate assay (2·28–2·36, two determinations), 2·72 mg/g acid glycosaminoglycan based on chondroitinase ABC chromogen (2·2–3·2, five determinations), 1·23 mg/g of dermatan sulphate (periodate–Schiff method, one determination) and 4·5 mg/g KS, based on galactose

assay (one determination). Measured by the periodate–Schiff colorimetric method, DS constituted about 50% of the total CS in rabbit cornea.

KS:total sulphated GAG ratios from the electrophoretic strip in HCl decreased markedly from rabbit to rat to mouse (41%, 19%, 0% respectively).

DISCUSSION

Composition of corneal GAGs

The corneas of the three species contained very different patterns of GAG (Fig. 3). Most notable was the absence of KS from mouse cornea, in contrast to rat and rabbit, which contained considerable quantities of oversulphated KS. The ratio of KS to total GAG increased with increasing size of cornea. In large animals (cow, human) KS is preponderant (60–70% of the total GAG; Borcherding *et al.* 1975; Anseth & Laurent, 1961; Breen *et al.* 1972). Stuhlsatz *et al.* (1981) demonstrated a direct relationship between degree of sulphation and chain length of corneal KS from calf and pig, most material being of longer chain length and higher sulphation. This agrees well with the electrophoretic pattern of chondroitinase ABC-resistant GAG (KS) in both rat and rabbit, which showed that most KS was more highly sulphated than the CS standard (Fig. 3).

Almost as striking were the different patterns of sulphation in CS-DS GAGs. Rabbit cornea contained almost no characteristic CS-DS, which was the main component of rat cornea GAG. The major rabbit CS-DS was less sulphated than most of the rat and mouse GAG. The pattern in rabbit cornea, of a highly sulphated KS and a very undersulphated CS-DS, was very similar to that of bovine cornea (Frånsson & Anseth, 1967). Our results confirm and extend those of Gregory, Cöster & Damle (1982), who found a major CS-DS-PG in rabbit cornea (PDS I), which was low in sulphate, and a minor CS-DS PG (PDS II) which was not analysed, but which eluted at a higher salt concentration from their anion exchanger, suggesting that it was more highly sulphated. PDS I must contain most of the undersulphated CS-DS (Fig. 3), while PDS II probably contains the more highly sulphated CS-DS, travelling in 0·1 M HCl just behind standard CS (Fig. 3).

There were considerable amounts of iduronate in the CS-DS GAGs by both periodate–Schiff (rabbit) and calcium acetate electrophoretic methods (all three species).

Our results are compatible with those of Venn & Mason (1985), who showed that a considerable part of rat corneal GAG was digested by keratanase and was resistant to chondroitinase ABC, but not with their data on mouse cornea, in which they found similar GAG, albeit in much smaller proportion than in rat. Their conclusions were based on immunohistochemical staining and on the sensitivity to enzymic digestion of ^{35}S-labelled GAGs. These procedures are, in principle, very sensitive but are not chemically quantitative. It is possible that there are small amounts of KS in mouse cornea that are below our levels of chemical detection. Electron microscopical evidence suggests that KS-PG may be present in very small amounts (see below).

Ultrastructure

Electron micrographs of mouse cornea showed large areas of orthogonally arrayed proteoglycan filaments, very regularly associated at the gap zone of the collagen fibrils. There was a strong resemblance to tendon, sclera and skin (Scott & Haigh, 1985*a*) – especially young tendon, in which the collagen fibrils are thin and of regular diameter

(Scott *et al.* 1981). Both tissues contained two kinds of CS-DS GAG, one of which was undersulphated (Results and Scott *et al.* 1981). In both tissues there were many PG filaments running parallel with the collagen fibrils. By analogy with tendon and sclera, mouse cornea might contain 'small' (PG II) and 'large' CS-DS PGs (PG I) (Cöster & Frånsson, 1981; Ward, Scott & Cöster, 1987). It was suggested (Scott & Haigh, 1986) that a small PG II would be found at the d and e bands of Type I collagen fibrils in all soft non-mineralising tissues. We therefore propose that in mouse cornea the gap zone-associated PG is a small DS-PG II. As in rabbit cornea, the DS-PG is associated at two fibril sites, the d and e bands, and it will be interesting to see if there are, as in rabbit, two distinct protein cores within the DS-PG population (Gregory *et al.* 1982), each associated with one fibril site (Scott & Haigh, 1985*b*).

One of the striking features of this investigation is the absence of PG at the a and c bands of mouse cornea, which in the rabbit cornea were identified as the binding sites for KS-PGs (Scott & Haigh, 1985*b*). This result strongly supports the hypothesis that binding sites are specific for their associated PG, and that in the absence of the appropriate PG the site will remain unoccupied (Scott & Haigh, 1985*b*; Scott, 1986). Conversely, in corneas at least, PGs at the a and c bands may be tentatively identified as KS-PGs. The small number of a, c band-associated PGs that were seen in mouse cornea may thus represent the KS detected by Venn & Mason (1985) by ^{35}S labelling and immunofluorescent staining, but not seen by chemical methods in the present investigation.

The increase in the ratio of a band:c band-associated PG after chondroitinase ABC treatment (Table 1), which implies selective removal of part of the c-band PG, is difficult to explain. The effect was seen in both rabbit and rat tissues (Table 1). The obvious conclusion, that part of the c-band GAG is CS-DS in nature, is incompatible with the finding that nearly all a and c band PG was removed by keratanase digestion (Scott & Haigh, 1985*b*).

Our data on control (non-digested) tissues are consistent with the idea that the percentage occupancy of the identifiable fibril binding sites reflects the percentage concentration of the PG in the tissue. The greater occupancy of a and c bands in the rabbit compared with rat parallels the higher content of KS in rabbit stroma. In rabbit cornea, 30% of the total tissue PG was PDS I and 6% PDS II (Gregory *et al.* 1982), which compares with our finding that d and e band-associated PG (i.e. CS-DS PGs) comprise 29% and 8%, respectively, of the total band occupancies (Table 1). The comparison of KS-PG tissue abundance (21% PKS I, 43% PKS II) (Gregory *et al.* 1982) with binding site occupancies is as striking – 21% at a, and 39% at c (Table 1). The relative tissue concentrations were based on hexosamine analyses, so the numerical coincidences may not be significant. Assuming that hexosamine/mol PG are similar for each of the PGs, these correlations suggest that each PG is bound at its specific binding site in an amount proportional to its tissue molar concentration. Alternatively, there may be an equilibrium between bound and free forms, in which the outcome is partly dependent on the affinity of each PG for its binding site (Scott, 1986). The basic features of such an equilibrium, namely free binding sites and unattached PG, are visible, and sometimes dominant, in most micrographs of rabbit corneal stroma. It remains to be seen how far this picture is an artefact of tissue preparation.

On either basis, it is reasonable to apportion the two PGs in each class (KS or CS-DS) to the two relevant binding sites (a, c or d, e respectively) according to the tissue concentration of each PG. Thus PDS I is at the d band, PDS II at the e band, PKS I is at the a band and PKS II is at the c band. This attribution of PKS I and

PKS II reverses the previous tentative assignment (Scott & Haigh, 1985*b*), which was based on X-ray density profiles of cupromeronic blue-stained cornea (Meek, Elliott & Nave, 1986).

It was pointed out (Scott & Haigh, 1985*b*; Scott, 1986) that the regular and specific binding of PGs to the collagen fibril could structure the interfibrillar sol or gel, helping to keep the collagen fibrils a constant distance apart, which is necessary for corneal transparency (Maurice, 1957). Our results demonstrate that KS-PG is not an invariant part of the fibril-spacing system, which in the mouse functions well without it. The essential feature, in all corneas, seems to be the presence of CS-DS PGs associated at the gap zone. Mouse is the first mammal that has thus been demonstrated to be able to dispense, in the adult, with corneal KS-PG, although squid, a non-mammal, apparently can (Anseth, 1961).

Control of keratan sulphate biosynthesis

Our results on cornea, and those on cartilage (Stockwell & Scott, 1965) emphasise the inverse relationship between tissue thickness and KS content. Results based on ^{35}S labelling strongly suggest that rat and mouse cartilages lack KS (Venn & Mason, 1984), in contrast to those of larger animals such as rabbit and guinea-pig. Moreover, these tissues provide parallel examples of the effects on KS tissue levels of a sudden worsening of nutrient supply during normal development. The deep zones of articular cartilage show a rise in KS content after the closure of the canals from the bone, which occurs in middle life in the human (Stockwell, 1970). These canals are a major route for the nutrition of deep articular cartilage. Similarly, during fetal development the atrophy of the hyaloid artery, which occurs at a relatively late period, is accompanied by a thickening of the cornea and the appearance of KS (Breen *et al.* 1972). A gradient of KS content in the cornea, increasing posteriorly, has been observed (Table 4 of Anseth, 1961; Bettelheim & Goetz, 1976), and this follows the declining O_2 tension across the cornea (Fatt, 1978).

We conclude that KS is characteristically produced in a restricted supply of nutrient, particularly oxygen. It is of the utmost interest, from the standpoints of tissue development, the ageing process, and the control of GAG biosynthesis, that the mechanisms behind these temporal and spatial distributions be elucidated.

To account for the almost reciprocal relationship between CS (DS) and KS contents in thick corneas or aged thick cartilage, it is assumed that the CS:KS ratio is predominantly determined by rates of synthesis. The converse, that rates of degradation are decisive, implies that in young thin tissues KS is synthesised only to be broken down, and that CS in very aged cartilage is broken down almost as soon as it is made. In view of the similar response of both cartilage and corneal KS content to environmental change, it is simplest to assume that it is in the GAG chain elongation mechanisms, that are common to both, rather than in the supply of protein precursors, which are very different (Stuhlsatz *et al.* 1981), that control is exercised.

The earlier hypothesis (Stockwell & Scott, 1965; Stockwell, 1970) was that CS biosynthesis, which is a net consumer of O_2 during the conversion of UDPGlc to UDPGlcUA, would be more seriously affected by poor O_2 supply than that of KS, which does not consume O_2 at any stage of biosynthesis. Work on liver hepatocytes has shown that hypoxia does dramatically reduce glucuronidation of acceptor molecules (Aw & Jones, 1984), by slowing production of UDPGlcUA from UDPGlc and NAD. An increase in the NADH–NAD ratio sufficient markedly to lower UDPGlcUA synthesis is brought about by substances which compete for available NAD, e.g. lactate and ethanol (Moldéus, Andersson & Norling, 1978). NADH itself

inhibits UDPGlc dehydrogenase (Goldberg (1963), cited by Moldéus *et al.* 1978). The effect of lactate on UDPG1cUA formation is particularly relevant to cornea, which, although in direct contact with air, and therefore not hypoxic in the normal sense, nevertheless in the rabbit converts most (84%) of its glucose into lactic acid, suggesting that there are difficulties in obtaining or making use of O_2 at the cellular level (Fatt, 1978). Most cells in the deep zones of mature thick cartilage metabolise in conditions of O_2-lack amounting almost to anaerobia (Stockwell, 1979; Silver, 1975).

The rates of reaction of the galactosyl transferase → KS and glucuronosyl transferase → CS are important in determining the balance of synthesis of KS and CS. The K_m of the galactosyl transferase → KS for UDPGal is considerably lower than those of typical glucuronosyl transferases (55 μM; Christner, Distler & Jourdian, 1979), as compared with several mM (Aw & Jones, 1984)). The K_ms of the UDPGlc dehydrogenases are also high ($\sim 10^{-3}$ M) (Balduini *et al.* 1973). The KS-building galactosyl transferase is therefore able to work at closer to optimum speeds than glucuronosyl transferase in conditions of restricted supply of precursor molecules. In principle, KS biosynthesis could continue fairly normally when CS biosynthesis is much reduced.

Although simple arguments account for the observed picture in 'hypoxic' tissues, the proposed scheme raises the question as to why KS is not made all the time, in young-thin, as well as old-thick tissues? One possibility, assuming the availability of protein precursor, is that the normal rate of synthesis of KS is low, and important only when CS synthesis is also low. Indeed, Balduini, Brovelli & Castellani (1970) showed that KS and CS (DS) biosynthetic rates in bovine cornea were inversely dependent. Inhibition of CS production by the addition of UDP xylose led to an increase in KS synthesis as a consequence of the increased UDPGlc available to the KS pathway. It is relevant that high KS levels in ageing cartilage are associated with a diminution in histochemically demonstrable UDPGlc dehydrogenase (Aureli, Rizzotti, Balduini & Castellani, 1969). If CS synthesis is much faster than that of KS, the available extracellular space is predominantly occupied by CSPGs. Since this space is limited, there may be a feedback mechanism which reduces GAG synthesis when interfibrillar pressures become critical (Handley & Lowther, 1977).

This view of KS-CS biosynthesis throws light on the difficulties experienced in getting cell cultures to synthesise KS (Dahl & Cöster, 1978; Christner, Distler & Jourdian, 1979). It would be interesting to use lower O_2 tensions than normal, with restricted glucose, and perhaps reduced pressure, over long time scales, to mimic tissue conditions, rather than well oxygenated, high nutrient media, in which CS and hyaluronate are freely produced. At unphysiologically high O_2 levels (95% by vol) bovine cornea synthesises CS three times faster than KS (Handley & Phelps, 1972), even though the tissue itself contains three times more KS than CS.

SUMMARY

Corneas from mouse, rat and rabbit were analysed quantitatively and/or qualitatively for collagen and acid glycosaminoglycans. They were examined by light and electron microscopy, using Alcian blue and Cupromeronic blue, in critical electrolyte concentration methods, with or without digestion by hyaluronidase, chondroitinases and keratanase, for their sulphated glycosaminoglycan distributions.

Glycosaminoglycan patterns were very different in the three species. Mouse lacked chemically detectable keratan sulphate, which was present in considerable amounts in rat and rabbit stroma.

Mouse corneal stroma proteoglycan filaments were located predominantly at the gap zone of the collagen fibrils, mainly at the d band, with few at the a and c bands. Rat and rabbit micrographs were more complicated, with many proteoglycan filaments at the a and c, as well as the d and e bands. These findings support the proposal that the a and c bands were specific binding sites for keratan sulphate proteoglycan (Scott & Haigh, 1985*b*).

Evidence from studies on cornea and cartilage suggests that keratan sulphate, rather than chondroitin sulphate is produced in conditions of O_2 lack. Metabolic mechanisms which could account for this balance are proposed The production of uridine diphosphate glucuronic acid is the key step, which is sensitive to hypoxia, lactate and NAD:NADH ratios.

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