The heterogeneity of the non-aggregating proteoglycans of the human intervertebral disc

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Non-aggregating proteoglycans of differing average hydrodynamic volumes were prepared from nuclei pulposi and anuli fibrosi of three human lumbar spines and characterized by biochemical and immunochemical analyses. The hexose-to-hexuronate and protein-to-hexuronate ratios increased with decreasing hydrodynamic volume. Analysis by composite agarose/polyacrylamide-gel electrophoresis has demonstrated two aggregating subpopulations [McDevitt, Jahnke & Green (1982) *Trans. Annu. Meet. Orthop. Res. Soc.* 7, 50]. In the present study, electrophoresis of the non-aggregating pools has shown three additional subpopulations, here named bands III, IV and V. The two smallest proteoglycan pools from each tissue contained two and three components respectively. These components were isolated by preparative electrophoresis and analysed. Band III was a proteoglycan richer in keratan sulphate than in chondroitin sulphate; band IV was a proteoglycan richer in chondroitin sulphate than in keratan sulphate; band V contained only chondroitin sulphate. Unsaturated disaccharides prepared from the chondroitin sulphate of all bands were predominantly 6-sulphated, with only 5–15% 4-sulphated. The molecular masses of the chondroitin sulphate and keratan sulphate did not differ between the bands. The amino acid composition of band III differed from that of band IV. Thus three distinct subpopulations of non-aggregating proteoglycan were demonstrated in the human intervertebral disc.

INTRODUCTION

Early work suggested that the proteoglycan of human intervertebral disc was a single polydisperse molecule containing both aggregating and non-aggregating species (Emes & Pearce, 1975; Pearce & Grimmer, 1976; Adams & Muir, 1976), the aggregate being formed by interaction of the proteoglycan with hyaluronate (Stevens *et al.*, 1979) and associated with a 'link protein' (Tengblad *et al.*, 1984). Three proteoglycan subpopulations were identified subsequently by CAPAGE of preparations of the baboon nucleus pulposus and anulus fibrosus (Stanescu *et al.*, 1980). McDevitt *et al.* (1982) reported similar findings in man and associated two of the components with aggregating proteoglycan and the third with non-aggregating proteoglycan populations. On the basis of this evidence for a single non-aggregating proteoglycan in the human intervertebral disc, a study of the structure of the non-aggregating proteoglycan was undertaken. These studies confirmed the presence of the two aggregating species, but, unexpectedly, identified three non-aggregating proteoglycans of differing electrophoretic mobilities in fractions of differing hydrodynamic volumes. The composition of the three non-aggregating fractions is the focus of this work.

METHODS

Unless stated otherwise, all chemicals were either of analytical-reagent grade or the best commercial grade available. The production and characterization of the monoclonal antibody 1/20/5D4, which recognizes keratan sulphate specifically, has been described elsewhere (Caterston *et al.*, 1983).

Analytical methods

Hexuronate was measured by using the carbazole/borosulphuric acid reaction (Bitter & Muir, 1962) with a sodium glucuronate standard (Corn Products Refining Co., New York, NY, U.S.A.), hexose by the anthrone reaction (Scott & Melvin, 1953) with a galactose standard (A grade; Calbiochem-Behring, San Diego, CA, U.S.A.), and protein by the method of Lowry *et al.* (1951), with bovine serum albumin monomer (Miles Laboratories, Elkhart, IN, U.S.A.) as standard. Hexosamines were assayed after hydrolysis *in vacuo* in 4 M-HCl at 100 °C for 16 h and conversion of the sugars into alditol acetates (Park *et al.*, 1985) using g.l.c. with a Hewlett-Packard 5890 gas chromatograph and a 15 m × 0.252 mm capillary column (DB17-15N). The following temperature gradient program was used:

Abbreviation used: CAPAGE, composite agarose/polyacrylamide-gel electrophoresis.

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180 °C for 2 min, then increasing at 5 °C/min to 220 °C. Amino acids were analysed in the laboratory of Dr. Paul G. Scott, Department of Oral Biology, University of Alberta, Edmonton, AL, Canada.

CAPAGE

Samples containing 10–20 nmol of hexuronate in 20–40 μ l of 7 M-urea containing 0.2 g of Bromophenol Blue/l were examined by electrophoresis in 10 mM-Tris/HCl buffer, pH 7.5, performed by the method of McDevitt & Muir (1971), modified to use vertical gels 3 mm thick on 140 mm $\times$ 160 mm frosted-glass plates (Heinegård *et al.*, 1985). A constant current of 15 mA for 30 min was followed by 50 mA for 3.0–3.5 h. After staining with Toluidine Blue, the gels were dried in air at room temperature on GelBond (FMC Corp., Marine Colloid Division Bioproducts, Rockland, ME, U.S.A.) and scanned at 525 nm with a recording absorptiometer (AutoScanner Flur-Vis; Helena Laboratories Corp., Beaumont, TX, U.S.A.). A chondroitin 4-sulphate standard prepared from bovine nasal cartilage as described by Wasteson (1971) was used as a reference for mobility; its average M_r , calculated from intrinsic viscosity, was 64000 (Wasteson, 1971).

Specimens

Three human lumbar spines, labelled X, Y and Z, were collected within 24 h of death from female donors aged 64, 67 and 72 years respectively, judged to have died from causes unlikely to have affected the spine. The spines were stored at –20 °C tightly wrapped in Saran Wrap (clingfilm) and aluminium foil until dissection, when they were thawed overnight at 4 °C. The L2-L3, L3-L4 and L4-L5 discs were removed in the plane of the cartilaginous end-plate and freed of the surrounding perichondrium. Their gross morphological appearances were grade 2 (Lewin, 1964). The anulus fibrosus and nucleus pulposus were dissected from three discs of each donor and the intervening transitional zone discarded. Separate pools of anulus and nucleus were prepared from each spine.

Preparation of A1 proteoglycan

The A1 proteoglycans from anuli fibrosi and nuclei pulposi were prepared as described previously (Tengblad *et al.*, 1984). Fractions containing proteoglycans of differing average hydrodynamic volumes were pooled as described in Fig. 2; the pools were dialysed against distilled water, freeze-dried and stored at –20 °C.

Preparative CAPAGE

Each proteoglycan pool showing two or more distinct bands by analytical CAPAGE was subjected to preparative-scale CAPAGE as described for the analytical procedure, except that a preparative comb (Bio-Rad) was used. Portions containing 2–4 μ mol of hexuronate were applied to the gel in 650 μ l of 50% (w/v) sucrose. The metachromatic bands were excised and homogenized, then extracted overnight with 10 ml of 4 M-guanidinium chloride containing 0.05 M-sodium acetate buffer, pH 5.8. The residue was removed by centrifugation and the supernatant fluid filtered through a cellulose membrane (5 μ m pore size; type SM; Millipore Corp., Bedford, MA, U.S.A.), dialysed against distilled water and freeze-dried. The dry residue was dissolved in 5 ml of 0.03 M- Na_2SO_4 and centrifuged

30 min at 3500 g (r_{av} 9.3 cm). The supernatant fluid was titrated to flocculation with 0.05% (w/v) cetylpyridinium chloride, the precipitate was washed once with distilled water, dissolved in 1 ml of 80% (v/v) propan-1-ol and, after clarification by centrifugation, the proteoglycans were recovered by the addition of 50 μ l of satd. sodium acetate and 6 ml of 100% ethanol. After being left overnight at 4 °C, the precipitate was washed with 80% (v/v) ethanol and dried *in vacuo* at 40 °C. The dried residues were dissolved in 1 ml of distilled water for analysis.

Radioimmunoassay of keratan sulphate

Samples of proteoglycan containing 9–10 nmol of hexuronate in 500 μ l of 0.1 M-NaCl/0.02 M-sodium orthophosphate buffer (pH 7.4)/0.2% (w/v) NaN_3 (PBS-azide) were analysed for keratan sulphate content by using a competitive binding radioimmunoassay, as shown in Fig. 1 (Caterson *et al.*, 1983).

M_r values for the glycosaminoglycans

Portions of the proteoglycan solution containing 0.14–1.44 μ mol of hexuronate were treated with 1 M- NaBH_4 /0.05 M-NaOH at 45 °C for 48 h (Carlson, 1968); 4 M-acetic acid was added dropwise to pH 5, the reaction mixture was diluted 10-fold with distilled water, applied to a 6 mm $\times$ 80 mm column of DEAE-cellulose (DE-52, Whatman) and eluted with 10 ml of 0.15 M-

Fig. 1. Competitive-binding radioimmunoassay for keratan sulphate proteoglycans from the anulus fibrosus of spine Z

A 1:200000 dilution of monoclonal antibody 1/20/5-D-4 and 50 ng of ^{125}I -labelled core protein of bovine nasal cartilage antigen (20000 c.p.m.) per tube were used. Duplicate 3-fold dilutions of stock solutions of bands III, IV or V were used to generate inhibitions in the range of 25–75%; these values were used to calculate the dilution corresponding to 50% inhibition, which was equated with the weight (18 ng) of skeletal keratan sulphate (KS II) giving 50% inhibition. Because of the weak inhibition shown by band V, the content of keratan sulphate in this solution could be estimated only approximately.

NaCl/4 mM-Tris/HCl buffer, pH 7.2, at 14 ml/h; elution was continued with 10 ml of 1 M-NaCl, then 10 ml of 2 M-NaCl, both in the 4 mM-Tris/HCl buffer, pH 7.2. The 1 M-NaCl eluates, which contained the hexuronate, were pooled, concentrated to 600 μ l by pressure ultrafiltration, applied to a 7 mm $\times$ 1200 mm column of Sephacryl S-200 (Pharmacia) and eluted with 0.2 M-NaCl/50 mM-Tris/HCl buffer, pH 7.0; the eluted fractions were analysed for hexuronate and hexose. The column was calibrated with chondroitin 4-sulphate fractions prepared from bovine nasal cartilage with average M_r values of 8500, 16000 and 30000, calculated from the intrinsic viscosities (Wasteson, 1971).

Chondroitin 4-sulphate/chondroitin 6-sulphate ratio

Portions of the glycosaminoglycans prepared by treatment with alkaline borohydride (see above) and containing 10–100 nmol of hexuronate, were exchanged into 0.1 M-sodium acetate/0.1 M-Tris/HCl, pH 8.0, by centrifugal ultracentrifugation (Centricon-10; Amicon Corp., Danvers, MA, U.S.A.) and treated twice with 20 μ l of chondroitinase ABC (2 units/ml; Miles Laboratories, Elkhart, IN, U.S.A.) at 37 °C. The digests were injected directly into a high-pressure liquid chromatograph (Waters Associates) and eluted from a 4 mm $\times$ 250 mm Lichrosorb RP8 (10 μ m) column with 0.5 M-sodium phosphate/2 mM-hexadecyltrimethylammonium bromide, pH 6.45, at 1.2 ml/min. A_{232} was monitored. Commercially available 4- and 6-sulphated unsaturated disaccharides (Miles Laboratories) were used as standards. The 4- and 6-sulphated species are separated by this procedure; the non-sulphated disaccharide is eluted in the void volume (H. Hughes, unpublished work).

RESULTS

Preparation of proteoglycans of differing hydrodynamic volumes

The A1 proteoglycans of nucleus pulposus from each spine were divided into six pools by Sepharose CL-2B chromatography (Fig. 2) believed to represent: aggre-

Fig. 2. Preparation by gel chromatography of proteoglycans of differing hydrodynamic volumes

A1 proteoglycans from the nucleus pulposus of spine Z were mixed with 20 mmol of hyaluronate hexuronate/mol of A1 hexuronate, equilibrated overnight at 4 °C, then a 15 ml portion containing 50 μ mol of hexuronate was applied to a 50 mm $\times$ 750 mm column of Sepharose CL-2B. The 15 ml fractions collected were analysed for hexuronate. Pool 0 is the excluded fraction and pools 1–5 are included fractions containing approximately equal amounts of hexuronate.

gated proteoglycans (pool 0), a mixture of aggregated and non-aggregated proteoglycans (pool 1), and four pools of non-aggregating proteoglycans (labelled pools 2–5 inclusive) containing equimolar amounts of hexuronate but of decreasing hydrodynamic volume.

The proteoglycan of the anulus fibrosus of spine Z was treated similarly. Because of the high proportion of aggregate in the anulus, the fractions corresponding to pools 2–5 were combined into a single pool that was

Table 1. Composition of proteoglycan pools from Sepharose CL-2B chromatography

Non-aggregating proteoglycans from both nucleus pulposus (NP) and anulus fibrosus (AF) were applied to a preparative column of Sepharose CL-2B, and fractions of differing hydrodynamic volumes were prepared by pooling the eluted fractions. Each such pool was analysed for hexuronate (HexA), hexose (Hex), protein (Prot) and the fractions from spine Y for the chondroitin 4-sulphate/chondroitin 6-sulphate ratio (Ch4S/Ch6S).

Spine	Tissue	Pool no.	K_{av} .	Analysis		Ch4S Ch6S (%)
				Hex/HexA (μ mol/ μ mol)	Prot/HexA (μ g/ μ mol)	
X	NP	2	0.35–0.47	1.00	310	–
		3	0.47–0.58	0.75	205	–
		4	0.58–0.70	0.94	180	–
		5	0.70–1.00	1.10	200	–
Y	NP	2	0.35–0.47	0.87	175	4
		3	0.47–0.58	0.81	170	6
		4	0.58–0.70	1.04	200	11
		5	0.70–1.00	1.10	215	10
Z	AF	2+3	0.35–0.58	1.01	201	–
		4	0.58–0.70	1.08	208	–
		5	0.70–1.00	1.20	251	–

subsequently concentrated by pressure ultrafiltration and re-chromatographed on the Sepharose CL-2B column. The eluate was divided into four pools containing equimolar amounts of hexuronate but of decreasing hydrodynamic volume (results not shown). Fractions corresponding in K_{av} range to nuclear pools 2 and 3 were combined and designated 'pool 2+3' (Table 1).

Each of the pools was analysed for hexuronate, hexose and 'Lowry' protein. With the exception of the pools 2 from the nuclei of spines X and Y, which may have been contaminated with aggregating proteoglycan of small hydrodynamic volume, the contents of hexose relative to hexuronate increased with decreasing hydrodynamic volume (Table 1). This result suggests that the smaller proteoglycans were relatively rich in keratan sulphate. The protein/hexuronate ratio showed a similar trend.

Electrophoretic analysis of the proteoglycan pools

Portions of each proteoglycan pool were analysed by CAPAGE. The following nomenclature was used to describe the several bands observed. Bands I and II corresponded to the two distinct subpopulations of the aggregated proteoglycans demonstrated previously by McDevitt *et al.* (1982) and confirmed in our aggregates (pools 0 and 1; Fig. 3), band I being the least mobile. Bands III, IV and V (of increasing electrophoretic mobility) were seen only in the non-aggregated-proteoglycan pools. Typical densitometric scans of a gel are shown in Fig. 3. Band III was present in pools 3–5 and band IV in pools 4–5. Band V was found only in pool 5. Pool 2 had a mobility intermediate between those of bands II and III and, although not obvious in Fig. 3, usually appeared to be a poorly resolved mixture of the two. The unfractionated mixture of non-aggregating proteoglycans appeared as a single major band with the suggestion of components of greater electrophoretic mobility. The distinct non-aggregating proteoglycans of bands III, IV and V were apparent only after fractionation by gel chromatography. There was a clear difference in the electrophoretic behaviour of the two major non-aggregated subpopulations (bands III and IV). The mobility of band III did not change with hydrodynamic size; that of band IV increased with decreasing size.

Preparation and characterization of the bands

The proteoglycan comprising each of the bands from pools 4 and 5 from the nuclei of spines X and Y and from the anulus of spine Z was isolated by preparative-scale CAPAGE. Hexosamine analysis of such bands from human costal cartilage had been reported by Pearson & Mason (1978). After examination for homogeneity by analytical CAPAGE, the products from several runs were combined to provide sufficient material for biochemical analysis and immunoassay. Typical analytical CAPAGE results for the bands are shown in Fig. 4. Materials with mobilities characteristic of bands III, IV and V were obtained minimally contaminated with other bands. The recovery of the hexuronate applied to the gel ranged from 60 to 80%. A major proportion of the hexuronate loss was attributable to the discarding of both the overlap region between the bands and the poorly resolved regions frequently encountered in the gel near the edges of the plate. The apparent recovery of hexose was higher than that of hexuronate, often

Fig. 3. Densitometric scans of CAPAGE of the proteoglycan pools

The pools from nucleus pulposus of spine Y are shown. The bands were numbered I to V as described in the text. Mobilities were calculated relative to that of the chondroitin 4-sulphate standard ($M_r \approx 64000$).

exceeding 100%; such high values were attributed to the contamination of the subfractions with agarose.

The mobility of band III relative to a chondroitin 4-sulphate standard was consistently between 0.72 and 0.77 for six preparations; that of band V was close to that of the chondroitin sulphate for three preparations (Table 2). However, the mobility of band IV from the smaller proteoglycan preparation was greater than that from the larger subfraction for each of the three spines (Table 2). Contamination of the proteoglycan preparations with agarose made it impossible to calculate their composition from hexuronate and hexose analyses. The relative proportions of keratan sulphate and chondroitin sulphate were assessed by measurement of the glucosamine/galactosamine ratios after g.l.c. of the alditol acetates (Table 2). The ratio of glucosamine to galactosamine for band III was higher than that of the other bands for all six preparations, exceeding a value of 3.0 for all except band III of pool 4 from spine X. If the latter is

Fig. 4. Densitometric scans of a CAPAGE gel after electrophoresis of bands derived from nucleus pulposus of spine Y

Portions of pools 4 and 5 (Fig. 1) were subjected to preparative CAPAGE. Samples from the bands recovered from the gel were examined by analytical CAPAGE. Mobilities were calculated relative to that of the chondroitin 4-sulphate standard ($M_r \approx 64000$).

ignored, the average galactosamine ($\pm$ s.d.) of band II was $20.4 \pm 2.5\%$ of the hexosamine and is higher for the annulus than the nucleus. By contrast, the average galactosamine ($\pm$ s.d.) of band IV was $79.0 \pm 10.6\%$ of the hexosamine. For each of three preparations, band V contained only galactosamine. As a control, the glucosamine/galactosamine ratios were determined for mixtures of purified intervertebral-disc keratan sulphate and chondroitin 6-sulphate. A mixture with a hexose/hexuronate ratio of 1.02 showed a glucosamine/galactosamine ratio of 0.69. Thus these two methods of estimating keratan sulphate/chondroitin sulphate ratio were clearly not equivalent. Nonetheless, the analyses indicated that band III was a proteoglycan richer in keratan sulphate than in chondroitin sulphate, band IV

was a proteoglycan richer in chondroitin sulphate than keratan sulphate, and band V contained chondroitin sulphate alone.

The low values for the glucosamine/galactosamine ratio relative to the hexose/hexuronate ratio suggested that the keratan sulphate/chondroitin sulphate ratio of the bands should be assayed by an independent method. Also, the presence of glucosamine and hexose in the samples might be attributable to the occurrence of both *N*- and *O*-linked oligosaccharides on the core protein. Thus an immunoassay procedure was used to measure the amount of keratan sulphate in bands III, IV and V of the non-aggregating proteoglycans. Typical results are shown in Fig. 1. Analyses obtained from all three spines are given in Table 2. Together these data indicate that the relative keratan sulphate contents of bands III, IV and V decreased progressively, confirming the trends seen on chemical analysis. In general, the proportion of keratan sulphate relative to hexuronate was high in relation to their glucosamine/galactosamine ratio.

Protein and chondroitin 4- and 6-sulphate contents of bands

The protein contents of the subfractions from spine Y were calculated from the amino acid analyses by summing the weights of the residues and correcting for the water lost with the formation of each peptide bond (Table 3). For both pools 4 and 5, the protein contents of band III were higher than those of band IV (Table 3).

The chondroitin 4-sulphate/chondroitin 6-sulphate ratio was determined for several preparations. The values for proteoglycan pools from spine Y ranged from 4 to 11% chondroitin 4-sulphate, with a suggestion of higher values for the proteoglycans of smaller hydrodynamic volume (Table 1). The relative chondroitin 4-sulphate contents of two different preparations of band IV and one of band V ranged from 11 to 14% (Table 3). The sulphate substitution pattern of the chondroitin sulphate isomers of band IV does not appear to differ from that of band V.

M_r values for the glycosaminoglycans

The glycosaminoglycans of the bands of non-aggregating proteoglycan were freed from the peptide core by treatment with alkaline borohydride and purified by ion-exchange chromatography. The sizes of the glycosaminoglycans were determined on a calibrated gel-chromatographic column, with analysis of the fractions for both hexuronate and hexose. Separation of the glycosaminoglycans was not necessary, because the cross-reactivity of the two manual absorptiometric procedures was negligible. The apparent average M_r values for the chondroitin sulphates ranged from 15700 to 17300 and those of the keratan sulphates from 7200 to 7900 (Table 3). The differing partition coefficients and electrophoretic mobilities of the proteoglycan fractions could not be explained by the minor differences in the M_r values for the glycosaminoglycan components.

Amino acid analyses

Bands III and IV from pools 4 and 5 were analysed for their amino acid contents (Table 4). Analysis of the two preparations of band III were similar, as were those of band IV. However, the differences between band III and band IV were striking, particularly with respect to the

Table 2. Properties of proteoglycan bands prepared by CAPAGE

Proteoglycan pools were separated by preparative CAPAGE and bands III, IV and V (if present) were eluted from the gel with 4 M-guanidinium chloride. The proteoglycan bands were recovered from the eluate as their cetylpyridinium salts. The electrophoretic mobility was expressed relative to that of a chondroitin 4-sulphate standard ($M_r \approx 64000$). Each band was analysed for its glucosamine/galactosamine ratio (GlcN/GalN) and keratan sulphate/hexuronate ratio (KS/HexA). The latter was expressed as a molar ratio calculated from the theoretical hexose (Hex) content of keratan sulphate.

Spine	Tissue	Pool no.	Band no.	Relative mobility	GlcN/GalN ($\mu\text{mol}/\mu\text{mol}$)	KS/HexA (μmol of Hex/ μmol)
X	Nucleus pulposus	4	II	0.75	1.38	3.63
			IV	0.82	0.10	1.64
		5	III	0.77	3.42	2.85
			IV	0.89	0.15	1.15
			V	1.02	0	0.16
Y	Nucleus pulposus	4	III	0.75	4.00	2.82
			IV	0.87	0.19	0.42
		5	III	0.72	3.35	3.18
			IV	0.89	0.30	1.08
			V	1.02	0	0.00
Z	Annulus fibrosus	4	III	0.75	4.50	3.59
			IV	0.84	0.60	1.73
		5	III	0.74	4.59	6.00
			IV	0.92	0.36	0.55
			V	1.03	0	0.01

Table 3. Analyses of proteoglycans bands from spine Y

Subfractions prepared by CAPAGE were analysed for amino acids; the protein content was calculated from the amino acid analysis by summing the weights of the residues and subtracting 1 mol of water for each peptide bond formed. The amino acid analyses were done on a fraction of known hexuronate content. The protein concentration relative to glycosaminoglycan, expressed as $\mu\text{g}/\mu\text{mol}$ of [Hex (hexose)+HexA (hexuronate)], was calculated by using the hexuronate analysis and the ratio KS/HexA from Table 2. The chondroitin 4-sulphate/chondroitin 6-sulphate ratio was used to calculate the percentage of chondroitin 4-sulphate (ChS). The average M_r values for the chondroitin sulphate (ChS) and keratan sulphate (KS) were determined by chromatography on a calibrated column of Sephacryl S-200 and analysis for hexuronate and hexose respectively.

Pool no.	Band no.	Protein [$\mu\text{g}/\mu\text{mol}$ of (Hex + HexA)]	Ch4S	$10^{-3} \times M_r$	
			Ch6S (%)	ChS	KS
4	III	37	—	—	—
	IV	26	12	15.7	7.2
5	III	47	—	16.5	7.9
	IV	23	14	15.7	7.2
	V	1	11	17.3	—

proportions of threonine, serine, proline, glycine, leucine, lysine and arginine.

DISCUSSION

As recognized by Stevens *et al.* (1979), the ability to model the structure of disc proteoglycans is limited by the extreme heterogeneity of the molecules. The presence of at least two principal components, band III and band IV, in the non-aggregated proteoglycans of human inter-

vertebral disc emphasizes this need for caution. The preliminary report of two proteoglycan subpopulations in the aggregating proteoglycan population (McDevitt *et al.*, 1982) is further evidence of heterogeneity. The development of meaningful models for disc proteoglycan will require the isolation, analysis and characterization of homogeneous species. One necessary criterion for homogeneity is the finding of a single band on CAPAGE,

Table 4. Amino acid analysis of proteoglycan bands from nucleus pulposus of spine Y

The proteoglycan samples are those in Table 3. Data for the non-aggregating proteoglycan fraction of Stevens *et al.* (1979) are included for comparison. Abbreviation: n.d., not determined.

Amino acid	Residues/1000 residues in band:				Stevens <i>et al.</i> (1979)
	III of pool:		IV of pool:		
	4	5	4	5	
Asp	71	78	85	86	64
Thr	111	108	66	72	91
Ser	90	88	110	112	98
Glu	136	144	133	136	135
Pro	123	114	83	89	124
Gly	91	85	137	129	113
Ala	74	71	67	62	76
Cys	14	n.d.	n.d.	41	—
Val	61	61	71	69	75
Met	15	n.d.	n.d.	12	—
Ile	30	31	38	36	36
Leu	68	66	100	96	83
Tyr	22	25	16	17	16
Phe	31	34	27	28	27
His	11	13	10	11	11
Lys	42	37	28	27	26
Arg	39	45	27	29	29

since this technique has proven to be a very sensitive test of proteoglycan heterogeneity.

In the present study, preparative-scale CAPAGE permitted the isolation of sufficient proteoglycan from each electrophoretic band to allow a limited biochemical characterization. The major glycosaminoglycan present in the proteoglycans of band III was keratan sulphate, with much smaller amounts of chondroitin sulphate. On the other hand, the proteoglycan of band IV contained predominantly chondroitin sulphate, with much smaller amounts of keratan sulphate. The composition of the core protein differed between band III and band IV. Band V proteoglycan contained chondroitin sulphate alone.

Keratan sulphate/chondroitin sulphate ratios were measured by three methods, each giving different results. No clear explanation can be offered for the difference between the hexose/hexuronate and the glucosamine/galactosamine ratios. The epitope for the antibody to keratan sulphate is a polysulphated poly(*N*-acetylactosamine) sequence at least seven monosaccharides in length (Mehmet *et al.*, 1986). Any difference in epitope content between the reference keratan sulphate and the isolated bands would affect the apparent keratan sulphate/hexuronate ratio.

Because of its low content of protein, band V differed from the small chondroitin sulphate-containing proteoglycan of other cartilages (Heinegård *et al.*, 1981). In addition, no differences were suggested between nucleus pulposus and annulus fibrosus except for the higher keratan sulphate contents of band III from the annulus. Analysis of the unsaturated disaccharides produced by the action of chondroitinase ABC demonstrated that 4–14% of the chondroitin sulphate disaccharides were 4-substituted, the remainder being 6-substituted. This procedure does not distinguish between, on the one hand, two glycosaminoglycan chains, one made up completely of 4-substituted disaccharides, the other completely by 6-substituted disaccharides and, on the other hand, a single chain with a mixture of 4- and 6-substituted disaccharides. With chondroitinase ABC, iduronate-containing disaccharides yield the same product as hexuronate-containing species, so that dermatan sulphate cannot be excluded. Thus the data indicate no more than a mixture of 6- and 4-substituted galactosaminoglycuronans.

The amino acid analyses of individual proteoglycan bands correlated with the glycosaminoglycan components of the subfractions. In bovine nasal cartilage, serine, glycine and glutamic acid were the predominant amino acids of a chondroitin sulphate-enriched fragment, and glutamic acid, proline and serine the predominant amino acids in a keratan sulphate-enriched fragment (Heinegård & Axelsson, 1977). Keratan sulphate can be linked to protein through either threonine or serine (Bray *et al.*, 1967), whereas chondroitin sulphate is linked through serine alone (Rodén, 1980). In our preparations, the keratan sulphate-rich band III contained more threonine and proline than band IV, whereas chondroitin sulphate-rich band IV contained more serine and glycine than did band III.

The possible relationship of the three non-aggregating species to the two aggregating proteoglycans is interesting. If these three fractions were degradation products

of an aggregating proteoglycan of structure similar to the model proposed by Stevens *et al.* (1979), band III might be regarded as a fragment arising from a region of the molecule near the hyaluronate-binding region, which is rich in keratan sulphate; band IV from a region of the molecule remote from the hyaluronate-binding region, which is rich in chondroitin sulphate; and band V from a short length of core protein, also from the chondroitin sulphate-rich portion of the molecule. However, the existence of two distinct precursor molecules (McDevitt *et al.*, 1982) argues against this simplistic interpretation of the data.

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