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The Structures of *N*- and *O*-Glycosidic Carbohydrate Chains of a Chondroitin Sulfate Proteoglycan Isolated from the Media of the Human Aorta[1]

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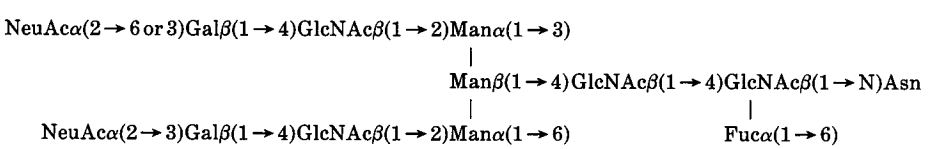
A large M_r chondroitin sulfate proteoglycan was extracted from the media of human aorta under dissociative conditions and purified by density-gradient centrifugation, ion-exchange chromatography, and gel filtration chromatography. Removal of a contaminating dermatan sulfate proteoglycan was accomplished by reduction, alkylation and rechromatography on the gel filtration column. After chondroitinase ABC treatment, the proteoglycan core was separated from a residual heparan sulfate proteoglycan by a third gel filtration chromatography step. As assessed by radioimmunoassay, the isolated proteoglycan core was free of link protein, but possessed epitopes that were recognized by antisera against the hyaluronic acid binding region of bovine cartilage proteoglycan as well as those that were weakly recognized by anti-keratan sulfate antisera. Following β-elimination of the protein core, the liberated low M_r oligosaccharides were partially resolved by Sephadex G-50 chromatography, and their primary structure was determined by 500-MHz ^{1}H NMR spectroscopy in combination with compositional sugar analysis. The *N*-glycosidic carbohydrate chains, which were obtained as glycopeptides, were all biantennary glycans containing NeuAc and Fuc; microheterogeneity in the NeuAc → Gal linkage was detected in one of the branches. The *N*-glycosidic glycans have the following overall structure:



The majority of the *O*-glycosidic carbohydrate chains bound to the protein core were found to be of the mucin type. They were obtained as glycopeptides and oligosaccharide alditols, and possessed the following structures: NeuAcα(2 → 3)Galβ(1 → 3)GalNAc-ol, [NeuAcα(2 → 3)Galβ(1 → 3)[NeuAcα(2 → 6)]GalNAc-ol, and NeuAcα-(2 → 3) Galβ(1 → 3)[NeuAcα(2 → 3)Galβ(1 → 4)GlcNAcβ(1 → 6)]GalNAc-ol.

The remainder of the *O*-glycosidic carbohydrate chains bound to the isolated proteoglycan were the hexasaccharide link regions of the chondroitin sulfate chains that remained after chondroitinase ABC treatment of the native molecule. These latter glycans,

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which were obtained as oligosaccharide alditols, had the following structure (with GalNAc free of sulfate or containing sulfate bound at either C-4 or C-6):

$$\Delta^{4,5}\text{GlcUA}\beta(1\rightarrow 3)\text{GalNAc}\beta(1\rightarrow 4)\text{GlcUA}\beta(1\rightarrow 3)\text{Gal}\beta(1\rightarrow 3)\text{Gal}\beta(1\rightarrow 4)\text{Xyl-ol.}$$

In previous investigations, we reported on quantitative changes in the four types of glycosaminoglycans of the intima and media of the human aorta with progressive atherosclerosis (1, 2). In the present investigation we have analyzed another carbohydrate moiety of aorta proteoglycans, the lower M_r carbohydrate chains. Since proteoglycans synthesized by chondrocytes (3–6), as well as by other cells (7–11) possess *N*- and/or *O*-glycosidic oligosaccharides, it was of interest to investigate the major large M_r chondroitin sulfate proteoglycan (ChS-PG)[6] of the media in terms of these carbohydrate units; we report here the structure of these units as determined by 500-MHz ^{1}H NMR spectroscopy in conjunction with compositional sugar analyses (12, 13). The use of this powerful ^{1}H NMR spectroscopic analytical technique has resulted in the first determination of the structures of the *N*- and *O*-glycosidic oligosaccharides derived from an aorta proteoglycan.

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[6] Abbreviations used: ChS-PG, chondroitin sulfate proteoglycan; GLC, gas–liquid chromatography; Fuc, fucose; GalNAc, *N*-acetylgalactosamine; GlcUA, glucuronic acid; NeuAc, *N*-acetylneuraminic acid; Xyl, xylose; -ol, alditol.

MATERIALS AND METHODS

Materials. Ninety-four human aortas with Grades II to III atherosclerotic involvement (14) were obtained 20–30 h postmortem and stored at −80°C until used.

Reagents. The following special reagents were utilized: ultrapure guanidine-HCl (Bethesda Laboratories); concanavalin A–Sepharose, Sepharose CL-4B, Sephadex G-50, and Sephadex G-25 (Pharmacia); DEAE-cellulose (Schleicher and Schuell Co.); cellulose acetate membranes (15 × 15 cm; Sepraphore II; Gelman Sciences, Inc.); Fuc, Gal, GalNAc, GlcNAc, Man, NeuAc, and Xyl (Pfanstiel); pepstatin, *N*-ethylmaleimide, phenylmethylsulfonyl fluoride, 6-aminohexanoic acid, iodoacetamide, α-methyl-D-glucoside, α-methyl-D-mannoside, and papain (Sigma Chemical Co.); pronase (Seikagaku Kogyo Co., Ltd., Tokyo); D_2O (99.96 atom % D), benzamidine hydrochloride (Aldrich Chemical Co.); Linbro enzyme-immunoassay (EIA) plates (Flow Laboratories); *Proteus vulgaris* chondroitinase ABC (Dr. Okuyama).

Extraction and purification of human aorta ChS-PG. Each aorta was thawed separately, and the intima was carefully dissected from the media to remove any region with Grade IV (14) atherosclerotic involvement, thereby minimizing autolysis of matrix proteoglycans and loss of oligosaccharides. The media were frozen in liquid nitrogen, pulverized in a Waring blender for 60 s, and pooled yielding 1.2 kg (wet wt) of starting material. Approximately 180-g portions of this pooled material were suspended in 900 ml of 4 M guanidine-HCl containing 10 mM EDTA, 0.10 M 6-aminohexanoic acid, 5 mM benzamidine hydrochloride, 1 mM iodoacetamide, and 50 mM Tris-HCl, pH 6.8, and extracted for 17 h at 4°C. The resulting extract was centrifuged at 1500*g* for 45 min to remove residual connective tissue and then subjected to dissociative CsCl density-gradient centrifugation at an initial density of 1.40 g/ml at 17°C and 150,000*g* for 48 h (15). The bottom third of the CsCl gradient which contained the majority of the extracted aorta proteoglycans was dialyzed at 4°C against 7 M urea containing 50 mM Tris-HCl, pH 6.8, and the above protease inhibitors. The density-gradient purified proteoglycans were then applied at 5°C to a DEAE-cellulose column (3 × 10 cm) previously equilibrated in the urea-containing

buffer (16). After washing the ion-exchange column with 300 ml of the latter buffer, proteoglycans were eluted with a 400-ml linear gradient from 0 to 3 M NaCl in the same urea-containing buffer. Proteoglycan-containing fractions, detected by uronic acid analysis (17), were pooled and chromatographed on a Sepharose CL-4B column (2.5 × 226 cm) which had been equilibrated with 4 M guanidine–HCl containing 10 mM 6-aminohexanoic acid, 10 mM EDTA, and 50 mM Tris–HCl, pH 6.8. Chondrosarcoma proteoglycan (~2,000,000 M_r) (18), bone marrow-derived mast cell proteoglycan (~200,000 M_r) (19), and [^{35}S]sulfate were used to standardize the gel filtration column. Distinct peaks containing proteoglycans of different hydrodynamic size were separately dialyzed against water and lyophilized. The major large M_r fractions from seven separate replicate gel filtration chromatomatograms were pooled, reduced with dithiothreitol, alkylated with vinylpyridine (20), and rechromatographed on Sepharose CL-4B to remove a residual low M_r dermatan sulfate PG.

Isolation of N- and O-linked glycans from human aorta ChS-PG. The purified ChS-PG (215 mg) was incubated at 37°C overnight with 15 units of chondroitinase ABC in albumin-free 10 mM Tris–HCl buffer, pH 8.1, containing 30 mM sodium iodoacetate, 10 mM EDTA, 10 mM *N*-ethylmaleimide, 5 mM phenylmethylsulfonyl fluoride, and 0.35 mM pepstatin (21). The chondroitinase ABC digest was again chromatographed on Sepharose CL-4B under dissociative conditions to separate the residual undigested heparan sulfate proteoglycan from the ChS-PG now devoid of its chondroitin sulfate chains. The hexose-containing protein core was dialyzed against 50 mM NH_4HCO_3, lyophilized to yield 32 mg, and then subjected to β-elimination (22) by incubating the sample in 5 ml of 0.05 M NaOH, 1 M $NaBH_4$ at 45°C for 26 h. The resulting alkaline cleavage products were neutralized with acetic acid and chromatographed on a Sephadex G-50 column (1.2 × 155 cm), previously equilibrated with 50 mM NH_4HCO_3. Alternatively, the chondroitinase-treated proteoglycan was dissolved in 5 mM NaCN, 10 mM cysteine–HCl, and 10 mM EDTA, pH 7.6, and incubated at 65°C for 20 h with 3% (w/w) papain (1). Solid calcium acetate was then added to give a final concentration of 20 mM, the pH was adjusted to 8.0, and then the papain-treated sample was incubated at 37°C for 20 h with 3% (w/w) pronase. The resulting papain–pronase digest was filtered on a Sephadex G-25 column (1.2 × 155 cm), equilibrated, and eluted with 50 mM NH_4HCO_3. Hexose-containing fractions were pooled, lyophilized, and the structures of the *N*- and *O*-glycosidic oligosaccharides determined. An attempt was made to fractionate a Sephadex G-50 fraction which contained both *N*- and *O*-glycosidic oligosaccharides by their differential binding to concanavalin A-Sepharose. Glycans (2.4 mg), obtained by Sephadex G-50 chromatography of alkali-treated proteoglycans, were suspended in 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.15 M NaCl, and 20 mM sodium acetate, pH 7.0, and applied to a 1.2 × 3.5-cm column of concanavalin A–Sepharose equilibrated with this same buffer. Lectin-bound glycans were eluted with 0.1 M α-methyl-D-glucoside, followed by 0.1 M α-methyl-D-mannoside.

Carbohydrate and protein analytical procedures. The glycosaminoglycans present in the different proteoglycan fractions were identified by two-dimensional electrophoresis on cellulose acetate (1, 23). For compositional sugar analysis, samples were methanolyzed (1 M HCl in methanol, 24 h at 85°C), *N*-(re)acetylated, and trimethylsilylated (20, 24). The resulting derivatized monosaccharides were analyzed by capillary gas–liquid chromatography on a CpSil 5 column (25 m × 0.32 mm). The oven temperature was programmed from 130 to 220°C at 2°C/min. The amino acid composition of the purified proteoglycan was determined using a Beckman Model 119 CLW/126 amino acid analyzer after hydrolysis with constant boiling 6 M HCl for 24 h at 110°C in a sealed tube under N_2. The effluents of the DEAE-cellulose, Sepharose, and Sephadex chromatography columns described above were monitored at 280 nm for protein and nucleic acid, at 530 nm for GlcUA (17), and at 490 nm for neutral hexose (25).

Immunological identification of the antigenic sites of ChS-PG. The antigenic sites of both native human aorta ChS-PG and ChS-PG devoid of its chondroitin sulfate were determined according to a previously described EIA method (26) utilizing the following monoclonal and polyclonal antibodies: 3-B-3 (anti-chondroitin 6-sulfate), 1-B-5 (anti-chondroitin), 9-A-2 (anti-chondroitin 4-sulfate), 5-D-4 (anti-keratan sulfate), 8-A-4 (anti-link protein), R-17 (anti-bovine nasal cartilage proteoglycan aggregates), R-13 (anti-bovine link protein), R-21 and SAH (anti-hyaluronic acid binding region of bovine cartilage proteoglycan). The preparation and specificity of the monoclonal and polyclonal antibodies used for the EIA analyses on the isolated human aorta proteoglycan have been described previously (26, 27).

500-MHz ^{1}H NMR spectroscopy. Approximately 1 mg of each Sephadex G-50 fraction was repeatedly dissolved in D_2O and lyophilized, and then analyzed with a Bruker WM-500 spectrometer equipped with a Brubaker Aspect-2000 computer (SON, hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating at 500 MHz in the Fourier transform mode at a probe temperature of 27°C. Resolution enhancement of the spectrum was achieved by Lorentzian to Gaussian transformation from quadrature phase detection followed by a complex Fourier transformation. Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured by reference to internal acetone in D_2O (δ 2.225 ppm)

(12). This method of analysis was chosen because of its increased sensitivity relative to differential enzymatic and/or chemical degradation of glycoconjugates and because of its ability to deduce the structures of distinct glycans in heterogeneous preparations (12, 13).

RESULTS

Purification of ChS-PG

Approximately 75% of the total uronic acid-containing macromolecules were extracted from human aorta media when treated with buffer containing 4 M guanidine-HCl. Upon fractionation of the extract by CsCl density-gradient centrifugation, approximately 65% of this material localized in the bottom third of the gradient. Chromatography of this high buoyant density material on a DEAE-cellulose column resulted in two uronic acid-containing fractions (Fig. 1). The minor, less acidic fraction (fraction I) contained considerable amounts of contaminating protein and was therefore stored at −30°C for future studies. Fraction II, which eluted at a NaCl concentration of approximately 1 M, included the major portion of the uronic acid-containing molecules and was further resolved into five subfractions by filtration through Sepharose CL-4B (Fig. 2, fractions A-E). Fraction A, eluting at the column's void volume, was relatively free of uronic acid. Since the absorbance of this fraction was greater at 260 nm than at 280 nm, it was concluded that it contained predominantly nucleic acids. Glycosaminoglycan analysis by two-dimensional cellulose acetate electrophoresis of the other fractions revealed that fraction B contained proteoglycans rich in chondroitin-6-sulfate glycosaminoglycans, although traces of heparan sulfate, dermatan sulfate, and hyaluronic acid were also present. As assessed by their 0.23 K_{av} on this Sepharose CL-4B column, the M_r of the proteoglycans in fraction B was estimated to be approximately 1×10^6. Fractions D and E contained proteoglycans rich in dermatan sulfate with small amounts of heparan sulfate and chondroitin 6-sulfate. Fraction C represented a mixture of fractions B and D. Thus, because of its higher degree of purity and its larger M_r, the proteoglycans in fraction B were selected for further investigation.

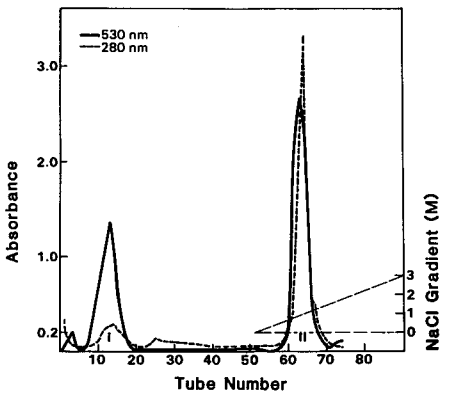


FIG. 1. Chromatography on DEAE-cellulose of the proteoglycans extracted from the human aorta media and purified by CsCl density-gradient centrifugation. The eluate was collected in 10-ml fractions and was monitored for protein and/or nucleic acid (---) and uronic acid (—).

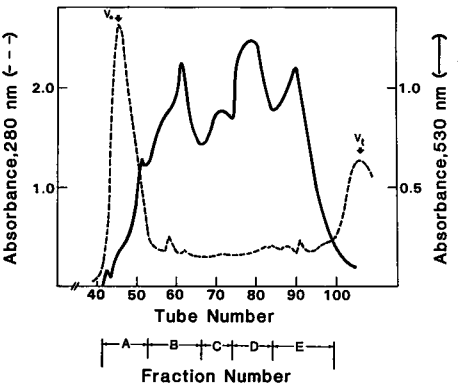


FIG. 2. Gel filtration through Sepharose CL-4B of the DEAE-fraction II (Fig. 1) proteoglycans derived from the aorta media. The eluate was collected in 10-ml fractions and was monitored for protein and/or nucleic acid (---) and for uronic acid (—). V_0 and V_t indicate the column's excluded volume and total volume, respectively.

The amino acid composition of fraction B was found to be rich in aspartic acid, threonine, serine, and glutamic acid (Table I). The amino acid composition of this purified proteoglycan was very similar to that reported for the large M_r chondroitin sulfate-containing proteoglycan of bovine

TABLE I

AMINO ACID COMPOSITION OF THE HUMAN AORTA ChS-PG AND THAT OF BOVINE AORTA CHONDROITIN SULFATE PG

Amino acid	Residues per 1000 residues	
	Human	Bovine[a]
Asx	98	108
Thr	92	83
Ser	98	95
Glx	135	137
Pro	81	94
Gly	78	75
Ala	60	66
Val	59	44
1/2 Cys	ND[b]	8
Met	7	14
Ile	74	29
Leu	83	83
Tyr	ND	21
Trp	ND	ND
Phe	52	35
Lys	35	49
His	18	26
Arg	30	32

[a] Values are those reported by Oegema *et al.* (28).
[b] ND, not determined.

aorta (28) (Table I) except for the former's higher isoleucine content.

Chondroitinase ABC Treatment of ChS-PG and Immunochemical Analyses

After reduction and alkylation of fraction B proteoglycans and rechromatography on the Sepharose CL-4B column, the ChS-PG was found to be free of dermatan sulfate. Upon incubation of the modified ChS-PG with chondroitinase ABC, the enzymatically treated proteoglycan decreased substantially in size, as evidenced by a change in K_{av} on the Sepharose CL-4B column from 0.23 before incubation to 0.43 after digestion. This separation step also resulted in the removal of essentially all of the residual chondroitinase ABC-resistant heparan sulfate proteoglycans.

Utilizing the monoclonal and polyclonal antibodies, it was determined that both the native fraction B proteoglycans and the chondroitinase ABC-treated proteoglycans contained a hyaluronic acid-binding site. Although the anti-chondroitin-6-sulfate antibody reacted with the chondroitinase ABC-treated sample, no substantial amount of unsulfated or 4-sulfated chondroitin could be detected immunologically. In addition, there was no reaction with either monoclonal or polyclonal antibodies that recognize link protein. Although the peptide core of the isolated human aorta ChS-PG was homologous immunologically to the large M_r aggregating proteoglycan present in cartilage, as assessed by the presence of a hyaluronic acid-binding region and chondroitin sulfate, only a weak cross-reaction with the 5-D-4 anti-keratan sulfate antibody was observed. This finding indicated that only small amounts of macromolecular keratan sulfate like glycosaminoglycans are present in this ChS-PG preparation.

Isolation of Glycans and Elucidation of Their Structures

After mild alkaline treatment of the chondroitinase ABC-treated ChS-PG, four oligosaccharide fractions were obtained by gel filtration (Fig. 3; fractions 3–6). Fractions 1, 2, and 7 were not investigated further as they contained predominantly nucleic acids, peptides, and salts, respectively. Sugar analysis of fractions 3 and 4 (Table II) revealed the presence of substantial amounts of Man, Gal, and NeuAc, lesser quantities of Fuc and GalNAc, and the lack of significant amounts of GlcNAc-ol and GalNAc-ol, suggesting that the carbohydrates in fractions 3 and 4 existed as glycopeptides. Application of ^{1}H NMR spectroscopy to fractions 3 and 4 readily revealed the presence of *N*-glycosidic glycopeptides. Although the spectrum of the glycopeptides in fraction 4 was somewhat simpler than that derived from fraction 3, both fractions were similar to each other in that they indicated the presence of glycans of the *N*-acetyllactosamine type. The spectrum of fraction 4 (Fig. 4A) is reminiscent of that obtained from analysis of a mixture of isomers of a biantennary

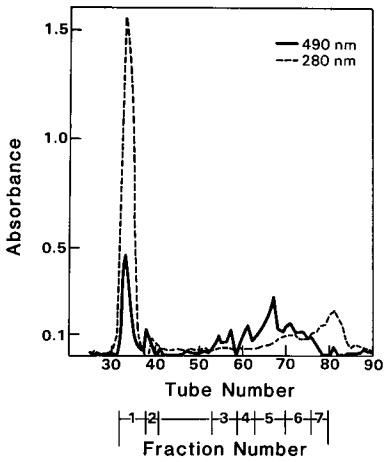


FIG. 3. Sephadex G-50 fractionation of the glycans liberated by reductive β-elimination from chondroitinase ABC-treated aorta ChS-PG (Fig. 2, fraction *B*). Fractions were monitored for protein and/or nucleic acid (---) and neutral hexose (—).

glycopeptide derived from horse pancreatic ribonuclease which contain NeuAc and Fuc [compounds R-N-44 and R-N-45 in Ref. (12)]. The ^1H chemical shifts of pertinent structural-reporter groups for the N-type glycopeptide found in this fraction (see Table III) are compared in Table IV to those of the corresponding R-N-44 reference compound. The pentasaccharide core, $Man_3GlcNAc_2$, of the human ChS-PG-derived Asn-linked glycan is typical of N-type glycopeptides. However, this glycan was found to contain a Fuc residue linked α-(1 → 6) to GlcNAc-1. Evidence for this assignment stems from the occurrence in the spectrum of the Fuc H-1 and CH_3 signals at $\delta \cong 4.87$ and 1.202 ppm, respectively, in conjunction with the *N*-acetyl methyl resonance of GlcNAc-2 being observed at δ 2.093 [compare Ref. (12)]. Because the set of chemical shifts of the Man H-2 atoms (Table IV) is identical to that obtained with the reference compound (12), it was deduced that this *N*-glycan is of the biantennary type. Both branches of this glycan end in sialic acid. That NeuAc is linked α(2 → 6) to Gal-6 in the Manα(1 → 3) branch and NeuAc is linked α(2 → 3) to Gal-6′ in the

TABLE II

CARBOHYDRATE COMPOSITIONS OF THE GLYCAN FRACTIONS (3–6) OBTAINED AFTER β-ELIMINATION, AND OF THAT OBTAINED AFTER PAPAIN–PRONASE DIGESTION OF CHONDROITINASE ABC-TREATED HUMAN AORTA ChS-PG

	Sephadex fraction[a]				
Monosaccharide	3 (1.5 mg)	4 (2.4 mg)	5 (6.0 mg)	6 (7.3 mg)	Protease digest
Fuc	0.9	1.2	+[b]	−[c]	1.0
Xyl	+	0.2	0.1	+	1.0
Man	3.0	3.0	+	−	3.0
Gal	6.4	4.8	2.1	1.4	5.8
GalNAc	1.9	1.9	0.5	0.2	5.3
GlcNAc	4.7	4.2	0.4	ND[d]	4.4
GalNAc-ol	ND	ND	1.0	1.0	−
GlcNAc-ol	−	−	−	−	−
GlcUA	−	−	0.3	+	1.1
NeuAc	4.8	4.2	2.5	1.2	4.7
Xyl-ol	−	−	0.2	0.2	−

[a] Values for fractions 3 and 4 and for the papain-pronase digest are expressed relative to 3 mol (assumed) of Man, and those of fractions 5 and 6 relative to 1 mol (assumed) of GalNAc-ol.

[b] +, traces.

[c] −, negligible.

[d] ND, could not be determined due to interference of contaminants.

Manα(1 → 6) branch were inferred from the chemical shifts of the H-3 signals of the sialic acid residues, the shifts of the H-1 signals of Man-4 and -4′, and the *N*-acetyl signals of GlcNAc-5 and -5′ (Table IV) (12). The structure of this glycopeptide, designated "Compound I," has been included in Table III.

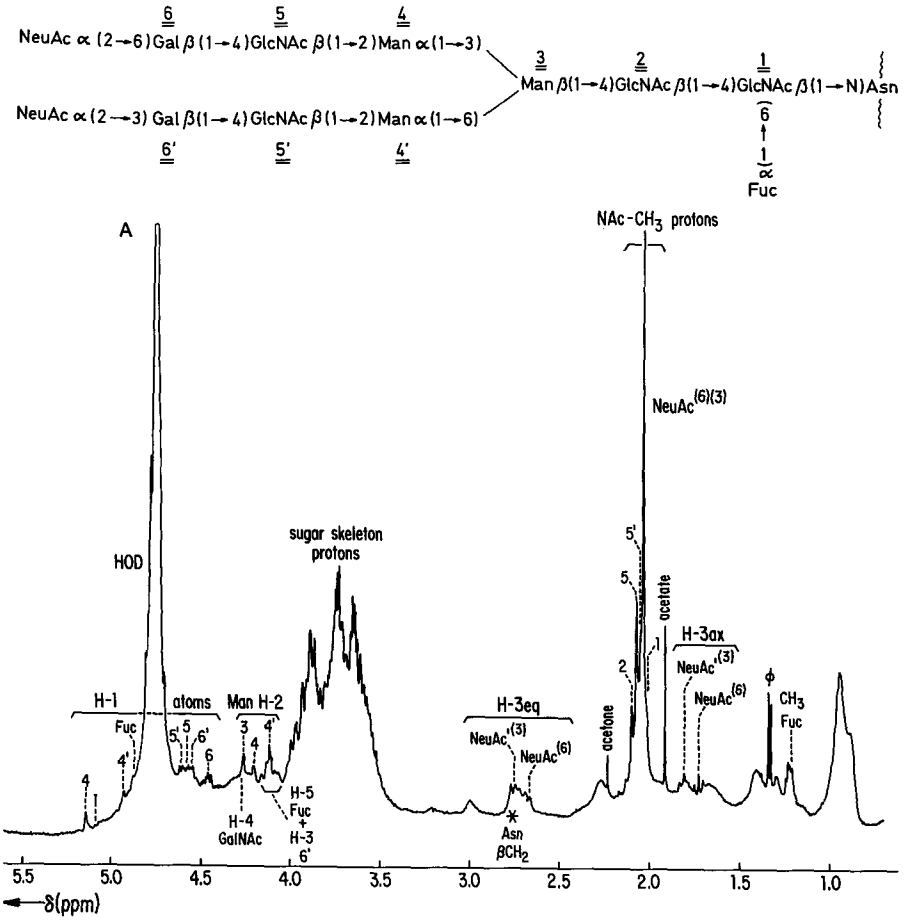


FIG. 4. 500-MHz ^{1}H NMR analyses of glycans derived from human aorta ChS-PG. (A) Structural reporter group regions of the 500-MHz ^{1}H NMR spectrum (D_2O, pD ≃7, 27°C) of Sephadex G-50, fraction 4, obtained by β-elimination of the chondroitinase ABC-treated chondroitin sulfate proteoglycan from human aorta. The numbers in the spectrum refer to the corresponding residues in the structure. The large peak at ~1.0 ppm is probably due to proton signals derived from amino acid residues of peptides. (B1) Overall 500-MHz ^{1}H NMR spectrum of Sephadex G-50, fraction 5, obtained by β-elimination of the chondroitinase ABC-treated chondroitin sulfate proteoglycan from human aorta. The 3.3 to 5.3 ppm region (B2) and the 1.3 to 3.0 ppm region (B2 cont.) of the overall spectrum have been enlarged to indicate the pertinent structural reporter groups. Assignments of the signals have been indicated above the spectrum for the three components of the mixture, each on a separate trace. The upper trace contains the assignments of the resonances for the major component, Compound III; the middle trace contains those for Compound IV; and the lower trace contains those for Compound V. For explanation of superscripts at the names of sugar residues, see Table V. The relative-intensity scale of the *N*-acetyl methyl proton signals deviates from that of the other parts of the spectrum, as indicated.

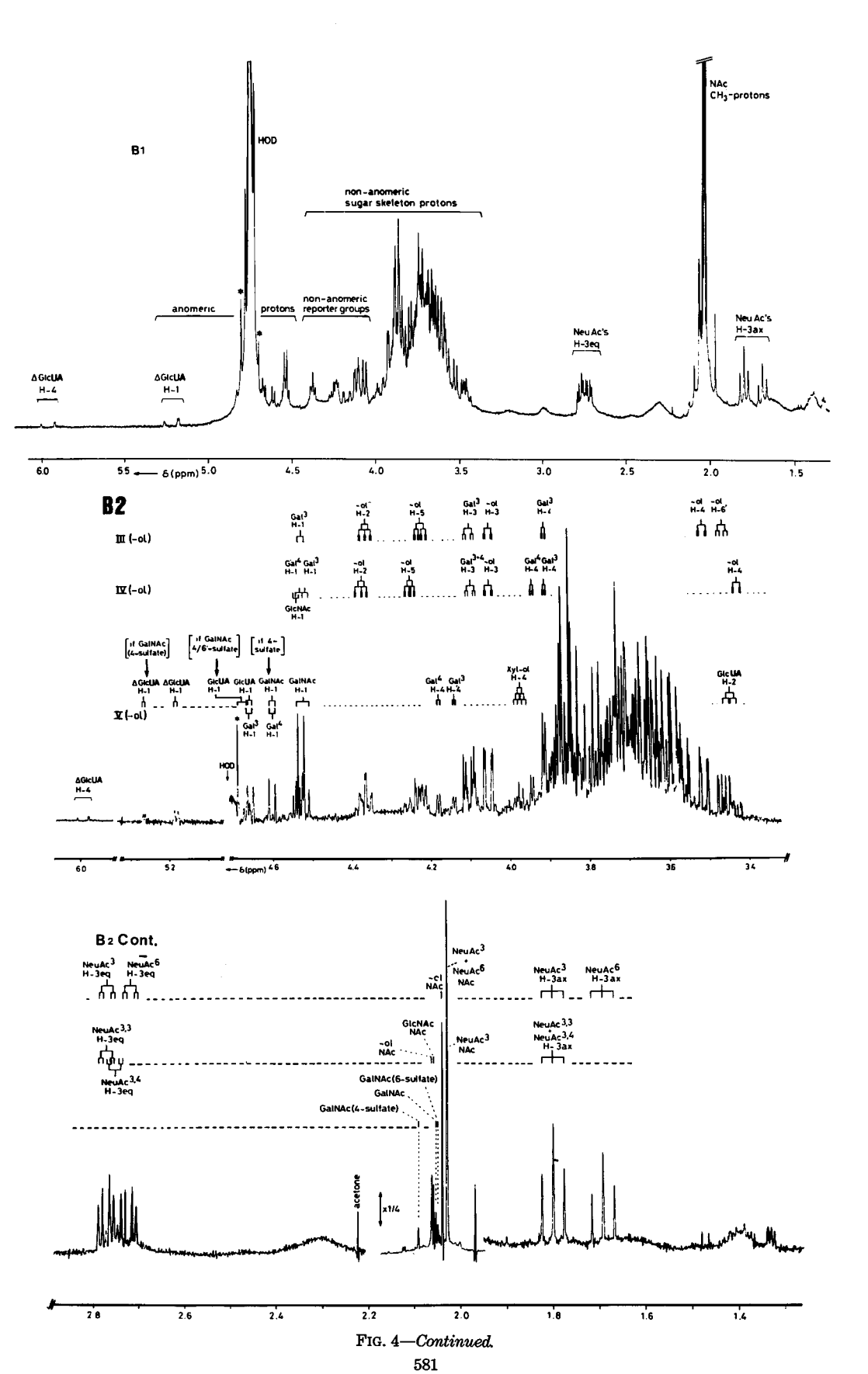
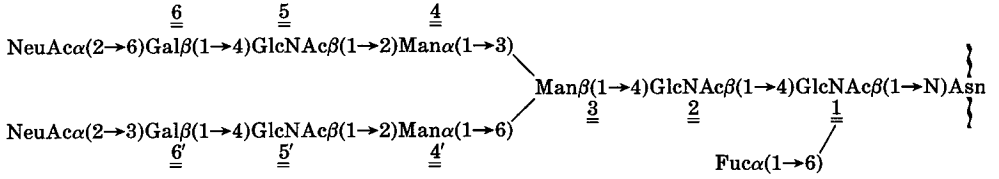
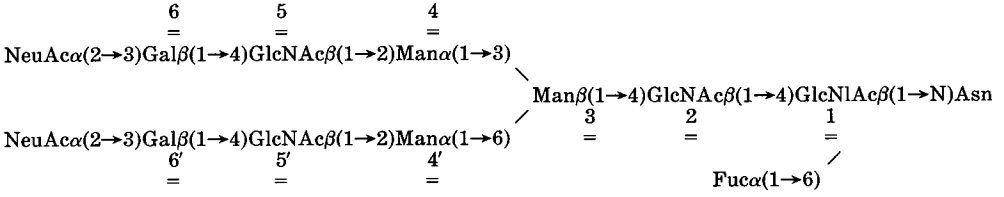
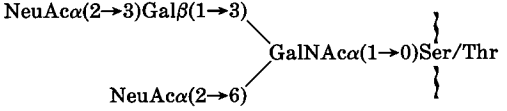
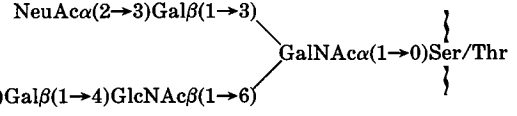
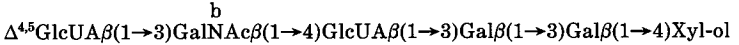
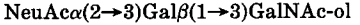


FIG. 4—*Continued.*

TABLE III

STRUCTURES OF HUMAN AORTA PROTEOGLYCAN N- AND O-LINKED OLIGOSACCHARIDES

Relative abundance[a] (%)	Oligosaccharide	Compound
13		I
1.5		II
18		III
11.5		IV
20.5		V
35.5		VI

[a] In mole %, calculated from the weights of fractions 3–6 (Table II), combined with the ratios derived from ^{1}H NMR and sugar analysis. The estimated error in the % of natural abundance is ±5.

[b] The GalNAc residue may carry a sulfate group either at c-4 or at C-6 (see text).

The spectrum of the glycopeptides present in fraction 3 (not shown) also revealed the presence of Compound I. However, in addition to the above-mentioned signals a lower intensity H-1 signal at δ 5.116 ppm (ratio 3:7, when compared to the signal at δ 5.130 ppm) was detected which was attributed to the H-1 signal of the Man-4-forming part of a branch that is terminated in NeuAcα(2 → 3) rather than in α(2 → 6). The 7:3 intensity ratios of the NeuAc H-3eq signals at δ 2.75 and 2.67 ppm, the NeuAc H-3ax signals at δ 1.80 and 1.72 ppm, and the GlcNAc *N*-acetyl signals at δ 2.045 and 2.068 ppm confirm the occurrence of this second *N*-glycan which has been designated as "Compound II" (Tables III and IV). Compound II has been obtained earlier from Sindbis virus membrane glycoproteins and the chemical shifts of its reporter groups (29) are in accordance with the data in Table IV for this ChS-PG-derived compound.

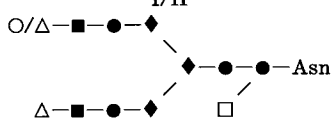
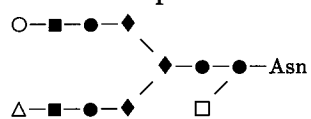
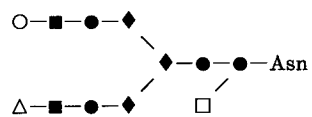
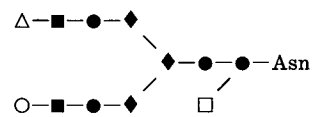
The molar ratios of the constituent monsaccharides present in fractions 3 and 4 (Table II) do not correspond exactly to the structure of the two biantennary N-type glycopeptides deduced by 500-MHz ^{1}H NMR analysis. The relative excess of Gal, GlcNAc and NeuAc, together with the presence of GalNAc, suggested β-elimination was incomplete and that glycopeptides of the GalNAc-containing *O*-glycosidic type probably are also present in these two gel filtration fractions. Even if the structural elements of the 0-type glycans present in these fractions were similar or identical to those present in Compounds I and II and resulted in most of the expected NMR resonances from the latter two compounds being overlapped by those originating from the former compounds, some of the expected resonances should be clearly observable. The presence of an H-4 signal at δ 4.254 ppm (Fig. 4A), which is a characteristic signal for peptide-linked GalNAc present in mucin-like glycans (30), in combination with the compositional sugar analyses of these two gel filtration fractions does suggest the presence of O-type chains containing NeuAcα(2 → 3)Galβ(1 → 4)GlcNAcβ(1 → O) and/or NeuAcα(2 → 3)Galβ(1 → 3).

Subsequent concanavalin A–Sepharose chromatography of fraction 3 and 4 glycans failed to separate the N-linked glycans from the O-linked glycans, most likely because both types of glycans were bound to a common peptide. As an alternative approach to mild alkali treatment for obtaining these glycans, the chondroitinase-treated ChS-PG was exhaustively digested with both papain and pronase. Upon Sephadex G-25 chromatography, the resulting peptides and glycopeptides eluted as a single hexose-containing fraction (Table II). ^{1}H NMR analysis of the glycopeptides in this fraction revealed the presence of such a complex mixture of glycans that their structures could not be determined conclusively.

Sugar analysis of Sephadex G-50 fractions 5 and 6 revealed the absence of Man and the presence of GalNAc-ol, Xyl-ol, GlcUA, and NeuAc, indicating the probable presence of peptide-free glycans of the mucin type and the linkage region glycans derived from the residual chondroitin sulfate chains. The ^{1}H NMR spectrum (Fig. 4B) of the glycans of fraction 5 confirmed the presence of mucin O-type glycans terminating in GalNAc-ol. The obtained spectrum revealed the characteristic chemical shifts (Table V) of the structural-reporter groups of the tetra- and hexasaccharide alditols which have been designated "Compounds III and IV" (Table III) [compare Refs. (31–33)]. Based on the intensity ratio of the NeuAc H-3 signals (Fig. 4B), the molar ratio of Compound III to Compound IV in fraction 5 was calculated to be 3:1. In addition to Compounds III and IV, further analysis of this ^{1}H NMR spectrum revealed the presence of $\Delta^{4,5}$GlcUA. This monosaccharide is known to be present at the terminal nonreducing site of the residual chondroitinase ABC-treated chondroitin sulfate chain (34–36). The H-1 signal at $\delta \cong 5.15$ ppm and the H-4 signal at $\delta \cong 5.85$ ppm are typical for $\Delta^{4,5}$GlcUA (37–39). Based on a comparison of the ^{1}H NMR data with that of a reference chondroitin sulfate-derived linkage region gly-

TABLE IV

^{1}H CHEMICAL SHIFTS OF STRUCTURAL-REPORTER GROUPS OF CONSTITUENT MONOSACCHARIDES FOR THE N-TYPE GLYCOPEPTIDES (COMPOUNDS I AND II) PRESENT IN SEPHADEX G-50 FRACTIONS 3 AND 4, OBTAINED AFTER β-ELIMINATION OF CHONDROITINASE ABC-TREATED HUMAN AORTA ChS-PG, TOGETHER WITH PERTINENT REFERENCE DATA

Reporter group	Residue	Chemical shift[a] in[b] 3-gp[c] I/II	4-gp I	R-N-44[d]	R-N-45[d]
					
H-1	GlcNAc-1	5.05	5.05	5.068	5.068
	Fucα(1→6)	4.876	4.863	4.876	4.876
	GlcNAc-2	ND[e]	4.69	4.682	4.682
	Man-3	ND	ND	4.77	4.77
	Man-4	5.130/5.116[c]	5.133	5.136	5.118
	GlcNAc-5	4.60/4.58	4.606	4.606	4.575
	Gal-6	4.441/4.542	4.444	4.444	4.545
	Man-4′	4.917	4.925	4.922	4.940
	GlcNAc-5′	4.58	4.58	4.575	4.606
	Gal-6′	4.542	4.537	4.549	4.447
H-2	Man-3	4.251	4.254	4.253	4.253
	Man-4	4.195	4.195	4.197	4.190
	Man-4′	4.107	4.110	4.11	4.11
H-3	Gal-6/6′	4.11	4.11	4.11	4.11
H-3ax	NeuAcα(2→6/3)	1.718/1.802	1.718	1.717	1.800
	NeuAc′α(2→3)	1.802	1.800	1.802	—[f]
H-3eq	NeuAcα(2→6/3)	2.669/2.753	2.67	2.669	2.758
	NeuAc′α(2→3)	2.753	2.758	2.760	—[f]
H-5	Fucα(1→6)	4.11	4.11	4.12	4.12

CH_3	Fucα(1→6)	1.206	1.202	1.202		1.202
NAc	GlcNAc-1	2.010	2.01	2.012		2.012
	GlcNAc-2	2.090	2.093	2.094		2.094
	GlcNAc-5	2.068/2.045	2.069	2.069		2.048
	GlcNAc-5′	2.045	2.043[g]	2.044		2.066
	NeuAc/NeuAc′	2.029	2.030	2.030		2.032

[a] Chemical shifts are expressed in ppm downfield from internal DSS in D_2O at 27°C, acquired at 500 MHz.

[b] In the table heading, structures are represented by symbolic notation [cf. Ref. (12)]: ◆, Man; ●, GlcNAc; ■, Gal; □, Fuc; ○, NeuAcα(2→6); △, NeuAcα(2→3). For complete structures and numbering of monosaccharide residues, see Table III.

[c] Glycopeptide fraction 3 consists of compounds I and II (Table III). The numbers in the left column are attributed to reporter groups of compound I, and those in the right column to compound II.

[d] Reference compounds R-N-44 and R-N-45 were obtained in a mixture from horse pancreatic ribonuclease (12).

[e] ND, not determined.

[f] This standard glycan has NeuAc′α(2→6) instead of (2→3).

[g] In addition, a low-intensity singlet at δ 2.048 ppm was observed, indicating the presence of a minute amount of glycopeptides with terminal, i.e., desialylated Gal-6′ in fraction 4.

can (Table VI), it was deduced that "Compound V" (Table III) was present in gel filtration fraction 5. Microheterogeneity of Compound V was observed with respect to the presence and site of attachment of the sulfate ester group on GalNAc. Because the three *N*-acetyl signals detected at δ 2.095, 2.057, and 2.052 ppm were observed earlier for ΔDi-4S, ΔDi-OS, and ΔDi-6S standards, respectively (Table VI) [compare Ref. (42)], it was concluded that the GalNAc residue of Compound V possessed sulfate bound to C-4, lacked sulfate, or had sulfate bound to C-6. This is in keeping with the doubling observed for the $\Delta^{4,5}$GlcUA H-1 and H-4 signals (Fig. 4). The H-1 and H-4 signals for both the nonsulfated and the C-6 sulfated GalNAc compounds are found at δ 5.183 and 5.813 ppm, respectively, whereas C-4 sulfated GalNAc results in an H-1 signal at δ 5.266 ppm and an H-4 signal at δ 5.953 ppm (Table VI).

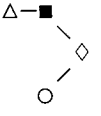
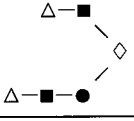
Sephadex G-50 fraction 6 consisted of lower M_r oligosaccharide alditols. The main component in this fraction ("Compound VI," Table III) was found to be a trisaccharide related to Compounds III and IV. The ^{1}H NMR spectral data of Compound VI, which are included in Table V, are identical to those obtained for this same compound obtained from other sources (13, 33).

DISCUSSION

This study describes the first structural determination of the N- and O-linked oligosaccharides of a ChS-PG purified from the human aortic media by a five-step procedure. As assessed immunologically, the ChS-PG contained a hyaluronic acid binding site. As assessed by enzyme-immunoassay, no link protein was present in the purified ChS-PG preparation. This finding indicates a high degree of purity of the ChS-PG, since link protein is often tightly associated with proteoglycan molecules containing a hyaluronic acid binding region. Carbohydrate composition analysis of the isolated chondroitinase ABC-treated ChS-PG suggested that this proteoglycan contained both N-type and O-type carbo-

TABLE V

[1]H CHEMICAL SHIFTS OF STRUCTURAL-REPORTER GROUPS OF CONSTITUENT MONOSACCHARIDES FOR THE MUCIN-TYPE OLIGOSACCHARIDE-ALDITOLS (COMPOUNDS III, IV AND VI) PRESENT IN SEPHADEX G-50 FRACTIONS 5 AND 6, OBTAINED AFTER β-ELIMINATION OF CHONDROITINASE ABC-TREATED HUMAN AORTA ChS-PG, TOGETHER WITH PERTINENT REFERENCE DATA

Residue[c]	Reporter group	Chemical shift[a] in[b]					
		Fraction 5				Fraction 6	
		III	R-O-1[d]	IV	R-O-2[d]	VI	R-O-3[e]
							
GalNAc-ol	H-2	4.378	4.378	4.387	4.389	4.389	4.390
	H-3	4.067	4.067	4.067	4.066	4.070	4.074
	H-4	3.524	3.524	3.45	3.439	3.497	3.498
	H-5	4.239	4.240	4.266	4.265	4.186	4.187
	H-6′	3.473	3.475	ND[f]	ND	ND	ND
	NAc	2.043	2.042	2.065	2.065	2.044	2.046
Gal[3]	H-1	4.541	4.541	4.529	4.530	4.544	4.547
	H-3	4.116	4.117	4.113	4.113	4.118	4.122
	H-4	3.927	3.927	3.927	3.927	3.927	3.931
GlcNAc[6]	H-1	—	—	4.553	4.552	—	—
	NAc	—	—	2.062	2.062	—	—
Gal[4]	H-1	—	—	4.546	4.546	—	—
	H-3	—	—	4.113	4.113	—	—
	H-4	—	—	3.957	3.956	—	—
NeuAc[3,3]	H-3ax	1.800	1.800	1.800	1.799	1.800	1.800
	H-3eq	2.775	2.774	2.775	2.775	2.773	2.774
	NAc	2.032	2.032	2.032	2.033	2.033	2.034
NeuAc[3,4]	H-3ax	—	—	1.800	1.799	—	—
	H-3eq	—	—	2.756	2.755	—	—
	NAc	—	—	2.032	2.031	—	—
NeuAc[6]	H-3ax	1.692	1.692	—	—	—	—
	H-3eq	2.722	2.723	—	—	—	—
	NAc	2.032	2.032	—	—	—	—

[a] Chemical shifts are expressed in ppm downfield from internal DSS in D_2O at 27°C, acquired at 500 MHz.

[b] In the table heading, structures are represented by symbolic notation: ◇, GalNAc-ol; ■, Gal; △, NeuAcα(2→3); ●, GlcNAc; ○, NeuAcα(2→6). See Table III for complete structures.

[c] The superscript for each sugar residue indicates to which carbon atom of the adjacent monosaccharide it is linked. For example, Gal[3] means Gal β(1→3)-linked.

[d] Compounds R-O-1 and R-O-2 were isolated from human plasma galactoprotein (31) and from human platelet glycocalicin (32).

[e] Compound R-O-3 was obtained from cow milk κ-casein (13, 33).

[f] ND, not determined.

hydrate chains attached to its protein backbone. Since alkaline borohydride reductive cleavage (β-elimination) is routinely used to degrade the peptide of glycoproteins that contain both N-type and O-type carbohydrate chains (43–46), this procedure was employed to degrade the proteoglycan for subsequent isolation of its glycans. β-Elimination of the ChS-PG under relatively mild conditions resulted in the recovery of most of the N-type chains as glycopeptides and most of the mucin O-type chains as peptide-free alditols. We demonstrate in this study that this hydrolysis technique in combination with ^{1}H NMR analysis can be also used to deduce the structure of the linkage region of O-linked glycosaminoglycans. The structures of the low M_r carbohydrates bound to human aorta ChS-PG, together with their relative abundances, are compiled in Table III and were deduced by a combination of 500-MHZ ^{1}H NMR spectroscopy and quantitative sugar analyses. Based on the evidence for the N-type structures (Compounds I and II), and by analogy to the structures of the alditols found in fractions 5 and 6, we propose that gel filtration fractions 3 and 4 (Fig. 3) consist of glycopeptides containing Compounds I, II, III, and IV in the approximate molar ratios of 7:3:2:5 and 10:0:1:4, respectively. The inability to separate the *N*-glycans from the *O*-glycans by subsequent concanavalin-A Sepharose chromatography even after pronase and papain treatments indicates that the different oligosaccharide chains in fractions 3 and 4 are probably attached to a common protease-resistant portion of the peptide core. Fraction 5 consists of the oligosaccharide alditols of Compounds III, IV, and V in the approximate molar ratio of 6:2:5, while fraction 6 contains Compounds V and VI in the ratio of 1:5. The finding that all Sephadex G-50 fractions derived from the human aorta ChS-PG contained more than one glycan raises the possibility that an unknown structural determinant of a compound in Table III may have been missed because of the complexity of the ^{1}H NMR analysis. Nevertheless, because the ^{1}H NMR spectrum for each of these glycans is known from earlier investigations on other glycoconjugates, we are reasonably certain of their structures. It should be noted that the structure of Compounds III and IV were deduced earlier when present in a 1:10 ratio in a mixture of β-elimination products from human plasma galactoprotein (31).

Proteoglycans of other tissue origins and of other species have also been reported to contain the same O-linked oligosaccharides (3, 4, 6) that are bound to the human aorta ChS-PG. Except for the presence of NeuAc, these latter oligosaccharides are identical in structure to the reducing terminus of skeletal keratan sulfate (47), which is the region of the keratan sulfate chain that is linked to the protein core. The weak recognition of the purified ChS-PG by the anti-keratan sulfate antibody indicates that the purified aorta-derived molecules differ from the proteoglycans present in cartilage in being relatively deficient in macromolecular keratan sulfate. This observation confirms those findings reported in our earlier investigation that little keratan sulfate of large M_r is present in the human aorta (1). The relative lack of biosynthesis of keratan sulfate beyond the first three monosaccharides suggests either the absence of the required glycosyltransferase system necessary to complete the biosynthesis of keratan sulfate or the presence of unusually high levels of specific sialic acid transferases that terminate further polymerization of the oligosaccharides.

In recent years considerable progress has been made in the isolation and characterization of aorta proteoglycans (16, 28, 48–56). However, no reports on the structures of the *N*- and *O*-glycosidic oligosaccharides bound to aorta proteoglycans have been made. The elucidation of the structures of these glycans will enable future studies to address the biologic relevance of these oligosaccharides on ChS-PG. At present their functions are unknown, but by analogy to circulating glycoproteins, one may speculate that they inhibit the proteolytic degradation of this important aorta matrix proteoglycan.

TABLE VI

^{1}H CHEMICAL SHIFTS OF STRUCTURAL-REPORTER GROUPS OF CONSTITUENT MONOSACCHARIDES FOR THE PROTEOGLYCAN-TYPE OLIGOSACCHARIDE-ALDITOLS (COMPOUND V) PRESENT IN SEPHADEX G-50 FRACTIONS 5 AND 6, OBTAINED AFTER β-ELIMINATION OF CHONDROITINASE ABC-TREATED HUMAN AORTA ChS-PG, TOGETHER WITH SOME REFERENCE DATA

Residue[c]	Reporter group	S at GalNAc	Chemical shift[a] in[b]								
			Fraction 5 V	GGXol	ΔdiOS[d]	Δdi4S[d]	Δdi6S[d]	R-P1[e]	R-P2[e]	Ch4S tetra[d]	Ch6S tetra[d]
Xyl-ol	H-4		3.985	3.989	—	—	—	—	—	—	—
Gal[4]	H-1		4.615	4.620	—	—	—	4.545	4.531	—	—
	H-4		4.194	4.200	—	—	—	4.185	4.190	—	—
Gal[3]	H-1		4.670	4.620	—	—	—	4.664	4.664	—	—
	H-4		4.154	3.926	—	—	—	4.155	4.161	—	—
GlcUA	H-1	0	4.670	—	—	—	—	4.664	—	—	—
		4/6	4.68	—	—	—	—	—	4.673	4.476[f]	4.513[f]
	H-2		3.46	—	—	—	—	3.454	3.455	3.394	3.339
GalNAc	H-1	0/6	4.54	—	4.706[g]	—	4.732[g]	4.538	—	—	4.543[i]
		4	4.615	—	—	ND	—	—	4.616	4.566[h]	—
	H-4	0/6	ND	—	4.17	—	4.23	4.098	—	—	4.169
		4	ND	—	—	4.62	—	—	4.623	4.799	—
	NAc	0	2.057	—	2.055	—	—	2.057	—	—	—
		4	2.095	—	—	2.089	—	—	2.096	2.044	—
		6	2.052	—	—	—	2.051	—	—	—	2.019
Δ[4,5]GlcUA	H-1	0/6	5.183	—	5.195[g]	—	5.189[g]	5.183	—	—	—
		4	5.266	—	—	5.275[g]	—	—	5.265	—	—
	H-4	0/6	5.813	—	5.906	—	5.886	5.894	—	—	—
		4	5.953	—	—	6.013	—	—	5.966	—	—

[a] Chemical shifts are expressed in ppm downfield from internal DSS in D_2O. They were acquired at 500 MHz and 27°C for compound V in fraction 5, GGXol, R-P1, and R-P2, and at 360 MHz and 25°C for the other compounds.

[b] For complete structure of compound V in fraction 5, see Table III. Abbreviations and structures of the reference compounds are as follows: GGXol, Galβ(1→3)Galβ(1→4)Xyl-ol(see Ref. 40); ΔdiOS, Δ[4,5]GlcUAβ(1→3)GalNAc; Δdi4S, Δ[4,5]GlcUAβ(1→3)GalNAc-4-sulfate; Δdi6S, Δ[4,5]GlcUAβ(1→3)GalNAc-6-sulfate; R-P1, Δ[4,5]GlcUAβ(1→3)GalNAcβ(1→4)GlcUAβ(1→3)Galβ(1→3)Galβ(1→4)Xylβ(1→0)Ser;

R-P2, Δ[4,5]GlcUAβ(1→3)GalNAcβ(1→4)GlcUAβ(1→3)Galβ(1→3)Galβ(1→4)Xylβ(1→0)Ser

4-sulfate

Ch4S-tetra, GlcUAβ(1→3)GalNAcβ(1→4)GlcUAβ(1→3)GalNAc
(4-sulfate, 4-sulfate)

Ch6S-tetra, GlcUAβ(1→3)GalNAcβ(1→4)GlcUAβ(1→3)GalNAc
(6-sulfate, 6-sulfate)

[c] For explanation of superscript notation, see Table V (footnote *c*).
[d] The unsaturated disaccharides and the tetrasaccharides from chondroitin sulfates were kindly provided by Dr. A. Kimura (41).
[e] The compounds R-P1 and R-P2 were isolated by Dr. K. Sugahara from rat chondrosarcoma proteoglycans. The NMR spectra of these compounds and related structures are to be published.
[f] For the internal GlcUA residue in the β-anomer of the oligosaccharide.
[g] For comparative purposes, only the values for the β-anomer of the oligosaccharide have been listed here.
[h] ND, not determined.
[i] For the internal GalNAc residue.

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