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Proteoglycans of bovine periodontal ligament and skin

Occurrence of different hybrid-sulphated galactosaminoglycans in distinct proteoglycans

Charles H. PEARSON and Gary J. GIBSON
Department of Oral Biology, University of Alberta, Edmonton, Alberta T6G 2N8, Canada

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A proteoglycan purified from 4M-guanidinium chloride extracts of bovine periodontal ligament closely resembled that of bovine skin, except for a rather lower protein content and a higher molecular weight (120000 compared with about 90000) by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The latter difference was explained by the molecular weights (29000 and 16000) of the respective dermatan sulphate components, each of which was rich in L-iduronate (about 75% of the total hexuronate). Significant amounts of other glycosaminoglycans did not occur in these proteoglycans, which were homogenous on gel chromatography and agarose/polyacrylamide-gel electrophoresis. Polydispersity was observed in sedimentation equilibrium experiments, but proteolysis or self-association of the proteodermatan sulphates may have affected these results. Ligament proteoglycans that were almost completely extracted with 0.1M-NaCl contained less protein of a completely different amino acid composition than the proteodermatan sulphates. They were heterogeneous in size but generally smaller than cartilage proteoglycans and L-iduronate was a component, comprising about 7% of the total hexuronate of the sulphated galactosaminoglycan chains. The latter consisted of two fractions differing in molecular weight, but a dermatan sulphate with a high L-iduronate content was not present. These proteoglycans had some resemblance to D-glucuronate-rich proteoglycans of other non-cartilaginous tissues. Such compounds, however, are difficult to categorize at present.

The periodontal ligament is vital to dental health but the ground substance of this tissue has received little attention. The glycosaminoglycan components have been studied (Munemoto *et al.*, 1970; Pearson *et al.*, 1975) but no proteoglycan has been isolated. More recently it was shown (Gibson, 1979) that bovine periodontal ligament contains approximately equal amounts of dermatan sulphates and CS(DS) hybrids, contrasting with pigskin (Fransson & Rodén, 1967) or mature bovine skin (C. H. Pearson & T. Sereda, unpublished work) in which CS(DS) hybrids are minor components and aorta (Fransson & Havsmark, 1970; Radhakrishnamurthy *et al.*, 1977), in which most if not all the hybrids are of this type. It is important to distinguish between the hybrid types, as the conformation of the chains and their interactions with collagen are probably influenced by the occurrence of L-iduronate residues, especially those that are sulphated (Fransson *et al.*, 1975). The distribution of the different hybrids in tissues and isolated proteoglycans has not been systematically studied, nor is there much firm data on the molecular weights of these proteoglycans or the glycans.

Abbreviations and definitions used: (CS)DS hybrids (Buddecke & von Figura, 1975), hybrid-sulphated galactosaminoglycans containing a greater number of D-glucuronate than L-iduronate residues, contrasting with dermatan sulphates (as defined by Fransson, 1976), where L-iduronate residues predominate (some authors refer to all such hybrids as dermatan sulphates); PG1 and PG2, periodontal ligament proteoglycans, extracted with 0.1M-NaCl and 4M-guanidium chloride respectively; CPC, cetyl pyridinium chloride; SDS, sodium dodecyl sulphate.

Materials and methods

Guanidinium chloride was either an ultra-pure grade (Schwartz–Mann, Orangeburg, NY, U.S.A.) or it was purified by stirring with activated charcoal. CsCl was obtained from Fisher Chemical Co., Fair Lawn, NJ, U.S.A. Solutions of urea were deionized on a mixed-bed ion-exchange resin column. All chemicals used in gel electrophoresis and a mixture of protein standards (mol.wt. 40000–250000) were from Bio-Rad Laboratories, Richmond, CA, U.S.A. Media for gel chromatography

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were purchased from Pharmacia, Uppsala, Sweden, DEAE-cellulose (DE-52) from Whatman Biochemicals, Maidstone, Kent, U.K., papain (twice crystallized) and CPC from Sigma, St. Louis, MO, U.S.A., and standard glycosaminoglycans from Miles Research Products, Elkhart, IN, U.S.A.

Bovine mandibles and dermis from cattle aged 1–2 years were obtained fresh from the abattoir. Periodontal ligaments were dissected as described (Pearson *et al.*, 1975), mainly from erupted incisor teeth but including some unerupted incisors (with prominent root formation). The pool of ligaments (86 g from about 400 incisors) was stored at −20°C befor freezing in liquid N_2. Small pieces of each tissue were ground in a Wiley cutter mill, cooling with liquid N_2. Exhaustive extractions of the milled ligament (86 g) or skin (300 g), first with 0.1 M-NaCl and then with 4 M-guanidinium chloride, were carried out at pH 7.6 and 6°C in the presence of several proteinase inhibitors essentially as described previously (Pearson *et al.*, 1978). Proteoglycans were extracted from bovine nasal cartilage directly with 4 M-guanidinium chloride and separated into aggregate and subunit, as described by Hascall & Sajdera (1969).

Purification of the ligament and skin proteoglycans

Components of the 0.1 M-NaCl extracts of the skin were not studied. Partly purified proteoglycans were isolated from the other extracts by chromatography on a DEAE-cellulose column (20 cm × 2.5 cm) as described by Antonopoulos *et al.* (1974) and exhaustively dialysed at 6°C against water, 1.0 M-NaCl and finally water, before freeze-drying. Further purification of the proteoglycans extracted with 4 M-guanidinium chloride was carried out by CsCl-density-gradient centrifugation under dissociating conditions as described by Hascall & Sajdera (1969), but at a starting density of about 1.4 g/ml (Toole & Lowther, 1968). This was followed by chromatography on a column (90 cm × 1 cm) of Sepharose 6B. Larger amounts of these proteoglycans, required for ultracentrifugation experiments, were purified by applying up to 21 mg of the material isolated by DEAE-cellulose chromatography directly to a column (90 cm × 2.3 cm) of Sepharose 6B. Proteoglycans extracted from the ligament with 0.1 M-NaCl and similarly isolated by DEAE-cellulose chromatography were also subjected to density-gradient centrifugation, but at higher starting densities and under non-dissociating conditions as given in Fig. 1(*c*). This was followed by chromatography on columns of Sepharose 2B and 6B and also under dissociating conditions on Sepharose CL-2B. Void volumes (V_0) were determined by using the cartilage proteoglycan aggregate for Sepharose 2B and Blue Dextran 2000 (Pharmacia) for Sepharose 6B columns. Total volumes (V_t) were found using ^3H_2O and $K_{av.}$ values were calculated from $(V_e - V_0/V_t - V_0)$, where V_e is the elution volume.

Methods used in characterizing the proteoglycans

Composite gel electrophoresis at pH 6.8 in 2% (w/v) polyacrylamide/0.6% agarose gels (10 cm), staining with Toluidine Blue and destaining were carried out as previously described (Pearson *et al.*, 1978). To detect protein, other gels were stained with Coomassie Blue R250 as described by Fairbanks *et al.* (1971). Molecular weight determinations by SDS-polyacrylamide gel electrophoresis in 5% gels (pH 7.2) were performed with conventional procedures (Weber & Osborn, 1975). A Gilford 252 spectrophotometer with a linear transporter was used to scan all gels and cellulose acetate strips (see below).

Sedimentation equilibrium. Proteoglycans were first dialysed at 4°C against several changes of 0.1 M-NaCl/0.12 mM-$NaHCO_3$, pH 6.8 (Preston, 1968). Ultracentrifugation at 20°C and 8°C was performed in a Beckman model E analytical ultracentrifuge equipped with Schlieren, Rayleigh interference and u.v.-absorption optics. A 12 mm double-sector cell was used with a charcoal-filled Epon centre piece and sapphire windows. Determinations were carried out by the conventional low-speed equilibrium technique and by the meniscus depletion method of Yphantis (1964). Point molecular weights were calculated as a function of concentration across the cell (Chervenka, 1969) using an IBM 360 computer and programmes supplied by Dr. C. Kay (Department of Biochemistry, University of Alberta). Partial specific volumes used in the calculations were obtained from the reciprocals of peak buoyant densities found in density-gradient centrifugation, giving $\bar{v}$ values of 0.69 and 0.71 ml/g for the 4 M-guanidinium chloride-extracted proteoglycans of ligament and skin respectively. The latter value was the same as calculated for heart-valve proteodermatan sulphate containing 60% protein (Preston, 1968) and the slightly lower value for the ligament proteoglycan was consistent with its lower protein content (about 50%).

Protein and amino acids. Protein was determined by the method of Lowry *et al.* (1951), with globulin-free albumin as standard and also from summation of amino acid contents. The latter were determined by using a Beckman 121 MB analyser after hydrolysis in constant-boiling HCl, under N_2 in the presence of 20 mM-phenol, at 110°C, for 21 h. Similar acid hydrolysates of performic acid-oxidized proteoglycans (Moore, 1963) were analysed for cystine.

Glycosaminoglycan components

Intact proteoglycans and isolated glycan chains

were analysed for hexuronate essentially as described by Heinegård (1973) with glucuronolactone (Sigma) as standard. In later work a Holochrome spectrophotometer (Gilson, Middleton, WI, U.S.A.) was employed, allowing accurate analyses down to 1 μg/ml. Galactosamine and glucosamine were determined, after hydrolysis in 4.0 M-HCl under N_2 for 18 h at 100°C, using the amino acid analyser with a modified programme developed by Mr. D. Fackre of this Department.

Isolation and fractionation of glycans and molecular-weight determinations

Freeze-dried proteoglycans, containing about 300 μg of uronic acid, were dissolved in 1.0 ml of 0.5 M-KOH/0.02 M-NaB^3H_4 (The Radiochemical Centre, Amersham, Bucks., U.K.) and stirred at 4°C for 10 days. The specific radioactivity of the alkaline borohydride solution was 2.1×10^{13} d.p.m./mol of NaB^3H_4 (calculated from the manufacturer's specification). After acidifying to pH 5 and desalting, the end-labelled glycosaminoglycans were fractionated with CPC essentially as described previously (Pearson *et al.*, 1975). The sulphated galactosaminoglycans (CPC fraction 2) were further fractionated in the presence of 5% calcium acetate/0.5 M-acetic acid (Meyer *et al.*, 1956; Rodén *et al.*, 1972) by adding ethanol to the following final concentrations: 18% (fraction I); 25% (II); 40% (III); and 50% (IV). Molecular weights of the major end-labelled ethanol fractions were then determined essentially as described by Hopwood & Robinson (1973). Ratios of d.p.m. to glycosaminoglycan in subfractions obtained by chromatography on Sephadex G-200 allowed number average ($\bar{M}_n$) and weight average ($\bar{M}_w$) molecular weights to be calculated.

The compositions of unlabelled glycans released from the proteoglycans by digestion with papain at 65°C (Pearson *et al.*, 1975) were investigated as described below.

Cellulose acetate electrophoresis. Electrophoresis on strips of Sepraphore III (Gelman Instruments, Ann Arbor, MI, U.S.A.) at pH 3.5, staining with 0.5% Alcian Blue (Terochem Laboratories, Edmonton, Alberta, Canada) and destaining were carried out as described by Habuchi *et al.* (1973). The strips were then immersed in *N*-methylpyrrolidine (40%) for 5 min, placed on glass slides and cleared at 80–90°C for 20 min before scanning at 600 nm.

L-Iduronate/total hexuronate ratios. L-Iduronate contents were determined by the periodate/Schiff method described by Di Ferrante *et al.* (1971). Schiff reagent was prepared from basic fuchsin (Serva, Heidelberg, Germany) and used within 12 h. Results were expressed initially as dermatan sulphate, employing the Miles product as standard. The proportions of L-iduronate and D-glucuronate present in the standard dermatan sulphate were determined from the changes in A_{232} (Saito *et al.*, 1968), after digestions with chondroitin ABC lyase and chondroitin AC lyase (Miles). This allowed the analytical results to be converted into L-iduronate/total hexuronate ratios, after correcting total hexuronate values for the lower colour yield (0.8) of L-iduronate (D-glucuronolactone = 1.0).

Results

The treatments with 0.1 M-NaCl and 4 M-guanidium chloride extracted at least 85% of the ligament and skin sulphated galactosaminoglycans. Chromatography of the extracts on DEAE-cellulose resulted in three protein peaks, as found by Antonopoulos *et al.* (1974), but cellulose acetate electrophoresis of papain digests showed that only peak 3 (eluted with 2 M-NaCl in 7 M-urea) contained sulphated galactosaminoglycans. Results of the further purification of DEAE-cellulose peak 3 isolated from the 4 M-guanidinium chloride extracts will be considered first.

The same results were obtained in density-gradient centrifugation of the skin proteoglycan whether 0.4 M- or 4 M-guanidinium chloride was present, indicating the absence of aggregates. However, dissociative conditions were employed in preparative runs for the proteoglycans of both tissues (Figs. 1*a* and 1*b*). Fractions that were homogenous in composite gel electrophoresis and contained most of the dermatan sulphate (skin, fractions 2, 3 and 4; ligament, fractions 2 and 3) were pooled for gel chromatography. Chromatography of the bovine skin proteoglycan on Sepharose-2B gave a single peak with a high $K_{av.}$ (0.89) and no indication of the very-high-molecular-weight forms reported by Öbrink (1972). On Sepharose-6B the skin proteoglycan gave a symmetrical peak ($K_{av.}$ 0.51) that was well separated from protein impurities. Similar results were obtained with the larger column (90 cm × 2.3 cm) of Sepharose-6B without prior density centrifugation. The ligament proteoglycan was eluted earlier ($K_{av.}$ 0.36), suggesting that it was larger and the leading edge of the peak had a small shoulder, which was excluded from the final pool ($PG2_{6B}$).

Protein contents and amino acid compositions of the purified proteoglycans are given in Table 1. The ligament proteoglycan contained less protein than that of skin but agreement in amino acid composition was remarkable. This composition was generally similar to those of dermatan sulphate proteoglycans of two other tissues, but compared with other proteoglycans lysine, half-cystine, the leucines and aspartate were exceptionally high, and glycine, serine and threonine were low (Table 1). Hydroxyproline was not detected. The skin proteo-

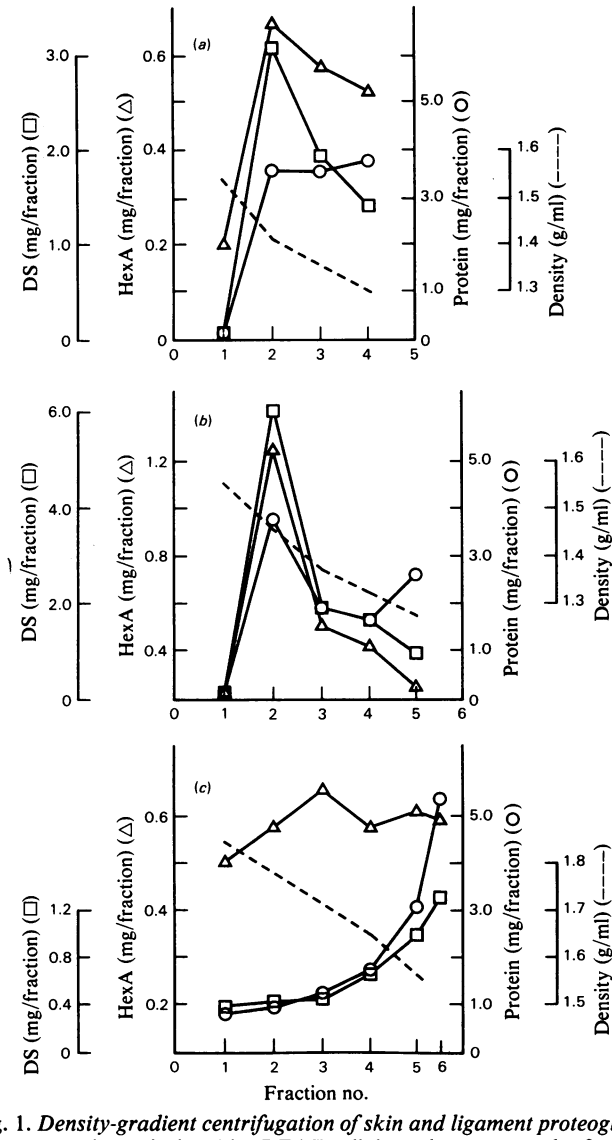

Fig. 1. *Density-gradient centrifugation of skin and ligament proteoglycans*
(*a*) Shows results for skin proteoglycan isolated by DEAE-cellulose chromatography from 4 M-guanidinium chloride extracts. Initial density was 1.39 g of CsCl/ml, in 4 M-guanidium chloride/0.05 M-sodium acetate/acetic acid buffer, pH 5.8. Centrifugation was at 190000 *g* for 48 h. (*b*) shows results for ligament proteoglycan (PG2) isolated by DEAE-cellulose chromatography from 4 M-guanidinium chloride extracts. Starting density was 1.41 g of CsCl/ml; other conditions were as for (*a*). (*c*) shows results for proteoglycan (PG1) isolated by DEAE-cellulose chromatography from 0.1 M-NaCl extracts of the ligament. Initial density was 1.63 g of CsCl/ml, in 0.4 M-guanidinium chloride/0.05 M-sodium acetate/acetic acid buffer, pH 5.8. Centrifugation conditions were as for (*a*). Fraction 6 was a floating gel. The fractions (about 2 ml) were exhaustively dialysed before determinations of protein (Lowry *et al.*, 1951), hexuronate (HexA) and L-iduronate (expressed as dermatan sulphate, DS) were performed.

glycan (DSPG[1]) also appeared to be well purified by direct chromatography on the larger Sepharose 6B column. This was confirmed by composite gel electrophoresis. When fresh solutions of the proteoglycans were examined by this method each preparation gave a sharp peak, whether Toluidine Blue or Coomassie Blue was used, but $PG2_{6B}$ consistently showed a lower mobility than the skin proteoglycan (Fig. 2). By using this technique or SDS/polyacrylamide-gel electrophoresis, (Fig. 2*c* and 2*f*), heterogeneity was observed only in preparation $PG2^{1}_{6B}$, isolated by direct gel chromato-

Table 1. *Amino acid compositions of proteoglycans*

Proteoglycans $PG2_{6B}$ and DSPG were purified by density-gradient centrifugation and on a column (90cm × 1cm) of Sepharose 6B. Skin DSPG[1] was purified directly on a column (90cm × 2.3cm) of Sepharose 6B. The NaCl-extracted proteoglycans $PG1_{Gu}$ and $PG1_{6B}$ were the pools indicated in Fig. 4. Analyses were not corrected for losses in acid hydrolysis. Results are also shown for tendon proteodermatan sulphate (Anderson, 1975), sclera proteoglycans (Cöster & Fransson, 1981) and D-glucuronate-rich proteoglycans of pigskin (Damle *et al.*, 1979), cartilage (Hardingham *et al.*, 1976) and aorta (Oegema *et al.*, 1979). Protein contents, determined by (*a*) the method of Lowry *et al.* (1951) or (*b*) summation of amino acid contents, were expressed as a percentage of protein plus glycosaminoglycan content (hexuronate content × 3.57 for PG2 and skin glycans, or × 3.0 for PG1 glycans). Hexosamine values were percentages on the basis of freeze-dried weight. Abbreviation used: ND, not determined.

	Content (residues/1000 amino acid residues)										
						Ligament					
	Ligament $PG2_{6B}$	Skin DSPG	Skin DSPG[1]	Tendon DSPG	Sclera PGII	$PG1_{Gu}$	$PG1_{6B}$	Pigskin	Cartilage	Aorta	Sclera PG1
Asx	125	126	122	110	123	74	83	57	77	103	96
Thr	39	39	38	43	49	59	78	51	58	86	58
Ser	74	68	62	79	68	168	155	136	123	90	92
Glx	108	108	109	104	122	174	157	150	145	140	138
Pro	67	69	70	87	74	51	61	97	92	93	83
Gly	80	81	74	89	84	135	135	151	136	67	110
Ala	49	49	50	53	54	72	69	58	77	61	65
Val	58	59	62	58	59	45	53	78	69	56	62
Met	9	9	9	10	7	4	6	‡	9	11	‡
Ile	57	60	65	54	55	28	32	35	38	40	41
Leu	123	122	124	119	115	43	56	86	78	88	88
Tyr	29	29	29	22	15	16	(16)*	9	16	19	16
Phe	33	33	34	28	34	24	(24)*	34	27	35	43
Lys	75	80	83	86	76	67	34	32	16	47	45
His	27	27	28	29	25	23	17	10	8	24	19
Arg	31	28	28	29	32	17	24	16	41	33	37
½CyS	(16)†	(13)†	13	ND	9	ND	ND	‡	6	7	8
Protein (%)											
(*a*)	48 (51†)	61	60			ND					
(*b*)	41	60	65			19					
GalN (%)	8.8	ND	7.3								
GlcN (%)	1.4	ND	2.0								

* Values obtained for $PG1_{2B}$ and $PG1_{Gu}$. Incomplete separation from hexosamines in analysis of $PG1_{6B}$.
† From analyses of proteoglycans purified by direct gel chromatography.
‡ Not detected.

graphy. The skin proteoglycan purified in the same way gave a single sharp peak in 5% (Fig. 2*f*) or 10% gels. Mobilities were not affected by prior reduction with 2-mercaptoethanol.

Molecular weights. Values obtained by SDS/polyacrylamide-gel electrophoresis are recorded in Table 2 with results of sedimentation equilibrium. In the latter experiments at lower speeds and in one of the runs on the ligament proteoglycan at the higher speed, the plots of log C versus r^2 (where C = concentration at point r and r = distance from the axis of rotation) were concave upwards and it appeared that two main molecular-weight species were present. Their molecular weights were found from the limiting slopes of the curve, two intersecting straight lines closely fitting the data, but the results are regarded as only approximate (Aune, 1978).

Ligament proteoglycans extracted with 0.1 M-NaCl (PG1)

Fig. 1(*c*) shows that the major proteoglycans extracted with NaCl exhibited larger buoyant densities (1.6–1.74g/ml), lower protein contents (22–44%) and much lower L-iduronate contents than the 4M-guanidinium chloride-extracted proteoglycans. The small amount of L-iduronate-rich proteoglycan extracted with 0.1M-NaCl was almost entirely confined to fractions 5 and 6, which were excluded from the pool (fractions 2–4) taken for gel chromatography. These fractions were also examined by gel electrophoresis (Fig. 3). A diffuse band of low mobility was obtained, rather similar to the band given by a cartilage proteoglycan subunit. The band was barely detectable with Coomassie Blue, probably because of a masking effect of the

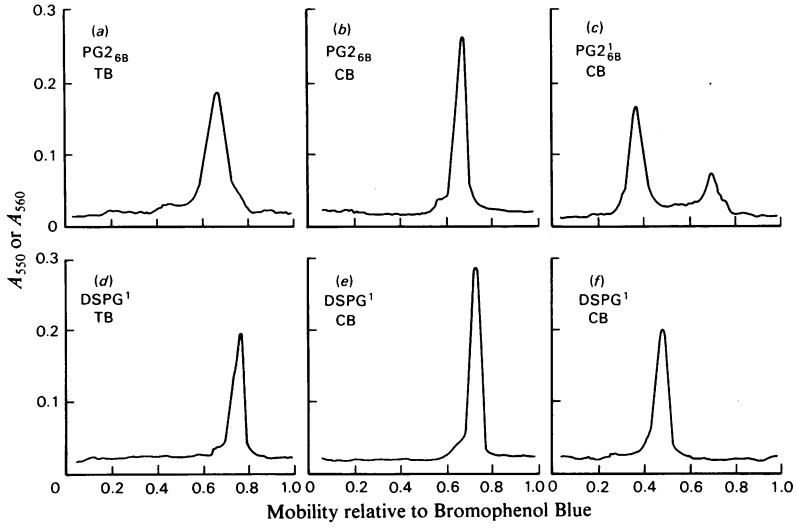

Fig. 2. *Gel electrophoresis of 4 M-guanidinium chloride-extracted proteoglycans*
(*a*), (*b*), (*d*) and (*e*) Show composite gel electrophoresis of about 20 μg of proteoglycan. (*c*) and (*f*) show SDS/5%-polyacrylamide-gel electrophoresis of about 10 μg of proteoglycan. Gels were stained with Toluidine Blue (TB) or Coomassie Blue (CB) and scanned at 550 nm or 560 nm respectively. Details are given in the Materials and methods section. Results are shown for the ligament proteoglycan, purified by density-gradient centrifugation and on a column (90 cm × 1 cm) of Sepharose 6B ($PG2_{6B}$) or directly on the larger column (90 cm × 2.3 cm) of Sepharose 6B ($PG2^1_{6B}$). The skin proteoglycan $DSPG^1$ was purified by the latter method.

Table 2. *Molecular weights of 4 M-guanidinium chloride-extracted proteoglycans*
The proteoglycans were purified by direct chromatography on a column (90 cm × 2.3 cm) of Sepharose 6B (see the Materials and methods section). Estimated molecular weights obtained by sedimentation equilibrium (see the text) are given for two components (A and B) detected in the conventional method (6800–9000 rev./min) and in one run using the meniscus depletion method (13 000–15 013 rev./min). When only one value is shown the 1_nC versus r^2 plot was linear. Molecular weights shown in parentheses in the first column were obtained by SDS/5% polyacrylamide-gel electrophoresis.

				10^{-3} × Molecular weights		
Proteoglycan	Rev./min	Temp. (°C)	Concn. (mg/ml)	A	B	Whole cell average
Skin $DSPG^1$	9000	20	1.3	75	129	100
[(85–90) × 10^3]	9000	8	2.0	104	141	120
	15013	8	2.0	—	—	137
Ligament $PG2^1_{6B}$	8000	20	0.5	62	174	105
(120 × 10^3 for major band)	6800	8	2.4	88	183	134
	15000	20	0.5	—	—	130
	13000	8	2.4	124	161	137

glycosaminoglycan chains, but protein impurities were detected with this stain (Fig. 3, CB).

Gel-chromatography profiles also demonstrated that the NaCl-extracted proteoglycans were heterogeneous in size but there was no indication of the presence of aggregates (cf. *a* and *c* in Fig. 4), although hyaluronate was present. The composition of the protein in these proteoglycans (Table 1) was competely different from the 4 M-guanidinium chloride-extracted proteoglycans. There was little difference between the amino acid compositions of the main components eluted with 4 M-guanidinium chloride from Sepharose CL-2B ($PG1_{Gu}$) and of $PG1_{2B}$ (amino acid composition not shown), although the dissociative conditions lowered the protein content from 33% ($PG1_{2B}$) to 19% ($PG1_{Gu}$). The protein in the fraction excluded from Sepharose 6B ($PG1_{6B}$) generally resembled that in $PG1_{2B}$ and $PG1_{Gu}$ but some differences, especially in lysine, other basic amino acids, threonine and leucine, were probably significant.

Glycan chains of the three proteoglycans

Fractionation with CPC showed that the glycans

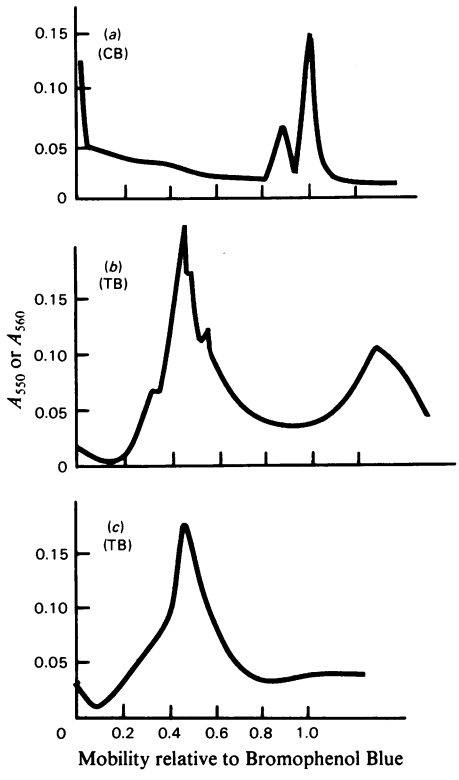

Fig. 3. *Composite gel electrophoresis of 0.1 M NaCl-extracted proteoglycans*

Density-gradient fractions (Fig. 2*c*) were dialysed and freeze-dried before gel electrophoresis. (*a*) and (*b*) show density-gradient fraction 4 (about 20 μg of proteoglycan) stained with Coomassie Blue (CB, A_{560}) or Toluidine Blue (TB, A_{550}). Fractions 2 and 3 gave similar results. Components with relative mobilities greater than 1.0 may have been derived from nucleic acids. (*c*) shows results for the bovine nasal cartilage proteoglycan subunit stained and scanned as for (*b*).

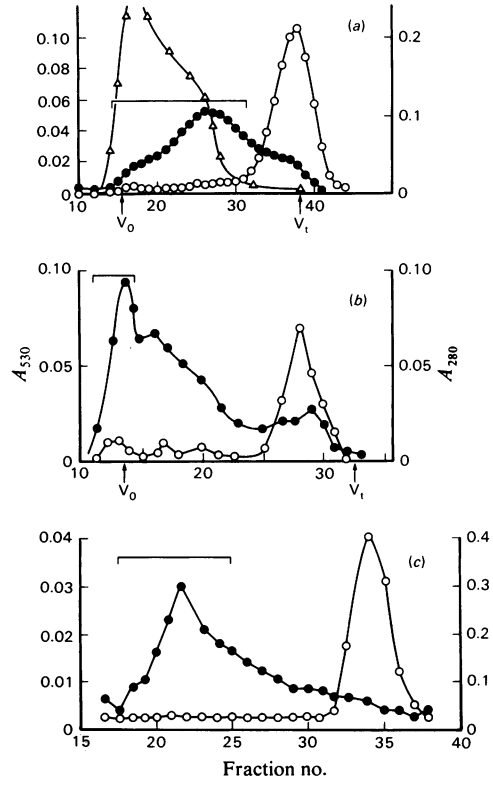

Fig. 4. *Gel chromatography of 0.1 M NaCl-extracted ligament proteoglycans*

Proteoglycan (about 1 mg) from the pool of density-gradient fractions 2, 3 and 4 (Fig. 1*c*) was applied to columns of Sepharose 2B (*a*), 6B (*b*) and CL-2B (*c*). The columns were eluted upwards at 4 ml/h at 4°C with 0.5 M-sodium acetate/acetic acid buffer, pH 6.8 (*a* and *b*) or 4 M-guanidinium chloride (*c*). Absorption of the fractions (2 ml) at 280 nm (O) and hexuronate (●, A_{530}) were determined. In (*c*) a Holochrome spectrophotometer was used (full-scale deflection for $0.10A_{530}$; see the Materials and methods section). In (*a*) △ shows the hexuronate profile of a nasal cartilage proteoglycan subunit. The pools indicated by the bars were designated $PG1_{2B}$ (*a*), $PG1_{6B}$ (*b*) and $PG1_{Gu}$ (*c*).

of $PG1_{2B}$ contained about 23% of hyaluronate, 16% of a fraction (CPC fraction 3) containing heparan sulphate (Gibson, 1979) and 61% of sulphated galactosaminoglycans, whereas the latter were the only glycosaminoglycans present in the proteoglycans extracted with 4 M-guanidinium chloride. Results given in Table 3, including a comparison with the standard glycosaminoglycan, strongly suggested that the great majority at least of the sulphated galactosaminoglycans of ligament proteoglycan $PG2_{6B}$, as well as those of the skin proteoglycan, were dermatan sulphates. This type of hybrid was absent from $PG1_{2B}$, which however was rich in CS(DS) hybrids with an average L-iduronate content of about 7% of the total hexuronate (Table 3). In the method of Di Ferrante *et al.* (1971) used for these analyses, standard chondroitin sulphate gave a response equivalent to less than 0.5% of an equal weight of the standard dermatan sulphate. The specifity of this method has been criticised (Scott & Tigwell, 1978), but the main problem seems to be in its application to mixtures of glycosaminoglycans, especially when heparan sulphate is present, rather than to purified sulphated galactosaminoglycans.

In cellulose acetate electrophoresis (Fig. 5) only dermatan sulphate was detected in the glycans of the 4 M-guanidinium chloride-extracted proteoglycans; no CS(DS) hybrids were present. Profiles obtained for the latter components of the NaCl-extracted

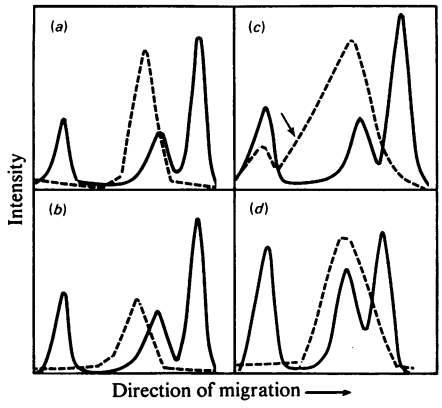

Fig. 5. *Cellulose acetate electrophoresis of ligament and skin glycosaminoglycans*

With a dual applicator the glycosaminoglycan solution (about 1.0 μg/μl) was applied to one side of a prepared strip and a mixture of standards to the other. Electrophoresis conditions: pyridine/acetic acid/water (1:9:115, by vol.), pH 3·5, 0.5 mA/cm width, 1h, Alcian Blue staining, scanned at 600nm. The continuous lines show the peak positions of the standards hyaluronate, dermatan sulphate and chondroitin (4- or 6-) sulphate, with peaks in order of increasing mobility. Broken-line profiles represent total glycosaminoglycan released from individual density-gradient fractions 2, 3 or 4 (Fig. 1) of (*a*) PG2, (*b*) skin proteoglycan and (*c*) PG1; the region indicated by the arrow probably contained heparan sulphate. (*d*) shows results for sulphated galactosaminoglycans (CPC fraction 2) of $PG1_{6B}$ (Fig. 4*b*).

proteoglycans were completely different from dermatan sulphates or chondroitin (4- or 6-) sulphates (standard compounds or a CPC fraction 2 isolated by us from the bovine nasal proteoglycan subunit). The broad bands of intermediate mobility were similar to those reported for CS(DS) hybrids of pigskin (Fransson & Rodén, 1967) and fibrous cartilage (Habuchi *et al.*, 1973). The profile obtained for CPC fraction 2 of $PG1_{6B}$ (Fig. 5*d*) was indistinguishable from that of ethanol fraction III isolated from the whole ligament and shown to consist predominantly of CS(DS) hybrids (Pearson & Gibson, 1979; Pearson, 1981).

The major dermatan sulphate components of the 4 M-guanidinium chloride-extracted proteoglycans had different molecular weights (Table 3), explaining their different ethanol solubilities. Two different molecular-weight species were present in CPC fraction 2 of $PG1_{2B}$. The end-labelling method employed to obtain these values avoids possible errors from self-association of the hybrid glycans (Fransson *et al.*, 1979).

Discussion

Sulphated galactosaminoglycan components of isolated proteoglycans had not previously been fractionated to distinguish hybrids of different hexuronate composition. The ethanol precipitation method used in the present paper gives only a limited resolution and is affected by molecular-weight variations. However, our results for periodontal ligament proteoglycans indicated that there were two main groups of hybrids of very different L-iduronate contents and these occurred in distinct proteoglycans. This uneven distribution of L-iduronate in glycans and proteoglycans raises questions about control processes that cannot be answered from present knowledge of the biosynthesis of these compounds (Lindahl, 1976; Rodén & Horowitz, 1978). The L-iduronate-rich proteodermatan sulphates are characteristic products of mature fibroblasts, which are numerous in periodontal ligament and presumably synthesized PG2. The origins of CS(DS) hybrid proteoglycans, a heterogenous group of compounds (see below), are less certain. Their prevalence in some cultures may be the result of a selective process favouring certain 'fibroblast-like' cells (Mourão *et al.*, 1980).

Recently Cöster & Fransson (1981) showed that in bovine sclera hybrid-sulphated galactosaminoglycans with different L-iduronate contents also occur in different proteoglycans. The difference in hexuronate composition of the unfractionated glycans was less marked than found here, as L-iduronate comprised about 20% and 52% respectively of the hexuronate of the two proteoglycans (PGI and PGII). Thus significant tissue variations may exist, but it is possible the separation of the two proteoglycans, after a simultaneous extraction with 4 M-guanidinium chloride, was not quite complete. In the present work a differential extraction procedure greatly facilitated the separation of ligament proteoglycans PG1 and PG2. Composite gel electrophoresis followed by Toluidine Blue staining, a sensitive test for the presence of PG1 (Fig. 3, TB), showed that a significant amount of the latter did not occur in preparations of PG2 (Fig. 2*a*). There was, however, more risk of proteoglycan degradation during the treatment with 0.1 M-NaCl, even though proteinase inhibitors were added.

The skin proteodermatan sulphate was homogeneous under different conditions of gel chromatography and gel electrophoresis and preliminary results (C. H. Pearson & N. Winterbottom, unpublished work) suggested that it had a single *N*-terminal amino acid residue. The polydispersity observed in the ultracentrifuge (Table 2), although less than found previously (Preston, 1968), was not easy to explain. Sedimentation equilibrium may have separated species containing different numbers of

Table 3. *Fractionation of sulphated galactosaminoglycan components of ligament and skin proteoglycans*
The proteoglycans were purified by density-gradient centrifugation and on a column (90cm × 1cm) of Sepharose 6B ($PG2_{6B}$ and DSPG) or as shown in Fig. 4 ($PG1_{2B}$). Preparations of CPC fraction 2 of end-labelled glycosaminoglycans, cleaved from the proteoglycans, were fractionated with ethanol to give four fractions at final ethanol concentrations of 18% (fraction I), 25% (II), 40% (III) and 50% (IV). Yields were determined from hexuronate (HexA) analyses. The molecular weights of the major fractions were then determined by the method of Hopwood & Robinson (1973). Unlabelled preparations of CPC fraction 2 (and ethanol fractions of the skin glycans), isolated from papain digests of the proteoglycans, were also analysed for L-iduronate (IdoA) by the method of Di Ferrante *et al.* (1971). The values given in parentheses were obtained for corresponding ethanol fractions (of closely similar molecular weights) isolated from papain digests of the ligament (Pearson, 1981).

Source of glycans	Fraction	HexA (% of total HexA)	(IdoA/HexA) × 100	10^{-3} × Mol.wt. $\overline{M}_n$	10^{-3} × Mol.wt. $\overline{M}_w$
Standard dermatan sulphate	Unfractionated		75‡		
	I	64	90		
	II	14	86		
	III*	19	62		
Bovine skin proteoglycan (DSPG)	CPC F2		76		
	I	18	95		
	II	49	83	16.0	16.5
	III*	30	71		
Ligament proteoglycan ($PG2_{6B}$)	CPC F2		75		
	I	65	(80)	29.0	29.1
	II	19	(66)		
	III*	13			
Ligament proteoglycan ($PG1_{2B}$)	CPC F2		7		
	III†	43	(14)	31.2	31.7
	IV	57	(4)	19.4	20.0

* Less then 3% of fraction IV was obtained.
† No fraction I or II was found.
‡ Determined by the method of Saito *et al.* (1968).

dermatan sulphate chains, but changes during ultracentrifugation were not ruled out. One possibility was self-association, which has been observed for this type of proteoglycan at lower temperatures (Cöster, 1979; C. H. Pearson & S. Lehocky, unpublished work) as well as at 37°C at acid (Pearson *et al.*, 1978) and neutral (Pearson, 1981) pH values. However, some proteolysis may have occurred, especially when ultracentrifugation was performed at 20°C rather than at 8°C (Table 2). Even highly purified proteodermatan sulphates apparently contain proteinase activity (Gibson, 1979; Pearson, 1981). These problems merit further investigation, particularly as self-association could influence the interaction of the proteoglycan with collagen fibres (Scott, 1980) or cell surfaces.

Determinations by SDS/polyacrylamide-gel electrophoresis, even with normal protein standards, may be the most reliable method at present for comparing molecular weights of proteodermatan sulphates of similar compositions. Results obtained by this technique suggest that the tendon proteoglycan (mol.wt. 120000; Anderson, 1975) and ligament proteodermatan sulphate (Table 2) are the same size. The bovine skin proteoglycan is smaller (Table 2; see also Fig. 2 and $K_{av.}$ values from gel chromatography), as is sclera proteoglycan II (mol.wt. 85000; Cöster & Fransson, 1981). Elution positions on Sepharose 2B columns indicate that the bovine skin proteoglycan ($K_{av.}$ 0.89) is also smaller than pigskin proteodermatan sulphate ($K_{av.}$ 0.63; Gregory & Damle, 1979).

The protein cores of the ligament and bovine skin proteodermatan sulphates had almost identical amino acid compositions and are likely to be similar in size. Calculations of the latter are complicated by the occurrence of a number of attached oligosaccharides (Gregory & Damle, 1979; Cöster & Fransson, 1981), which probably accounted for the glucosamine in our preparations (Table 1). However, we estimate that each protein core had a molecular weight of $(6–7) \times 10^4$ and carried two to three dermatan sulphate chains. Cöster & Fransson (1981) obtained a value of 46000 for the molecular weight of the core protein of sclera proteoglycan II after deglycosylation with chondroitin ABC lyase. Proteinase activity is, however, a problem in this procedure (Oike *et al.*, 1980). The relative sizes of bovine skin and ligament dermatan sulphates (Table 3) satisfactorily explained the difference in molecular weight of the respective proteoglycans. More firm data are needed on the sizes of dermatan

sulphates in other isolated proteoglycans before tissue or species variations can be generally evaluated.

The proteoglycans extracted from the ligament with 0.1M-NaCl ($K_{av.}$ 0.5 on Sepharose-2B) were significantly larger than ligament proteodermatan sulphate. Most of them, however, appeared to be smaller than bovine nasal cartilage proteoglycans (Fig. 4*a*), although mobilities in composite gel electrophoresis were similar (Fig. 3). There was some resemblance in composition to sclera proteoglycan I (Cöster & Fransson, 1981) and other non-cartilaginous proteoglycans containing either CS(DS) hybrids of very similar hexuronate composition (L-iduronate being 7–9% of the total hexuronate) to those isolated here, or chondroitin sulphate (Lowther *et al.*, 1970; Radhakrishnamurthy *et al.*, 1977; Oegema *et al.*, 1979; Damle *et al.*, 1979). Protein contents of the non-cartilaginous proteoglycans have been variable but generally much higher than those of typical cartilage proteoglycans (5–10%). In this respect the ligament proteoglycans $PG1_{Gu}$ (19% protein, Table 1) were similar to some fractions of aorta proteoglycans (Oegema *et al.*, 1979; McMurtrey *et al.*, 1979) but not to sclera proteoglycan I (45% protein; Cöster & Fransson, 1981). Not surprisingly amino acid compositions have also been variable (Table 1). The NaCl-extracted proteoglycans of the ligament had lower contents of proline and leucine than the other proteoglycans and were particularly rich in serine and glutamate. More complete purification of D-glucuronate-rich proteoglycans of non-cartilaginous tissues and additional precautions against proteolysis may give more consistent amino acid compositions.

Table 3 shows that two different molecular-weight fractions of sulphated galactosaminoglycans occurred in PG1. Fraction III resembled ligament dermatan sulphate in molecular weight and low polydispersity. It may be smaller than CS(DS) hybrids of bovine aorta, which were estimated as mol. wt. 40000 by gel chromatography (Oegema *et al.*, 1979). The hybrid nature of the lower-molecular-weight fraction IV has not been definitely established, but as indicated in Table 3 the corresponding fraction isolated from the whole ligament contained a small amount of L-iduronate. We have been unable to demonstrate the presence of authentic chondroitin sulphate in any glycan fraction of the whole ligament or of the isolated proteoglycans studied in the present paper.

The ease of extraction of ligament PG1 at low salt concentrations, which contrasts with data obtained for the somewhat similar pigskin proteoglycan (Damle *et al.*, 1979), is consistent with an interfibrillary location (Plecash, 1974; Pearson, 1981) and a low affinity for collagen. Ligament PG2 is almost certainly more firmly associated with the collagen fibres. The functional implications of the different characteristics and probable distributions of these proteoglycans and their quantitative variations during tooth eruption have been discussed previously (Gibson, 1979; Pearson, 1981).

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