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Identification of specific binding sites for keratan sulphate proteoglycans and chondroitin–dermatan sulphate proteoglycans on collagen fibrils in cornea by the use of Cupromeronic Blue in 'critical-electrolyte-concentration' techniques

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Proteoglycans (PGs) in bovine corneal stroma were stained with Cupromeronic Blue in 'critical-electrolyte-concentration' (CEC) methods for electron microscopy, and were located vis-à-vis collagen fibril a–e banding patterns. Keratanase and chondroitin ABC lyase digestion showed that a + c-band- and d + e-band-associated PGs were keratan sulphate-rich and chondroitin (dermatan) sulphate-rich respectively. The CEC pattern proved that the keratan sulphate PGs at the a and c bands differed. Comparison of their CECs with their behaviour on anion-exchange chromatography confirmed previous (indirect) attempts at identification [Scott & Haigh (1985) Biosci. Rep. **5**, 765–774]. Similar arguments were applied to the dermatan sulphate PGs at the d and e bands. These results strongly support the one-PG–one-binding-site hypothesis [e.g. Scott (1988) Biochem. J. **252**, 313–323]. Remarkable inter-species variations in the keratan sulphate PG patterns contrast with the relatively constant picture of dermatan sulphate PG–collagen fibril interactions.

INTRODUCTION

The organization and mechanical stability of animal bodies are preserved by their connective tissues: systems of insoluble fibrils and soluble polymers, which respectively resist tensile or compressive forces, internally or externally generated [1]. The matrix of inextensible fibres (usually collagen) is inflated by the soluble polymer gel or sol, in which proteoglycans (PGs) and/or hyaluronate are characteristically present. It was often speculated that interactions between PGs and collagen are not merely mechanical, but are determined by chemical factors, i.e. that there is an element of specificity. Neither the techniques nor the chemistry of the participants had developed to a point where meaningful statements could be made until recently, when ultrastructural methods of high resolving power for locating PGs in tissues by electron microscopy became available (see [2] for review), and at the same time relevant data on the 'small' PGs (see [3] and [4] for reviews) began to accumulate. It became clear that collagen fibrils carried a set of binding sites for small PGs [2,5]. Dermatan sulphate PGs were found at the d and e bands of type-1-collagen-rich tissues (tendon [5], skin [6] and sclera [7]), whereas keratan sulphate PGs were present at the a and c bands of cornea collage fibrils (probably also type 1) [8]. A 'map' was drawn up [8] implying that each PG had its own binding site, and that in the absence of this PG the site would be unoccupied [9]. The interactions probably occur between the PG protein cores and the collage fibril [2].

The identification in the cornea of d and e bands and a and c bands as dermatan sulphate PG- and keratan sulphate PG-binding sites respectively depended on the use of Cupromeronic Blue as electron-microscopical histochemical stain at a 'critical electrolyte concentration' (CEC) high enough to ensure that only sulphated PGs were stained, together with the enzymes keratanase and chondroitin ABC lyase, which selectively digested keratin sulphate and dermatan sulphate respectively [8]. The existence of two binding sites for each type of PG (the d + e bands for dermatan sulphate PG and the a + c bands for keratan sulphate PG) was linked [8] to the polymorphism of the PGs [10]. It was suggested that the two fractions of keratan sulphate PG (differing in protein cores [10]) each had a separate binding site (a or c), and similarly in the case of the dermatan sulphate PGs [8]. A parallel was noted between the tissue concentrations of dermatan sulphate PGs, PDS I and PDS II, and the relative frequency of occupancy of bands d and e, and on this basis PDS I was assigned to band d and PDS II to band e. Similar reasoning led to PKS I and PKS II (now PKS A and PKS B; J. D. Gregory, personal communication) being assigned to bands c and a respectively [8]. These assignments were recognized as being tentative, and those for PKS I and PKS II were reconsidered when more data were forthcoming [11].

In view of the importance of the one-PG–one-binding-site hypothesis [8,9], it is desirable that there should be independent evidence for these assignments. In the present paper we apply the CEC approach, and demonstrate a direct link between the properties of the PGs on anion-exchange columns and their CECs in the tissue, which confirms the original assignment to their collagen binding sites. We also show, for the first time at the electron-microscope level, that chondroitin/dermatan sulphate PGs can be distinguished from keratan sulphate PGs by using a CEC method.

Abbreviations used: CEC, critical electrolyte concentration; PG, proteoglycan.

EXPERIMENTAL

Tissues and staining

Rabbit or bovine corneas were taken soon after death into isopentane cooled with liquid N_2. Sections (10–15 μm thick) were cut, stained with Cupromeronic Blue and processed for electron microscopy, with or without enzyme digestion by the technique of Haigh & Scott [12]. Collagen a–e banding was demonstrated by UO_2^{2+} staining [5,6]. Alcian Blue CEC staining was by the method of Scott & Dorling [13].

Enzymes and digestions

Keratanase (keratan sulphate 4-galactopyranosyl-glycan hydrolase), chondroitin ABC lyase (EC 4.2.2.4) and hyaluronidase were from Sigma Chemical Co. Digestions of sections were performed as described by Scott & Haigh [11,12].

Dyes

Cupromeronic Blue was from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Alcian Blue was from I.C.I. Dyestuffs, Blackley, Manchester, UK.

Occupancy of collagen bands by PG filaments

This was determined by counting the number of PG filaments that could be unambiguously localized at a–e bands along the collagen fibrils, as described previously [8]. Occasionally (< 5% of the total), orthogonal PG filaments occurred between, for example, bands b and c, bands c and d, bands d and e or bands e and a. In such cases the count was divided equally between the neighbouring bands.

RESULTS

Rabbit cornea stained with Cupromeronic Blue at 0.4 M-$MgCl_2$ presented a similar picture to that already described [8] obtained at 0.3 M-$MgCl_2$. The a, c, d and e bands were associated with PG filaments. The percentage occupancy of d+e bands (based on 458 counts) was decreased markedly at 0.4 M-$MgCl_2$ as compared with that at 0.3 M-$MgCl_2$ (Table 1), indicating that staining of a+c-band-associated PG filaments was the more resistant to increased $MgCl_2$ concentrations.

The pattern in bovine cornea was qualitatively similar (Fig. 1), in that (1) the a, c, d and e bands were the predominant binding sites for PGs, (2) the a+c-band-associated PGs were sensitive to keratanase and resistant to chondroitin ABC lyase, and (3) the d+e-band-associated PGs were resistant to keratanase but sensitive to chondroitin ABC lyase. As in the rabbit cornea, the a+c-band-associated PGs are therefore keratan sulphate-rich and the d+e-band associated PGs are chondroitin/dermatan sulphate-rich [8,11]. Quantitatively, however, there were striking differences in the ratio of the a-band/c-band occupancies, from 0.54 in the rabbit at 0.3 M-$MgCl_2$ to 1.62 in the cow (Table 1).

Two bovine corneas showed similar percentage occupancy patterns in the anterior (epithelial), middle and posterior (endothelial) thirds of the central stroma at 0.3 M-$MgCl_2$, but a marked decrease in a+c-band occupancy in the anterior third was seen in a further sample. This heterogeneity in keratan sulphate distribution was confirmed in this one specimen, but not in the others, by Alcian Blue staining in the CEC mode [13] for light microscopy. The results in Table 1 are pooled

Table 1. Percentage occupancy by PG filaments of corneal collagen fibril a, c, d and e bands, and ratios of percentage occupancies at bands a and c with increasing CECs

'Occupancy' means that PG was observed at the relevant band. 'Percentage occupancy' is the total number of observed occupancies at the relevant band expressed as a percentage of the total number of observed occupancies at all bands. Data are from Scott & Haigh [11]. Data for bovine cornea were accumulated from blocks taken from posterior, middle and anterior thirds of the stroma. *, One cornea; **, two corneas; ***, three or more corneas.

CEC of staining solution (M-$MgCl_2$)	Percentage occupancy																				
	Rabbit								Bovine												
	Undigested						Undigested						Chondroitin ABC lyase-digested						Keratanase-digested				
	a	c	d	e	a/c ratio		a	c	d	e	a/c ratio		a	c	d	e	a/c ratio		a	c	d	e	d/e ratio
0.05													44.4	33.1	7	5.2	1.34*		3	3	79.5	13.7	5.8*
0.2							37.5	21.6	22.6	14.2	1.71*												
0.3	21	39	29	8	0.54***		32.6	20.1	24	13.8	1.62***		47.3	27.4	7	7	1.72*		5	4	70.2	19.1	3.7*
0.4	50.6	23.3	9	7	2.17*																		
0.5							62.3	19.4	12	6.8	3.21**												

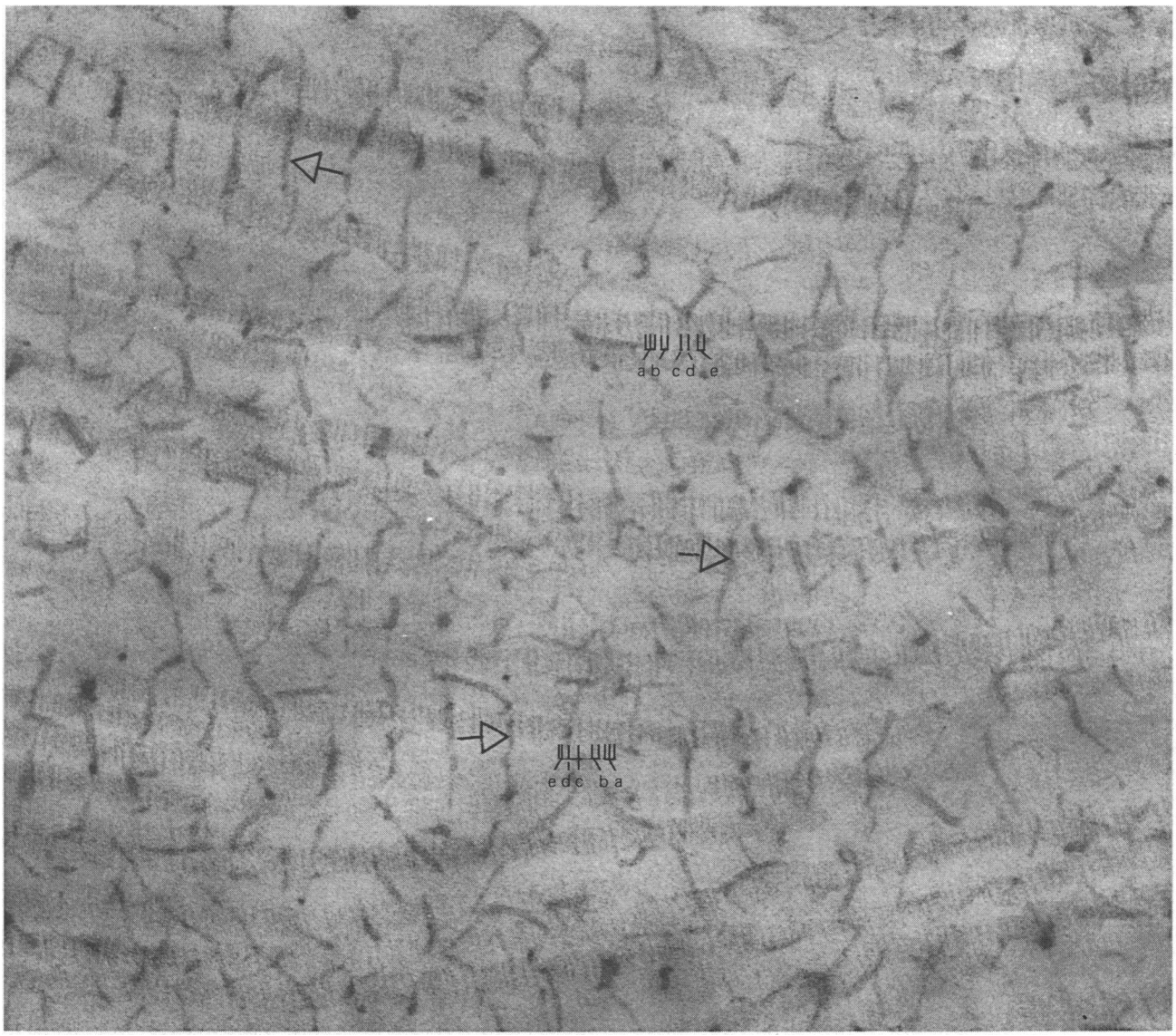

Fig. 1. Electron micrograph of bovine corneal stroma, stained with Cupromeronic Blue at 0.05 M-$MgCl_2$, after digestion with keratanase [8,12]

Glutaraldehyde was present in the staining solution to prevent translocation of PG [14]. The collagen fibril bands are stained with UO_2^{2+}. The a–e banding patterns within the D period are labelled. Magnification × 160000. PG filaments (arrowed) are frequently regularly spaced, orthogonal to the fibrils, separated by one D period. They were demonstrated by chondroitin ABC lyase and keratanase digestion to be chondroitin/dermatan sulphate-rich, and located in the gap zone at the d and e bands. See the text for details.

from the three zones of three corneas (a total of 1174 counts from 99 micrographs).

The increase in d-band/e-band ratios after keratanase digestion, implying selective removal of part of the e-band-associated PG, might suggest that the latter contained keratan sulphate. An analogous finding in rabbit cornea after chondroitin ABC lyase digestion, implying that the c-band-associated PG could contain chondroitin (dermatan) sulphate as well as keratan sulphate [11], was without a parallel in the bovine cornea.

The a-band/c-band ratios rose sharply with increasing $MgCl_2$ molarity, indicating that the a-band-associated PG had a higher CEC than the c-band-associated PG in both the rabbit and the cow. Similarly the decrease in d-band/e-band ratios with increasing $MgCl_2$ concentration implies that d-band-associated PG has a lower CEC than e-band PG in the rabbit and the cow. The d+e-band/a+c-band ratios were much lower at 0.4 M- and 0.5 M-$MgCl_2$ in rabbit and bovine corneas respectively compared with those at 0.3 M-$MgCl_2$, showing that d+e-band-associated PGs had lower CECs than a+c-band-associated PGs. The ratios were almost identical in the cow cornea at $MgCl_2$ concentrations of 0.2 M and 0.3 M (Table 1), as expected, since both concentrations are below the CECs of typical keratan sulphates and chondroitin sulphates.

DISCUSSION

Our data extend those reported on rat, rabbit and mouse [8,11] and confirm the general picture that collagen

fibril bands a and c are associated with keratan sulphate whereas bands d and e are chondroitin/dermatan sulphate-associated. Nevertheless, remarkable quantitative differences are emerging with this broad framework, particularly concerning the keratan sulphate PGs. Keratan sulphate was not detected chemically in the mouse [11], but constitutes over 65% of the total glycosaminoglycans in bovine cornea [15] (J. E. Scott & M. Haigh, unpublished work). Almost as striking is the switch from a dominant c band, almost 3 times as frequently populated as the a band, in the rat, to a dominant a band, 60–70% more frequently populated than the c band, in the cow. The rabbit data lie between the two. It is probable that the greater part of the dramatic increase in corneal keratan sulphate content from mouse to cow is due to the rise in the proportion of a-band-associated PG.

By contrast, the d+e-band-associated PG picture is more stable. It is present in all species so far examined, and the d-band/e-band ratio of occupancies is always much greater than 1.0, with a downward trend from mouse to rat, to rabbit and then to cow. These findings reinforce the suggestion that the constant d+e-band association with chondroitin/dermatan sulphate PGs is of the utmost importance in most tissues and in many species [2,6,9].

The very different behaviour of the a-band-associated and c-band-associated PGs with changing $MgCl_2$ molarity (Table 1) demonstrates directly and for the first time that they are not identical, i.e. they are different PGs, specifically located. This finding strongly supports the one-PG–one-binding-site hypothesis [2,8,9], which was based on indirect evidence that the band occupancies paralleled tissue PG concentrations in rabbit cornea. Further, the simple theoretical background to the CEC phenomenon permits a direct comparison between the salt concentration at which the PG is eluted from an anion-exchanger [16,17]: the higher the CEC, the higher the eluting salt concentration. The different CECs of PKS I and PKS II (as well as PDS I and PDS II), due to differences in their glycosaminoglycans, give rise to different staining patterns (i.e. percentage band occupancies) as the $MgCl_2$ concentrations change. Thus PKS II (PKS B), eluted at a higher salt concentration than PKS I (PKS A) [10], corresponds to band-a-associated PG, with the higher CEC, and band-c-associated PG to PKS I (PKS A). These attributions agree with those proposed on the basis of tissue PG concentrations [12].

Similar reasoning confirms the attributions of PDS I and PDS II to bands d and e respectively, also as originally proposed on the basis of tissue PG concentrations [8].

The analogous behaviour in the CEC system of the PGs from the rabbit and the cow suggests that the 'map' of PG-binding sites on the collagen fibril applies in detail to a wide variety of species.

CEC procedures with Alcian Blue in $MgCl_2$ solutions have been much used in light microscopy to differentiate between keratan sulphates and chondroitin (or dermatan) sulphates [13,18]. Keratan sulphate retains stain at the higher CECs. The present paper demonstrates that d+e-band-associated PGs differ from a+c-band-associated PGs in that they almost cease to stain at 0.4 M- or 0.5 M-$MgCl_2$, as expected from the behaviour of chondroitin (dermatan) sulphate and keratan sulphate in CEC systems with Cupromeronic Blue and $MgCl_2$ [19]. This result, obtained on tissues that had not previously been digested with enzymes, further confirms the assignments of d and e bands and e and c bands to chondroitin/dermatan sulphate-rich and keratan sulphate-rich PGs respectively.

As used in the present paper, the CEC principle dealt with just a few molecules of PG, thus approaching the ultimate limit of sensitivity available to a fractionation method.

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