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Two linkage-region fragments isolated from skeletal keratan sulphate contain a sulphated *N*-acetylglucosamine residue

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Peptido-keratan sulphate fragments were isolated from the nucleus pulposus of bovine intervertebral discs (6-year-old animals) after chondroitin ABC lyase digestion followed by digestion of A1D1 proteoglycans by diphenylcarbamoyl chloride-treated trypsin and gel-permeation chromatography on Sepharose CL-6B. Treatment of these peptido-keratan sulphate fragments with alkaline NaB^3H_4 yielded keratan sulphate chains with [^{3}H]galactosaminitol end-labels, and these chains were further purified by gel-permeation chromatography on Sephadex G-50 and ion-exchange chromatography on a Pharmacia Mono-Q column in order to exclude any contamination with *O*-linked oligosaccharides. The chains were then treated with keratanase, and the digest was chromatographed on a Bio-Gel P-4 column followed by anion-exchange chromatography on a Nucleosil 5 SB column. Two oligosaccharides, each representing 18% of the recovered radiolabel, were examined by 500 MHz ^{1}H-n.m.r. spectroscopy, and shown to have the following structures:

GlcNAc(6SO_3)1–6GalNAc-ol
3
|
1
NeuAc2–3Gal
(I)

GlcNAc(6SO_3)1–6GalNAc-ol
3
|
1
Gal
(II)

The structure of oligosaccharide (I) confirms the *N*-acetylneuraminylgalactose substitution at position 3 of *N*-acetylgalactosamine in the keratan sulphate–protein linkage region found by Hopwood & Robinson [(1974) Biochem. J. **141**, 57–69] but additionally shows the presence of a 6-sulphated *N*-acetylglucosamine. Electron micro-probe analysis specifically confirmed the presence of sulphur in this sample. This sulphate ester group differentiates the keratan sulphate linkage region from similar structures derived from *O*-linked oligosaccharides [Lohmander, De Luca, Nilsson, Hascall, Caputo, Kimura & Heinegård (1980) J. Biol. Chem. **255**, 6084–6091].

INTRODUCTION

The large cartilage proteoglycan contains a protein core that is glycosylated with numerous chondroitin sulphate and keratan sulphate chains as well as *O*-linked and *N*-linked oligosaccharides (Hardingham, 1986). Recently the keratan sulphate-enriched region of the bovine cartilage proteoglycan core protein has been shown (Antonsson *et al.*, 1989) to contain a repeating hexapeptide motif.

The complete primary structure of cartilage keratan sulphate (skeletal keratan sulphate II, after the notation of Meyer, 1970) and that of its linkage region to protein is not yet fully elucidated (but for reviews see Stuhlsatz *et al.*, 1989; Hascall & Midura, 1989). It is known that the chains are *O*-linked from *N*-acetylgalactosamine to either threonine or serine (Bray *et al.*, 1967) in the protein core, and that the disaccharide *N*-acetylneuraminylgalactose is attached to C-3 of the *N*-acetylgalactosamine residue (Hopwood & Robinson, 1974). By analogy with the structures of the *O*-linked oligosaccharides isolated from proteoglycans of the Swarm rat chondrosarcoma (Lohmander *et al.*, 1980), it is generally understood that the major branch of the keratan sulphate chain is linked to C-6 of the *N*-acetylgalactosamine residue.

In the present study two keratanase-derived fragments from the carbohydrate linkage region are isolated and examined by ^{1}H-n.m.r. spectroscopy. There have been several previous n.m.r.-spectroscopic studies of keratan sulphates: a ^{1}H-n.m.r. investigation of corneal keratan sulphate (Hounsell *et al.*, 1986), a ^{1}H-n.m.r. and ^{13}C-n.m.r. examination of shark keratan sulphate (Cockin *et al.*, 1986), and ^{1}H-n.m.r. (Thornton *et al.*, 1989*a*) and ^{13}C-n.m.r. (Thornton *et al.*, 1989*b*) studies of bovine articular-cartilage keratan sulphate. The three small *O*-linked oligosaccharides found in proteoglycans (Lohmander *et al.*, 1980) have also been derived from human chorionic gonadotrophin and their ^{1}H-n.m.r. spectra have been published (Damm *et al.*, 1987).

EXPERIMENTAL

Materials

Chemicals and enzymes used in this study are as described in Thornton *et al.* (1989*b*), except that $LiClO_4$ (A.C.S. grade) and piperazine (99%) were from Aldrich Chemical Co. (Gillingham, Dorset, U.K.), Ecoscint A was from Mensura Technology (Parbold, Wigan, Lancs., U.K.) and NaB^3H_4 was from Amersham International (Amersham, Bucks., U.K.). The Nucleosil 5 SB column, manufactured by Macherey–Nagel (Düren, Germany), was purchased from H.P.L.C Technology (Macclesfield, Cheshire, U.K.), and Mono-Q HR 5/5 was from Pharmacia (Milton Keynes, Bucks., U.K.). Bio-Gel P-4 was purchased from Bio-Rad Laboratories (Watford, Herts., U.K.). The enzymes chondroitin ABC lyase (EC 4.2.2.4; from *Proteus vulgaris*) and keratanase (EC 3.2.1.103; from *Pseudomonas* sp.) were purchased from ICN Biomedicals (High Wycombe, Bucks., U.K.) and Sigma Chemical Co. (Poole, Dorset, U.K.) respectively.

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Analytical methods

All assay procedures were performed as described in Thornton *et al.* (1989*b*). Radioactivity in samples was determined in a Packard Tri-Carb 300 scintillation counter, 5 μl or 10 μl samples being taken and diluted with 2 ml of Ecoscint A.

Preparation of peptido-keratan sulphates

Proteoglycan monomers were extracted from the comminuted nucleus pulposus of bovine intervertebral discs (6-year-old animals) in 4 M-guanidinium chloride in the presence of proteolytic inhibitors, dialysed into associative conditions and subjected to associative followed by dissociative CsCl-density-gradient-centrifugation steps (as described in detail in Thornton *et al.*, 1989*b*). The A1D1 fraction was dialysed against 0.1 M-Tris/acetate buffer, pH 7.3, and then digested with chondroitin ABC lyase (0.5 unit/300 mg of proteoglycan) followed by diphenylcarbamoyl chloride-treated trypsin (2 mg/1 g of proteoglycan). The digest was partially freeze-dried and then chromatographed on a Sepharose CL-6B column (152 cm × 3.2 cm) eluted with 10 mM-EDTA/0.5 M-sodium acetate buffer, pH 6.8. This produced peptido-keratan sulphate fragments as described in Thornton *et al.* (1989*b*).

Alkaline-borohydride reduction

Keratan sulphate chains were prepared by alkaline-borohydride reduction (Carlson, 1968) of the peptido-keratan sulphates. These fragments (60 mg) were dissolved in 3 ml of 0.05 M-NaOH, and NaB^3H_4 (100 mCi; 5 Ci/mmol dissolved in 200 μl of 0.05 M-NaOH) was added. After 1 h a further 3 ml of 0.05 M-NaOH was added and sufficient $NaBH_4$ to produce a 1 M-solution.

Non-radioactive alkaline-borohydride reduction was performed on 165 mg of peptido-keratan sulphate (5 mg of peptido-keratan sulphate/ml of 1 M-$NaBH_4$ in 0.05 M-NaOH).

In both cases the reduction was terminated by the dropwise addition of acetic acid and the mixtures were dialysed against frequent changes of 0.1 M-Tris/acetate buffer, pH 7.3.

Purification of keratan sulphate

The reduced chain preparations were then digested with chondroitin ABC lyase (0.5 unit/300 mg) and chromatographed on a Sephadex G-50 column (82 cm × 1.5 cm) eluted with 0.2 M-NH_4HCO_3. The reduced keratan sulphate chains were recovered by freeze-drying after extensive dialysis against 1.0 M-NaCl and then water, and the radioactive end-labelled keratan sulphate chains were also further purified to exclude totally any *O*-linked oligosaccharides on a Pharmacia Mono-Q HR 5/5 column eluted with a linear gradient of 0–0.5 M-$LiClO_4$/10 mM-piperazine, pH 5.0.

Keratanase digestion

Reduced keratan sulphate chains (35 mg) were spiked with 1 mg of the reduced radiolabelled keratan sulphate chains, dissolved in 0.2 M-sodium acetate buffer, pH 7.4, and digested for 24 h with keratanase (1 unit/2.8 mg of keratan sulphate) at 37 °C. The digest was then chromatographed in water on a Bio-Gel P-4 column (131 cm × 0.85 cm) eluted at 3.6 ml/h. The eluent was analysed for hexose, sialic acid and radioactivity and the fractions were pooled as indicated (Fig. 1).

H.p.l.c.

H.p.l.c. was performed on a Bio-Rad series 700 HRLC titanium gradient system with u.v. and refractive-index detectors.

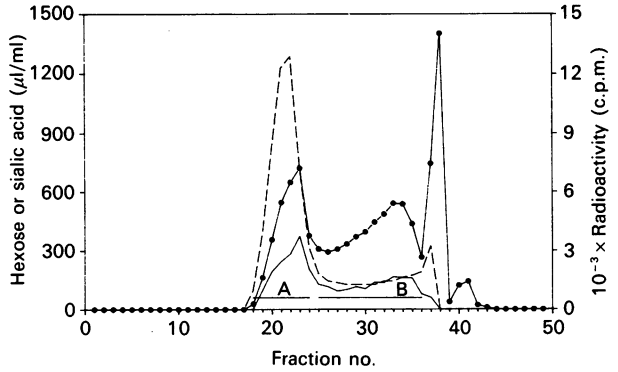


Fig. 1. Bio-Gel P-4 gel-chromatography profile of the keratanase digest of bovine intervertebral-disc keratan sulphate chains spiked with ^{3}H-labelled keratan sulphate chains

The column (131 cm × 0.85 cm) was eluted at a flow rate of 3.6 ml/h with water and 1.2 ml fractions were assayed for hexose (----), sialic acid (——) and radioactivity (●—●). Fractions P-4-A and P-4-B were pooled as indicated.

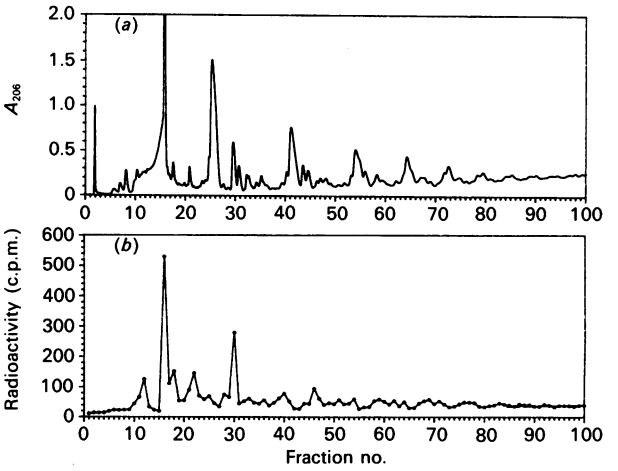


Fig. 2. H.p.l.c. profile of fraction P-4-A

Chromatogram (*a*) shows the trace produced by on-line monitoring of the Nucleosil 5 SB column eluent by using u.v. detection at 206 nm. The gradient program was as follows: 10 min of buffer A (10 mM-piperazine, pH 5.0) and then 230 min of 0–100% buffer B (0.5 M-$LiClO_4$, 10 mM-piperazine, pH 5.0). Fractions (2.4 min) were collected and monitored (*b*) for radioactivity.

Fraction P-4-A (25 mg) was chromatographed in two separate runs (12.5 mg each) on a Nucleosil 5 SB column eluted with a linear gradient of 0–0.5 M-$LiClO_4$/10 mM-piperazine, buffered to pH 5.0 (Fig. 2). Fractions 16 containing 18% of the recovered radioactivity were combined from the two Nucleosil runs, desalted on a Bio-Gel P-2 column (11.2 cm × 1 cm), eluted with water and freeze-dried. This material was examined by 500 MHz ^{1}H-n.m.r. spectroscopy and found to contain several components. Hence it was re-chromatographed on Nucleosil 5 SB but eluted with a shallower gradient, 0–0.25 M-$LiClO_4$, pH 5.0. This experiment resolved the original fraction 16 into three components (Fig. 3). The third peak contained radiolabel and was desalted on Bio-Gel P-2, eluted with water and freeze-dried to yield the sample referred to below as oligosaccharide I.

Fraction P-4-B, after being desalted on a Bio-Gel P-2 column, was chromatographed on Nucleosil 5 SB, and eluted with a linear gradient of 0–0.25 M-$LiClO_4$, pH 5.0. Fraction P-4-B-II (Fig. 4) containing 18% of the recovered radioactivity was

desalted on a Bio-Gel P-2 column and freeze-dried to yield the sample referred to below as oligosaccharide II.

Oligosaccharides I and II were then examined by ^{1}H-n.m.r. spectroscopy.

N.m.r. spectroscopy

Samples were buffered to pH 7 and referenced with sodium 3-trimethylsilyl[$^2H_4$]propionate as internal standard for ^{3}H-n.m.r. spectroscopy as previously described (Sanderson *et al.*, 1987). ^{1}H-n.m.r. spectra were obtained at 500.14 MHz with a Bruker AM500 spectrometer with 5 mm variable-temperature probes at room temperature and 60 °C. COSY-45 and one-step relayed-COSY two-dimensional measurements were performed at room temperature on a Bruker WH-400 instrument operating at 400.13 MHz. Spectra were re-processed for presentation by using the computer program NMR1 (Lab One NMR1 Spectroscopic Data Analysis System, Release 3.92; New Methods Research, Syracuse, NY, U.S.A.).

Electron micro-probe analysis

Oligosaccharide I was examined by using electron micro-probe analysis. The sample (500 μg) was dissolved in 30 μl of distilled water and two 1 μl drops were placed on top of a polished pyrolytic carbon stub, with a take-off angle of 45 °. The specimen was quench-frozen in melting N_2 (−210 °C) and then transferred to the pre-chamber/air-lock of a JEOL JSM 840A scanning electron microscope and placed on the cold pedestal (−180 °C). After a high vacuum had been attained the specimen was uncovered and transferred to the cold stage (−176 °C) of the scanning electron microscope. The specimen was then coated with 50 nm of aluminium and freeze-dried for 2 h in the microscope, the stage temperature during this time being permitted to rise slowly to 0 °C to allow sample concentration by freeze-drying. The specimen was probed at an accelerating voltage of 15 kV and a beam current of 0.5 nA until a pre-set integral of 80000 counts for aluminium was reached.

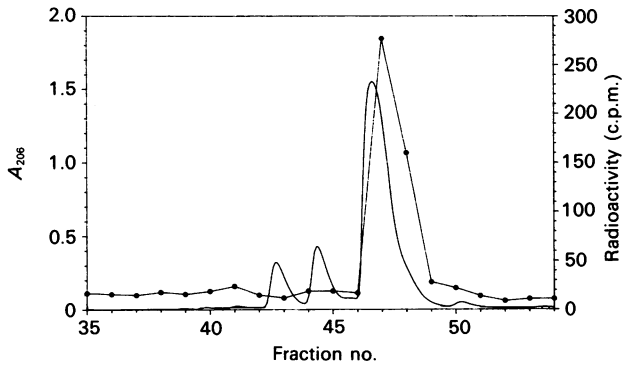


Fig. 3. H.p.l.c. profile of fraction 16

Fraction 16 (Fig. 2) was re-chromatographed on a Nucleosil 5 SB column at a flow rate of 0.75 ml/min. The column eluent was monitored on-line by using u.v. detection at 206 nm. (Note the slight off-set between the continuous u.v. trace and the radioactivity data, which were only determined for whole fractions.) The $LiClO_4$ gradient rose from 0.027 M to 0.048 M across the section shown. Fractions (0.75 ml) were monitored for radioactivity (●—●), and then fractions 47 and 48 were pooled as oligosaccharide I.

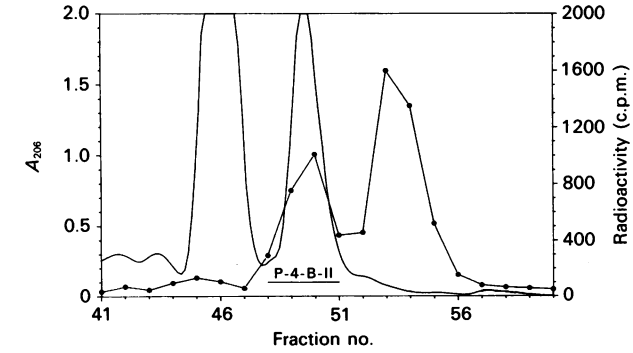


Fig. 4. H.p.l.c. profile of fraction P-4-B

Fraction P-4-B was chromatographed on a Nucleosil 5 SB column at a flow rate of 0.75 ml/min. The column eluent was monitored on-line by using u.v. detection at 206 nm. (Note the slight off-set between the continuous u.v. trace and the radioactivity data, which were only determined for whole fractions.) The $LiClO_4$ gradient rose from 0.011 M to 0.022 M across the section shown. Fractions (0.0375 ml) were monitored for radioactivity (●—●), and fractions 48–51 (P-4-B-II) were pooled as oligosaccharide II.

RESULTS

In this study keratan sulphate chains from bovine intervertebral disc were used specifically because they are of high M_r (Hopwood & Robinson, 1973) and this factor facilitates their separation from the much smaller *O*-linked oligosaccharides.

The enzyme keratanase (from *Pseudomonas* sp.) is a form of endo-β-galactosidase that cleaves at an unsubstituted galactose residue and 'requires at least one *N*-acetylglucosamine 6-sulphate residue located at either of the nearest sides of the galactose residue participating in the galactosidic linkage to be attacked' (Nakazawa *et al.*, 1989).

The chromatogram on Bio-Gel P-4 of the keratanase digest of reduced end-labelled keratan sulphates from bovine intervertebral disc is shown in Fig. 1. As described above, oligosaccharides I and II were purified from fractions P-4-A and P-4-B respectively via chromatography on Nucleosil 5 SB, as shown in Figs. 3 and 4. These oligosaccharides are homogeneous as judged by their chromatographic behaviour and clearly derive from the keratan sulphate carbohydrate-to-protein linkage, since they both contain radiolabel.

Analysis of the complete ^{1}H-n.m.r. spectra of both samples confirms that they are homogeneous and that they both contain *N*-acetylgalactosaminitol. Partial ^{1}H-n.m.r. spectra for the oligosaccharides are shown in Fig. 5. The n.m.r. signals were assigned (see Table 1) by using a combination of approaches. Preliminary shift data were obtained through comparison with previously assigned spectra from related molecules, such as the

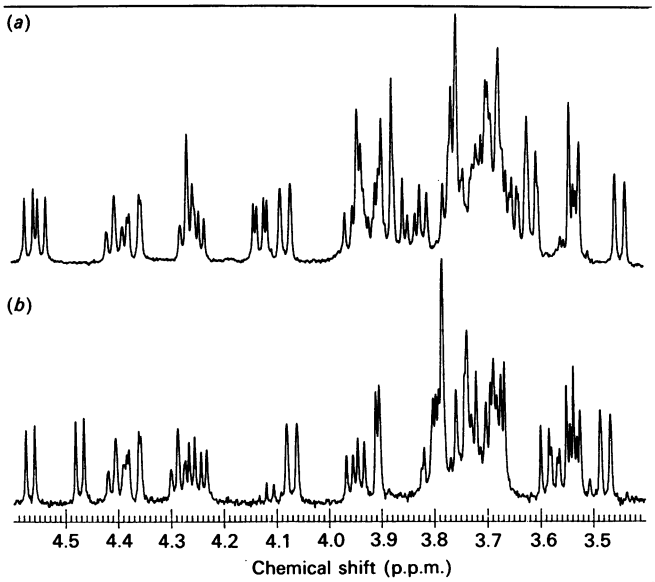


Fig. 5. Partial 500 MHz ^{1}H-n.m.r. spectra of (*a*) oligosaccharide I and (*b*) oligosaccharide II

Table 1. Oligosaccharide ^{1}H-n.m.r. chemical-shift data

^{1}H-n.m.r. chemical shifts of oligosaccharides I and II, together with oligosaccharides OL-2 and OL-3 isolated from hen ovomucin by Strecker *et al.* (1987), are given. The first superscript at the name of a sugar indicates to which position of the adjacent monosaccharide it is glycosidically linked. Key: ◇, *N*-acetylgalactosaminitol; ■, galactose; ●, *N*-acetylglucosamine 6-sulphate; △, *N*-acetylneuraminic acid.

		Chemical shift (p.p.m.)			
					
Residue	Proton	Oligosaccharide I (23 °C)	Oligosaccharide OL-3*	Oligosaccharide II (23 °C)	Oligosaccharide OL-2*
GalNAc-ol	H-1	3.76		~ 3.75	
	H-1′	3.826		~ 3.80	
	H-2	4.403	4.400	4.403	4.401
	H-3	4.080	4.082	4.071	4.074
	H-4	3.445	3.453	3.475	3.487
	H-5	4.266	4.268	4.287	4.287
	H-6	3.94	3.953	3.950	3.959
	H-6′	3.69		3.68	
	Me	2.082	2.082	2.080	2.081
GlcNAc(6SO_3)	H-1	4.566	4.597	4.567	4.599
	H-2	3.74		3.74†	
	H-3	3.52–3.54		3.54–3.55†	
	H-4	3.52–3.54		3.54–3.55†	
	H-5	~ 3.68†		~ 3.69†	
	H-6	4.249	4.428	4.249	4.435
	H-6′	4.365	4.389	4.369	4.365
	Me	2.077	2.080	2.080	2.081
Gal 3	H-1	4.541	4.543	4.472	4.478
	H-2	3.621	3.625	3.581	3.587
	H-3	4.127	4.127	3.68	3.693
	H-4	3.93†	3.941	3.907	3.914
	H-5				
	H-6				
	H-6′				
Gal 4	H-1	–	4.553	–	4.554
	H-2	–	3.544	–	3.571
	H-3	–	3.691	–	3.693
	H-4	–	3.941	–	3.943
NeuAc	$H\text{-}3_{ax.}$	1.812	1.817	–	–
	$H\text{-}3_{eq.}$	2.783	2.790	–	–
	H-4	~ 3.87†		–	–
	H-5	~ 3.68†		–	–
	Me	2.040	2.048	–	–

* Shifts corrected to sodium 3-trimethylsilylpropionate referencing by addition of 0.016 p.p.m.
† These signals were assigned by comparison of COSY-45 and relayed COSY two-dimensional spectra.

O-linked oligosaccharides from human chorionic gonadotropin (Damm *et al.*, 1987), from the oligosaccharides derived from corneal keratan sulphates (Hounsell *et al.*, 1986) and, most notably, from the sulphated tetra- and penta-saccharides derived from hen ovomucin (Strecker *et al.*, 1987). Confirmations were made by approximate measurements of spin–spin coupling constants where the signals were reasonably first-order in character, and by using data from two-dimensional COSY and relayed-COSY spectra. The signals corresponding to the *N*-acetylgalactosaminitol residue were visually similar in appearance to those observed by Korrel *et al.* (1984) for *O*-linked oligosaccharides derived from glycocalicin, but in both these systems and the earlier ones significant distortions of multiplet line spacings could be seen, making definite assignments problematical. Preliminary simulations using reasonable assumed values for shifts and spin couplings afforded calculated spectra similar in form to the observed signals, suggesting that distortions are due to second-order effects and that the assignments are correct.

From the spectra (see Fig. 5) it is clear that oligosaccharide I contains an *N*-acetylneuraminic acid residue that is missing from oligosaccharide II. The linkage type and position of this *N*-acetylneuraminic acid residue is α(2–3), as shown by the peaks at 1.812 and 2.783 p.p.m. (see Table 1), which are recognizable as the $H(3)_{ax.}$ and $H(3)_{eq.}$ signals in such a linkage (Vliegenthart *et al.*, 1983). This *N*-acetylneuraminic acid residue may be assumed to be linked to galactose, as the resonance at 4.12 p.p.m. is characteristic of H(3) of galactose when it is substituted at this position. In oligosaccharide II, which is unsubstituted, the galactose H(3) signal occurs at 3.68 p.p.m. The shift position of H(1) in galactose is also sensitive to the presence of *N*-acetylneuraminic acid, the resonance positions being 4.541 and 4.472 p.p.m. for oligosaccharides I and II respectively. These shift positions compare closely (see Table 1) with those obtained for the oligosaccharides OL-3 and OL-2 isolated from hen ovomucin by Strecker *et al.* (1987).

The *N*-acetylgalactosaminitol residue in both spectra shows clear signals for H(2), H(3), H(4) and H(5), which again compare

well with those reported by Strecker *et al.* (1987) (Table 1). The signals arising from the H(6) methylene protons of *N*-acetylgalactosaminitol in oligosaccharides I and II occur at around 3.945 p.p.m. and 3.685 p.p.m., indicating that the *N*-acetylgalactosaminitol residue is substituted at this position, whereas methylene protons from *N*-acetylgalactosaminitol unsubstituted at position 6 display signals at 3.7 p.p.m. and 3.67 p.p.m. (Korrel *et al.*, 1984).

The presence of *N*-acetylglucosamine is indicated by the H(1) resonance at 4.566 and 4.567 p.p.m. for oligosaccharides I and II respectively. This residue is sulphated, as indicated by the relatively downfield positions of the two H(6) methylene proton resonances, which occur at about 4.25 and 4.37 p.p.m. compared with 4.012 and 3.844 p.p.m. for the unsulphated residue (Strecker *et al.*, 1987).

Finally, in the spectrum of oligosaccharide I there is a group of signals at 2.082, 2.077 and 2.040 p.p.m., which correspond to the acetyl methyl resonances in *N*-acetylgalactosaminitol, *N*-acetylglucosamine and *N*-acetylneuraminic acid respectively. These are present in equal proportions, indicating that the residues are in the proportions 1:1:1.

The presence of the sulphate group in oligosaccharide I was additionally confirmed by electron micro-probe analysis (Goldstein *et al.*, 1981), which showed a strong K_α sulphur peak at 2.307 keV.

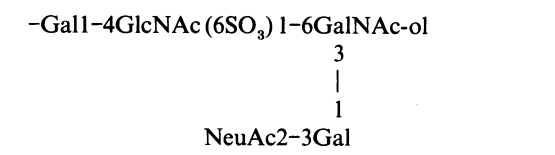
DISCUSSION

Oligosaccharides I and II account for 36% of the incorporated radiolabel. Much of the remaining radiolabel occurs in other fragments obtained from fraction P-4-B for which there is n.m.r. evidence of signals from unsaturated compounds. It seems likely that these are Kuhn chromogens as proposed by Hopwood & Robinson (1974), but apparently complexed with proteinase inhibitors used earlier in the preparation. Currently there are insufficient quantities of these samples for complete structural analysis.

The major finding in this study has been the identification of a 6-sulphate on the linkage-region *N*-acetylglucosamine residue. The sulphation of this residue would seem to be the first biosynthetic event that would distinguish a keratan sulphate from an *O*-linked oligosaccharide. No fragment corresponding to the non-sulphated version of oligosaccharide I has been isolated in amounts sufficient for structural analysis.

The two linkage oligosaccharides I and II are clearly related to one another, although it is uncertain whether the absence of sialic acid from oligosaccharide II results from non-sialylation during biosynthesis, degradation *in vivo* in the tissue or loss during extraction and purification. Analysis of ^{1}H-n.m.r. spectra of reduced keratan sulphate chains prepared in our laboratory reveals that the α(2–3)-linked *N*-acetylneuraminic acid component in this environment is generally present in proportions significantly less than one residue per chain.

The specifity of the keratanase used (only unsubstituted galactose residues are susceptible) enables further structural detail to be deduced, namely that the galactose residue adjacent to the *N*-acetylglucosamine is non-sulphated, The overall structure of this keratan sulphate linkage region is shown below:



This study, together with the elucidation of the 3-*O*-sulphation in the antithrombin-binding site of heparin (Meyer *et al.*, 1981) and the 4-*O*-sulphation of the second galactose residue in the chondroitin sulphate linkage region (Sugahara *et al.*, 1988), demonstrate the potential of high-field n.m.r. spectroscopy in the detailed structural analysis of glycosaminoglycans.

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REFERENCES

Antonsson, P., Heinegård, D. & Oldberg, A. (1989) J. Biol. Chem. **264**, 16170–16173

Bray, B. A., Lieberman, R. & Meyer, K. (1967) J. Biol. Chem **242**, 3373–3380

Carlson, D. M. (1968) J. Biol. Chem. **243**, 616–626

Cockin, G. H., Huckerby, T. N. & Nieduszynski, I. A. (1986) Biochem. J. **236**, 921–924

Damm, J. B. L., Kamerling, J. P., Van Dedem, G. W. K. & Vliegenthart, J. F. G. (1987) Glycoconjugate J. **4**, 129–144

Goldstein, J. I., Newbury, D. E., Echlin, P., Joy, D. C., Fiori, C. & Lifshin, E. (eds.) (1981) Scanning Electron Microscopy and X-Ray Microanalysis, pp. 589–614, Plenum Press, New York

Hardingham, T. E. (1986) Rheumatology **10**, 143–183

Hascall, V. C. & Midura, R. J. (1989) in Keratan Sulphate: Chemistry, Biology, Chemical Pathology (Greiling, H. & Scott, J., eds.), pp. 66–75, The Biochemical Society, London

Hopwood, J. J. & Robinson, H. C. (1973) Biochem. J. **135**, 631–637

Hopwood, J. J. & Robinson, H. C. (1974) Biochem. J. **141**, 57–69

Hounsell, E. F., Feeney, J., Scudder, P. & Feizi, T. (1986) Eur. J. Biochem. **157**, 375–384

Korrel, S. A. M., Clemetson, K. J., Van Halbeek, H., Kamerling, J. P., Sixma, J. J. & Vliegenthart, J. F. G. (1984) Eur. J. Biochem. **140**, 571–576

Lohmander, L. S., De Luca, S., Nilsson, B., Hascall, V. C., Caputo, C. B., Kimura, J. H. & Heinegård, D. (1980) J. Biol. Chem. **255**, 6084–6091

Meyer, B., Thunberg, L., Lindahl, U., Larm, O. & Leder, I. G. (1981) Carbohydr. Res. **88**, C1–C4

Meyer, K. (1970) in Chemistry and Molecular Biology of the Intercellular Matrix (Balazs, E. A., ed.), vol. 1, p. 15, Academic Press, New York

Nakazawa, K., Makoto, I., Yamagata, T. & Suzuki, S. (1989) in Keratan Sulphate: Chemistry, Biology, Chemical Pathology (Greiling, H. & Scott, J., eds.), pp. 99–110, The Biochemical Society, London

Sanderson, P. N., Huckerby, T. N. & Nieduszynski, I. A. (1987) Biochem. J. **243**, 175–181

Strecker, G., Wieruszeski, J.-M., Martel, C. & Montreuil, J. (1987) Glycoconjugate J. **4**, 329–337

Stuhlsatz, H. W., Keller, R., Becker, G., Oeben, M., Lennartz, L., Fischer, D. C. & Greiling, H. (1989) in Keratan Sulphate: Chemistry, Biology, Chemical Pathology (Greiling, H. & Scott, J., eds.), pp. 1–11, The Biochemical Society, London

Sugahara, K., Yamashina, I., De Waard, P., Van Halbeek, H. & Vliegenthart, J. F. G. (1988) J. Biol. Chem. **263**, 10168–10174

Thornton, D. J., Morris, H.G., Cockin, G. H., Huckerby, T. N., Nieduszynski, I. A., Carlstedt, I., Hardingham, T. E. & Ratcliffe, A. (1989*a*) Biochem. J. **260**, 277–282

Thornton, D. J., Morris, H. G., Cockin, G. H., Huckerby, T. N. & Nieduszynski, I. A. (1989*b*) Glycoconjugate J. **6**, 209–218

Vliegenthart, J. F. G., Dorland, L. & Van Halbeek, H. (1983) Adv. Carbohydr. Chem. Biochem. **41**, 209–374

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