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Structural Studies on Sulfated Oligosaccharides Derived from the Carbohydrate-Protein Linkage Region of Chondroitin 6-Sulfate Proteoglycans of Shark Cartilage

I. SIX COMPOUNDS CONTAINING 0 OR 1 SULFATE AND/OR PHOSPHATE RESIDUE*

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Shark cartilage proteoglycans bear predominantly chondroitin 6-sulfate. After exhaustive protease digestion, reductive β-elimination, and subsequent chondroitinase ABC digestion, 13 hexasaccharide alditols, which are nonsulfated, sulfated, and/or phosphorylated, were obtained from the carbohydrate-protein linkage region. Six compounds, containing 0 or 1 sulfate and/or phosphate residue, represent approximately 40% of the isolated linkage hexasaccharide alditols. They were analyzed by chondroitinase ACII or alkaline phosphatase digestion in conjunction with high performance liquid chromatography, and by 500 MHz one- and two-dimensional ^{1}H NMR spectroscopy. All six compounds have the conventional structure in common.

$\Delta_{4,5}$-GlcAβ1–3GalNAcβ1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl-ol

One compound has no sulfate nor phosphate. Two of the monosulfated compounds have an *O*-sulfate on C-6 or on C-4 of the GalNAc residue. The third monosulfated compound has a novel *O*-sulfate on C-6 of the Gal residue attached to xylitol. The two phosphorylated compounds have *O*-phosphate on C-2 of Xyl-ol, and one of them has in addition sulfate on C-6 of GalNAc.

The studies by Lindahl and co-workers on various proteoglycans (1) revealed a common carbohydrate-protein linkage structure, GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser.[1] Since then, the basic principles of the biosynthesis as to chain initiation and elongation have been established (2): monosaccharide residues are transferred stepwise from the corresponding nucleotide sugars to growing carbohydrate chains. This process starts with the transfer of Xyl to Ser residues of a protein core and is governed largely by the substrate specificities of the glycosyltransferases involved. Recently, Esko *et al.* (3) demonstrated that Chinese hamster ovary cell mutants deficient in xylosyl- or galactosyltransferase do not produce chondroitin sulfate nor heparan sulfate, emphasizing the importance of the common linkage region.

The question arises how different glycosaminoglycans are synthesized on the common structure. Recent evidence obtained by analysis of the gene and the mRNA for the mast cell proteoglycan serglycin, which can bear either chondroitin sulfate and/or heparin, clearly indicates that the attachment of these chains is not encoded by the amino acid sequence of the core protein (4, 5). The concept of a multienzyme complex has been introduced for the synthesis of the linkage region of chondroitin sulfate chains (6, 7) and for the modification reactions of heparin glycosaminoglycan chains (8). It is unkown, however, if this concept is applicable to the sorting of different glycosaminoglycan chains.

Our studies, focused on the enigmatic sorting mechanisms in the biosynthesis of different glycosaminoglycan chains, started from the working hypothesis that differences in the structures of the linkage regions of the various glycosaminoglycan chains may exist, which determine the character of the glycosaminoglycan species to be synthesized (9). Careful enzymatic preparation of the linkage glycopeptides under mild conditions led to the identification by ^{1}H NMR spectroscopy of the novel structure, $\Delta_{4,5}$-GlcAβ1–3GalNAc(4-*O*-sulfate)β1–4GlcAβ1–3Gal(4-*O*-sulfate)β1–3Galβ1–4Xylβ1-*O*-Ser, in the carbohydrate-protein linkage region of chondroitin 4-sulfate

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[1] The abbreviations used are: GlcA, glucuronic acid; $\Delta_{4,5}$-GlcA, 4,5-unsaturated glucuronic acid; GalNAc, 2-deoxy-2-*N*-acetylamino-D-galactose; ΔDi-0S, $\Delta_{4,5}$-GlcA(β1–3) GalNAc; ΔDi-6S, $\Delta_{4,5}$-GlcA(β1–3)GalNAc (6-*O*-sulfate); ΔDi-4S, $\Delta_{4,5}$GlcA(β1–3)GalNAc(4-*O*-sulfate); ΔDi-diS$_D$, $\Delta_{4,5}$-GlcA(2-*O*-sulfate)(β1–3)GalNAc(6-*O*-sulfate); ΔDi-diS$_E$, $\Delta_{4,5}$-GlcA(β1–3)GalNAc(4,6-*O*-disulfate); ΔDi-triS, $\Delta_{4,5}$-GlcA(2-*O*-sulfate)(β1–3)GalNAc(4,6-*O*-disulfate); HOHAHA, homonuclear Hartmann-Hahn; ROESY, rotating frame Overhauser enhancement spectroscopy; TQF-COSY, triple quantum filtered correlation spectroscopy; SED spectroscopy, spin-echo difference spectroscopy; RESED, relayed spin-echo difference spectroscopy; 2D, two-dimensional.

proteoglycans from Swarm rat chondrosarcoma (9). It was suggested that this sulfation could be a sorting signal for the biosynthesis of chondroitin 4-sulfate or sulfated galactosaminoglycans (9). Recently, we discovered the presence of this structure also in whale cartilage chondroitin 4-sulfate (10). Thus, the 4-*O*-sulfated Gal residue is present in chondroitin 4-sulfate both from tumorous as well as from normal cartilage. The linkage regions of heparan sulfate (11) and probably heparin (12) contain xylose-2-*O*-phosphate, but it is unknown if they also bear Gal-4-*O*-sulfate. In our recent studies we could not demonstrate the presence of sulfate in the linkage region, Galβ1–3Galβ1–4Xylβ1-*O*-Ser, of porcine intestinal heparin (13).

In the present investigation we determined the structure of 13 hexasaccharides from the carbohydrate-protein linkage region of shark cartilage chondroitin sulfate, which contains predominantly 6-*O*-sulfated GalNAc residues (14). In the first paper of this series the nonsulfated compound and the monosulfated compounds, together with two compounds containing phosphate are reported. The other seven hexasaccharides will be reported in the second article of this series (15).

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources: Actinase E, previously supplied as Pronase P, from Kaken Pharmaceutical Co., Tokyo; chondroitinase ACII (EC 3.1.6.9), the conventional and protease-free preparations of chondroitinase ABC (EC 4.2.2.4) from Seikagaku Kogyo Co., Tokyo; special quality of calf intestine alkaline phosphatase (EC 3.1.3.1) for molecular biology from Boehringer Mannheim; $NaBH_4$ from Wako Pure Chemical Industry, Osaka; a 0.93 mM, 0.1 M NaOH solution of $NaB[^3H]_4$ (21.5 Ci/mmol) from Amersham Corp.

Preparation of Peptidoglycans—Shark scapular cartilage (400 g) was kept in boiling water for 30 min, minced, and digested with 2 g of Actinase E in the presence of a small amount of toluene in 600 ml of 0.1 M sodium borate, pH 8.0, containing 10 mM calcium acetate. After 24 h of incubation at 54 °C, again 2 g of enzyme was added, and the incubation was continued for another 24 h. During the incubation the mixture was adjusted to pH 8.0 with 1 M NaOH intermittently. The digest was cooled to room temperature and filtered through Celite. To the filtrate sodium acetate and then ethanol were added to give final concentrations of 3 and 54% (w/w), respectively. The precipitate was collected by centrifugation, dissolved in 3% (w/v) sodium acetate solution, pH 6.0, and precipitated with ethanol (final 45%, w/v). The sample was redigested with Actinase E, and the precipitate was recovered by ethanol precipitation (final 45%, w/v), then dissolved in 1% sodium acetate solution, pH 6.0, and mixed with 6 g of activated charcoal at 50 °C for 1 h. The mixture was filtered through Celite. Peptidoglycans were precipitated with ethanol (final 85%) and dried. The yield was 19.2 g. The contents of protein and GlcA were 0.61 and 32.4% (w/w), respectively. Based on the amino acid/amino sugar analysis the molar ratios of the major amino acids and amino sugars were as follows. Ser/Glu/Pro/Gly/Val/Lys/$GalNH_2$/$GlcNH_2$ = 1.00:1.16:1.41:1.23:0.46:0.29:46:0.25. The amounts of other amino acids were negligible.

Reduction of the Peptidoglycans—The chondroitin sulfate peptidoglycan fraction (2 mg) was treated with 20.8 mM $NaB[^3H]_4$ (500 mCi/mmol), 0.46 M NaOH in a total volume of 85 μl overnight at room temperature. Acetone (1 ml) was added to the incubation mixture to decompose excess $NaB[^3H]_4$. Following repeated evaporations with water, the residue was chromatographed on a column (1 × 52 cm) of Sephadex G-25 (fine) with 0.05 M pyridine-acetate, pH 5.0. The flow-through fractions containing ^3H-labeled polysaccharides were pooled, concentrated to dryness by evaporation, and reconstituted in water. A total radioactivity of 5.7×10^6 dpm was incorporated into this fraction.

For preparation of nonlabeled reduced glycans, the peptidoglycan fraction (4.0 g dry weight containing 79 μmol of Ser) was treated with 1 M $NaBH_4$, 0.05 M NaOH in a total volume of 80 ml for 27 h at room temperature. The sample was acidified with acetic acid, neutralized with 1 M NaOH, dialyzed against water, and lyophilized. The yield of the polysaccharides was 3.73 g. Only 9.3% of Ser remained in the preparation, indicating efficient release of glycans from the peptides. The release (91%) of glycan chains by the β-elimination reaction after exhaustive protease digestion is effective yet incomplete. This is probably due to the fact that β-elimination requires that both amino and carboxyl groups of glycosylated Ser residues are substituted (36), which is not the case for all glycopeptides in the mixture.

Chondroitinase ABC Treatments—The reduced nonlabeled (2.0 g) and ^3H-labeled (4.5×10^6 dpm) polysaccharide preparations were mixed and digested with 1.5 units of chondroitinase ABC (protease-free) in a total volume of 28 ml of 0.05 M Tris-HCl buffer, pH 8.0, containing 50 mM sodium acetate and 100 μg/ml of bovine serum albumin as an enzyme stabilizer for 29 h at 37 °C; additional 0.25 and 0.125 unit of the enzyme were added after 19 and 26 h, respectively. The enzymatic reaction reached a plateau after approximately 24 h of incubation as monitored by UV absorbance at 232 nm. Then, the mixture was adjusted to pH 6.5 with 1 M HCl and boiled for 3 min. The digest was lyophilized, dissolved in 7 ml of 1 M NaCl, and subjected to gel filtration on a column of Sephadex G-15.

Chondroitinase ABC (a conventional preparation) digestion of fraction I (177 μmol as $\Delta_{4,5}$-GlcA) (Fig. 1, *lower panel*) was carried out with 1.5 units of the enzyme overnight in a total volume of 2 ml, otherwise as described above.

Chondroitinase ACII Treatments—The isolated linkage oligosaccharides (4 nmol) were incubated with 14 munits of the enzyme in a total volume of 40 μl of 0.03 M sodium acetate buffer, pH 6.0, at 37 °C for 10 min. The incubation mixtures were treated at 100 °C for 1 min, cooled to room temperature, mixed with 960 μl of 16 mM NaH_2PO_4 (pH 4.50); 200-μl aliquots were analyzed by HPLC.

Alkaline Phosphatase Treatment—The isolated linkage oligosaccharides (0.5 nmol) were incubated with 3 units of the enzyme in a total volume of 60 μl of 0.07 M glycine-NaOH buffer, pH 9.9, containing 0.5 mM $MgCl_2$ at 37 °C for 10 min. The incubation mixtures were mixed with 140 μl of 23 mM NaH_2PO_4 and analyzed by HPLC.

HPLC—Fractionation of the linkage oligosaccharide alditols and analysis of the chondroitinase ACII digests of the isolated oligosaccharides were carried out by HPLC as previously reported for the separation of the chondro-disaccharides (16, 17). The lower limit for the detection of unsaturated oligosaccharides on HPLC was 0.1–0.2 nmol. Chromatography was performed on a 4.6 × 250-mm polyamine-bound silica PAO3 column (YMC Co., Kyoto) in an LC6A HPLC system (Shimadzu Corp., Kyoto) using a linear gradient from 16 to 530 mM NaH_2PO_4 over a 60-min period at a flow rate of 1.0 ml/min at room temperature. Samples dissolved in 16 mM NaH_2PO_4 were treated with a C3HV membrane filter (Millipore) and injected. Eluates were monitored by UV absorbance at 232 nm.

For preparative purposes up to 3.2 μmol (as $\Delta_{4,5}$-GlcA) of oligosaccharide alditols were applied to the analytical column by a single injection. Separated fractions were concentrated in a vacuum concentrator (Savant Instruments, Inc., Farmingdale, New York) and desalted through a column (0.8 × 56 cm) of Sephadex G-25 (fine) with glass-distilled water as effluent. Oligosaccharide fractions detected by UV absorbance at 232 nm were pooled, concentrated to dryness, and rechromatographed as above. This process was repeated several times until the preparations became homogeneous as judged by HPLC.

500-MHz ^1H NMR Spectroscopy—Linkage oligosaccharides were repeatedly exchanged in ^2H_2O (99.96% ^2H, Aldrich Chemical Co.) with intermediate lyophilization. 500-MHz ^1H NMR spectra were recorded on a Bruker AM-500 spectrometer (Department of NMR spectroscopy, Utrecht University, The Netherlands) at a probe temperature of 15 °C to prevent disturbance by the HOD resonance. Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly to acetone in ^2H_2O (δ 2.225) (18). Resolution enhancement of the one-dimensional spectra was achieved by Lorentzian-to-Gaussian transformation (19). For the 2D spectra 480 (fraction 4), 512 (fraction 6), or 360 (fraction 8–1) experiments of 2048 data points were recorded. The HOD signal was suppressed by presaturation during 1 s, the 90° ^1H pulse width was 30 μs. 2D HOHAHA spectra (20) were recorded with 32 scans (fractions 4 and 6) or 16 scans (fraction 8–1)/t_1 value, using a Malcom Levitt-17 mixing sequence of 120 ms for isotropic mixing. A 2D ROESY spectrum of fraction 8–1 (21) was recorded with 64 scans/t_1 value; the spinlock mixing pulse with a duration of 200 ms was given as a series of 32° pulses (10 μs) with in-between delays (30 μs) to attenuate the power of the amplifier (22). A triple quantum filtered COSY (TQF-COSY) spectrum (23) was recorded with 40 scans/t_1 value. The time domain data were multiplied with a phase shifted sine-bell; phase-sensitive Fourier transformation was performed after zero filling to a 2048 × 1048 data matrix size. The RESED (24, 25) and the SED (26) spectra of fraction 8–1 were recorded at 27 °C. For the RESED spectrum, a Malcom Levitt-17 mixing sequence of 100

ms was supplied through the decoupling channel.

Other Analytical Methods—Chondroitinase-produced oligosaccharides were quantified based on the UV absorbance (E_{232} = 5500 M^{-1} cm^{-1}) (27) caused by the Δ4,5 sites of the uronic acid at the nonreducing ends. Uronic acid was determined by the carbazole method (28) using GlcA as standard. Amino sugars and amino acids were analyzed with an amino acid analyzer after hydrolysis in 6 M HCl at 100 °C for 3 h and at 110 °C for 20 h, respectively (29). Protein was determined according to Lowry *et al.* (30). Analysis of sulfate and phosphate was performed after pyrolysis by ion chromatography (31) using an ion chromatography HIC-6A system (Shimadzu, Kyoto) equipped with an anion-exchange Shim-pack IC-A1 column (4.6 × 100 mm) using 7.5 mM Tris, 6 mM boric acid buffer, pH 8.0, containing 18 mM mannitol and 5 mM tri-*n*-butylphosphine oxide (Nacalai Tesqui Inc., Kyoto). Negative ions were detected conductometrically. The radioactivity was determined by liquid scintillation counting with a Beckman LS-7500 scintillation counter.

RESULTS

Isolation of the Linkage Oligosaccharides—To investigate the structure of the carbohydrate-protein linkage region of chondroitin 6-sulfate proteoglycans, a peptidoglycan fraction was prepared from shark cartilage by exhaustive Actinase E digestion. Disaccharide analysis of the chondroitinase ABC digest of the peptidoglycan fraction by HPLC shows the ΔDi-6S component to be prevailing (Table I). Isolation of glycoserines as previously achieved for the linkage regions of chondroitin 4-sulfate proteoglycans from Swarm rat chondrosarcoma (9) was unsuccessful because of the difficulty to get complete protease digestion of the peptide moieties. This is probably due to the occurrence of clusters of glycans attached to the peptide backbone. Therefore, the peptidoglycan fraction was subjected to a reductive β-elimination. A small portion was treated separately with alkaline $NaB[^3H]_4$ to radiolabel the reducing ends of the polysaccharides. The nonradiolabeled (2 g, 3.34 mmol as GlcA) and ^3H-labeled fractions (4.54×10^6 dpm, 1.6 mg) were mixed and digested exhaustively by a protease-free preparation of chondroitinase ABC. Based on the UV absorbance, approximately 2.45 mmol of $\Delta_{4,5}$-GlcA was found, which demonstrates a 73% digestion. The reduced fractions correspond to 36.8 μmol of Ser.

The chondroitinase ABC digest was fractionated by gel filtration on a Sephadex G-15 column using 1 M NaCl as effluent. The chromatogram shows two major UV absorbing peaks, tetra- and disaccharide fractions, respectively, in a molar ratio of 1:1.8, and a radiolabeled peak eluted slightly ahead of the tetrasaccharide fraction (Fig. 1, *upper panel*). The radioactive fractions, presumed to contain the linkage oligosaccharides and the majority of the tetrasaccharides, were pooled, concentrated, and desalted by gel filtration. A portion (28.6%) of the incubated mixture corresponding to 533 μmol of $\Delta_{4,5}$-GlcA and 1.30×10^6 dpm of ^3H was rechromatographed on the same column with 0.25 M NH_4HCO_3, 7% 1-propanol as effluent. With this solvent the radioactive peak was separated into a major peak and a shoulder, and the former was now better separated from the UV-absorbing fraction containing tetrasaccharides (Fig. 1, *lower panel*). Peaks I and II containing 81 and 19% of the total radioactivity, respectively, were separately pooled, concentrated, and desalted by repeated evaporations with water. The major fraction (I) was collected as above and used for isolation of the linkage oligosaccharides. Fraction II was not investigated in the present study. Fraction I was incubated with a conventional preparation of chondroitinase ABC (see "Experimental Procedures"). The UV absorbance was doubled (1.99-fold), indicating the completion of the digestion of the above tetrasaccharides composed of two repeating disaccharide units. Upon gel filtration on Sephadex G-15, the digest gave two oligosaccharide fractions (data not shown), one of them radiolabeled, the other representing the newly produced disaccharides. The radiolabeled fraction containing 85% (8.84×10^5 dpm) of the applied radioactivity and 22.7 μmol of oligosaccharides (as $\Delta_{4,5}$-GlcA) was recovered and fractionated by HPLC (Fig. 2). Among the obtained fractions, the fractions 4, 6, 7, 8, 10, 11, 13, 14, 15, and 16 are labeled with ^3H (Table II), indicating that they probably contain oligosaccharide alditols derived from the linkage region. In the present study the fractions 4, 6, and 7 were further purified by repeated preparative chromatography, yielding 156, 63, and 99 nmol (as $\Delta_{4,5}$-GlcA), respectively, per 100 mg of the peptidoglycans containing 1.84 μmol of Ser. Fraction 8 was well separated into two subfractions, 8–1 and a slower-eluting fraction 8–2 when a new PA03 column or a slightly shallow gradient was used for HPLC. They correspond to 80 and 25 nmol (as $\Delta_{4,5}$-GlcA), respectively, per 100 mg of peptidoglycan. Fraction 13

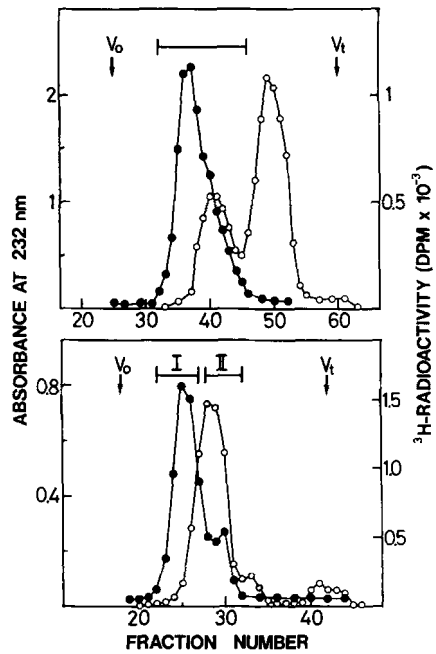


FIG. 1. **Gel filtration of the chondroitinase ABC (protease-free) digest.** The chondroitinase ABC digestion was carried out as described under "Experimental Procedures." The digest was chromatographed on a column (2.15 × 147 cm) of Sephadex G-15 with 1 M NaCl as eluent (*upper panel*). The fraction size was 7 ml, and 50-μl aliquots were used for determination of the radioactivity (●). Another aliquot was diluted 100-fold to measure the UV absorbance (○). The fractions indicated by the *bar* were pooled and rechromatographed on the same column, but with 0.25 M NH_4HCO_3, 7% 1-propanol as eluent (*lower panel*). The fraction size was 10 ml. The radioactivity and the UV absorbance were determined as above.

TABLE I

Disaccharide composition of peptidoglycans

Disaccharide composition was determined by chondroitinase ABC digestion followed by HPLC analysis as previously reported (17). The values indicate a percentage of the total.

Component	Chondroitin sulfate peptidoglycans
	%
ΔDi-0S	1.9
ΔDi-6S	75.8
ΔDi-4S	14.6
$\Delta Di\text{-}diS_D$	7.2
$\Delta Di\text{-}diS_E$	0.5
ΔDi-triS	ND[a]

[a] ND, not detected.

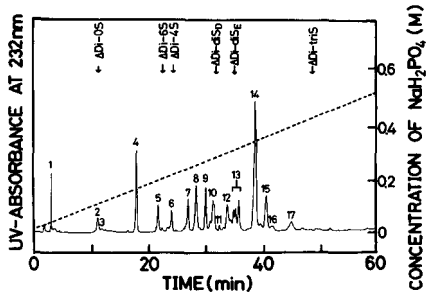


FIG. 2. **HPLC of the oligosaccharide alditol fraction prepared from the carbohydrate-protein linkage region of shark cartilage chondroitin sulfate.** The oligosaccharide alditol fraction corresponding to 12 nmol of $\Delta_{4,5}$-GlcA was chromatographed on a polyamine column. The elution positions of authentic unsaturated chondro-disaccharides are indicated.

TABLE II

Oligosaccharides isolated from the carbohydrate-protein linkage fraction of chondroitin 6-sulfate

Fraction no.	Recoveries	
	A_{232}	^3H
	%	%
1	1.9	ND[a]
2	1.6	ND
3	2.3	ND
4	7.0	12.6
5	2.9	ND
6	2.2	3.7
7	4.2	6.2
8	6.5	3.8
9	4.2	0.6
10	7.2	7.7
11	1.6	1.2
12	2.9	1.0
13	5.5	1.8
14	18.9	25.8
15	5.5	8.1
16	1.4	1.8
17	3.2	0.7
Total	79.0	75.1

[a] ND, not detected.

was separated into four subfractions as can be seen in Fig. 2, three minor poorly radiolabeled fractions (13–1, 13–2, and 13–3), and a major labeled fraction 13–4. Several rechromatographic steps yielded a purified fraction 13–4 corresponding to 40 nmol (as $\Delta_{4,5}$-GlcA) per 100 mg of peptidoglycan.

Characterization of the Oligosaccharides—As shown in Table III, each fraction contains per mol of oligosaccharide approximately 1 mol of $GalNH_2$ and 2 mol of uronic acid, one of which is $\Delta_{4,5}$-GlcA. Fraction 4 contains no sulfate or phosphate, whereas fractions 6, 7, and 8–2 contain 1 mol of sulfate, per mol of oligosaccharide, but no phosphate. Fraction 8–1 contains 1 mol of phosphate, while fraction 13–4 contains one mol each of sulfate and phosphate. Small amounts of phosphate observed for fractions 4 and 7 were probably derived from NaH_2PO_4 used for HPLC.

Aliquots of fractions 4, 6, 7, 8–1, 8–2, and 13–4 were subjected to chondroitinase ACII digestion followed by HPLC analysis. They were each degraded into equimolar amounts of an unsaturated disaccharide and a presumed unsaturated tetrasaccharide (Fig. 3). The former gave a slightly broad peak and the latter a sharp peak, because the former is composed of α- and β-anomers while the latter is an alditol. For ΔDi-0S (*i.e.* nonsulfated) and ΔDi-6S, the α- and β-anomers gave rise to double peaks. From these results shown in Fig. 3, it can be

TABLE III

Chemical composition of linkage oligosaccharides from shark cartilage chondroitin sulfate

The values are expressed by molar ratio to the $GalNH_2$ values which were determined using an amino acid analyzer and have been corrected for degradation (22.7%) during acid hydrolysis. $\Delta_{4,5}$-GlcA and GlcA were determined by UV absorption and the carbazole reaction, respectively. Sulfate was quantitated by ion chromatography.

Fraction	$GalNH_2$	$\Delta_{4,5}$-GlcA	GlcA[a]	Sulfate	Phosphate
Fraction 4	0.84	0.79	*2.00*	0.08	0.24
Fraction 6	0.82	0.75	*2.00*	0.85	ND[b]
Fraction 7	1.21	0.84	*2.00*	0.75	0.08
Fraction 8-1	1.20	1.00	*2.00*	0.10	1.17
Fraction 8-2	0.88	1.01	*2.00*	1.17	ND
Fraction 13-4	0.92	0.92	*2.00*	1.31	1.19

[a] Including $\Delta_{4,5}$-GlcA.
[b] ND, not detected.

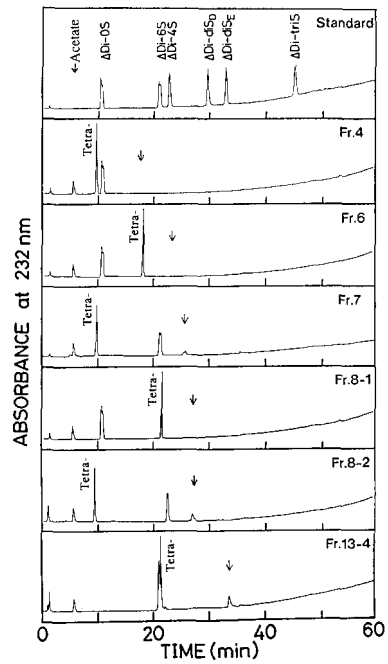


FIG. 3. **HPLC of the chondroitinase ACII digests of the isolated hexasaccharide alditols.** *Top column*, the authentic chondro-disaccharides; *lower columns*, the chondroitinase ACII digests of fractions 4, 6, 7, 8–1, 8–2, and 13–4. The *arrows* indicate the elution positions of the corresponding intact hexasaccharide alditols.

deduced that in fraction 4 $\Delta_{4,5}$-GlcA-GalNAc-GlcA-Gal-Gal-xylitol is present. Fractions 7 and 8–2 were degraded into a nonsulfated tetrasaccharide and ΔDi-6S or ΔDi-4S, indicating sulfate on C-6 or C-4 of GalNAc, respectively. Fractions 8–1 and 13–4 gave rise to a presumed common tetrasaccharide core and ΔDi-0S or ΔDi-6S, respectively. The tetrasaccharide is assumed to be phosphorylated because both parent hexasaccharides contain 1 mol of phosphate (Table III) and were converted into fraction 4 or 7, respectively, by treatment with alkaline phosphatase, as judged by HPLC (data not shown). The common tetrasaccharide cochromatographed on HPLC (data not shown) with authentic $\Delta_{4,5}$-GlcAβ1–3Galβ1–3Galβ1–4xylitol(2-*O*-phosphate), which was isolated from chondroitin sulfate produced by Engelbreth-Holm-Swarm mouse tumor and characterized by periodate oxidation (32), indicating the phosphate group on C-2 of xylitol. Fraction 6 was degraded into ΔDi-0S and an unsaturated tetrasaccharide which has a longer retention time than that derived from fractions 4, 7, and 8–2, but a shorter retention time than that

of $\Delta_{4,5}$-GlcAβ1–3Gal(4-*O*-sulfate)β1–3Galβ1–4xylitol obtained from whale cartilage chondroitin 4-sulfate proteoglycans (10). Therefore, fraction 6 presumably has sulfate on yet another position in its tetrasaccharide core.

500-MHz ^1H NMR Spectroscopy—The ^{1}H NMR spectrum of fraction 4 is presented in Fig. 4*A*, and the ^{1}H NMR data are summarized in Table IV. Comparison of the spectral data with those of the conventional core structure $\Delta_{4,5}$-GlcA-GalNAc-GlcA-Gal-Gal-Xyl-Ser (fraction D-1 in Ref. 9) reveals that fraction 4 contains a similar structure, except for the reduced Xyl residue. Compared to the ^{1}H NMR spectrum of the non-reduced reference compound the Gal-2 H-1 has a different chemical shift (δ 4.617). The Xyl H-1 is absent in the anomeric region and the Xyl-ol H-4 is found at δ 3.988. The differences between the reduced and non-reduced compounds are in accordance with earlier ^{1}H NMR spectroscopic studies on fragments of the proteoglycan linkage region (33). Assignments of resonances hidden in the bulk region between 3 and 4 ppm were made on guidance of a 2D HOHAHA spectrum (data not shown). The structure of the compound

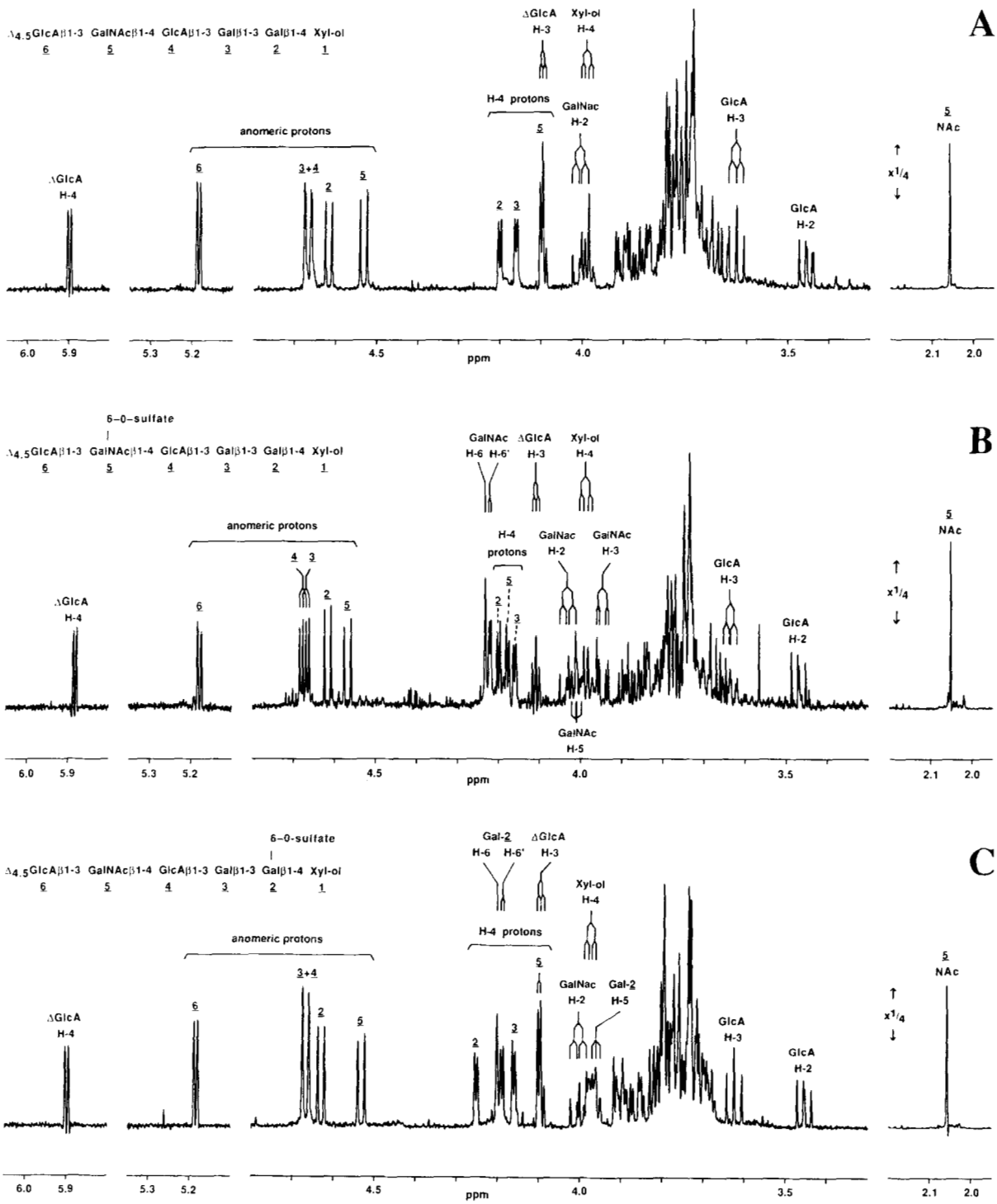


FIG. 4. **Structural-reporter-group regions of the resolution-enhanced 500-MHz ^{1}H NMR spectra of fraction 4 (*A*), 7 (*B*), and 6 (*C*) recorded in ^2H_2O at 15 °C.** The *numbers* and *letters* in the spectra refer to the corresponding residues in the structures.

TABLE IV

^1H chemical shifts of structural-reporter groups of the constituent monosaccharides of linkage oligosaccharides together with those for a reference compound R (fraction D-3 in 9)

Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly to acetone in ^2H_2O (δ 2.225) at 15 °C (reference compound at 22 °C).

Residue	Reporter group	Chemical shift in						
		R	4	7	6	8-2	8-1	13-4
Xyl-ol	H-1		ND[a]	ND	ND	ND	3.785	ND
	H-1′		ND	ND	ND	ND	3.872	ND
	H-2		ND	ND	ND	ND	4.267	4.279
	H-3		ND	ND	ND	ND	3.89	ND
	H-4		3.988	3.987	3.974	3.989	4.034	4.034
	H-5		ND	ND	ND	ND	3.89	ND
	H-5′		ND	ND	ND	ND	3.89	ND
Gal-*2*	H-1	4.530	4.617	4.617	4.629	4.618	4.628	4.625
	H-2	3.675	3.720	3.720	3.719	ND	3.736	ND
	H-3	3.826	3.842	3.843	3.863	ND	3.849	ND
	H-4	4.188	4.200	4.200	4.252	4.202	4.186	4.189
	H-5	ND	ND	ND	3.960	ND	ND	ND
	H-6	ND	ND	ND	4.202	ND	ND	ND
	H-6′	ND	ND	ND	4.189	ND	ND	ND
Gal-*3*	H-1	4.664	4.668	4.669	4.667	4.672	4.666	4.666
	H-2	3.744	3.745	3.745	3.745	ND	3.744	ND
	H-3	3.800	3.800	3.801	3.802	ND	3.806	ND
	H-4	4.160	4.160	4.160	4.160	4.167	4.161	4.161
GlcA-*4*	H-1	4.672	4.668	4.677	4.667	4.672	4.666	4.673
	H-2	3.454	3.455	3.470	3.454	3.456	3.455	3.469
	H-3	3.635	3.624	3.636	3.624	3.636	3.625	3.635
	H-4	ND	3.778	3.74	3.778	ND	3.781	ND
	H-5	ND	3.719	3.74	3.719	ND	3.722	ND
GalNAc-*5*	H-1	4.615	4.531	4.568	4.532	4.610	4.532	4.568
	H-2	4.071	4.003	4.032	4.003	4.074	4.003	4.030
	H-3	4.149	3.901	3.947	3.902	4.152	3.905	3.946
	H-4	4.621	4.098	4.178	4.099	4.623	4.098	4.178
	H-5	ND	ND	4.010	ND	ND	ND	4.009
	H-6	ND	ND	4.232	ND	ND	ND	4.230
	H-6′	ND	ND	4.220	ND	ND	ND	4.220
	NAc	2.095	2.057	2.052	2.058	2.097	2.059	2.053
ΔGlcA-*6*	H-1	5.265	5.184	5.179	5.184	5.269	5.184	5.178
	H-2	3.833	3.793	3.780	3.795	ND	3.798	ND
	H-3	3.943	4.094	4.109	4.095	3.945	4.095	4.108
	H-4	5.966	5.899	5.881	5.898	5.971	5.899	5.881

[a] ND, not determined.

present in fraction 4 is as follows.

$$\Delta_{4,5}\text{-GlcA}\beta 1\text{-}3\text{GalNAc}\beta 1\text{-}4\text{GlcA}\beta 1\text{-}3\text{Gal}\beta 1\text{-}3\text{Gal}\beta 1\text{-}4\text{Xyl-ol}$$
$$\underline{6} \quad \underline{5} \quad \underline{4} \quad \underline{3} \quad \underline{2} \quad \underline{1}$$

The ^1H NMR spectrum of fraction 7 is given in Fig. 4*B*, and the corresponding NMR data are presented in Table IV. In comparison to the spectrum of fraction 4 signals are observed at δ 4.23 which have shifted out of the bulk region under influence of a sulfate group (34). In the 2D HOHAHA spectrum (Fig. 5), cross-peaks are observable between the signals at δ 4.23 and δ 4.010. By means of a TQF-COSY spectrum (data not shown), which reveals cross-peaks of protons involved in three or more couplings, the signals at δ 4.23 could be assigned to the H-6 and H-6′ atoms and the signal at δ 4.010 to the H-5 atom of a residue sulfated at C-6. The unusual multiplet structure at δ 4.23 is due to strong coupling effects between the overlapping H-6 signals, as was proven by simulating this part of the spectrum. Comparison with the data of fraction 4 shows that the H-1 signals of GlcA-*4*, GalNAc-*5*, and $\Delta_{4,5}$-GlcA-*6* have shifted. Therefore, it is most likely that GalNAc-*5* is 6-*O*-sulfated. Owing to the small coupling between H-4 and H-5 of GalNAc-5 no cross-peak between these signals is observed in the 2D HOHAHA spectrum (Fig. 5). However, the H-5 signal can be assigned on the basis of a 2D ROESY spectrum, since the closeness in space of the H-4 and H-5 atoms affords a cross-peak in this spectrum (Fig. 5). The deduced pathway from H-1 to H-6/H-6′ proves that GalNAc is 6-*O*-sulfated. The structure of the compound in fraction 7 is as follows.

$$\begin{array}{c} \text{6-}O\text{-Sulfate} \\ | \\ \Delta_{4,5}\text{-GlcA}\beta 1\text{-}3\text{GalNAc}\beta 1\text{-}4\text{GlcA}\beta 1\text{-}3\text{Gal}\beta 1\text{-}3\text{Gal}\beta 1\text{-}4\text{Xyl-ol} \\ \underline{6} \quad \underline{5} \quad \underline{4} \quad \underline{3} \quad \underline{2} \quad \underline{1} \end{array}$$

The ^1H NMR spectrum of fraction 6 is given in Fig. 4*C* and the corresponding ^1H NMR data are summarized in Table IV. In this spectrum a similar strong coupling pattern is observed at δ 4.20 as in the spectrum of fraction 7, probably belonging to H-6 signals of a 6-*O*-sulfated residue. Since only the H-1, H-3, and H-4 of Gal-*2* together with the Xyl-ol H-4 have shifted compared to the spectrum of fraction 4, it is reasonable to suggest that Gal-*2* is sulfated. In the TQF-COSY spectrum

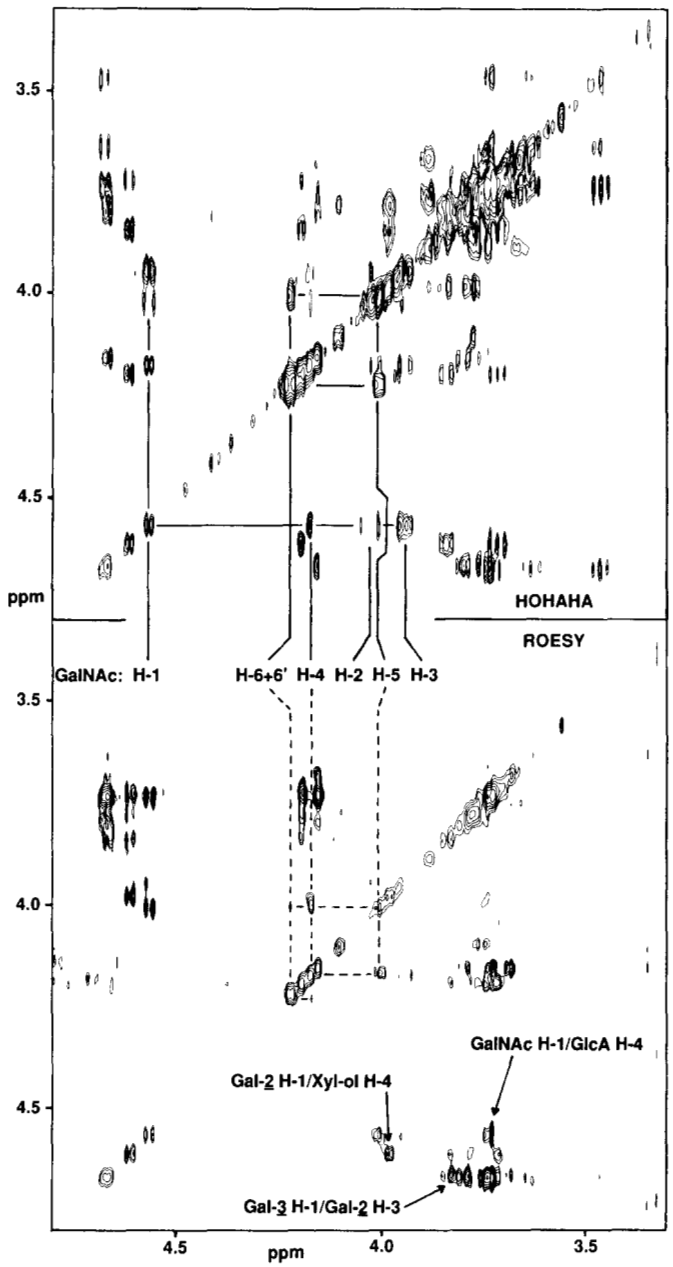


FIG. 5. **2D HOHAHA spectrum (*upper panel*) and 2D ROESY spectrum (*lower panel*) of fraction 7 recorded at 500 MHz in ^2H_2O at 15 °C.** The mixing time for the HOHAHA spectrum was 120 ms and for the ROESY spectrum 200 ms. In the figure the assignment pathway for the GalNAc-*5* residue is drawn in the HOHAHA spectrum (——) and the ROESY spectrum (- - -). Interresidue ROEs are indicated in the ROESY spectrum by *arrows*.

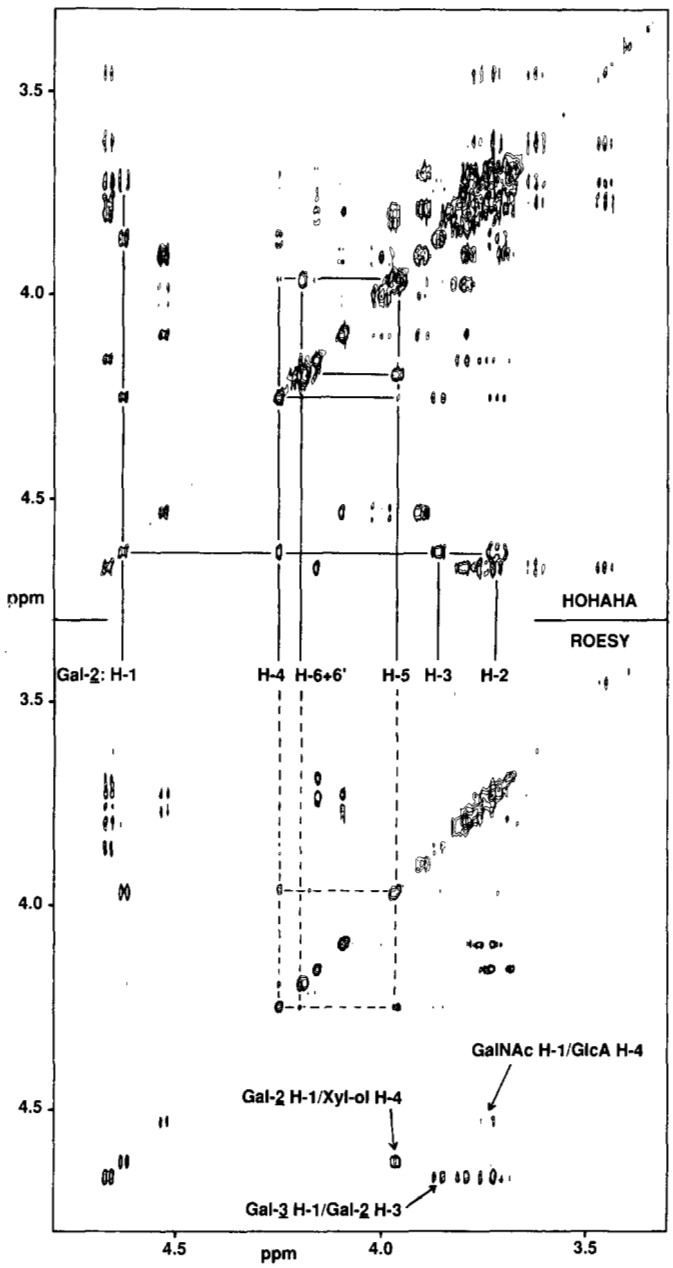
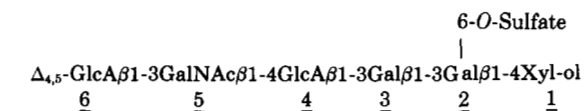


FIG. 6. **2D HOHAHA spectrum (*upper panel*) and 2D ROESY spectrum (*lower panel*) of fraction 6 recorded at 500 MHz in ^2H_2O at 15 °C.** The mixing time for the HOHAHA spectrum was 120 ms and for the ROESY spectrum 200 ms. In the figure the assignment pathway for the Gal-*2* residue is drawn in the HOHAHA spectrum (——) and the ROESY spectrum (- - -). Interresidue ROEs are indicated in the ROESY spectrum by *arrows*.

(data not shown) as well as in the 2D HOHAHA spectrum (Fig. 6) of fraction 6 cross-peaks are found between H-6/H-6′ signals at δ 4.20 and a H-5 signal at δ 3.960. A cross-peak between the H-5 at δ 3.960 and the H-4 is observable in the 2D ROESY spectrum, whereas the connectivity of this H-4 with the Gal-2 H-1 is evident from the 2D HOHAHA spectrum (Fig. 6). The resulting assignment pathway from H-1 to H-6/H-6′ proves that Gal-*2* is sulfated at C-6. The longer measuring time applied for fraction 6 resulted in small, but distinct cross-peaks between Gal-*2* H-4 and H-5 in the 2D HOHAHA spectrum (Fig. 6). Furthermore, the assignment is substantiated by ROESY cross-peaks between H-4 and H-6/H-6′ of Gal-2. The structure of the compound in fraction 6 is as follows.



In the ^1H NMR spectrum (not shown) of fraction 8–2, the GalNAc H-4 has shifted downfield to a position in the anomeric region in the same way as in the spectrum of $\Delta_{4,5}$-GlcA-GalNAc(4-*O*-sulfate)-GlcA-Gal-Gal-Xyl-Ser (fraction D-3 in Ref. 9, Table IV). Furthermore, the chemical shifts of GalNAc H-1, H-2, H-3, and $\Delta_{4,5}$-GlcA H-1, H-3, and H-4 are similar to those of the reference compound. Therefore, fraction 8–2

contains a 4-*O*-sulfated GalNAc (32).

4-*O*-Sulfate
|
$\Delta_{4,5}$-GlcAβ1-3GalNAcβ1-4GlcAβ1-3Galβ1-3Galβ1-4Xyl-ol
6 5 4 3 2 1

The ^{1}H NMR spectrum of fraction 8–1 is given in Fig. 7*A*, and the corresponding NMR data are presented in Table IV. In comparison to the spectrum of fraction 4, a signal is observed at δ 4.267 which has shifted out of the bulk region. By using SED spectroscopy (26) which reveals only proton signals coupled to phosphor, it was proven that the signal at δ 4.267 belongs to a proton attached to an *O*-phosphorylated carbon atom. The spectra recorded initially of this fraction had a low resolution, but this could be improved by treatment of this fraction with Dowex (Na^+). In RESED spectroscopy (24, 25) the magnetization of the proton coupled to phosphate is spread out to other protons belonging to the same residue through isotropic mixing. This revealed a subspectrum of the Xyl-ol residue, being phosphorylated at C-2, consistent with the chemical and enzymatic analysis. Owing to the low amount of material the SED and RESED spectra have a low signal to noise ratio. Therefore, the assignments of the Xyl-ol residue are substantiated by 2D HOHAHA spectroscopy. The position of the H-4 signal of Xyl-ol is confirmed by a cross-peak with Gal-*2* H-1 in the 2D ROESY spectrum (not shown). Under the influence of phosphate, the chemical shifts of the Gal-*2* residue are altered compared to those of fraction 4. The structure of the compound in fraction 8–1 is as follows.

2-*O*-Phosphate
|
$\Delta_{4,5}$-GlcAβ1-3GalNAcβ1-4GlcAβ1-3Galβ1-3Galβ1-4Xyl-ol
6 5 4 3 2 1

The ^{1}H NMR spectrum of fraction 13–4 is given in Fig. 7*B*, and the corresponding ^{1}H NMR data are summarized in Table IV. Comparison of the chemical shifts of the Xyl-ol and Gal-*2* residues with those of fraction 8–1 also reveals that the compound in fraction 13–4 is phosphorylated at C-2 of Xyl-ol. This fraction was not treated with Dowex (Na^+); the presence of another counter ion to phosphate probably accounts for the slightly different chemical shift of the H-2 in fraction 13–4 compared to that of fraction 8–1. Comparison of the chemical shifts of GalNAc and $\Delta_{4,5}$-GlcA of fraction 13–4 with those of fraction 7 indicates that the compound in fraction 13–4 also contains an *O*-sulfate on C-6 of GalNAc. Therefore, the structure of the compound in fraction 13–4 is

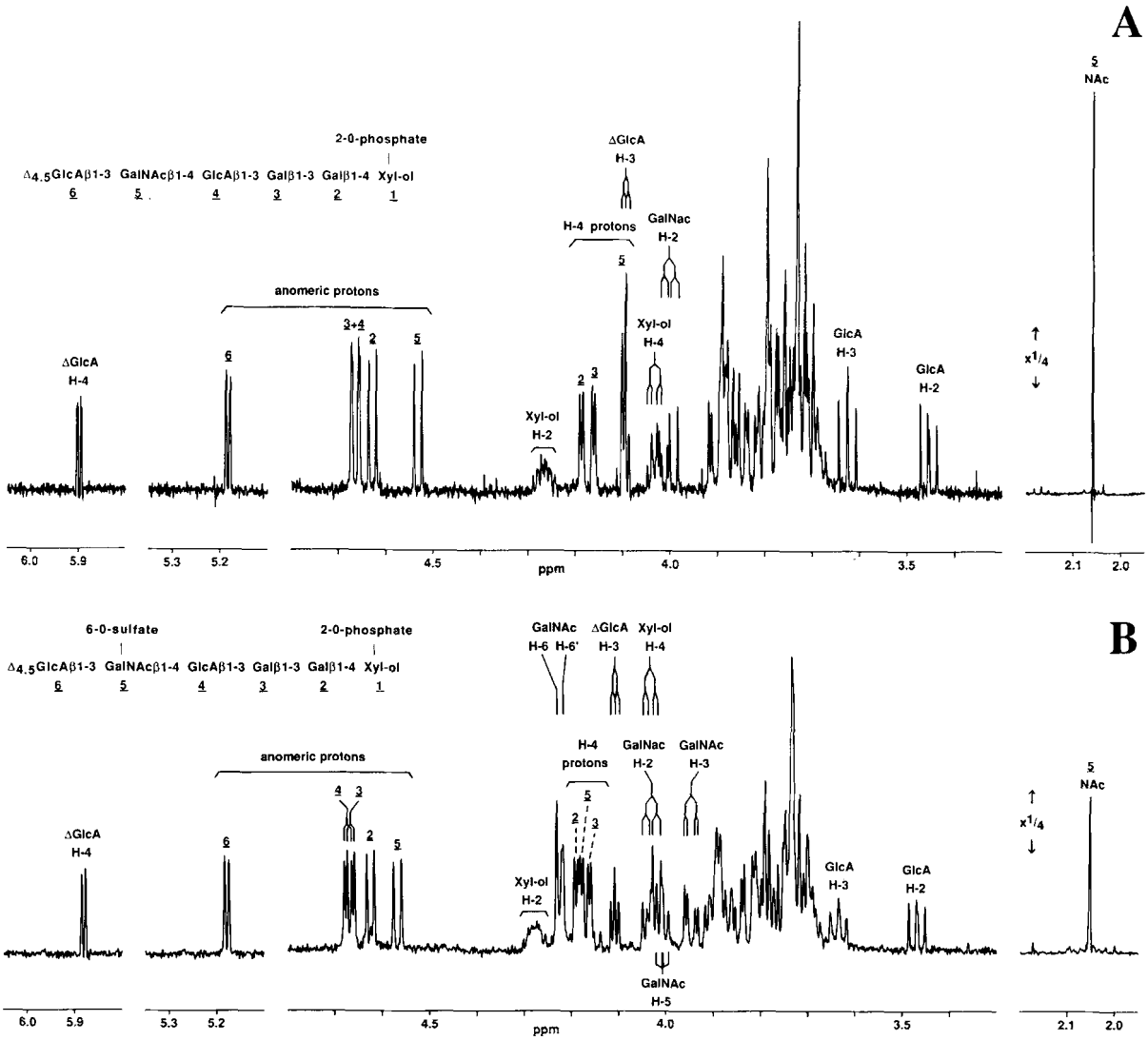
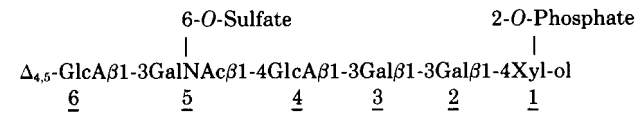


FIG. 7. **Structural-reporter-group regions of the resolution-enhanced 500-MHz ^{1}H NMR spectra of fraction 8–1 (*A*), and 13–4 (*B*) recorded in ^2H_2O at 15 °C.** The *numbers* and *letters* in the spectra refer to the corresponding residues in the structures.

as follows.



For the compounds in fractions 6, 7, and 8–1 the interresidue ROESY cross-peaks (see Figs. 5 and 6) between Gal-*2* H-1 and Xyl-ol H-4, Gal-*3* H-1 and Gal-*2* H-3, as well as between GalNAc-*5* H-1 and GlcA-*4* H-4, confirm the sequential arrangement of these residues.

DISCUSSION

Our discovery of the 4-*O*-sulfated Gal-*3* residue (9) in the carbohydrate-protein linkage region of chondroitin 4-sulfate proteoglycans prompted us to systematically investigate structures of the linkage regions of various proteoglycans. In the present study procedures were established for the isolation and analysis of the linkage regions, which are widely applicable to investigate sulfated proteoglycans. The isolation procedure involved degradation with alkaline/NaB[^{3}H]$_4$ instead of proteolytic preparation of glycoserines. By HPLC several ^{3}H-labeled hexasaccharide alditols derived from the linkage regions were obtained, and six compounds representing approximately 40% of the isolated linkage oligosaccharide alditols were characterized in this paper: one nonsulfated/nonphosphorylated, three monosulfated, one monophosphorylated, and one monosulfated/monophosphorylated compounds.

Modification of the linkage region was first demonstrated by the finding of xylose-2-*O*-phosphate which seems to be common to the linkages of chondroitin 4-sulfate (35), heparan sulfate (11), and probably heparin (12). It remains to be determined whether the considerable number of structures in the linkage region of chondroitin 6-sulfate reflects variable synthetic modifications by phosphorylation and/or sulfation or result from degradation by dephosphorylation and/or desulfation. One of the two phosphorylated linkage oligosaccharides isolated in the present study also has a sulfate group on C-6 of GalNAc-*5*. Thus, phosphorylation of Xyl occurs also on chondroitin 6-sulfate and does not seem to be directly involved in the sorting mechanisms in biosynthesis of different glycosaminoglycans. So far phosphorylation of Xyl and sulfation of Gal have not been demonstrated on the same oligosaccharide.

^{1}H NMR analysis of the purified fraction 6 revealed the presence of a novel sulfated compound, containing a 6-*O*-sulfated Gal-*2* residue. The Gal-4-*O*-sulfate (9, 10) and the Gal-6-*O*-sulfate residues in the linkage region seem to be characteristic of chondroitin 4- and 6-sulfate isomers, respectively, since we could not find these sulfated Gal structures in the linkage region, Galβ1–3Galβ1–4Xylβ1-*O*-Ser, isolated from porcine intestinal heparin (13). Although the biological significance of these structures is unknown as yet, it is tempting to suggest that they may play essential roles in the biosynthesis of glycosaminoglycans, such as a contribution to the segregation of galactosaminoglycans (chondroitin sulfate and dermatan sulfate) and glucosaminoglycans (heparin and heparan sulfate), or to the triggering of sulfation of the repeating units of chondroitin sulfate chains. Evaluation of this hypothesis requires at least characterization of the substrate specificities and of the subcellular localization of the hexosaminyltransferases and the sulfotransferases involved. Since the committing biosynthetic step in sorting of galactosaminoglycans and glucosaminoglycans is the transfer of the first *N*-acetylhexosamine unit to the linkage tetrasaccharide region, it is especially important to examine whether or not the sulfated Gal structures could stimulate the *N*-acetylgalactosaminyltransferase and/or inhibit the *N*-acetylglucosaminyltransferase.

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