Previously we isolated a tetrasaccharide-serine and a hexasaccharide-serine from the carbohydrate-protein linkage region of porcine intestinal heparin after digestion with a mixture of Flavobacterium heparinase and heparitinases I and II (Sugahara, K., Yamada, S., Yoshida, K., de Waard, P., and Vliegenthart, J. F. G. (1992) J. Biol. Chem. 267, 1528-1533). In this study we isolated four longer carbohydrate sequences (I-IV) attached to Ser or a dipeptide (Ser-Gly or Gly-Ser), which accounted for at least 18.2% of the total linkage region, were isolated from the same heparin preparation after digestion with heparinase only. IV was successfully isolated only after subsequent digestion with glycuronate-2-sulfatase. 

Their structures were determined by chemical and enzymatic analyses and 3H NMR spectroscopy and found to be the following octa- and decasaccharide sequences attached to Ser in a molar ratio of 1:1:2:3:1:0:1:3: 

\[ \Delta \text{HexA}(2S)\alpha 1-4 \text{GlcN}(NS,6S)\alpha 1-4 \text{GlcA}\beta 1-3 \text{Gal}(1-4)\text{Xyl(1-0-Ser)}(II), \Delta \text{HexA}(2S)\alpha 1-4 \text{GlcN}(NS,6S)\alpha 1-4 \text{daGlcA}\beta 1-4 \text{GlcNAc}\beta 1-3 \text{Gal}(1-4)\text{Xyl(1-0-Ser)}(III), \Delta \text{HexA}(2S)\alpha 1-4 \text{GlcN}(NS,6S)\alpha 1-4 \text{GlcA}\beta 1-3 \text{Gal}(1-4)\text{Xyl(1-0-Ser)}(IV). \]

(1-4)GlcA\beta 1-4GlcN(6-sulfate); 3S, 3-O-sulfate; 3S, 3-O-sulfate; 3S, 3-O-sulfate. I and II contained 1 mol of Gly in addition to Ser. The four structures indicated that sulfation in heparin chains takes place on the monosaccharide residues located in close vicinity to the core protein than found for heparan sulfate chains and that there exist at least several heparin sub-class chains with different linkage region structures. The significance of the isolated structures is discussed in relation to the biological functions and the biosynthetic mechanisms of heparin.

Heparin exerts a variety of biological activities such as inhibition of blood coagulation (Marcum and Rosenberg, 1989), modulation of cellular proliferation (Clowes and Karnovsky, 1977; Thornton et al., 1983), potentiation of angiogenesis (Folkman and Ingber, 1989), and interactions with acidic and basic fibroblast growth factors (Maciag et al., 1984; Shing et al., 1984; Klagsbrun and Shing, 1985). Some of these activities seem to reside within the complex fine structure of heparin. It is generally accepted that heparin expresses most of these activities by mimicking in vitro the physiological activities of heparan sulfate through its structure, similar to that of heparan sulfate. However, the structure-function relationships of heparin/heparan sulfate are not fully understood.

The basic polymeric common structure of heparin and heparan sulfate is an alternating repeat sequence of 1-4GlcA\beta 1-4GlcN(6-sulfate); 1-4GlcA\alpha 1-4GlcN\beta 1-3Gal(1-4)\text{Xyl(1-0-Ser)}(IV), which can be variably sulfated (for reviews see Roden (1980), Gallagher and Lyon (1989), and Lindahl (1989)). Heparin contains more sulfate and daGlcA but less N-acetyl groups and GlcA as compared with heparan sulfate. Sulfate groups can be located at C-2 of hexuronic acid and C-2, C-3, and/or C-6 of glucosamine residue and add the structural complexity to the carbohydrate backbone to form various active domain structures responsible for a number of biological activities. Recent structural studies of the binding domains to antithrombin III (for review see Lindahl (1989)) and basic fibroblast growth factor (Habuchi et al., 1992; Turnbull et al., 1992; Tyrrell et al., 1993; Maccarana et al., 1993) are the best examples showing the relationships between the fine structure and biological functions.

Heparin and heparan sulfate are synthesized on the specific serine residues of the core polypeptides through the unique carbohydrate-protein linkage region, 3Gal(1-3Gal(1-4)Xyl(1-0-Ser), which is also shared by chondroitin sulfate and dermatan sulfate.
dermatan sulfate. It has not been clarified yet how these glycosaminoglycans diverge in biosynthesis into different structures from the same trisaccharide sequence. The biosynthetic sorting mechanism of glycosaminoglycans (heparin/heparan sulfate) and galactosaminoglycans (chondroitin sulfate and dermatan sulfate) has been an enigma. Although unique modifications by phosphorylation and sulfation of the linkage trisaccharide sequence have been demonstrated (Oegema et al., 1988; Fransson et al., 1985; Sugahara et al., 1988, 1991, 1992b; de Waard et al., 1992), no evidence has been presented for the involvement of these modifications in the biosynthetic sorting mechanism. The importance of the amino acids near the heparan sulfate attachment site has also been pointed out (Zhang and Esko, 1994), but whether peptide sequences are the primary determinants for heparan sulfate synthesis remains to be determined. Differences between biosynthesis of heparin and heparan sulfate are not well understood either. Although they share a number of common structural features, there are several structural differences. Heparan sulfate has a long nonsulfated sequence consisting of at least eight repeating units (-4GlCα1-4GlCα1-3Galβ1-4Xylβ1-O-Ser (glycoserine I) and -4GlCα1-3Galβ1-4Xylβ1-O-Ser (glycoserine II)) from the linkage region of heparin after exhaustive digestion with a mixture of heparinase and heparitinases I and II (Sugahara et al., 1992a). However, the exact modified structure of this region of a heparin chain has not been investigated in detail yet.

We have been analyzing the structure of the carbohydrate-protein linkage region of various sulfated glycosaminoglycans to investigate the structure-function relationships and the biosynthetic mechanisms of these glycosaminoglycans (Sugahara et al., 1988, 1991, 1992a, 1992b, 1994, 1995; de Waard et al., 1992). Previously we isolated two glycoserines, ΔHexaα1-3Galβ1-3Galβ1-4Xylβ1-O-Ser (glycoserine I) and ΔHexaα1-4GlCα1-3Galβ1-3Galβ1-4Xylβ1-O-Ser (glycoserine II) from the linkage region of heparin after exhaustive digestion with a mixture of heparinase and heparitinases I and II (Sugahara et al., 1992a). In the present study we isolated and characterized larger glycoserines and glycopeptides after digestion with only heparinase in order to investigate the structure above the tetra- and heparosaccharide sequences.

**EXPERIMENTAL PROCEDURES**

**Materials—** Stage 14 heparin was purchased from American Diagnostica (New York) and purified by DEAE-cellulose chromatography as previously reported (Sugahara et al., 1992a), and the preparation should have been devoid of heparan sulfate. Cellulofine gels, 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. ΔHexa-Glycuronate-2-sulfatase (EC 3.1.6.-), abbreviated as 2-sulfatase, was purified from Flavobacterium heparinum (McLean et al., 1984). Six standard unsaturated disaccharides were prepared from heparin as previously reported (Yamada et al., 1992). Fragmentation of Heparin and Size Fractionation of the Fragments— The purified heparin (140 mg) was digested by 5 IU of heparinase in a total volume of 10 ml of 30 mM acetate-NaOH buffer, pH 7.0, containing 3 mM Ca(OAc)₂ and 1% bovine serum albumin. When the reaction reached a plateau after 7 h as monitored by absorption at 232 nm, it was terminated by heating at 100 °C for 2 min. The digest was adjusted to 0.2 M NaCl and fractionated on a column (3 × 96 cm) of Cellulofine GCL-90-m equilibrated with 0.2 M NaCl. Elution was performed with the same solution at a flow rate of 30 ml/h. Fractions (7.5 ml) were collected and monitored by absorption at 232 nm. Eluates were separated into fractions a-d as shown in Fig. 1. The separated fractions were concentrated, desalted by gel filtration on a column (0.8 × 58 cm) of Cellulofine GCL-25-m, and lyophilized. HPLC and Capillary Electrophoreses— Fractionation and analysis of unsaturated disaccharides were carried out by HPLC on an amine-bound silica PA03 column using a linear gradient of NaH₂PO₄ at a flow rate of 1 ml/min basically as described previously (Sugahara et al., 1992b). Eluates were monitored by absorption at 232 nm. The separated fractions were concentrated and desalted through a column of Sephadex G-25. Capillary electrophoresis was carried out to examine the purity of each isolated fraction using a fused silica capillary in a Waters capillary ion analyzer (Sugahara et al., 1994). A new capillary (60 cm total length, 75 μm internal diameter; Millipore Corp.) was activated by sequential washings with 0.1 M sodium hydroxide, distilled water, and 25 mM sodium phosphate buffer, pH 3.0, before use. The electrophoretic fractions were detected by absorption at 185 nm caused by OH groups, since that of the test compounds (the linkage hexasaccharide alditols) was at least 5-fold higher than that obtained at 232 nm. Samples (1 nmol/10 μl), prepared in distilled water, were injected using an automatic pressure to a total volume of 24 nl of solution. The electrophoresis was performed using constant voltage of 15 kV for a period of 20 min. Negative polarity power supply was used.

Quantification of the Linkage Region Gycosaminoglycans— The content of the linkage region in fractions a–d was quantified by exhaustive digestion with a mixture of heparinase and heparitinases I and II followed by determination of the resultant glycoserine I by HPLC on an amine-bound silica column as described previously (Sugahara et al., 1992a). The glycoserine II content was below the detection limit of HPLC; the molar ratio of glycoserines I and II in the heparin preparation used was 96:4 (Sugahara et al., 1992a).

Digestion of Fraction b-10 with 2-Sulfatase and Subfractionation of the Digest— Fraction b-10 (120 nmol) was incubated with 60 μl of 2-sulfatase in a total volume of 6.7 M NaCl, containing 0.05% bovine serum albumin at 37 °C for 1 h. The reaction was terminated by boiling for 1 min, and the digest was fractionated by HPLC on an amine-bound silica column. Otherwise the HPLC conditions were the same as described above.

Digestion of the Isolated Oligosaccharides with Heparinase, Heparitinase I, and 2-Sulfatase— Oligosaccharides (0.05 μmol) were digested using 2 μl of heparinase at 37 °C for 20 min in a total volume of 40 μl of 100 mM acetate-NaOH buffer, pH 7.0, containing 3 mM Ca(OAc)₂. For exhaustive heparitinase I digestion, an incubation was conducted using 2.4 μl of the enzyme and 0.3 nmol of each substrate at 37 °C for 150 min in a total volume of 30 μl of 20 mM acetate-NaOH buffer, pH 7.0, containing 0.5 mM Ca(OAc)₂. The digestion was carried out using 1 μl of the enzyme and 0.5 nmol of an oligosaccharide at 37 °C for 5 h for (fraction b-5) or 60 min for (fraction b-6) in a total volume of 50 μl of the buffer described above. Heparitinase II digestion was performed using 0.5 μU of the enzyme and 0.5 nmol of a substrate at 37 °C for 20 min in a total volume of 50 μl of the buffer described above for heparitinase I. 2-Sulfatase digestion was carried out using 5 μU of the enzyme and 1.0 nmol of a substrate at 37 °C for 60 min in a total volume of 65 μl of the buffer described above for 2-sulfatase. Enzymatic reactions were terminated by boiling for 1 min, and the digests were analyzed by HPLC on an amine-bound silica column as reported (Sugahara et al., 1992b).

**1H NMR Spectroscopy—** Oligosaccharides for NMR analysis were fully sodiated by cation-exchange chromatography through a column of Dowex 50-X8 (Na⁺ form) (7 × 18 mm) and then repeatedly exchanged in H₂O with intermediate lyophilization.

NMR spectra of fractions b-5 and b-6 were recorded on a Bruker AMX-500 or AMX-600 spectrometer (Department of NMR spectroscopy, Utrecht University) operated at a probe temperature of 292, 295, or 300 K. One-dimensional spectra and a double quantum-filtered correlation spectrometry spectrum of fraction b-5 were recorded as described previously (Piantini et al., 1982; Derome and Williamson, 1990; Hård et al., 1992). Two-dimensional NOESY experiments were performed with a mixing time of 150–200 ms (J eener et al., 1979). Two-dimensional TOCSY spectra were recorded using a clean-MLEV-17 spin-lock pulse sequence of 100 ms, preceded by a 2.5 ms-tripulse (Braunschweiler and Ernst, 1983; Bax and Davis, 1985; Griesinger et al., 1988). In all two-dimensional experiments the HO²H resonance was presaturated during the relaxation delay and additionally during the NOE-mixing time in NOESY-experiments. Phase-sensitive detection was achieved by the time-proportional phase increment method (Marion and Wüthrich, 1982). Two-dimensional NOESY spectra were recorded with mixing times of 300–512 τ₁, experiments, and 80–160 free induction decays of 2048 or 4096 data points were collected per τ₁ increment. Data sets were processed using the Bruker UXNMR software package. In short, time domain data were zero-filled, multiplied by a phase-shifted sine bell function, and after Fourier transformation base line-corrected with fifth order polynomial fits.

The spectra of fraction b-10-II were recorded on a Varian VXR-500 spectrometer (Kobe Pharmaceutical University) at a probe temperature of 299 K as previously reported (Yamada et al., 1992).
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Fig. 1. Fractionation of the heparinase digest by gel filtration. Purified stage 14 heparin was exhaustively digested by heparinase and gel-filtered on a column of Cellulofine GCL-90-m. Fractions were monitored by absorption at 232 nm (●) and the carboxyl reaction (○) and pooled as indicated.

Other Analytical Methods—Uronic acid was determined by the carboxyl method (Bitter and Muir, 1962). Unsaturated uronic acid was spectrophotometrically quantified based upon an average millimolar absorption coefficient of 5.5 at 232 nm (Yamagata et al., 1968). Amino acids and amino sugars were quantified after acid hydrolysis in 6 M HCl at 110 °C for 20 h and 3 M HCl at 100 °C for 16 h, respectively, using a Beckman 6300E amino acid analyzer (Sugahara et al., 1987).

RESULTS

Isolation of the Linkage Glycoserines—Heparinase specifically cleaves the glucosaminide linkage in N-GlcN(4S)-L-iduronic acid (Linker and Hovingh, 1984; Linhardt et al., 1990; Yamada et al., 1994), and heparinase-resistant structures containing a few or several N-acetylgalactosamine residues have been claimed to occur in the vicinity of the linkage region (Lindahl, 1966; Rosenfeld and Danishefsky, 1988). Therefore, to isolate linkage region fragments larger than the previously isolated glycoserines I and II, stage 14 heparin purified from porcine intestine, which contains Ser as the predominant amino acid (Lindahl and Rodén, 1972), was exhaustively digested with heparinase only. A heparinase digest was fractionated by gel filtration into fractions a–d as indicated in Fig. 1. The recoveries of serine in fractions a, b, c, and d were 67, 24, 2, and 1%, respectively, of that contained in the starting heparin. Fractions c and d mainly contained tetra- and disaccharides, respectively, which were derived from the repeating disaccharide region as judged from the ratio of uronic acid and GlcN to their sensitivity to 2-sulfatase was examined to evaluate their purity and structural characteristics. 2-Sulfatase acts only on the 4,5-unsaturated hexuronic acid 2-sulfate structure at the nonreducing end of a saccharide chain (McLean et al., 1984). Fractions b-5, -6, and -10 were all sensitive to the enzyme, as expected from the linkage specificity of heparinase (see above), indicating that the major compound in each fraction has a corresponding parent compound on HPLC (data not shown). The separated fractions b-5, -6, and -10 gave a single symmetrical peak upon HPLC. In the preliminary experiments their sensitivity to 2-sulfatase was examined to evaluate their purity and structural characteristics. 2-Sulfatase acts only on the 4,5-unsaturated hexuronic acid 2-sulfate structure at the nonreducing end of a saccharide chain (McLean et al., 1984). Fractions b-5, -6, and -10 were all sensitive to the enzyme, as expected from the linkage specificity of heparinase (see above), indicating that the major compound in each fraction has a sulfate group at the C-2 position of the GlcN (not shown).

After 2-sulfatase digestion, fractions b-5 and -6 gave a single peak, which eluted approximately 10 min earlier than the corresponding parent compound on HPLC (not shown), supporting the homogeneity of fractions b-5 and -6. In contrast, a 2-sulfatase digest of fraction b-10, designated fraction b-10S, gave three peaks, fractions b-10S-I, -II, and -III, in a molar ratio of 21:58:21, which eluted approximately 8 min earlier than the parent fraction (Fig. 2, inset), indicating that fraction b-10 was a mixture of at least three components. Since it was not possible to resolve fraction b-10 into its subcomponents preparatively, it was first digested with 2-sulfatase, and then the digest was fractionated by HPLC. The major peak b-10S-II, the yield of which was about 90 nmol from 100 mg of the starting heparin, was isolated and subjected to structural analysis.
Fractions 10S-I and -III were not analyzed due to their limited amounts.

Characterization of Fraction b-5—Capillary electrophoresis resolved fraction b-5 into two fractions, b-5-I and b-5-II, in a molar ratio of 1.02:2.2 (Fig. 3A), indicating that it contains at least two components. However, it was not separable on a large scale into each component and was therefore analyzed without further purification.

As shown in Table I, chemical analysis showed that fraction b-5 contained \( \Delta \text{HexA, HexA, GlcN, Ser, and Gly} \) in a molar ratio of 1.00:1.73:3.17:2.46:2.38. The disaccharide analysis of fraction b-5 was performed by heparitinase I digestion followed by HPLC analysis on an amine-bound silica column, using a linear gradient of NaH2PO4 from 16 to 530 mM over 60 min. Elution positions of the standard disaccharides isolated from heparin/heparan sulfate are indicated in panel A. 1. \( \Delta \text{DiHS-0S, 2. DiHS-diS}, 3. \Delta \text{DiHS-triS, 4. DiHS-tetraS, 5. DiHS-pentaS, 6. DiHS-triS, Glycoserine I is the tetrasaccharide-serine } \Delta \text{HexA-1-3Gal-1-4GlcNAc-4Xyl-Ser} \text{ reported previously (Sugahara et al., 1992a). The peak marked by an asterisk at around 35 min is often observed upon high sensitivity analysis and is due to an unknown substance eluted from the column.}

Figure 4. HPLC analysis of the enzyme digests of fraction b-5. Fraction b-5 was digested by heparitinase I (panel B) or by heparitinase II alone (panel C) as described under "Experimental Procedures." The digest was subjected to HPLC on an amine-bound silica column using a linear gradient of NaH2PO4 from 16 to 530 mM over 60 min. Elution positions of the standard disaccharides isolated from heparin/heparan sulfate are indicated in panel A. 1. \( \Delta \text{DiHS-0S, 2. DiHS-diS, 3. DiHS-triS, 4. DiHS-tetraS, 5. DiHS-pentaS, 6. DiHS-triS, Glycoserine I is the tetrasaccharide-serine } \Delta \text{HexA-1-3Gal-1-4GlcNAc-4Xyl-Ser} \text{ reported previously (Sugahara et al., 1992a). The peak marked by an asterisk at around 35 min is often observed upon high sensitivity analysis and is due to an unknown substance eluted from the column.}

Fraction 10S-II contained \( \Delta \text{HexA, HexA, GlcN, and Ser} \) in a molar ratio of 1.00:2.38:1.40, indicating that the major compound in fraction b-6 is a trisulfated decasaccharide-serine \( \Delta \text{HexA-2S, 4GlcNAc-1-4GlcA-3Gal-1-4Xyl-Ser} \text{ derived from heparan sulfate (Sugahara et al., 1994), and which therefore was assumed to be a nonsulfated octasaccharide-serine } \Delta \text{HexA-1-4GlcNAc-1-4HexA-1-3Gal-1-4Xyl-Ser} \text{ reported previously (Sugahara et al., 1992a). The peak marked by an asterisk at around 35 min is often observed upon high sensitivity analysis and is due to an unknown substance eluted from the column.}

Characterization of Fraction b-6—Fraction b-6 gave a single peak on HPLC (not shown) and is more than 85% pure as judged by capillary electrophoresis (Fig. 3B). Chemical analysis showed that fraction b-6 contained \( \Delta \text{HexA, HexA, GlcN, and Ser} \) in a molar ratio of 1.00:3.17:2.46:1.13 (Table I). The disaccharide analysis of fraction b-6 was performed by heparitinase I digestion followed by HPLC. Exhaustive heparitinase I digestion gave rise to three unsaturated components, \( \Delta \text{DiHS-0S, DiHS-triS and glycoserine I} \), in a molar ratio of 2:1:1, based upon peak areas (Table II). The results indicate that fraction b-6 contains a decasaccharide-serine composed of 2 mol of the nonsulfated disaccharide units, 1 mol each of the trisulfated disaccharide unit and glycoserine I. Heparitinase II digestion of fraction b-6 yielded equimolar amounts of two components, namely \( \Delta \text{DiHS-triS and glycoserine I} \). The results are summarized in Table II and are consistent with the previous observation that the hexasaccharide structure is resistant to heparitinase II (Fig. 5). (Sugahara et al., 1994). Together the above results indicate that the two components in fraction b-6 share the common structure, \( \Delta \text{HexA-2S, 4GlcNAc-1-4GlcA-3Gal-1-4Xyl-Ser} \text{ derived from the carbohydrate-protein linkage region of bovine kidney heparan sulfate (Sugahara et al., 1994) (Fig. 4C). These results indicate that fraction b-6 probably contains two components, namely \( \Delta \text{DiHS-triS and glycoserine I} \). The results indicate that fraction b-6 contains a decasaccharide-serine composed of 2 mol of the nonsulfated disaccharide units, 1 mol each of the trisulfated disaccharide unit and glycoserine I.

Characterization of Fraction b-5—Fraction b-5 was subjected to electrophoresis as described under “Experimental Procedures.” The peak at around 4 min is presumably due to a non-carbohydrate contaminant.

Characterization of Fraction b-6—Fraction b-6 gave a single symmetrical peak both on HPLC (data not shown) and on capillary electrophoresis (Fig. 3C), indicating its homogeneity. Chemical analysis showed that fraction b-6 contained \( \Delta \text{HexA, HexA, GlcN, and Ser} \) in a molar ratio of 1.00:1.23:1.40 (Table I). Upon HPLC analysis of the
TABLE II
Enzymatic analysis of the isolated linkage fractions

| Digestion          | Fraction b-5 | Fraction b-6 | Fraction b-105-II |
|--------------------|--------------|--------------|------------------|
| Heparitinase I     |              |              |                  |
| ∆DIHS-OS (107%)    | ∆DIHS-OS (195%)* | ∆DIHS-6S (88%)* |
| ∆DIHS-triS (89%)  | ∆DIHS-triS (91%)* | ∆DIHS-diS (94%)* |
| Nonsulfated tetrasaccharide linkage component (97%) | Nonsulfated tetrasaccharide linkage component (90%) | Nonsulfated tetrasaccharide linkage component (92%) |
| Heparitinase II    |              |              |                  |
| ∆DIHS-triS (106%) | ∆DIHS-triS (148%) | ∆DIHS-diS (136%)* |
| Nonsulfated hexasaccharide linkage component (103%) | Nonsulfated octasaccharide linkage component (101%) | Monosulfated hexasaccharide linkage component (111%) |

* A few minor products were also observed.

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After each fraction was incubated with heparitinase I or II, the reaction products were analyzed by HPLC. Recoveries of the disaccharides calculated based on absorbance at 232 nm are shown in parentheses. The absorptions obtained with intact compounds are taken as 100%.

![Diagram](image)

**Fig. 5. Specificities of heparitinases I and II.** Enzymatic action of heparitinases I and II on the isolated linkage compounds are shown by arrows with the Roman numerals I and II above and below each structure, respectively. A thick arrow shows a preference for the indicated linkage over the other(s). a, the octasaccharide-peptides in fractions b-5 and b-5-II; b, the disaccharide-serine in fraction b-6; c, the octasaccharide-serine in fraction b-105-II. * It is noted that this linkage in the octasaccharide-serine in fraction b-105-II was cleaved by heparitinase I, whereas the corresponding linkage in glycoserine II ∆HexA-GlcNAc(6S)-GlcA-Gal-Xyl-Ser was not (Sugahara et al. 1992a, and see 'Discussion').

heparitinase I digest of fraction b-105-II, three major UV-absorbing peaks of glycoserine I, ∆DIHS-6S, and ∆DIHS-diS1 were observed in a molar ratio of 1.00:0.96:1.02 with a few minor peaks (Table II), indicating that the major compound in fraction b-105-II was a trisulfated octasaccharide-serine composed of equimolar amounts of the above three components.

When digested with heparitinase II, this compound yielded equimolar amounts of ∆DIHS-diS1 and the component eluted at the position of glycoserine II (Sugahara et al., 1992a) (Table II), indicating that the disulfated disaccharide unit, ∆DIHS-diS2, was located at the nonreducing terminus. Thus, the structure of the compound in fraction b-105-II is ∆HexA1-4Glcn(6S)1-4HexA1-3Galβ1-3Galβ1-4Xylβ1-O-Ser.

**1H NMR Spectroscopy**—The structures in fractions b-5 and b-6 were determined using one- and two-dimensional 1H correlation spectroscopy, TOCSY, and NOESY spectra. The anomic region of the one-dimensional 1H NMR spectrum (Fig. 5A) of fraction b-5 shows two sets of signals differing in intensity, reflecting the presence of two compounds in an approximately 2:1 ratio. Resonances stemming from the protons of the core monosaccharide residues Xyl-1, Gal-2, Gal-3, and GlcA-4 were readily assigned by their characteristic TOCSY patterns on the H-1 tracks (Fig. 7A), and the close resemblance of their chemical shifts with those of the common core region GlcA-Gal-Xyl-Ser (Table III) (van Halbeek et al., 1982). The presence of this partial structure is corroborated by NOEs between GlcA-4 H-1 and Gal-3 H-3, between Gal-3 H-1 and Gal-2 H-3, and between Gal-2 H-1 and Xyl-1 H-4 (Fig. 7B). The presence of a single series of proton resonances stemming from this partial structure indicates that both compounds contain this common core region. Almost identical sets of signals are observed for the terminal ∆HexA residue (Fig. 6A), the chemical shifts being indicative of sulfation at the 2-position (Table III) (Horne and Gettins, 1991), showing that both structures contain a terminal ∆HexA(2S) residue.

The similar intensities of the resonances at δ 5.377, δ 5.335, and δ 4.948 suggest that they belong to the major compound (b-5-I). The resonance at δ 5.377 stems from the anomic proton of an N,6-disulfated glucosamine residue (Fig. 6A). This assignment is based on the upfield shift out of the bulk region of the H-2 resonance (δ 3.279) reflecting N-sulfation (Horne and Gettins, 1991; Yamada et al., 1994), and the downfield shifts out of the bulk region of the H-5 (δ 3.985) and the hydroxymethyl-proton signals (δ 4.203, 4.353), indicating sulfation at the 6-position (Fig. 6A) (Horne and Gettins, 1991; Sugahara et al., 1992a; Yamada et al., 1994). The presence of an NAc resonance (δ 2.034), and the characteristic TOCSY pattern observed on the H-1 track (Fig. 7A) show that the signal at δ 5.335 stems from the anomic proton of a nonsulfated glucosamine residue (Sugahara et al., 1994). The presence of an iduronic acid residue is deduced from the small coupling constant (2 Hz) observed on the signal at δ 4.948 and its characteristic TOCSY pattern observed on the H-1 track (Fig. 7A) (Sugahara et al., 1994). The sequence of the major compound b-5-I is unequivocally established by NOEs between ∆HexA2S-8 H-1 and GlcN(N5,6S)-7 H-4 and H-6, between GlcN(N5,6S)-7 H-1 and IdoA-6 H-3 and H-4, between IdoA-6 H-1 and GlcNac-5 H-4, and between GlcNac-5 H-1 and GlcA-4 H-4 (Fig. 7B).

The minor compound (b-5-II) also contains an N,6-disulfated and a nonsulfated glucosamine residue as judged from the TOCSY patterns on their H-1 tracks at δ 5.566 and δ 5.359, respectively (Fig. 7A) (Sugahara et al., 1994; Yamada et al., 1994). NOEs between GlcNac-5 H-1 and GlcA-4 H-4 and the one between ∆HexA(2S)-8 H-1 and GlcN(N5,6S) H-4 and H-6, locate these glucosamine residues at positions 5 and 7 in the oligosaccharide sequence. The resonance at δ 4.510 ppm, dis-
FIG. 6. One-dimensional 600-MHz $^1$H NMR spectra of the structures in fractions b-5 and b-6, recorded in $^2$H$_2$O at 300 K. The Arabic numerals in the spectra refer to the corresponding residues in the structures. A, fraction b-5; B, fraction b-6.
**Fig. 7.** Two-dimensional TOCSY (A) and NOESY (B) spectra of the structures in fraction b-5 recorded at 300 and 292 K, respectively. Anomeric protons are indicated by vertical lines, and the number at the top corresponds to the monosaccharide number. Resonances of the core region overlap and are indicated in italics. Cross-peaks on the H-1 tracks are labeled by proton number in the TOCSY spectrum. In the NOESY spectrum only trans-glycosidic NOEs on the H-1 tracks are labeled. The double digit number represents the monosaccharide unit followed by the proton number.
playing a coupling constant of 8 Hz, and its characteristic TOCSY-pattern on the H-1 track show the presence of a GlcA residue (Sugahara et al., 1994; Yamada et al., 1994). The NOE between GlcN(\(\text{N}^\text{S,6S}\))-7 H-1 and GlcA H-4 and the one between GlcA H-1 and H-4 of GlcNAc locate this residue at position 6 in the sequence. The resonance of the anomeric proton of GlcN(\(\text{N}^\text{S,6S}\))-7 (\(\delta 5.566\)) has shifted downfield by \(\Delta \delta 0.19\) with respect to the corresponding signal of the major compound, in agreement with the previously reported observation that the GlcN(\(\text{N}^\text{S,6S}\))-1 reports on the identity of the preceding hex-
Linkage Oligosaccharides from Porcine Intestinal Heparin

In this study, we identified one deca- and three octasaccharide structures in the linkage-derived fractions b-5, b-6, b-10, and b-10S-II. They represent most likely the molecules from which glycoserines I and II were previously produced by exhaustive digestion with a mixture of heparinase and heparitinases I and II (Sugahara et al., 1992a). Presumably, glycoserine I had been produced under harsh conditions, only the middle linkage was cleaved by the enzyme unsulfated stretch of more than eight repeating disaccharide units, which are assumed to contain only GlcA (Gallagher and Lyon, 1989; Lindblom et al., 1991; Lyon et al., 1994) and therefore would be rather rigid in the proximal portion of the linkage region but plastic in the distal portion.

The present study indicates that there are at least four subclasses of heparin chains different in structure of the linkage region and/or in length of the nonsulfated sequence proximal to the protein core. It is likely that there exist other subclass chains in fraction a, and it is possible that different chains have different patterns of modification. It remains to be determined whether biologically active domain structures such as the binding domains to the antithrombin III and basic fibroblast growth factor are found on a specific subclass chain and where along a heparin chain they are embedded. Since the linkage region is first constructed in biosynthesis, differences in the structure of the linkage region may influence that of the repeating disaccharide region to be synthesized thereafter. It should be noted that the anticoagulant-conferring area appears to occur about 20 disaccharide units away from the linkage region (Rosenfeld and Danishefsky, 1988). The observed heterogeneity in the linkage region also raises questions of whether the different chains are derived from different core proteins and whether they come from identical or different sites of a single core protein. Answers to these questions require further investigation.

The present work provided some useful information about the substrate specificities of heparitinases I and II, which are essential tools for structural studies of heparin/heparan sulfate. Previously, heparitinase I was shown to cleave glucosaminidic linkages bound to nonsulfated GlcA and IdoA except for two glucosaminidic linkages: the one linked to the GlcA residue substituting the 3-sulfated glucosamine residue in the antithrombin III-binding sequence (Yamada et al., 1993) and the one linked to the GlcA residue located between the Gal and the 6-sulfated GlcNAc found in glycoserine II (Sugahara et al., 1992a). In this study, however, all the glucosaminidic linkages in the four molecules including the octasaccharide in fraction b-10 were cleaved by this enzyme (Fig. 5c). It may be that the enzyme acts on the glucosaminidic linkage in the sequence -GlcNAc(6S)-GlcA-Gal- when it is located in an octasaccharide (b-10S-II) but not in a hexasaccharide (glycoserine II). Digestibility of the glucosaminidic linkages in fraction b-6 suggests some linkage preference of this enzyme. Although all of the three glucosaminidic linkages were cleaved by the enzyme under harsh conditions, only the middle linkage was cleaved but never IdoA in these four molecules, which is in contrast to the recent finding of both GlcA and IdoA at this position in dermatan sulfate from bovine aorta (Sugahara et al., 1995). The second and the third uronic acid could be IdoA when located adjacent to the trisulfated disaccharide unit as in the compounds in fractions b-5, b-10, and b-6. However, the uronic acid next to the trisulfated disaccharide unit was not always GlcA but could be GlcA as in the compound in fraction b-5-II. The above structural characteristics of these four molecules may have some implications in the expression of biological functions and in the biosynthetic mechanisms of heparin.
under milder conditions for partial digestion (see “Experimental Procedures”), yielding a tetrasaccharide and a linkage hexosaminidic bond in heparin except for the two unique hexosaminidic bonds: the one in the structure -4GalNAc(6S)-4GlcAβ1-3Galβ1-4GlcN(5S,3S,6S) found within the antithrombin III-binding domain (Yamada et al., 1993) and the one in the structure -4HexA1-4GalNAc(6S)-4GlcAβ1-3Galβ1- of the carbohydrate-protein linkage region (Fig. 5) (Sugahara et al., 1994). In this study it was demonstrated that this enzyme cleaves the hexosaminidic linkage adjacent to a trisulfated disaccharide unit more preferentially than the one next to a nonsulfated disaccharide unit (Fig. 5b).

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