Fractionation of Proteoglycans from Bovine Corneal Stroma

By INGE AXELSSON and DICK HEINEGÅRD

Department of Physiological Chemistry 2, University of Lund, P.O. Box 750,
S-220 07 Lund, Sweden

(Received 2 July 1974)

Proteoglycans were extracted from bovine corneal stroma with 4M-guanidinium chloride, purified by DEAE-cellulose chromatography (Antonopoulos *et al.*, 1974) and fractionated by precipitation with ethanol into three fractions of approximately equal weights. One of these fractions consisted of a proteoglycan that contained keratan sulphate as the only glycosaminoglycan. In the other two fractions proteoglycans that contained chondroitin sulphate, dermatan sulphate and keratan sulphate were present. Proteoglycans which had a more than tenfold excess of galactosaminoglycans over keratan sulphate could be obtained by further subfractionation. The gel-chromatographic patterns of the glycosaminoglycans before and after digestion with chondroitinase AC differed for the fractions. The individual chondroitin sulphate chains seemed to be larger in cornea than in cartilage. Oligosaccharides, possibly covalently linked to the protein core of the proteoglycans, could be isolated from all fractions. The corneal proteoglycans were shown to have higher protein contents and to be of smaller molecular size than cartilage proteoglycans.

Approx. 60% of the corneal glycosaminoglycans of man, cattle and most other species investigated is keratan sulphate. The remaining 40% is chondroitin 4-sulphate (Meyer *et al.*, 1953; Gardell, 1957; Anseth, 1961; Greiling & Stuhlsatz, 1966). Bovine cornea also contains dermatan sulphate (Stuhlsatz *et al.*, 1972) as well as small amounts of chondroitin 6-sulphate (Handley & Phelps, 1972) and chondroitin (Davidson & Meyer, 1954; Greiling *et al.*, 1967*b*), which is, however, never completely free from sulphate.

Little is known about the overall structures of corneal proteoglycans, although descriptions of the polysaccharide part of the molecules have been given (Berman, 1970; Stuhlsatz *et al.*, 1971). The extraction and purification procedures used in those earlier investigations (high-speed homogenization in hypotonic media and/or exposure to Pronase and alkaline solutions) are known to degrade proteoglycans. The present investigation was therefore initiated to study the structure of corneal proteoglycans obtained by extraction, purification and fractionation by using mild methods.

Experimental

Materials

Eyes from adult cows were obtained directly from the abattoir and immediately cooled to 0–4°C. They were used within a few hours of slaughter. The corneas were excised and the endothelium, epithelium and Descemet's membrane were scraped off with a surgical scalpel. The corneal stroma was cut into thin

slices and subsequently put directly into the extraction solvent.

DEAE-cellulose and ECTEOLA-cellulose were obtained from Serva Entwicklungslabor, Heidelberg, West Germany; Sepharose and Sephadex gels were from Pharmacia Fine Chemicals, Uppsala, Sweden; fibrous cellulose powder (Whatman CF 11) was from W. and R. Balston Ltd., Maidstone, Kent, U.K.; papain (twice-crystallized) and chondroitinases AC and ABC (Seikagaku) were from Sigma Chemical Co., St. Louis, Mo., U.S.A., and Miles Laboratories Inc., Elkhart, Ind., U.S.A., respectively.

Analytical methods

Hexuronic acid, hexose and protein. If not otherwise stated in the text, column effluents were analysed, with a Technicon autoanalyser, for hexuronic acid, hexose and protein by automated versions (Heinegård, 1973) of the carbazole (Dische, 1947; Bitter & Muir, 1962), anthrone (Goa, 1955) and Folin (Lowry *et al.*, 1951) procedures respectively. The protein contents of the different fractions were calculated from the amino acid analysis or measured by the Folin procedure (Lowry *et al.*, 1951) with bovine crystalline albumin as standard.

Amino acid and hexosamine composition. Amino acids, hexosamine contents and ratios of glucosamine to galactosamine were determined as described elsewhere (Antonopoulos *et al.*, 1974).

Neutral sugar composition. Neutral sugars were determined by g.l.c. of their alditolacetates, essentially as described by Lindahl (1970) with some modifica-

tions described below (I. Carlstedt, personal communication). The materials (about 1.0mg) were hydrolysed in 500 μ l of 2M-trifluoroacetic acid at 100°C for 4h under N₂. Rhamnose (40 μ g) was added as an internal standard. The samples were evaporated and the sugars were purified on micro-columns (0.5cm $\times$ 1.5cm) of Dowex 1 (X2; acetate form) eluted with water. The sugars were reduced in 500 μ l of NaBH₄ (1mg/ml) in 1M-NH₃ at room temperature (21°C) for 2h and neutralized with 6M-acetic acid. The alditols formed were purified on Dowex 50W (X8; H⁺ form) micro-columns (0.5cm $\times$ 1.5cm) eluted with water. The effluents were evaporated and transferred with methanol to small test tubes and acetylated at 90°C for 1h in 200 μ l of a mixture of acetic anhydride and pyridine (1:1, v/v). After evaporation to dryness the alditol acetates were dissolved in 10 μ l of chloroform and 1 μ l portions were chromatographed on a column (internal diameter 2mm, length 1.80m) of 1.5% (w/w) ethylene glycol succinate and 1.5% (w/w) silicone oil (XF-1150) on Gas-Chrom P (100–120 mesh). The temperature was 170°C and N₂ was used as carrier gas.

Gel chromatography. A column of Sephadex G-25 (1.6cm $\times$ 209cm) was eluted at a flow rate of 10ml/h with 0.25M-pyridine buffered with acetic acid to pH6.5, and 6ml fractions were collected. A column of Sephadex G-150 (1.3cm $\times$ 130cm) was eluted with the same buffer at a flow rate of 7ml/h, and 2ml fractions were collected. A Sephadex G-200 column (0.9cm $\times$ 138cm) was eluted with a 0.5M-sodium acetate buffer (pH7.0) at a flow rate of about 2ml/h to give 1.3ml fractions. For desalting, a Sephadex G-15 column (1.3cm $\times$ 70cm) eluted with water was used. Agarose-gel chromatography was performed on Sepharose 4B columns (0.9cm $\times$ 130cm) eluted with the sodium acetate buffer. The flow rate was 3ml/h, and the fraction size was 1.25ml.

A large proportion of the chondroitin sulphate-rich proteoglycan (called 50P below) was virtually insoluble in the neutral acetate buffers used for chromatography. Therefore fraction 50P was first dissolved in 4M-guanidinium chloride buffered with 0.05M-sodium acetate (pH5.8) and then the material remained in solution even after extensive dialysis against the elution buffer. Before the sample was applied to the chromatography column small amounts of insoluble material were removed by centrifugation at 25000g and 4°C for 20min. All the other samples were dissolved directly in the elution buffer and centrifuged in the same way. The pellets obtained contained only 0–5% of the total hexosamine in the samples and they were therefore discarded.

Enzymic digestions

Papain digestion. Unless otherwise stated in the text, papain digestion was performed at 65°C for 4h

in 0.1M-sodium phosphate buffer (pH7.0) containing 0.005M-EDTA (disodium salt) and 0.005M-cysteine hydrochloride. Proteoglycan samples of 5–10mg/ml were digested with 0.2–0.4mg of papain/ml.

Chondroitinase digestion. Digestions with chondroitinases AC and ABC were performed at 37°C for about 20h in a buffer of 0.1M-sodium acetate–0.1M-Tris–acetate, pH7.3, with a few drops of toluene as a bacteriostatic agent. The sample concentration was about 10mg/ml and about 0.04 unit nominal of chondroitinase was used per mg of sample. [One enzyme unit is defined as the amount of chondroitinase that catalyses the release of 1 μ mol of unsaturated disaccharide/min under the conditions described by Yamagata *et al.* (1968).] Half of the chondroitinase was added at the beginning of the incubation, the rest after 3–6h.

Preparation of proteoglycans

Proteoglycans from bovine cornea were extracted with guanidinium chloride and purified by DEAE-cellulose chromatography as described by Antonopoulos *et al.* (1974) with one modification. The corneas were only sliced before extraction, since the extraction yield was the same whether the tissue was sliced with a scalpel or ground under liquid N₂. The material that was eluted from the DEAE-cellulose column with 2M-NaCl in 7M-urea–0.05M-Tris (pH6.5) was dialysed and freeze-dried. This material will be referred to as the proteoglycan preparation.

Density-gradient centrifugation

For density-gradient centrifugation solid CsCl was added to a solution of 4M-guanidinium chloride in 0.05M-sodium acetate, pH5.8 (Sajdera & Hascall, 1969) to give a density of 1.39g/ml and 17ml of this solution was used to dissolve 50mg of the proteoglycan preparation. Gradients were established by centrifugation at 34000rev./min for 48h in an 8 $\times$ 25ml angle rotor at 18°C in an MSE Superspeed 65 centrifuge. Tubes were emptied into 2ml fractions with the aid of an MSE tube piercer. The last (eighth) fraction contained 3ml. Densities of the fractions were determined with a 200 μ l constriction pipette as a pycnometer. The fractions were dialysed against several changes of water and freeze-dried. The distribution of oligosaccharides and polysaccharides was determined as described below.

Alcohol fractionation

Alcohol fractionation of the proteoglycan was performed in a cold-room (+4°C) with all solutions pre-cooled. Samples of the proteoglycan preparation (5mg/ml) were dissolved in 4M-guanidinium chloride–

0.1M-barium acetate, pH5.8. Ethanol (99.5%, v/v) was added dropwise during vigorous magnetic stirring until the required concentration of ethanol was reached. The mixture was then left without stirring overnight in the cold-room and centrifuged in a Sorvall RC2-B centrifuge at 38000g and 4°C for 30min. The supernatant was decanted and further fractionated by stepwise addition of more ethanol. Each precipitate was harvested by centrifugation as described above. Thus several precipitates and one final supernatant were obtained in each experiment. In some cases, small amounts of buffer salts were precipitated. The pH, however, was not changed significantly. The precipitates were dissolved and dialysed against water and then freeze-dried. The final supernatant was reduced in volume by rotary evaporation to about one-fifth of the original volume. It was then diluted tenfold and dialysed against water and freeze-dried. The fractions were analysed for contents of hexosamine, protein and amino acids. The oligosaccharide/glycosaminoglycan and keratan sulphate/galactosaminoglycan ratios were determined by the ECTEOA-technique described below.

Detection and quantitative analysis of dermatan sulphate

Recently the presence of considerable amounts of dermatan sulphate in calf cornea was briefly reported (Stuhlsatz *et al.*, 1972), and the present studies have confirmed this report. Thirty calf corneas were cut into small pieces and digested with papain. The galactosaminoglycans were isolated by chromatography on an ECTEOA-cellulose column (Cl⁻ form; bed size 2cm×8cm; equilibrated with water) eluted first with 3 bed volumes of 0.02M-HCl followed by 1 bed volume of water and then with 3 bed volumes of 2.5M-sodium formate. The last fraction, which contained the galactosaminoglycans (Antonopoulos *et al.*, 1967), was dialysed, freeze-dried, digested with chondroitinase AC and chromatographed on a column (1.6cm×209cm) of Sephadex G-25 (superfine grade) eluted with the pyridinium acetate buffer. The

developed with isobutyric acid-0.5M-NH₃ (5:3, v/v) for 48h as described by Yamagata *et al.* (1968). *N*-Acetylchondrosine* and ΔDi4-S (obtained from chondroitinase AC digestion of chondroitin sulphate) were chromatographed as standards. The sugars were detected with a silver-dip reagent (Smith, 1960).

Iduronic acid was identified by ion-exchange chromatography with the automated system described by Fransson *et al.* (1968) after hydrolysis of the sample (2mg/ml) for 2h at 100°C in 1M-H₂SO₄. H₂SO₄ was neutralized with BaCO₃ after hydrolysis and the suspension was filtered through Celite on a sintered-glass filter. The filtrate and washings were concentrated to about 2ml in a rotary evaporator before application to the column. Under these conditions the recovery of the uronic acids is 30–50% (A. Malmström, personal communication). Thus only an approximate estimate of the content of iduronic acid in the sample could be obtained. Chromatography was performed twice, first with hydrolysate only and then with iduronic acid added as an internal standard.

The assay procedure for chondroitinase AC and ABC activities described by Hascall *et al.* (1972) was used to determine the dermatan sulphate/chondroitin sulphate ratio of galactosaminoglycans (I. Sjöberg, personal communication). From each sample, six portions were taken and digested with chondroitinase AC for 20h (conditions are given above). Three of these portions were then digested with chondroitinase ABC for 20h. The other three portions were not exposed to chondroitinase ABC. The amount of unsaturated disaccharides liberated by the enzymes was measured by a modification of the periodic acid-thiobarbituric acid reaction (Hascall & Heinegård, 1974). It was assumed that chondroitin sulphate but not dermatan sulphate was degraded to disaccharides by chondroitinase AC, whereas both chondroitin sulphate and dermatan sulphate were degraded to disaccharides by chondroitinase ABC. The dermatan sulphate/chondroitin sulphate ratio was calculated as follows:

$$\text{ratio} = \frac{(\Delta\text{UA formed by chondroitinases AC+ABC}) - (\Delta\text{UA formed by chondroitinase AC})}{\Delta\text{UA formed by chondroitinase AC}}$$

(ΔUA = unsaturated uronic acid.)

material in the void volume was freeze-dried, re-digested with chondroitinase AC and rechromatographed on the Sephadex G-25 column. The material that was again eluted in the void volume was further analysed by paper chromatography before and after chondroitinase ABC digestion.

Whatman 3MM paper was used for descending paper chromatography. The chromatograms were

Isolation of chondroitin sulphate

It has been suggested that bovine cornea contains a co-polymer of chondroitin sulphate and heparan

* Abbreviations: *N*-acetylchondrosine, 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose; ΔDi4-S, 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-4-*O*-sulpho-D-galactose (Yamagata *et al.*, 1968).

sulphate (Greiling *et al.*, 1967a). To reveal the presence of such hybrid polysaccharides, chondroitin sulphate was isolated from fraction 50P (see below) by a procedure using a combination of alkaline β -elimination and proteolysis of proteoglycans. Approx. 5 mg of fraction 50P was dissolved in 0.5 ml of 0.5 M-NaOH-0.3 M-NaBH₄ and left for 48 h at room temperature (21°C). The pH of the solution was then adjusted to approx. 6.5 by addition of the calculated volume of 0.5 M-acetic acid. Cysteine hydrochloride and EDTA were added, each to a final concentration of 0.005 M. The sample was digested with 0.15 mg of papain at 65°C for 4 h. The digest was diluted twofold with water and applied to an ECTEOLA-cellulose column (bed volume 3.5 ml), which was first eluted with 7 ml of 0.02 M-HCl and then with 7 ml of 1 M-sodium formate. These fractions, which mainly contain glycopeptides (Antonopoulos *et al.*, 1967), were discarded. Chondroitin sulphate was then eluted with 2.5 M-sodium formate, desalted by chromatography on the Sephadex G-15 column, freeze-dried, redissolved in 200 μ l of 0.05 M-NaCl and applied to a cetylpyridinium chloride-cellulose column (0.8 cm $\times$ 5 cm) (Antonopoulos *et al.*, 1964). Keratan sulphate and remaining glycopeptides were eluted with 10 ml of 1% cetylpyridinium chloride, and hyaluronic acid was eluted with 10 ml of 0.3 M-NaCl in 0.05% cetylpyridinium chloride. Finally the chondroitin sulphate was eluted with 10 ml of 0.6 M-MgCl₂ in 0.05% cetylpyridinium chloride (Antonopoulos *et al.*, 1964). The latter fraction was diluted fivefold with 0.05% cetylpyridinium chloride and left overnight at room temperature. The precipitate was collected by centrifugation and its glucosamine/galactosamine ratio was determined after hydrolysis in 8 M-HCl.

Determination of oligosaccharide, galactosaminoglycan and keratan sulphate contents

Glycosaminoglycans and oligosaccharides were separated by a micro-modification (Anseth, 1961) of the procedure of Ringertz & Reichard (1960). The samples were digested with papain and then applied to ECTEOLA-cellulose micro-columns. The oligosaccharides (glycopeptides) were eluted with water and 0.02 M-HCl. The glycosaminoglycans were then eluted with 2 M-HCl and the hexosamine contents of the fractions were determined. As an estimate of the keratan sulphate content of the samples the glucosamine/galactosamine ratios of the glycosaminoglycan fractions were determined.

Identification of keratan sulphate in fraction 50P

To demonstrate the presence of keratan sulphate in fraction 50P (see below), 24 mg of this fraction was

Fig. 1. Gel chromatography on Sephadex G-150 of chondroitin sulphate-rich proteoglycans (fraction 50P) after digestion with chondroitinase AC and papain

Experimental details are given in the text. —, Uronic acid (carbazole absorbance at 530 nm); ----, hexose (anthrone absorbance at 620 nm); ····, protein (Folin absorbance at 680 nm). The fractions indicated by the horizontal bar were pooled and analysed as described in the text. The arrow indicates the void volume of the column.

digested with chondroitinase AC, dialysed and then digested with papain. The digest was freeze-dried and applied to the Sephadex G-150 column. The effluent was analysed for hexose, uronic acid and protein (Fig. 1). Fractions were pooled as indicated in Fig. 1, freeze-dried and chromatographed on ECTEOLA-cellulose micro-columns eluted with 0.02 M-HCl and 2 M-HCl as described above. The 2 M-HCl effluent was hydrolysed and its glucosamine/galactosamine ratio was determined.

Results and Discussion

Demonstration of dermatan sulphate in bovine cornea

The galactosaminoglycans present in calf cornea were isolated and digested with chondroitinase AC and chromatographed on Sephadex G-25 (Fig. 2a). Several peaks were observed. It seems likely that the material eluted in the void volume (Fig. 2a) was dermatan sulphate, since it contained uronic acid and was not degraded by chondroitinase AC in spite of repeated treatment with the enzyme (Fig. 2b). To identify dermatan sulphate, samples of the material in the peak indicated in Fig. 2(b) were digested with chondroitinase ABC or AC and chromatographed on paper. Redigestion with chondroitinase AC did not change the paper-chromatographic behaviour of the material, whereas the chondroitinase ABC-digested material chromatographed like an unsaturated disaccharide with one 4-sulphate group (Δ Di-4S). Further, the uronic acid component was identified as iduronic acid by ion-exchange chromatography

Fig. 2. Gel chromatography on Sephadex G-25 of chondroitinase AC-digested glycosaminoglycans from calf cornea

(a) shows the carbazole elution profile (measured at 530nm) of galactosaminoglycans from calf cornea after chondroitinase AC digestion. The material eluted with the void volume was pooled as indicated by the horizontal bar, redigested with chondroitinase AC and rechromatographed on the Sephadex G-25 column (b). The material indicated by the horizontal bar in (b) was pooled and further analysed. The arrow indicates the void volume of the column.

(Fransson *et al.*, 1968). In this chromatogram only one peak was obtained, which was at the position expected for iduronic acid. Addition of iduronic acid to the hydrolysate still gave only one (but a larger) peak that was eluted at the same position.

From the data presented it appears that calf cornea contains dermatan sulphate. The amount of dermatan sulphate was calculated to be about 5–8% of the total glycosaminoglycans of the cornea. This value is very approximate, mainly owing to the variable yield of uronic acid on hydrolysis of glycosaminoglycans (A. Malmström, personal communication). However, it is closely similar to that (9%) reported by Stuhlsatz *et al.* (1972).

Density-gradient centrifugation

The higher protein contents of the proteoglycans from cornea result in much lower buoyant densities

Fig. 3. Dissociative gradient centrifugation of the proteoglycan preparation

Centrifugation was performed as described in the text. Fractions 1–7 were 2ml; fraction 8 was 3ml. ○, Protein measured by the Folin procedure (Lowry *et al.*, 1951); △, glucosamine in glycosaminoglycans; □, galactosamine in glycosaminoglycans; ●, hexosamine in oligosaccharides; ----, density.

than for the proteoglycans from aorta, sclera and cartilage (Antonopoulos *et al.*, 1974). The composition of the fractions recovered at various densities were similar (Fig. 3). The glycoprotein hexosamine constituted between 13 and 19% of the total hexosamine in the fractions except in the one recovered at the lowest density. In this fraction 26% of the hexosamines were recovered in the glycoprotein fraction. Approximately one-third of the glycosaminoglycans in all the fractions were galactosaminoglycans. Thus there is neither any separation of proteoglycans that contain keratan sulphate from those that contain galactosaminoglycans nor any separation of proteoglycans from glycoproteins. Neither was any separation obtained by ion-exchange chromatography. The corneal proteoglycans were therefore fractionated by ethanol precipitation.

Alcohol fractionation

Attempts were first made to fractionate corneal proteoglycans with ethanol by using either calcium acetate buffer, used by Meyer *et al.* (1953) for fractionation of corneal glycosaminoglycans, or barium acetate buffer, used by Gardell (1957) for the same purpose. The fractions obtained showed no significant differences in the ratios of keratan sulphate to galactosaminoglycans. However, when alcohol precipitation was performed in the presence of guanidinium chloride, fractions with different ratios of keratan sulphate to galactosaminoglycans were obtained. In a typical experiment, 200mg of the proteoglycan preparation was dissolved in 40ml of the 4M-guanidinium chloride–barium acetate solution and fractionated with ethanol as described in the Experimental

Table 1. *Composition of the fractions obtained by alcohol fractionation of 200mg of the proteoglycan preparation*

The values are not corrected for ash and moisture contents of the samples. Glycosaminoglycan/oligosaccharide ratios were measured as hexosamines (for details, see the text).

Fraction	Weight (mg)	Protein (% w/w)	Hexosamine (% w/w)	Glycosaminoglycan/oligosaccharide ratios	Glucosamine/galactosamine ratios	
					Glycosaminoglycans	Oligosaccharides
50P	66.5	41.2	9.2	80:20	11:89	87:13
60P	65.2	42.0	8.5	76:24	54:46	90:10
70P	48.6	39.3	11.0	77:23	99:1	87:13
70 Super	3.7	Not analysed	0.3	Not analysed	Not analysed	Not analysed

Table 2. *Amino acid composition of corneal proteoglycans*

The fractions were obtained by ethanol precipitation. Values are expressed as residues per 1000 residues.

Amino acid	Proteoglycan preparation	Fraction 50P	Fraction 60P	Fraction 70P
Asx	112	101	127	139
Thr	42	37	48	40
Ser	67	58	67	77
Glx	111	106	118	110
Pro	76	84	71	67
Gly	110	178	89	48
Ala	69	87	65	47
Cys	5	1	5	8
Val	55	58	31	53
Met	2	2	2	3
Ile	47	39	56	51
Leu	117	84	127	154
Tyr	28	19	30	38
Phe	36	30	40	41
Lys	58	53	59	65
His	20	18	20	23
Arg	43	48	44	36

section. Fractions were taken at ethanol concentrations of 50, 60 and 70% (v/v) respectively. The three precipitated fractions were denoted 50P, 60P and 70P and the final supernatant was called 70 Super. The total recovery on a weight basis was 92%. The compositions of the fractions are shown in Table 1. The fractionation procedure has been repeated several times with good reproducibility. Fraction 70 Super was small and contained less than 0.1% of the total hexosamine and was not further analysed. The 50P, 60P and 70P fractions, which contained approx. 33, 33 and 24% respectively of the starting material, had similar contents of protein and hexosamine (Table 1) but differed notably in their amino acid compositions (Table 2) and ratios of keratan sulphate to galactosaminoglycan (Table 1). For all three fractions, 76–80% of the hexosamines were found in the glycosaminoglycan fraction (Table 1) and the residual 20–24% in oligosaccharides which may be covalently bound to the protein cores of the proteoglycans. Less likely, these oligosaccharides may be derived from

Table 3. *Neutral sugar composition of the glycosaminoglycans isolated from corneal proteoglycans*

The proteoglycans were separated into fractions 50P, 60P and 70P by alcohol precipitation. From each fraction, a sample was digested with papain, and oligosaccharides and glycosaminoglycans were separated on ECTEOLA-cellulose micro-columns (Anseth, 1961) and the neutral sugar composition of the glycosaminoglycans was determined by g.l.c. (Lindahl, 1970). The values are expressed as mol% of the total contents of neutral sugars of the sample. Glucose is at least partially a contamination from the DEAE-cellulose and ECTEOLA-cellulose columns.

Sugar	Glycosaminoglycans		
	50P	60P	70P
Fucose	4	4	2
Xylose	29	5	2
Mannose	11	7	8
Galactose	51	80	88
Glucose	4	4	Traces

impurities mixed with the proteoglycans or entangled in the proteoglycan molecules. Low-sulphated and/or low-molecular-weight chondroitin sulphate and keratan sulphate, however, could be constituents of the oligosaccharide fractions, since such substances would be eluted from the ECTEOLA-cellulose column with the weak acid (0.02M-HCl) used. The amounts of uronic acid and galactosamine are, however, small.

The analytical data for each fraction will be discussed separately.

Fraction 50P. Glycine, aspartic acid and glutamic acid were the predominant amino acids in acid hydrolysates of fraction 50P, and the content of serine was low as compared with cartilage proteoglycans (Table 2). Galactose and xylose were the predominant neutral sugars of the glycosaminoglycans in fraction 50P (Table 3). These sugars occur at the chondroitin sulphate–protein linkage region (Rodén, 1970) and galactose is also a major component of keratan sulphate.

Fig. 4. Gel chromatography on Sepharose 4B of the proteoglycan preparation and the three proteoglycan fractions obtained by alcohol precipitation

(a) The proteoglycan preparation. (b) Fraction 50P. The four peaks (A–D) were pooled as indicated by the horizontal bars, dialysed and freeze-dried and the material was digested with papain and chromatographed on ECTEOLA-cellulose columns (Anseth, 1961). The glycosaminoglycans isolated by this procedure were analysed for glucosamine/galactosamine ratios (A, 18:82; B, 13:87; C, 11:89; D, 7:93) in order to estimate the keratan sulphate/galactosaminoglycan ratios. (c) Fraction 60P. (d) Fraction 70P. —, Uronic acid (carbazole absorbance at 530 nm); ----, hexose (anthrone absorbance at 620 nm); ····, protein (Folin absorbance at 680 nm). The arrow indicates the void volume of the column.

The glucosamine/galactosamine ratio of the glycosaminoglycans of fraction 50P was 11:89 (w/w) (Table 1), which would suggest the presence of both galactosaminoglycans and keratan sulphate, although corneal chondroitin sulphate is reported to contain glucos-

amine (Greiling *et al.*, 1967a). Therefore to identify the glucosamine-containing polysaccharide a sample of fraction 50P was digested with chondroitinase AC and papain and then chromatographed on a Sephadex G-150 column (Fig. 1). The major part of the anthrone-positive material appeared before the total volume in a broad peak that was pooled (Fig. 1). Since the glycosaminoglycans of this fraction have glucosamine/galactosamine ratio of 89:11 (w/w) and since they gave a strong colour in the anthrone reaction, it appears that their predominant constituent is keratan sulphate. Most of the carbazole- and Folin-positive material, in contrast, was eluted in the total volume of the column. The anthrone-positive peak in the total volume most probably represented unspecific interference in the anthrone reaction by oligosaccharides containing uronic acid (Heinegård, 1973) and possibly some colour was obtained from chondroitin sulphate linkage regions.

To determine whether or not corneal chondroitin sulphate free from glucosamine can be isolated, the galactosaminoglycans of fraction 50P were prepared from alkali-treated and papain-digested fraction 50P by chromatography on ECTEOLA-cellulose and cetylpyridinium chloride-cellulose as described in the Experimental section. The galactosaminoglycans purified showed a ratio of glucosamine to galactosamine of less than 0.01. Therefore it appears that the galactosaminoglycans in fraction 50P did not contain glucosamine, in contrast with the results of Greiling *et al.* (1967a).

A sample of fraction 50P was chromatographed on Sepharose 4B. The elution pattern (Fig. 4b) indicated polydispersity and heterogeneity of the material. The effluent was pooled as indicated (Fig. 4b) and the ratio of glucosamine to galactosamine of the glycosaminoglycans in the fractions was determined. The values (Fig. 4b) suggested that smaller molecules contain less keratan sulphate. Thus in peak D, there seems to be a more than tenfold excess of galactosaminoglycans over keratan sulphate. However, no keratan sulphate-free proteoglycans could be isolated with the methods used. Whether or not the keratan sulphate and the galactosaminoglycans were bound to the same or different protein cores remains to be investigated.

Gel-chromatographic analyses of papain digests of corneal proteoglycans revealed that the galactosaminoglycan chains from fraction 50P (Fig. 5a) have larger average hydrodynamic volumes and show greater polydispersity than chondroitin sulphate chains from bovine tracheal cartilage (Fig. 5d) prepared as described by Heinegård & Hascall (1974).

To determine the dermatan sulphate/chondroitin sulphate ratio in fraction 50P a sample was digested with papain and the galactosaminoglycans were isolated by ECTEOLA-cellulose chromatography. The

Fig. 5. Gel chromatography on Sephadex G-200 of papain-digested proteoglycans from cornea and tracheal cartilage

(a) Corneal proteoglycans, fraction 50P, after papain digestion. (b) Corneal proteoglycans, fraction 60P, after papain digestion. Two peaks, 60P-PAP-A and 60P-PAP-B, were pooled as indicated by the horizontal bars and analysed for (1) ratio of dermatan sulphate/chondroitin sulphate (see Table 4), and (2) susceptibility to their degradation by chondroitinase AC (Fig. 6). (c) Corneal proteoglycans, fraction 70P, after papain digestion. (d) Papain-digested proteoglycan from bovine tracheal cartilage (Heinegård & Hascall, 1974). —, Uronic acid (carbazole absorbance at 530 nm); ----, hexose (anthrone absorbance at 620 nm). The arrow indicates the void volume of the column.

Table 4. Dermatan sulphate/chondroitin sulphate ratios of the corneal galactosaminoglycans

The ratios were determined with the thiobarbituric acid assay (Hascall & Heinegård, 1974). 50P and 60P are fractions from the ethanol fractionation of corneal proteoglycans. 60P-PAP-A and 60P-PAP-B refer to the subfractions indicated in Fig. 5(b).

Fraction	Dermatan sulphate/chondroitin sulphate ratio (w/w)
50P	9:91
60P-PAP-A	12:88
60P-PAP-B	14:86

column was eluted with formate buffers as described by Antonopoulos *et al.* (1967). Samples of the galactosaminoglycan fraction (eluted with 2.5M-formate) were digested with chondroitinase AC or ABC. The relative colour yield in the thiobarbituric acid procedure (Hascall & Heinegård, 1974) obtained with the two digests indicated that the ratio of dermatan sulphate/chondroitin sulphate disaccharide units in the fraction was 9:91 (w/w) (Table 4).

Fraction 60P. Fraction 60P is intermediate to fractions 50P and 70P with respect to amino acid composition (Table 2). Leucine, aspartic acid and glutamic acid are the predominant amino acids in acid hydrolysates, and serine is comparatively low in amount. Galactose is the major neutral sugar of the glycosaminoglycans in the fraction (Table 3). The glucosamine/galactosamine ratio (54:46, w/w) of the glycosaminoglycans of fraction 60P (Table 1) indicates that this fraction contains keratan sulphate also. The keratan sulphate chains from fractions 60P and 70P show similar Sephadex G-200 gel-chromatographic patterns, as shown by the anthrone reactivity (Figs. 5b and 5c).

The great polydispersity of the material in fraction 60P is shown by the gel chromatogram in Fig. 4(c). It is also demonstrated that the tracings for anthrone- and carbazole-positive material have maxima at different elution volumes, indicating that the hydrodynamic volume of the proteoglycans changes with their glycosaminoglycan composition.

The galactosaminoglycan chains from fraction 60P show a larger average hydrodynamic volume on Sephadex G-200 chromatography than the galactosaminoglycan chains from fraction 50P or chondroitin sulphate chains from tracheal cartilage (Figs. 5a, 5b and 5d). No significant differences in the dermatan sulphate/chondroitin sulphate ratio could be demonstrated for galactosaminoglycans of different molecular sizes, since the materials in fractions 60P-PAP-A and 60P-PAP-B indicated in Fig. 5(b) have similar ratios (Table 4). Portions of the material in these two peaks (60P-PAP-A and 60P-PAP-B) were digested with chondroitinase AC and chromatographed on Sephadex G-200 (Fig. 6). Almost all of the glycosaminoglycan chains of fraction 60P-PAP-A were degraded to small pieces which were eluted with the total volume of the column (Fig. 6a). It seems likely that the disaccharide units which contain the iduronic acid are intercalated between the glucuronic acid-containing disaccharide units along the polysaccharide chain and that the chains do not contain long disaccharide sequences that are chondroitinase AC-resistant, i.e. contain iduronic acid. The chromatograms of chondroitinase AC-digested fraction 60P-PAP-B (Fig. 6b), however, show that some carbazole-positive material is eluted only slightly more retarded than the undegraded material. There

Fig. 6. Gel chromatography on Sephadex G-200 of corneal glycosaminoglycans before and after digestion with chondroitinase AC

(a) E_{530} for 60P-PAP-A (Fig. 5b) in the carbazole test for uronic acid before (—) and after (····) digestion with chondroitinase AC. (b) E_{530} for 60P-PAP-B (Fig. 5b) in the carbazole test for uronic acid before (—) and after (····) digestion with chondroitinase AC. The arrow indicates the void volume of the column.

is also a large peak of uronic acid in the column total volume. The carbazole colour in the least-retarded peak could result from unspecific interference from keratan sulphate, since fraction 60P-PAP-B contains much more keratan sulphate than fraction 60P-PAP-A, as indicated by tracing of the anthrone-reactive material in Fig. 5(b). However, this peak could also contain large segments of disaccharide units that contain iduronic acid. Such segments are resistant to digestion with chondroitinase AC.

Fraction 70P. Leucine, aspartic acid and glutamic acid are the predominant amino acids in acid hydrolysates of fraction 70P, but the content of glycine, the predominant amino acid of fraction 50P, is much lower (Table 2). Galactose and mannose comprise 96 mol % of the neutral sugar of the polysaccharides (Table 3). The glycosaminoglycans of fraction 70P contain only negligible amounts of galactosamine, 1% or less of the total hexosamine content. Keratan sulphate peptides isolated after papain digestion of bovine cornea have been reported to have glucosamine/galactosamine ratios of 50 (Seno *et al.*, 1965) and 160 (Greiling & Stuhlsatz, 1966). Thus fraction 70P seems to contain a keratan sulphate proteoglycan free from galactosaminoglycans. Such a proteoglycan has not been demonstrated before.

The gel chromatogram of fraction 70P (Fig. 4d) shows two peaks which are only partially separated.

It has so far not been possible to demonstrate any significant differences in the amino acid or hexosamine composition of the proteoglycans in the two peaks.

Gel chromatography after papain digestion (Fig. 5c) shows that the keratan sulphate chains have an average hydrodynamic volume which is smaller than the average hydrodynamic volume of the galactosaminoglycan chains. The anthrone-positive material eluted with the total volume could be either glycoprotein-type oligosaccharides or traces of cellulose from the ion exchanger used for the purification of the proteoglycans.

Evaluation of the extraction, purification and fractionation procedure

High-speed homogenization of fresh bovine corneas in water yields only about 45% of the total tissue hexosamines in solution (Stuhlsatz *et al.*, 1971). The extracted proteoglycans show extreme polydispersity on analytical ultracentrifugation and gel chromatography, and the gel-chromatographic behaviour is partly irreproducible (Stuhlsatz *et al.*, 1971). During such an extraction procedure there is a great risk of degradation of proteoglycans owing to high shear forces (Sajdera & Hascall, 1969) and liberation of cathepsins (Lucy *et al.*, 1961; Morrison *et al.*, 1973). The yield of extracted corneal glycosaminoglycans can be increased to 85% of the total when homogenization in water and 0.1 M- K_2CO_3 is used (Berman, 1970). However, at the high pH of such solutions, β -elimination of chondroitin sulphate chains is likely to occur. Purification of the extract with a method involving a Pronase-digestion step as described by Berman (1970) will most likely split the proteoglycans into glycosaminoglycan peptides.

The extraction, purification and fractionation procedure presented in this and in a preceding paper (Antonopoulos *et al.*, 1974) yields extracts that contain 85–90% of the proteoglycans of the tissue (measured as hexosamines in glycosaminoglycans). The conditions used are mild, and high shear forces, alkaline pH and hypo-osmotic solutions are avoided to minimize the risk of degradation of the proteoglycans. Further, the high concentrations of urea and guanidinium chloride in this procedure will probably inactivate most tissue proteinases (Siegel *et al.*, 1972). The alcohol fractionation subsequently used permits the isolation of, on the one hand, a keratan sulphate proteoglycan that contains no galactosaminoglycan, and on the other hand, a chondroitin sulphate-dermatan sulphate proteoglycan with very low content of keratan sulphate. These molecules, considering the methods used, most probably represent proteoglycans with intact primary structures. Stuhlsatz *et al.* (1971) suggested, from the results of metabolic

studies, that pure keratan sulphate proteoglycans are present in bovine corneas, although they did not isolate molecules of this type.

To show that the results obtained with the alcohol fractionation were not artifacts caused by exposure of the proteoglycans to urea, a crude guanidinium chloride extract was fractionated with alcohol with no exposure to urea or ion-exchange resins. The distribution of keratan sulphate and chondroitin sulphate in the fractions obtained was identical with the distribution obtained with proteoglycans which were previously purified by the DEAE-cellulose procedure.

The presence of the glycoprotein type of oligosaccharides in all fractions of cornea proteoglycans is not explained, but it is likely that these oligosaccharides are bound to the same protein core as the glycosaminoglycans.

Comparison with proteoglycans from other tissues

When data from the present investigation are compared with data on proteoglycans from various types of tissues it can be deduced that corneal proteoglycans have much higher protein contents and smaller molecular size than do, for instance, cartilage proteoglycans (Heinegård, 1972; Antonopoulos *et al.*, 1974). The individual chondroitin sulphate chains are larger in corneal proteoglycans (Fig. 5) and corneal chondroitin sulphate is partially hybridized with dermatan sulphate, in contrast with chondroitin sulphate from hyaline cartilage. Further, corneal proteoglycans appear to contain glycoprotein-type oligosaccharides not found in cartilage proteoglycans. Another point of difference is that it has not been possible to demonstrate a cartilage proteoglycan that contains keratan sulphate but no chondroitin sulphate.

We are very grateful to Dr. Ingemar Carlstedt, Mr. Peter Ericson and Dr. Anders Malmström for help with various analytical techniques. I. A. is recipient of a predoctoral scholarship from the Faculty of Medicine, University of Lund. This work was supported by grants from the Swedish Medical Research Council (project no. B74-13X-139-10A), the Faculty of Medicine at the University of Lund, Konung Gustaf V:s 80-årsfond, the Swedish Society of Medical Research, the Royal Physiographic Society of Lund, Harald Jeansson's Stiftelse and Harald and Greta Jeansson's Stiftelse.

References

- Anseth, A. (1961) *Exp. Eye Res.* **1**, 106–115
 Antonopoulos, C. A., Gardell, S., Szirmai, J. A. & de Tyssens, E. R. (1964) *Biochim. Biophys. Acta* **83**, 1–19

- Antonopoulos, C. A., Fransson, L.-Å., Heinegård, D. & Gardell, S. (1967) *Biochim. Biophys. Acta* **148**, 158–163
 Antonopoulos, C. A., Axelsson, I., Heinegård, D. & Gardell, S. (1974) *Biochim. Biophys. Acta* **338**, 108–119
 Berman, E. R. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 2, pp. 879–886, Academic Press, New York
 Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330–334
 Davidson, E. A. & Meyer, K. (1954) *J. Biol. Chem.* **211**, 605–611
 Dische, Z. (1947) *J. Biol. Chem.* **167**, 189–198
 Fransson, L.-Å., Rodén, L. & Spach, M. L. (1968) *Anal. Biochem.* **23**, 317–330
 Gardell, S. (1957) *Acta Chem. Scand.* **11**, 668–675
 Goa, J. (1955) *Scand. J. Clin. Lab. Invest.* **7**, Suppl. **22**, 19–25
 Greiling, H. & Stuhlsatz, H. W. (1966) *Hoppe-Seyler's Z. Physiol. Chem.* **345**, 236–248
 Greiling, H., Stuhlsatz, H. W. & Plageman, L. (1967a) *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 121–128
 Greiling, H., Stuhlsatz, H. W. & Kisters, R. (1967b) *Hoppe-Seyler's Z. Physiol. Chem.* **348**, 970–978
 Handley, C. J. & Phelps, C. F. (1972) *Biochem. J.* **128**, 205–213
 Hascall, V. C. & Heinegård, D. (1974) *J. Biol. Chem.* **249**, 4232–4241
 Hascall, V. C., Riolo, R. L., Hayward, J. & Reynolds, C. C. (1972) *J. Biol. Chem.* **247**, 4521–4528
 Heinegård, D. (1972) *Biochim. Biophys. Acta* **285**, 181–192
 Heinegård, D. (1973) *Chem. Scripta* **4**, 199–201
 Heinegård, D. & Hascall, V. C. (1974) *Arch. Biochem. Biophys.* **165**, 427–441
 Lindahl, U. (1970) *Biochem. J.* **116**, 27–34
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
 Lucy, J. A., Dingle, J. T. & Fell, H. B. (1961) *Biochem. J.* **79**, 500–508
 Meyer, K., Linker, A., Davidson, E. A. & Weissman, B. (1953) *J. Biol. Chem.* **205**, 611–616
 Morrison, R. I. G., Barrett, A. J., Dingle, J. T. & Prior, D. (1973) *Biochim. Biophys. Acta* **302**, 411–419
 Ringertz, R. & Reichard, P. (1960) *Acta Chem. Scand.* **14**, 303–311
 Rodén, L. (1970) in *Chemistry and Molecular Biology of the Intercellular Matrix* (Balazs, E. A., ed.), vol. 2, pp. 797–821, Academic Press, London and New York
 Sajdera, S. W. & Hascall, V. C. (1969) *J. Biol. Chem.* **244**, 77–87
 Seno, N., Meyer, K., Anderson, B. & Hoffman, Ph. (1965) *J. Biol. Chem.* **240**, 1005–1010
 Siegel, S., Brady, A. H. & Awad, W. M. (1972) *J. Biol. Chem.* **247**, 4155–4159
 Smith, I. (1960) in *Chromatographic and Electrophoretic Techniques*, vol. 1, p. 252, Interscience, New York
 Stuhlsatz, H. W., Kisters, R., Wollmer, A. & Greiling, H. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* **352**, 289–303
 Stuhlsatz, H. W., Muthiah, P. L. & Greiling, H. (1972) *Scand. J. Clin. Lab. Invest.* **29**, Suppl. **123**, 31
 Yamagata, T., Saito, H., Habuchi, O. & Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1523–1535