Carbohydrate Research, 69 (1979) 151–164

ISOLATION AND DESULPHATION OF KERATAN SULPHATES*

MARY M. U. MCCARTHY† AND JOHN R. BAKER§
Department of Biochemistry, Trinity College, Dublin 2 (Ireland)
(Received December 23rd, 1977; accepted for publication, March 30th, 1978)

ABSTRACT

Keratan sulphates I and II have been isolated and purified from bovine cornea and nasal cartilage, respectively. Upon desulphation, keratan sulphate I yielded both water-soluble and -insoluble products, whereas keratan sulphate II gave only water-soluble products. Analyses of the desulphated keratan sulphates support the view that oligosaccharides derived from the main chain of the glycosaminoglycan associate to form the water-insoluble species and glycopeptides from the protein-linkage region are components of the water-soluble form. All other glycosaminoglycans of mammalian origin, which differ from keratan sulphate by having a glycosyluronic residue in their repeat disaccharide sequence, give only water-soluble desulphated products. Some evidence indicates that reduction of such glycosyluronic residues of a glycosaminoglycan, followed by desulphation, allows for the formation of some water-insoluble product.

INTRODUCTION

Keratan sulphates have been isolated from a number of mammalian connective tissue sources[1–3]. Basically, two types are recognised: keratan sulphate I (KS I, from cornea) and keratan sulphate II (KS II, from cartilage and other tissues). Commonly, they have been purified from tissues after extensive proteolysis. Invariably, both forms are found to be highly polydisperse with reported molecular weights[4,5] ranging from 4×10^3 to 2×10^4. KS I tends to have a higher molecular weight than KS II.

Both types of KS consist of alternating D-galactopyranosyl and 2-acetamido-2-deoxy-D-glucopyranosyl residues. Through the work of Meyer and associates[6–8], the structure of the repeating disaccharide sequence has been determined as: (1→3)-(β-D-galactopyranosyl)-(1→4)-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl). Analyses indicate that KS bears approximately one ester sulphate group per disaccharide,

*Keratan Sulphates I.
†Present address: Regional Technical College, Galway, Ireland.
§To whom correspondence should be addressed. Present address: Institute of Dental Research, School of Dentistry and Department of Medicine, University of Alabama in Birmingham, Birmingham, AL 35294, U.S.A.

although preparations of widely different sulphate content has been obtained by fractionation. Some sulphate groups (40–50%) esterified C-6 of the D-galactose residues and the remainder occur at C-6 of the 2-acetamido-2-deoxy-D-glucose residues[8].

Both KS I and II contain small proportions of L-fucose, sialic acid, and D-mannose[8]. It has been reported that some sialic acid and galactose residues occur as side branches of sialylgalactose along the KS II chain[9], which may explain the small excess of galactose over glucosamine in this KS. The isolation[10] of sialyl-galactosyl-(2-acetamido-2-deoxyglucosyl)galactose from enzymatic digests of KS II lends support to these earlier findings. At present, there is no evidence that the small amount of sialic acid residues found in KS I is similarly located. Evidently, the D-mannose residue(s) occur near the linkage of KS to protein, as the isolation of small glycopeptides containing mannose has been reported[11,12]. KS II contains a small proportion of 2-acetamido-2-deoxy-D-galactose residues[13] that link some chains by alkali-labile bonds to seryl or threonyl residues of the protein core[5]. Other linkages of KS II to protein are alkali-stable and have not been characterised. The KS I chain is linked to protein *via* a 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine residue[14].

KS I and II have been desulphated by Bhavanandan and Meyer[8], who reported that both compounds yield water-soluble and -insoluble products. The carbohydrate and amino acid compositions of the two fractions were described as similar and could not account for their different water solubilities.

In this paper, the purification and desulphation of KS I and II are described. The water-soluble and -insoluble, desulphated keratan sulphates are compared on the basis of amino acid analyses, neutral sugar determinations, and gel filtration on Sephadex G-200. An explanation is offered for some evident differences between these two products.

EXPERIMENTAL

Materials. — Bovine eyes and nasal septa were obtained immediately after slaughter of the animals and stored at −20° until required. Granular papaya latex was a generous gift of Wallerstein Laboratories, Ltd. Subsequently, crystalline papain was prepared from this source by the procedure of Kimmel and Smith[15]. Its specific activity (1.66 enzyme units/mg protein) was determined by estimating the liberation of free amino groups from 2-*N*-benzoyl-L-arginamide. Pronase B was purchased from Calbiochem San Diego, CA 92122. The chondroitin 4-sulphate and KS I standards were generously provided by Dr. J. A. Cifonelli. Their mol. wts. were 1.2×10^4 and 1.6×10^4, respectively. The chondroitin 4-sulphate used in the reduction and desulphation experiment was a gift from Dr. Cifonelli.

3-(3-Dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride was purchased from the Ott Chemical Co. Benzene-free ethanol, carbazole, formamide, and ascorbic acid were purchased from E. Merck (Darmstadt, Germany). Potassium rhodizonate

was obtained from I.C.N./K & K Life Science Group, Plainview, NY 11803, and cellulose acetate strips from the Oxoid Div. of Oxo Ltd. (London, England). All sugars used as standards in quantitative assays were purchased from Pfanstiehl Laboratories Inc., Waukegan, IL 60085, or from British Drug Houses, Poole, England. Sephadex G-200 (superfine) and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and ion-exchange resins from Bio-Rad Laboratories, Richmond, CA 94804.

Analytical methods. — Total neutral sugars were determined by the primary cysteine–sulphuric acid reaction[16], uronic acid residues by a carbazole method[17], and glucosamine and galactosamine residues by the Elson–Morgan reaction as modified by Ludowieg and Benmaman[18] following hydrolysis with 4M hydrochloric acid at 100° for 14 h under nitrogen. When amounts of sample were limited, hexosamine residues were estimated on a microscale by the Morgan–Elson reaction as described by Brownlee and Wheat[19]. Sulphate groups in KS were determined by the rhodizonate method[20] following hydrolysis with M hydrochloric acid at 100° for 2 h, and free amino groups and ammonia by a ninhydrin procedure[21]. Protein content was determined by the biuret reaction[22], and the approximate sulphate content of KS fractions by electrophoresis on cellulose acetate membranes in 0.1M hydrochloric acid[23].

Amino acid analyses were performed with a Jeolco JAH5 Automatic Analyser following hydrolysis with 6M hydrochloric acid at 110° for 20 h *in vacuo*. Norleucine was the internal standard.

Individual neutral sugars were identified and quantitatively determined with the Technicon Sugar Chromatography System following hydrolysis with M hydrochloric acid for 3 h at 100°, neutralisation with Dowex 3 (HCO_3^-) cation-exchange resin, passage through AG 50W-X8 (H^+, 200–400 mesh) cation-exchange resin, and concentration of eluant and water wash. L-Rhamnose was included as internal standard and was added to the sample after hydrolysis.

Infra-red spectra of keratan sulphate samples in potassium bromide discs were obtained with a Perkin–Elmer KB 157 spectrophotometer.

Gel filtration. — Samples (0.2 ml) containing ~1 mg of keratan sulphate were applied to columns (1.0 × 60 cm) of Sephadex G-200 and eluted at 2.5–3.5 ml/h with 0.2M sodium chloride or with formamide. Fractions (1.0 ml) were collected and aliquots assayed for neutral sugar, hexosamine, or uronic acid. Columns were calibrated with Blue Dextran 2000, [^{35}S]sulphate, and the acid glycosaminoglycan standards.

Purification of KS I. — Bovine eyes were semi-thawed and the corneas rapidly cut out with a scalpel. One thousand corneas (930 g wet wt.) were digested with papain (461 mg) in 0.05M sodium acetate (5 L), 5mM EDTA, 5mM L-cysteine, pH 5.5, for 60 h at 65°. The enzyme was pre-incubated with the buffer for 15 min at 65°. Digestion was followed by measuring the liberation of free amino groups, and when it appeared to be completed (30 h), further papain (230 mg) was added. After 60 h, the cooled solution was filtered through Whatman No. 541 paper and the acid

glycosaminoglycans precipitated by the addition of 3 vol. of ethanol. After 30 h at 4°, the precipitate was collected by centrifugation, dissolved in 0.01M calcium acetate (800 ml), pH 7.5, and digested with Pronase (100 mg) for 48 h at 55°. The pH was maintained at 7.5 throughout and a further addition of Pronase (100 mg) made after 24 h. The solution was cooled to 4° and filtered as described before, the concentration of sodium acetate was adjusted to 1%, and the acid glycosaminoglycans were precipitated with ethanol (3 vol.). The precipitate was collected by centrifugation, dissolved in 0.5M acetic acid containing 5% calcium acetate (700 ml), and ethanol added sequentially to concentrations of 30%, 40%, and 66%. Precipitates (E30, E40, and E66, respectively) were collected by centrifugation, washed with 80% aqueous ethanol (twice) and absolute ethanol, and dried with ether. To further purify E66, it was dissolved in 1% sodium chloride, applied to a column (40 × 5 cm) of AG 1-X2(Cl^-) anion-exchange resin, and eluted successively with 1.5M, 2.0M, 3.0M, and 4.0M sodium chloride. The four fractions obtained (KS I–S1.5, –S2.0, –S3.0, and –S4.0, respectively) were exhaustively dialysed against water, concentrated by rotary evaporation at 37°, and precipitated from 1% sodium chloride with ethanol (3 vol.). The precipitates were washed with 80% aqueous ethanol (twice), absolute ethanol (twice), and ether. Ether was removed *in vacuo* over shavings of paraffin wax.

Purification of KS II. — Bovine nasal septa (1 kg) were cleaned of adhering connective tissue, sliced (0.5-mm thick), and then extracted by gentle shaking in 3M magnesium chloride (15 l) for 48 h at room temperature. The extracted proteoglycan was precipitated with ethanol (2 vol.) and collected by centrifugation after being kept for 20 h at 4°. Following washing with 80% aqueous ethanol (twice), absolute ethanol (twice), and ether, the precipitate was dried under an i.r. lamp. This material (90 g) was digested with papain (1 g) for 40 h at 65° in 0.5M sodium citrate, 0.2M EDTA, 0.01M L-cysteine, pH 6.5 (1.8 L). After filtration, the acid glycosaminoglycans were precipitated with ethanol (2 vol.), as before, and then redissolved in 0.5M sodium chloride (12 l). Chondroitin sulphate was precipitated by the addition of 10% aqueous cetyl pyridinium chloride (CetPyCl) until ~2.5 g of CetPyCl per g of chondroitin sulphate had been added. After centrifugation, the supernatant solution was recovered and exhaustively dialysed against water to remove CetPyCl. Following concentration by rotary evaporation at 40°, calcium acetate and acetic acid were added to final concentrations of 5% and 0.5M, respectively, and the acid glycosaminoglycans precipitated by addition of ethanol (3 vol.). The precipitation with ethanol was repeated twice to ensure removal of final traces of CetPyCl. The material was then applied to a column (5 × 20 cm) of AG 1- X2 (Cl^-, 200–400 mesh) anion-exchange resin and eluted stepwise with 1.0M, 1.5M, 2.0M, and 4.0M sodium chloride. The four fractions obtained were subsequently digested with Pronase for 48 h (as described for KS I), and the concentrations of acetic acid and calcium acetate adjusted to 0.5M and 5%, respectively. Precipitates were collected by centrifugation after adding ethanol to concentrations of 50% and then 80%. All eight precipitates were washed and dried as described before. The 50%-ethanol fractions were discarded as they contained mainly chondroitin sulphate. The 80%-ethanol fractions, obtained

from the AG 1 column at 1.0M, 1.5M, 2.0M, and 4.0M salt concentrations, were dissolved in water and passed through small columns of AG 50W- X2 (Na^+) cation-exchange resin. The eluates were lyophilised (fractions KS II–S1.0, –S1.5, –S2.0, and –S4.0, respectively).

Reduction of chondroitin 4-sulphate. — The D-glucopyranosyluronic acid residues of chondroitin 4-sulphate were reduced to D-glucopyranosyl residues according to the method of Taylor and Conrad[24]. 3-(3-Dimethylaminopropyl)-1-ethyl-carbodiimide hydrochloride (957.5 mg) was added to a solution of chondroitin 4-sulphate (416 mg) in water (50 ml) at room temperature. The pH was maintained at 4.75 by continuous addition of 0.1M hydrochloric acid for 2 h. The reaction had ceased by this time as no further addition of acid was necessary. Then 2M sodium borohydride (115 ml) was added and the reduction allowed to proceed for 30 min at 50°. After dialysis against de-ionised water at 4°, ethanol (4 vol.) was added to the concentrated retentate, adjusted to a sodium acetate concentration of 1%. The gel-like precipitate was recovered by centrifugation after being kept overnight at 4°, washed with 80% aqueous ethanol, redissolved in water, and lyophilised. This material (300 mg) was subjected to a second reduction under identical conditions, and the reduced polymer (250 mg) recovered.

Desulphation of KS I, KS II, and reduced chondroitin 4-sulphate. — KS I–S1.5 (500 mg), KS I–S2.0 (500 mg), KS I–S3.0 (500 mg), KS II–S1.0 (300 mg), KS II–S1.5 (400 mg), KS II–S2.0 (150 mg), and reduced chondroitin 4-sulphate (290 mg) were desulphated essentially as described by Kantor and Schubert[25]. They were suspended in 0.06M methanolic hydrogen chloride and shaken for 24 h at room temperature. Following centrifugation and removal of the supernatant solution, the remaining insoluble glycosaminoglycans were subjected to the methanolic hydrogen chloride treatment two more times. The three supernatant solutions and any insoluble residue from each preparation were combined and carefully neutralised with M sodium hydroxide, and the methanol was removed by rotary evaporation at 34°. KS I fractions were treated with aqueous 0.1M sodium hydroxide for 36 h at room temperature to de-esterify any methyl sulfate residues. KS II and reduced chondroitin 4-sulphate preparations were not treated with alkali in order to avoid cleavage of their carbohydrate–protein linkages. All preparations were desalted by ultrafiltration on an Amicon UM-2 membrane and lyophilised. Each desulphated preparation was identified by the designation K, which replaces KS, *eg.*, desulphated KS I-S1.5 is subsequently termed K I–S1.5.

Analyses of these preparations and of the UM-2 filtrates showed that in all cases more than 90% of the neutral sugar of the samples was recovered in the retentates. Only 2–5% was found in the filtrates, which indicates little loss of low-molecular-weight oligosaccharides. Desulphation was effective (Table III), with more than 85% of ester sulphate groups removed from the recovered fractions. Removal of sulphate ester groups by the methanolic hydrogen chloride treatment of KS I fractions was confirmed qualitatively by i.r. spectrometry. The strong absorption band at 1220–

1280 cm^{-1}, which can be assigned to ester sulphate groups, was abolished or drastically diminished.

RESULTS

Characterisation of KS I fractions. — Following exhaustive proteolysis of corneas, glycosaminoglycans were fractionally precipitated by the addition of ethanol to concentrations of 30%, 40%, and 66%. The precipitates obtained (E30, E40, and E66, respectively) were analysed for hexosamine and uronic acid content (Table I). The high uronic acid contents and nearly equimolar uronic acid to hexosamine

TABLE I

ANALYSIS OF GLYCOSAMINOGLYCAN FRACTIONS OBTAINED FROM BOVINE CORNEAS BY ETHANOL PRECIPITATION

Components	*Fraction*		
	E30	*E40*	*E66*
Glucosamine[a]	125	95	1652
Galactosamine[a]	510	215	91
Uronic acid[a]	587	230	180
Uronic acid: Hexosamine[b]	1.04:1	0.93:1	0.13:1

[a]Results are expressed as mg per 930 g of wet weight of corneas. [b]Molar ratios.

TABLE II

ANALYSES OF PURIFIED CORNEAL KERATAN SULPHATE (KS I) FRACTIONS

Yield and components	*Fraction*			
	S1.5	*S2.0*	*S3.0*	*S4.0*
Yield (g)	1.26	1.28	1.43	0.22
Uronic Acid (%)[a]	3.5	0.0	0.0	0.0
Glucosamine (%)[a]	17.7	19.9	17.4	9.1
Galactosamine (%)[a]	4.5	0.0	0.0	0.0
Galactose (%)[a]	14.7	20.9	21.4	
Mannose (%)[a]	2.9	2.0	1.8	
Fucose (%)[a]	1.1	0.2	Trace	
Total hexose (%)[a]	18.7	23.1	23.2	11.7
Total amino acids (%)[a]	13.7	1.8	1.3	0.9
Galactose/glucosamine[b]	0.83	1.05	1.23	1.28
Galactose/mannose[b]	4.10	10.30	12.00	
Sulphate/hexosamine[b,c]	0.89	0.98	1.19	1.30
Galactose/aspartic acid[b]	2.17	21.87	27.91	

[a]Percent of dry weight. [b]Molar ratios. [c]These results were obtained using the electrophoretic method of Wessler[23].

ratios of E30 and E40 indicate that these fractions were enriched in chondroitin sulphate. E66 contained little uronic acid or galactosamine, but relatively much more glucosamine. This analysis of E66 is consistent with the presence of KS, which is known to precipitate at and above a concentration of 40% of ethanol[22]. For this reason, E66 was used for further purification of KS. Purification by ion-exchange chromatography yielded four fractions (S1.5, S2.0, S3.0, and S4.0) for which analyses are given in Table II. The yield of the S4.0 fraction was small, which prevented its complete analysis. Fraction S1.5 contained small proportions of uronic acid and galactosamine, whereas neither sugar was detectable in any other fraction. Therefore, the dermatan or chondroitin sulphates present in E66 are confined to S1.5. In this fraction, glucosamine and galactose predominate, and are in approximately equimolar amounts (Table II). Also, the sulphate to hexosamine ratio is near unity, which indicates that keratan sulphate may be a major component of S1.5. Judging from the total amino acid content, a considerable proportion of peptide remained in this fraction despite the extensive proteolysis employed in its preparation. Small proportions of mannose and fucose may be associated with keratan sulphate or a contaminating glycoprotein. Glucosamine and galactose were the predominant sugars in the other three fractions, S2.0, S3.0, and S4.0. Trace amounts of mannose and fucose were present. Again, no uronic acid nor galactosamine was detected, which clearly demonstrates the absence of dermatan or chondroitin sulphates. The near-equimolar proportions of glucosamine, galactose, and sulphate in these fractions is a good indicator of the presence of keratan sulphate. In all cases, the amounts of peptide were reduced to minimal levels (Table II), leaving aspartic acid as the predominant residue (27–35% of the total amino acids). Evidently all salts had not been removed from the fractions as their compositions, expressed as percentages of dry weight, do not approach the theoretical figures for keratan sulphate.

Certain compositional trends are noticeable with increase in concentration of salt required to elute each fraction from AG 1 resin (*i.e.*, from fractions S1.5 to

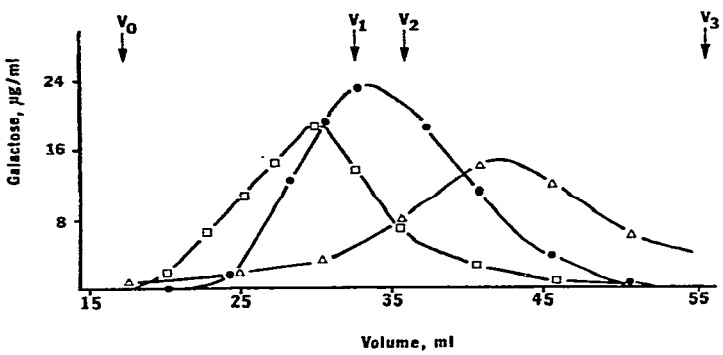

Fig. 1. Elution pattern of gel filtration of KS I fractions on a Sephadex G-200 column. Profiles shown are of KS I–S1.5, △; KS I–S2.0, ●; and KS I–S3.0, □. The column had been pre-calibrated with Blue Dextran (V_0), KS I (mol. wt. 1.6×10^4, V_1), chondroitin 4-sulphate (mol. wt. 1.2×10^4, V_2), and sodium sulphate (V_3). The column was eluted with 0.2M sodium chloride. Other chromatographic details are as given in "Methods".

S4.0): (a) The ratio of galactose to glucosamine increases (a ratio approaching 1.3 is common for keratan sulphate). (b) The ratio of galactose to mannose increases. As galactose is a constant component of the main keratan sulphate chain, and mannose is a component only of the linkage region, this change indicates an increase in size of the keratan sulphate chain from fraction S1.5 to S4.0. (c) The ratio of sulphate to hexosamine increases. Not unexpectedly, the degree of sulphation of keratan sulphate increases from S1.5 to S4.0.

To estimate the average molecular weights of the fractions, these were chromatographed on Sephadex G-200 (Fig. 1) as described in "Methods". The approximate molecular weights estimated from K_{av} for the S1.5, S2.0, and S3.0 fractions were 4.9×10^3, 1.3×10^4, and 1.9×10^4, respectively. The K_{av} for S1.5 and S3.0 were not sufficiently close to those of the standards to permit more precise calculations of their molecular weights. It is evident that KS fractions of increasing molecular weight have been eluted from AG 1 with increase in salt concentration. The trend in the galactose to mannose ratio (Table II) is in agreement with these results.

Desulphated KS I. — Samples of K I–S1.5, K I–S2.0, and K I–S3.0 were suspended in water at a concentration of 10 mg/ml, shaken at room temperature for 3 h and centrifuged. The supernatants were carefully withdrawn and the residues resuspended in an equal volume of water. The water–soluble fractions are termed K I–S1.5–1, etc. and the water-insoluble suspensions K I–S1.5–2, etc. They were analysed for hexose to quantitatively determine the proportion of water-soluble and insoluble subfractions. Accordingly, the "soluble" fractions K I–S1.5–1, –S2.0–1, and –S3.0–1 constituted 96%, 74%, and 38%, respectively, of each desulphated fraction (Table III). These striking differences between the fractions clearly indicate that KS of increasing molecular weight yields, after desulphation, a decreasing proportion of water-soluble polymer. The sulphate contents of the insoluble products were especi-

TABLE III

COMPOSITION OF DESULPHATED CORNEAL KERATAN SULPHATE (KI) FRACTIONS

Components	*Fraction*					
	S1.5–1	*S1.5–2*	*S2.0–1*	*S2.0–2*	*S3.0–1*	*S3.0–2*
Total hexose (%)[a]	96.0	4.0	73.7	26.3	38.4	61.6
Hexosamine[b]	1.00	1.00	1.00	1.00	1.00	1.00
Galactose[b]	0.92	1.00	0.99	0.99	1.05	1.22
Mannose[b]	0.21	0.15	0.14	0.05	0.11	trace
Fucose[b]	0.05	trace	0.03	0.00	0.02	0.00
Sulphate[b]	0.10	0.08	0.20	0.00	0.34	0.01
Aspartic acid[b]	0.43		0.05	0.03	0.05	0.02
Total amino acids[b]	0.92		0.14	0.17	0.21	0.11
Sulphate removed (%)	86.3	89.2	80.1	100.0	73.0	99.2

[a]The percentage distribution of total hexose of each desulphated fraction (*e.g.*, K I–S1.5) between soluble and insoluble fraction (*e.g.*, K I–S1.5–1 and –S1.5–2) is given. [b]Molar ratios.

ally low, whereas some residual sulphate groups were present in the water-soluble desulphated KS (Table III). Nevertheless, it is not considered likely that the remaining ester sulphate groups are responsible for solubility in water, as repeated methanolic hydrogen chloride treatment of "soluble" desulphated KS fractions did not yield more insoluble product.

Table III shows that the galactose to hexosamine ratio remains at approximately 1.0:1.0 for all fractions. Mannose is in relatively higher concentration in the soluble subfractions K I–S1.5–1, –S2.0–1, and –S3.0–1 than in their insoluble counterparts K I–S1.5–2, –S2.0–2, and –S3.0–2, respectively (Table III). The same is true for the distribution of aspartic acid. It is evident that the KS chains have been partially cleaved during the methanolic hydrogen chloride treatment of the desulphation procedure, as different parts of the KS chain can concentrate in "soluble" or "insoluble" subfractions: the protein-linkage region containing aspartic acid and mannose is preferentially in the "soluble", and the remainder of the chain, mainly composed of galactose and 2-acetamido-2-deoxyglucose, primarily in the "insoluble" part.

In order to determine the effect of desulphation of KS on its size, the soluble and insoluble, desulphated subfractions of K I–S2.0 and K I–S3.0 were chromatographed on Sephadex G-200. Samples were dissolved in formamide, applied to the chromatographic column, and eluted with the same solvent. Elution profiles were determined by analysis for 2-acetamido-2-deoxyglucose and are illustrated in Fig. 2. Chromatographed in this manner, K I–S3.0–1 and –S3.0–2 both gave two peaks, one at the void volume and the other in a well-included position. K I–S3.0–2 contained a much larger void peak (Peak 1) and a smaller included peak (Peak 2) than K I–S3.0–1.

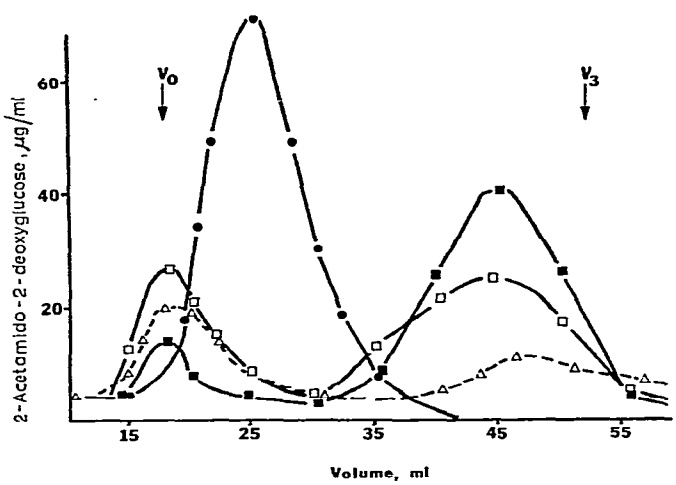

Fig. 2. Elution pattern of gel filtration of desulphated KS I fractions on a Sephadex G-200 column. Samples (0.5–1.5 mg) were dissolved in formamide (200 µl) and applied to the column, which was developed with formamide. Fractions were evaporated to dryness at 100° *in vacuo* before hydrolysing and analysing for 2-acetamido-2-deoxyglucose. Profiles shown are: KS I–S3.0, ●; K I–S3.0–1, ■; K I–S3.0–2, □; and K 1–S2.0–2, △. V_0 and V_3 are as in legend to Fig. 1.

TABLE IV

COMPOSITION OF PURIFIED KERATAN SULPHATE FRACTIONS FROM BOVINE NASAL SEPTUM (KS II)

Yield and components	*Fraction*			
	S1.0	*S1.5*	*S2.0*	*S4.0*
Yield (mg)	526	702	277	95
Uronic Acid (%)[a]	10.0	5.2	2.8	1.8
Glucosamine (%)[a]	14.9	18.2	19.2	17.2
Galactosamine (%)[a]	11.8	7.3	5.6	3.1
Total hexosamine (%)[a]	26.7	25.5	24.8	20.3
Total hexose (%)[a]	20.0	20.9	25.5	16.4
Hexose/glucosamine[b]	1.30	1.13	1.30	1.17
Hexose/uronic acid[b]	2.00	4.02	9.11	9.11

[a]Percent of dry weight. [b]Molar ratios.

The keratan sulphate fraction KS I–S3.0, from which these desulphated subfractions had been derived, gave an elution pattern that closely resembled that of the same sample chromatographed in 0.2M sodium chloride (Fig. 1). This data indicates that during desulphation some cleavage of KS occurs. Thus, some fragments (Fig. 2, Peak 2) have a smaller size than the parent KS (Figs. 1 and 2). Then, desulphation must also permit some aggregation of chains (Fig. 2, Peak 1). A much higher proportion of this aggregated material was found in the water-insoluble than in the water-soluble subfraction (Fig. 2). The chromatographic profiles of K I–S2.0–1 and –S2.0–2 are similar to those of K I–S3.0–1 and –S3.0–2, respectively, and may be interpreted in the same manner.

Characterisation of KS II and *desulphated KS II fractions.* — Following isolation as described in the Experimental section, four fractions termed KS II–S1.0, –S1.5, –S2.0, and –S4.0 were obtained. Analyses of these preparations are given in Table IV. From the uronic acid analyses, it is clear that all fractions contained chondroitin sulphate, but the proposition decreased from KS II–S1.0 to –S4.0. Galactosamine followed the same trend. Each fraction contained an excess of galactosamine over uronic acid, which is presumably due to galactosamine being a component of the alkali-labile linkage of KS II to protein. An excess of hexose over glucosamine is apparent from the analyses of each fraction. The fractions contained ~8% of peptide, and glutamic acid, proline, serine, threonine, and aspartic acid predominated. Further proteolysis did not markedly affect the amino acid content, and proteolysis followed by repurification of the fractions did not improve the separation of KS from chondroitin sulphate. As expected, the higher-salt fractions (*i.e.*, KS II–S2.0 and –S3.0) appeared to have the highest KS content. Chromatography on Sephadex G-200 (not shown) revealed that the fractions were highly polydisperse and had approximate mol. wts. of 2.9×10^3 (KS II–S1.0), 4.2×10^3 (KS II–S1.5), and 4.9×10^3 (KS II–S2.0).

Fractions were desulphated as described in the section on Methods, and analyses

TABLE V

COMPOSITIONS OF CHONDROITIN SULPHATE PREPARATIONS

Components	*Preparation*[a]				
	1	*2*	*3*	*4*	*5*
Hexosamine (%)[b]	25.3	19.3	22.5	16.4	8.9
Hexose (%)[b]	0.5	16.0	22.3	13.9	8.0
Uronic Acid (%)[b]	25.0	2.9	0.0	0.0	0.0
Sulphate (%)[b]	12.5		12.6	0.6	0.2
Sulphate/hexosamine[c]	1.1		1.2	0.07	0.04

[a](1) Chondroitin sulphate without modification. (2) Chondroitin sulphate after one cycle of reduction. (3) "Reduced chondroitin sulphate" (*i.e.*, after two cycles of reduction). (4) Water-soluble fraction of desulphated "reduced chondroitin sulphate". (5) Water-insoluble fraction of desulphated "reduced chondroitin sulphate". [b]Percent of dry weight. [c]Molar ratios.

indicated the removal of ~86.0, 83.0, and 77.3% of ester sulphate groups from KS II–S1.0, –S1.5, and –S2.0, respectively. At a concentration of 10 mg/ml of water, the desulphated KS II fractions were completely soluble. Chromatography on Sephadex G-200 showed that no material was eluted at the void volume (*cf.*, desulphated KS I fractions, Fig. 2). A single, broad peak, more included than the parent KS II fraction, was obtained from each desulphated KS II fraction. In Fig. 3 the elution patterns of KS II–S2.0 and of its desulphated product are shown, which is also illustrative of the elution patterns of KS II–S1.0 and –S1.5, and of their desulphated derivatives.

Characterisation of chondroitin 4-sulphate and "reduced chondroitin 4-sulphate". — After one cycle of reduction, the uronic acid content of chondroitin sulphate was diminished to 2.9% and its hexose content raised to 16.0% (Table V). A second cycle of reduction removed all uronic acid, as determined by the carbazole procedure, and raised the hexose content to approximate equimolarity with hexosamine. Treatment with methanolic hydrogen chloride removed 94% of the ester sulphate groups, *i.e.*, from 12.5% to 0.8% (Table V). Equilibration of the desulphated polymer with water at a concentration of 10 mg/ml permitted the separation into water-soluble (64%) and water-insoluble (36%) fractions. By contrast, desulphated chondroitin sulphate (*i.e.*, without reduction of the D-glucopyranosyluronic residues) does not contain any water-insoluble product. Therefore, it is evident that the conversion of the carboxylic-bearing D-glucopyranosyluronic residues of chondroitin sulphate into uncharged D-glucopyranosyl residues, followed by desulphation, is required for association of the desulphated polymer chains into a water-insoluble product.

DISCUSSION

Among the known glycosaminoglycans, keratan sulphate is the least understood structurally. It is both polydisperse and heterogeneous, and even, for example,

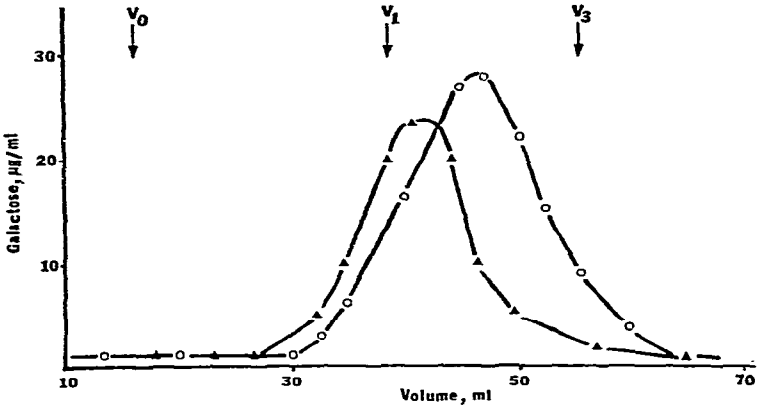

Fig. 3. Elution pattern of gel filtration of KS II–S2.0 and its desulphated product, K II–S2.0 on a Sephadex G-200 column. Profiles shown are: KS II–S2.0, ▲; and K II–S2.0, ○. The column was calibrated and developed as described in the legend to Fig. 1.

possesses more than one type of linkage to protein. Those of KS II still remain to be elucidated. The first problem in the study of keratan sulphate is its isolation from tissues and separation from glycoproteins. In cornea, and possibly in cartilage, are present glycoproteins that have structural features in common with keratan sulphate, and which complicate the process of purifying this glycosaminoglycan. In this work, preparations from cornea and cartilage were exhaustively proteolysed in order to convert glycoproteins to glycopeptides and proteoglycans to glycosaminoglycans. This was followed by fractionation with increasing concentrations of ethanol, which precipitates glycosaminoglycans in the order: dermatan sulphate, chondroitin sulphate, and keratan sulphate. Subsequent fractionation on AG 1 resin by stepwise elution with increasing concentrations of salt released the smallest, least charged, polyanions (*i.e.*, glycopeptides) first. The polyglycosyluronic acids, dermatan sulphate or chondroitin sulphate, and finally keratan sulphate followed. Chromatography on AG 1 resin also fractionated the keratan sulphate (*i.e.*, as the salt concentration of the eluent increased from 1.5M to 4.0M, keratan sulphates of increasing mol. wt. were eluted), as shown by gel chromatography (Fig. 1). D-Mannose and L-aspartic acid residues are components of the linkage region[11,12,14] of KS I and D-galactose residues occur in the main chain[6–8]. Therefore, the observed increase of the ratios of galactose to mannose and of galactose to aspartic acid with the concentration of salt required to elute keratan sulphate from AG 1 resin (Table II) also supports the view that the keratan sulphate chains of largest mol. wt. are eluted last. This fractionation by size of KS I permitted investigation of the effect of chain length on the relative yields of water-soluble and -insoluble desulphated products.

The analytical results of fractions KS I-S1.5, –S2.0, –S3.0, and, as far as sample size allowed, –S4.0, were consistent with keratan sulphate being the major carbohydrate constituent. Approximately equal molar ratios of galactose, glucosamine, and sulphate, and small proportions of mannose and fucose typical of keratan sulphate, were found (Table II). The low galactose to aspartic acid ratio of –S1.5

indicates that only KS having very short chain-lengths is present. Axelsson and Heinegård[26] have previously reported the presence of "glycoprotein-type oligosaccharides" attached to protein in corneal proteoglycans. It is probable that sulphated glycopeptides and keratan sulphate of low molecular-weight are similar in composition and structure, no clear distinction between the two being observed.

In our work, desulphation of KS I was obtained by the procedure of Kantor and Schubert[25], and a fraction of the product was water insoluble. This procedure did not lead to the isolation of an "intractable" product as reported by Hirano *et al.*[6], who desulphated keratan sulphate with Dowex 50 (H^+) and sodium chloride in anhydrous methanol[8]. By this procedure also, some of the product was water insoluble. In our study, the fractions from KS having the highest molecular weight gave the highest proportion of insoluble desulphated KS. Furthermore, judging from the results of chemical analysis (Table III), the glycopeptides tended to be concentrated in the water-soluble, and the oligosaccharides in the water-insoluble fractions. This result is, of course, in accord with the finding that the longer the KS chain, the larger the relative amount of insoluble, desulphated product obtained from it. Bhavanandan and Meyer[8] concluded that there were no compositional differences between the water-soluble and -insoluble products. However, their results showed a slightly higher sulphate content in the water-soluble than in the -insoluble fraction, and also indicated that the carbohydrate to protein ratio was significantly higher in the insoluble (37) than in the soluble (20.5 fraction); these results are basically in agreement with ours. Additionally, we have shown that water-soluble, desulphated KS contains relatively more mannose than water-insoluble, desulphated KS, which is indicative of the relative increase of linkage region components in the former fraction.

Apparently, some intermolecular association of desulphated polymer chains is responsible for water insolubility. A strong indication of this is provided by the elution of some desulphated keratan sulphate at the void volume of a Sephadex G-200 column. In contrast, keratan sulphate which had not been subjected to desulphation, was invariably included by a column developed under identical conditions. It would be expected that oligosaccharides derived from the main chain of keratan sulphate would have a more regular structure than glycopeptides from the linkage region. This more regular structure should assist intermolecular association, thus, more oligosaccharide than glycopeptide would be expected in the water-insoluble, desulphated keratan sulphate. Both forms of desulphated KS gave, on Sephadex G-200, peaks at V_0 and peaks well retarded, indicating that separation of the two forms was not complete. The insoluble form gave relatively more excluded material than the soluble, indicating the presence of more aggregated material in this fraction.

Upon desulphation, KS II from bovine nasal cartilage gave no water-insoluble product. This result is not surprising as KS I of similarly low-average-molecular-weight would have yielded no perceptible water-insoluble, desulphated product. However, Bhavanandan and Meyer[8] reported that desulphation of KS II from human rib cartilage does give some insoluble product. The milder conditions of

desulphation employed by these authors, (which would have led to a desulphated product of higher molecular-weight than ours) are the likely cause for this different finding.

Keratan sulphate is unique among mammalian glycosaminoglycans in not being a glycuronoglycan (*i.e.*, it possesses a galactosyl rather than a glycosyluronic acid residue in its disaccharide repeat sequence). All glycosaminoglycuronoglycans give fully water-soluble products upon desulphation. Apparently, it is necessary for a desulphated glycosaminoglycan to be uncharged for it to be water insoluble. This conclusion is supported by the finding that chondroitin sulphate, upon reduction of its glycosyluronic acid residues and after desulphation, gives a water-insoluble product.

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