

Studies on the Polydispersity and Heterogeneity of Cartilage Proteoglycans

IDENTIFICATION OF THREE PROTEOGLYCAN STRUCTURES IN BOVINE NASAL CARTILAGE

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1. Three chondroitin sulphate components were isolated from adult bovine nasal cartilage after treatment with alkaline NaB^3H_4 . Average molecular weights of 13000, 18600 and 28000 were obtained for chondroitin sulphate species representing 10, 52 and 38% (w/w) of the total chondroitin sulphate respectively. Each chondroitin sulphate pool has a narrow molecular-weight distribution. 2. A proteoglycan subunit preparation, isolated from one nasal cartilage by extraction and density-gradient fractionation in dissociative solvents, partitioned on a CsCl density gradient according to size and composition. Variation of proteoglycan molecular weight across the gradient was directly related to the average chondroitin sulphate chain length, which in turn reflected the relative proportion of the three chondroitin sulphate pools in each proteoglycan fraction. Consideration of proteoglycan molecular parameters, compositions and behaviour on sedimentation leads to a proposal that nasal cartilage contains three distinct proteoglycan pools, each of which has a constant number of chondroitin sulphate side chains of different average molecular weight. 3. Molecular-weight distribution parameters for these proteoglycan preparations indicate that all serine residues on the protein core capable of initiating chondroitin sulphate biosynthesis are occupied and that proteoglycan polydispersity results directly from the polydispersity of the attached chondroitin sulphate component.

Proteoglycans, major extracellular components of cartilage, consist mainly of chondroitin and keratan sulphate chains glycosidically linked to serine and threonine residues on a protein core. There have been many reports concerned with the molecular size, composition and shape of these proteoglycans and there is considerable evidence that proteoglycans are heterogeneous and polydisperse (Mathews & Lozaityte, 1958; Malawista & Schubert, 1958; Marler & Davidson, 1965; Muir & Jacobs, 1967; Luscombe & Phelps, 1967*a,b*; Eyring & Yang, 1968; Dunstone & Franek, 1969; Sajdera & Hascall, 1969; Hascall & Sajdera, 1969, 1970; Rosenberg *et al.*, 1970; Mashburn & Hoffman, 1971; Woodward *et al.*, 1972).

Sajdera & Hascall (1969) and Hascall & Sajdera (1969) introduced a new isolation procedure by which proteoglycans, extracted from cartilage with high-ionic-strength salt solutions, are fractionated on CsCl density gradients in the presence of guanidinium chloride to yield proteoglycan subunit structures. The same authors demonstrated that this subunit interacts with a specific glycoprotein

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to promote its aggregation to more complex proteoglycan structures. They also suggested that much of the size and compositional variation previously found in proteoglycan preparations reflected varying degrees of proteoglycan aggregation.

Hascall & Sajdera (1970) reported that the polydispersity and heterogeneity of the subunit structure stems from a variation in the number of chondroitin sulphate chains attached to the protein core. It has been reported that chondroitin chains, isolated from bovine nasal cartilage, exhibited a bimodal chain-size distribution (Hopwood & Robinson, 1973). This finding suggests that part of the polydispersity and heterogeneity of nasal cartilage proteoglycan may result from the presence of two different chondroitin sulphate species. It seems important to establish the cause of polydispersity in the proteoglycan subunit structure, since such findings may provide insight into the mechanism of proteoglycan biosynthesis.

This present study suggests that much of the polydispersity and heterogeneity of nasal-cartilage proteoglycan may result from the presence of at least three difference proteoglycan species, each with narrow molecular-size distributions, rather than a continuous polydisperse series of structures.

Materials and Methods

Materials

Cartilage powder. Whole intervertebral discs and nasal cartilage from ten adult steers were collected, pooled and milled as described previously (Robinson & Hopwood, 1973). The nasal cartilage from a single adult steer was milled as described before to yield 20 g wet wt. of powdered cartilage.

Alkaline stock solution of NaB³H₄. Two separate stock solutions were prepared from different batches of NaB³H₄ from The Radiochemical Centre, Amersham, Bucks., U.K. The first stock solution contained 100 mCi of NaB³H₄ (502 mCi/mmol) and 1.84 g of NaBH₄ in 250 ml of 0.5 M-KOH; the second batch contained 100 mCi of NaB³H₄ (800 mCi/mmol) and 0.92 g of NaBH₄ in 125 ml of 0.5 M-KOH. Both stock solutions were prepared at 4°C and stored at -20°C. The specific radioactivities of the first and second stock solutions, assayed as described by Robinson & Hopwood (1973), were 6.65×10^8 d.p.m./mmol and 1.76×10^9 d.p.m./mmol respectively.

Other materials. Guanidinium chloride was purified by recrystallization from ethanol-benzene (2:1, v/v). Caesium formate monohydrate was prepared by acidification of an aqueous solution of caesium carbonate (BDH, Poole, Dorset, U.K.) with formic acid; water was removed by azeotropic distillation with 98% (v/v) ethanol. The solid product was dried for 5 h at 50°C *in vacuo*. Collagenase from *Clostridium histolyticum* (purified grade) was purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Chondroitin sulphate ABC lyase (EC 4.2.2.4) was prepared, and gel and ion-exchange materials were purchased and used, as described previously (Robinson & Hopwood, 1973; Hopwood & Robinson, 1973). All other reagents were of analytical grade.

Methods

Isolation of chondroitin sulphate from the nasal cartilage of one steer. (a) Cartilage extraction with KOH in NaB³H₄. Milled cartilage (3 g wet wt.) from a single steer was stirred with a mixture of 22.5 ml of 0.5 M-KOH and 2.5 ml of 0.2 M-NaB³H₄ stock solution (1.76×10^9 d.p.m./mmol) at 4°C for 10 days. The reaction mixture was acidified to pH 5.0 with acetic acid and the cartilage residue removed by centrifugation and washed with 25 ml of water. The combined supernatants (extract plus washings) were desalted on a column (28 cm × 5 cm diam.) of Bio-Gel P-4 and the combined polysaccharide fraction eluted at the void volume of the column was applied to a column (36 cm × 2 cm diam.) of ECTEOLA-cellulose (Cl⁻ form), which was eluted with a linear gradient of NaCl as described by Robinson & Hopwood (1973). The flow rate was 42 ml/h, and 12 ml fractions were collected and

assayed for hexuronic acid content and radioactivity.

(b) Alkaline cleavage of LiBr-solubilized proteoglycan. Milled cartilage (10 g wet wt.) from a single steer was extracted for 4.5 h at 4°C with 100 ml of 5.3 M-LiBr containing imidazole-HCl buffer, pH 7.2 (0.02 M in Cl⁻). The residue was removed by centrifugation and the extraction and centrifugation were repeated. The supernatants from both extractions were dialysed against water at 4°C and freeze-dried (yield: first extract 0.93 g, second extract 0.05 g). Proteoglycan (300 mg) from the first extract was stirred at 4°C for 11 days with 22.5 ml of 0.5 M-KOH and 2.5 ml of 0.2 M-NaB³H₄ stock solution (1.76×10^9 d.p.m./mmol). The reaction mixture was acidified with acetic acid and fractionated by the methods described above.

(c) Alkaline cleavage of the LiBr-insoluble residue. The residue remaining after LiBr extraction of powdered cartilage was stirred in a mixture of 22.5 ml of 0.5 M-KOH and 2.5 ml of 0.2 M-NaB³H₄ stock solution (1.76×10^9 d.p.m./mmol) at 4°C for 12 days. After acidification the reaction mixture was fractionated by the method described above.

Isolation of proteoglycan and chondroitin sulphate from pooled nasal cartilage and pooled intervertebral-disc tissue from ten steers. Proteoglycan was extracted with 5 M-LiBr and purified by sedimentation in a LiBr solution at a density of 1.51 g/ml (Robinson & Hopwood, 1973). Chondroitin sulphate was extracted from 3.0 g (dry wt.) of milled nasal cartilage with 45 ml of 0.5 M-KOH and 5 ml of stock borohydride solution (6.65×10^8 d.p.m./mmol) for 6 days at 4°C, and after acidification the reaction mixture was desalted and fractionated on ECTEOLA-cellulose by the method described above.

Fractionation of proteoglycan from adult bovine nasal cartilage. Proteoglycan from milled nasal cartilage from a single steer was extracted and purified by centrifugation in LiBr solution of density 1.52 g/ml as described previously (Robinson & Hopwood, 1973). A sample of this preparation (300 mg) was centrifuged at 50000 rev./min at 4°C for 36 h (8 × 25 ml titanium rotor, MSE Super-Speed 65 centrifuge) in 160 ml of 4 M-guanidinium chloride containing CsCl to give a density of 1.50 g/ml and 25 mM-imidazole-HCl buffer, pH 6.0. The tube contents were divided into two equal fractions and the bottom fraction was mixed with an equal volume of 4.0 M-guanidinium chloride in CsCl ($\rho = 1.5$ g/ml) and re-centrifuged at 50000 rev./min for 36 h (8 × 25 ml titanium rotor, MSE Super-Speed 65 centrifuge). The tube contents were divided once again into equal fractions and the bottom fractions from all tubes were combined and dialysed against water at 4°C until the diffusate was free of Cl⁻ (tested with AgNO₃ solution), and then against 0.02 M-imidazole-HCl buffer, pH 7.2 (2 litres), at 4°C for 24 h. A sample of this proteoglycan solution

(Preparation A), which contained 120mg of dry material in 30ml, was concentrated to 15ml by ultrafiltration (McKenzie & Murphy, 1970) at 4°C and dialysed at 4°C against two changes (each 75 ml) of 4M-guanidinium chloride–0.02M-imidazole–HCl buffer, pH6.0. The proteoglycan solution was removed from the dialysis sac and adjusted to 1.62g/ml by the addition of CsCl (0.83g of CsCl/g of solution). The solution was then centrifuged at 26000rev./min for 66h at 20°C (3×5ml Spinco SW 50.1 rotor). The tube contents were divided into 20 fractions, each 0.25ml (Flamm *et al.*, 1969). Density was determined with a 200 μ l constriction pipette as pycnometer. Each fraction was assayed for hexuronic acid content by the method of Bitter & Muir (1962). Fractions 1–3, 4–7, 8–12 and 13–20 were pooled and designated CsCl fractions 1, 2, 3 and 4 respectively, and each pooled fraction was dialysed against water at 4°C until the diffuseate was free of Cl⁻. Samples from CsCl fractions 1, 2, 3 and 4 were used for sedimentation-equilibrium molecular-weight estimations, measurements of amino acid and hexosamine content and alkaline-NaB³H₄-cleavage experiments.

Approximately half of each CsCl fraction was freeze-dried after dialysis and dissolved by gentle stirring at 4°C in 0.5ml of 0.2M-NaB³H₄ stock solution (1.76×10⁹d.p.m./mmol) and 4.5ml of 0.5M-KOH. Each reaction mixture was acidified after 8 days by the addition of acetic acid (0.2ml) and desalted on a column of Bio-Gel P-4. All of the hexuronate-positive material was eluted close to the void volume of the column and these fractions were combined and applied to a column of ECTEOLA-cellulose and eluted with a linear NaCl gradient as previously described.

Centrifugation of proteoglycan in high-density caesium formate solutions. A second sample of the proteoglycan solution (Preparation A; 60mg in 15 ml) was concentrated to approx. 2ml by ultrafiltration at 4°C (McKenzie & Murphy, 1970). The density was adjusted to 2.20g/ml by the addition of caesium formate monohydrate and 0.5ml was then carefully pipetted into the bottom of a 5ml centrifuge tube. A discontinuous gradient of caesium formate was formed by manual addition of nine portions of caesium formate solution (0.5ml) with densities ranging from 2.15g/ml to 1.75g/ml. In another tube a similar caesium formate gradient was formed except that the proteoglycan sample was applied at the top of a similar gradient in 0.5ml of caesium formate solution of density 1.75g/ml. The tubes were centrifuged at 37000rev./min at 22.5°C (3×5ml Spinco SW 50.1 rotor). The tube contents were fractionated and the density and hexuronic acid content of each fraction determined as described above.

Isolation of proteoglycan from cartilage residue after extraction with LiBr solution. The cartilage

residue (0.6g dry wt.) after exhaustive extraction of powdered nasal cartilage with 5.3M-LiBr was stirred at 37°C for 70h in 50ml of 0.1M-Tris-acetate buffer pH7.4 (0.1M in acetate), containing 0.01M-CaCl₂ and two drops of toluene. Collagenase (1.0mg) was added at the beginning and at approx. 24h intervals during the digestion. The digest was centrifuged at 38000g (rotor 8×50ml; MSE High-Speed 18 centrifuge) to remove a trace of insoluble residue. As a control, 6mg of soluble proteoglycan was incubated with collagenase (1 mg) in 7ml of the Tris-acetate buffer described above for 70h at 37°C. Both collagenase digestions were kindly performed by Dr. W. H. Murphy (Monash University, Department of Biochemistry). Each digest was dialysed against 2×2litres of 0.02M-imidazole–HCl buffer, pH7.2, at 4°C for 40h and then adjusted to a density of 1.65g/ml by the addition of CsCl. These solutions were centrifuged at 120000g for 40h at 4°C (rotor 50-Ti, Beckman Spinco preparative ultracentrifuge). The tube contents were divided into three equal fractions and the corresponding fractions from each tube pooled. Each pooled fraction was dialysed against water at 4°C until the diffuseate was chloride-free and then dialysed against 0.02M-imidazole–HCl buffer, pH7.2, and assayed for hexuronic acid content. More than 90% of the total hexuronate-positive material in the collagenase digests was present in the bottom fractions.

Analytical methods. The cysteine content of proteoglycans was measured by an adaptation of the procedure of Glazer & Smith (1961, 1965). Proteoglycan (approx. 3mg) was dissolved in 10ml of 9.6M-HCl containing 6mg of di-Dnp-cystine and was incubated at 39°C. Samples (1.0ml) were removed and extracted with diethyl ether; the aqueous layer was then diluted to 10ml and its absorption determined at 357nm. Reaction was complete after 200h.

Hydroxyproline was determined by the method of Stegemann & Stalder (1967) by using proteoglycan samples that had been hydrolysed with 6M-HCl (Robinson & Hopwood, 1973).

Hexuronate, hexosamine, protein, hexose and amino acids were determined by methods described previously (Robinson & Hopwood, 1973). Hexuronate was also determined by the method of Bitter & Muir (1962). Sedimentation studies were performed as described by Robinson & Hopwood (1973). Values of 0.52cm³/g and 0.54cm³/g were assumed for the partial specific volume of sulphated glycosaminoglycans and proteoglycan respectively.

End-group estimation of chondroitin sulphate molecular weight and molecular-weight distributions used procedures described by Robinson & Hopwood (1973) and Hopwood & Robinson (1973) respectively.

Paper chromatography was performed on What-

man 3MM paper with descending development in isobutyric acid-aq. 2.0M-NH₃ (5:3, v/v).

Results

Chondroitin sulphate polydispersity

A sample of milled nasal cartilage from a single steer and a sample of milled nasal cartilage pooled from 10 adult steers were treated separately with alkaline NaB³H₄ at 4°C as described under 'Methods'. The reaction mixtures were fractionated by gel chromatography on Bio-Gel P-4 and by ion-exchange chromatography on ECTEOA-cellulose. The elution profiles from ECTEOA-cellulose of a reaction mixture containing milled nasal cartilage from a single steer and of a reaction mixture containing milled nasal cartilage pooled from 10 steers are shown in Figs. 1(a) and 1(b) respectively. The elution profile in Fig. 1(b) shows a single broad peak of labelled chondroitin sulphate (Robinson & Hopwood, 1973), but in Fig. 1(a) the polysaccharide was eluted as three sharp peaks of hexuronate and of ³H label. Material from the peak tube (fraction 39, Fig. 1a) applied once again to ECTEOA-cellulose was eluted by an identical gradient as a single peak of radioactivity. This result supports the idea that the three peaks in Fig. 1(a) represent separate species of chondroitin sulphate and are not due to an experimental artifact arising from chromatography on ECTEOA-cellulose.

The three radioactive components in Fig. 1(a) were collected separately as follows: fractions 35-37 were combined and labelled H-1, fractions 38-41 were combined and labelled H-2 and fractions 42-44 were combined and labelled H-3. Each sample was then characterized as greater than 90% chondroitin sulphate by digestion with chondroitin sulphate lyase and identification of the disaccharides produced by paper chromatography (Robinson & Dorfman, 1969).

The resolution of chondroitin sulphate in ECTEOA-cellulose was only observed with tissue isolated from a single animal. We conclude that the separation is masked with samples derived from pooled tissue owing to a broadening of the peaks as a result of variation from one animal to the next.

Approx. 80% of the total proteoglycan present in the nasal cartilage of one steer could be isolated by extraction with LiBr solution. This proteoglycan fraction and the cartilage residue remaining after LiBr extraction were incubated separately with alkaline NaB³H₄ to label the chondroitin sulphate (Robinson & Hopwood, 1973). Three distinct hexuronate-positive peaks similar to those shown in Fig. 1(a) resulted when chondroitin sulphate from either reaction was chromatographed over ECTEOA-cellulose. Molecular-weight values of 13000, 18500 and 28000 were obtained by end-group estimation

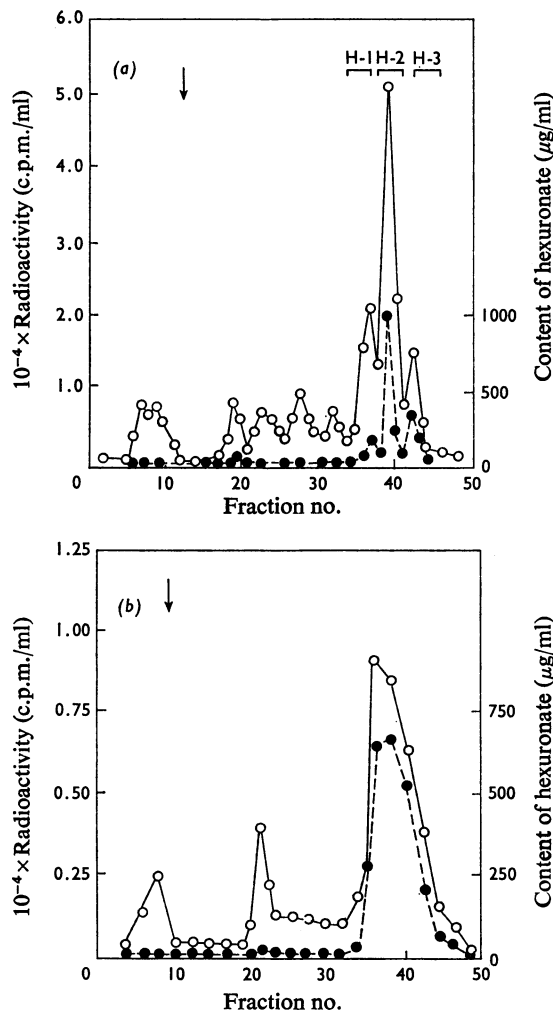


Fig. 1. Elution from ECTEOA-cellulose of the products obtained by treating proteoglycan with 0.02M-NaB³H₄ in 0.5M-KOH

Proteoglycan, isolated from (a) one nasal cartilage and (b) a pool of nasal cartilage derived from 10 animals, was treated with alkaline NaB³H₄ and the products excluded from Bio-Gel P-4 were eluted from ECTEOA-cellulose with a linear gradient of NaCl as outlined under 'Methods'. Fractions were volume 12 ml; hexuronate (●) and radioactivity (○) are shown. Arrow indicates start of salt gradient.

of the chondroitin sulphate component in fractions H-1, H-2 and H-3 from both the LiBr-soluble and -insoluble fractions, compared with a value of 21 500 for the total alkali-soluble chondroitin sulphate (Table 1). The average molecular weights (M_n , M_w and M_z) for the chondroitin sulphate component in fractions H-1, H-2 and H-3, determined by chromato-

Table 1. Molecular-weight data for the major peaks of hexuronate-positive material isolated from nasal cartilage by chromatography on ECTEOLA-cellulose

The hexuronate content of fractions H-1, H-2 and H-3, isolated from LiBr-soluble and -insoluble fractions from a single nasal cartilage (Fig. 1), are given as a percentage of the total hexuronate extracted with alkali from the same tissue. The values in parentheses are derived values adjusted for overlap between the three peaks which was estimated from the profile of molecular weights obtained by fractionation of each ECTEOLA-cellulose peak, on Sephadex G-200 (Hopwood & Robinson, 1973). $M_n(A)$, number-average molecular weights, by end-group determination (Robinson & Hopwood, 1973); $M_n(B)$, number-average, $M_w(B)$, weight-average, and $M_z(B)$, z-average molecular weight by Sephadex G-200 fractionation (Hopwood & Robinson, 1973). N.D., Not determined.

ECTEOLA fraction % of total hexuronate	LiBr-insoluble fraction			LiBr-soluble fraction			Alkali-soluble
	H-1 4.3 (2)2	H-2 9.9 (9.7)	H-3 5.8 (8.1)	H-1 16.0 (8.0)	H-2 43.4 (42.0)	H-3 20.6 (30.0)	H-1+H-2+H-3 100
Molecular weight							
$10^{-3} \times M_n(A)$	12.6	18.5	29.0	13.2	18.6	28.0	21.5
$10^{-3} \times M_n(B)$	N.D.	18.7	29.6	12.5	18.5	28.5	21.1
$10^{-3} \times M_w(B)$	N.D.	19.3	30.3	13.6	19.2	29.7	22.6
$10^{-3} \times M_z(B)$	N.D.	20.5	31.5	14.5	20.1	30.1	23.4
$M_w(B)/M_n(B)$	—	1.03	1.02	1.09	1.03	1.04	1.07

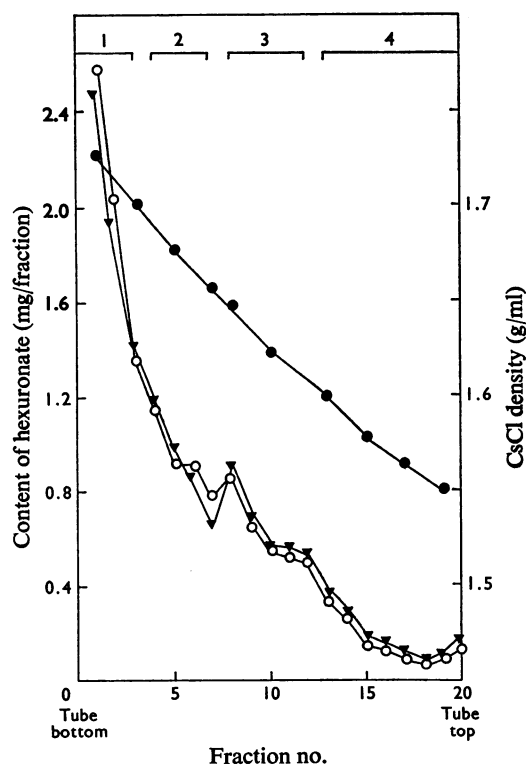


Fig. 2. Partial fractionation by density-gradient centrifugation in a CsCl solution of the proteoglycan from the nasal cartilage of one steer

Proteoglycan (120mg) was centrifuged in CsCl-guanidinium chloride solution with a density of 1.62g/ml as described under 'Methods'. The hexuronate content (▼ and ○) of each fraction from two separate centrifuge tubes are shown. The density of the CsCl solution across the gradient at the completion of the centrifugation is shown (●). CsCl fractions 1-4 are indicated.

graphy on Sephadex G-200 (Hopwood & Robinson, 1973), are also recorded in Table 1.

The content of chondroitin sulphate in fractions H-1, H-2 and H-3 is expressed as a percentage of the total chondroitin sulphate solubilized from milled cartilage after treatment with dilute alkali (Table 1). The weight ratio of the three chondroitin sulphate species was the same in both the LiBr-soluble and -insoluble fractions. This was 1.0:5.2:3.8 for the chondroitin sulphate fractions with molecular weights of 13000, 18500 and 28000 respectively.

Fractionation of proteoglycan in caesium chloride solution

Proteoglycan isolated from a single nasal septum was subjected to centrifugation in CsCl solution containing 4.0M-guanidinium chloride at an initial density of 1.62g/ml. The results in Fig. 2 show that hexuronate-positive material was distributed throughout the solution after centrifugation. The tube contents were divided into four fractions, as indicated in Fig. 2 and designated CsCl fractions 1-4.

Analytical data recorded in Tables 2 and 3 indicate that the contents of amino acid and glucosamine increase across the gradient from fraction 1 to fraction 4, whereas the content of galactosamine decreases. Amino acid contents of each CsCl fraction (Table 3) show that the amounts of serine, proline and glycine change across the gradient from CsCl fraction 1 to fraction 4. The presence of hydroxyproline and higher glycine and alanine contents in CsCl fraction 4, compared with the other fractions, suggests that approx. 10% of the protein in this fraction is collagen.

Samples of each CsCl fraction 1-4 were treated with alkaline NaB^3H_4 and the reaction products chromatographed on ECTEOLA-cellulose. As shown in Fig. 3, three or four peaks of hexuronate-positive

Table 2. *Molecular weight, hexosamine and protein analytical values for CsCl fractions 1-4*

Galactosamine and glucosamine values are expressed as residues per 1000 amino acid residues (Table 3). Chondroitin sulphate-galactosamine/serine indicates the ratio of total galactosamine to the number of serine residues lost after treatment of each CsCl fraction with dilute alkali (Table 3), and keratan sulphate-glucosamine/threonine indicates the ratio of the number of glucosamine residues from keratan sulphate (total glucosamine minus hyaluronic acid-glucosamine) to the number of threonine residues lost on dilute alkali treatment (Table 3). Hyaluronic acid-glucosamine was estimated by assuming that all of the hexuronate-positive material present in fraction A (Fig. 3) is hyaluronic acid. Chondroitin sulphate-galactosamine/serine and keratan sulphate-glucosamine/threonine ratios multiplied by 500 and 760 yield the shown M_n (C) chondroitin sulphate and M_n (E) keratan sulphate molecular weights respectively. Molecular weights of the chondroitin sulphate M_n (D) and keratan sulphate M_n (F) components in each CsCl fraction were estimated by the end-group method described under 'Methods'. Amino acid content of each CsCl fraction is given as a percentage of total dry weight. N.D., Not determined.

	Caesium chloride fraction			
	1	2	3	4
Total galactosamine	4360	3546	2570	544
Chondroitin sulphate-galactosamine/serine	49.0	46.0	41.5	N.D.
M_n (C)	24500	23000	20700	N.D.
M_n (D)	24400	22300	19500	19700
Total glucosamine	278	278	345	208
Hyaluronic acid-glucosamine	87	95	136	163
Keratan sulphate-glucosamine/threonine	9.1	5.9	9.1	N.D.
M_n (E)	6900	4500	6900	N.D.
M_n (F)	6800	4900	N.D.	N.D.
Amino acids	4.2	5.0	6.5	about 25
Chondroitin sulphate-galactosamine/keratan sulphate-glucosamine	24.1	19.4	12.3	12.1

Table 3. *Amino acid composition of CsCl fractions 1-4*

CsCl fractions 1B-3B were obtained by treating approx. 1 mg of CsCl fractions 1-3 respectively with dilute alkali (0.7 ml of 0.5M-KOH at 4°C for 10 days) before acid hydrolysis. All values except those for the alkali-treated samples are expressed as residues per 1000 residues. For the alkali-treated samples (fractions 1B-3B) the number of amino acid residues was calculated by adjusting the galactosamine content in the acid hydrolysate from the alkali-treated samples (fractions 1B-3B) to the same value found in the untreated samples (fractions 1-3). Hydroxyproline, expressed as residues per 1000 other amino acid residues, was determined separately as described under 'Methods'. N.D., Not determined.

Amino acids	Caesium chloride fraction							
	1	1B	2	2B	3	3B	4	
Lys	20	20	21	17	20	16	38	
His	21	20	21	23	16	18	17	
Arg	35	36	27	29	35	36	50	
Asp	66	66	68	66	77	75	88	
Thr	57	36	69	37	60	37	52	
Ser	145	56	138	61	115	53	71	
Glu	147	146	151	151	149	148	120	
Pro	82	80	100	92	109	104	104	
Gly	138	136	131	132	124	127	161	
Ala	64	67	68	71	70	70	93	
Val	60	64	52	60	62	60	50	
Ile	35	33	36	30	35	41	31	
Leu	77	80	73	76	74	73	76	
Tyr	17	20	18	17	19	20	18	
Phe	36	31	27	36	35	34	31	
Residue totals	1000	891	1000	898	1000	912	1000	
Hyp	<1.0	N.D.	<1.0	N.D.	<1.0	N.D.	8.1	

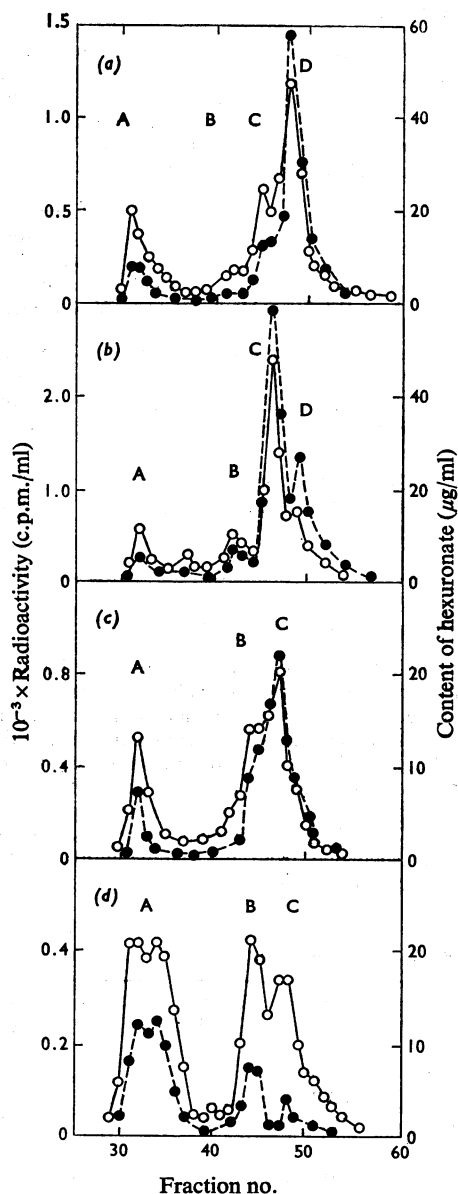


Fig. 3. Elution from ECTEOLA-cellulose of the products obtained by treating CsCl fractions 1-4 with $0.02M\text{-NaB}^3\text{H}_4$ in $0.5M\text{-KOH}$

CsCl fractions 1-4, obtained by sedimentation in a CsCl density gradient (Fig. 2), were each treated with alkaline NaB^3H_4 and the products excluded from Bio-Gel P-4 were eluted from ECTEOLA-cellulose with a linear gradient of NaCl as outlined under 'Methods'. Fractions were volume 12ml; hexuronate content (●) measured by the method of Bitter & Muir (1962) and radioactivity (○) are shown. (a) CsCl fraction 1; (b) CsCl fraction 2; (c) CsCl fraction 3; (d) CsCl fraction 4.

material were obtained from each reaction mixture. Fractions were combined as shown in Fig. 3 to give fractions A, B, C and D. Glucosamine was the only hexosamine detected in fraction A. Digestion of fraction A with chondroitin sulphate ABC lyase yielded a product with the same R_F value on paper chromatography as the disaccharide produced from authentic hyaluronic acid. Fractions B, C and D (Fig. 3) contain both chondroitin sulphate and keratan sulphate.

The amount of hexuronate-positive material in each of fractions A, B, C and D (Fig. 3) as a percentage of the total hexuronate in each CsCl fraction is recorded in Table 4. The molecular weights of the chondroitin sulphate components in fractions B, C and D from each CsCl fraction were determined by end-group estimation and are shown in Table 4. The chondroitin sulphate components of fractions B, C and D from each CsCl fraction have average molecular weights of about 14200, 20600 and 28200 respectively. These values are close to the values for the three chondroitin sulphate species isolated from the nasal cartilage of one steer (Table 1). The relative concentration of the three chondroitin sulphate pools in each CsCl fraction is different. The chondroitin sulphate components with average molecular weights of 28200, 20600 and 14200 are concentrated in CsCl fractions 1, 2 and 3 respectively. The average molecular weight of the total chondroitin sulphate in each CsCl fraction decreases from 24400 to 19700 across the CsCl gradient from fraction 1 to fraction 4 respectively. The proteoglycan molecular weights also decrease from 1.206×10^6 to 0.990×10^6 across the CsCl gradient from fraction 1 to fraction 3 respectively (Table 5). The molecular weight of proteoglycan in CsCl fraction 4, which represents only 5% of the total proteoglycans in fractions 1-4, was not determined because of the presence of relatively large amounts of hyaluronic acid and collagen in this fraction. The hyaluronic acid component (fraction A), concentrated in CsCl fraction 4 and representing about 4% of the total unfractionated proteoglycan hexuronate, has, after treatment with dilute alkali, a molecular weight of 56000 estimated by end-group analysis (Table 4).

The average molecular weight of the chondroitin sulphate component in each CsCl fraction was calculated from the number of serine residues lost on treatment of each CsCl fraction with dilute alkali. Values of 24500, 23000 and 20700 were obtained for CsCl fractions 1, 2 and 3 respectively and are consistent with the values obtained by the alkaline NaB^3H_4 technique (Tables 2 and 4). Molecular weights of keratan sulphate present in CsCl fractions 1, 2 and 3 were calculated from the number of threonine residues lost after treatment of each CsCl fraction with dilute alkali. Values of 6900,

Table 4. *Molecular-weight data for the peaks of hexuronate-positive material isolated from CsCl fractions 1-4*

Each CsCl fraction was treated with alkaline NaB^3H_4 and fractionated on ECTEOLA-cellulose into four hexuronate-positive fractions A, B, C and D as described under 'Methods' (Fig. 3). The hexuronate contents of fractions A, B, C and D are expressed as a percentage of the total hexuronate in each CsCl fraction. The molecular weight (M_n) and the hexuronate content of fractions A-D shown for the unfractionated proteoglycan were calculated from the determined values recorded in this Table for CsCl fractions 1-4. Determined molecular-weight and percentage composition values shown in the bottom line of this Table were obtained from the data recorded in Table 1. N.D., Not determined.

	ECTEOLA-cellulose fraction								% of total hexuronate	Calculated M_n of total chondroitin sulphate in CsCl fraction
	A		B		C		D			
	Wt. %	M_n	Wt. %	M_n	Wt. %	M_n	Wt. %	M_n		
Caesium chloride fraction										
1	2	46000	2	14000	36	19000	60	28000	42	24400
2	3	13000	2	13000	65	20800	26	28200	36	22300
3	5	46000	31	14000	54	21000	10	28500	17	19500
4	30	62000	35	14500	19	22000	16	28300	5	19700
Unfractionated proteoglycan										
Calculated	4	56000	10.0	14200	48	20600	38	28200	100	23000
Determined	N.D.	N.D.	10.0	13000	52	18600	38	28000	100	20800

Table 5. *Molecular weight and calculated composition of the proteoglycan in CsCl fractions 1-3*

The weight per cent of protein, chondroitin sulphate and keratan sulphate in CsCl fractions 1-3 were estimated by applying factors of 100, 500 and 750 respectively to the content of amino acid, chondroitin sulphate-galactosamine and keratan sulphate-glucosamine given in Table 2 for each CsCl fraction. The number of chondroitin sulphate and keratan sulphate chains per molecule of proteoglycan in CsCl fractions 1-3 was calculated from the proteoglycan sedimentation-equilibrium molecular weight recorded in this Table, by using the end-group molecular weights of chondroitin sulphate [$M_n(D)$] and keratan sulphate [$M_n(F)$] recorded in Table 2 and by assuming that the protein core is present as a single chain. The molecular weight of keratan sulphate in CsCl fraction 3 was calculated from the number of threonine residues lost after dilute alkali treatment (Table 3). The molecular-weight values are means of 30 point measurements along the sedimentation interference pattern \pm S.E.M. M_w/M_n ratios for proteoglycans were calculated from the sedimentation-equilibrium molecular-weight values. M_w/M_n ratios for the chondroitin sulphate component of each fraction were estimated as described under 'Methods'. N.D., Not determined.

	CsCl fraction 1		CsCl fraction 2		CsCl fraction 3		Unfractionated proteoglycan	
	Weight %	Number of chains	Weight %	Number of chains	Weight %	Number of chains	Weight %	Number of chains
Proteoglycan component								
Protein	4.2	1.0	5.0	1.0	6.5	1.0	5.0	1.0
Chondroitin sulphate	89.8	44.2	88.1	43.6	83.3	42.3	88.0	44.3
Keratan sulphate	6.0	10.7	6.9	15.6	10.2	14.8	7.0	11.3
Proteoglycan molecular weight								
$10^{-6} \times M_n$	1.206 \pm 0.012		1.102 \pm 0.008		0.990 \pm 0.011		1.156	
$10^{-6} \times M_w$	1.277 \pm 0.031		1.160 \pm 0.035		1.029 \pm 0.042		1.223	
M_w/M_n ratio								
Proteoglycan	1.06		1.05		1.04		1.06	
Chondroitin sulphate	1.05		1.04		N.D.		1.07	

4500 and 6900 were obtained for the keratan sulphate component present in CsCl fractions 1, 2 and 3 respectively (Table 2). Keratan sulphate isolated from each CsCl fraction by treatment with alkaline NaB^3H_4 and chondroitin sulphate lyase was purified on Sephadex G-50 and ECTEOLA-

cellulose (Hopwood & Robinson, 1973). The molecular weights of these preparations were determined by the end-group procedure previously described (Hopwood & Robinson, 1973) and found to be 6800 (CsCl fraction 1) and 4800 (CsCl fraction 2). Insufficient material prevented a similar end-

group estimation of the molecular weight of the keratan sulphate components in CsCl fractions 3 and 4.

Fractionation of proteoglycan in caesium formate solution

Fig. 4 shows the distribution of hexuronate-positive material obtained when proteoglycan (8 mg, Preparation A) was sedimented through a pre-formed caesium formate gradient. After 24h proteoglycan formed a narrow band at a density of 2.02g/ml at 22°C when the proteoglycan was applied initially at the bottom or at the top of the gradient. Centrifugation of a small quantity of proteoglycan (2mg) for shorter periods in a shallow caesium formate gradient (bottom 2.10/ml, top 1.90g/ml) gave the hexuronate profiles shown in Fig. 5. After 6h centrifugation three distinct peaks of hexuronate-positive material were present. The minor (less than 10% of the total hexuronate) and rapidly moving peak of hexuronate-positive material moving to the top of the tube

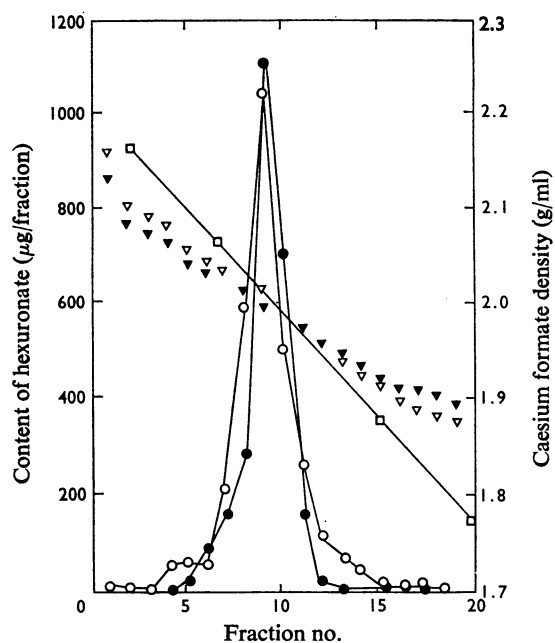


Fig. 4. Distribution of hexuronate-positive material obtained after sedimentation of proteoglycan through density gradients of caesium formate

Proteoglycan (8mg total) from one nasal cartilage was centrifuged in caesium formate solutions as outlined under 'Methods'. Fractions (0.25ml) were collected from the tube bottom; hexuronate content after 24h (○) and 48h (●) is shown; CsCl density initially (□), after 24h (▽) and 48h (▼) is also shown.

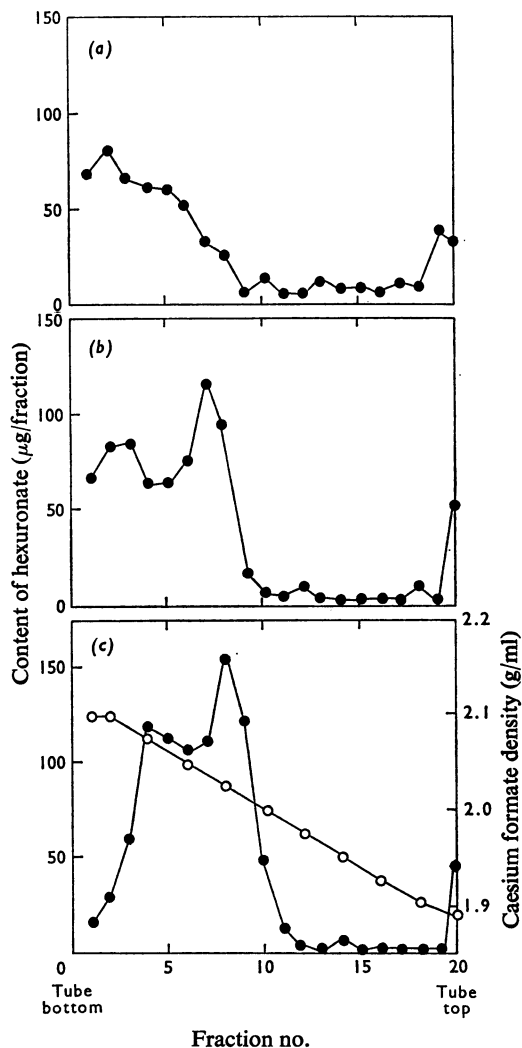


Fig. 5. Distribution of hexuronate-positive material obtained after sedimentation of proteoglycan through caesium formate density gradients

Proteoglycan (2mg total) from one nasal cartilage was applied to the bottom of a pre-formed caesium formate gradient and centrifuged as outlined under 'Methods' for (a) 2.5h, (b) 6h and (c) 9h. Fractions (0.25ml) were collected from the tube bottom; hexuronate content (●) and initial caesium formate density (○) are shown.

after 2.5h centrifugation was shown to contain glucosamine and no galactosamine and is therefore probably hyaluronic acid. The two major peaks of hexuronate-positive material present after 6h and 9h centrifugation moved together after 24h and banded in the density range 1.99–2.03 g/ml.

Proteoglycan from intervertebral disc was centri-

Table 6. Analytical values for proteoglycan from milled cartilage

Samples 1 and 2 were isolated from the pool of intervertebral disc and nasal cartilage from 10 steers respectively by extraction and purification in LiBr solutions as described under 'Methods'. Sample 3 was isolated from the nasal cartilage of one steer by the procedure described for Preparation A under 'Methods'. Samples 4 and 5 were isolated from the collagenase digest of sample 2 and the insoluble cartilage residue respectively. Protein, galactosamine and glucosamine content were determined by using the amino acid analyser. Results are expressed as percentage by weight of the dry sodium salt of the proteoglycan. Molecular-weight values were estimated by equilibrium sedimentation.

Sample	Tissue extracted	Proteoglycan composition (%)			$10^{-6} \times$ Proteoglycan mol.wt.	
		Protein	Galactosamine	Glucosamine	M_n	M_w
1	Intervertebral disc	7.2	7.7	16.9	0.60	0.69
2	Nasal septum	5.1	32.2	2.6	1.15	1.25
3	Nasal septum	5.0	34.8	2.6	1.16	1.22
4	Nasal septum	5.1	31.7	3.1	0.32	0.35
5	Nasal septum	5.2	30.7	2.9	0.23	0.28

fused in a caesium formate gradient (bottom density 2.15g/ml, top density 1.70g/ml) in the same way. The proteoglycan was applied at the top or the bottom of the gradient, and during centrifugation the hexuronate-positive material migrated towards the centre of the gradient as a single but broad band. Unlike the proteoglycan from nasal cartilage the material applied at the top and the bottom of the gradient failed to reach a common density after 24h of centrifugation. This result, however, is consistent with the lower molecular weight and the broader molecular-weight distribution (M_w/M_n ratio) found for this proteoglycan preparation from intervertebral disc (Table 6).

Proteoglycan molecular size and shape

The molecular-weight values for proteoglycan isolated from nasal cartilage and from intervertebral disc were estimated by the method of equilibrium sedimentation to be 1.15×10^6 and 0.60×10^6 respectively (Table 6). The sedimentation coefficients extrapolated to infinite dilution, $s_{20,w}^0$, for the same preparations were 26 and 12S respectively (Fig. 6). At concentrations below 1.0mg/ml the sedimentation behaviour of the proteoglycan preparation from nasal cartilage changed from that giving a single boundary to one giving a bimodal schlieren pattern with $s_{20,w}^0$ values of approx. 24 and 31S. Dialysis of proteoglycan from nasal cartilage and intervertebral disc against 0.05M-sodium bisulphite caused a considerable but similar change in the concentration-dependence of the sedimentation coefficients for both preparations (Fig. 6). Bisulphite treatment failed to alter $s_{20,w}^0$ or the molecular-weight values determined by sedimentation equilibrium.

Exhaustive extraction of milled nasal cartilage with 5M-LiBr solutions failed to extract approx. 18% of the total proteoglycan. The residual proteoglycan was solubilized by treatment of the LiBr-insoluble fraction with collagenase and then purified by

centrifugation of the digest on a CsCl density gradient as described under 'Methods'. LiBr-soluble proteoglycan was similarly treated with collagenase and centrifuged on a CsCl gradient. The amino acid profile, galactosamine and glucosamine content and molecular weights of both preparations are given in Tables 6 and 7. Results suggest that proteoglycan present in the LiBr-soluble and -insoluble fractions have similar molecular weights and compositions. The substantial decrease from 1.2×10^6 to 0.32×10^6 for the molecular weight of proteoglycan, after collagenase treatment, would require only three or four breaks within the proteoglycan protein core.

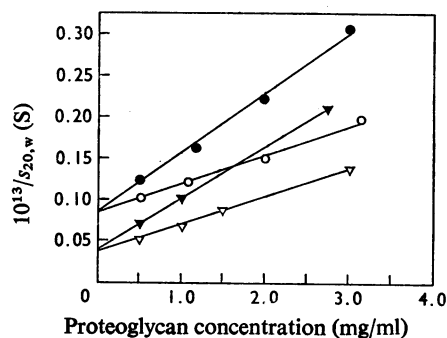


Fig. 6. Plots of reciprocals of sedimentation coefficients against proteoglycan concentration

Proteoglycans isolated from nasal cartilage and intervertebral disc by using the LiBr technique described by Robinson & Hopwood (1973) were dialysed and then sedimented in either 0.1M-NaCl containing 0.12mM-NaHCO₃ (buffer A) or 0.05M-NaHSO₃ containing 0.12mM-NaHCO₃ (buffer B). Both buffers were at pH7.0. Proteoglycan from nasal septum in buffers A (▽) and B (▼) and from intervertebral disc in buffers A (○) and B (●) are shown

Such breaks could result from the action of trace amounts of contaminating proteinase in the collagenase or in the cartilage residue or from the presence in the proteoglycan protein core of bonds susceptible to collagenase.

Discussion

Proteoglycan biosynthesis and proteoglycan polydispersity and heterogeneity

Despite numerous attempts, proteoglycan isolated from nasal cartilage has never been purified into a major homogeneous fraction (Pal *et al.*, 1966; Hascall & Sajdera, 1970; Mashburn & Hoffman, 1971). Owing to this lack of success a general conclusion has been that this proteoglycan is heterogeneous and polydisperse.

Sajdera & Hascall (1969) and Hascall & Sajdera (1969) have pointed out that much of the heterogeneity and polydispersity previously found in the proteoglycan isolated from nasal cartilage stems either from degradation by enzymes or shear during proteoglycan isolation or from the presence of various amounts of proteoglycan subunit aggregates and contaminating cartilage glycoproteins. However,

even after minimizing these sources of variation, proteoglycan preparations still appear to contain a broad population of structures with differences in size and composition (Hascall & Sajdera, 1970; Tsiganos *et al.*, 1971). Hascall & Sajdera (1970) have suggested that this polydispersity results from a variable number of chondroitin sulphate chains attached to the proteoglycan protein core. They report a range of molecular weights for proteoglycan from 1×10^6 to 4×10^6 and conclude that this distribution could occur if a variable number of chondroitin sulphate chains are synthesized on each protein core. This conclusion implies: (1) that the protein core is synthesized as a single chain from which an average of 90 chondroitin sulphate chains are propagated, (2) that chondroitin sulphate chain initiation is a variable process and (3) that, on average, only half of the serine residues programmed as chondroitin sites on the protein core are utilized.

This proposal of Hascall & Sajdera (1970) was based on the finding reported by Luscombe & Phelps (1967b) that the average molecular weight of chondroitin sulphate in nasal cartilage is 25000 and that the chains are essentially monodisperse. However, evidence presented in the present paper

Table 7. *Amino acid composition for proteoglycan from milled cartilage*

Proteoglycan samples 1 and 2 were isolated from adult bovine intervertebral disc and nasal cartilage respectively. Samples 1B and 2B were obtained by treating approx. 1 mg of samples 1 and 2 with dilute alkali (0.7 ml of 0.5M-KOH at 4°C for 10 days) before acid hydrolysis. Proteoglycan samples 4 and 5 were isolated from a collagenase digest of sample 2 and the LiBr-insoluble nasal cartilage residue respectively. All values except those for the alkali-treated samples are expressed as residues per 1000. For the alkali-treated samples the number of amino acid residues was calculated by adjusting the galactosamine content in the acid hydrolysate from the alkali-treated proteoglycan preparations to the same values found for the untreated preparations. Hydroxyproline and cysteine values, expressed as residues per 1000 other amino acid residues, were determined separately as described under 'Methods'. N.D., Not determined.

	Proteoglycan sample					
	1	1B	2	2B	4	5
Lys	35	37	30	32	32	31
His	14	14	14	15	14	16
Arg	34	32	29	26	27	28
Asp	81	83	69	70	69	72
Thr	95	43	60	38	60	61
Ser	77	53	145	60	145	140
Glu	144	140	143	140	139	138
Pro	95	91	80	86	82	79
Gly	120	118	138	137	142	143
Ala	70	69	75	73	74	75
Val	64	59	58	60	61	63
Met	3	5	5	4	N.D.	N.D.
Ile	33	34	31	30	33	31
Leu	72	70	76	74	74	75
Tyr	25	24	17	18	19	18
Phe	38	39	30	35	33	32
Residue totals	1000	911	1000	898	1000	1000
Hyp	2	N.D.	2	N.D.	3	1
Cys	19	N.D.	27	N.D.	N.D.	N.D.

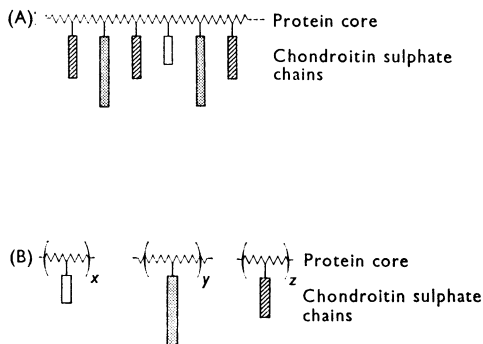


Fig. 7. Proposed structures for the proteoglycan of nasal cartilage

Scheme A is a model in which a random mixture of three different chondroitin sulphate pools of different average molecular weight is attached to a common protein core. Scheme B is a model in which there are as many proteoglycan structures as there are chondroitin sulphate pools of different average molecular weight. The number of chondroitin sulphate chains per proteoglycan molecule shown as x , y or z could be the same or different.

indicates that nasal cartilage contains at least three separate chondroitin sulphate species with average molecular weights of 13000, 18600 and 28000, contributing 10, 52 and 38% respectively of the total chondroitin sulphate weight. Fig. 7 shows two possible schemes in which three chondroitin sulphate species could be incorporated into a common proteoglycan structure. Scheme A represents a model where a random mixture of the three different chondroitin sulphate pools is attached to a common protein core. Scheme B illustrates a model in which there are as many proteoglycan structures as there are chondroitin sulphate pools of different average molecular weight. The number of chondroitin sulphate chains per proteoglycan molecule in Schemes A and B could be the same or different.

Proteoglycan subunit isolated from a single nasal cartilage and fractionated by a procedure essentially the same as that described by Hascall & Sajdera (1970) resulted in a similar distribution of proteoglycan structures to that reported by these authors; proteoglycan molecular weight increased as the protein and glucosamine concentrations decreased within the three major fractions obtained by this procedure. However, estimation of the size and amount of chondroitin sulphate moiety in each proteoglycan fraction indicates that proteoglycan molecular weight is directly related to the average chain length of the chondroitin sulphate component and not, as Hascall & Sajdera (1970) have suggested, to the number of chondroitin sulphate chains attached to the proteoglycan unit. The average chain length of the chondroitin sulphate in each proteo-

glycan fraction was determined by the relative proportion of the three different chondroitin sulphate species present (Table 4). These results, together with the conclusion that there seems to be a constant number of chondroitin sulphate chains per proteoglycan fraction (Table 5) and that the amino acid content is different (Table 3) in each proteoglycan fraction, strongly suggest that nasal cartilage contains at least three different proteoglycan populations, as illustrated by Scheme B in Fig. 7, and that a constant number of chondroitin sulphate chains are bonded to each proteoglycan molecule ($x = y = z$ in Fig. 7).

The protein core of the proteoglycan isolated from nasal cartilage has approx. 60% of its serine residues substituted with chondroitin sulphate chains; the remaining serine residues do not appear to function as initiation sites for chondroitin sulphate biosynthesis (Tables 3 and 7). Baker *et al.* (1972) concluded from studies with a cell-free xylosyltransferase preparation that approximately half of the serine residues of nasal-cartilage proteoglycan could initiate chondroitin sulphate biosynthesis and that all of these sites are normally substituted. These results suggest that the amino acid sequence around the substituted serine residues must determine those serine residues in the protein core which act as substrates of the xylosyltransferase and chondroitin sulphate synthesis.

Theoretical values for the chemical composition and the average molecular weight of each of the three proposed proteoglycan species in nasal cartilage were calculated as shown in Table 8. From these data, it is apparent that although the molar ratios of chondroitin sulphate, keratan sulphate and protein in the proteoglycan remain constant, the percentage of these three components by weight shows considerable variation. These values were calculated by varying the molecular weight of the chondroitin sulphate component only. It must be stressed, however, that in the proteoglycans in CsCl fractions 1, 2 and 3 the keratan sulphate chains had average molecular weights of 6800, 4500 and 6800. Thus the three proteoglycan structures proposed for nasal cartilage are certainly more complicated than the simplified structures indicated in Table 8. The ratio of M_w to M_n , a measure of polymer polydispersity, was calculated from the sedimentation-equilibrium data for each of the proteoglycan fractions (Table 5). The results indicate that the polydispersity of this crudely fractionated proteoglycan mixture is quite low. For example, the M_w/M_n ratio of the proteoglycan component in CsCl fractions 1 and 2 is 1.06 and 1.05 compared with ratios of 1.05 and 1.04 for the corresponding chondroitin sulphate components (Table 5). The closeness of the chondroitin sulphate and proteoglycan M_w/M_n ratios fits with the proposal that each serine residue in the protein core

Table 8. *Theoretical molecular weight and composition of the three proposed proteoglycan species of adult bovine nasal cartilage*

For these calculations the protein and keratan sulphate chains were assumed to have average molecular weights 55000 and 7000 respectively. Molecular weights of 28200, 20600 and 14200 (Table 4) were used for the chondroitin sulphate components of proteoglycans PG-1, PG-2 and PG-3 respectively. Proteoglycan compositions in terms of weight per cent of protein, keratan sulphate and chondroitin sulphate were calculated by assuming 1, 10 and 45 chains respectively for each proteoglycan molecule. The molecular weight of proteoglycans PG-1, PG-2 and PG-3 were calculated from the molecular weight and number of protein, keratan sulphate and chondroitin sulphate chains per proteoglycan unit.

Proteoglycan type	Proteoglycan composition (%)			$10^{-6} \times$ Proteoglycan mol.wt.
	Protein	Keratan sulphate	Chondroitin sulphate	
PG-1	4.0	5.0	91.0	1.40
PG-2	5.2	6.7	88.1	1.05
PG-3	7.2	9.2	83.6	0.77

capable of initiating chondroitin sulphate is substituted and that the polydispersity in the proteoglycan results from the polydispersity of the chondroitin sulphate chains, which appears to arise during chondroitin sulphate biosynthesis (Hopwood & Robinson, 1973).

Two major proteoglycan species are observed when the proteoglycan subunit preparation was centrifuged at low concentrations in a caesium formate gradient or in the analytical ultracentrifuge. However, at high concentrations of proteoglycan, the material sediments as a single narrow band or with a single boundary. This discrepancy suggests that at high proteoglycan concentrations, where the sedimentation rate has been found to be independent of molecular weight (Ogston & Woods, 1954), a continuous three-dimensional network of molecules sediments rather than single molecules.

Molecular size and shape of the proteoglycan unit

The molecular weight of proteoglycan preparations, isolated from nasal cartilage and intervertebral disc by methods utilizing dissociating solvents and conditions of minimum degradation, were measured by using the method of Yphantis (1964). Equilibrium sedimentation is probably the most accurate physical method available for the determination of proteoglycan molecular weights if low proteoglycan concentrations are used to minimize non-ideal effects. At proteoglycan concentrations of 0.2mg/ml and below, the molecular weight of proteoglycan from both tissues did not significantly vary with concentration. Therefore the values reported in Tables 5 and 6 are probably accurate molecular-weight estimates of the dissociated or subunit proteoglycan structure present in both tissues.

The conditions of extraction and purification were similar to those used by Sajdera & Hascall (1969) and Hascall & Sajdera (1969, 1970) to isolate and purify proteoglycan from adult bovine nasal cartilage. Hascall & Sajdera (1970) reported a considerably different molecular weight (2.5×10^6) from the value

(1.2×10^6) given here, although similar s^0 values were obtained. The difference probably arises from the different methods of determination; our values were determined by sedimentation equilibrium, which is an absolute method, independent of any assumptions about shape, whereas the values calculated by Hascall & Sajdera (1970) are dependent on a relationship between shape, sedimentation coefficient and intrinsic viscosity developed by Scheraga & Mandelkern (1953). There are several reported examples where this relationship has been shown not to follow theory (Schachman, 1959).

Bisulphite treatment of proteoglycans isolated from nasal cartilage and intervertebral disc caused a marked but similar change in the concentration-dependence of the sedimentation coefficients for both proteoglycan preparations. However, s^0 and equilibrium-sedimentation-determined molecular-weight values were not altered by the bisulphite treatment. Thus bisulphite probably cleaves intramolecular disulphide bonds in the proteoglycan protein core, causing a conformational change in the proteoglycan molecule. This observation is consistent with a proposal that the proteoglycan structure of both nasal cartilage and intervertebral disc have conformations in solution that are restricted by intramolecular disulphide bonds, which when cleaved enable some unfolding of the proteoglycan molecule. This proposal is consistent with the suggestion by Hascall & Sajdera (1969) that cysteine residues are directly involved in folding the conformation of proteoglycan molecules into a favourable shape for aggregation to more complex structures.

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