A Major Common Trisulfated Hexasaccharide Core Sequence, Hexuronic Acid(2-Sulfate)-Glucosamine(N-Sulfate)-Iduronic Acid-N-Acetyglucosamine-Glucuronic Acid-Glucosamine(N-Sulfate), Isolated from the Low Sulfated Irregular Region of Porcine Intestinal Heparin*

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The major structure of the low sulfated irregular region of porcine intestinal heparin was investigated by characterizing the hexasaccharide fraction prepared by extensive digestion of the highly sulfated region with Flavobacterium heparinase and subsequent size fractionation by gel chromatography. Structures of a tetrasaccharide, a pentasaccharide, and eight hexasaccharide components in this fraction, which accounted for approximately 19% (w/w) of the starting heparin representing the major oligosaccharide fraction derived from the irregular region, were determined by chemical and enzymatic analyses as well as 1H NMR spectroscopy. Five compounds including one penta- and four hexasaccharides had hitherto unreported structures. The structure of the pentasaccharide with a glucuronic acid at the reducing terminus was assumed to be derived from the reducing terminus of a heparin glycosaminoglycan chain and may represent the reducing terminus exposed by a tissue endo-β-glucuronidase involved in the intracellular post-synthetic fragmentation of macromolecular heparin. Eight out of the 10 isolated oligosaccharides shared the trisaccharide sequence, -4IdceA1–4GlcNAc1–4GlcAβ1–4Glc(N-sulfate)α1–4HexA(2-sulfate)1–4, and its reverse sequence, -4GlcAβ1–4GlcNAc1–4IdceA1–, was not found. The latter has not been reported to date for heparin/heparan sulfate, indicating the substrate specificity of the β-glucuronoyl C-5 epimerase. Furthermore, seven hexasaccharides shared the common trisulfated hexasaccharide core sequence ΔHexA(2-sulfate)α1–4Glc(N-sulfate)α1–4IdceA1–4GlcNAc1–4GlcAβ1–4Glc(N-sulfate)α1–4HexA(2-sulfate)1–4, which contained the above trisaccharide sequence (ΔHexA, IdecA, GlcN, and GlcA represent 4-deoxy-a-threo-hex-4-ene-pyranosyluronic acid, β-iduronic acid, β-glucosamine, and β-glucuronic acid, respectively) and additional sulfate groups. The specificity of the heparinase used for preparation of the oligosaccharides indicates the occurrence of the common pentasulfated octasaccharide core sequence, -4Glc(N-sulfate)α1–4HexA(2-sulfate)1–4

GlcN(N-sulfate)α1–4IdceAα1–4GlcNAcα1–4GlcAβ1–4Glc(N-sulfate)α1–4HexA(2-sulfate)1–4, where the central hexasaccharide is flanked by Glc(N-sulfate) and HexA(2-sulfate) on the nonreducing and reducing sides, respectively. The revealed common sequence constituted a low sulfated trisaccharide representing the irregular region sandwiched by highly sulfated regions and should reflect the control mechanism of heparin biosynthesis.

Heparin is a highly sulfated co-polymer of glucosamine and uronic acid residues that are alternatively 1→4-linked. Most of the heparin molecule is accounted for by the major trisulfated disaccharide repeating unit, -4IdceA(2-sulfate)α1→4GlcNα1→6. This repeating sequence forms highly sulfated regions and represents at least 75% heparin from porcine intestine (1). Under sulfation and substantial structural variability are observed in the rest of the region which is called the irregular region and distributed along the chain flanked by the fully sulfated region composed of the trisulfated disaccharide units, accounting for approximately one-quarter of the heparin polysaccharide chain (for reviews see Refs. 2–4).

Heparin exhibits a wide range of biological activities such as inhibition of blood coagulation (5), modulation of cellular proliferation (6, 7), potentiation of angiogenesis (8), and interactions with various growth factors (9–12). These activities result from the ability of heparin to interact with various proteins causing their activation, deactivation, or stabilization. Interactions between heparin and proteins generally depend on the presence of sulfate groups. Some proteins such as lipoprotein lipase (13), thrombin (14), and platelet factor 4 (15) bind to the highly sulfated region consisting exclusively of the trisulfated disaccharide unit in a seemingly nonspecific fashion. Still, many other proteins are thought to require specific sequences for binding, and the precise requirement for individual sulfate groups may vary from one protein to another. However, it should be noted that heparin is oversulfated and contains not only the essential sulfate groups but also additional nonessential ones for protein binding. The contribution of the irregular region to the biological activities of heparin is not well understood mainly because of the difficulty in analyzing the variably sulfated structure. However, it is well known that the antithrombin-binding minimum pentasaccharide sequence GlcN(6S)1

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1The abbreviations used are: 6S, 6-O-sulfate; NS, 2-N-sulfate; 2S, 2-O-sulfate; 8S, 3-O-sulfate; HPLC, high performance liquid chromatography; HexA, hexuronic acid; ΔHexA, 4-deoxy-a-threo-hex-4-ene-pyranosyluronic acid; MALDI TOF, matrix-assisted laser desorption ionization time-of-flight.
Major Common Sequence in the Low Sulfated Region of Heparin

α1-4GlcAβ1-4GlcN(NS,3S)α1-4IdcaA(2S)α1-4GlcN(NS,6S) (16, 17) is embedded over the connecting border of the high and low sulfated regions and contains both low and high sulfated disaccharide units. Hence, it is conceivable that the low sulfated irregular region is involved in some other active domains as well.

Biosynthetic reactions required to generate heparin sequences include the formation of an initial, simple polysaccharide structure, →4GlcAβ1-4GlcNα1-4, that is subsequently modified through N-deacetylation/N-sulfation of GlcNAc units, C-5 epimerization of GlcA to Idca units, and O-sulfation at C-2 of Idca and C-6 of GlcN units (for review see Ref. 4). The undersulfated region of the final product heparin is thought to have escaped such modifications. However, the mechanism by which certain residues are selected for modification is not understood, partly because of a lack of sequence information on relatively large fragments although certain rules about the sequential arrangement of various disaccharide units have been noted (4).

In this study we isolated and systematically characterized 10 oligosaccharide structures from the undersulfated region of porcine intestinal heparin after extensive digestion with heparinase, which acts only on the highly sulfated repeating region, to investigate the variability and/or regularity, if any, of the low sulfated region. A majority of these oligosaccharides were revealed to share a common trisulfated hexasaccharide core sequence.

EXPERIMENTAL PROCEDURES

Enzymes and Oligosaccharides—Stage 14 heparin was purchased from American Diagnostica, New York, and purified by DEAE-cellulose chromatography as reported (18). Heparinase (EC 4.2.2.7) and purified heparitinases I (EC 4.2.2.8) and II (no EC number) were obtained from Seikagaku Corp., Tokyo, Δ5-6-Glycuronate-2-sulfatase (EC 3.1.6.-), abbreviated as ΔHexuronate-2-sulfatase, was purified from Flavobacterium heparinum (19). β-Glucoronidase (EC 3.2.1.31) purified to homogeneity from Ampullaria (freshwater apple shell) hepatopancreas (20) was obtained from Tokyo Zouki Chemical Co., Tokyo. Human liver α-iduronidase (EC 3.2.1.76) was a gift from Dr. J. J. Hopwood, Adelaide Children’s Hospital (21). The hexasaccharide fraction was prepared from stage 14 heparin after heparinase digestion as described (22). A peptide (Arg-Gly)_15 was custom-synthesized by Peptide Institute, Inc., Osaka.

Digestion of the Isolated Oligosaccharides with Heparinase, Heparitinases, or ΔHexuronate-2-Sulfatase—Each isolated oligosaccharide (0.5–1.0 nmol) was digested using 1–5 mIU of heparinase I and/or II, or ΔHexuronate-2-sulfatase as described previously (18, 23, 24). Digestive enzymatic digestion of a given oligosaccharide with ΔHexuronate-2-sulfatase and then heparitinase I or II was also carried out as reported (22). Reactions were terminated by boiling for 1 min, and the reaction mixture was analyzed by HPLC as reported (18).

α-Iduronidase and β-Glucuronidase Digestion of Tetrasaccharides Obtained by Deamination—Tetrasaccharides obtained by deamination of the hexasaccharides in fractions 6-26 and 6-27 were tested for their sensitivities to α-iduronidase and β-glucuronidase to determine the isomer type of the uronic acid residue exposed at the nonreducing termini. Each heparin hexasaccharide (2.0 nmol) was treated at room temperature with HNO₂ at pH 1.5 for 30 min (25), and the resultant diand/or tetrasaccharides were reduced under alkaline conditions using [H]sodium borohydride (0.50 mCi) as reported (22). Each resultant [H]tetrasaccharide (2.6 pmol) corresponding to approximately 2.9 × 10⁶ dpm was digested using 17.7 mIU α-iduronidase or 48.9 mIU β-glucuronidase as reported (22).

500-MHz 1H NMR Spectroscopy—Oligosaccharides for NMR analysis were fully sodiated using a Dowex 50-X8 (Na⁺ form) column (7 × 18 mm) and then repeatedly exchanged in D₂O with intermediate lyophilization. 500-MHz 1H NMR spectra of hexasaccharides were measured on a Varian VXR-500 at a probe temperature of 26 °C as reported (26, 27). Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly relative to acetone (δ 2.225) in D₂O (28).

Matrix-assisted Laser Desorption Ionization (MALDI) Time-of-flight (TOF) Mass Spectrometry—MALDI TOF mass spectra of a sulfated heparin oligosaccharide were recorded on a Compact MALDI 1 (Shimadzu/Kratos) linear instrument in the positive ion mode at Toray Research Center, Kanagawa, Japan. Ultraviolet MALDI experiments were carried out using an Nd:YAG laser (Laser Science, Newton, MA, 337-nm wavelength, 3-n pulse width). The ions were accelerated to 20 keV energy. Caffeic acid was used as a matrix at a concentration of 10 mg/ml in 50% water/methanol mixture. A synthetic peptide (Arg-Gly)₉, was used as a complexing agent to shield the negatively charged groups of a sulfated oligosaccharide according to Juhasz and Biemann (29). Aqueous solutions of a heparin oligosaccharide (10 pmol/μl) and the peptide (10 pmol/μl) were mixed in advance and then diluted with an equivolum proportion of the matrix solution. Of this sample/matrix solution, 0.5–1.0 μl was placed on the probe surface and dried under a stream of air.

RESULTS

Isolation of the Oligosaccharides—Purified stage 14 heparin from porcine intestine was exhaustively digested with heparinase and fractionated into fractions 1–8 by gel filtration on a column of Bio-Gel P-10 (22). Fraction 8 contained disaccharides, and the major fraction 7, which contained tetrasaccharides, was characterized previously (22). In this study fraction 6 was characterized. Fractions 6, 7, and 8 represented approximately 19, 24, and 32% (w/w) of the starting heparin, respectively. Amino sugar and uronic acid analyses showed that fraction 6 contained hexasaccharides. Fraction 6 was subfractionated by HPLC on an amine-bound silica column to fractions 6-1 to -37, as indicated in Fig. 1. Twelve fractions, 6-1, -6, -17, -22, -23, -25, -26, -27, -30, -31, -32, and -34, were further purified by rechromatography. They altogether accounted for 61 mol% (as ΔHexA) of the oligosaccharides obtained from fraction 6. These individual fractions gave a single peak on HPLC but were 98, 91, 81, 57, 75, 95, 66, 89, 82, 80, 93, and 99% pure, respectively, when examined by capillary electrophoresis (results not shown). Characterization of fractions 6-1 and 6-6, which turned out to contain dermatan sulfate-derived oligosaccharides, will be described elsewhere. In this article, analyses of the other fractions are described.

Enzymatic Characterization of the Isolated Oligosaccharides—Disaccharide compositions of the isolated oligosaccharides were determined by digestion with heparitinases I and/or II, followed by HPLC analysis on an amine-bound silica column. Substrate specificities of the three heparin lyases are shown in Fig. 2. Most of the fractions except for fractions 6-32 and 6-34 were degraded into disaccharides by the enzymes, although fractions 6-32 and 6-34 were degraded into approximately 1 mol each of a di- and a tetrasaccharide unit. The results are summarized in Table I. Recoveries of the oligosaccharides in Table I were calculated taking the absorbance of the parent oligosaccharide in each fraction at 100%. Excess or insufficient recoveries of the products were observed in some cases partly due to the products derived from possible impurities and partly due to the products derived from possible impurities and partly due to the use of the average millimolar absorption coefficient of 5.5 × 10⁶ obtained from unsaturated disaccharides (31) for quantification of oligosaccharides. Millimolar absorption coefficients of individual oligosaccharides derived from the average value due to various degrees. As representative chromatograms, those obtained with fractions 6-23 and 27 are shown in Fig. 3. Fractions 6-23 and 27 yielded ΔHexA-GlcNAc(6S) and ΔHexA(2S)-GlcN(6S,8S) in a molar ratio of 1.0:1.4 and ΔHexA-GlcNAc(6S), ΔHexA-GlcN(6S,8S), and ΔHexA(2S)-GlcN(6S,8S) in a molar ratio of 0.9:1.0:1.1, respectively, upon heparitinase I digestion. A sequential arrange-
are summarized in Table II. The NMR data obtained in this study for the oligosaccharides were also analyzed by 500-MHz $^1$H NMR, and individual monosaccharide units were identified based on the chemical shifts of the proton signals and the coupling constants $J_{1,2}$. Chemical shifts were assigned by two-dimensional homonuclear Hartmann-Hahn and correlation spectroscopy analyses (data not shown) as reported for the sulfated oligosaccharides isolated previously from heparin (26) and heparan sulfate (33). Although some fractions were still mixtures as revealed by capillary electrophoresis, it was possible to extract sequence information about the major compound in these fractions by taking advantage of $^1$H NMR spectroscopy. Since peak heights of resonances reflect molar ratios of the components, signals for the major compound in the mixtures could be easily distinguished from those of the minor impurities. The internal uronic acid residues of each isolated oligosaccharide were unambiguously identified based on the chemical shifts of the anomeric proton signals and the coupling constants $J_{1,2}$. Anomeric proton signals of a GlcA residue in heparin/heparan sulfate oligosaccharides are observed around $\delta 5.2-5.5$ and 4.7-4.5, respectively (22, 35). The coupling constants $J_{1,2}$ of a GlcA residue in heparin/heparan sulfate oligosaccharides are approximately 3.0 and 8.0 Hz, respectively (22, 36). The NMR data obtained in this study for the oligosaccharides are summarized in Table II.

Fraction 6-25—When fraction 6-25 was digested with heparitinase II, it yielded equimolar amounts of $\Delta$HexA-GlcN(NS,6S) and a component that eluted near the elution position of a trisulfated tetrasaccharide (Table I), indicating that $\Delta$HexA-GlcN(NS,6S) was derived from the reducing end. Therefore, the compound in fraction 6-25 is a pentasulfated hexasaccharide with a sequence of $\Delta$HexA-(2S)-GlcN(NS)-HexA(2S)-GlcNAc-HexA-GlcN(NS,6S).

In the spectrum of fraction 6-25, six individual saccharide residues were readily identified. Two internal uronic acid residues were determined as IdceA-4 and GlcA-2 based on the chemical shifts of the anomeric proton signals, at $\delta 5.192$ and 4.574, respectively, as well as the coupling constants $J_{1,2}$ of 2.0 and 8.0 Hz, respectively. The chemical shifts of H-1 and H-2 of the IdceA residue of the compound in fraction 6-25 were shifted downfield by approximately 0.2 and 0.6 ppm, respectively, when compared with those of the nonsulfated IdceA residue of the oligosaccharides isolated from bovine kidney heparan sulfate or porcine intestinal heparin (22, 33). In contrast, the proton chemical shifts of the GlcA residue of the compound in this fraction were very similar to those of the nonsulfated GlcA residue. These results indicated the 2-sulfation of IdceA and GlcA-2 of this compound. Based upon these NMR data and the sequential arrangement of the disaccharide units determined by enzymatic analysis, the structure of the major compound in fraction 6-25 has been determined as the following.

$$\Delta$$HexA(2S)$\alpha1-4$(GlcN(NS)$\alpha1-4$(IdceA(2S)$\alpha1-4$GlcNAc)

$\alpha1-4$(GlcA)$\alpha1-4$(GlcN(NS,6S))

**Structure 1. Fraction 6-25.**

Fraction 6-30—When fraction 6-30 was digested with heparitinase II, it yielded approximately equimolar amounts of $\Delta$HexA-(2S)-GlcN(NS,6S), $\Delta$HexA-GlcN(NS,6S), and $\Delta$HexA-(2S)-GlcNAc (Table I). Upon successive digestion with hexuronate-2-sulfatase and then heparitinase II, it yielded equimolar amounts of $\Delta$HexA-(2S)-GlcNAc, $\Delta$HexA-GlcN(NS,6S), and $\Delta$HexA-GlcN(NS) (Table I). These results indicated that the disaccharide unit on the nonreducing terminus was $\Delta$HexA(2S)-GlcN(NS). When digested with heparitinase I, fraction 6-25 gave rise to equimolar amounts of $\Delta$HexA-GlcN(NS,6S) and a component that eluted near the elution position of a trisulfated tetrasaccharide (Table I), indicating that $\Delta$HexA-GlcN(NS,6S) was derived from the reducing end. Therefore, the compound in fraction 6-25 is a pentasulfated hexasaccharide with a sequence of $\Delta$HexA-(2S)-GlcN(NS)-HexA(2S)-GlcNAc-HexA-GlcN(NS,6S).

In the spectrum of fraction 6-25, six individual saccharide residues were readily identified. Two internal uronic acid residues were determined as IdceA-4 and GlcA-2 based on the chemical shifts of the anomeric proton signals, at $\delta 5.192$ and 4.574, respectively, as well as the coupling constants $J_{1,2}$ of 2.0 and 8.0 Hz, respectively. The chemical shifts of H-1 and H-2 of the IdceA residue of the compound in fraction 6-25 were shifted downfield by approximately 0.2 and 0.6 ppm, respectively, when compared with those of the nonsulfated IdceA residue of the oligosaccharides isolated from bovine kidney heparan sulfate or porcine intestinal heparin (22, 33). In contrast, the proton chemical shifts of the GlcA residue of the compound in this fraction were very similar to those of the nonsulfated GlcA residue. These results indicated the 2-sulfation of IdceA and the nonsulfation of GlcA-2 of this compound. Based upon these NMR data and the sequential arrangement of the disaccharide units determined by enzymatic analysis, the structure of the major compound in fraction 6-25 has been determined as the following.

$$\Delta$$HexA(2S)$\alpha1-4$(GlcN(NS)$\alpha1-4$(IdceA(2S)$\alpha1-4$GlcNAc)

$\alpha1-4$(GlcA)$\alpha1-4$(GlcN(NS,6S))

**Structure 1. Fraction 6-25.**
4.572, respectively, as well as the coupling constants $J$ and then heparitinase II, it yielded D$_D$D$D$1866 results indicated the 2-sulfation of IdceA residues were very similar to those of the nonsulfated GlcA residue. These enzymes can cleave both glucosaminidic linkages (marked by II (heparinase II). These enzymes can cleave both glucosaminidic linkages (marked by asterisk) adjacent to the disaccharide cleavage site has to be free for sensitivity to heparitinases I and II (26).

In the spectrum of fraction 6-30, two internal uronic acid residues were identified as IdceA-4 and GlcA-2 based on the chemical shifts of the anomeric proton signals, at $\delta$ 5.203 and 5.472, respectively, as well as the coupling constants $J_{1,2}$ of 1.5 and 7.5 Hz, respectively. The chemical shifts of H-1 and H-2 of the IdceA residue of the compound in fraction 6-30 were shifted downfield by approximately 0.2 and 0.6 ppm, respectively, when compared with those of the nonsulfated GlcA residue. These results indicated the 2-sulfation of IdceA-4 and the nonsulfation of GlcA-2 of this compound. Based upon these NMR data and the sequential arrangement of the disaccharide units determined by enzymatic analysis, the structure of the major compound in fraction 6-30 has been determined as the following.

$$\Delta\text{HexA(2S)\alpha1-4GlcN(\alphaS,\betaS)\alpha1-4\text{IdceA(2S)\alpha1}}$$

4GlcNAc-1-4GlcA\beta1-4GlcN(\alphaS,\betaS)

**STRUCTURE 2.** Fraction 6-30.

 Fraction 6-22—Fraction 6-22 was resolved into several subcomponents by capillary electrophoresis (data not shown), the major component accounting for only 57% of the UV absorbing materials in this fraction. However, it was not possible to fractionate it preparatively into its subcomponents. Therefore, it was first digested with $\Delta$hexuronate-2-sulfatase and then the digest was analyzed by HPLC. The $\Delta$hexuronate-2-sulfatase digest gave four peaks in a molar ratio of 7:24:54:15, all of which eluted 11–16 min earlier than the parent fraction (data not shown), indicating that fraction 6-22 was a mixture of at least four different compounds. The major product, designated as fraction 6-22S-1, was isolated and subjected to structural analysis. The yield of fraction 6-22S-1 was 42 nmol/100 mg starting heparin. When examined by capillary electrophoresis, fraction 6-22S-1 was 69% pure (results not shown). Upon heparitinase II digestion, fraction 6-22S-1 yielded $\Delta$HexA-GlcN(NS) and $\Delta$HexA(2S)-GlcNAc with the recoveries of 220 and 66%, respectively, taking the UV absorbance of the total parent oligosaccharides in fraction 6-22S-1 as 100% (Table I). Amino sugar and uronic acid analyses showed that fraction 6-22S-1 contained a heparinase as a major compound. These results altogether indicate that the major compound in this fraction was composed of 2 mol of $\Delta$HexA-GlcN(NS) and 1 mol of $\Delta$HexA(2S)-GlcNAc. The excess recovery of $\Delta$HexA-GlcN(NS) upon heparitinase II digestion over the other product was probably due to the disaccharide produced by degradation of contaminating oligosaccharide(s). Since this fraction was isolated after $\Delta$hexuronate-2-sulfatase digestion, the disaccharide unit on the nonreducing terminus of the parent hexasaccharide was not $\Delta$HexA(2S)-GlcNAc but $\Delta$HexA-GlcN(NS). Heparitinase I digestion of both fractions 6-22 and 6-22S-1 resulted in mainly two unsaturated components, the monosulfated disaccharide $\Delta$HexA-GlcN(NS) and a presumable tetrasaccharide component that eluted near the elution position of the tri- or disulfated tetrasaccharide. Recoveries of the di- and tetrasaccharide components from fraction 6-22 or 6-22S-1 were 56 and 49% or 51 and 83%, respectively (Table I). The low recoveries of the components from fraction 6-22 were due to the corresponding low content of the major component in the fraction. Since the presumable tetrasaccharide peak from fraction 6-22 was shifted by the prior $\Delta$hexuronate-2-sulfatase digestion to the position corresponding to the loss of one sulfate group on HPLC, the tetrasaccharide component was derived from the nonreducing terminus. Therefore, the structure of the compound in fraction 6-22S-1 is $\Delta$HexA-GlcN(NS)-HexA(2S)-GlcNAc-HexA-GlcN(NS). Consequently, the major compound in the parent fraction 6-22 is a tetrasulfated hexasaccharide with a sequence of $\Delta$HexA(2S)-GlcN(NS)-HexA(2S)-GlcNAc-HexA-GlcN(NS).

In the spectrum of fraction 6-22S-1, two internal uronic acid residues were identified as IdceA-4 and GlcA-2 based on the chemical shifts of the anomeric proton signals, at $\delta$ 5.217 and 4.542, respectively, as well as the coupling constants $J_{1,2}$ of 2.0 and 8.0 Hz, respectively. The chemical shifts of H-1 and H-2 of the IdceA residue of the compound in fractions 6-22S-1 were shifted downfield by approximately 0.2 and 0.6 ppm, respectively, when compared with those of the nonsulfated IdceA resi-
After each oligosaccharide fraction was incubated with heparitinase I, with 2-sulfatase and then heparitinase II, or with heparitinase II, the reaction products were characterized by HPLC. Recoveries of the disaccharides calculated based on absorption at 232 nm taking the absorbance of the parent oligosaccharide(s) in each fraction as 100% are shown in parentheses.

### Table I

Enzymatic analysis of the isolated oligosaccharides

| Fraction | Yield* | Heparitinase I digest | Heparitinase II digest | 2-Sulfatase/Heparitinase II digest |
|----------|--------|-----------------------|------------------------|-----------------------------------|
| 6–17     | 86     | \(\Delta\text{HexA-GlcNAc(6S)}\) (63%); \(\Delta\text{HexA(2S)-Glc(NS)}\) (62%) | ND                     | \(\Delta\text{HexA-GlcNAc(6S)}\) (62%); \(\Delta\text{HexA-Glc(NS)}\) (67%) |
| 6–22     | 77     | \(\Delta\text{HexA-Glc(NS)}\) (56%); and a trisulfated tetrasaccharide (49%) | ND                     | ND                                 |
| 6–22S–1  | 42     | \(\Delta\text{HexA-Glc(NS)}\) (91%); and a disulfated tetrasaccharide (83%) | \(\Delta\text{HexA-Glc(NS)}\) (220%); \(\Delta\text{HexA(2S)-Glc(NS)}\) (66%) | ND                                 |
| 6–23     | 284    | \(\Delta\text{HexA-GlcNAc(6S)}\) (88%); and \(\Delta\text{HexA(2S)-Glc(NS)}\) (121%) | ND                     | ND                                 |
| 6–25     | 65     | \(\Delta\text{HexA-Glc(NS,NS)}\) (72%); and a trisulfated tetrasaccharide (83%) | \(\Delta\text{HexA(2S)-GlcNAc(6S)}\); \(\Delta\text{HexA-Glc(NS,NS)}\) (82%); and \(\Delta\text{HexA(2S)-Glc(NS)}\) (82%) | ND                                 |
| 6–26     | 142    | \(\Delta\text{HexA-GlcNAc(6S)}\) (53%); \(\Delta\text{HexA-Glc(NS)}\) (67%); and \(\Delta\text{HexA(2S)-Glc(NS)}\) (67%) | ND                     | \(\Delta\text{HexA-GlcNAc(6S)}\) (53%); \(\Delta\text{HexA-Glc(NS)}\) (53%); and \(\Delta\text{HexA(2S)-Glc(NS)}\) (94%) |
| 6–27     | 273    | \(\Delta\text{HexA-GlcNAc(6S)}\) (92%); \(\Delta\text{HexA-Glc(NS,NS)}\) (104%); and \(\Delta\text{HexA(2S)-Glc(NS,NS)}\) (109%) | ND                     | \(\Delta\text{HexA-GlcNAc(6S)}\) (69%); and \(\Delta\text{HexA-Glc(NS,NS)}\) (179%) |
| 6–30     | 186    | \(\Delta\text{HexA-Glc(NS,NS)}\) (118%); and a tetrasulfated tetrasaccharide (102%) | \(\Delta\text{HexA(2S)-GlcNAc(6S)}\); \(\Delta\text{HexA-Glc(NS,NS)}\) (100%); and \(\Delta\text{HexA(2S)-Glc(NS,NS)}\) (138%) | ND                                 |
| 6–31     | 121    | \(\Delta\text{HexA-Glc(NS,NS)}\) (155%); and \(\Delta\text{HexA(2S)-Glc(NS,NS)}\) (92%) | ND                     | \(\Delta\text{HexA-Glc(NS,NS)}\) (227%) |
| 6–32     | 122    | \(\Delta\text{HexA(2S)-Glc(NS,NS)}\) (107%); and a trisulfated tetrasaccharide (69%) | \(\Delta\text{HexA(2S)-Glc(NS,NS)}\) (127%); and a trisulfated tetrasaccharide (73%) | \(\Delta\text{HexA-Glc(NS,NS)}\) (136%); and a trisulfated tetrasaccharide (77%) |
| 6–34     | 264    | \(\Delta\text{HexA(2S)-Glc(NS,NS)}\) (158%); and a tetrasulfated tetrasaccharide (110%) | \(\Delta\text{HexA(2S)-Glc(NS,NS)}\) (147%); and a tetrasulfated tetrasaccharide (119%) | \(\Delta\text{HexA-Glc(NS,NS)}\) (129%); and a tetrasulfated tetrasaccharide (127%) |

* nmol/100 mg of heparin.

\(\Delta\) ND, not determined.

of the oligosaccharides isolated from bovine kidney heparan sulfate or porcine intestinal heparin (22, 33). In contrast, the proton chemical shifts of the GlcA residue of the compound in this fraction were very similar to those of the nonsulfated GlcA residue. These results indicated the 2-sulfation of IdecA-4 and the nonsulfation of GlcA-2 of this compound. Based upon these NMR data and the sequential arrangement of the disaccharide units determined by enzymatic analysis, the structure of the major compound in fraction 6-22S-1 has been determined as follows: fraction 6-22S-1, \(\Delta\text{HexA-1–4Glc(NS)}\); \(\Delta\text{IdcA-1–4Glc} \); \(\Delta\text{GlcaAc1–4Glc} \); \(\Delta\text{GlcAp1–4Glc(NS)}\). Since fraction 6-22S-1 was isolated after hexuronate-2-sulfatase digestion as described above, the major compound in the parent fraction 6-22 contained the following structure.

\[ \Delta\text{HexA(2S)-IdcA-1–4Glc(NS)} \]

Structure 3. Fraction 6-22.

Fractions 6-17, -27, -32, and -34—The spectral data of fraction 6-27 (Table II) were indistinguishable from those of fraction b-15 obtained from porcine intestinal heparin reported previously (24). Hence, the major compound in fraction 6-27 was identical with \(\Delta\text{HexA(2S)-1–4Glc(NS)}\); \(\Delta\text{IdcA-1–4Glc} \); \(\Delta\text{GlcaAc1–4Glc} \); \(\Delta\text{GlcAp1–4Glc(NS)}\). Likewise, \(\Delta\) H NMR data of fractions 6-17, -32, and -34 (Table II) were also indistinguishable from those of fractions VII, b-20, and b-22, respectively, that were obtained from porcine intestinal heparin as reported previously (22, 24), indicating that the major compounds in fractions 6-17, -32, and -34 were identical with those in the compounds in the above fractions, respectively. These structures are in good agreement with the results obtained from enzymatic characterization (Table I). Therefore, the struc-

![Fig. 3. HPLC analysis of the heparitinase I and/or II digests of the isolated oligosaccharides.](Image)

The oligosaccharides were digested with heparitinases I and/or II and fractionated by HPLC on an aminobound silica column. A, the heparitinase I digest of fraction 6-23 (0.5 nmol); B, the heparitinase I digest of fraction 6-27 (0.5 nmol). Elution positions of the standard disaccharides isolated from heparin/heparan sulfate are indicated in A as follows: 1, \(\Delta\text{HexA-GlcNAc} \); 2, \(\Delta\text{HexA-GlcNAc(6S)} \); 3, \(\Delta\text{HexA(2S)-GlcNAc} \); 4, \(\Delta\text{HexA-Glc(NS)} \); 5, \(\Delta\text{HexA-Glc(NS,NS)} \); 6, \(\Delta\text{HexA(2S)-Glc(NS)} \); 7, \(\Delta\text{HexA(2S)-Glc(NS,NS)} \); 8, \(\Delta\text{HexA(2S)-Glc(NS,NS)} \). The peak marked by an asterisk around 35 min is often observed upon high sensitivity analysis and is due to an unknown substance eluted from the column resin.
Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly relative to acetone (δ 2.225 ppm) in H₂O at 26 °C. The estimated error for the values to two decimal places was only ±0.01 ppm because of partial overlap of signals. That for the values to three decimal places was ±0.002 ppm. Coupling constants J₁₂ (in Hz) are given in parentheses.

| Residue group | fraction 6-17 | fraction 6-22-1 | fraction 6-23 | fraction 6-25 | fraction 6-26 | fraction 6-27 | fraction 6-30 | fraction 6-31 | fraction 6-32 | fraction 6-34 |
|---------------|--------------|----------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| GlcN-1 | H₁ | 5.519 (3.1) | 5.539 (2.3) | 5.539 (2.3) | 5.519 (4.0) | 5.367 (4.0) | 5.379 (4.0) | 5.347 (2.5) | 5.433 (2.6) | 5.433 (2.6) | 5.448 (3.5) |
| H₂ | 5.376 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₃ | 5.327 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₄ | 5.332 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₅ | 5.348 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₆ | 5.327 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₇ | 5.332 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₈ | 5.348 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₉ | 5.327 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₀ | 5.332 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₁ | 5.348 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₂ | 5.327 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₃ | 5.332 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₄ | 5.348 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₅ | 5.327 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₆ | 5.332 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₇ | 5.348 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₈ | 5.327 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₉ | 5.332 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₂₀ | 5.348 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |

a Symbols represent the following: open triangle, DHexA; closed triangle, DHexA(2S); open square, GlcA; open diamond, IdceA; closed diamond, IdceA(2S); closed circle, GlcN(NS,6S); circle half-closed on the left side, GlcNAc(6S); circle half-closed on the right side, GlcN(NS); double underline, GlcN(3S).

b Fraction 6–23 contains GlcA-1, GlcN-2, IdceA-3, GlcN-4, and DHexA-5.

c For fractions 6–17 and –23 values of DHexA-4 and -5, respectively, are presented.

d ND, not determined.

e —, not occurring.

**Table I**

| 1H chemical shifts of the constituent monosaccharides of the isolated oligosaccharides derived from heparin |
|---------------------------------------------------------------|
| Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly relative to acetone (δ 2.225 ppm) in H₂O at 26 °C. The estimated error for the values to two decimal places was only ±0.01 ppm because of partial overlap of signals. That for the values to three decimal places was ±0.002 ppm. Coupling constants J₁₂ (in Hz) are given in parentheses. |
| Residue group | fraction 6-17 | fraction 6-22-1 | fraction 6-23 | fraction 6-25 | fraction 6-26 | fraction 6-27 | fraction 6-30 | fraction 6-31 | fraction 6-32 | fraction 6-34 |
|----------------|--------------|----------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| GlcN-1 | H₁ | 5.519 (3.1) | 5.539 (2.3) | 5.539 (2.3) | 5.519 (4.0) | 5.367 (4.0) | 5.379 (4.0) | 5.347 (2.5) | 5.433 (2.6) | 5.433 (2.6) | 5.448 (3.5) |
| H₂ | 5.376 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₃ | 5.327 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₄ | 5.332 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₅ | 5.348 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₆ | 5.327 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₇ | 5.332 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₈ | 5.348 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₉ | 5.327 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₀ | 5.332 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₁ | 5.348 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₂ | 5.327 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₃ | 5.332 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₄ | 5.348 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₅ | 5.327 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₆ | 5.332 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₇ | 5.348 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₈ | 5.327 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₁₉ | 5.332 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| H₂₀ | 5.348 | 3.76 | ND | ND | ND | ND | ND | ND | ND | ND | ND |

a Symbols represent the following: open triangle, DHexA; closed triangle, DHexA(2S); open square, GlcA; open diamond, IdceA; closed diamond, IdceA(2S); closed circle, GlcN(NS,6S); circle half-closed on the left side, GlcNAc(6S); circle half-closed on the right side, GlcN(NS); double underline, GlcN(3S).

b Fraction 6–23 contains GlcA-1, GlcN-2, IdceA-3, GlcN-4, and DHexA-5.

c For fractions 6–17 and –23 values of DHexA-4 and -5, respectively, are presented.

d ND, not determined.

e —, not occurring.
a pentasulfated hexasaccharide composed of equimolar amounts of the above three components and accounted for only 53%, as judged by the recovery of one of the three major disaccharides. The sensitivity of this fraction to Δhexuronate-2-sulfatase indicated that the disaccharide unit on the nonreducing terminus was ΔHexA(2S)-GlcN(NS,6S) (Table I). Thus, the major compound in this fraction was a pentasulfated hexasaccharide with a sequence of either ΔHexA(2S)-GlcN(NS,6S)-HexA-GlcNAc(6S)-HexA-GlcN(NS) or ΔHexA(2S)-GlcN(NS,6S)-HexA-GlcN(NS)-HexA-GlcNAc(6S).

The two internal uronic acid residues of the hexasaccharide in fraction 6-26 were identified as IdceA and GlcA, based on the chemical shifts (δ 4.997 and 4.538) of the anomeric proton signals and the coupling constants J1,2 (1.5 and 8.0 Hz), respectively. Analysis of the deamination products identified the location of internal uronic acid residues. A tetrasaccharide component derived from the reducing side of the parent hexasaccharide was obtained by nitrous acid degradation. It was sensitive to α-iduronidase but resistant to β-glucuronidase (results not shown), indicating that the uronic acid HexA-4 at the nonreducing end was IdceA, and the GlcA residue was in turn localized at position 2 of the hexasaccharide. Thus, the sequence of the major compound in fraction 6-26 is ΔHexA(2S)-GlcN(NS,6S)-IdceA-GlcNAc(6S)-GlcA-GlcN(NS), which was confirmed by 500 MHz 1H NMR spectroscopy (Table II). The proton chemical shifts of ΔHexA-6, GlcN-5, IdceA, GlcNAc-3, and GlcA of the compound in fraction 6-26 were very similar to those of ΔHexA-6, GlcN-5, IdceA-4, GlcNAc-3, and GlcA-2 of the compound in fraction 6-27. Those of GlcN-1 were also analogous to those of GlcN-1 of the compound in fraction 6-27 except for the upfield shifts of H-5, H-6, and H-6'. These results indicated that the compound in fraction 6-26 lacks a sulfate group on C-6 of the GlcN-1 in the structure of the compound in fraction 6-27. Therefore, the structure of the major compound in fraction 6-26 is the following:

ΔHexA(2S)α1–4GlcN(NS,6S)α1–4IdceAα1–4GlcNAc(6S)

\( α1–4\) GlcAβ1–4GlcN(NS)

**Structure 9. Fraction 6-26.**

Fraction 6-23—Amino sugar and uronic acid analyses showed that fraction 6-23 contained 1.8 mol of HexA and 1.9 mol of GlcN/mol of ΔHexA, where the GlcN value has been corrected for the degradation (16%) during acid hydrolysis, indicating that the major compound in this fraction is a pentasaccharide. Upon exhaustive heparitinase I digestion of fraction 6-23, the absorption at 232 nm doubled, and ΔHexA-GlcNAc(6S) and ΔHexA(2S)-GlcN(NS,6S) were observed with recoveries of 88 and 121%, respectively, taking the UV absorbance of the total parent oligosaccharides in fraction 6-23 as 100% (Fig. 3A and Table I). The expected unsaturated uronic acid residue to be derived from the reducing end was not detected probably since it was labile and decomposed into an α-keto acid as reported previously for the enzymatic digestions of unsaturated di- and trisaccharide from chondroitin sulfate (37, 38). The excess recovery of ΔHexA(2S)-GlcN(NS,6S) over the other products was probably due to the disaccharide derived from contaminating oligosaccharides. When fraction 6-23 was digested successively with Δhexuronate-2-sulfatase and then heparitinase II, it yielded ΔHexA-GlcN(6S) and ΔHexA-GlcN(NS,6S) (Table I). These results indicated the presence of the nonreducing end tetrasaccharide sequence of ΔHexA(2S)-GlcN(NS,6S)-HexA-GlcNAc(6S)-. However, it remained to be determined whether the reducing terminal uronic acid was sulfated or not.

To define the pentasaccharide structure, fraction 6-23 was analyzed by mass spectrometry. However, it was not possible to obtain spectra of good quality by fast atom bombardment-ionization mass spectrometry unlike for heparin tetrasaccharides (23) probably due to the high negative charge. The fraction was successfully analyzed by MALDI TOF mass spectrometry, where the negatively charged groups of the oligosaccharide were shielded with a synthetic peptide as a complexing agent (Arg-Gly)_{15} (29). Internal calibration by the peptide yielded a molecular ion signal of the protonated 1:1 complex at m/z 4434 (data not shown). After subtracting the contribution of the protonated peptide (m/z 3217), the molecular mass of the oligosaccharide was calculated to be 1217 Da, in reasonable agreement with the theoretical value (1213 Da) for an unsat-
urated tetrasulfated pentasaccharide $\Delta$Hex$_A$Hex$_A$HexNaC$_1$-HexN$_1$(OSO$_3$H)$_6$. The mass accuracy of the present method could be lower ($\pm 0.2\%$–$0.3\%$) (29) than that obtained using a spectrometer equipped with the recently developed delayed ion extraction device (39) but still sufficed to determine the number of saccharide units and sulfate groups present. Hence, the major compound in this fraction is a pentasaccharide with a sequence of $\Delta$HexA(2S)-GlcN(NS,6S)-IdcA-GlcNAc(6S)-GlcA.

The spectrum of fraction 6-23 (Fig. 4) contained additional H-1 signals in the anomeric region when compared with fraction 6-17 containing tetrasaccharides. Characteristic H-1 resonances at 5.52 and 4.6 led to identification of $\alpha$GlcA and $\beta$GlcA residues at the reducing end, respectively (38), confirming that the reducing terminal sugar residue was GlcA. The signals of the nonreducing terminal trisaccharide region were very similar to those of fraction 6-27, showing the presence of the structural element, $\Delta$HexA(2S)-GlcN(NS,6S)-IdcA-. Compared with the chemical shifts of the protons belonging to the reducing terminal GlcA residue of the reference compound, $\Delta$HexAa1–3GalNAc(4-sulfate)$\beta$1–4GlcA, isolated from commercial pig skin dermatan sulfate (38), no significant differences were observed, confirming the presence of a GlcA residue at the reducing terminus of the compound in fraction 6-23. The NMR data indicated that the compound in fraction 6-23 has the pentasaccharide structure which contains a GlcA residue extension on the reducing side. Based upon the NMR data, the MALDI TOF mass data, and the sequential arrangement of the disaccharide units determined by the enzymatic analysis, the structure of the compound in fraction 6-23 was deduced as the following tetrasulfated pentasaccharide structure.

$$\Delta$HexA(2S)$\alpha$1–4GlcN(NS,6S)$\alpha$1–4IdcAa1–4GlcNaC(6S)$\alpha$1–4GlcA$

### TABLE III

**Structures of the unsaturated oligosaccharides isolated from the low sulfated region of porcine intestinal heparin**

| Fraction | Structure | Ref. |
|----------|-----------|------|
| 6–17     | $\Delta$HexA(2S)$\alpha$-GlcN(NS,6S)$\alpha$-IdcA-GlcNAc(6S) | 22   |
| 6–22     | $\Delta$HexA(2S)$\alpha$-GlcN(NS,6S)$\alpha$-IdcA-GlcNAc-GlcA-GlcNaC(NS,6S) | 22, 24, 56 |
| 6–23     | $\Delta$HexA(2S)$\alpha$-GlcN(NS,6S)$\alpha$-IdcA-GlcNaC(6S)-GlcA | 22   |
| 6–25     | $\Delta$HexA(2S)-GlcN(NS)-IdcA(2S)-GlcNaC-GlcA | 22   |
| 6–26     | $\Delta$HexA(2S)-GlcN(NS,6S)-IdcA-GlcNAc-GlcA | 22   |
| 6–27     | $\Delta$HexA(2S)-GlcN(NS,6S)-IdcA-GlcNAc(6S)-GlcA-GlcNaC(NS,6S) | 24, 56 |
| 6–30     | $\Delta$HexA(2S)-GlcN(NS,6S)-IdcA(2S)-GlcNaC-GlcA | 24   |
| 6–31     | $\Delta$HexA(2S)-GlcN(NS,6S)-GlcA-GlcNaC-GlcA(GlcA) | 24   |
| 6–32     | $\Delta$HexA(2S)-GlcN(NS,6S)-IdcA-GlcNaC-GlcA | 24   |
| 6–33     | $\Delta$HexA(2S)-GlcN(NS,6S)-IdcA-GlcNAc-GlcA | 24   |
| 6–34     | $\Delta$HexA(2S)-GlcN(NS,6S)-IdcA-GlcNaC-GlcA | 24   |

*References for the previously isolated structures.*

*2S, 3S, 6S, or NS represents 2-O-, 3-O-, 6-O-, or 2-N-sulfate, respectively.*

*The characteristic trisaccharide sequence shared in the isolated compounds are underlined.*

have been isolated from the heparinase digestes of both bovine lung heparin (41, 42) and porcine intestinal heparin (43). Two others obtained by heparinase digestion of porcine intestinal heparin have been determined as $\Delta$HexA(2S)-GlcN(NS,6S)-IdcA(2S)-GlcNaC(6S)-GlcA(GlcA) and $\Delta$HexA(2S)-GlcN(NS,6S)-IdcA(2S)-GlcNaC-6S)-IdcA-GlcNaC(6S) (43). Another, IdcA(2S)-GlcN(NS,6S)-IdcA(2S)-GlcNaC(6S)-IdcA(2S)-2,5-anhydromannitol(6S), which is an HNO$_2$ degradation product of porcine mucosal heparin, has also been isolated (44). However, all these are considered to be derived from the highly sulfated region. A few larger oligosaccharides have also been isolated from the highly sulfated region (41, 45–47). In contrast, the oligosaccharides isolated in this study are derived from the irregular region of heparin. The isolation of hexa- and larger oligosaccharides from the irregular region has been limited to those derived from the antithrombin III-binding site, and the structural variability of several such oligosaccharides has been summarized and discussed (24, 26, 48).

The isolated pentasaccharide appears to be derived from the reducing end of a parent heparin glycosaminoglycan chain. During biosynthetic processing, heparin glycosaminoglycan chains are synthesized on the core protein as part of a proteoglycan, released from macromolecular proteoglycans by an endo-$\beta$-glucuronidase, and stored in mast cell granules that may be discharged from cells suitably stimulated (49–51). The pentasaccharide in fraction 6-23 could be derived from the newly formed reducing end exposed by the specific endo-$\beta$-glucuronidase. Endoglycosidase activities toward heparin/heparan sulfate have also been found in various other cells and tissues (for a review, see Ref. 52). Some heparanases are secreted from cells to play a role in remodeling basement membranes after injury or at inflammation sites. Other heparanases are intracellular and important for degrading cell surface heparan sulfate proteoglycans once they have been internalized. One glucuronidic linkage cleavable by the endoglycosidase of human platelets is in the sequence -GlcNaC-GlcA-GlcN(NS) (53). However, the substrate specificities of most of the endoglycosidases for heparin/heparan sulfate have been only partially characterized. One approach to investigate substrate specificity of endo-$\beta$-glucuronidases is to analyze the reducing and nonreducing terminal structures of heparin glycosaminoglycan chains, which would reflect the specific sites cleaved by endoglycosidases. The nonreducing terminal of a heparin glycosaminoglycan chain will be isolated as a saturated oligosaccharide after digestions with heparin lyases (23, 54). The oligosaccharides in fractions 6-26, 27, 32, and 34, which contain the pentasaccharide sequence of the compound in fraction 6-23, may be useful for elucidating the structural requirement for recognition by an endo-$\beta$-glucuronidase. Bame and Robson (55) have recently characterized the reducing terminal structures of heparanase-derived shorter heparan sulfate chains isolated from **DISCUSSION**

In this study, we determined the structures of a tetra-, a penta-, and eight hexasaccharides isolated from the heparin hexasaccharide fraction, which was prepared by the extensive digestion of porcine intestinal heparin with *Flavobacterium* heparinase. Five subfractions, 6-22, 23–25, 26–28, and -30, were isolated for the first time as discrete structures. Since heparinase cleaves most glucosaminidic linkages in the highly sulfated region (22, 40), which accounts for three-quarters of a heparin polysaccharide chain, but does not cleave chains in the less sulfated irregular region scattered along the polysaccharide chain being flanked by the highly sulfated region, the isolated oligosaccharides are derived from the irregular region. Isolation of the heparin hexa- and larger oligosaccharides has been limited. Two hexasaccharides, $\Delta$HexA(2S)-GlcN(NS,6S)-IdcA(2S)-GlcNaC(6S)-GlcA, and $\Delta$HexA(2S)-GlcN(NS,6S)-IdcA(2S)-GlcNaC(6S)-GlcA-GlcNaC(6S),
Chinese hamster ovary cells and proposed a model for the heparanase action.

As many as 8 of the 10 isolated oligosaccharide components shared the trisaccharide sequence -4IdcaA1-4GlcNAc1-4GlcB1 (Table III). In striking contrast, its reverse sequence -4GlcA1-4GlcNAc1-4IdcaA1- was not found and has not been reported to date for heparin/heparan sulfate to our knowledge. Although Lindahl (4) previously referred in a review to the lack of identification of the disaccharide sequence -4GlcNAc1-4IdcaA1- in heparin/heparan sulfate, this is the first demonstration of the common trisaccharide sequence in the oligosaccharides isolated from the irregular region of heparin. These findings indicate that the -4GlcNAc1-4GlcB1- sequence does not serve as a substrate for the epimerase involved in heparin/heparan sulfate biosynthesis. Alternatively or in addition, GlcNAc N-deacetylase/N-sulfotransferase may not attack the -4IdcaA1-4GlcNAc1-4GlcB1- sequence. It should be noted that the trisaccharide sequence was found to be sulfated either at C-2 of the Idca residue (fractions 6-22, -25, and -30) or at C-6 of the GlcNAc residue (fractions 6-23, -26, -27, -32, and -34), suggesting that the -4GlcB1-4GlcNAc1-4IdcaA1- sequence serves as a substrate for Idca 2-O-sulfotransferase or GlcNAc 6-O-sulfotransferase. One of the five heparin hexasaccharides reported previously (24) contained the disulfated trisaccharide sequence -4IdcaA2(S)α1-4GlcNAc6Sω1-4GlcA, indicating a possible monosulfated to the disulfated trisaccharide sequence. Seven hexasaccharides isolated in this study shared the common trisulfated hexasaccharide core sequence Hexa(2S1-4GlcN(NS)α1-4IdcaA1-4GlcNAc1-4GlcAβ1-4GlcN(NS) which contained the above trisaccharide sequence. The specificity of the heparinase used for preparation of the oligosaccharides strongly indicates the occurrence of the common penta- sulfated octasaccharide core sequence -4GlcN(NS)α1-4Hexa(2S1-4GlcN(NS)α1-4IdcaA1-4GlcNAc1-4GlcAβ1-4GlcN(NS) which is flanked by Hexa(2S) on the nonreducing and reducing sides, respectively. The common sequence revealed for the first time by the present systematic analysis turned out to be a low sulfated trisaccharide representing the irregular region sandwiched by highly sulfated regions and should reflect the control mechanism of heparin biosynthesis.

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