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Isolation and partial characterization of proteoglycans from rat incisors

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Newly synthesized proteoglycans of rat incisors were labelled *in vivo* for 6h with [^{35}S]-sulphate in order to facilitate their detection during purification and characterization. Proteoglycans were extracted from non-mineralized portions (predentine) of rat incisors with 4M-guanidinium chloride and subsequently from dentine by demineralization with a 0.4M-EDTA solution containing 4M-guanidinium chloride. Both extractions were performed at 4°C in the presence of proteinase inhibitors. Purification of proteoglycans was achieved with a procedure involving gel-filtration chromatography, selective precipitation of phosphoproteins, affinity chromatography and ion-exchange chromatography. Two proteoglycan populations were found in the initial extract (Pd-PG I and Pd-PG II), whereas only one fraction (D-PG) was obtained after demineralization. The minor proteoglycan fraction from the first extract, Pd-PG I, although not totally characterized, differed sharply from the other proteoglycans in that it had a larger molecular size with larger glycosaminoglycan chains composed of chondroitin 4- and 6-sulphate isomers. In contrast, the major proteoglycans Pd-PG II and D-PG had smaller hydrodynamic sizes with smaller glycosaminoglycan chains (but larger than those from bovine nasal cartilage proteoglycans) composed exclusively of chondroitin 4-sulphate. The major proteoglycans were incapable of interacting with hyaluronic acid. In general, the amino acid compositions of the major proteoglycans of rat incisors resembled that of bovine nasal cartilage proteoglycans, but the former had lower proline, valine, isoleucine, leucine, and higher aspartic acid, contents.

Proteoglycans are important structural components of all connective tissues (Hascall & Hascall, 1981). Whereas the chondroitin sulphate proteoglycans, particularly those of bovine nasal cartilage, have been studied extensively and often serve as reference standards, the proteoglycans of most other tissues are less well characterized. In particular, this is true for the proteoglycans of mineralized tissues.

Although a number of investigators have shown that various types and amounts of glycosaminoglycans are present in the teeth of several species (Pincus, 1948; Clark *et al.*, 1965; Engfeldt & Hjerpe, 1972; Linde, 1973; Embery, 1974; Branford-White, 1978; Hjerpe *et al.*, 1983), little information is available regarding the nature of the intact proteoglycans. Jones & Leaver (1974) showed that proteoglycans occur as a minor component of human dentine, and Hjerpe & Engfeldt (1976) demonstrated that the quality and quantity of proteoglycans differ between predentine and dentine of rachitic dogs. Smalley & Embery (1980) observed that the metabolism and size of chondroitin 4-sulphate proteoglycans were altered in fluorotic rat incisors.

Previous studies from our laboratory have shown that continuously erupting rat incisors provide a useful model for the study of proteoglycans and other non-collagenous proteins involved in dentinogenesis (Linde *et al.*, 1980; Butler *et al.*, 1981). In these studies, a sequential extraction procedure was employed that resulted in two pools of proteoglycans: an initial 4M-GdmCl-extraction-

Abbreviations used: GdmCl, guanidinium chloride; ΔDi0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose; ΔDi6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose; SDS, sodium dodecyl sulphate.

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solubilized material from non-mineralized portions of the tissue, and a second pool, which was 'mineral-bound', was obtained only after demineralization of the tissue (Termine *et al.*, 1980, 1981). In pursuit of our aim to investigate the role of proteoglycans in the mineralization process, we have developed methods to study the chemistry and metabolism of proteoglycans of rat incisors (Rahemtulla *et al.*, 1981) and calvaria (Prince *et al.*, 1983) and using an '*in vivo*' radiolabelling technique. In the present study we report the isolation and partial characterization of the proteoglycans extractable from rat incisors.

Experimental procedures

Materials

GdmCl, 6-aminohexanoic acid, benzamidine hydrochloride, sodium iodoacetate, phenylmethanesulphonyl fluoride, soya-bean trypsin inhibitor, pepstatin, papain (twice-crystallized), bovine serum albumin, D-glucuronic acid and urea were purchased from Sigma. Bio-Rad Protein Assay Kit and Bio-Gel P-2 were from Bio-Rad Laboratories. Sepharose CL-6B, DEAE-Sephacel, CNBr-activated Sepharose 4B and Sephadex PD-10 columns were from Pharmacia. Ultrafiltration cells and membranes (YM-5) were purchased from Amicon. Precoated microcrystalline cellulose t.l.c. plates were obtained from Quantum Industries (Fairfield, NJ, U.S.A.). Carrier-free sodium [^{35}S]-sulphate (25–40 Ci/mg) was obtained from Amersham International and ScintiVerse I was from Fisher. Chondroitinase ABC, ΔDi0S, ΔDi4S, ΔDi6S and antibodies against rat serum proteins were purchased from Miles Laboratories. An affinity column was prepared as follows: antibodies against rat serum proteins were dissolved in 0.1 M-$NaHCO_3$, pH 8.3, containing 0.5 M-NaCl; the coupling reaction was carried out at 4°C overnight by using 10 mg of protein/ml of gel and a gel-to-buffer ratio of 1:2 (v/v).

Bovine nasal cartilage proteoglycan (fraction A1D1) was a gift from Dr. Bruce Caterson and Dr. John R. Baker of this University, and had been prepared as described by them (Baker & Caterson, 1979). High-M_r hyaluronic acid, prepared from rooster comb by the the method of Swann (1968), was generously given by Dr. James E. Christner of this University. Chondroitin sulphate from cartilage was a gift of Dr. Katherine Rostand of this University, and had been prepared by papain digestion of fraction A1D1.

Analytical methods

Quantification of hexuronic acid was carried out by the procedure of Blumenkrantz & Asboe-Hansen (1973), with D-glucuronic acid as standard. Protein was measured by the method of Lowry *et al.* (1951) or by the Bio-Rad Protein Assay, following the manufacturer's suggested procedure, with bovine serum albumin as standard.

Hexosamines were determined after hydrolysis in 6 M-HCl for 4 h by a modification of the Askenasi (1973) procedure that is routinely used for quantification of hydroxylysine glycosides from collagen. The 0.28 cm × 39 cm column (AA-20 spherical resin) on a Beckman 121M amino acid analyser was eluted with 0.13 M-sodium citrate buffer, pH 4.74, at 47°C, and a flow rate of 8 ml/h. Under these conditions glucosamine was eluted at 58.4 min and galactosamine at 66.1 min. For amino acid analysis, samples were hydrolysed in constant-boiling HCl (108°C) for 24 h and analysed on the Beckman 121M amino acid analyser by the method of Butler *et al.* (1977). Radioactivity was measured with a Beckman LS 8000 liquid-scintillation counter with 1 ml of aqueous sample and 2.5 ml of ScintiVerse I.

Isolation of [^{35}S]sulphate-labelled proteoglycans

Each of twenty male Sprague–Dawley rats weighing 50–150 g was injected intraperitoneally with 1 mCi of carrier-free $Na_2{}^{35}SO_4$ per 100 g of body wt. The rats were killed 6 h after the injection. Mandibular and maxillary incisors were removed and immediately placed in ice-cold buffer containing proteinase inhibitors (Rahemtulla *et al.*, 1981). After thorough removal of pulp and adhering soft tissue, the incisors were broken into small pieces and sonicated twice for 2 min in buffer containing proteinase inhibitors. The supernatant, containing finely suspended cell debris, was discarded and the sediment was rinsed briefly with distilled water and freeze-dried.

Non-collagenous proteins and [^{35}S]sulphate-labelled proteoglycans were extracted at 4°C by a modification of the procedure described previously (Linde *et al.*, 1980; Butler *et al.*, 1981; Termine *et al.*, 1980, 1981; Rahemtulla *et al.*, 1981). Briefly, 2.6 g of freeze-dried tissue was suspended in 52 ml of 4 M-GdmCl, pH 6.5, containing 50 mM-sodium acetate, 50 mM-EDTA, 0.1 M-6-aminohexanoic acid, 1 mM-sodium iodoacetate, 5 mM-benzamidine hydrochloride, 10 mM-phenylmethanesulphonyl fluoride, 1 mg of soya-bean trypsin inhibitor/litre and 5 mg of pepstatin/litre. After stirring for 14–16 h, the suspension was centrifuged at 3000 rev./min ($r_{av.}$ 18 cm) for 20 min and the supernatant, which contained components of the non-mineralized tissue, was concentrated to a small volume by ultrafiltration through a YM-5 membrane with addition of 500 ml of 0.05 M-Tris/HCl, pH 7.4, containing 4 M-GdmCl. The insoluble residue containing dentine was demineralized by extraction for 72 h with 104 ml of a solution of the same composition as above except that the EDTA concentration

was 0.4M. The solubilized material was recovered as described above. In order to remove dentine phosphoproteins, the concentrated material was dialysed overnight against 50vol. of 0.05M-Tris/HCl, pH7.4, containing 1M-$CaCl_2$, plus the proteinase inhibitors listed above. Phosphoproteins which precipitated during dialysis were removed by centrifugation at 27000*g* for 15min, and the supernatant solution was concentrated to a small volume by ultrafiltration through a YM-5 membrane; then GdmCl was added to a final concentration of 4M.

Each extract was chromatographed on a column (2cm × 150cm) of Sepharose CL-6B, which was eluted with 0.05M-Tris/HCl, pH7.4, containing 4M-GdmCl. The radiolabelled material in the eluates was pooled (see the Results section), concentrated, and desalted on a column (2cm × 40cm) of Sephadex G-25, which was eluted with 0.2M-NH_4HCO_3 and freeze-dried.

Serum proteins were then removed by affinity chromatography of each extract on Sepharose 4B linked to antiserum against rat serum proteins. The radiolabelled material was dissolved in 2.5ml of 0.2M-phosphate-buffered saline, pH7.2, and loaded on to a column (2cm × 5cm) of affinity matrix, which was eluted with the same buffer, and then subsequently with 0.05M-Tris/HCl, pH7.4, containing 4M-GdmCl. The first eluate was dialysed against 0.05M-Tris/HCl buffer, pH7.4, containing 4M-urea, and applied to a column (2cm × 15cm) of DEAE-Sephacel that had been equilibrated with the same buffer. After a 100ml buffer wash the column was eluted over a total volume of 500ml with a linear gradient of 0–1M-NaCl in the Tris/urea buffer. The GdmCl eluate (serum proteins) from the affinity-chromatography step was desalted on Sephadex G-25 as described above.

Enzymic digestion of proteoglycans

Conditions for digestion with chondroitinase ABC were similar to those described by Saito *et al.* (1968). Samples containing 5000–10000c.p.m. of [^{35}S]sulphate were dissolved in 1–2ml of 0.1M-Tris/HCl, pH7.0, and were digested with 0.1 unit of enzyme at 37°C for 2h; after addition of another 0.1 unit of enzyme, the incubations were continued overnight. Each chondroitinase digest was applied to a column (1cm × 40cm) of Bio-Gel P-2, and was eluted with 0.2M-NH_4HCO_3. Disaccharides, which were eluted close to the total bed volume of the column, were freeze-dried and then dissolved in 150–200μl of 0.2M-sodium acetate buffer, pH5.4, containing 3μg of each standard disaccharide. Portions of this solution (2000–4000c.p.m.) were then analysed by high-pressure liquid chromatography on a Waters Carbohydrate Analysis column eluted with 0.2M-acetate buffer, pH5.4, at a flow rate of 1.5ml/min (Hjerpe *et al.*, 1979). Fractions (0.2min) were collected and analysed for radioactivity as described above. Alternatively, the chondroitinase digests and the disaccharide standards were chromatographed on thin-layer cellulose plates (Wasserman *et al.*, 1977) and stained with aniline phthalate (Partridge, 1949).

Proteoglycans (~5000c.p.m.) were dissolved in 2ml of 0.05M-sodium acetate/0.05M-cysteine hydrochloride/0.2M-EDTA, pH6.5, and digested with papain (0.6mg) at 65°C for 16–18h. The papain digests were applied to columns (1cm × 100cm) of Sepharose CL-6B and were eluted with 0.05M-Tris/HCl, pH7.4, containing 4M-GdmCl. Fractions (1.2ml) were collected and analysed for radioactivity as described above.

Electrophoresis

Slab-gel electrophoresis was performed as described by Butler *et al.* (1981), the discontinuous Tris/glycine buffer system of Laemmli (1970) being used. Protein bands were stained with Coomassie Brilliant Blue.

Results

Proteoglycan extraction and purification

The sequential-extraction procedure employed resulted in three radioactive fractions (Table 1). Of the total radioactivity incorporated into the proteoglycan fractions, 70% was recovered in the initial 4M-GdmCl extract, whereas about 25% was extracted during EDTA demineralization of dentine. Only 2–5% of the radioactivity was released after papain digestion of the insoluble dentine matrix.

The purification scheme for each extract involved sequential passage through Sepharose CL-6B (dissociative conditions), an affinity column to remove serum proteins, and DEAE-Sephacel in 4M-urea. The chromatographic profile of the GdmCl extract (from uncalcified tissue) on Sepharose CL-6B revealed three [^{35}S]sulphate-labelled peaks (results not shown); most of the [^{35}S]sulphate-labelled material eluted at, or close

Table 1. *Distribution of macromolecular* [^{35}S]*sulphate incorporated into rat incisors*

Twenty rats were injected with 1mCi of [^{35}S]sulphate/100g body wt. After 6h of incorporation, rats were killed and [^{35}S]sulphate proteoglycans were extracted as described in the text.

Dry weight of incisors (g)	Treatment ...	[^{35}S]Sulphate radioactivity (c.p.m./mg of tissue) GdmCl	EDTA	Papain
2.6		510.7	180.2	18.4
2.8		610.4	218.0	18.1

to, the V_0 of the column as two broad, unresolved peaks, whereas a small amount of [^{35}S]sulphate-containing material was eluted with $K_{av.}$ 0.7. A number of u.v.-absorbing protein peaks were also observed. The fractions containing the major [^{35}S]-sulphate-labelled proteoglycans were pooled for further purification. The minor [^{35}S]sulphate-containing material was not characterized.

When the EDTA extract from dentine was chromatographed on Sepharose CL-6B, the [^{35}S]-sulphate-labelled material eluted in a broad peak with $K_{av.}$ 0.30. This peak was pooled for further purification.

Portions of the two pools were analysed for neutral-proteinase activity by zymography using co-polymerized gelatin/acrylamide (Heussen & Dowdle, 1980) and by proteolysis of [^{3}H]acetyl-fibrin (Unkeless *et al.*, 1974) as modified by Birkedal-Hansen & Taylor (1983). None of these assays gave positive results; therefore, in subsequent fractionation steps, buffers without the denaturating agents and inhibitors could be used. The pools were also analysed by SDS/polyacrylamide-gel electrophoresis. In both pools several Coomassie Blue-stainable components were observed, including a prominent band co-migrating with the bovine serum albumin standard (results not shown).

In order to separate the [^{35}S]sulphate-labelled proteoglycans from contaminating serum proteins, each sample was then passed through an affinity column to which antibodies against rat serum proteins were coupled. The [^{35}S]sulphate-labelled proteoglycans were eluted in the non-binding fraction, whereas the rat serum proteins, as revealed by SDS/polyacrylamide-gel electrophoresis (results not shown), eluted with 4M-GdmCl (Fig. 1).

After ion-exchange chromatography on DEAE-Sephacel in 4M-urea, the material obtained from the predentine (GdmCl) extract was resolved into two radioactive peaks and several other protein peaks (Fig. 2*a*). The minor proteoglycan fraction (Pd-PG I) was eluted with 0.15M-NaCl and was associated with other proteins, as indicated by the u.v. absorption profile and by SDS/polyacrylamide-gel electrophoresis. The major proteoglycan fraction (Pd-PG II) was eluted at 0.5M-NaCl. The [^{35}S]sulphate-labelled proteoglycans of dentine (D-PG) were eluted with 0.5M-NaCl as a single peak (Fig. 2*b*). Recover of radioactivity in excess of 80%

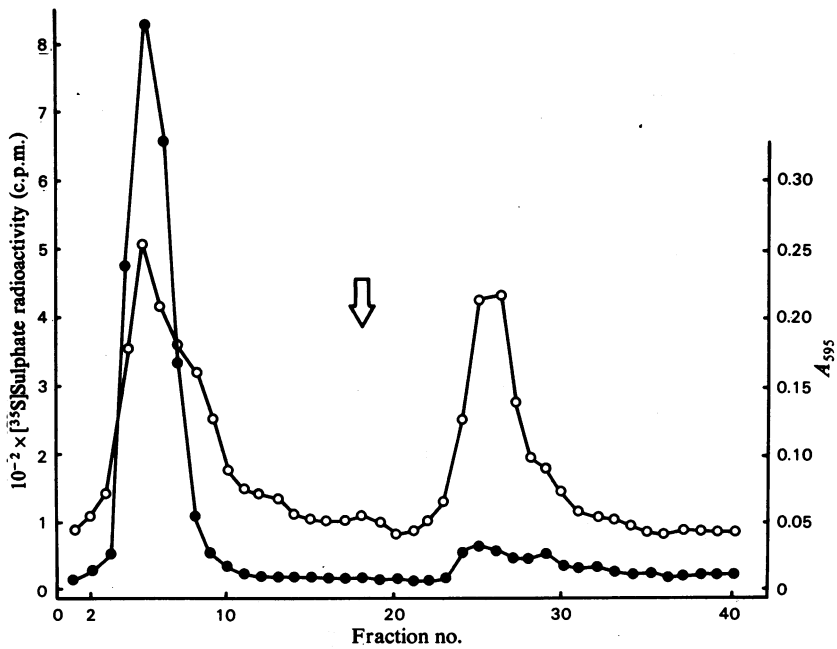

Fig. 1. *Affinity chromatography of pooled* [^{35}S]*sulphate-labelled macromolecules obtained after Sepharose CL-6B chromatography of predentine extract*

Radiolabelled material containing (15–30) $\times 10^3$ c.p.m. of [^{35}S]sulphate was dissolved in 0.2M-phosphate buffered saline, pH 7.2, and was then applied to an affinity column which was prepared as described in the text. The column was eluted at room temperature with 40ml of 0.2M-phosphate-buffered saline, pH 7.4, and then with 40ml of 0.05M-Tris/HCl buffer, pH 7.4, containing 4M-GdmCl (where indicated by the arrow). Fractions (2ml) were collected and analysed for radioactivity and protein as described in the text. Chromatography of the dentine-derived sample produced a similar elution profile. ●, [^{35}S]sulphate radioactivity; ○, A_{595}.

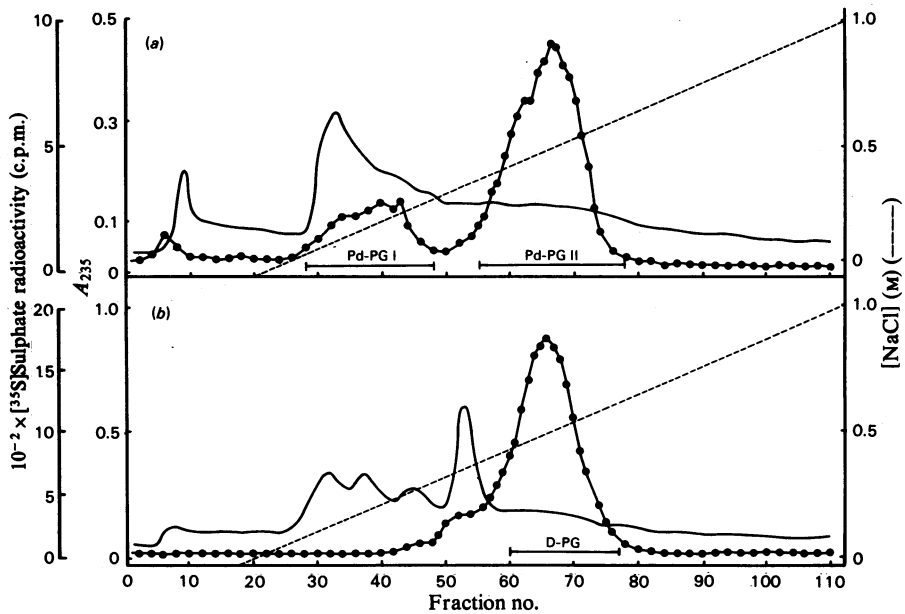

Fig. 2. *DEAE-Sephacel chromatography of [^{35}S]sulphate-labelled macromolecules obtained from predentine (a) and dentine (b)*

Fractions eluted from the affinity column (Fig. 1) with phosphate-buffered saline were dialysed against 0.05M-Tris/HCl buffer, pH7.4, containing 4M-urea and then applied to the ion-exchange column (2cm × 15cm). The column was washed with 100ml of the same buffer and then eluted with a linear gradient of 0–1M-NaCl (----) over a total volume of 500ml. A flow rate of 20ml/h was used and the column was continuously monitored at 235nm. Fractions (5ml) were collected and radioactivity was determined as described in the text. Fractions were pooled as indicated by the bars. ——, A_{235}; ●, [^{35}S]sulphate radioactivity.

Table 2. *Chemical analyses of proteoglycans of rat incisors*

Bovine serum albumin was the standard in the Lowry assay. The results are means for duplicate samples.

Fraction ...	Weight (% of total) Pd-PG II	D-PG
Total hexosamine	21.0	25.5
Galactosamine	17.0	20.5
Glucosamine	4.0	5.0
Hexuronic acid	20.3	18.2
Protein (Lowry)	20.0	19.0

was observed in all chromatographic runs. The radiolabelled proteoglycans obtained from ion-exchange chromatography were pooled separately, concentrated and freeze-dried.

Chemical composition of proteoglycans

Because of the small amount of material and impure nature of the minor radioactive fraction (Pd-PG I) obtained from the GdmCl extract, chemical analyses of this fraction were not performed.

Analysis of the major proteoglycan fractions isolated from predentine and dentine (Table 2) indicated that galactosamine was the major hexosamine, with only 5% glucosamine present. The hexuronic acid contents of Pd-PG II and D-PG were 20% and 18% respectively. The nearly equimolar contents of galactosamine and hexuronic acid are indicative of the chondroitin sulphate nature of the proteoglycans. The small amount of glucosamine may reflect the presence of *N*-linked oligosaccharides. About 20% protein was present in both preparations. The amino acid analyses of the two proteoglycan preparations were similar, with a preponderance of aspartic acid, serine, glutamic acid and glycine (Table 3).

Digestion with chondroitinase ABC

To establish further the identity of the chondroitin sulphate in these proteoglycans, they were separately digested with chondroitinase ABC. It has been reported previously that dentine contains small amounts of dermatan sulphate, representing 2% or less of the total glycosaminoglycans (Jones & Leaver, 1974; Hjerpe *et al.*, 1983). Therefore we use the term 'chondroitin sulphate' recognizing that these glycosaminoglycans may contain trace amounts of iduronic acid. The susceptibility of

Table 3. *Amino acid composition of predentine and dentine proteoglycans obtained from rat incisors*
For comparison, data for intact bovine nasal cartilage A1D1 proteoglycan (Heinegård, 1977) and its chondroitin sulphate-containing region (Yanagishita *et al.*, 1979) are included.

	Composition (residues/1000 residues)			
Amino acid / Proteoglycan ...	Pd-PG II	D-PG	A1D1	Chondroitin sulphate-containing region
Aspartic acid	180	177	64	56
Threonine	59	61	59	42
Serine	125	128	121	181
Glutamic acid	171	172	148	165
Proline	48	47	101	100
Glycine	127	118	131	171
Alanine	61	59	63	67
Cysteine	0	0	0	0
Valine	24	40	69	58
Methionine	Trace	Trace	3	0
Isoleucine	17	21	37	38
Leucine	41	45	80	83
Tyrosine	28	16	17	7
Phenylalanine	24	12	35	31
Lysine	39	42	18	Trace
Histidine	21	29	2	Trace
Arginine	31	32	34	Trace
Hydroxylysine	0	0	0	
Hydroxyproline	0	0	0	

[^{35}S]sulphate-labelled proteoglycans to this specific enzyme was evaluated by passing the digests through a column of Bio-Gel P-2 eluted with 0.2M-NH_4HCO_3. Essentially all (95–97%) of the radioactivity was recovered in the included volume of the column, indicating complete digestion of the polysaccharide chains. After chondroitinase ABC digestion, a modified chondroitin sulphate disaccharide remains attached to the linkage-region oligosaccharide of chondroitin sulphate (Hascall *et al.*, 1972). This fact would account for the small amount (3–5%) of radioactive material eluting in the void volume of the column.

The unsaturated disaccharides resulting from chondroitinase ABC digestion of Pd-PG II and D-PG were fractionated by high-pressure liquid chromatography. For both fractions, all the radioactivity was recovered in the elution position of the ΔDi4S standard. Similarly, when analysed by t.l.c., the chondroitinase ABC digests of both preparations exhibited only one spot with an R_F value similar to the ΔDi4S standard.

When the chondroitinase ABC digest of fraction Pd-PG I was analysed by high-pressure liquid chromatography, about 20% of the radioactivity was eluted in the position of the ΔDi6S standard, whereas 80% of the labelled material was recovered in the position of the ΔDi4S standard. We therefore conclude that the glycosaminoglycan chains of Pd-PG I consist of chondroitin 4-sulphate and chondroitin 6-sulphate in a ratio of 4:1 (w/w), whereas those of the major proteoglycan fractions, Pd-PG II and D-PG, consist exclusively of chondroitin 4-sulphate.

Gel-filtration chromatography of proteoglycans and glycosaminoglycans

For further characterization, proteoglycans obtained from the DEAE-Sephacel fractionation (Fig. 2) were chromatographed on a column of Sepharose CL-6B under dissociative conditions. From fraction Pd-PG I, about 18% of the labelled material was recovered in the void volume of the Sepharose CL-6B column (Fig. 3*a*), whereas the remainder eluted as a broad peak with $K_{av.}$ ~0.6. Fractions Pd-PG II and D-PG were eluted as a single broad peak with $K_{av.}$ 0.33 (Figs. 3*b* and 3*c*).

In order to demonstrate the proteoglycan nature of these preparations, as well as to estimate the chain size of the polysaccharides in the preparations, the three radiolabelled fractions from Fig. 2 were separately digested with papain and chromatographed on a column of Sepharose CL-6B. The [^{35}S]sulphate-labelled glycosaminoglycan chains of Pd-PG I eluted with $K_{av.}$ 0.44, whereas a value of 0.48 was observed for the [^{35}S]sulphate-labelled glycosaminoglycan chains of Pd-PG II and D-PG respectively. In all three chromatographic runs of the papain digests about 15–20% of the radioactivity was recovered at the total bed

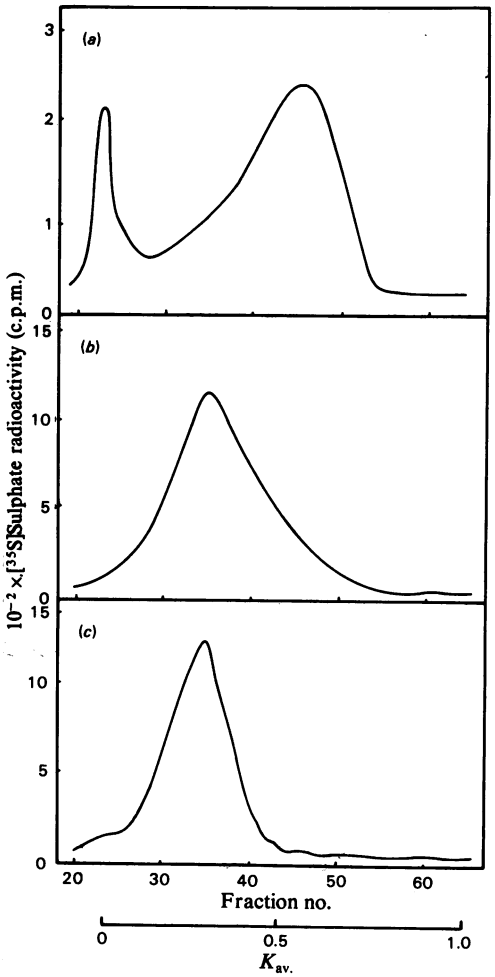

Fig. 3. *Sepharose CL-6B chromatography of (a) Pd-PG I, (b) Pd-PG II and (c) D-PG*

Samples containing 5000–10000c.p.m. of [^{35}S]-sulphate-labelled proteoglycans were dissolved in 0.05M-Tris/HCl, buffer, pH7.5, containing 4M-GdmCl and were applied to a column of Sepharose CL-6B (1cm × 100cm). The column was eluted at 4ml/h at room temperature with 0.05M-Tris/HCl buffer, pH7.4, containing 4M-GdmCl. Fractions (1.5ml) were collected and radioactivity was determined as described in the text.

volume of the column. These observations suggest that a few polysaccharide chains of small size may be present in these proteoglycans. Further characterization of these fractions was not carried out in the present study.

When chondroitin sulphate chains, obtained by papain digestion of bovine nasal cartilage, were chromatographed on the same column of Sepharose CL-6B, a $K_{av.}$ of 0.51 was obtained. Thus the glycosaminoglycan chains of rat incisor proteoglycans are larger than those of bovine nasal cartilage, which have an estimated M_r of 22000 (Wasteson, 1971).

Discussion

In previous experiments we demonstrated that the proteoglycans of rat incisors can be obtained in two pools by using a sequential-extraction procedure in conjunction with extensive precautions to avoid 'artefactual' degradation (Linde *et al.*, 1980; Butler *et al.*, 1981; Rahemtulla *et al.*, 1981). An initial 4M-GdmCl extraction solubilized proteoglycans and other components of the non-mineralized portions of the tooth, whereas macromolecules trapped within the mineralized tissue are removed during subsequent demineralization (Termine *et al.*, 1980). The following lines of evidence support the conclusion that the first extract is from predentine and the second from dentine. Having dissected predentine from non-erupted permanent bovine teeth and extracted this material with 4M-GdmCl, Jontell & Linde (1983) found that the major non-collagenous component of predentine was a proteoglycan. Fisher *et al.* (1983) have shown by indirect immunofluorescence that a proteoglycan extractable with 4M-GdmCl from bovine subperiosteal bone is located in the non-mineralized portion of the tissue, whereas another biochemically and immunologically distinct proteoglycan, extractable only upon demineralization, was localized in mineralized bone trabeculae. Finally, several studies have demonstrated that a variety of non-collagenous proteins (e.g. phosphoproteins, osteocalcin, osteonectin) which are closely associated with bone or tooth mineral, can be extracted from these tissues *only upon dimineralization* (Termine *et al.*, 1980, 1981; Linde *et al.*, 1980, 1983; Butler *et al.*, 1981). In fact, the absence of these components from predentine has been shown (Jontell & Linde, 1983). We therefore conclude that proteoglycans present in the 4M-GdmCl and EDTA extracts of rat incisors were derived from predentine and dentine respectively.

Owing to the small amount of proteoglycans in rat incisors, we have employed an '*in vivo*' radiolabelling technique (Rahemtulla *et al.*, 1981) to facilitate their detection during purification. With this method we have observed a high-M_r proteoglycan (Pd-PG I) extractable from predentine that was eluted in the void volume of Sepharose CL-6B run under dissociative conditions (Fig. 3*a*). In a separate study (Rahemtulla *et al.*, 1981) we observed that the relative proportion of this high-M_r proteoglycan decreased with increasing time of incorporation. The detection of this minor component illustrates the utility of radiolabelling in studying proteoglycans, since it would not have been observed by extraction of unlabelled tissues.

The present study established that rat incisors contain three populations of proteoglycans which differ in their size, chemistry and susceptibility to extraction from the tissue. A minor [^{35}S]sulphate-labelled fraction (Pd-PG I) and a major radiolabelled proteoglycan (Pd-PG II) were obtained in the initial 4M-GdmCl extraction (i.e. from predentine), whereas a third proteoglycan, D-PG, was extracted only after demineralization. The chemical composition of Pd-PG I, which co-eluted from the DEAE-Sephacel column with other non-collagenous proteins, was not established, but it differed from the other two proteoglycans. The glycosaminoglycan chains for Pd-PG I were larger than those of Pd-PG II and D-PG, and they consisted of chondroitin 4-sulphate and chrondroitin 6-sulphate in a ratio of 4:1. These results are consistent with those of previous studies by Engfeldt & Hjerpe, (1972) who showed that the glycosaminoglycans of predentine contained chondroitin 4-sulphate and 6-sulphate isomers. Proteoglycans Pd-PG II and D-PG were similar in chemical composition; additionally the glycosaminoglycan chains released by papain digestion of these two proteoglycans were similar in size and composition. The glycosaminoglycan chains of both fractions were slightly larger than those of bovine nasal cartilage and contained exclusively chondroitin 4-sulphate. These data also agree with the previous findings of chondroitin 4-sulphate in dentine (Engfeldt & Hjerpe, 1972; Linde, 1973; Embery, 1974).

Fisher *et al.* (1983) have isolated and characterized proteoglycans from foetal bone and have shown that the major proteoglycans are relatively small (M_r 80000–120000). They consist of one or two glycosaminoglycan chains of M_r 40000 attached to a core protein of M_r 38000. Antibodies against foetal bone proteoglycans cross-reacted with components of dentine but not with soft tissue macromolecules.

The data we report here suggest that proteoglycans of dentine are also relatively small (M_r 70000–120000) with few glycosaminoglycan chains that are larger than those of cartilage proteoglycans. The size estimates are based on the elution volumes of dentine proteoglycans and glycosaminoglycans chains from Sepharose CL-6B columns compared with that of fraction A_1TA_1 (M_r 126000) which is eluted from the same column as two unresolved peaks: one at the void volume and the other with $K_{av.}$ 0.15.

The amino acid compositions of Pd-PG II and D-PG were similar to that published by Smalley & Embery (1980), but they differed in containing lower aspartic acid and higher glycine values. However, the amino acid data presented here differed sharply from that obtained by Hjerpe & Engfeldt (1976); their analysis showed very high values for aspartic acid and serine, a finding that is possibly due to contamination of their preparation by dentine phosphoproteins (Butler *et al.*, 1981).

We observed that Pd-PG II and D-PG were unable to form complexes with hyaluronic acid (results not shown), in contrast with cartilage proteoglycans, which readily aggregate with hyaluronic acid. It is interesting to note that most of the cysteine residues are found in the hyaluronic acid-binding region of cartilage proteoglycans (Heinegård & Axelsson, 1977) as well as in rat chondrosarcoma proteoglycans (Oegema *et al.*, 1977). Lack of cysteine residues in Pd-PG II and D-PG lend support to the absence of a hyaluronic acid-binding region.

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