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The Alkaline Cleavage and Borohydride Reduction of Cartilage Proteoglycan

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A method for the rapid isolation and purification of proteoglycan by using neutral solutions of LiBr for extraction and density-gradient centrifugation is described. The effect of 0.5M-KOH on isolated proteoglycan has been studied by using NaB^3H_4 to reduce and label the chondroitin sulphate chains released. This study has established: (*a*) that at least 95% of the chondroitin sulphate chains are attached to the proteoglycan by alkali-labile bonds between xylose and serine; (*b*) that random degradation of the chondroitin sulphate chains does not occur to any significant extent; (*c*) that the method is convenient for the determination of polysaccharide number-average molecular weights.

The extraction of proteoglycan from cartilagenous tissues in an undegraded state appears to be a virtually insurmountable problem. Methods involving high-speed disruption of the tissue at low temperatures are suspect owing to the deleterious effects of shear on the macromolecules (Sajdera & Hascall, 1969; Hascall & Sajdera, 1969), whereas long periods of extraction under less-vigorous conditions are to be avoided because of the likelihood of proteolytic attack by endogenous enzymes within the tissue (Buddecke & Platt, 1966).

The use of concentrated salt solutions of high ionic strength, which facilitate the extraction of proteoglycan (Sajdera & Hascall, 1969), provides a method that avoids the difficulties mentioned above, but because the effect of such high salt concentrations is poorly understood this method may well cause undetermined changes in the native structure of the proteoglycan molecules.

In the present paper, we report a simple and rapid procedure for the extraction of proteoglycan from bovine nasal cartilage and bovine intervertebral disc in concentrated LiBr solutions that avoids mechanical shear and minimizes the chance of proteolytic attack. Structural studies on the isolated proteoglycan confirm that no breakdown of the polysaccharide chains occurs during the extraction.

The structure of proteoglycan from bovine nasal cartilage has been investigated by many laboratories (Sajdera & Hascall, 1969; Hascall & Sajdera, 1969; Pal *et al.*, 1966; Franek & Dunstone, 1967; Rodén, 1970; Tsiganos & Muir, 1969; Luscombe & Phelps, 1967).

The polysaccharide chains are predominantly chondroitin 4-sulphate and occur covalently bound to a protein core through a well-defined linkage oligosaccharide composed of two galactose residues and a xylose residue. The xylose residue is glycosidically linked to the OH groups of serine in the protein. A minor quantity of keratan sulphate and of chondroitin 6-sulphate is also associated with the same molecule, but the nature of the association is not clear despite a great deal of investigation (Rodén, 1970).

The studies outlined in the present paper were undertaken to answer the following three questions about the structure of the proteoglycan. (*a*) Are all of the chondroitin sulphate chains attached to protein by glycosidic bonds between xylose and serine or do other types of linkage occur? (*b*) Does treatment with alkali, which is known to cleave the bond between xylose and serine by a β-elimination reaction, result in any non-specific cleavage of the polysaccharide chains themselves? (*c*) What is the nature of the molecular-weight distribution of the free chondroitin sulphate chains?

These questions were prompted by the difficulties associated with determining the molecular weight of isolated chondroitin sulphate chains. The xylose content of proteoglycan preparations has been used to calculate a number-average molecular weight (Tsiganos & Muir, 1969), yet such a calculation would be in error if any other type of chondroitin sulphate–protein linkage, not involving xylose, were present. Many studies have employed alkaline cleavage of the proteoglycan, which is believed to be more reliable than proteolytic digestion for releasing single chondroitin sulphate chains (Seno *et al.*, 1965), but indiscriminate cleavage of the polysaccharide chains during extraction of the proteoglycan or during the alkaline treatment itself would render subsequent physical determinations of molecular weight most unreliable.

The present study demonstrates that non-specific cleavage of chondroitin sulphate does not occur during alkaline treatment of proteoglycan from bovine

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nasal cartilage at 0–4°C and that more than 95% of the chains are linked by a glycosidic bond between xylose and serine (or possibly threonine).

A preliminary communication of these results has been published (Hopwood & Robinson, 1970).

Materials and Methods

Materials

Cartilage powder. Bovine nasal septum cartilage and intervertebral discs were obtained from adult steers within 1 h of slaughter and chilled in ice. The cartilage was cleaned of adhering tissues and sliced to give coarse fragments which were immediately frozen by stirring with powdered solid CO_2 and then immersed in liquid N_2. The frozen tissue was ground to a fine powder by means of a Wiley Intermediate Mill (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.) with a 20-mesh attachment. The powdered tissue was stored at −20°C.

Cartilage proteoglycan. (*a*) Extraction with LiBr. Milled cartilage (10 g wet wt., 1.99 g dry wt.) was extracted for 4 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 cartilage slurry was mixed during the extraction with gentle rocking agitation. The residue was separated by centrifugation at 4°C for 60 min at 10000*g* and the supernatant solution was adjusted to a density of 1.51 g/ml by addition of 98 g of LiBr, $2H_2O$/100 ml. The resulting solution was fractionated by density-gradient centrifugation at 120000*g* for 48 h at 4°C (rotor 50-Ti, Beckman Spinco preparative ultracentrifuge, 44000 rev./min). The tube contents were divided into three equal fractions and the corresponding fractions from each tube were pooled. Samples of each fraction were taken for analysis and the remainder of the bottom fraction was mixed with 2 vol. of LiBr solution ($\rho = 1.51$ g/ml) and re-centrifuged for a further 24 h at 120000*g*. The bottom fraction from the second centrifugation was dialysed against water at 4°C until the diffusate was free of LiBr and stored frozen at −20°C (yield = 0.53 g).

(*b*) Extraction with NaCl. Milled cartilage (135 g wet wt., 26.8 g dry wt.) was extracted as described above for 4 h at 4°C with 1400 ml of 0.15 M-NaCl containing imidazole–HCl buffer, pH 7.2 (0.05 M in Cl^-). The insoluble residue was removed by centrifugation and the proteoglycan was isolated from the supernatant solution by density-gradient centrifugation in potassium citrate solution, pH 7.0, at an initial density of 1.46 g/ml (rotor 30, Beckman Spinco model L preparative ultracentrifuge, 27000 rev./min for 24 h at 4°C). The contents of each centrifuge tube were divided into four equal fractions and the corresponding fractions from each tube were combined, dialysed exhaustively against water and assayed for hexuronate content, protein content and dry weight. The bottom fraction contained the bulk of the applied hexuronate in the form of a gelatinous plug of proteoglycan which redissolved during dialysis. This fraction was stored frozen at −20°C (yield = 1.6 g).

Other materials. Reagent-grade LiBr from British Drug Houses Ltd. (Poole, Dorset, U.K.) was recrystallized as the dihydrate. Cyclohexanone and acetylacetone were redistilled before use. $NaBH_4$ (assayed as 92% by weight) was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) and sodium [^{3}H]borohydride (502 mCi/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Chondroitin sulphate lyase from *Proteus vulgaris* was prepared as described previously (Robinson & Dorfman, 1969). Galactose dehydrogenase was purchased from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany) and Pronase from Calbiochem (Los Angeles, Calif., U.S.A.).

Sephadex G-50 (fine grade) was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and Bio-Gel P-4 (50–100 mesh), Dowex 50 (X4, 100–200 mesh) and Dowex 1 (X4, 200–400 mesh) from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Whatman Chromedia ET-II cellulose (ECTEOLA) was purchased from W. and R. Balston Ltd., Maidstone, Kent, U.K. (nominal capacity, 0.5 mequiv./g). All ion-exchange materials were thoroughly washed and treated to remove fine material before use.

All other reagents were of analytical grade.

Methods

Extraction of proteoglycan from milled tissue. The optimum conditions for extraction were determined by using a standard procedure. Milled tissue (0.6 g wet wt.) was extracted at 4°C for a fixed interval in 12 ml of the appropriate electrolyte solution with gentle rocking agitation. At the end of the extraction period, the residue was removed by centrifugation and the proteoglycan was preciptitated from a sample of the supernatant solution by the addition of 2 vol. of ethanol.

The precipitated proteoglycan was washed thoroughly with ethanol and then dried under vacuum for 16 h at room temperature (25°C) over anhydrous silica gel. The dry material was redissolved in water and assayed for hexuronate content.

Total tissue hexuronate was determined in the same way on a separate sample of tissue after complete digestion with Pronase. The efficiency of extraction was then expressed as a percentage of the total hexuronate released from the tissue by Pronase.

Preparation of alkaline borohydride solution. A stock solution of alkaline borohydride was prepared by dissolving 100 mCi of NaB^3H_4 (502 mCi/mmol) in 250 ml of freshly prepared 0.5 M-KOH containing 1.84 g of $NaBH_4$. The solution was prepared at 4°C and stored frozen at −20°C. The stock solution was

diluted with 9vol. of 0.5M-KOH immediately before use to yield a solution approx. 0.02M in borohydride. This solution exhibited no loss of borohydride content after 15 days at 4°C.

The specific radioactivity of the borohydride was determined by reductive labelling of 0.21ml of cyclohexanone with 50ml of the dilute (0.02M) borohydride solution for 18h at room temperature. The reaction was followed by determining the disappearance of borohydride. At the end of the reaction, the excess of borohydride was destroyed by acidification with acetic acid (approx. 1.5ml) and the cyclohexanol was extracted into diethyl ether, evaporated to dryness and esterified with excess of 3,5-dinitrobenzoyl chloride (1.0g) by refluxing in 20ml of anhydrous benzene containing 0.2ml of anhydrous pyridine. The benzene solution was extracted several times with 40ml of 1M-HCl, 40ml of 1M-NaOH and finally with 50ml of water (two extractions). The benzene solution was then evaporated to dryness and the cyclohexyl-3,5-dinitrobenzoate (yield = 0.108g; m.p. 112°C) was recrystallized to constant specific radioactivity (6.65×10^5 d.p.m./μmol) from 50% (v/v) aq. ethanol.

The specific radioactivity was also measured by reducing xylose to xylitol under the same conditions. The xylitol was isolated as xylitol penta-acetate which failed to crystallize. Nevertheless, the measured specific radioactivity of the isolated xylitol penta-acetate was essentially the same as that of the cyclohexanol derivative (6.2×10^5 d.p.m./μmol).

Alkaline cleavage of proteoglycan. Proteoglycan solution containing 1.0–1.5g of the extracted polysaccharide was freeze-dried and the dry material was dissolved by gentle mixing in 50ml of dilute borohydride (0.02M) in 0.5M-KOH at 4°C. Weighed samples (0.5–0.8ml) were removed at periods during the reaction and assayed for borohydride concentration. At the end of the reaction, the excess of borohydride was destroyed by acidification to pH5.0 with acetic acid.

Similar reactions were performed by using 3.0g dry wt. of milled cartilage instead of the isolated proteoglycan preparations.

Reactions were also performed at 4°C with higher concentrations of borohydride. Proteoglycan (0.3g dry wt.) or milled cartilage (0.6g dry wt.) was treated with 10ml of the stock borohydride (0.2M) solution in 0.5M-KOH. The reaction was stopped after 10–11 days by acidification with acetic acid.

Isolation of chondroitin sulphate. The acidified proteoglycan reaction mixtures were applied to a column (28cm×5cm diam.) of Bio-Gel P-4 equilibrated with pyridine–acetic acid buffer, pH5.2 (0.2M in acetic acid). The column was eluted with the same buffer at a flow rate of 48ml/h and 12ml fractions were collected and assayed for hexuronate content and radioactivity. All 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 (Cl^- form; 36cm×2cm diam.) equilibrated with imidazole–HCl buffer, pH5.7 (0.02M in Cl^-). The column was eluted with a linear gradient of NaCl in 0.02M-imidazole–HCl buffer, pH5.7, generated from 500ml of 0.02M-imidazole–HCl buffer and 500ml of 2.0M-NaCl in 0.02M-imidazole–HCl buffer. The flow rate was 48ml/h and 12ml fractions were collected and assayed for protein content, hexuronate content and radioactivity. The chondroitin sulphate was eluted as a single hexuronate-positive peak (Fig. 4, fractions 30–40); it was desalted on Bio-Gel P-4 in pyridine–acetic acid buffer as described above and freeze-dried. When milled cartilage was used in the alkaline cleavage reaction the cartilage residue was removed by centrifugation at 38000*g* (rotor 8×50ml; MSE high-speed 18 centrifuge) and re-extracted with 30ml of 0.15M-NaCl for 3h at 4°C. After an additional centrifugation, the two supernatant fractions were combined and chondroitin sulphate was isolated as described above. The residue was digested for 24h at 37°C with 25mg of Pronase in 55ml of Tris–HCl buffer, pH7.9 (0.1M in Cl^-), containing 0.5mM-$CaCl_2$ and 2 drops of toluene. Any insoluble material remaining after digestion was removed by centrifugation and chondroitin sulphate was isolated from the supernatant solution as described above.

Measurement of the specific radioactivity of chondroitin sulphate. Chondroitin sulphate was further purified by precipitation with cetylpyridinium chloride from 0.05M-LiBr solution. The precipitate was then redissolved in 3.0M-LiBr and the chondroitin sulphate was finally isolated as its lithium salt by precipitation with 3vol. of ethanol. The white precipitate was washed thoroughly with ethanol and with ether and dried to constant weight at 70°C over P_2O_5 *in vacuo*. Samples were weighed directly into glass scintillation vials and the radioactivity was determined as described below.

Isolation of the chondroitin sulphate-linkage region. The chondroitin sulphate fraction from ECTEOLA-cellulose was digested exhaustively with chondroitin sulphate lyase from *P. vulgaris* in Tris–HCl buffer, pH8.0 (0.025M in Cl^-), as previously described (Robinson & Dorfman, 1969). The enzyme preparation contained no detectable sulphatase activity and was used at a final concentration of 1 unit/ml. The reaction was followed by measuring the change in absorbance at 235nm and was complete after 20h at room temperature.

The digest was concentrated to approx. 5.0ml at room temperature and applied to a column (80cm×2cm diam.) of Sephadex G-50 equilibrated with 0.2M-pyridine–acetate buffer, pH5.2. The column was eluted with the same buffer at a rate of 36ml/h and 12ml fractions were collected and assayed for hexuronate content and radioactivity

(Fig. 6). The chondroitin sulphate-linkage region was eluted as a single retarded peak (fractions 18–21), well separated from a large peak of disaccharide product (fractions 22–25) and a smaller peak of radioactivity which was eluted immediately after the void volume of the column (fractions 9–14). The pooled fractions containing the linkage region were freeze-dried and subjected to chemical analysis.

Fractionation of the chondroitin sulphate-linkage region. The linkage region was further fractionated by ion-exchange chromatography on Dowex 1 (X4; formate form; 38cm × 1.5cm diam.). The column was eluted with a linear concentration gradient of formic acid generated from 500ml of 0.05M-formic acid and 500ml of 2.5M-formic acid followed by a linear gradient of ammonium formate generated from 500ml of 2.5M-formic acid and 500ml of 2.0M-ammonium formate in 2.5M-formic acid. The column was eluted at a rate of 36ml/h and 12ml fractions were collected and assayed for hexuronate content and radioactivity. For accurate analysis of hexuronate content the formic acid was removed from the pooled fractions by freeze-drying and ammonium formate by de-salting on Sephadex G-50 in pyridine–acetic acid buffer, pH5.2.

Acid hydrolysis of the chondroitin sulphate-linkage region. A sample of the linkage region equivalent to approx. 1.0μmol of xylitol was heated at 100°C for 9h with 0.8ml of 0.05M-HCl and 250mg of Dowex 50 (H^+ form) in a tube sealed under vacuum. The mixture was mixed thoroughly every 2h during the hydrolysis.

After hydrolysis the neutral sugars were recovered by passing the hydrolysate over a column of Dowex 50 (H^+ form; 20cm × 1cm diam.) and then over a column of Dowex 1 (acetate form; 20cm × 1cm diam.).

Analytical methods. Hexuronate was determined by the carbazole method of Dische (1947) and hexosamine by the Boas (1953) modification of the Elson–Morgan reaction, omitting the Dowex treatment. Protein was determined by the Folin method of Lowry *et al.* (1951) with bovine serum albumin (Commonwealth Serum Laboratories, Melbourne, Australia) as standard. The serum albumin standard was dried for 16h under vacuum over P_2O_5 at room temperature.

Total hexose was assayed by the method of Trevelyan & Harrison (1952) with galactose as standard. Galactose was also assayed enzymically with galactose dehydrogenase as described by Lindahl & Rodén (1965). SO_4^{2-} was determined by the method of Antonopoulos (1962). Amino acid analysis was performed after hydrolysis in 6M-HCl for 24h at 100°C in tubes sealed under vacuum by using a Beckman Spinco automatic amino acid analyser (model 120C). Borohydride concentration was assayed by a modification of the method of Lyttle *et al.* (1952). A sample containing approx. 10μmol of borohydride was mixed with 1.0ml of 0.04M-KIO_3 for 30s and then 1.0ml of 25% (w/v) KI in water and 1.0ml of 2M-H_2SO_4 were added and the mixture was stored in darkness for 3min. The liberated I_2 was determined by titration with 0.1M-$Na_2S_2O_3$.

G.l.c. was performed by using a modification of the method described by Clamp *et al.* (1967). Samples of carbohydrate (10–100μg) were mixed with 25μg of D-mannitol and subjected to methanolysis for 24h under reflux with 1.0M-HCl in dry methanol. The methyl glycosides were isolated, converted into trimethylsilyl derivatives and separated by using glass columns (4mm internal diam. × 2.0m) packed with solid support Gas Chrom Q (100–120 mesh, Applied Science Laboratories, Inglewood, Calif., U.S.A.) coated with 2.5% SE-30 ultraphase (Pierce Chemical Co., Rockford, Ill., U.S.A.) in a F & M gas chromatograph model 810 (F & M Scientific Corp., Avondale, Pa., U.S.A.) equipped with hydrogen flame-ionization detectors and temperature programming. Dual-column technique was employed to avoid baseline drift during temperature programming. Peak areas were measured manually and a response factor for each monosaccharide was determined relative to the internal standard of D-mannitol. A multiple-peak pattern was obtained for all monosaccharides except D-mannitol. Positive identification of sugars was made only if both the relative retention and peak pattern corresponded with those of the standard. The amount of each monosaccharide was calculated from the total peak area of monosaccharide corrected with the response factor relative to the peak area of the internal standard of D-mannitol.

Radioactivity was assayed by using a Packard Tri-Carb liquid-scintillation spectrometer model 3003. Aqueous samples (0.5ml) were mixed with 10ml of scintillation mixture prepared as described by Bruno & Christian (1961). Under these conditions, ^3H was determined at 11.3% efficiency.

Dry weight was determined on samples after exhaustive dialysis against water. Samples were dried at 100°C to constant weight and cooled in a desiccator over anhydrous silica gel before weighing.

Paper chromatography was performed on Whatman 3MM paper with descending development in the following solvents: A, ethyl acetate–pyridine–water (8:2:1, by vol.); B, butan-2-one–acetic acid–water saturated with boric acid (9:1:1, by vol.); C, isobutyric acid–aq. 2.0M-NH_3 (5:3, by vol.). Sugars and sugar alcohols were detected by means of alkaline $AgNO_3$. Papers run in solvent B were washed with a solution of HF in acetone (1ml of conc. HF solution in 40ml of acetone) before treatment with alkaline $AgNO_3$.

Sedimentation studies. All experiments were performed in a Spinco model E ultracentrifuge equipped with Schlieren and Rayleigh interference optics. The temperature was maintained at 20°C.

Molecular weights were determined from sedimentation-equilibrium experiments by using the meniscus-depletion method of Yphantis (1964). Filled epon 12mm double-sector cells with sapphire windows were used with fluorocarbon oil [FC-43: Minnesota Mining and Manufacturing (Aust.) Pty. Ltd., Melbourne, Vic., Australia] as an immiscible base fluid. Solutions of chondroitin sulphate (0.2mg/ml) and proteoglycan (0.02mg/ml) in 0.1M-NaCl (buffered at pH6.8 with 0.12mM-$NaHCO_3$) were centrifuged at rotor speeds of 31410rev./min and 4908rev./min respectively for time sufficient to attain equilibrium.

Molecular weights were calculated as described by Yphantis (1964); the values reported are those for the initial solutions before any centrifugal fractionation has occurred (Creeth & Pain, 1967). A value of 0.52 was assumed for the partial specific volume of chondroitin sulphate in this technique.

Sedimentation-velocity experiments were performed at a rotor speed of 50740rev./min as described by Preston (1968).

Results

Extraction and purification of proteoglycan from milled cartilage

Fig. 1 illustrates the percentage of total tissue hexuronate extracted from nasal cartilage by different concentrations of NaCl, $CaCl_2$ and LiBr. Extraction with $CaCl_2$ or LiBr solutions, in contrast with extraction with NaCl, displays an optimum concentration for maximum yield of proteoglycan. These optimum concentrations are similar to those reported by Sajdera & Hascall (1969), but the rate of extraction is considerably faster than that reported by these authors (Fig. 2). This difference is no doubt due to the greater degree of tissue disintegration and the greater surface area of the milled cartilage compared with the sliced tissue that they employed.

Analysis values for the extracted proteoglycan preparations after purification by density-gradient centrifugation are shown in Table 1.

The procedure outlined in Scheme 1 whereby extraction and density-gradient centrifugation are performed in LiBr solutions represents an efficient and straightforward procedure for the isolation of proteoglycan. The analysis values obtained for nasal cartilage proteoglycan at each step are shown in Table 1. With this method, proteoglycan is extracted under conditions of low shear and degradation by proteases is minimized by the short extraction times.

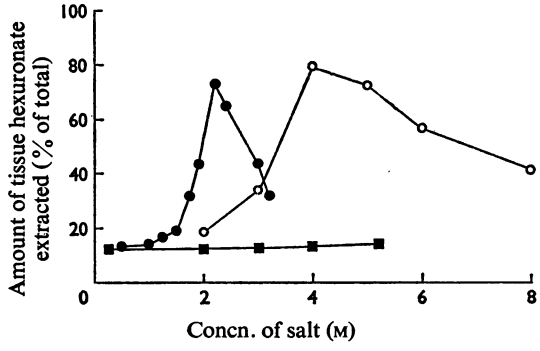


Fig. 1. *Extraction of milled bovine nasal cartilage with neutral salt solutions*

Samples (0.6g wet wt.) of milled cartilage were extracted with 12ml of solution for 4h at 4°C and the extent of extraction of hexuronate-positive material was determined as outlined under 'Methods'. Results were obtained with different concentrations of NaCl (■), $CaCl_2$ (●) and LiBr (○).

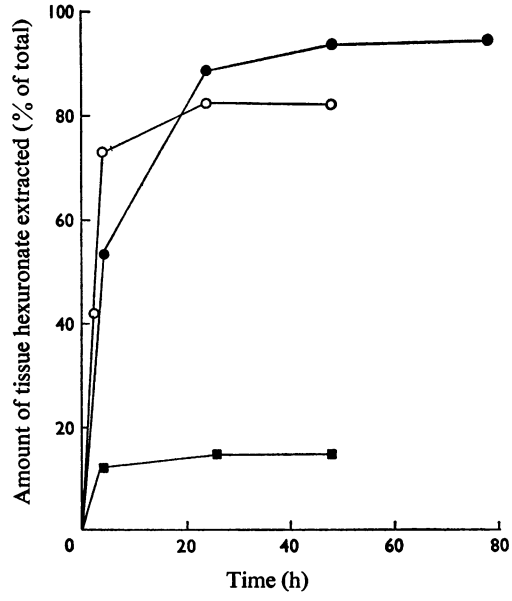


Fig. 2. *Extraction of milled bovine nasal cartilage with neutral salt solutions and with alkali*

Samples (0.6g wet wt.) of milled cartilage were extracted with 12ml of solution at 4°C for different times as shown in the figure. The extent of extraction of hexuronate-positive material was determined as outlined under 'Methods'. Results were obtained with 4.0M-LiBr (○), 0.15M-NaCl (■) and 0.5M-KOH (●).

Table 1. *Analytical values for proteoglycan from milled cartilage*

Sample 1 was a crude extract of bovine nasal septum in 5.3M-LiBr. Samples 2 and 3 were derived from sample 1, after an initial (sample 2) and a final (sample 3) fractionation by centrifugation in LiBr solution ($\rho = 1.51$ g/ml) as shown in Scheme 1. Sample 4 was proteoglycan isolated from bovine nasal septum by extraction with 0.15M-NaCl solution as outlined under 'Materials'. Sample 5 was proteoglycan isolated from bovine intervertebral disc in the same manner as sample 3. Total tissue hexuronate was determined by Pronase digestion and protein, hexuronate, hexose and hexosamine were assayed as described under 'Methods'. Results are expressed as percentage by weight in the dry sodium salt of the proteoglycan. N.D. = not determined.

Sample	Tissue extracted	Tissue hexuronate recovered (% of total)	Proteoglycan composition (%)			
			Protein	Hexuronate	Hexose	Hexosamine
1	Nasal septum	82	15.2	22.0	8.1	N.D.
2	Nasal septum	66	10.3	24.9	N.D.	N.D.
3	Nasal septum	63	6.6	27.5	8.6	29.0
4	Nasal septum	12	13.3	26.2	7.5	27.8
5	Intervertebral disc	72	9.1	15.2	18.7	28.1

High yields of proteoglycan are consistently obtained, but only 82% of the total tissue hexuronate can be extracted. Scheme 1 also shows the subsequent steps during the alkaline cleavage and NaB^3H_4 reduction of proteoglycan and the fractionation and identification of the products.

Alkaline cleavage of the proteoglycan

Fig. 3 shows the time-course of disappearance of borohydride when nasal septum proteoglycan was incubated with 0.02M-NaB^3H_4 in 0.5M-KOH as outlined under 'Methods'. The reaction was judged to be complete after 4 days at 4°C when the loss of borohydride ceased. The rate of the reaction was similar to the rate obtained with a sample of free xylose and considerably slower than the rate of extraction of hexuronate from milled cartilage which was complete after 2 days (Fig. 2), which suggests that the reduction of the reducing sugar released by the alkaline cleavage of the proteoglycan is the rate-determining step.

The reaction mixture obtained after acidification of the alkali-cleaved proteoglycan was chromatographed on Bio-Gel P-4. All the hexuronate was recovered close to the void volume of the column, coinciding with a peak of radioactivity. The pooled void-volume material was then subjected to ion-exchange chromatography on ECTEOLA-cellulose as described under 'Methods'. Fig. 4 shows the elution profile obtained from that procedure. Three main peaks of radioactivity are apparent, labelled E-1, E-2 and E-3 respectively. At least 80% of the protein was eluted in fractions E-1 and E-2 whereas the hexuronate-positive material was confined to fraction E-3.

Table 2 shows the results obtained from this and several other reactions, in which different proteoglycan preparations and milled cartilage were treated with alkaline borohydride. With each reaction carried out with an initial borohydride concentration of 0.02M, the elution profile from ECTEOLA-cellulose was essentially the same as that shown in Fig. 4. Fig. 5, on the other hand, shows a typical elution profile for a reaction carried out in 0.2M-borohydride. At the higher borohydride concentration, a greater percentage of the radioactivity was found in fractions E-1 and E-2; nevertheless, the specific radioactivity (d.p.m./mg of hexuronate) of peak E-3 remained unchanged. Quantitative recovery of the applied radioactivity was obtained for each preparation.

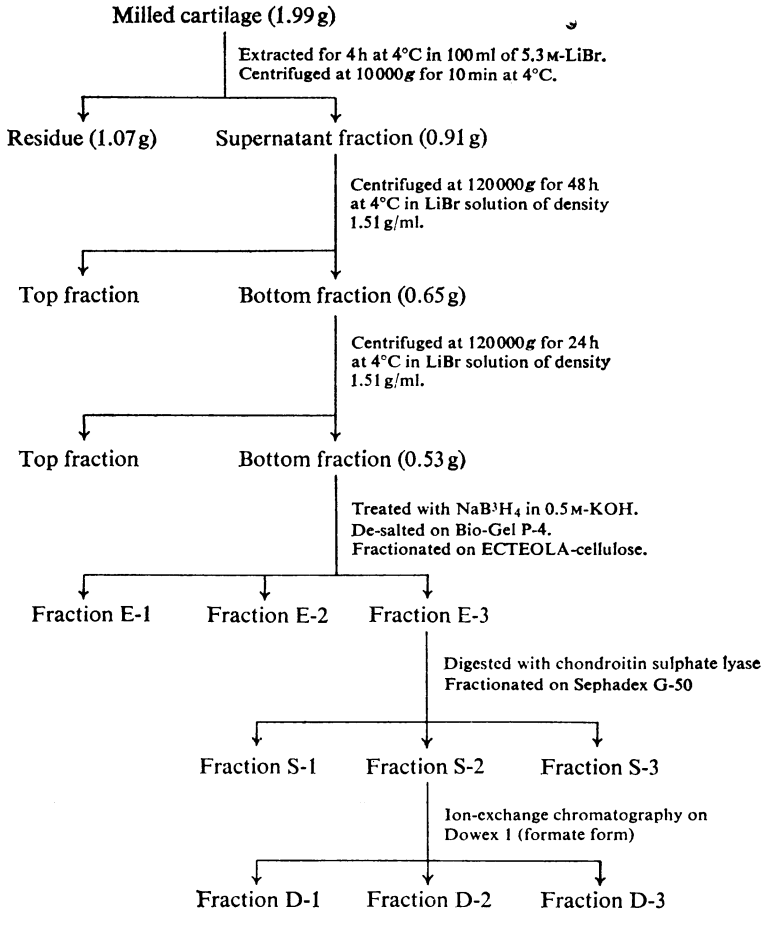
Essentially similar results were obtained when proteoglycan from bovine intervertebral disc was treated in the same way.

The ^3H label associated with fractions E-1 and E-2 after reaction in 0.02M-borohydride was associated with a variety of oligosaccharide units presumably derived from cartilage glycoproteins. Only trace amounts of label were associated with amino acids after acid hydrolysis and amino acid analysis.

The increase in ^3H label in fractions E-1 and E-2 after reaction of purified proteoglycan with 0.2M-borohydride, on the other hand, was directly attributable to incorporation into the amino acids alanine and α-aminobutyric acid. The ratio of ^3H label in these amino acids was 10:1 respectively.

Isolation of the chondroitin sulphate-linkage region

The chondroitin sulphate fraction E-3 from ECTEOLA-cellulose chromatography was de-salted



Scheme 1. *Isolation of proteoglycan from milled cartilage and its subsequent treatment with alkaline NaB^3H_4*

The methods used for each step are detailed under 'Methods'. Dry weight values (in parentheses) are for the non-diffusible material present in each fraction after exhaustive dialysis against water.

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on Bio-Gel P-4 and digested with chondroitin sulphate lyase from *P. vulgaris* as outlined under 'Methods'. The digest was fractionated by gel filtration on Sephadex G-50 into three main fractions as shown in Figs. 6 and 7. The first peak of radioactivity (S-1) contained hexose and hexosamine in approximately equimolar proportions. Ion-exchange chromatography of the hexosamines liberated by hydrolysis for 8h at 100°C in 4M-HCl indicated that glucosamine and galactosamine were present in a molar ratio of approx. 25:1 respectively. This fraction has been identified as keratan sulphate.

The second radioactive fraction (S-2) contained the rest of the ^3H label and has been identified as the chondroitin sulphate-linkage region. This material was hydrolysed in dilute acid and the neutral sugar fraction was isolated as described under 'Methods'. All of the radioactivity of this fraction was recovered in the neutral sugar fraction and paper chromatography in solvents A and B established that all of the ^3H label moved to the same position as standard xylitol.

The conditions of hydrolysis in this step were critical. When the hydrolysis was performed in 4M-HCl, 13M-acetic acid or 1.0M-HCl in anhydrous methanol for various times at 100°C additional radioactive components were observed after paper chromatography. This component appeared to be produced by

acid degradation of [^{3}H]xylitol since the same radioactive band was observed when pure [^{3}H]-xylitol was heated with 4M-HCl before paper chromatography.

The chromatography solvents employed for the identification of xylitol do not separate this sugar alcohol from lyxitol (arabinitol). However, g.l.c. of the monosaccharides in fraction S-2 has demonstrated the presence of 0.15–0.20mol of lyxitol/mol of oligosaccharide (see below).

Unlabelled galactose was the only other neutral sugar detected in fraction S-2 (Fig. 6). The molar ratio galactose/xylitol was measured by g.l.c. and was approx. 2.0. The identification of galactose was confirmed by using galactose dehydrogenase as described under 'Methods'. By using this assay, and assuming a specific radioactivity of 6.65×10^5 d.p.m./μmol for the labelled xylitol, the molar ratio galactose/xylitol was found to be 2.12.

Fraction S-3 contained no radioactivity but the bulk of the hexuronate was eluted in this fraction. It has been identified as the unsaturated disaccharide produced from the chondroitin sulphate chains by chondroitin sulphate lyase by comparison with the pure disaccharide (Robinson & Dorfman, 1969).

Further fractionation of the chondroitin sulphate-linkage-region oligosaccharides in fraction S-2 could be obtained by ion-exchange chromatography on Dowex 1 as outlined under 'Methods'. The results obtained for nasal septum chondroitin sulphate are shown in Fig. 8 and corresponding fractions, but in different proportion, were obtained from intervertebral disc chondroitin sulphate. Three main peaks are

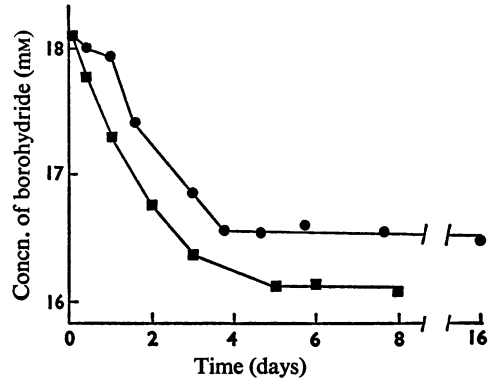

Fig. 3. *Time-course of reaction of proteoglycan with* 0.02M-*NaB^{3}H$_4$ in* 0.5M-*KOH*

Proteoglycan (●; 30mg/ml) and D-xylose (■; 0.79mg/ml) were incubated with 18.0mM-NaB^{3}H$_4$ in 0.5M-KOH at 4°C and samples were taken at the times indicated in the figure. Borohydride concentration was determined as outlined under 'Methods'.

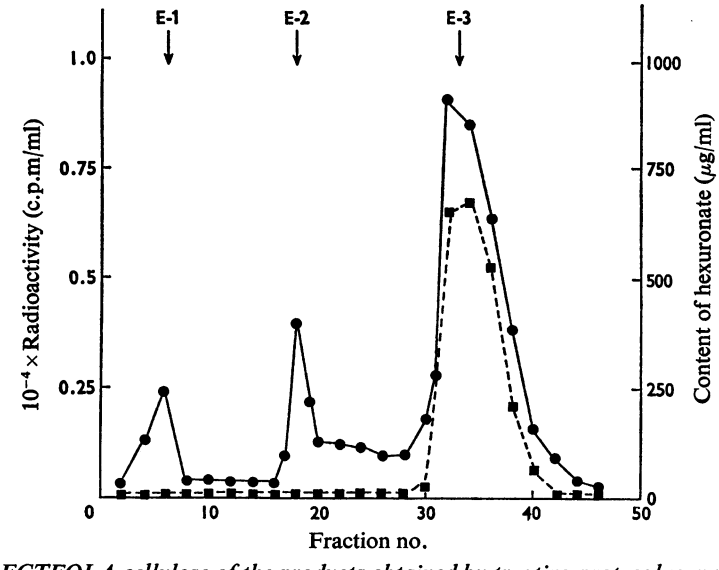

Fig. 4. *Elution from ECTEOLA-cellulose of the products obtained by treating proteoglycan with* 0.02M-*NaB^{3}H$_4$ in* 0.5M-*KOH*

Proteoglycan from bovine nasal cartilage was treated with alkaline NaB^{3}H$_4$ 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 12ml; hexuronate content (■) and radioactivity (●) are shown.

Table 2. *Reaction of proteoglycan and whole tissue with alkaline NaB^3H_4*

Proteoglycan and tissue samples were stirred at 4°C in 50ml of 0.5M-KOH containing NaB^3H_4 as shown in the table. Samples 1 and 2 were proteoglycan extracted from bovine nasal septum with LiBr (sample 3, Table 1) whereas sample 3 was proteoglycan extracted from the same tissue with 0.15M-NaCl (sample 4, Table 1). Samples 4 and 5 were milled bovine nasal cartilage. Sample 6 was milled bovine intervertebral disc whereas sample 7 was proteoglycan extracted from the same tissue with LiBr (sample 5, Table 1). After treatment the samples were subjected to ion-exchange chromatography on ECTEOLA-cellulose and number-average molecular weights (±S.D.) were calculated as outlined under 'Methods' assuming a specific radioactivity of 6.65×10^{11} d.p.m./mol.

Sample no.	Borohydride concentration (M)	Extraction time (days)	Dry weight of sample (g)	Elution of products from ECTEOLA-cellulose: Recovery of applied radioactivity (%)	Distribution of radioactivity in fractions (%) E-1	E-2	E-3	$10^{-4}\times$Calculated number-average molecular weight of chondroitin sulphate
1	0.02	4	0.54	99	12.0	17.2	70.8	2.12±0.08 (5)
2	0.20	10	0.30	98	17.8	35.0	47.2	2.10±0.10 (5)
3	0.02	4	1.30	98	10.3	16.7	73.0	2.20
4	0.02	6	3.00	96	12.7	18.2	69.1	2.07±0.05 (5)
5	0.20	11	0.60	97	24.0	35.2	40.8	2.08
6	0.02	6	4.0	97	14.5	24.0	61.5	1.32
7	0.20	11	0.22	98	23.0	25.8	51.2	1.37

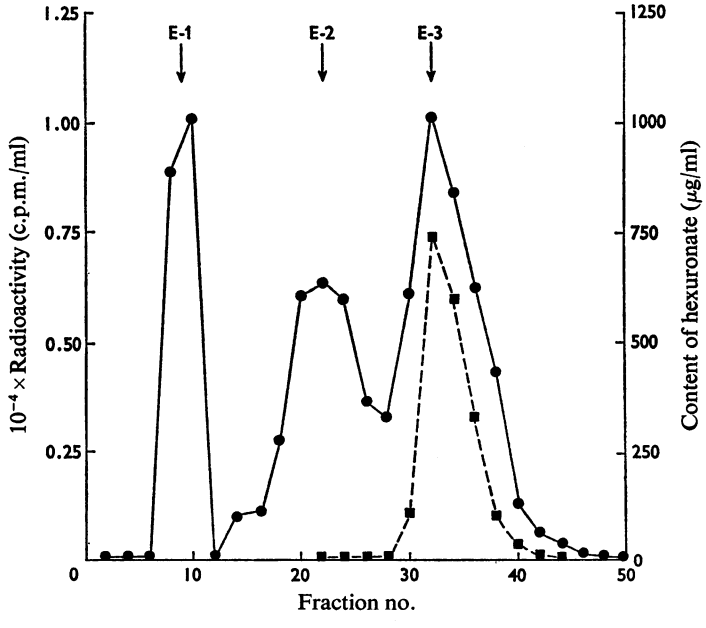

Fig. 5. *Elution from ECTEOLA-cellulose of the products obtained by treating proteoglycan with* 0.2M-*NaB^3H_4 in* 0.5M-*KOH*

Proteoglycan from bovine nasal cartilage was 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 12ml; hexuronate content (■) and radioactivity (●) are shown.

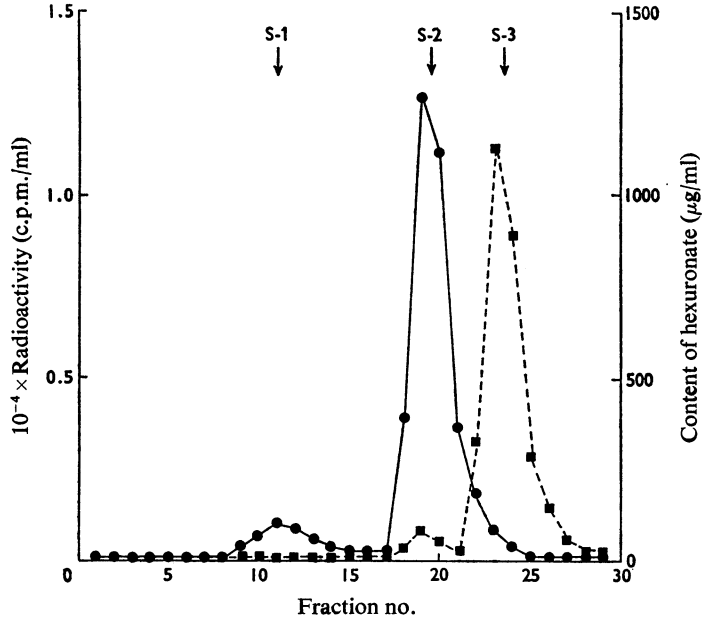

Fig. 6. *Elution from Sephadex G-50 of the products obtained after chondroitin sulphate lyase digestion of fraction E-3 from proteoglycan from bovine nasal cartilage*

Fraction E-3 (see Fig. 4) was de-salted on Bio-Gel P-4, digested with chondroitin sulphate lyase from *P. vulgaris* and applied to a column (80cm × 2cm diam.) of Sephadex G-50 equilibrated with pyridine–acetic acid buffer, pH 5.2, as outlined in 'Methods'. Fractions were 12ml; hexuronate content (■) and radioactivity (●) are shown.

observed, D-1, D-2 and D-3; the relative amounts of radioactivity in each and the results of chemical analyses are shown in Table 3.

By assuming a specific radioactivity of 6.65×10^5 d.p.m./μmol of xylitol, it has been possible to determine the number-average molecular weight of the chondroitin sulphate chains. The results in Table 2 were obtained with E-3 fractions after precipitation with cetylpyridinium chloride to remove the small amount of keratan sulphate present. The results obtained are in good agreement with values obtained by equilibrium sedimentation; these values were $\bar{M}_n = 2.17 \times 10^4$ and $\bar{M}_w = 2.23 \times 10^4$ for chondroitin sulphate from bovine nasal septum.

When milled nasal cartilage was treated with alkali and borohydride approx. 6% of the tissue hexuronate was not extracted. This material was isolated by Pronase digestion of the residue and fractionated on ECTEOLA-cellulose as outlined under 'Methods'. The specific radioactivity of the chondroitin sulphate fraction (E-3) was 1.51×10^4 d.p.m./mg dry wt., a value only 50% that of the extracted chondroitin sulphate (approx. 3.1×10^4 d.p.m./mg.).

Discussion

Previous investigations have established that a large proportion of the chondroitin sulphate chains in proteoglycan is linked by *O*-glycosidic bonds to serine residues of the core protein. The sugar involved in this immediate linkage is xylose and this is at the reducing end of a linkage oligosaccharide which has the structure: GlcA$p\beta$1–3Gal$p\beta$1–3Gal$p\beta$1–4Xyl (Rodén, 1970). Rapid cleavage of the xylose–serine bond in dilute alkali has been demonstrated by Muir (1958) and shown to be a β-elimination reaction by Anderson *et al.* (1965). The alkaline cleavage of *O*-glycosidically linked carbohydrate side chains from serine and threonine residues in ovine and bovine submaxillary mucins has been shown to be at least 95% complete (Bertolini & Pigman, 1970).

There is, however, some evidence to suggest that as much as 20% of the chondroitin sulphate chains in pig costal cartilage may be linked to protein by a *N*-glycosylamine bond to the γ-carboxyl group of glutamic acid (Katsura & Davidson, 1966). This finding indicates some heterogeneity in the mode of linkage; such heterogeneity has also been reported in

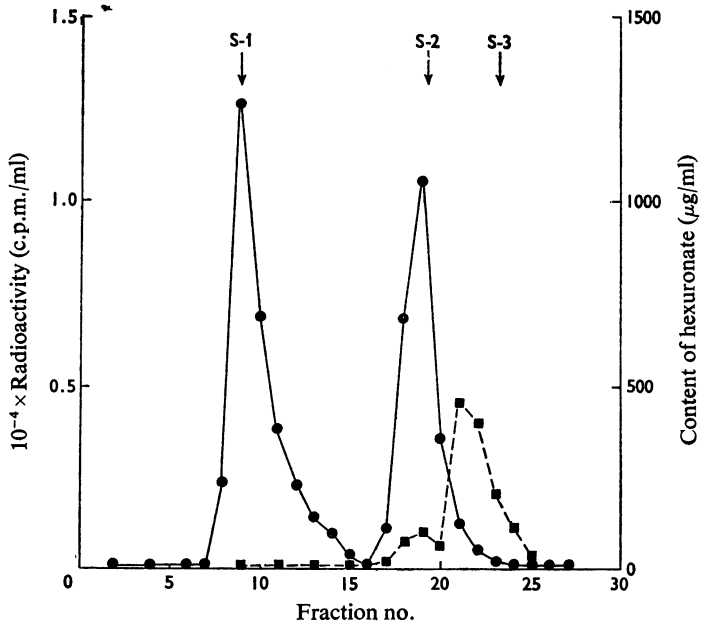

Fig. 7. *Elution from Sephadex G-50 of the products obtained after chondroitin sulphate lyase digestion of fraction E-3 from proteoglycan from bovine intervertebral disc*

Fraction E-3 derived from intervertebral disc proteoglycan was treated as described in Fig. 6. Fractions were 12ml; hexuronate content (■) and radioactivity (●) are shown.

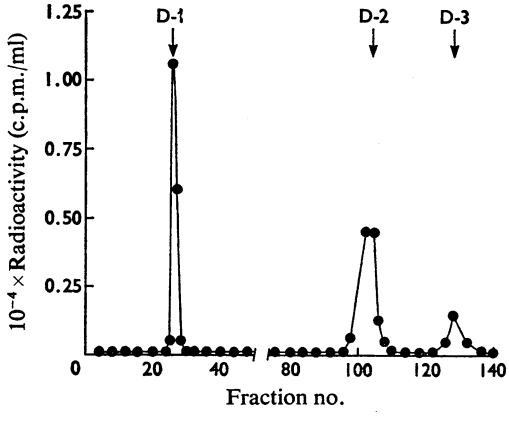

Fig. 8. *Elution from Dowex 1 (X4) of the components in fraction S-2 derived from proteoglycan from bovine nasal cartilage*

Fraction S-2 (see Fig. 6) was applied to a column of Dowex 1 (X4; formate form; 38cm×1.5cm diam.) and eluted with a linear gradient of formic acid followed by a linear gradient of ammonium formate in formic acid as outlined under 'Methods'. Fractions were 12ml.

glycoproteins (Sentandreu & Northcote, 1968; Descamps *et al.*, 1968).

The results in the present paper obtained by alkaline cleavage of chondroitin sulphate from milled tissue and from extracted proteoglycan indicate that at least 95% of the polysaccharide is linked by an alkali-labile bond and that xylose is the only monosaccharide involved in the linkage, with the possible exception of lyxose or even arabinose (see below). Complete cleavage by alkali was supported by the following observations: (1) the linkage-oligosaccharide fraction (S-2) had a negligible amino acid content, (2) the number-average molecular weight of the chondroitin sulphate calculated from its specific radioactivity after reduction with NaB^3H_4 was virtually the same as that obtained by sedimentation equilibrium; an agreement which indicates that the majority of the polysaccharide chains had been reduced and had undergone no further degradation during isolation.

The results also provide useful information on another point. It is possible that alkali might cause random depolymerization of glycosaminoglycans; a β-elimination reaction between *N*-acetylhexosamine and hexuronate would be favoured by a tautomeric

Table 3. *Analytical values for chondroitin sulphate-linkage oligosaccharides*

Sample 1 was the chondroitin sulphate-linkage-region-oligosaccharide fraction isolated from proteoglycan from bovine nasal septum (sample 2, Table 2) and sample 2 was the corresponding fraction from bovine intervertebral disc (sample 7, Table 2). Each sample was isolated and fractionated by ion-exchange chromatography on Dowex 1 as outlined under 'Methods' and yielded three subfractions D-1, D-2 and D-3. Hexuronate was assayed by using the carbazole method of Dische (1947); the numbers in parentheses give the values obtained after pretreatment with Br_2 (Robinson & Dorfman, 1969). Hexose was assayed with the anthrone reaction as described under 'Methods'; the values in parentheses correspond to the galactose content determined by using g.l.c. SO_4^{2-} was assayed by using the method of Antonopoulos (1962). All results are expressed as mol/mol (6.65×10^{11} d.p.m.) of oligosaccharide. Glucuronate, galactosamine, xylitol, lyxitol and xylose were determined by using g.l.c. Radioactivity is expressed as the label in each peak as % of the total applied to the column. N.D. = not determined.

Sample	Dowex fraction	Radio-activity (%)	Composition [mol/mol (6.65×10^{11} d.p.m.) of oligosaccharide]							
			Hexuronate	Glucuronate	Hexose	Sulphate	Galactosamine	Xylitol	Lyxitol	Xylose
1	D-1	48	2.14 (1.00)	0.95	2.02 (2.10)	0	0.99	0.81	0.16	0.03
1	D-2	30	2.24 (1.03)	0.82	1.98 (2.16)	0.91	0.97	0.83	0.12	0.05
1	D-3	17	3.20 (N.D.)	N.D.	2.06	1.89	N.D.	N.D.	N.D.	N.D.
2	D-1	9	2.18 (1.01)	0.84	2.10 (2.01)	0	0.96	0.79	0.19	0.03
2	D-2	68	2.13 (1.04)	0.86	2.05 (2.04)	0.96	0.97	0.79	0.18	0.03
2	D-3	23	3.45 (N.D.)	N.D.	2.12 (N.D.)	2.01	N.D.	N.D.	N.D.	N.D.

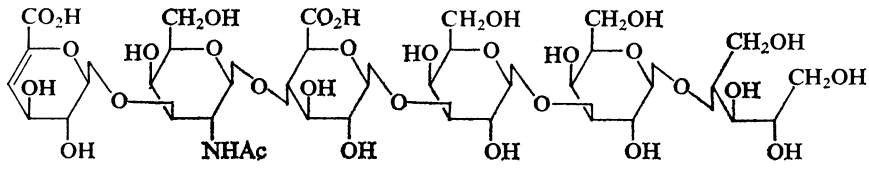

Fig. 9. *Proposed structure of the linkage oligosaccharide in fraction D-1 (see Fig. 8)*

modification of the uronic acid structure (Whistler & Rowell, 1966) and, in addition, stepwise degradation of the polysaccharide chains from the reducing end has been observed with blood-group substances (Lloyd *et al.*, 1966). In the latter reaction, the extent of this peeling would be governed by the relative rates of cleavage and reduction in the presence of borohydride. The conditions employed in the present study gave no indication of significant random degradation or peeling since all of the ^3H label was in xylitol. The absence of such peeling indicates a 4- or 2-substituted xylose residue, consistent with the β-1–4-glycosidic linkage between galactose and xylose reported for chondroitin sulphate-linkage region (Rodén, 1970).

The absence of any detectable depolymerization of chondroitin sulphate in alkali establishes that the extracted chondroitin sulphate chains represent in size and composition at least 95% of the tissue polysaccharide. For chondroitin sulphate derived from nasal cartilage, the calculated molecular weights ($\bar{M}_n$) were in the range 2.07×10^4–2.20×10^4 and did not vary greatly from one proteoglycan preparation to another regardless of the conditions used to isolate the proteoglycan or the efficiency of the extraction. The values are in reasonable agreement with previously reported values (Luscombe & Phelps, 1967). Chondroitin sulphate derived from intervertebral disc was smaller with a molecular weight ($\bar{M}_n$) of approx. 1.35×10^4. This polysaccharide is predominantly chondroitin 6-sulphate whereas chondroitin sulphate from nasal septum is predominantly the 4-sulphate, but both polysaccharides behave similarly during alkaline cleavage as demonstrated by the similarity of molecular weights determined by labelling and by sedimentation.

The keratan sulphate fraction (S-1) isolated after digestion with chondroitin sulphate lyase and gel filtration on Sephadex G-50, was also labelled with ^3H. This indicates that an alkali-labile linkage occurs within this polysaccharide as well, and from the specific radioactivity of each peak values of 7.5×10^3 and 20.5×10^3 were calculated for the molecular weights ($\bar{M}_n$) of keratan sulphate from bovine nasal septum and intervertebral disc respectively. The latter value is in reasonable agreement with a value ($\bar{M}_w$) of 18.6×10^3 obtained by equilibrium sedimentation.

The linkage oligosaccharides isolated by gel filtration were further fractionated by chromatography on Dowex 1. Two main components were isolated, fractions D-1 and D-2 (Fig. 8), which appeared to differ only in SO_4^{2-} content. The analysis of fraction D-1 was consistent with the structure shown in Fig. 9 whereas fraction D-2 presumably bears a SO_4^{2-} group on the lone hexosamine residue. The analysis of fraction D-3 indicates the presence of an additional disaccharide residue at the non-reducing end of the oligosaccharide. The distribution of ^3H label in these three fractions was different in the two tissues studied; with nasal septum chondroitin sulphate 48% of the total label was in the non-sulphated oligosaccharide D-1, 30% in fraction D-2 and 17% in fraction D-3, whereas in the intervertebral disc chondroitin sulphate only 9% was in the non-sulphated fraction D-1, 68% in fraction D-2 and 23% in fraction D-3.

The two samples, therefore, differ considerably in the extent of sulphation of the first hexosamine in the chain. Paper chromatography of these oligosaccharide fractions on Whatman 3MM paper for 90h in solvent C failed to resolve the D-1 components from nasal septum and intervertebral disc. Both samples moved as single radioactive peaks with identical R_F values. The D-2 fractions were, however, resolved into two radioactive components which were present in different proportions in each tissue sample. The component with the higher R_F value has been tentatively identified as the oligosaccharide sulphated at position 4 of the galactosamine residue by comparison with the mobility in the same solvent of known sulphated disaccharides (Robinson & Dorfman, 1969). This compound accounted for 94% of the D-2 fraction from nasal septum and 30% of the D-2 fraction from intervertebral disc. The second component with a smaller R_F value, was assumed to be the corresponding 6-sulphated oligosaccharide.

The analysis of these linkage oligosaccharides (Table 3) indicates the presence of low amounts of xylose (0.03–0.05mol/mol of oligosaccharide), and lyxitol or arabinitol (0.12–0.19mol/mol of oligosaccharide). The presence of these minor components

indicates incomplete reduction by borohydride during the course of the reaction and significant rearrangement of the 4-substituted xylose residue in the presence of alkali before reduction with borohydride. The most likely products obtained by alkaline rearrangement of xylose would be D-xylulose and D-lyxose (Speck, 1958) which on reduction with borohydride would yield D-xylitol and D-lyxitol (L-arabinitol). The presence of these sugar alcohols does not, therefore, necessarily indicate the existence of any other linkage sugar in chondroitin sulphate besides D-xylose.

The reduction of aldehydes and ketones with borohydride follows the equation:

$$4\,R\text{-}CHO + BH_4^- + 2H_2O \rightarrow 4\,R\text{-}CH_2OH + BO_2^-$$

In a reaction of this type, a kinetic-isotope effect can be anticipated owing to the relative rates of ^3H and ^1H in the nucleophilic attack on the carbonyl carbon. The relative incorporation of ^3H and ^1H into cyclohexanol, under the standard conditions of reduction outlined under 'Methods', was calculated to be 0.60:1.00 respectively. Errors owing to this effect were minimized in all subsequent reductions by using a large excess of borohydride over the amount of carbonyl compound present and by employing constant conditions of reduction.

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