

The Nature of the Protein Moieties of Cartilage Proteoglycans of Pig and Ox

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Proteoglycans extracted with 4M-guanidinium chloride from pig laryngeal cartilage and bovine nasal septum were purified by density-gradient centrifugation in CsCl under 'associative' followed by 'dissociative' conditions [Hascall & Sajdera (1969) *J. Biol. Chem.* **244**, 2384-2396]. Proteoglycans were then digested exhaustively with testicular hyaluronidase, which removed about 80% of the chondroitin sulphate. The hyaluronidase was purified until no proteolytic activity was detectable under the conditions used for digestion. The resulting 'core' proteins of both species were fractionated by a sequence of gel-chromatographic procedures which gave four major fractions of decreasing hydrodynamic size. Those that on electrophoresis penetrated 5.6% (w/v) polyacrylamide gels migrated as discrete bands whose mobility increased with decreasing hydrodynamic size. The unfractionated 'core' proteins had the same *N*-terminal amino acids as the intact proteoglycan, suggesting that no peptide bonds had been cleaved during hyaluronidase digestion. Alanine predominated as the *N*-terminal residue in all the fractions of both species. Fractions were analysed for amino acid, amino sugar, uronic acid and neutral sugar compositions. In pig 'core' proteins, the glutamic acid content increased significantly with hydrodynamic size, but in bovine 'core' proteins this trend was less marked. Significant differences in amino acid composition between fractions suggested that in each species there was more than one variety of proteoglycan. The molar proportions of xylose to serine destroyed on alkaline β -elimination were equivalent in most fractions, indicating that the serine residues destroyed were attached to the terminal xylose of chondroitin sulphate chains. The ratio of serine residues to threonine residues destroyed on β -elimination, was similar in all fractions of both species. Since the fractions of smallest hydrodynamic size contained less keratan sulphate than those of larger size, it implies that in the former the keratan sulphate chains were shorter than in the latter.

The proteoglycans of cartilage consist of a central protein core to which chains of chondroitin sulphate (Mathews & Lozaityte, 1958; Partridge *et al.*, 1961) and keratan sulphate (Tsiganos & Muir, 1967; Heinegård, 1972*b*) are attached by covalent bonds to serine (Muir, 1958; Anderson *et al.*, 1965), and to threonine or serine (Anderson *et al.*, 1965; Seno *et al.*, 1965; Bray *et al.*, 1967; Tsiganos & Muir, 1967) respectively. The use of efficient and reproducible methods of extraction and purification of proteoglycans introduced by Sajdera & Hascall (1969) has greatly advanced knowledge of the structure of cartilage proteoglycans, and enabled the phenomenon of their aggregation (Hascall & Sajdera, 1969) to be elucidated. These two authors found that proteoglycan aggregates could be dissociated by 4M-guanidinium chloride. By using CsCl density-gradient centrifugation, in the presence of 4M-guanidinium chloride, they were able to

separate the components of the aggregate at different buoyant densities, and thus showed that the aggregates were not formed by simple self-association of proteoglycans, but by interaction with other specific non-proteoglycan components. It was originally thought that a 'protein-link' (first called 'glycoprotein-link') constituent linked together proteoglycans into aggregates (Hascall & Sajdera, 1969; Rosenberg *et al.*, 1970, 1973). It has since been recognized, however, that aggregation depends on a highly specific interaction of proteoglycans with hyaluronic acid (Hardingham & Muir, 1972*a*), by which many proteoglycan molecules interact with a single chain of hyaluronic acid. Hyaluronic acid was subsequently shown to be present in cartilage, where it accounted for less than 1% of the total uronic acid (Hardingham & Muir, 1973) and could be separated from proteoglycan aggregates during density-gradient centrifugation in the presence of 4M-guanidinium

chloride (Hardingham & Muir, 1974; Hascall & Heinegård, 1974a).

The 'protein-link' component functions in stabilizing the proteoglycan-hyaluronate complex (Hascall & Heinegård, 1974a; Hardingham & Muir, 1975) and may be removed by density-gradient centrifugation in the presence of 4M-guanidinium chloride by the procedure of Hascall & Sajdera (1969). Proteoglycans prepared in this way were not homogeneous, however, and could be separated into fractions of differing amino acid composition, in which the ratio chondroitin sulphate/protein varied considerably (Tsiganos *et al.*, 1971; Hardingham & Muir, 1974; Heinegård, 1972a). Proteoglycans have also been separated in other ways into fractions of differing overall composition, amino acid composition and *N*-terminal groups (Muir & Jacobs, 1967; Serafini-Fracassini *et al.*, 1967; Brandt & Muir, 1969, 1971a,b; Tsiganos & Muir, 1969; Mashburn & Hoffman, 1971; Simunek & Muir, 1972). Nevertheless, it has been suggested that proteoglycans are a single, albeit polydisperse, population of molecules (Hascall & Sajdera, 1969, 1970; Hascall *et al.*, 1972). The possibility has been examined here that the 'core' protein of proteoglycans may not be a single polypeptide, which differs only in the degree of substitution by glycosaminoglycan chains, but may consist of several closely related polypeptides. Proteoglycans in common use from bovine nasal septum and pig larynx were prepared, and, since they were extracted with 4M-guanidinium chloride, which is a particularly effective solvent (Sajdera & Hascall, 1969), they were representative of the proteoglycans in the tissue as a whole and hence were not selected fractions. The proteoglycans were treated with highly purified testicular hyaluronidase to remove the bulk of the chondroitin sulphate to expose differences between 'core' proteins that might otherwise be masked by the preponderance of chondroitin sulphate.

Materials and Methods

Materials

All reagents were of analytical grade, except glucuronolactone, galactosamine hydrochloride, casein (BDH Chemicals Ltd., Poole, Dorset, U.K.), haemoglobin (Sigma Chemical Co., St. Louis, Mo., U.S.A.), bovine serum albumin (Armour Pharmaceutical Co., Hastings, Sussex, U.K.) and guanidinium chloride (BDH), which was purified by stirring with activated charcoal (Norit GSX: Hopkin and Williams, Romford, Essex, U.K.) and Celite (BDH). Sephadex G-50 (fine grade), G-150 and Sepharose 6B (6% agarose) were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Bio-Gel A-0.5m (200-400 mesh; 8% agarose) and Bio-Gel A-1.5m (200-400 mesh; 10% agarose) were

obtained from Bio-Rad Laboratories, St. Albans, Herts., U.K.

Methods

Preparation of disaggregated proteoglycans. The procedure followed for the extraction of proteoglycans was essentially that of Sajdera & Hascall (1969) and the preparation and isolation of disaggregated proteoglycans that of Hascall & Sajdera (1969).

Cartilage was dissected from fresh pig larynges and ox nasal septa, and after removal of the perichondrium, was diced finely with a 'Surform' woodworking tool. The diced cartilage was suspended in 4M-guanidinium chloride containing 50mM-sodium acetate, pH 5.8, and placed on a roller for 24h at room temperature (20°C) (yield, 65mg of proteoglycan/g of wet cartilage). The insoluble residue was removed by filtration on a coarse sintered-glass filter, and the extracts were dialysed overnight at 4°C against 7vol. of water. Solid CsCl was added to give a starting density of 1.69g/ml and the solution centrifuged in 25ml tubes at 95000 *g*_{av} for 48h at 18°C in an MSE Super-Speed 65 with a titanium T825 angle rotor. At the completion of the run, the contents of the centrifuge tubes were frozen by immersion in a mixture of methanol-solid CO₂, and the bottom 5ml, containing the bulk of the proteoglycans, was removed by cutting the tubes at a measured distance from the rim. The proteoglycans thus isolated were free of collagen and other contaminants, and consisted of both aggregated and non-aggregated molecules. The proteoglycans were then disaggregated and separated from other components of the aggregate by a second CsCl-density-gradient centrifugation in the presence of 4M-guanidinium chloride. The starting density was adjusted to 1.5g/ml with CsCl and the solution centrifuged at 34 000 rev./min for 48h in the same rotor. The tubes were frozen as before and cut so that the bottom 5ml [which contained 90-95% of the proteoglycans (Hascall & Sajdera, 1969)] was separated.

Purification of testicular hyaluronidase. Sheep testicular hyaluronidase (EC 3.2.1.3.5) (Koch-Light Ltd., Colnbrook SL3 0B2, Bucks., U.K.; 560units/mg) was purified by the procedure of Borders & Raftery (1968), except that to minimize inactivation, enzyme solutions were not dialysed to low ionic strength during the purification procedure, and were concentrated by ultrafiltration in Visking tubing, rather than by freeze-drying. Enzyme activity was measured as turbidity-decreasing units with chondroitin 4-sulphate as substrate. No proteolytic activity in the purified enzyme preparations could be demonstrated by the release of any trichloroacetic acid-soluble peptide from denatured haemoglobin (2%,

w/v) or casein (1%, w/v) after incubation at 37°C for 24h under the reaction conditions used to prepare 'core' proteins.

Preparation and isolation of 'core' proteins. (a) Digestion with testicular hyaluronidase. Highly purified hyaluronidase was added in the proportion of 20000 turbidity-decreasing units/g of proteoglycan to a solution of proteoglycan (0.2%, w/v) in 0.1M-NaH₂PO₄, adjusted to pH 5.0 with NaOH and containing 0.15M-NaCl, 0.002% benzylpenicillin and 0.005% streptomycin sulphate. The solution was incubated at 37°C for 24h in a flask that had been silicone-treated to minimize inactivation of the enzyme by interaction with glass. A further 10000 turbidity-decreasing units/g of proteoglycan were then added and incubation was continued for another 24h.

(b) Isolation of 'core' protein components. Digests of large volume were concentrated to about 100ml by ultrafiltration through dialysis tubing under negative pressure, and were then passed through a column (3cm×40cm) of Biorex 70 (-400 mesh; Na⁺ form; Bio-Rad Laboratories, Richmond, Calif., U.S.A.). It was essential to remove fine particles from the resin by suspending in water and decanting several times. The resin was then equilibrated with 0.1M-NaH₂PO₄ and adjusted to pH 6.0 with NaOH before use. The 'core' proteins, unlike the enzyme, were not bound to the resin, and were washed through in 1-2 column vol. of equilibrating buffer, which was concentrated by ultrafiltration. Any oligosaccharides and buffer salts were removed by passing the solution through a column (2cm×58cm) of Sephadex G-50, eluted with 0.1M-NaCl, from which the 'core' proteins were excluded.

Disc electrophoresis. The 5.6% acrylamide gels were prepared by mixing the following stock solutions: (A) 36.6g of Tris and 0.46ml of NNN'N'-tetramethylethylenediamine dissolved in 48ml of 1M-HCl and diluted to 100ml with water; (B) 30% (w/v) acrylamide, 0.8% (w/v) NN-methylenebisacrylamide in water; (C) 0.14% (w/v) solution of ammonium persulphate. Solutions A, B, C and water were mixed respectively in the proportions 2:3:8:3 by vol. The reservoir buffer was prepared from a stock solution of 0.6% (w/v) Tris, 2.8% (w/v) glycine, pH 8.3, which was diluted 1:10 with water. A Pleuger disc-electrophoresis chamber was used with a current of 4mA/tube and 0.01% Bromophenol Blue as a marker. Samples containing about 10µg of protein were applied to each tube. Protein bands were fixed in trichloroacetic acid and stained with Coomassie Blue (Gurr Products, Romford, Essex, U.K.) following the procedure of Chrambach *et al.* (1967).

Gel chromatography. All gel columns were eluted with 0.1M-NaCl and the elution of protein was followed by its absorption at 230nm; protein (0.5-2.0mg) [bovine serum albumin equivalents,

assayed by the procedure of Lowry *et al.* (1951)] was applied to the columns.

Analytical methods. Protein was measured by an automated modification (Heinegård, 1973) of the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V; Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) as standard. Uronic acid was measured by an automated modification (Heinegård, 1973) of the method of Bitter & Muir (1962) with glucuronolactone as standard.

For the determination of total hexosamine and molar ratios of galactosamine/glucosamine, duplicate samples were hydrolysed in sealed glass tubes under N₂ in 8M-HCl for 3h at 95°C (Swann & Balazs, 1966). Excess of acid was removed by drying at 40°C on a rotary evaporator. The hydrolysed samples were dissolved in water and applied to the 15cm column of a Locarte amino acid analyser (Tsiganos & Muir, 1969), each sample being analysed twice. Amino acid analyses were also performed on the above analyser (Tsiganos & Muir, 1969). Duplicate samples were hydrolysed in sealed glass tubes under N₂ in 6M-HCl at 105°C for 24h, and the excess of acid was removed by rotary evaporation at 40°C. The residues were dissolved in 1ml of water and 0.5ml was applied to the column. Each sample was analysed twice.

Alkaline β-carbonyl elimination. Samples of 'core' protein (about 50µg of protein) were dissolved in 1ml of 0.5M-NaOH and kept under N₂ at 18-20°C for 24h, after which 1.4ml of concentrated HCl was added. The sample was then hydrolysed and the amino acid composition was determined as described above.

N-Terminal amino acids. N-Terminal amino acids were identified in the intact proteoglycans, before hyaluronidase digestion, essentially as described by Woods & Wang (1967), by using dansyl chloride. The dansylated proteoglycans were precipitated from the reaction mixture with acetone, washed, dried, redissolved in the appropriate buffer, and digested with testicular hyaluronidase before acid hydrolysis; this was done to decrease the amount of carbohydrate present during hydrolysis, and so minimize the destruction of dansylated amino acids. The oligosaccharides liberated by hyaluronidase were removed by ultrafiltration through a Diaflow UM 10 membrane (Amicon, High Wycombe, Bucks., U.K.). 'Core' proteins that had already been digested with hyaluronidase were hydrolysed directly after dansylation.

G.l.c. of sugars. Duplicate samples were subjected to methanolysis and each was analysed twice. Neutral sugars and sialic acid were determined by preparation of the trimethylsilyl derivatives of their methyl glycosides as described by Clamp *et al.* (1967). These were fractionated and measured by chromatography on a Hewlett Packard F and M 5750B

research gas chromatograph by using a column (3.0m×0.6cm; 10ft×0.25in) containing Diaport S, coated with a liquid phase of silicone.

Results

Exhaustive digestion with testicular hyaluronidase of the disaggregated proteoglycans from pig laryngeal cartilage and ox nasal septum removed about 80–85% of the chondroitin sulphate from both preparations. The resulting 'core' proteins contained some residual fragments of chondroitin sulphate chains, as well as the keratan sulphate originally present. The pattern of *N*-terminal amino acids in each unfractionated 'core'-protein preparation was the same as in the original proteoglycans before hyaluronidase treatment (Tables 1 and 2), implying that no proteolysis had occurred during the digestion, and that all the enzyme had been removed by the isolation procedure.

Table 1. *Distribution of N-terminal amino acids (as dansyl derivatives) in fractions of 'core' proteins*

'Core' proteins were obtained, as shown in Scheme 1 and Fig. 3, from disaggregated proteoglycans of pig laryngeal cartilage after hyaluronidase digestion. +++, ++, +, Relative amounts (arbitrary scale).

	Alanine	Glycine	Serine	Glutamic acid
Intact proteoglycan	+++	++	+	+
Unfractionated 'core' proteins	+++	++	+	+
Fractions				
E	++	+	—	—
IP	++	—	+	—
IIIP	++	Trace	Trace	—
IVP	++	+	—	+

Table 2. *Distribution of N-terminal amino acids (as dansyl derivatives) in fractions of core proteins*

Core proteins were obtained, as shown in Scheme 2 and Fig. 4, from disaggregated proteoglycans of bovine nasal septum after hyaluronidase digestion. +++ and +, Relative amounts (arbitrary scale).

	Alanine	Glycine
Intact proteoglycans	+++	+
Unfractionated 'core' proteins	+++	+
Fractions		
IB	+++	—
IIB	+++	—
IIIB	+++	Trace
IVB	+++	—

Fractionation of 'core' protein components

(a) *Disc electrophoresis*. Disaggregated proteoglycans are too large to penetrate 5.6% polyacrylamide gels. However, after hyaluronidase treatment, some components of the 'core'-protein preparations from both species were small enough to migrate into these gels during electrophoresis, although in each preparation some material remained at the surface of the gels. Two major components from the pig 'core'-protein preparation (Fig. 1a), and three from the ox preparation (Fig. 2a), migrated as separate bands. Thus together with the material remaining at the gel surface, electrophoresis demonstrated at least three fractions in the pig preparation and four in the ox preparation.

(b) *Gel chromatography*. By using a series of gel-chromatographic fractionations, it was possible to separate four major fractions of diminishing hydrodynamic size from the 'core' proteins of both species (see Schemes 1 and 2 and Figs 3 and 4). The percentage distribution of 230nm-absorbing material between fractions was: IP, 20.0; IIP, 27.0; IIIP, 35.0; IVP, 14.2; IVP(a), 4.3; IB, 49.0; IIB, 27.5; IIIB, 9.0; and IVB, 14.5. These fractions corresponded to components that separated on disc electrophoresis (Figs. 1b–1d and 2b–2e), the fractions of smallest

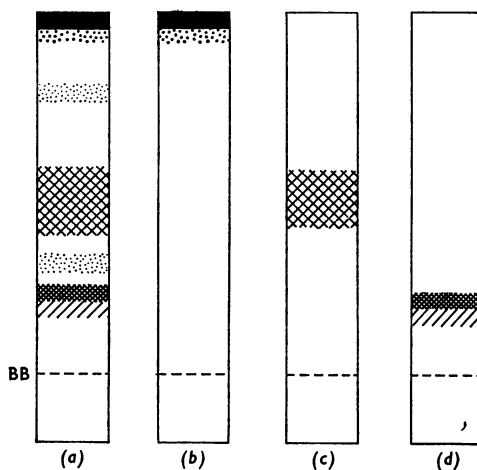


Fig. 1. *Electrophoresis on 5.6% polyacrylamide gels of fractions of 'core' proteins obtained, as shown in Fig. 3, from disaggregated proteoglycans of pig cartilage after hyaluronidase digestion (see Scheme 1)*

(a) Unfractionated 'core' proteins; faintly stained bands not evident in purified subfractions. (b) fraction E; (c) fraction IIIP; (d) fraction IVP; BB, Bromophenol Blue marker band.

hydrodynamic size corresponding to those with the greatest electrophoretic mobility. Two gel-chromatographic fractions from the pig preparation, namely fraction IP, which was excluded from 6% agarose, and fraction IIP, which was retarded by 6% agarose but excluded from 8% agarose, did not penetrate the disc-electrophoresis gel (Fig. 1*b*), and neither did one chromatographic fraction from the bovine preparation, namely fraction IB, which was retarded by 6% agarose but excluded from 8% agarose (Fig. 2*b*).

separation of the four *N*-terminal amino acids present in the pig 'core'-preparation fractions (Table 1). Alanine, the major *N*-terminal amino acid (qualitative assessment), was present in each fraction, whereas the other three *N*-terminal amino acids were unevenly distributed between fractions. All the fractions from the ox preparation contained

General composition

The proportions of uronic acid, galactosamine, glucosamine and protein were determined in order to assess the amounts of residual chondroitin sulphate and keratan sulphate in each fraction (Tables 3 and 4).

The fractions of smallest hydrodynamic size, fraction IVP(a) of the pig preparation, and fractions IIIB and IVB of the ox preparation, contained less glucuronic acid and galactosamine relative to protein, hence there was less residual chondroitin sulphate than in the other fractions. Since they also had considerably less glucosamine, they contained less keratan sulphate than proteoglycans of larger hydrodynamic size, whose overall composition was remarkably similar (Tables 3 and 4), even though their size varied considerably.

Distribution of N-terminal amino acids in 'core' protein fractions

Fractionation of 'core' proteins on the basis of hydrodynamic size did not result in a complete

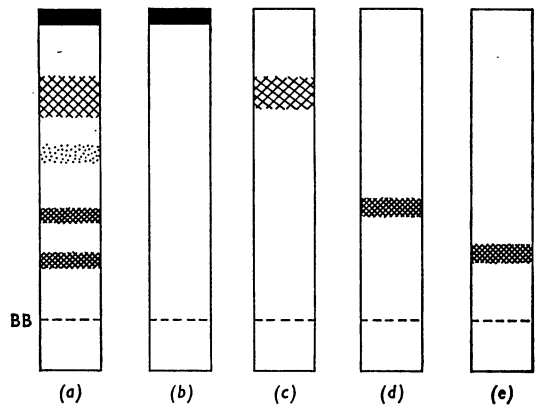
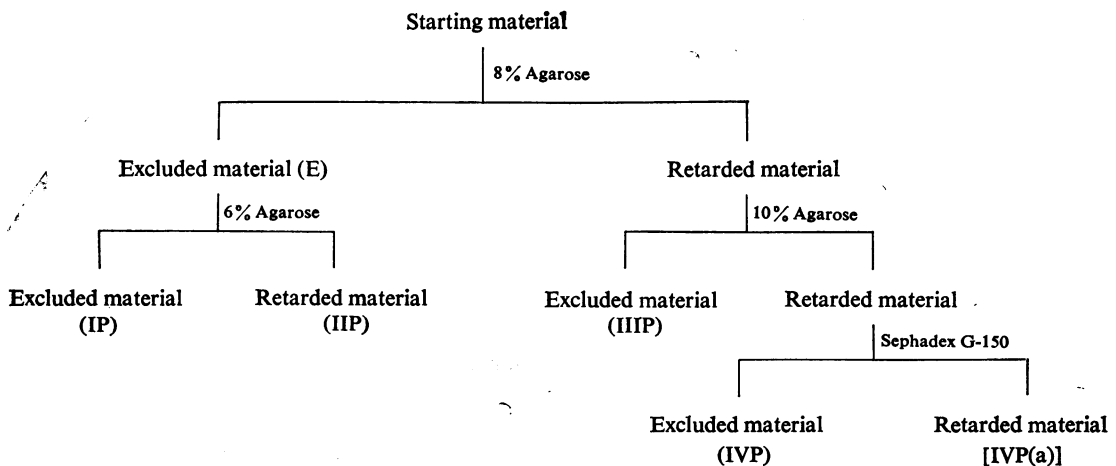


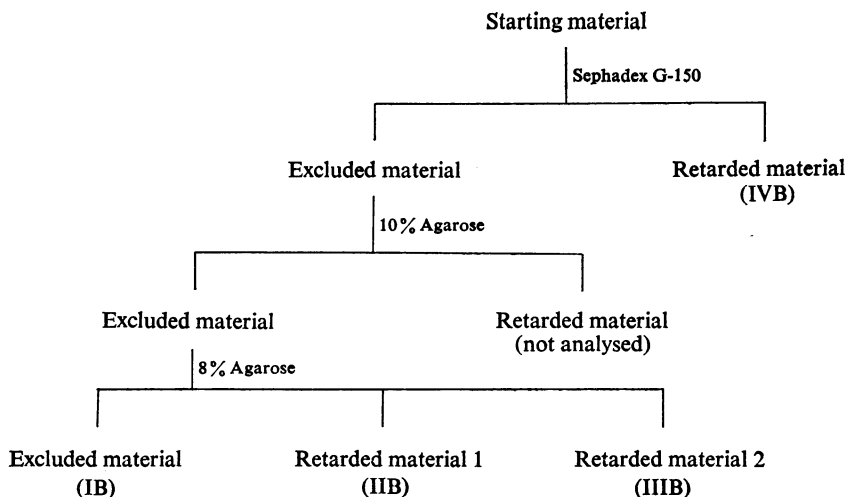
Fig. 2. Electrophoresis on 5.6% polyacrylamide gels of fractions of 'core' proteins obtained, as shown in Fig. 4, from disaggregated proteoglycans of bovine nasal cartilage after hyaluronidase digestion (see Scheme 2)

(a) Unfractionated 'core' proteins; faintly stained band not evident in purified subfraction. (b) fraction IB; (c) fraction IIB; (d) fraction IIIB; (e) fraction IVB.



Scheme 1. Gel-chromatographic fractionation of pig 'core' proteins

Fractions IP-IVP(a) are in order of decreasing hydrodynamic size. For further details see the text and Fig. 3.



Scheme 2. Gel-chromatographic fractionation of bovine 'core' proteins

Fractions IB–IVB are in order of decreasing hydrodynamic size. Note that in this separation, gels of increasing porosity were used. For further details see the text and Fig. 4.

alanine, and only one fraction, namely IIB, contained glycine, the only other *N*-terminal amino acid detected in the original preparation (Table 2).

Amino acid composition

(a) *Pig 'core' proteins.* The amino acid composition of the four 'core'-protein fractions of the pig preparations were similar, and typical of proteoglycans in general (Table 5). Four amino acids, namely serine, glutamic acid, proline and glycine, accounted for 50–55% of the total residues in each fraction. Despite these similarities, however, there were distinct differences between fractions. The glutamic acid content increased with the hydrodynamic size of the fractions. Thus whereas the fraction of largest hydrodynamic size, fraction IP, had 170 residues of glutamic acid/1000 residues, the fraction of smallest size that was analysed, fraction IVP, had 135 residues of glutamic acid/1000 residues. Under the conditions of hydrolysis used, only the two larger components contained detectable amounts of methionine or cysteine. Fraction IP contained measurable amounts of methionine, but no detectable cysteine, whereas fraction IIP contained detectable cysteine, but no methionine.

(b) *Ox 'core' proteins.* The four major 'core' protein fractions from the ox preparation were also very similar in amino acid composition (Table 6). As with the pig 'core' proteins, serine, glutamic acid, glycine and proline were the major amino acids and together accounted for 49–52% of the total residues

in each fraction. However, there were again differences between the fractions, although the size-related trend of glutamic acid content found in the pig 'core'-protein fractions was less apparent in the ox 'core'-protein fractions. The fraction of smallest hydrodynamic size had 131 residues of glutamic acid/1000 residues, whereas the other three fractions had 149–155 residues of glutamic acid/1000 residues. The four fractions differed in glycine, aspartic acid and alanine contents (Table 6). Under the conditions of hydrolysis used, no methionine was detected in any fraction, and cysteine was detectable only in fraction IB of larger hydrodynamic size.

Carbohydrate-protein linkage

(a) *β -Elimination.* Treatment of the pig 'core'-protein fractions with 0.5M-NaOH resulted in the destruction of 85–90 residues of serine/1000 residues in fractions IIP and IVP and 70 residues of serine/1000 residues in fraction IIIP (Table 7). At the same time, 19–24 residues of threonine/1000 residues were destroyed in each fraction and, although the proportion of the total residues of threonine destroyed diminished with increasing hydrodynamic size, the ratio of serine/threonine residues destroyed remained fairly constant in each fraction (Table 7).

The situation with the ox 'core'-protein fractions was, however, somewhat different (Table 8). Although the larger fractions IB or IIB were of similar general analysis, there was an almost twofold difference between them in the amounts of

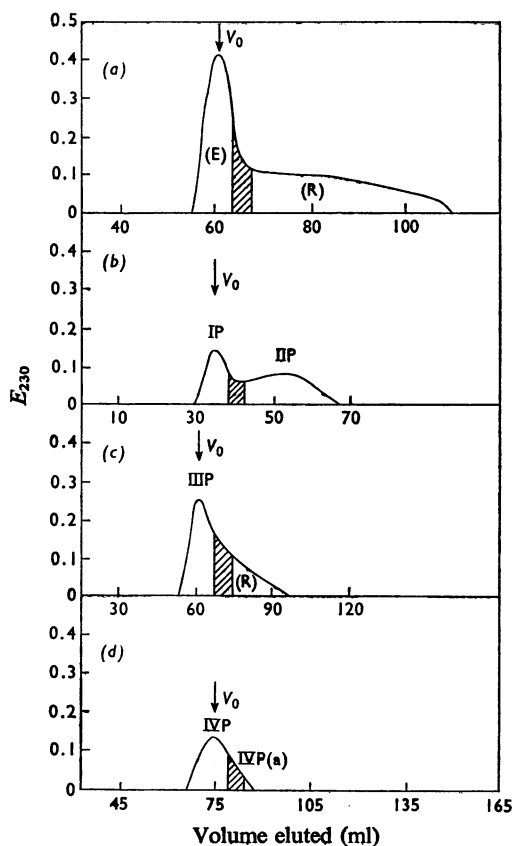


Fig. 3. Fractionation of 'core' proteins obtained from disaggregated proteoglycans of pig laryngeal cartilage after hyaluronidase digestion, by a series of gel-chromatographic procedures (see Scheme 1).

(a) Initial fractionation on a column (1.2cm \times 170cm) of 8% agarose. (b) Further fractionation of excluded material (E) from (a) on a column (1.1cm \times 95cm) of 6% agarose to give peaks IP and IIP. (c) Further fractionation of retarded material (R) from (a) on a column (1.4cm \times 130cm) of 10% agarose giving peak IIIIP. (d) Further fractionation of retarded material (R) from (c) using a column (1.6cm \times 110cm) of Sephadex G-150 giving peaks IVP and IVP(a). The fractions represented by the shaded areas were rechromatographed. The columns were equilibrated and eluted with 0.1M-NaCl at 4°C. Note that the scales shown in the abscissa are all different. V_0 , Void volume.

serine and threonine destroyed, although like the pig 'core' proteins, the ratio of serine/threonine residues destroyed was similar.

(b) *Sugars of the linkage region and other minor sugar components.* Neutral sugars and sialic acid in the 'core'-protein fractions were determined by g.l.c. As shown in Table 9, the proportion of xylose was

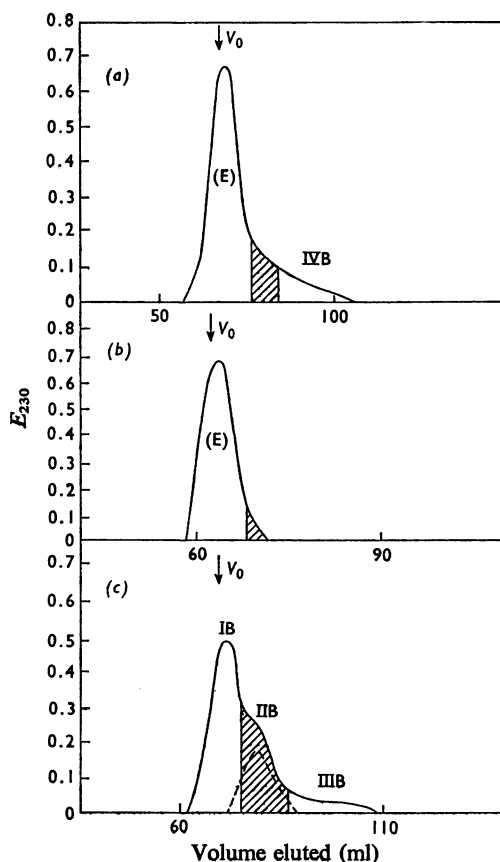


Fig. 4. Fractionation of 'core' proteins obtained from disaggregated proteoglycans of bovine nasal cartilage after hyaluronidase digestion, by using a sequence of gels of increasing porosity (see Scheme 2)

(a) Initial fractionation on a column (1.4cm \times 120cm) of Sephadex G-150 giving fraction IVB. (b) Further fractionation of excluded material (E) from (a) on a column (1.4cm \times 130cm) of 10% agarose. (c) Final fractionation of excluded material from (b) on a column (1.6cm \times 110cm) of 8% agarose gave peaks IB, IIB and IIIB. Material in central area was rechromatographed to give fraction IIB, which was eluted as shown by the dotted lines. Materials in shaded areas were rechromatographed. All columns were equilibrated and eluted with 0.1M-NaCl at 4°C. Note that the scales shown in the abscissa are all different.

approximately equivalent to the number of serine residues destroyed by β -elimination, which is consistent with the structure of the chondroitin sulphate-protein linkage region, where each chain is attached to serine residues of the protein core by the trisaccharide sequence galactosyl-galactosyl-xylosyl-

Table 3. Analysis of fractions of 'core' proteins obtained, as shown in Scheme 1 and Fig. 3, from disaggregated proteoglycans of pig laryngeal cartilage after hyaluronidase digestion

Protein is expressed as bovine serum albumin equivalents, hexosamines as hydrochlorides, and uronic acid as glucuronolactone.

Intact proteoglycan Fractions	Weight (relative to protein)				Molar ratio galactosamine/ glucosamine
	Hexosamine	Uronic acid	Glucosamine	Galactosamine	
	2.44	1.7	0.17	2.27	13.30
IP	0.54	0.43	0.27	0.27	1.02
IIP	0.44	0.31	0.23	0.21	0.94
IIIP	0.49	0.40	0.27	0.22	0.84
IVP	0.41	0.38	0.22	0.19	0.83
IVP(a)	0.29	0.21	0.11	0.18	1.70

Table 4. Analysis of fractions of 'core' proteins, obtained as shown in Scheme 2 and Fig. 4, from disaggregated proteoglycans of bovine nasal septum after hyaluronidase digestion

Protein is expressed as bovine serum albumin equivalents, hexosamines as hydrochlorides and uronic acid as glucuronolactone.

Fractions	Weight (relative to protein)				Molar ratio galactosamine/ glucosamine
	Hexosamine	Uronic acid	Glucosamine	Galactosamine	
IB	0.59	0.40	0.33	0.26	0.78
IIB	0.55	0.40	0.35	0.20	0.57
IIIB	0.15	0.25	0.06	0.09	1.64
IVB	0.20	0.20	0.08	0.12	1.51

Table 5. Amino acid composition of fractions of 'core' proteins obtained, as shown in Scheme 1 and Fig. 3, from disaggregated proteoglycans of pig laryngeal cartilage after hyaluronidase digestion

Compositions are mean values of duplicate analyses; underlined values differ from two or more of the other fractions by an amount greater than that of the largest difference between duplicate analyses in that line.

Fractions ...	Composition (residues/1000 residues)			
	IP	IIP	IIIP	IVP
Asp	60.6	60.0	58.1	62.5
Thr	54.2	61.4	56.9	66.0
Ser	142.7	140.2	141.0	140.8
Glu	170.1	162.8	152.8	135.3
Pro	88.6	89.8	85.2	81.2
Gly	154.3	152.4	158.5	149.0
Ala	75.5	79.3	77.7	80.0
$\frac{1}{2}$ -Cys		4.7		
Val	53.2	51.0	64.0	65.0
Met	1.0			
Ile	33.8	30.1	33.6	32.8
Leu	69.4	68.1	74.2	82.0
Tyr	19.6	20.6	16.1	19.0
Phe	22.1	25.4	23.8	30.0
His	6.9	8.0	10.2	9.9
Lys	10.4	9.5	13.7	11.0
Arg	32.3	31.5	36.0	34.4

Table 6. Amino acid composition of fractions of 'core' proteins, obtained as shown in Scheme 2 and Fig. 4, from disaggregated proteoglycans of bovine nasal septum after hyaluronidase digestion

Compositions are mean values of duplicate analyses; underlined values differ from two or more of the other fractions, by an amount greater than that of the largest difference between duplicate analyses in that line.

Fractions ...	Composition (residues/1000 residues)			
	IB	IIB	IIIB	IVB
Asp	63.3	51.1	54.8	75.9
Thr	53.0	57.4	61.8	62.2
Ser	110.8	121.2	136.2	116.1
Glu	152.1	155.9	149.4	131.9
Pro	102.7	120.3	105.9	112.0
Gly	152.2	119.4	132.3	125.2
Ala	74.2	68.0	69.2	75.9
$\frac{1}{2}$ -Cys				
Val	60.5	60.8	65.8	60.7
Met	2			
Ile	29.4	33.8	36.2	35.7
Leu	76.5	76.5	85.2	75.1
Tyr	19.7	20.2	18.2	24.2
Phe	34.5	36.6	32.7	30.8
His	12.3	12.5	11.8	15.1
Lys	29.6	21.3	15.8	23.6
Arg	26.9	34.4	35.0	43.0

Table 7. Destruction of serine and threonine in pig laryngeal cartilage 'core' proteins by alkaline β -elimination

Results are mean values of duplicate analyses.

Fractions	Composition (residues/1000 residues)						Ratio of serine/ threonine residues destroyed
	Threonine			Serine			
	Original	After β -elimination	Destroyed	Original	After β -elimination	Destroyed	
IP	54	30	24 (45%)	143	53	90 (65%)	3.8
IIP	61	39	22 (37%)	140	50	90 (64%)	4.1
IIIP	57	38	19 (33%)	141	71	70 (49%)	3.7
IVP	66	44	22 (33%)	141	56	85 (60%)	3.9

Table 8. Destruction of serine and threonine in bovine cartilage 'core' proteins by alkaline β -elimination

Results are mean values of duplicate analyses.

Fractions	Composition (residues/1000 residues)						Ratio of serine/ threonine residues destroyed
	Threonine			Serine			
	Original	After β -elimination	Destroyed	Original	After β -elimination	Destroyed	
IB	53	41	12 (22%)	111	80	30 (28%)	2.6
IIB	57	35	22 (38%)	121	48	73 (59%)	3.3
IIIB	62	—	—	136	—	—	—
IVB	62	47	15 (25%)	116	76	40 (35%)	2.7

Table 9. Neutral sugar composition of fractions of 'core' proteins obtained, as shown in Fig. 3, from disaggregated proteoglycans of pig laryngeal cartilage after hyaluronidase digestion

Note that linkage-region sugars are xylose and galactose, whose molar ratios to serine residues destroyed on β -elimination are shown in parentheses. Results are mean values of duplicate analyses.

Fractions	Composition [mmol/g of protein (bovine serum albumin equivalents)]							
	Amino acid destroyed by β -elimination calculated from Table 7							
	Serine	Threonine	Xylose	Galactose	Mannose	Glucose	Sialic acid	Fucose
IP	11.0	2.8	12.2 (1.1)	36.6 (3.3)	1.6	—	0.1	0.5
IIP	7.7	2.0	9.0 (1.2)	28.0 (3.6)	1.2	—	0.21	0.5
IIIP	10.4	2.8	13.1 (1.3)	20.7 (2.0)	1.7	1.3	—	—
IVP	7.9	1.9	5.4 (0.7)	33.86 (4.3)	1.9	1.8	2.7	—
IVP(a)	—	—	5.5	8.25	0.9	1.6	0.55	Trace

serine (Rodén & Armand, 1966; Rodén & Smith, 1966; Helting & Rodén, 1968).

The excess of galactose over the predicted ratio of 2:1 to xylose in fractions IP, IIP and IVP indicated the presence of keratan sulphate. If it is assumed that galactose and glucosamine are present in keratan sulphate in approximately equimolar proportions, the galactose due to keratan sulphate may be calculated from the glucosamine analyses (Table 3). When this is subtracted, the molar ratio of the remaining galactose to xylose was 1.98 and 1.94 in

fractions IP and IIP respectively. However, the analyses of the other fractions were less consistent, since in fractions IIIP and IVP(a) the molar ratio galactose/xylose was below 2, even without taking into consideration the small amounts of keratan sulphate that were present. In addition to the sugars of the chondroitin sulphate-protein linkage region, four other sugars, namely mannose, glucose, sialic acid and fucose, were detected in the various fractions. Three of these sugars, namely sialic acid (Gregory & Rodén, 1961; Seno *et al.*, 1965), fucose

Table 10. *Neutral sugar composition of two fractions of 'core' proteins obtained, as shown in Fig. 4, from disaggregated proteoglycans of bovine nasal cartilage after hyaluronidase digestion*

Note that linkage-region sugars are xylose and galactose, whose molar ratio to serine residues destroyed on β -elimination are shown in parentheses. Results are mean values of duplicate analyses.

Composition [mmol/g of protein (bovine serum albumin equivalents)]								
Fractions	Amino acid destroyed by β -elimination calculated from Table 8		Xylose	Galactose	Mannose	Glucose	Sialic acid	Fucose
	Serine	Threonine						
IB	2.93	1.1	2.6 (0.89)	16.6 (5.7)	0.99	2*	0.63	—
IVB	7.1	2.6	11.6 (1.6)	16.5 (2.3)	1.9	3.8	—	—

* Interference by unidentified component giving elevated value.

(Seno *et al.*, 1965) and mannose (Bhavanandan & Meyer, 1968), are minor constituents of keratan sulphate. However, since glucose, mannose and sialic acid were found in fractions of smaller hydrodynamic size [fractions IIIP, IVP and IVP(a)], which contained only very small amounts of keratan sulphate, it is possible that some of these sugars may be attached in some other way. In contrast, the fractions of largest hydrodynamic size (fractions IP and IIP) contained appreciable amounts of fucose, but no detectable glucose. Although the amounts of detectable sugars were of the same low order, they were not uniformly distributed amongst the various fractions, and it therefore seems improbable that they arose simply from contamination by the chromatographic gels.

Only two of the bovine 'core'-protein fractions (IB and IVB) were analysed by g.l.c. (Table 10). There was good agreement between the number of xylose residues present in fraction IB and the number of serine residues destroyed by β -elimination. The ratio of galactose to xylose residues exceeded the predicted value of 2:1, which was consistent with the presence of keratan sulphate, although the excess was less than expected from the glucosamine content. The analysis of fraction IVB was more anomalous because considerably more xylose residues were present than serine residues destroyed by β -elimination (1.6:1). On the other hand, when the galactose attributable to keratan sulphate (assessed by glucosamine content) was subtracted, the ratio of galactose residues to serine residues destroyed was 1.8:1, which is close to the theoretical ratio of 2:1. Fraction IB contained sialic acid and both fractions IB and IVB contained mannose and glucose, but neither contained fucose.

Discussion

Treatment of disaggregated proteoglycans with the purified testicular hyaluronidase used here

removed approx. 85% of the chondroitin sulphate without apparent cleavage of peptide bonds (because *N*-terminal amino acids were the same before as after enzyme treatment). The resultant 'core' proteins were not homogeneous on gel chromatography, and separated into discrete bands on electrophoresis. These results are similar to those of Heinegård (1972*b*), who obtained three fractions on gel chromatography after hyaluronidase digestion of bovine tracheal proteoglycans. It seems unlikely that cleavage of peptide bonds had occurred during the extraction procedure, because the proteoglycans were extracted with 4M-guanidinium chloride, which would render most enzymes inactive, since it is a strongly denaturing solvent. Moreover, there is considerable indirect evidence from studies of the structure (Tsiganos & Muir, 1969; Hardingham & Muir, 1974), immunological properties (Tsiganos & Muir, 1969; Brandt *et al.*, 1973) and the biosynthesis (Hardingham & Muir, 1972*b*) of proteoglycans that the heterogeneity does not arise during the extraction but exists in the tissues initially. In the present study, a general similarity in the range of sizes of 'core' proteins from each species and in the number of components was found. Analytical differences between fractions also showed similar trends, which suggest that the proteoglycans were comparable in the two species.

In both species, the core proteins of smallest hydrodynamic size [fractions IVP(a) and IVB] and greatest electrophoretic mobility, contained little keratan sulphate (Tables 3 and 4). They probably arose from the small proportion of proteoglycans in cartilage that are of smaller hydrodynamic size and contain less keratan sulphate than the majority, and which may be extracted with solutions of low ionic strength such as 0.15M-sodium acetate or -NaCl (Tsiganos & Muir, 1969; Brandt & Muir, 1971*a*; Simunek & Muir, 1972; Mayes *et al.*, 1973; Hardingham & Muir, 1974). Proteoglycans in such extracts were predominantly non-aggregated,

and were not associated with hyaluronic acid or protein-link components (Hardingham & Muir, 1974). These non-aggregated proteoglycans were unable to interact with hyaluronic acid (Hardingham & Muir, 1974). Likewise, Hascall & Heinegård (1974b) found that only 70–75% of proteoglycan 'core' proteins (produced by chondroitinase digestion) would bind to hyaluronic acid.

Proteoglycans of smallest hydrodynamic size differ from larger proteoglycans in reacting directly with antibody, whereas larger proteoglycans, even after disaggregation, must first be digested with hyaluronidase before they will react (Brandt *et al.*, 1973). Small proteoglycans also differ in having fewer antigenic determinants than larger proteoglycans, possessing only determinants common to all proteoglycans (Brandt *et al.*, 1973), which are situated on the region of the 'core' protein to which chondroitin sulphate chains are attached (Baxter & Muir, 1972). Isotope experiments *in vitro* showed that such proteoglycans were neither precursors nor breakdown products of the majority of proteoglycans (Hardingham & Muir, 1972b).

In contrast with proteoglycans of smallest hydrodynamic size, the 'core' proteins of those of larger hydrodynamic size contained appreciable amounts of keratan sulphate (Tables 3 and 4). A consistent finding was that an increase in the amount of keratan sulphate in proteoglycans is accompanied by an increase in protein content (Muir & Jacobs, 1967; Pedrini, 1969; Tsiganos & Muir, 1969, 1970; Brandt & Muir, 1969, 1971a,b; Tsiganos *et al.*, 1971; Simunek & Muir, 1972). Thus although the ratio of chondroitin sulphate to protein may vary in different fractions, the ratio of keratan sulphate to protein is generally remarkably constant (Hoffman *et al.*, 1967; Brandt & Muir, 1969; Tsiganos *et al.*, 1971). This was also true for the larger 'core' proteins even though individually they differed considerably in size.

The linkages between skeletal keratan sulphate and protein have yet to be established in detail. Many of the linkages are labile to alkali and involve the hydroxyl groups of threonine, and to a lesser extent those of serine residues (Anderson *et al.*, 1965; Seno *et al.*, 1965; Bray *et al.*, 1967; Tsiganos & Muir, 1967). The threonine residues destroyed by β -elimination (Tables 7 and 8) would represent keratan sulphate-threonine linkages. It is notable that the ratio of serine residues destroyed to threonine residues destroyed was similar in all fractions. This suggests that in the smaller 'core' proteins, which had mainly chondroitin sulphate side chains, the small amount of keratan sulphate that is present may consist of rather short chains. There is evidence for differences in the size of keratan sulphate isolated from sub-fractions prepared from hyaluronidase digests of

disaggregated proteoglycans of bovine tracheal cartilage (Heinegård, 1972b).

The majority of proteoglycans interact with hyaluronic acid. From the stoichiometry of the interaction, it can be deduced that they possess a single binding site (Hardingham & Muir, 1972a,b). This region of the molecule is largely devoid of glycosaminoglycans (Heinegård & Hascall, 1974), its conformation being maintained by disulphide bonds. Thus reduction of disulphide bonds interfered with aggregation (Hascall & Sajdera, 1969), and reduction and alkylation of disaggregated proteoglycans prevented their interaction with hyaluronic acid (Hardingham & Muir, 1974).

Partial proteolysis of aggregated proteoglycans shows that some keratan sulphate chains are situated near the hyaluronic acid-binding site (Heinegård & Hascall, 1974). Whether the remainder is a simple substituent of the 'core' proteins, or links together smaller units, is a matter of conjecture (Rodén, 1970).

Several workers (Toda & Seno, 1970; Hascall & Riolo, 1972; Heinegård, 1972b) have found considerable amounts of glutamic acid associated with the keratan sulphate isolated by alkaline and proteolytic degradation of proteoglycans, and it is suggested that glutamic acid or a derivative is involved in an alkali-stable linkage between keratan sulphate and protein. It is notable that the larger 'core' proteins, which are richer in keratan sulphate than other fractions, contained more glutamic acid.

Heinegård & Hascall (1974) have suggested that proteoglycans are constructed of an invariant region that constitutes the hyaluronic acid combining site and a variable region to which the glycosaminoglycans are attached. So far as the larger proteoglycans are concerned, the present results are in keeping with this model. Smaller proteoglycans, which do not interact with hyaluronic acid, may be of simpler construction, lacking all or part of the combining site. These have a simpler amino acid composition and, although considerably smaller than other proteoglycans, the ratio of carbohydrate to protein is higher (Tsiganos & Muir, 1969; Hardingham & Muir, 1974), which is not compatible with the proposed model, where carbohydrate content should decrease with size. There would thus appear to be two classes of proteoglycans in cartilage; the majority which are able to aggregate and interact with hyaluronic acid, having a more complex partially variable structure as proposed by Heinegård & Hascall (1974), and a minority of simpler structure that do not aggregate or interact with hyaluronate, which can be extracted from cartilage with solutions of low ionic strength. That these may have some special function in articular cartilage is suggested by the changes in their relative proportions during development (Brandt &

Muir, 1969; Simunek & Muir, 1972). The present results in two species provide further evidence that there is more than one variety of proteoglycan in cartilage.

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