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Characterization of the Keratan Sulphate Proteoglycans from Bovine Corneal Stroma

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The keratan sulphate proteoglycans that can be prepared from bovine corneal stroma [Axelsson & Heinegård (1975) *Biochem. J.* **145**, 491–500] were characterized by gel chromatography, gel electrophoresis and analytical ultracentrifugation in associative (0.6M-NaCl) and dissociative (6M-guanidinium chloride) solvents. The proteoglycans aggregated at low salt concentrations and pH. The weight-average molecular weight of the monomer proteoglycan was 72000 in dissociative solvent. The presence of disulphide bonds in the proteoglycans was established. Keratan sulphate peptides and oligosaccharide peptides were isolated after proteolysis. Their composition indicated that both are linked to protein via asparagine residues. A tentative model for corneal keratan sulphate proteoglycans is suggested.

About one-third of the proteoglycans obtained by extraction of bovine corneal slices with 4M-guanidinium chloride can be isolated in a fraction that contains a large proportion of the keratan sulphate of the tissue and small amounts of oligosaccharides, but no galactosaminoglycans (Axelsson & Heinegård, 1975). Such a keratan sulphate proteoglycan has not been identified in any other tissue. In comparison, all the keratan sulphate in cartilaginous tissues is attached to the protein core, which also contains chondroitin sulphate chains. Some of the properties of the unique keratan sulphate proteoglycan prepared from cornea were studied in an attempt to identify the structural differences from cartilage proteoglycans. A preliminary account of some of this work has been given (Axelsson & Heinegård, 1976).

Experimental

Materials

Corneas from adult cows were prepared as described previously (Axelsson & Heinegård, 1975). DEAE-cellulose (DE-23) and ECTEOLA-cellulose (ET-11) were from Whatman, Springfield Mill, Maidstone, Kent, U.K. Dowex ion-exchange resins were from Bio-Rad Laboratories, München, Germany. Sepharose and Sephadex gels were from Pharmacia Fine Chemicals, Uppsala, Sweden. Papain (twice-crystallized) was from Sigma Chemical Co., St. Louis, MO, U.S.A. Solutions of guanidinium chloride (practical grade; Sigma) were shaken with active charcoal overnight and filtered through filter paper before use to remove u.v.-absorbing material. High-molecular-weight hyaluronate from rooster comb was a gift from Dr. Endre A. Balazs, Eye Research Institute, Columbia University, New York, NY, U.S.A.

Abbreviation used: SDS, sodium dodecyl sulphate.

Analytical methods

Column effluents. The effluents from gel columns were analysed for hexose and protein contents by automated versions (Heinegård, 1973) of the methods of Goa (1955) (A_{620}) and Lowry *et al.* (1951) (A_{680} or A_{750}) respectively, except when guanidinium chloride solutions were used for elution. In these cases, hexoses were determined by the manual procedure described by Goa (1955), and proteins were detected by their A_{280}.

Neutral sugar. The neutral-sugar composition of samples was determined by g.l.c. of their alditol acetates as described previously (Axelsson & Heinegård, 1975). Methanol was added to the alditols before acetylation and removed by evaporation. This procedure was repeated several times to ensure complete removal of borate (Albersheim *et al.*, 1967).

Uronic acids. The oligosaccharide peptides were analysed for uronic acid contents by using the carbazole procedure of Bitter & Muir (1962).

Amino acids and hexosamines. Amino acids and hexosamines were determined by using a Biocal BC 200 automatic amino acid analyser after hydrolysis of samples under argon in 6M-HCl at 110°C for 24h and in 8M-HCl at 95°C for 3h respectively. Norleucine was used as the internal standard for amino acid analysis.

Cysteine and cystine. The total amount of cysteine

and cystine was determined as cysteic acid by using the amino acid analyser after oxidation of samples with performic acid as described by Moore (1963) and subsequent hydrolysis as described above. Cysteine (SH groups) was determined by the colorimetric reaction described by Grassetti &Murray (1967). Reduced glutathione was used as the standard; it gave a molar absorbance coefficient close to that described by Grassetti & Murray (1967).

Isolation of keratan sulphate proteoglycans

Keratan sulphate proteoglycans were extracted with 4M-guanidinium chloride (pH5.8), purified by DEAE-cellulose chromatography and fractionated by ethanol precipitation as described previously (Antonopoulos *et al.*, 1974; Axelsson & Heinegård, 1975). This preparation (referred to as 'fraction 70P'; Axelsson & Heinegård, 1975) was in some cases further purified by gel chromatography in denaturing solvent, but was usually used without further purification.

Isolation of keratan sulphate peptides and oligosaccharides from proteoglycan preparations

A portion (25mg) of the proteoglycan preparation was dissolved in 3ml of 0.1M-sodium phosphate buffer (pH7.0) containing 5mM-EDTA and 5mM-cysteine hydrochloride. Papain (0.4mg) was added and the sample was incubated at 65°C for 4h. A papain blank was prepared by incubating a solution containing no sample under identical conditions. After incubation the samples and the blank were diluted with 3ml of water and applied to ECTEOLA-cellulose columns (Cl^- form; bed size 1.3cm×6cm) equilibrated with water. About 95% of the oligosaccharide-peptides were eluted with 2 bed-volumes of water and about 5% with 2 bed-volumes of 0.02M-HCl; these two oligosaccharide–peptide fractions were pooled. They contained all the oligosaccharides (Anseth, 1961) and, in addition, free amino acids and oligopeptides. The keratan sulphate peptides were eluted from the ion-exchange resin with 6M-HCl (Anseth, 1961; Antonopoulos *et al.*, 1967) and a portion was hydrolysed for amino acid analysis. The values were corrected for the small amounts of amino acids found in the corresponding fraction from the column with the papain blank applied. The oligosaccharide–peptide fraction was freeze-dried, dissolved in 1.5ml of a pyridinium acetate buffer (0.25M-pyridine buffered with acetic acid to pH6.5) and applied to a column of Sephadex G-50 (superfine grade) (see Fig. 12 below) eluted with the same pyridinium acetate buffer. Samples (0.2ml) from each fraction were freeze-dried, redissolved in 0.5ml of water and analysed for hexose and protein by automated methods (Heinegård, 1973). The anthrone-positive fractions were pooled as indicated in Fig. 12, freeze-dried and analysed for contents of amino acids, hexosamines and neutral sugars.

Reduction and alkylation of proteoglycans

Reduction of proteoglycan samples was performed with dithiothreitol and followed by alkylation with iodoacetic acid as described by Sajdera & Hascall (1969), with minor modifications. Samples (5–6mg/ml) were dissolved in 4M-guanidinium chloride/0.1M-Tris/HCl buffer, pH8.5, and 1M-dithiothreitol (10μl/ml of solution) was added. The samples were incubated in a 37°C water bath for 4h. Then 1M-iodoacetic acid (40μl/ml solution) was added and the samples were stirred with a magnetic stirrer in darkness at room temperature (20°C) overnight (16h). They were then dialysed for 3 days at 4°C against the buffer used in the gel chromatography that followed.

Gel chromatography

Details of the conditions used for gel chromatography are given in the text and in the Figure legends. The methods used for calibration of gels with well-characterized proteins are described elsewhere (Axelsson, 1978). When the elution buffer was changed, at least 2 bed-volumes of the new buffer were passed through the column before the sample was applied. Buffers containing guanidinium chloride or SDS will be referred to as dissociative solvents, since guanidinium chloride and SDS break non-covalent bonds and dissociate, e.g. cartilage proteoglycan aggregates. Other buffers will be referred to as 'associative' buffers. The columns packed in and equilibrated with dissociative buffers were never eluted with associative buffers, and vice versa. Most associative buffers contained 0.02% NaN_3 as a bacteriostatic agent. Control chromatographic runs without NaN_3 showed that NaN_3 does not affect the gel-chromatographic pattern of the proteoglycans at neutral or acid pH.

SDS/polyacrylamide-gel electrophoresis

Electrophoresis was performed on 7% (w/v) polyacrylamide gels in 0.1% SDS as described by Neville (1971). The gels were stained with Coomassie Brilliant Blue (Weber & Osborn, 1969) and scanned for A_{605} by using a Gilford model 222 photometer equipped with a Beckman model DU monochromator and a gel-scanning device (constructed by Dr. Sven Björnsson and Mr. Runo Svensson of this laboratory).

Determination of partial specific volume

The partial specific volume of the proteoglycans was determined. This required estimation of the dry weights of the proteoglycan samples. Attempts to determine the dry weight by drying samples in vacuum over P_2O_5 followed by weighing at atmospheric pres-

sure were unsuccessful, since the sample weight increased rapidly during the first 5min owing to water uptake by the sample, as described by Laurent & Anseth (1961). The dry-weight determinations were therefore performed with a Cahn RG electrobalance (Ventron Instruments Corp., Paramount, CA, U.S.A.), with the weighing device inside a high-vacuum system. The air was evacuated with a Speedivac high-vacuum pump (model ES 50) and a Speedivac oil-vapour-diffusion pump (model E 02). The pressure was monitored with a Penning gauge head (model 6). The pumps and the manometer were from Edwards High Vacuum Ltd., Crawley, Sussex, U.K. The weight of the sample was continually registered by means of a Servogor recorder. The air pressure was maintained at 1mPa (10nmHg) for several days until the weight of the sample was constant. This weight was considered to be the dry weight of the sample. When air was allowed to enter the system, the sample weight increased by 5–10% within 15min. The sample was utilized for preparation of solutions with known proteoglycan concentration in 0.15M-NaCl/5mM-Tris/HCl (pH7.0). Corrections were made for the small amounts (1–5%) of sample that were not dissolved in the buffer. The densities of the solutions were determined by means of a mechanical oscillator (Precision density meter DMA 02C from Anton Paar K.G., Graz, Austria) and the partial specific volume of the sample was calculated (Kratky *et al.*, 1973).

Molecular-weight determination

The weight-average molecular weight of fraction 70P was determined by long-column meniscus-depletion sedimentation-equilibrium centrifugation (Chervenka, 1970). Three solutions with different sample concentrations were centrifuged at two different speeds in 12mm double-sector capillary synthetic-boundary cells in an An-D rotor in a Beckman–Spinco model E centrifuge. Photographs of the interference optics patterns were analysed in a Nikon comparator and the apparent weight-average molecular weights were calculated from the plots of ln(fringe displacement) versus the square of the radial distance (Chervenka, 1969). Attempts were made to follow the fringes to the bottom of the cell, although this is technically difficult. The effect of 6M-guanidinium chloride on the partial specific volume and the molecular weight of proteins is small (Mann *et al.*, 1970; Bryce & Crichton, 1971; Reisler *et al.*, 1977) and therefore no correction was made for this effect.

Sedimentation-velocity ultracentrifugations

Sedimentation-velocity centrifugations were carried out with an An-D rotor in a Beckman–Spinco model E centrifuge or with an MSE six-place analytical rotor in an MSE Centriscan 75 centrifuge. Schlieren patterns were recorded in both cases. Sedimentation coefficients ($s_{20,w}$) were calculated as described by Schachman (1957).

Polydispersity analysis by transport centrifugations

Polydispersity of proteoglycans was studied by using the transport-centrifugation method described by Pita & Müller (1973). A Sorvall OTD-2 centrifuge and a Beckman SW 50.1 swinging-bucket rotor equipped with the adaptors described by Pita & Müller (1973) were used. Centrifugation was at 20°C for various times and rotor speeds. The volume of the sample solution was 0.55ml, and 20 fractions (25μl each) were removed by aspiration from the top of the tube after centrifugation. The distance from the rotor centre to the meniscus was 6.95cm. The length of the part of the liquid column that was fractionated for analysis was 3.00cm. The fractions were analysed for protein (A_{280}) in a Zeiss PMQ II spectrophotometer fitted with a 5μl micro-cuvette (light-path 1.0cm; J. C. Pita, personal communication).

Results and Discussion

Characterization of proteoglycans by gel chromatography

The corneal keratan sulphate proteoglycans (fraction 70P) can be fractionated to give two major peaks (fractions 70PA and 70PB; Axelsson & Heinegård, 1975) by chromatography on 4% agarose at neutral pH. The proportions of fraction 70PA and fraction 70PB show some variation between different batches of proteoglycans even if they are prepared under as reproducible conditions as possible. The material in fraction 70PA may represent partially denatured proteoglycans (see below). In most preparations there is a slight excess of fraction 70PB over fraction 70PA. The K_D values of the two peaks are always the same. A small proportion of the material is eluted in the void volume. When the pH of the elution buffer is lowered, the relative size of fraction 70PA increases, whereas fraction 70PB decreases (Fig. 1). It is possible then that the molecules eluted in fraction 70PB change their conformation and/or form aggregates. The amounts of material completely excluded from the gel show only a minor increase. The hexose profiles (not shown in Fig. 1) always follow the protein curves very closely. Therefore the hexose/protein ratio is similar in all three peaks. When the samples are preincubated in elution buffer for 24h before chromatography they show the same elution pattern as samples that were not preincubated. It is thus likely that the chromatograms represent equilibrium or near-equilibrium. The changes in the gel-chromatographic behaviour are reversible (----, Fig. 1). The material in peaks 70PA and 70PB was isolated from a preparative Sepharose 4B column and rechromatographed on

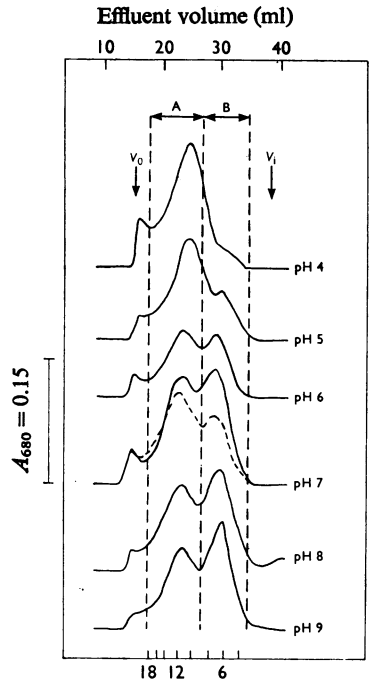

Fig. 1. *Chromatography of keratan sulphate proteoglycans (fraction 70P) on Sepharose 4B at different pH values*
Samples (0.25–0.30mg) were dissolved in 200μl of elution buffer and applied to the column. The curves show the protein concentration (A_{680}); the broken curve (----) is for a sample incubated in the pH4 buffer for 24h, then dialysed against pH7 buffer for 24h and finally run on the column at pH7. Elution buffers were 0.6M-NaCl/0.05M-acetic acid adjusted with NaOH to pH4, 5, 6 and 7, and 0.6M-NaCl/0.05M-barbitone sodium adjusted with HCl to pH8 and 9. The scale showing Stokes radius was obtained by calibration with globular proteins. The bed size of the column was 0.6cm×134cm and the fraction size 0.57ml. In this and the other Figures, V_0 is the void volume of the gel, and V_i is the total solvent volume in the gel.

an analytical Sepharose 4B column. They chromatographed as two separate peaks (Figs. 2*a* and 2*b*), with the same elution volume as the original peaks. It is most likely, then, that the material in peak 70PA either represents aggregated peak-70PB molecules or that they are an unrelated molecular species. When the material from peak 70PB was separately chromatographed at the lower pH, however, the 'peak' was eluted at essentially the same position as at pH7 (Fig. 2*c*). Since no 'peak' was eluted at this position when fraction 70P was chromatographed at acid pH, the simplest explanation is that the molecules in fraction 70PA can interact with the molecules in fraction 70PB to form aggregates at acid pH, whereas isolated fraction 70PB cannot form aggregates.

The effects of different salt concentrations and of dissociating agents on the gel-chromatographic pattern of the proteoglycans were studied. The same elution patterns were obtained with 0.15, 0.3, 0.6 and 0.9M-NaCl in 0.05M-sodium acetate buffer (pH7.0) (results not shown). Most of the proteoglycans were eluted from a Sepharose 4B column with 4M-guanidinium chloride in a peak with K_D about 0.63 (Fig. 3*a*), i.e. close to that of peak 70PB (0.61) eluted with associative buffers from Sepharose 4B. The effluent was pooled as indicated in Fig. 3(*a*), recovered and rechromatographed under associative conditions (Fig. 3*b*). The elution curves at pH7 and 4 are similar to the original patterns (cf. Fig. 1). It is

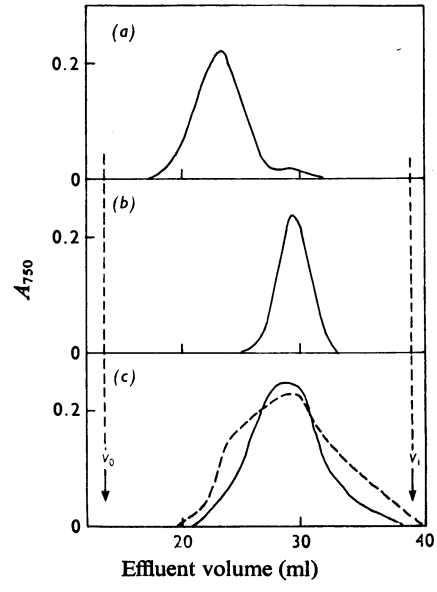

Fig. 2. *Rechromatography on Sepharose 4B of fractions 70PA and 70PB*
Fractions 70PA and 70PB were isolated from a chromatographic run of fraction 70P on a preparative Sepharose 4B column (bed size 1.6cm×128cm; fraction size 4.6ml), eluted with 0.5M-sodium acetate (pH7.0), dialysed against water, freeze-dried and portions (about 0.8mg) were applied to an analytical Sepharose 4B column (bed size 0.6cm×134cm; fraction size 0.84ml) eluted with the same buffer. The effluent was analysed for protein (A_{750}). (*a*) Fraction 70PA; (*b*) fraction 70PB. (*c*) Chromatography on the same column of another preparation of fraction 70PB isolated by the same procedure. Elution was with 0.15M-NaCl/5mM-barbitone sodium buffer/0.02% NaN_3 (pH7.0) (——) or 0.15M-NaCl/5mM-sodium acetate buffer/0.02% NaN_3 (pH4.0) (----).

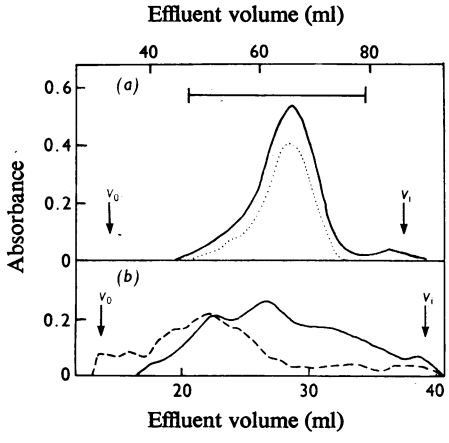

Fig. 3. *Chromatography of proteoglycans on Sepharose 4B under dissociative and associative conditions*

(*a*) A portion (20mg) of fraction 70P was applied to a Sepharose 4B column (bed size 0.8cm×150cm) eluted with 4M-guanidinium chloride/0.05M-sodium acetate, pH5.8. ——, Protein (A_{280}); ····, hexose (A_{620}). (*b*) Rechromatography on a Sepharose 4B column (bed size 0.6cm×134cm) of the material indicated by the horizontal bar in Fig. 3(*a*). The material was first dialysed against water and freeze-dried. ——, Protein (A_{750}) curve from a 1.15mg sample eluted with 0.15M-NaCl/5mM-barbitone sodium buffer (pH7.0)/0.02% NaN_3; ----, protein (A_{750}) curve from a 0.85mg sample eluted with 0.15M-NaCl/5mM-sodium acetate buffer (pH4.0)/0.02% NaN_3.

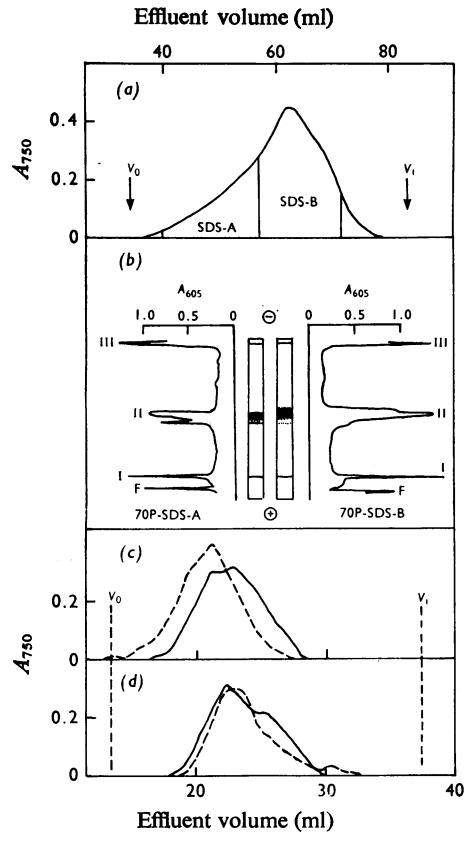

Fig. 4. *Gel chromatography and gel electrophoresis of proteoglycan–SDS complexes*

(*a*) Chromatography of fraction 70P (18mg) on a Sepharose CL-4B column (bed size 0.9cm×141cm; fraction size 1.1ml) eluted with 1% (w/v) SDS in 5mM-barbitone sodium buffer, pH7.0, after pre-incubation of the sample at 37°C for 2h in this solvent. The effluent was analysed for protein (A_{750}). The indicated fractions 70P-SDS-A and 70P-SDS-B were dialysed against water and freeze-dried. (*b*) SDS/polyacrylamide-gel electrophoresis of fractions 70P-SDS-A and 70P-SDS-B. Peak F (front) was marked by inserting a steel needle at the position of Bromophenol Blue, which was included in the samples. (*c*) and (*d*) Rechromatography of portions (0.7–0.9mg) of fractions 70P-SDS-A and 70P-SDS-B respectively on a Sepharose 4B column (bed size 0.6cm×134cm; fraction size 0.58ml) eluted with 0.15M-NaCl/5mM-barbitone sodium/0.02% NaN_3 (——, pH7.0) or with 0.15M-NaCl/5mM-sodium acetate/0.02% NaN_3 (----, pH4.0) and analysed for protein (A_{750}).

likely then that gel chromatography in 4M-guanidinium chloride does not remove any extraneous protein that can cause aggregation. The same results were obtained with chromatography in 6M-guanidinium chloride. When the proteoglycans were chromatographed on a Sepharose 4B column equilibrated with 1% SDS (pH7.0) they also showed one major retarded peak with K_D 0.57 (Fig. 4*a*), i.e. a slightly lower K_D value than that of peak 70PB eluted with associative buffer. SDS will bind to proteins to a much larger extent than guanidinium chloride, and unfolded proteins show partial 'stiff-rod' properties in SDS solutions, but almost pure 'random-coil' properties in guanidinium chloride solutions (Reynolds & Tanford, 1970; Nozaki *et al.*, 1976; Mattice *et al.*, 1976). It is therefore possible that solutions of SDS cause such conformational changes in the proteoglycans that fraction-70PB molecules are eluted with a larger molecular size because of a partial 'stiff-rod' character. The two fractions isolated from the Sepharose 4B column eluted with SDS solution were rechromatographed under associative conditions on a Sepharose 4B column (Figs. 4*c* and 4*d*). Both fractions were eluted in the region of peak 70PA at both pH4 and pH7 (K_D 0.32–0.40; K_D for peak 70PA is about 0.36 in 0.6M-NaCl, pH7). It may be that the remaining, tightly bound SDS cannot dissociate

proteoglycans, but may cause a conformational change that facilitates aggregation of proteoglycans. The same conformational change may be caused by low pH or reduction and alkylation. It is also possible that the SDS may directly serve as a link between proteoglycan molecules. The effects observed cannot be attributed to an effect of SDS or guanidinium chloride on the gel, since such solutions do not change the properties of Sepharose 4B (Fish *et al.*, 1970). Also, the various batches of Sepharose 4B used in the present investigation showed negligible differences in chromatographic properties.

The proteoglycan fractions isolated from the Sepharose 4B column eluted with SDS solution (Fig. 4*a*) were heterogeneous, as indicated by SDS/polyacrylamide-gel electrophoresis (Fig. 4*b*). Most of the material migrated with intermediate mobility (fraction II). Fraction II contained more than one component. No attempt was made to characterize the subfractions.

Reduction and alkylation of the proteoglycans gave a drastic decrease in polydispersity; all reduced and alkylated proteoglycans were eluted at the position of peak 70PA in associative buffers at both pH4 and pH7 (Fig 5). With 6M-guanidinium chloride (pH4 and 7) as eluent the reduced and alkylated proteoglycans showed the same retarded elution pattern as did native proteoglycans in 4M-guanidinium chloride (Fig. 3*a*). These results indicate that the conformation of the protein core in the proteoglycans is important for the gel-chromatographic behaviour. The native conformation, which probably is more ordered, only allows interaction at acid pH. Unfolding of the protein by reduction may expose charged groups, and it is possible that some of the interactions studied are of electrostatic nature and perhaps non-specific. A series of experiments were therefore done to test interactions of fraction 70P with other proteoglycans and glycosaminoglycans. Portions (2mg) of fraction 70P were dissolved in 1ml of 4M-guanidinium chloride/0.05M-sodium acetate (pH5.8) containing 20μg of hyaluronate or 200μg of bovine nasal-cartilage proteoglycan monomer (A1-D1; Heinegård, 1972) and incubated at room temperature for 3–4h. Each portion was then divided into two equal fractions, which were dialysed against 100ml of 0.15M-NaCl/5mM-sodium acetate buffer/0.02% NaN_3 (pH4.0) and 0.15M-NaCl/5mM-barbitone sodium buffer/0.02% NaN_3 (pH7.0) respectively. The samples were separately chromatographed on Sepharose 4B eluted with the respective buffers described. A third 2mg portion of fraction 70P was treated in the same way, but without addition of hyaluronate or cartilage proteoglycans, and served as a control. The six chromatograms obtained are shown in Fig. 6. The fraction-70P preparation used in these experiments contained a larger proportion of fraction-70PB molecules, as shown in Fig. 6. This material therefore probably represents more native molecules. Hyaluronate and fraction A1-D1 were eluted in the void volume of the Sepharose 4B. Almost all of the protein in the chromatograms came from corneal proteoglycans, since neither hyaluronate nor fraction A1-D1 contains appreciable amounts of protein. The curves suggest then that a portion of fraction 70P can bind to hyaluronate or fraction A1-D1 at pH4, but not at pH7, since the void volume contained a large proportion of material, particularly when fraction A1-D1 was chromatographed at pH4. As discussed above, it is therefore likely that at lower pH, positively charged groups in the proteoglycan protein may interact with the negative charges of the glycosaminoglycan chains. The pH-dependent changes in elution pattern may therefore be caused by unspecific proteoglycan–proteoglycan interactions.

The Sepharose 4B column used for the experiments presented in Fig. 1 was calibrated with globular proteins (Axelsson, 1978). The average molecular size of fractions 70PA and 70PB could then be determined; these fractions have the same K_D as proteins with Stokes radius about 11nm and 7nm respectively. These values are very approximate, since the calibration curve was extrapolated for values for Stokes radius above that of the largest

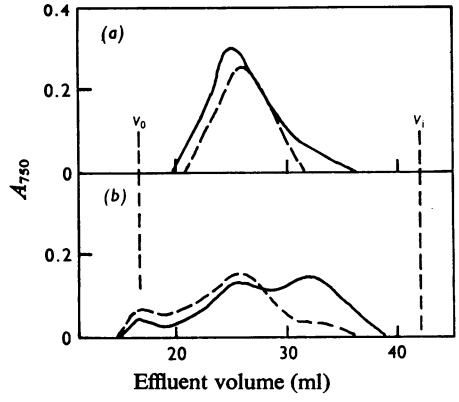

Fig. 5. *Gel chromatography of reduced and alkylated proteoglycan*

A Sepharose 4B column (bed size 0.6cm×134cm; fraction size 0.98ml) was eluted with 0.6M-NaCl/0.05M-sodium acetate buffer, pH4.0 or pH7.0, and the effluent was analysed for protein (A_{750}). The same preparation of fraction 70P was used for all experiments. (*a*) Reduced and alkylated fraction 70P, and (*b*) untreated control, chromatographed at pH7 (——) and pH4 (----). The control sample was incubated in the same guanidinium chloride solution as the other sample, but no reducing or alkylating agents were added.

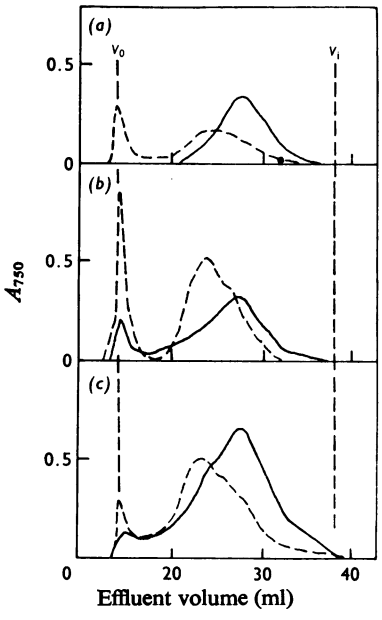

Fig. 6. *Gel chromatography of proteoglycans after incubation with hyaluronate and with proteoglycan monomers from bovine nasal cartilage*

The same preparation of fraction 70P was used in all experiments; this preparation contained only small amounts of fraction 70PA at pH7. A Sepharose 4B column (bed size 0.6cm×134cm; fraction size 0.6ml) was eluted with 0.15M-NaCl/5mM-barbitone sodium buffer/0.02% NaN_3 (pH 7.0) or 0.15 M-NaCl/5 mM-sodium acetate buffer/0.02% NaN_3 (pH4.0). —— and ----, Protein (A_{750}) curves obtained at pH7.0 and pH4.0 respectively. (*a*) Fraction 70P and hyaluronate; (*b*) fraction 70P and nasal cartilage proteoglycan monomers; (*c*) fraction 70P control incubated in guanidinium chloride solution like the other samples but without addition of other macromolecules.

protein used (8nm). Further, K_D is dependent on the shape of the molecules (Nozaki *et al.*, 1976; Axelsson, 1978).

In summary, two populations (fractions 70PA and 70PB) of corneal keratan sulphate proteoglycans (fraction 70P) can be prepared by chromatography on Sepharose 4B in 0.15–0.6M-NaCl. Fraction 70PB predominates in most batches of corneal proteoglycans. It has an elution volume between those of jack-bean urease and bovine thyroglobulin (Stokes radii 6.5 and 8.6nm respectively) (Axelsson, 1977), which means that it is much smaller than cartilage proteoglycans. At low pH or after reduction and alkylation it forms aggregates. This aggregation requires the presence of fraction 70PA. Most of fraction 70PA is dissociated by SDS and guanidinium chloride to monomers with approximately the same K_D as fraction 70PB. Other explanations for the relations between fractions 70PA and 70PB are, however, possible. Therefore further studies using ultracentrifugation, determination of disulphide bonds and proteolytic fragmentation were made.

Characterization of proteoglycans by ultra centrifugation

Sedimentation-velocity runs of fraction 70P in associative buffers indicated that the material was very polydisperse. Therefore fractions 70PA and 70PB were analysed separately in associative buffers (Fig. 7) and fraction 70P was centrifuged only in 6M-guanidinium chloride (Fig. 8). Fraction 70PB showed a distinct schlieren peak with $s_{20,w}$ 2.7S (sample concentration 3.6g/l; the partial specific volume for fraction 70P was found to be 0.67ml/g; this value was used in the subsequent calculations) (Fig. 7*a*). The schlieren pattern obtained with fraction 70PA contained a corresponding peak ($s_{20,w}$ 3.1S; sample concentration 3.6g/l), but most of the material sedimented faster and showed a large

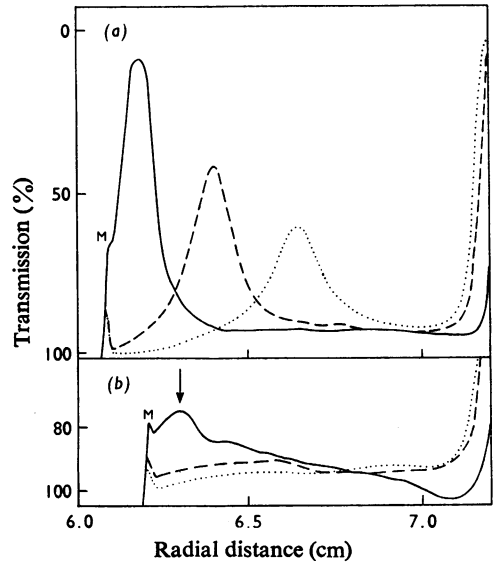

Fig. 7. *Sedimentation-velocity centrifugation of fractions 70PA and 70PB in associative buffers*

Centrifugation was performed in an MSE Centriscan 75 centrifuge at 50000rev./min. The samples (3.6mg/ml) were dissolved in and dialysed against 0.6M-NaCl/5mM-barbitone sodium/0.02% NaN_3 (pH7.0). 'M' indicates the meniscus. (*a*) Schlieren patterns (wavelength 550nm) of fraction 70PB obtained 40s after the curves in (*b*). $s_{20,w}$ for the main peak was 2.7S. (*b*) Schlieren patterns (wavelength 550nm) of fraction 70PA obtained 30, 120 and 210min after reaching full speed. $s_{20,w}$ for the peak indicated by the arrow was 3.1S.

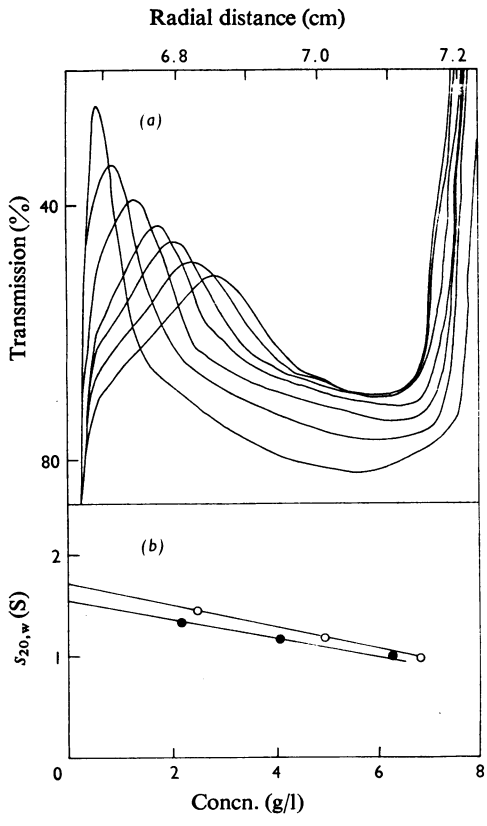

Fig. 8. *Sedimentation-velocity centrifugation of the proteoglycans (70P) in dissociating buffer*

The samples were dissolved in and dialysed against 6M-guanidinium chloride/0.05M-Tris/HCl, pH7.0. (*a*) Schlieren curves (wavelength 550nm) from a run of untreated fraction 70P in an MSE Centriscan 75 centrifuge at 60000rev/min. The sample concentration was 4.9g/l and the curves were obtained 40, 80, 120, 165, 210, 255 and 300min after reaching full speed. (*b*) Determination of sedimentation constants for untreated (○) and reduced and alkylated (●) fraction 70P. By extrapolation of the best least-squares regression lines the $s^0_{20,w}$ values 1.7S and 1.5S were obtained for untreated and reduced and alkylated fraction 70P respectively.

polydispersity (Fig. 7*b*). In 6M-guanidinium chloride solution, a single boundary was obtained with fraction 70P (Fig. 8*a*); the $s^0_{20,w}$ value was 1.7S for the untreated proteoglycan and 1.5S for the reduced and alkylated proteoglycan (Fig. 8*b*). The difference is small, but suggests that the reduced and alkylated proteoglycans are more unfolded than the untreated proteoglycans. Neither sedimentation analysis nor gel chromatography indicated that peptides were released by reduction. It may be concluded from Fig. 8(*a*) that there are small amounts of faster-sedimenting material even in 6M-guanidinium chloride, but most of the proteoglycans seem to be dissociated into monomers. Fraction 70PA contains molecules of higher molecular weight than does fraction 70PB, since fraction 70PA has a larger molecular size (according to gel-chromatographic data) and higher average $s_{20,w}$ (according to Fig. 7). Therefore it is possible that the changes induced in fraction 70PB by, e.g., low pH, involves aggregation. Further evidence was obtained by analysis of the polydispersity by the transport-centrifugation method described by Pita & Müller (1973) (Fig. 9). The protein concentration (measured as A_{280}) at the plateau region (20–30S) was 70% of the initial protein concentration at both pH4 and pH7. This decrease in protein cannot be fully explained by radial dilution. Therefore it can be estimated that about 20% of the material sedimented so fast that it was not detected under the experimental conditions. Of the remaining material, about 70% showed apparent s values above 5S at pH4, whereas at pH7 only about 15% has s values above 5S. These results suggest that low pH causes aggregation of the proteoglycans, corroborating the gel-chromatographic data.

The weight-average molecular weight of the proteoglycan monomer was 72000, as determined by sedimentation-equilibrium centrifugation at 22000rev./min with dissociating solvent (Fig. 10). The plots of the ln(fringe displacement) versus the square of the radial distance were perfectly linear to the bottom of the cell, suggesting monodispersity of the sample. However, a considerable polydispersity is suggested by the gel-chromato-

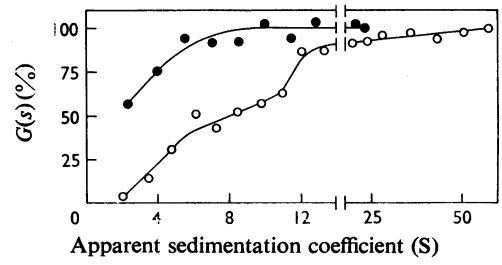

Fig. 9. *Polydispersity analysis of proteoglycans (70P) by transport centrifugation in associative buffers*

The fractions were analysed for protein (A_{280}), and integral-distribution function [$G(s)$] curves were calculated as described by Pita & Müller (1973). A portion of fraction 70P (1.8g/l) in 0.15M-NaCl/5mM-sodium acetate, pH4.0, was centrifuged at 52950rev./min for 83.3min or at 48930rev./min for 30min (○). Another portion (1.8g/l) was centrifuged in 0.15M-NaCl/5mM-sodium phosphate, pH7.0, at 44600rev./min for 100min (●). Centrifugation times were measured from the start of acceleration to the start of retardation.

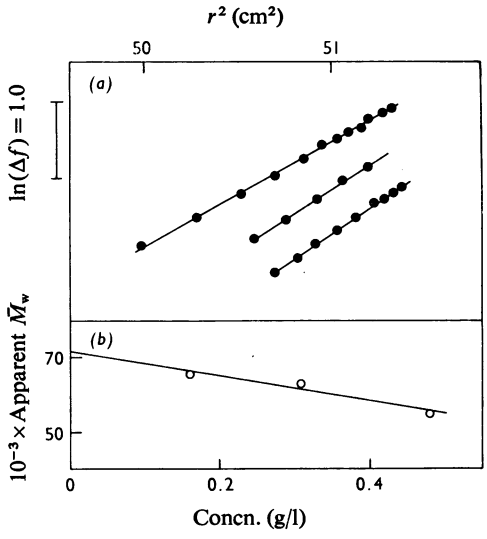

Fig. 10. *Molecular-weight determination of proteoglycans* (*a*) Plot of ln(fringe displacement) (Δf) versus the square of the radial distance (r) in cm. The three curves correspond to sample concentration 0.48g/l (top curve), 0.32g/l and 0.16g/l (bottom curve) in 6M-guanidinium chloride/0.05M-Tris/HCl, pH7.0. The rotor speed was 22000rev./min. (*b*) Apparent weight-average molecular weights ($\bar{M}_w$) plotted versus concentration. The best least-squares line intersects the ordinate at $\bar{M}_w = 72000$.

graphy pattern in guanidinium chloride. Therefore the sample was centrifuged at a higher speed (28000rev./min). The plots were again linear, but gave a lower molecular weight (53000). Thus there is considerable polydispersity of the proteoglycans, despite the linear fringe-displacement plots. Similar observations were made with keratan sulphate peptides from cartilage proteoglycans (Heinegård & Axelsson, 1977). It was not possible to reach meniscus depletion at speeds significantly lower than 22000rev./min. There is a trend in the sedimentation-equilibrium method to underestimate the molecular weight of polydisperse samples, e.g. by accumulation of fast-sedimenting material at the bottom of the cell. Therefore we consider 72000 to be the better approximate weight-average molecular-weight value for the proteoglycan monomers.

The physical parameters for proteoglycan monomers obtained in the present investigation (mol.wt. approx. 72000; sedimentation coefficient 1.7S; partial specific volume 0.67ml/g) were used to calculate Stokes radius (12nm) and frictional ratio (4.5) by Svedberg's equation (Svedberg & Pedersen, 1940) and equations discussed elsewhere (Axelsson, 1978). The value for Stokes radius (12nm) is higher than that obtained by gel chromatography (7nm; Figs. 1 and 3). The role of asymmetry may be considered (Nozaki *et al.*, 1976; Axelsson, 1977); gel chromatography underestimates Stokes radius for the monomers if they are asymmetric. The value for frictional ratio (4.5) reflects the high degree of hydration shown by proteoglycans, but may also reflect molecular asymmetry.

In summary, data from analytical ultracentrifugation confirm that corneal keratan sulphate proteoglycans consist of a major population of monomers, which form aggregates under certain conditions. There is also evidence that a small population of larger proteoglycans does not dissociate readily. [In fact, gel-chromatographic data (Fig. 2*c*) suggest that these large molecules are necessary for a high degree of aggregation of monomers.] Very approximate values for average molecular weight and Stokes radius of the monomers are 72000 and 12nm respectively. Proteoglycan monomers from bovine hyaline cartilage are much larger and have average mol.wts. of 2000000–3000000 (Hascall & Sajdera, 1970) and average molecular lengths around 300nm (Thyberg *et al.*, 1975). Thus the size of corneal keratan sulphate proteoglycan monomers is only about 3–7% of the size of cartilage proteoglycan monomers.

The corneal proteoglycan monomers, like cartilage proteoglycan monomers, are much less compact than most globular proteins. Calf thyroglobulin, for example, has a molecular weight (669000) ten times that of the corneal proteoglycan monomers but its Stokes radius (8.6nm) is smaller (data on thyroglobulin from Edelhoch, 1960).

Demonstration of disulphide bonds

Reduction and alkylation of corneal proteoglycan monomers alter their size (Figs. 5 and 8). It is therefore likely that disulphide bonds are important for the three-dimensional structure of the proteoglycans. Chemical analysis was used to identify disulphide bonds (cystine) in the proteoglycans, but no thiol groups (cysteine) could be demonstrated (Table 1). The cystine content of 37nmol/mg of proteoglycan can be used to calculate the number of disulphide bonds per molecule. It is assumed that the average mol.wt. is 72000, as discussed above. Each proteoglycan molecule then contains an average of two to three disulphide bonds. Reduction of the samples could not be used to liberate any small peptides or to cleave the molecules, as indicated by gel chromatography or analytical ultracentrifugation (Figs. 5 and 8). It is therefore probable that the disulphide bonds connect different parts of the same polypeptide chain. In support of this it appears that reduction of the proteoglycans results in changes in their conformation and ability to form aggregates (Figs. 5 and 8). The most simple explanation for such

Table 1. *Cysteine and cystine contents of the proteoglycans*
The buffers used were: Krebs–Ringer phosphate buffer (pH7.4; Grassetti & Murray, 1967); 0.15M-NaCl/5mM-sodium acetate (pH4.0); and 6M-urea in Krebs–Ringer phosphate buffer (pH7.4).

	Amount (nmol/mg)
Thiol content measured at pH7.4	0.0
at pH4.0	0.4
in 6M-urea, pH7.4	0.0
Calculated cysteine content	0
Cysteic acid content after performic acid oxidation	74
Calculated cystine content	37
Molecular weight of proteoglycan corresponding to one cystine residue per molecule: 27000	

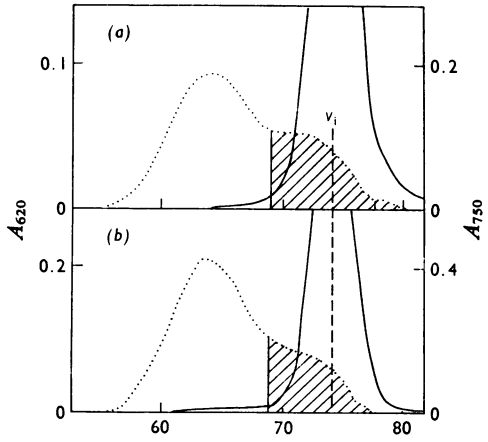

Fig. 11. *Chromatography on Sepharose 4B of papain-digested proteoglycan fractions*
Fractions A and B were obtained from a run on a preparative Sepharose 4B column (cf. Fig. 2). An analytical Sepharose 4B column (bed size 0.86cm× 128cm, void volume 28ml, fraction size 1.25ml) was eluted with 0.5M-sodium acetate, pH7.0, and analysed for hexose (····, A_{620}) and for protein (——, A_{750}). The fractions in the shaded areas were pooled, dialysed, freeze-dried, separated into oligosaccharides and keratan sulphate on ECTEOLA-cellulose microcolumns and analysed for hexosamine. (*a*) Fraction A; oligosaccharide-bound hexosamine/keratan sulphate-bound hexosamine ratio 0.61:1. (*b*) Fraction B; ratio as in (*a*), 0.92:1.

a behaviour is that the molecule is unfolded when reduced.

Composition of fractions 70PA and 70PB

Analysis of the isolated fractions 70PA and 70PB did not reveal any significant differences in keratan sulphate chain size (Fig. 11), oligosaccharide content (Fig. 11, legend) or keratan sulphate/protein ratio (Axelsson & Heinegård, 1975). Some differences between fractions 70PA and 70PB in cysteine and methionine contents are indicated in Table 2. The yield of these amino acids, however, varies considerably between different analyses, because the hydrolysis conditions are not optimal for the sulphur-containing amino acids; only the performic acid oxidation of cysteine and cystine to cysteic acid used in this investigation gives reliable values. The other amino acids show the same values in fractions 70PA and 70PB (Table 2) and in the starting material (Axelsson & Heinegård, 1975). In summary, no certain differences in the composition of fractions 70PA and 70PB could be demonstrated by chemical analyses.

Degradation of the proteoglycans

The amino acid composition of keratan sulphate peptides purified by ECTEOLA-cellulose chromatography of papain-digested proteoglycans was determined (Table 3). Asparagine has been established as the amino acid to which the *N*-acetylglucosamine in the reducing terminal of the keratan sulphate chain is attached (Baker *et al.*, 1969, 1975; Stuhlsatz *et al.*, 1971). Our preparation contains equal amounts of asparagine (and/or aspartic acid) and glutamine (and/or glutamic acid). It is possible that there is one glutamine or glutamic acid residue adjacent to each asparagine residue in the linkage region. It is also possible that there are several glutamic acid/glutamine residues in some linkage regions but none

Table 2. *Amino acid composition of fractions A and B*
The fractions were obtained by gel chromatography on Sepharose 4B (compare with Fig. 2).

	Composition (residues/1000 residues)	
	A	B
Asx	140	153
Thr	38	35
Ser	67	74
Glx	102	106
Pro	62	62
Gly	46	48
Ala	45	43
Cys	29	18
Val	53	52
Met	6	11
Ile	52	47
Leu	154	158
Tyr	38	40
Phe	36	34
Lys	67	63
His	24	21
Arg	32	27

Table 3. *Amino acid composition of keratan sulphate peptides*

The amino acids are arranged in order of decreasing abundance.

	Composition (residues per 1000 residues)
Asx	223
Glx	221
Ser	86
Leu	79
Gly	75
Pro	51
Cys	46
Thr	38
Ala	34
Tyr	33
Ile	26
Phe	24
Lys	24
Val	19
Met	14
His	5
Arg	Traces

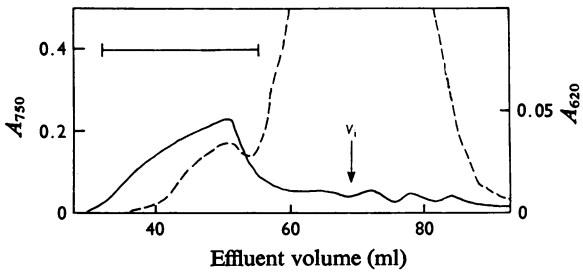

Fig. 12. *Chromatography of oligosaccharide peptides on Sephadex G-50 (superfine grade)*

The column (bed size 0.8cm×138cm, void volume 30ml, fraction size 1.6ml) was eluted with a pyridinium acetate buffer (0.25M-pyridine solution adjusted with acetic acid to pH6.5). The fractions were analysed for hexose (——, A_{620}) and protein (----, A_{750}). The fractions indicated by the horizontal bar were pooled and analysed as described in the text.

in others. Other amino acids are present in much smaller amounts. The sequence

GlcNAc
|
-Glx-Asn-

has been isolated from human immunoglobulin M and sheep pituitary lutropin (Marshall, 1972). Corneal keratan sulphate may have a similar structure in the linkage region. Leucine is the predominant amino acid in the proteoglycans (Axelsson & Heinegård, 1975). The low content of leucine and of other amino acids with hydrophobic side chains (isoleucine, valine) (Table 3) in the keratan sulphate peptides suggests that the non-polar amino acids are located in regions other than those in the vicinity of the keratan sulphate chains.

A considerable fraction of the hexosamines in corneal proteoglycan preparations is not found in glycosaminoglycans, but in neutral or weakly acidic glycans or oligosaccharides. After digestion of proteoglycans with proteinases the glycopeptides can be recovered in a fraction that does not bind to ECTEOLA-cellulose resin under neutral or weakly acidic conditions (Axelsson & Heinegård, 1975). These glycopeptides are partly included in Sephadex G-50 gels (Fig. 12). It can therefore be concluded that their molecular size is small, and 'oligosaccharide' is probably a more adequate term than 'glycan'. Two questions can be raised. Firstly, are these oligosaccharides covalently bound to the proteoglycan molecules? Secondly, are these oligosaccharides low-sulphated, low-molecular-weight glycosaminoglycans, or are they completely different oligosaccharides? Enzymic-degradation experiments indicate that corneal chondroitin sulphate proteoglycans contain oligosaccharides covalently bound to the protein cores of the proteoglycans (I. Axelsson & D. Heinegård, unpublished work). For keratan sulphate proteoglycans there is no such direct evidence. It has not been possible to separate the oligosaccharide-containing peptides from the proteoglycans by gel chromatography in various buffers, e.g. 6M-guanidinium chloride or 1% SDS, or by density-gradient centrifugation in a solution of CsCl and 4M-guanidinium chloride (Axelsson & Heinegård, 1975; I. Axelsson & D. Heinegård, unpublished work). The oligosaccharides are thus closely associated with the proteoglycans and cannot be removed by the dissociative solvents tested. It is possible, however, that glycopeptides and proteoglycans are separated by SDS/polyacrylamide-gel electrophoresis (Fig. 4*b*).

To determine their composition, oligosaccharide–peptides were isolated after papain digestion of the proteoglycan preparation. The digest was chromatographed on an ECTEOLA-cellulose column and a Sephadex G-50 (superfine grade) column as described in the Experimental section. The anthrone-positive peak was pooled (Fig. 12). The isolated oligosaccharide–peptides contained the following predominant constituents: mannose, galactose, glucosamine and aspartic acid/asparagine (molar proportions 2.1:1.9:1.5:1.0; Table 4). Stuhlsatz *et al.* (1971) have proposed the following structure for corneal keratan sulphate: $[GlcNAc\text{-}Gal(SO_4)]_n$-Man-Man-GlcNAc-Asn. A mixture of the oligosaccharides Gal-GlcNAc-Gal-Man-Man-GlcNAc-Asn, GlcNAc-Gal-Man-Man-GlcNAc-Asn and Gal-Man-Man-GlcNAc-Asn could

Table 4. *Composition of the oligosaccharide peptides isolated from papain-digested keratan sulphate proteoglycans*

It was not possible to obtain a reliable value for the xylose content (see the text). Uronic acids were not detectable.

Amino acids	Amount (μmol/g)
Asx	324
Thr	117
Ser	73
Glx	181
Pro	162
Gly	66
Ala	58
Cys	0
Val	37
Met	5
Ile	91
Leu	144
Tyr	19
Phe	20
Lys	75
His	6
Arg	Traces
Hexosamines	
Glucosamine	483
Galactosamine	34
Neutral sugars	
Fucose	79
Xylose	<130
Arabinose	Traces
Mannose	694
Galactose	601
Glucose	204

give the molar proportions found in our preparation. The slight excess of galactose and the presence of some xylose may be due to oligosaccharides with a structure similar to that of the linkage region of corneal galactosaminoglycans, which have the following structure (Stuhlsatz *et al.*, 1971): $[GalNAc(SO_4)\text{-}GlcA]_n$-Gal-Gal-Xyl-Ser. (Some of the glucuronic acid residues may be replaced by iduronic acid residues.) The rather low content of serine suggests that the latter type of oligosaccharide cannot be a major component of the oligosaccharide fraction. It was not possible to obtain a reliable value for the xylose content, since the samples contained an unidentified compound that did not separate completely from xylose in the system used for g.l.c. (results not shown). This compound was not eliminated when a hydrolysate was purified by ion-exchange chromatography with AG1 (X8) resin. It was not present in intervertebral-disc samples analysed in parallel with the corneal samples, but appeared again when the whole analytical procedure was repeated. Glucose is at least partly a contaminant from the cellulose ion-exchange column (Axelsson & Heinegård, 1975).

To summarize, the oligosaccharide–peptide fraction has a composition similar to a mixture of corneal glycosaminoglycan linkage regions; it may represent such a mixture. The low-sulphated keratan sulphate oligosaccharides from pig cornea described in a preliminary communication by Hirtzel *et al.* (1976) and the structural glycoproteins isolated from calf cornea by Moczar *et al.* (1969) show similar analytical results, with mannose and glucosamine as predominant sugars. There are also several glycoproteins that have a similar sugar composition.

Tentative model for corneal keratan sulphate proteoglycans

Preparations of corneal keratan sulphate proteoglycans contain monomers with mol.wts. about 72000 and Stokes radii around 7–12nm, and, in addition, polydisperse larger molecules with Stokes radii above 10nm and the same chemical composition as the monomers. From analytical data (Axelsson & Heinegård, 1975, Table 1; the present paper, Table 4) and values for moisture content (5–10%; see above) it may be roughly estimated that the proteoglycans consist of about 45% protein, 30% keratan sulphate and 10–12% oligosaccharides; the rest is probably mostly counter-ions. Thus in an average proteoglycan molecule the molecular weight of the protein core is about 32000, and the total molecular weights of the keratan sulphate chains and the oligosaccharides are about 22000 and about 8000 respectively. Experiments described above suggest that the molecules contain intrachain disulphide bonds and only one polypeptide chain. The molecular weight of bovine corneal keratan sulphate has been estimated to vary between 4000 and 20000, with most of the chains distributed between 9000 and 19000 (Laurent & Anseth, 1961; Greiling & Stuhlsatz, 1966). The average keratan sulphate proteoglycan would then contain one, two or three keratan sulphate chains, but there may also be some proteoglycan molecules with several chains, since there is a wide variation in the molecular weights of both proteoglycans and keratan sulphate chains. The oligosaccharide structures discussed above have mol.wts. about 700–1000; a very rough estimate of the number of oligosaccharide chains in the average molecule is thus less than 12. It should be re-emphasized that the oligosaccharides are closely associated with the proteoglycans but have not been proved to be covalently linked.

The proteoglycan monomers may form aggregates. Aggregation is promoted by a large-molecular-size fraction of the keratan sulphate proteoglycan preparation and by denaturation of the proteoglycans. It has not been possible to isolate any non-proteoglycan factor promoting aggregation. It is not known at present whether aggregation occurs *in vivo* or

whether it is an artefact caused by the purification procedure.

Bettelheim & Plessy (1975) have used methods similar to ours (Antonopoulos *et al.*, 1974) for isolation of corneal proteoglycans. Their recovery of proteoglycans was only about 15% of the tissue content, compared with about 85% in the present study. Their lower yield may be due to omission of urea from the dialysis and ion-exchange-chromatography buffers. Despite this, our data are in good agreement with theirs. They did not isolate pure keratan sulphate proteoglycans free from other glycosaminoglycans, but they obtained two proteoglycan fractions with more keratan sulphate than chondroitin sulphate. The weight-average molecular weights of these fractions were 57000 and 90000 and the partial specific volume was 0.68 ml/g.

The only proteoglycans that have been carefully characterized previously are proteoglycans from cartilage and intervertebral discs. All the present data related to the size, molecular weight and composition of the proteoglycans are very approximative average values, since the proteoglycans show considerable polydispersity and heterogeneity. The present investigation, however, clearly shows that corneal keratan sulphate proteoglycans are very different from skeletal proteoglycans. The molecular weight, molecular size and number of glycan chains are orders of magnitude smaller than the corresponding values for skeletal proteoglycans. In addition, there are considerable amounts of oligosaccharides closely associated with corneal proteoglycans, whereas skeletal proteoglycans are free from oligosaccharides; the protein content is much higher in corneal proteoglycans than in skeletal proteoglycans.

The present paper gives results for only one-quarter to one-third of the total proteoglycan population of cornea. However, further investigations (Axelsson & Heinegård, 1975; I. Axelsson & D. Heinegård, unpublished work) have shown that the corneal galactosaminoglycan-containing proteoglycans are much smaller than the skeletal proteoglycans and that the former contain considerable amounts of closely associated oligosaccharides and have higher protein contents than the skeletal proteoglycans.

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