Biochem. J. (1987) 248, 85–93 (Printed in Great Britain)

Constant and variable domains of different disaccharide structure in corneal keratan sulphate chains

Marion OEBEN, Ruprecht KELLER, Helmut W. STUHLSATZ,* and Helmut GREILING
Department of Clinical Chemistry and Pathobiochemistry, Medical Faculty, Aachen University of Technology (RWTH Aachen), Pauwelsstrasse, D-5100 Aachen, Federal Republic of Germany

Four peptidokeratan sulphate fractions of different M_r and degree of sulphation were cut from the pig corneal keratan sulphate distribution spectrum. After exhaustive digestion with keratanase, the fragments were separated on DEAE-Sephacel and Bio-Gel P-10 and analysed for their M_r, degree of sulphation and amino sugar and neutral sugar content. It was found that every glycosaminoglycan chain is constructed of a constant domain of non-sulphated and monosulphated disaccharide units and a variable domain of disulphated disaccharide units. Total neuraminic acid of the four peptidokeratan sulphates was recovered from their isolated linkage-region oligosaccharides. In kinetic studies, the four peptidokeratan sulphates were investigated for M_r distribution after various incubation times with keratanase. There was a continuous shift towards lower M_r and no appearance of a distinct intermediate-sized product at any degradation time. The linkage-region oligosaccharide was already being liberated after a very short incubation period. From the results of these kinetic investigations in connection with the results of neuraminic acid analyses it is suggested that there exists only one disaccharide chain per peptidokeratan sulphate molecule. A model of corneal keratan sulphate is postulated. One of the α-mannose residues in the linkage region is bound to an oligosaccharide consisting of a lactosamine and a terminal sialic acid. The other α-mannose residue is attached to the disaccharide chain. This chain contains one or two non-sulphated disaccharide units at the reducing end, followed by 10–12 monosulphated disaccharide units. The disulphated disaccharide moiety of variable length is positioned at the non-reducing end of the chain.

INTRODUCTION

The M_r distribution of the polysaccharide moiety in pig and bovine corneal peptidokeratan sulphates ranges from about 10000 to 26000 (Stuhlsatz *et al.*, 1981). The keratan sulphate polysaccharide chain has been shown to be *N*-asparagine-linked to the core protein (Baker *et al.*, 1969; Greiling *et al.*, 1970; Stuhlsatz *et al.*, 1971) via a linkage-region oligosaccharide, which is of the same structure as 'complex-type' glycoprotein moieties (Keller *et al.*, 1981; Stein *et al.*, 1982; Nilsson *et al.*, 1983; Yamaguchi, 1983*a*,*b*, 1984). This was first shown for bovine corneal proteokeratan sulphate by our group (Keller *et al.*, 1981; Stein *et al.*, 1982), confirmed by Yamaguchi (1983*a*,*b*, 1984), and for monkey corneal proteokeratan sulphate by Nilsson *et al.* (1983). The group of Mersmann, however, found only one *N*-acetylglucosamine residue instead of the chitobiose unit in the linkage region (Brekle & Mersmann, 1983), but no sulphate (Ziegler & Mersmann, 1983).

Studies on the homogeneity and polydispersity of the polysaccharide portions of pig and bovine proteokeratan sulphates have shown that the polysaccharides exist in an M_r distribution that is unimodal, but distorted to low M_r (Stuhlsatz *et al.*, 1981).

From investigations on the sulphation reaction in corneal keratan sulphate biosynthesis we postulated a 'negative-feedback' model of corneal keratan sulphate biosynthesis (Keller *et al.*, 1983*a*,*b*) and suggested that the chain-elongation reaction is inhibited by sulphation of polysaccharide chain after a certain chain length is achieved.

However, some of the structural prerequisites for postulating this model of non-DNA-controlled chain elongation needed to be confirmed. It was still unknown whether corneal keratan sulphate contains one or two disaccharide chains linked to the linkage-region oligosaccharide. Furthermore, the dependency between degree of sulphation and chain length was, until now, only determined as the average value for mol of sulphate/mol of disaccharide. The increase of this value with chain length was interpreted by our group as an accumulation of higher-sulphated disaccharides towards the non-reducing end of the keratan sulphate chain.

In the present paper we describe experiments that answer the questions whether corneal keratan sulphate contains one or two disaccharide chains per linkage region and whether there exist domains of mono-and di-sulphated disaccharide units within the disaccharide chains.

EXPERIMENTAL

Materials

The following materials were purchased from the companies indicated: keratanase (*Pseudomonas* sp.) (EC 3.2.1.103) from Seikagaku (Tokyo, Japan); neuraminidase and *N*-acetylneuraminic acid from Sigma (Munich, Germany); Bio-Gel P-2 (200–400 mesh), Bio-Gel P-10 (100–200 mesh) and Dowex 1-X2 (200–400 mesh; Cl^- form) from Bio-Rad Laboratories (Munich, Germany); DEAE-Sephacel from Deutsche Pharmacia (Freiburg, Germany); NaB^3H_4 (10 Ci/mmol) from Amer-

Abbreviations used: h.p.g.p.c., high-performance gel-permeation chromatography.
* To whom correspondence should be sent.

sham International (Amersham, Bucks., U.K.); HPX-87H (300 mm × 7.8 mm), Bio-Sil TSK-250 (300 mm × 7.5 mm), Bio-Sil TSK-125 (300 mm × 7.5 mm) and pre-column Bio-Sil TSK-250 (75 mm × 7.5 mm) from Bio-Rad (Munich, Germany); Lumagel from Lumac (Basel, Switzerland). All reagents used were of analytical grade. Corneal peptidokeratan sulphates were from a preparation previously described (Stuhlsatz *et al.*, 1981).

Analytical methods

Galactose (Rommel *et al.*, 1968), sulphate (Antonopoulos, 1962) and mannose (Gawehn, 1974) were measured as described in the references cited. In the case of galactose and mannose determination samples were hydrolysed for 2 h at 105 °C in 1 M-HCl, and for sulphate they were hydrolysed for 24 h at 105 °C in 25% (v/v) formic acid. Glucosamine, galactosamine and amino acids were determined with the automatic LKB Alpha Plus amino acid analyser. Samples were hydrolysed for 15 h at 105 °C in 3 M-HCl.

H.p.g.p.c. and h.p.l.c.

H.p.g.p.c. and h.p.l.c. were performed on an LC 31A apparatus from Bruker (Bremen, Germany). H.p.g.p.c. was performed on Bio-Sil TSK-250 and Bio-Sil TSK-125 with a pre-column cartridge TSK-250 equilibrated with 0.2 M-NaCl/0.04 M-Tris/HCl buffer, pH 6.0. The HPX-87H column was equilibrated with 0.025 M-H_2SO_4. The flow rate was 0.5 ml/min, and the absorbance was monitored at 206 nm. The radioactivity was measured with an LKB (Gräfelfing, Germany) model 1217 Rack Beta liquid-scintillation counter.

Keratanase digestion of pig corneal peptidokeratan sulphates

Degradation with keratanase was performed in accordance with Nakazawa & Suzuki (1975) and Nakazawa *et al.* (1983), with slight modifications. A 30 μmol (as glucosamine) portion of pig corneal peptidokeratan sulphate was dissolved in 1 ml of 0.05 M-Tris/HCl buffer, pH 7.2, and incubated with 15 units of keratanase at 37 °C. After 15, 24 and 42 h, an additional 5 units were added. The incubation was terminated after 60 h by heating at 95 °C for 2 min.

DEAE-Sephacel chromatography of keratanase-digested pig corneal peptidokeratan sulphates

The incubation mixtures from the keratanase digestions were applied to the top of a DEAE-Sephacel column (2.0 cm × 22 cm) equilibrated with 0.05 M-Tris/HCl buffer, pH 6.8. The column was first eluted with 140 ml of 0.05 M-Tris/HCl buffer, pH 6.8, then with a linear gradient of 0–0.75 M-NaCl in 0.05 M-Tris/HCl buffer, pH 6.8 (total 300 ml), and finally with 3 M-NaCl in 0.05 M-Tris/HCl buffer, pH 6.8. Fractions (3 ml) were collected. The elution of the material was monitored by the orcinol reaction (Kesler, 1967), which was performed in an autoanalyser version (H. W. Stuhlsatz, unpublished work). The fractions were pooled as displayed in Fig. 1. The pooled fractions of the bound material from the DEAE-Sephacel chromatography were concentrated to about 10 ml by evaporation *in vacuo*, desalted on a Bio-Gel P-2 column (2.7 cm × 25 cm) and freeze-dried.

Separation of the linkage-region oligosaccharide from the oligosaccharide with one sulphate group per disaccharide unit

The unbound fractions of DEAE-Sephacel chromatography were freeze-dried, dissolved in 2 ml of 0.4 M-ammonium acetate buffer, pH 5.5, and applied to a column (1.2 cm × 115 cm) of Bio-Gel P-10, equilibrated with 0.4 M-ammonium acetate buffer, pH 5.5. The column was eluted with the same buffer, and 1.5 ml portions were collected. Samples of each portion were assayed by the orcinol reaction. The portions were pooled as shown in Fig. 2 and freeze-dried.

Neuraminic acid determination in the pig corneal peptidokeratan sulphates KSI–KSIV

Peptidokeratan sulphate (1 mg of each) was digested with 0.6 unit of neuraminidase in 200 μl of 0.075 M-sodium acetate buffer, pH 5.0, at 37 °C for 5 h. Samples of the incubation mixtures were analysed by h.p.l.c. on an HPX-87H column, with *N*-acetylneuraminic acid (0.1 mg/ml) as standard.

Neuraminic acid determination in the linkage-region oligosaccharides of peptidokeratan sulphates KSI–KSIV

Peptidokeratan sulphate (1 mg of each) was digested with 5 units of keratanase for 2 h as described above. The incubation mixtures were applied to a column (0.35 cm × 16 cm) of Dowex 1-X2 equilibrated with 0.15 M-NaCl. The column was eluted with 2 ml of 0.15 M-NaCl (to give fraction 1) and then with 2 ml of 0.5 M-NaCl (to give fraction 2). A 50 μl portion of 0.75 M-sodium acetate buffer was added to a 450 μl portion of each of fraction 1 and fraction 2. The solutions were each incubated with 0.6 unit of neuraminidase for 5 h at 37 °C. Then portions of each sample were applied to a Bio-Sil TSK-125 column and the neuraminic acid-containing portions separated by h.p.g.p.c. Their neuraminic acid content was determined by h.p.l.c. on HPX-87H, with *N*-acetylneuraminic acid (0.1 mg/ml) as standard.

SDS/polyacrylamide-gradient-gel electrophoresis

An LKB horizontal-slab-gel electrophoresis cell was used. Gels 0.5 mm thick were cast between glass plates (12 cm × 25 cm). Acrylamide stock solution contained 30% (w/v) acrylamide and 3% (w/v) *NN'*-methylenebisacrylamide. The reservoir buffer and the gel buffer were prepared as follows: a 10-fold concentration of Tris/glycine/SDS (25 M-Tris base/0.2 M-glycine/5 mM-SDS/0.2 M-NaN_3) and 2-fold concentration Tris/SDS (0.4 M-Tris base/5 mM-SDS/0.4 mM-NaN_3). The gradient gel was prepared as follows: the heavy solution was made up of 1.05 ml of acrylamide stock solution, 3.35 ml of glycerol and 2 ml of gel buffer, and the slight solution was made up of 6 ml of acrylamide stock solution and 2 ml of gel buffer; for polymerization 0.4 ml of 10% (w/v) ammonium persulphate and 7 μl of *NNN'N'*-tetramethylenediamine were added. The oligosaccharide samples, containing 30 nmol/10μl, were dissolved in 0.01 M-sodium phosphate buffer, pH 7, containing 1% (w/v) SDS. Portions (10 μl of each) were applied to the gel and electrophoresis was done for 2–3 h at 600 V. After electrophoresis the gel was transferred to a fix solution [50% (v/v) ethanol]. After 30 min of fixation, the gel was stained with Toluidine Blue [0.2 g/l in 3%

Table 1. Analyses of the four pig corneal peptidokeratan sulphates KSI–KSIV

	Composition (μmol/g of peptidokeratan sulphate)			
	KSI	KSII	KSIII	KSIV
Glucosamine	1161.2	1216.4	1052.5	1183.5
Galactosamine	0	0	0	0
Sulphate	1080.0	1362.4	1305.1	1668.7
Mannose	122.8	104.0	74.6	56.2
Galactose	1021.6	1080.7	1032.9	1017.7
Neuraminic acid	42.3	29.8	23.3	18.8
Amino acids	394.8	243.3	162.3	130.1
M_r	11400	16300	20700	25600

(v/v) acetic acid]. After destaining overnight, the gel was colourless with the exception of the fixed oligosaccharides. Oligosaccharides smaller than 18 disulphated disaccharide units were washed out partially or totally overnight. The stained patterns were scanned at 570 nm with an Elscript densitometer (Hirschmann, Unterhaching/Munich, Germany).

Determination of reducing end groups with NaB^3H_4

To determine the molecular size of the oligosaccharides from the peptidokeratan sulphates KSI–KSIV the reducing method of Robinson & Hopwood (1973) was modified as follows. A 0.5 nmol portion (related to glucosamine) of oligosaccharide and 0.5 nmol of galactose (as internal and external standard) were reduced and ^{3}H-labelled by dissolving at 4 °C in 50 μl of 0.1 M-sodium carbonate, pH 11.6, containing 0.5 μmol of NaB^3H_4 and incubating at this temperature for 16 h. One drop of acetic acid was added. The material was dried under a stream of N_2, and the residues were dissolved in 30 μl of 1 M-acetic acid and again evaporated to dryness. This dissolving–evaporation process was repeated twice with 30 μl of water each. The residue was dissolved in 100 μl of water and chromatographed on a Bio-Gel P-2 column (0.5 cm × 60 cm) equilibrated with 0.2 M-NaCl/0.04 M-Tris/HCl buffer, pH 6. Fractions were monitored for radioactivity by scintillation counting (1 μl samples). The number of reducing end groups was calculated from the ratio of the radioactivity of the external standard to that of the ^{3}H-labelled oligosaccharide alditols.

Kinetic studies on the keratanase digestion of pig corneal peptidokeratan sulphates

Four pig corneal peptidokeratan sulphates (1 mg each), of M_r 11400 with 0.93 mol of sulphate/mol of disaccharide, M_r 16300 with 1.12 mol of sulphate/mol of disaccharide, M_r 20700 with 1.24 mol of sulphate/mol of disaccharide and M_r 25600 with 1.41 mol of sulphate/mol of disaccharide, were incubated with 1.5 units of keratanase at 37 °C in 1 ml of 0.05 M-Tris/HCl buffer, pH 7.2. At 0 s, 30 s, 1 min, 2 min, 4 min, 16 min, 32 min and 60 min samples were removed and mixed immediately with 200 μl of h.p.g.p.c.-elution buffer at 95 °C. Portions (20 μl) were analysed by h.p.g.p.c. on Bio-Sil TSK-250.

RESULTS

The four pig corneal peptidokeratan sulphates KSI–KSIV that were utilized in this study are from a preparation described previously (Stuhlsatz *et al.*, 1981). All corneal peptidokeratan sulphate preparations have been analysed for their relative molecular mass, degree of sulphation, glucosamine, galactosamine, mannose and neuraminic acid content and amino acid composition (Table 1). Since galactosamine is not detectable in these preparations under the conditions used, contamination with peptidochondroitin sulphates can be neglected. No contamination with other biological material was observed. The peptide moieties contain between seven and nine amino acid residues.

Portions (30 μmol) (related to glucosamine) of each of peptidokeratan sulphates KSI–KSIV (for molecular mass and degree of sulphation see the Experimental section) were exhaustively digested with keratanase and the digests were chromatographed on DEAE-Sephacel (Fig. 1). Similar elution patterns were observed for all preparations. The unbound material that was eluted in front of the gradient (peak 1) was pooled and further separated on Bio-Gel P-10 into Ia and Ib fractions (Fig. 2). The bound material was eluted from the DEAE-Sephacel column with a 0–0.75 M-NaCl gradient and a final wash with 3 M-NaCl. Five fractions were separated by this method (peaks II–VI). The proportions of all fragments released by keratanase digestion are listed in Table 2.

The material Ia is the linkage-region oligosaccharide, as it contains mannose and amino acids but no sulphate (Table 3). Quantitative analyses show glucosamine/mannose/galactose/neuraminic acid relative proportions of 4:3:1:1 for fraction Ia. A very important point of this finding is that the linkage-region oligosaccharide still contains galactose and neuraminic acid in the ratio 1:1, indicating the presence of a lactosamine attached to a neuraminic acid residue. It was shown by neuraminic acid determinations of the intact peptidokeratan sulphate and its linkage-region oligosaccharide under optimized conditions that this neuraminic acid residue is the single one in the peptidokeratan sulphate molecule. Table 4 shows that 82–103 % of total neuraminic acid has been found in the linkage-region oligosaccharide isolated after degradation of the peptidokeratan sulphate by keratanase.

The M_r values for fractions Ia were estimated from their elution positions on h.p.g.p.c. and calculated from the quantitative analysis of their components as shown in Table 3, based on the known structure, which contains three mannose residues and one fucose residue per linkage region. The calculated values $M_{theor.}$ were in

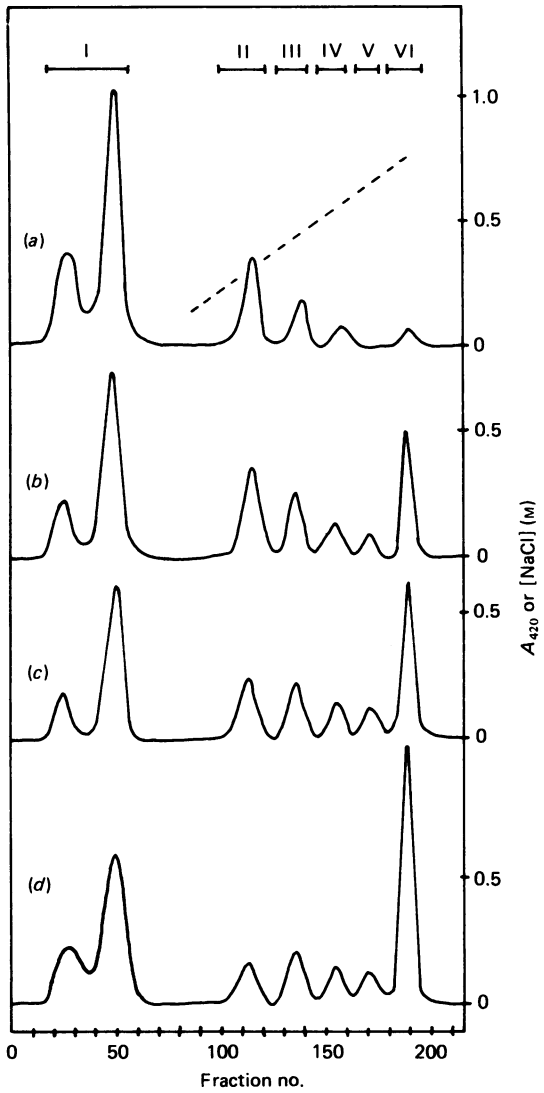


Fig. 1. DEAE-Sephacel chromatography of keratanase digests from four pig corneal peptidokeratan sulphates

Portions (30 μmol, related to hexosamine) of pig corneal peptidokeratan sulphates were digested with 15 units of keratanase in 1 ml of 0.05 M-Tris/HCl buffer, pH 7.2, at 37 °C for 60 h. The peptidokeratan sulphates were of (*a*) M_r 11400 with 0.93 mol of sulphate/mol of disaccharide, (*b*) M_r 16300 with 1.12 mol of sulphate/mol of disaccharide, (*c*) M_r 20700 with 1.24 mol of sulphate/mol of disaccharide and (*d*) M_r 25600 with 1.41 mol of sulphate/mol of disaccharide. The digests were applied to the top of a DEAE-Sephacel column (2.0 cm × 22 cm), which was first washed with 70 ml of 0.05 M-Tris/HCl buffer, pH 6.8. Chromatography was continued by applying a gradient of 0–0.75 M-NaCl in 0.05 M-Tris/HCl buffer, pH 6.8 (150 ml + 150 ml), after which the column was eluted with 3 M-NaCl in 0.05 M-Tris/HCl buffer, pH 6.8. Fractions (3 ml) were collected and portions were analysed by the orcinol reaction (——) and for conductivity (------, expressed as concentration of NaCl).

accordance with the data obtained from the elution position in h.p.g.p.c.

Substance Ib, which is the most prominent oligosaccharide in the peptidokeratan sulphates KSI–KSIII has a degree of sulphation of about 1, whereas that of the

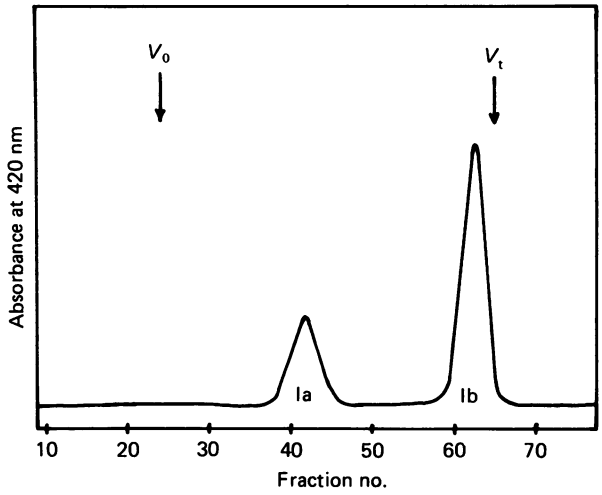


Fig. 2. Separation of the linkage-region oligosaccharide (Ia) from the oligosaccharide with one sulphate group per disaccharide unit (Ib) by chromatography on Bio-Gel P-10

Peak I material from DEAE-Sephacel chromatography of a keratanase digest from pig corneal peptidokeratan sulphate KSI was chromatographed on a Bio-Gel P-10 column (1.2 cm × 115 cm) in 0.4 M-ammonium acetate buffer, pH 5.5. Fractions (1.5 ml) were collected and portions were analysed by the orcinol reaction. Arrows indicate V_0 and V_t. Chromatography of peak I material derived from the other peptidokeratan sulphate preparations (KSII–KIV) yielded similar elution profiles on Bio-Gel P-10.

fractions II–VI is around 2 (Table 2). With the exception of fractions Ib the M_r values of the fragments II–VI were determined by h.p.g.p.c. on a Bio-Sil TSK-250 column calibrated with peptidokeratan sulphates of known M_r values. The M_r values in Table 2 correspond to octa- (II), deca- (III), dodeca- (IV), tetradeca- (V) and hexadecasaccharides (VI), as shown by the $M_{calc.}$ and $M_{theor.}$ values.

In order to examine whether there exist larger oligosaccharides, fraction VI from peptidokeratan sulphate KSIV was subjected to SDS/polyacrylamide-gel electrophoresis. By this, a ladder-like distribution pattern of the oligosaccharides was achieved in which adjacent bands correspond to oligosaccharides differing in chain length by one disulphated disaccharide unit. Fig. 3 demonstrates the existence of larger oligosaccharides after exhaustive keratanase digestion up to chain lengths of 34 disaccharide units.

Since in h.p.g.p.c. the fragments Ib are eluted close to V_t, their M_r values could not be determined reliably. Therefore the size of fragments Ib was ascertained by the reducing method with NaB^3H_4, which was also applied to the oligosaccharides II–VI. Table 5 gives the chain lengths of the different fragments expressed as numbers of disaccharides. In the case of fragments II–VI, the molecular sizes established by h.p.g.p.c. (M_r in Table 2) were confirmed by the NaB^3H_4 method ($M_{calc.}$ in Table 2). Fragments Ib from peptidokeratan sulphates KSI–KSIII were monodispersed by Bio-Gel P-2 chromatography ($K_{av.}$ 0.74) and proven to be trisulphated hexasaccharides, whereas fraction Ib from peptidokeratan sulphate KSIV seems to be a mixture of

Table 2. Proportions, degree of sulphation and M_r values (M_r, $M_{calc.}$ and $M_{theor.}$) of fragments released by keratanase from the peptidokeratan sulphates KSI–KSIV

M_r was determined by h.p.g.p.c. on Bio-Sil TSK-250 calibrated with peptidokeratan sulphates of known M_r (Stuhlsatz *et al.*, 1981). $M_{calc.}$ was calculated the following way. The number of disaccharides was taken from the reducing-terminal labelling (Table 5). In the case of the oligosaccharides II–VI the $M_{calc.}$ values were calculated from the number of disaccharide units assuming that each monosaccharide is sulphated despite the reducing galactose residue. For the value marked with an asterisk (*) the averaged $M_{calc.}$ value of fraction Ib was calculated for the homogeneous fractions of peptidokeratan sulphates KSI–KSIII from the averaged disaccharide number assuming one sulphate group per disaccharide unit. With regard to fragments Ib–VI $M_{theor.}$ was calculated as described for $M_{calc.}$ but using rounded figures of disaccharide numbers obtained from labelling experiments. $M_{theor.}$ for fragments Ia, however, was calculated from the quantitative analysis of their components (Table 3) based on the known structure, which contains three mannose residues, one fucose residue and the other constituents as listed in Table 3. Abbreviation: N.D., not determinable by h.p.g.p.c. on Bio-Sil TSK-250 since eluted too close to the V_t.

	Proportions of fragments (mol of glucosamine/100 mol of glucosamine)						
	Ia	Ib	II	III	IV	V	VI
KSI	19.2	49.5	10.0	11.7	7.0	0	2.6
KSII	12.7	37.0	16.0	12.0	7.7	4.0	10.6
KSIII	10.3	28.7	15.3	13.3	9.3	7.4	15.7
KSIV	8.6	21.0	9.0	11.3	8.1	7.3	34.7
	Degree of sulphation (mol of sulphate/mol of glucosamine)						
	Ia	Ib	II	III	IV	V	VI
KSI	0	1.12	1.91	1.85	2.01	0	2.12
KSII	0	1.02	1.90	2.03	2.14	2.07	2.00
KSIII	0	1.10	2.10	2.01	2.00	2.10	2.10
KSIV	0	1.06	1.87	1.85	1.91	2.11	2.04
	M_r value						
	Ia	Ib	II	III	IV	V	VI
M_r	2400	N.D.	2300	2700	3200	4000	4200
$M_{calc.}$	–	1323*	2174	2686	3312	3938	4507
$M_{theor.}$	2400	1401	2174	2743	3312	3881	4450

Table 3. Composition of linkage-region oligosaccharides (Ia) derived from peptidokeratan sulphates KSI–KSIV each normalized to three mannose residues

	Composition (mol/3 mol of mannose)			
	Ia (KSI)	Ia (KSII)	Ia (KSIII)	Ia (KSIV)
Glucosamine	4.18	3.90	4.10	4.04
Galactosamine	0	0	0	0
Sulphate	0	0	0	0
Mannose	3.00	3.00	3.00	3.00
Galactose	0.96	0.95	0.99	0.99
Neuraminic acid	1.12	0.86	0.93	1.00
Amino acids	9.2	7.7	7.0	7.2

disaccharides and tetrasaccharides after chromatography on Bio-Gel P-2 (results not shown) with one sulphate group per disaccharide unit.

The proportion of oligosaccharides containing disulphated disaccharides increases with the M_r of the peptidokeratan sulphates KSI–KSIV (Table 6). In contrast, the number of monosulphated disaccharides per linkage region equal to 11 ± 1 disaccharide units is constant in all peptidokeratan sulphates (Table 6). The results in Table 6 show that the increase in size from peptidokeratan sulphate KSI to peptidokeratan sulphate KSIV corresponds to an enlargement of the disulphated disaccharide region. If this is produced by chain growth, then it suggests that the low-sulphated region is attached to the linkage region.

In addition to the exhaustive digestion of peptidokeratan sulphates KSI–KSIV with keratanase, kinetic degradation studies were performed. Portions were removed after various incubation times and analysed by h.p.g.p.c. on a Bio-Sil TSK-250 column, calibrated with keratan sulphate M_r standards. Fig. 4 shows that the linkage-region oligosaccharide is already being released

Table 4. Neuraminic acid contents of peptidokeratan sulphates KSI–KSIV

Neuraminic acid was split by neuraminidase from the peptidokeratan sulphates KSI–KSIV (A) and from their linkage-region oligosaccharide peptides (B) isolated after degradation of peptidokeratan sulphates KSI–KSIV by keratanase. Determinations of neuraminic acid were performed by h.p.l.c. on an HPX-87H column (300 mm × 7.8 mm) eluted with 0.025 M-H_2SO_4; *N*-acetylneuraminic acid was used as standard. Values are expressed as μmol/g of peptidokeratan sulphate.

	A (μmol/g)	B (μmol/g)	B/A ratio
KSI	42.3	38.6	0.91
KSII	29.8	24.7	0.83
KSIII	23.3	23.9	1.03
KSIV	18.8	15.4	0.82

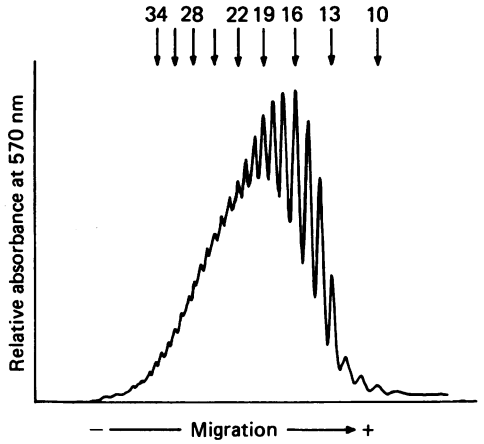


Fig. 3. Densitometric scan of gel-electrophoretic pattern for fragment VI from peptidokeratan sulphate KSIV

SDS/polyacrylamide-gradient-gel electrophoresis was in 4–22% polyacrylamide gel in Tris/glycine buffer at 60 V/cm. The gel was fixed with 50% (v/v) ethanol and subsequently stained with Toluidine Blue. The number of repeating disulphated disaccharide units is indicated. Calibration was performed with keratan sulphate oligosaccharides of known disulphated disaccharide numbers. Oligosaccharides with chain lengths between 18 and eight disulphated disaccharide units had been washed out of the gel by repeating destaining procedure.

after 30 s. There was no intermediate-sized degradation product detectable at any incubation time, but a continuous shift towards lower-M_r substances.

DISCUSSION

We have investigated the disaccharide composition of four peptidokeratan sulphates from pig cornea that differ in their M_r and degree of sulphation. These peptidokeratan sulphates were taken from a preparation described previously (Stuhlsatz *et al.*, 1981). Corneal keratan sulphates appear as a polydisperse mixture (average M_r 19000) that is heterogeneous with regard to

Table 5. Determination of labelled reducing end groups of oligosaccharides (OS) from the four keratanase-treated peptidokeratan sulphates (KSI–KSIV) after reaction with NaB^3H_4

The amounts of the oligosaccharides examined contained 0.5 nmol of glucosamine; 0.5 nmol of galactose was used as external standard.

	10^{-5} × Labelling (d.p.m.)	Radioactivity of galactose standard/radioactivity of labelled oligosaccharide
Galactose	20.6	–
OS-Ib (KSI)	7.26	2.8
OS-Ib (KSII)	7.32	2.8
OS-Ib (KSIII)	7.00	2.9
OS-Ib (KSIV)	12.80	1.6
OS-II (KSII)	5.18	4.0
OS-III (KSI)	4.21	4.9
OS-IV (KSIV)	3.41	6.0
OS-V (KSIV)	2.90	7.1
OS-VI (KSIV)	2.53	8.1

the degree of sulphation. The four peptidokeratan sulphate fractions that were used for this study were cut from the distribution spectrum and are much less polydisperse in size and less heterogeneous in degree of sulphation compared with the original substance.

The structure of the linkage-region oligosaccharide, which is positioned between the core protein and the keratan sulphate disaccharide chain, had been determined previously by our group and by others. Furthermore, we have measured the M_r distribution of the keratan sulphate chains and the dependency between degree of sulphation and chain length. There were two main questions remaining with regard to the structure of the corneal peptidokeratan sulphate polysaccharide portion, as follows.

1. What is the distribution of sulphate groups on the polysaccharide chain?
2. Does the corneal peptidokeratan sulphate contain one or two keratan sulphate chains per linkage region?

Question 1

Since the polysaccharide chain grows in a polar fashion starting from the reducing end of the chain, we define any position on the chain as distance from the reducing end. Two antithetical assumptions can be made, as follows.

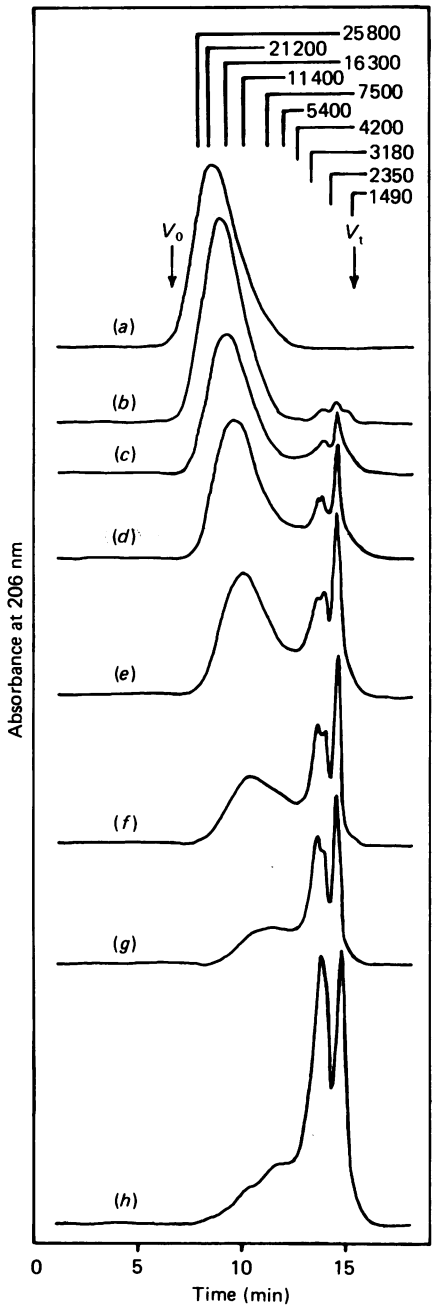
(a) The keratan sulphate biosynthesis takes place as a chain elongation in close association with sulphation (Keller *et al.*, 1983*a*,*b*). Under these circumstances an increase of the statistical sulphation of keratan sulphate with chain length can only be achieved by introducing two sulphate groups per disaccharide unit towards the non-reducing end. For the same reason the non- and low-sulphated region needs to be positioned close to the reducing end (Figs. 5*b* and 5*d*).

(b) The keratan sulphate biosynthesis takes place in two independent steps: chain elongation and sulphation. Under these circumstances the degree of sulphation

Table 6. Domains of keratan sulphate from pig cornea

The numbers of glucosamine residues per keratan sulphate molecule are calculated from the M_r (Table 1). Their distribution in the different domains is derived from the percentage values in Table 2, where Ia represents the linkage region, Ib represents the monosulphated disaccharide region and the sum of II–VI represents the disulphated disaccharide region.

	Distribution (mol of glucosamine/mol)			
	Linkage region	Monosulphated disaccharide region	Disulphated disaccharide region	Total in peptidokeratan sulphate
KSI	4.3	11.2	7.1	22.6
KSII	4.1	12.0	16.3	32.4
KSIII	4.1	11.5	24.6	40.2
KSIV	4.2	10.2	34.1	48.5



would grow in average with chain length; this means that all sulphate groups are distributed statistically on the polysaccharide chain and that there are no regular domain structures (Figs. 5*a* and 5*c*).

Many objections can be made against assumption (b), as follows. (i) The sulphotransferase activity decreases with increasing chain length and degree of sulphation (Keller *et al.*, 1983*a*,*b*). This is contradictory to the finding that larger chains are overproportionally sulphated. (ii) The amount of monosulphated keratan sulphate disaccharides remains constant relative to the amount of the linkage region in all peptidokeratan sulphate preparations investigated (Table 6). It would be difficult to explain how in statistical sulphation, which would be independent from chain elongation, this number of monosulphated disaccharides can be regulated. (iii) In earlier studies (Stuhlsatz *et al.*, 1981) we have described a low-sulphated low-M_r keratan sulphate fraction (M_r 7500, degree of sulphation 0.59) from the same preparation as peptidokeratan sulphates KS-I–KSIV. We did not find any disulphated disaccharide in this peptidokeratan sulphate (results not shown). This is compatible with the notion that this fraction represents the non- and mono-sulphated domain within the whole M_r distribution, as it results from the hypothetical pathway described under (a).

In our model for the total peptidokeratan sulphate distribution pattern there is a highly sulphated polydisperse domain at the non-reducing end of the molecule

Fig. 4. Molecular-size profiles during peptidokeratan sulphate degradation by keratanase after h.p.g.p.c. on Bio-Sil TSK-250

A 1 mg portion of pig corneal peptidokeratan sulphate KSIII of M_r 20700 with 1.24 mol of sulphate/mol of disaccharide was incubated with 1.5 units of keratanase in 1 ml of 0.05 M-Tris/HCl buffer, pH 7.2, at 37 °C. Portions (50 μl) were removed at 0 s (*a*), 30 s (*b*), 1 min (*c*), 2 min (*d*), 4 min (*e*), 16 min (*f*), 32 min (*g*) and 60 min (*h*) and chromatographed on Bio-Sil TSK-250 (300 mm × 7.5 mm) with a pre-column Bio-Sil TSK-250 (75 mm × 7.5 mm) in 0.2 M-NaCl/0.04 M-Tris/HCl buffer, pH 6. The flow rate was 0.5 ml/min. Absorbance was monitored at 206 nm. Arrows indicate V_0 and V_t. The column was calibrated with keratan sulphate standards from preparations described previously (Stuhlsatz *et al.*, 1981).

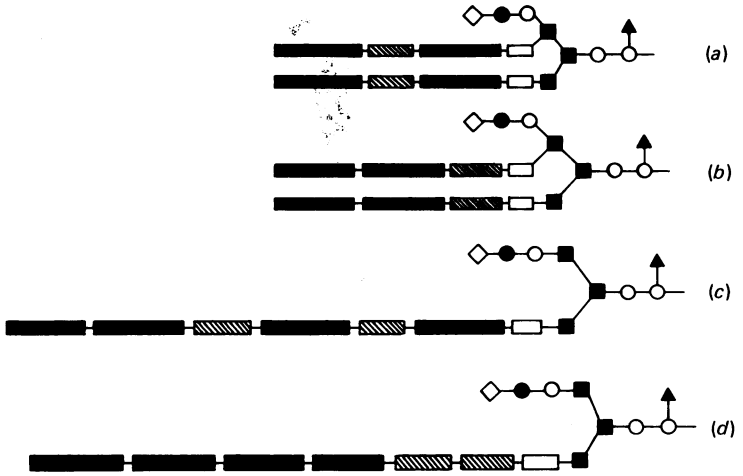


Fig. 5. Model of the structure of corneal keratan sulphate

Key: ▬, $[GlcNAc(SO_3^-)–Gal(SO_3^-)]_x$; ▨, $[GlcNAc–Gal(SO_3^-)]_y$; ▭, $[Gal–GlcNAc]_z$; ○, GlcNAc; ●, Gal; ■, Man; ◇, NeuAc; ▲, Fuc.

(Figs. 5*b* and 5*d*). This domain is also heterogeneous, since there may be a different number of sulphate gaps in the variable disulphated part of the molecule. Assuming no sulphate gap within this part of the molecule, one should find in small amounts a fully extended domain of disulphated disaccharides not degradable by keratanase. In peptidokeratan sulphate KSIV this domain of the molecule is expected to contain more than 30 disaccharide units (Table 6) on the assumption that there exists only one keratan sulphate chain per linkage region (Fig. 5*d*). Fig. 3 shows that this domain of peptidokeratan sulphate KSIV indeed contains more than 30 disulphated disaccharide units.

Question 2

Until now, those groups (Keller *et al.*, 1981; Stuhlsatz *et al.*, 1981; Nilsson *et al.*, 1983; Yamaguchi, 1984) that have worked on the corneal keratan sulphate structure have favoured a structure with two repeating unit chains, as is displayed in Figs. 5(*a*) and 5(*b*). However, there has never been any experimental proof for this.

In the present study we describe for the first time that corneal keratan sulphate contains one *N*-acetylneuraminic acid residue per molecule. With the exception of colominic acid, a bacterial polycondensate of *N*-acetylneuraminic acid, this carbohydrate is always positioned at the non-reducing terminus of poly- or oligo-saccharides. It cannot be located at the non-reducing end of the keratan sulphate disaccharide chain, since we detected it in the linkage-region oligosaccharide after keratanase treatment. Therefore it may be that there is another branching point close to the linkage region to which a lactosamine-linked *N*-acetylneuraminic acid residue is connected (Figs. 5*a* and 5*b*); or there may be only one repeating unit chain linked to one of the two (α)-mannose residues. The other (α)-mannose would then carry a lactosaminyl-*N*-acetylneuraminic acid group, as described for *N*-linked glycoprotein oligosaccharides. The existence of a branching point in the polysaccharide portion of corneal proteokeratan sulphate in addition to the 1,3,6-substituted mannose would require one sugar (galactose, glucosamine or mannose) to be substituted in an unusual fashion. Permethylation studies of the entire polysaccharide, of its chemically desulphated derivative and of the enriched linkage region (Keller *et al.*, 1981; Ohst, 1981; Stein *et al.*, 1982) clearly exclude this possibility.

Furthermore, the relative proportions of the constituents glucosamine/mannose/galactose/neuraminic acid of the linkage region liberated by keratanase treatment are 4:3:1:1, which is not in agreement with structures such as displayed in Fig. 5(*a*) and 5(*b*). In the linkage-region oligosaccharide we did not find sulphated glucosamine.

Since keratanase cleavage only occurs at non-sulphated galactose residues, this requires the existence of a non-sulphated disaccharide at the reducing end of the repeating unit chain.

For these reasons we suggest a model of corneal keratan sulphate as shown in Fig. 5(*d*): a non-sulphated disaccharide is positioned at the reducing end of the chain followed by a constant number of monosulphated disaccharide units and a varied number of disulphated disaccharide units.

There is also more evidence for the proposed model from kinetic degradation studies with keratanase. The linkage region was liberated from the entire peptidokeratan sulphate molecule immediately after addition of the enzyme. If there are two keratan sulphate chains linked to one linkage region, one should find the immediate appearance of a keratan sulphate of intermediate size. Such a case has, however, never been demonstrated with any of the keratan sulphates investigated. Furthermore, the probability of a simultaneous attack by two enzyme molecules on two keratan sulphate chains seems very low.

The structural data, presented in this study, give some more evidence for a 'negative-feedback' model of keratan sulphate biosynthesis (Keller *et al.*, 1983*a*,*b*), which was also based on assumption (a). On the basis of this model we have performed a computer simulation study of keratan sulphate biosynthesis, which yielded plausible

results on the regulation of corneal keratan sulphate biosynthesis and structure.

Scudder *et al.* (1986) has described oligosaccharides from bovine corneal keratan sulphate generated by an endo-β-galactosidase from *Bacteroides fragilis* that differs in substrate-specificity from the keratanase (*Pseudomonas* sp.) (Nakazawa & Suzuki, 1975) used in the present paper. Furthermore, Scudder *et al.* (1986) started with only one peptidokeratan sulphate fraction of high polydisperity. The preceding arguments and perhaps the species may be responsible for some differences between their and our results. They found 3% unsulphated disaccharides, 35% monosulphated disaccharides and 40% tetra-, hexa-, octa- and deca-saccharides with three, five, seven and nine sulphate groups, respectively, and about 15% oligosaccharides of greater chain lengths. If the average disaccharide number per peptidokeratan sulphate molecule in the Scudder *et al.* (1986) preparation (25 disaccharide units) is compared with the disaccharide number of the peptidokeratan sulphates used by us, it becomes obvious that their material resembles our peptidokeratan sulphate KSII, with 28 disaccharide units. The proportions of the mono- and di-sulphated disaccharide moieties, 35% and 55% (Scudder *et al.*, 1986) compared with 37% and 50% (Table 2), are also comparable. The degrees of sulphation of the oligosaccharide fragments II–VI, ranging from 1.85 to 2.14 (Table 2), are in accordance with data from fast-atom-bombardment m.s. studies (Scudder *et al.*, 1986) that each monosaccharide in the oligosaccharides is sulphated except the reducing galactose residue.

At one time glycosaminoglycans were designed as 'mucopolysaccharides', an expression that just by the name 'muco' suggested a heterogeneous and polydisperse material, difficult to define by any other means than structural elements and statistical data such as overall degree of sulphation and average M_r. In contrast, our studies on the pig corneal keratan sulphate structure have shown that these molecules are well defined, contain domains of different charge and structure in reproducible positions of the polysaccharide moiety and have a narrow M_r distribution. This offers the opportunity to investigate the biological role of glycosaminoglycans such as corneal keratan sulphate on the basis of structure–function relationship.

The expert technical assistance of Ms. Eggels, Ms. Kalf and Mr. Delvoux is greatly appreciated. This work was supported by grants of the Deutsche Forschungsgemeinschaft.

Received 8 January 1987/26 May 1987; accepted 3 August 1987

REFERENCES

Antonopoulos, C. A. (1962) Acta Chem. Scand. **16**, 1521–1522

Baker, J. R., Cifonelli, J. A., Mathews, M. B. & Rodén, L. (1969) Fed. Proc. Fed. Am. Soc. Exp. Biol. **28**, 605

Brekle, A. & Mersmann, G. (1983) Hoppe-Seyler's Z. Physiol. Chem. **361**, 31–39

Gawehn, K. (1974) in Methoden der Enzymatischen Analyse, 3rd edn. (Bergmeyer, H. U., ed.), vol. 2, pp. 1308–1313, Verlag Chemie, Weinheim/Bergstr.

Greiling, H., Stuhlsatz, H. W. & Kisters, R. (1970) in Chemistry and Molecular Biology of the Intracellular Matrix, vol. 2 (Balàzs, E. A., ed.), pp. 873–877, Academic Press, London and New York

Keller, R., Stein, T., Stuhlsatz, H. W., Greiling, H., Ohst, E., Müller, E. & Scharf, H. D. (1981) Hoppe-Seyler's Z. Physiol. Chem. **362**, 327–336

Keller, R., Driesch, R., Stein, T., Momburg, M., Stuhlsatz, H. W. & Greiling, H. (1983*a*) Hoppe-Seyler's Z. Physiol. Chem. **364**, 239–252

Keller, R., Driesch, R., Stein, T., Momburg, M., Stuhlsatz, H. W. & Greiling, H. (1983*b*) Hoppe-Seyler's Z. Physiol. Chem. **364**, 253–260

Kesler, R. B. (1967) Anal. Chem. **39**, 1416–1422

Nakazawa, K. & Suzuki, S. (1975) J. Biol. Chem. **250**, 912–917

Nakazawa, K., Newsome, D. A., Nilsson, B., Hascall, V. C. & Hassell, J. R. (1983) J. Biol. Chem. **258**, 6051–6055

Nilsson, B., Nakazawa, K., Hassel, J. R., Newsome, D. A. & Hascall, V. C. (1983) J. Biochem. (Tokyo) **258**, 6056–6063

Ohst, E. (1981) Ph.D. Thesis, RWTH-Aachen

Robinson, H. C. & Hopwood, J. J. (1973) Biochem. J. **133**, 457–470

Rommel, K., Bernd, E., Schmitz, F. & Grimmel, K. (1968) Klin. Wochenschr. **46**, 936–940

Scudder, P., Tang, P. W., Hounsell, E., Lawson, A., Mehmet, H. & Feizi, T. (1986) Eur. J. Biochem. **157**, 365–373

Stein, T., Keller, R., Stuhlsatz, H. W., Greiling, H., Ohst, E., Müller, E. & Scharf, H. D. (1982) Hoppe-Seyler's Z. Physiol. Chem. **363**, 825–833

Stuhlsatz, H. W., Kisters, R., Wollmer, A. & Greiling, H. (1971) Hoppe-Seyler's Z. Physiol. Chem. **352**, 289–303

Stuhlsatz, H. W., Hirzel, F., Keller, R., Cosma, S. & Greiling, H. (1981) Hoppe-Seyler's Z. Physiol. Chem. **362**, 841–852

Yamaguchi, H. (1983*a*) J. Biochem. (Tokyo) **94**, 207–213

Yamaguchi, H. (1983*b*) J. Biochem. (Tokyo) **94**, 215–221

Yamaguchi, H. (1984) J. Biochem. (Tokyo) **95**, 601–604

Ziegler, C. & Mersmann, G. (1983) Hoppe-Seyler's Z. Physiol. Chem. **364**, 97–100