

Search for the Heparin Antithrombin III-binding Site Precursor*

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The last step of heparin biosynthesis is thought to involve the action of 3-*O*-sulfotransferase resulting in the formation of an antithrombin III (ATIII) binding site required for heparin's anticoagulant activity. The isolation of a significant fraction of heparin chains without antithrombin III-binding sites and having low affinity for ATIII suggests the presence of a precursor site, lacking the 3-*O*-sulfate group. Porcine mucosal heparin was depolymerized into a mixture of oligosaccharides using heparin lyase. One of these oligosaccharides was derived from heparin's ATIII-binding site. In an effort to find the ATIII-binding site precursor, the structures of several minor oligosaccharides were determined. A greater than 90% recovery of oligosaccharides (on a mole and weight basis) was obtained for both unfractionated and affinity-fractionated heparins. An oligosaccharide arising from the ATIII-binding site precursor was found that comprised only 0.8 mol % of the oligosaccharide product mixture. This oligosaccharide was only slightly enriched in heparin having a low affinity for ATIII and only slightly disenriched in high affinity heparin. The small number of these ATIII-binding site precursors, found in unfractionated and fractionated heparins, suggests the existence of a low ATIII affinity heparin may not simply be the result of the incomplete action of 3-*O*-sulfotransferase in the final step in heparin biosynthesis. Rather these data suggest that some earlier step, involved in the formation or placement of these precursor sites, may be primarily responsible for high and low ATIII affinity heparins.

Heparin is a polydisperse sulfated copolymer of 1→4-linked glucosamine and uronic acid residues (Casu, 1985). It has been medically important as an anticoagulant since its introduction over a half-century ago (Linhardt, 1991), and recently a number of new biological activities has been ascribed to heparin (Linhardt and Loganathan, 1990; Lane and Lindahl, 1989). Despite both medical and scientific interest in heparin, its precise chemical structure remains unclear. An improved understanding of heparin's structure has resulted from two complementary approaches, direct structural studies and biosynthetic studies.

Structural studies have relied on spectroscopic (Gatti *et al.*, 1979; Loganathan *et al.*, 1990; Mallis *et al.*, 1989), chemical (Bienkowski and Conrad, 1984; Roden, 1989), and enzymatic (Linker and Hovingh, 1984; Nader *et al.*, 1987; Linhardt *et al.*, 1986) methods. Pioneering studies from a number of

laboratories demonstrated that heparin's major repeating unit was the trisulfated disaccharide, →4)α-D-GlcNp2S6S(1→4)α-L-IdoAp2S(1→¹ (Roden, 1989). The integrated use of spectroscopic, chemical, and enzymatic techniques have led to the discovery of the structure of oligosaccharides that represent variants in the primary structure of the heparin polymer (Loganathan *et al.*, 1990; Rice and Linhardt, 1989; Merchant *et al.*, 1985). Oligosaccharide mapping techniques (Linhardt *et al.*, 1988; Linhardt *et al.*, 1990a; Turnbull and Gallagher, 1988) and computer and mathematical simulation studies (Radoff and Danishefsky, 1984; Rosenfeld and Danishefsky, 1988; Oscarsson *et al.*, 1989; Linhardt *et al.*, 1989; Cohen and Linhardt, 1990) are leading to an understanding of the placement of these structural variants with the heparin polymer. Recently, studies have begun (Linhardt *et al.*, 1990b; Sugahara *et al.*, 1988; Fransson *et al.*, 1990; Turnbull and Gallagher, 1991) that are aimed at sequencing the glycosaminoglycan polysaccharides from their site of attachment to core protein.

Biosynthetic studies represent a second, equally important approach toward understanding heparin's structure (Lindahl *et al.*, 1986; Lindahl, 1989). The biosynthesis of the heparin proteoglycan in mastocytoma begins with the assembly of core protein and the *O*-glycosylation of multiple serine residues to form a linkage region. From this linkage region the polymer chain grows by the alternate addition of 1→4-linked α-D-GlcNpAc and β-D-GlcAp residues. This leads to a group of closely spaced, long ($M_r \sim 60,000$), linear polysaccharide chains attached to the protein core. The sequential action of enzymes that *N*-deacetylate, *N*-sulfate, C5-epimerize, and *O*-sulfate the homocopolymer chain gives heparin its complex structure (Lindahl *et al.*, 1986; Lindahl and Kjellen, 1987). Complete modification by the action of each of these enzymes would result in a simple repeating polymer of heparin's major trisulfated disaccharide. The structural variation found in heparin, which complicates its structure, is thought to be the result of incomplete modification during its biosynthesis.

In the final step of heparin biosynthesis (Fig. 1), GlcNp2S6S residues located within specific, incompletely modified, sequences are 3-*O*-sulfated (Lindahl *et al.*, 1986; Kusche *et al.*, 1988). This step is particularly noteworthy as it generally results in sites at which the serine protease inhibitor ATIII binds (Pejler *et al.*, 1987; Kusche *et al.*, 1988). This specific pentasaccharide binding site has been shown to be

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¹ The abbreviations used are: GlcNp, 2-deoxy-2-amino-α-D-glucopyranose; IdoAp, α-L-idopyranosyluronic acid; GlcAp, β-D-glucopyranosyluronic acid; ΔUAp, 4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid; S, sulfate; Ac, acetate; p, pyranose; ATIII, antithrombin III; TSP-d4, 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt; SAX, strong anion exchange; HPLC, high pressure liquid chromatography; AUFS, absorbance units full scale; PAGE, polyacrylamide gel electrophoresis; COSY, correlation spectroscopy; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy; FAB-MS, fast atom bombardment-mass spectrometry; NOE, nuclear Overhauser enhancement.

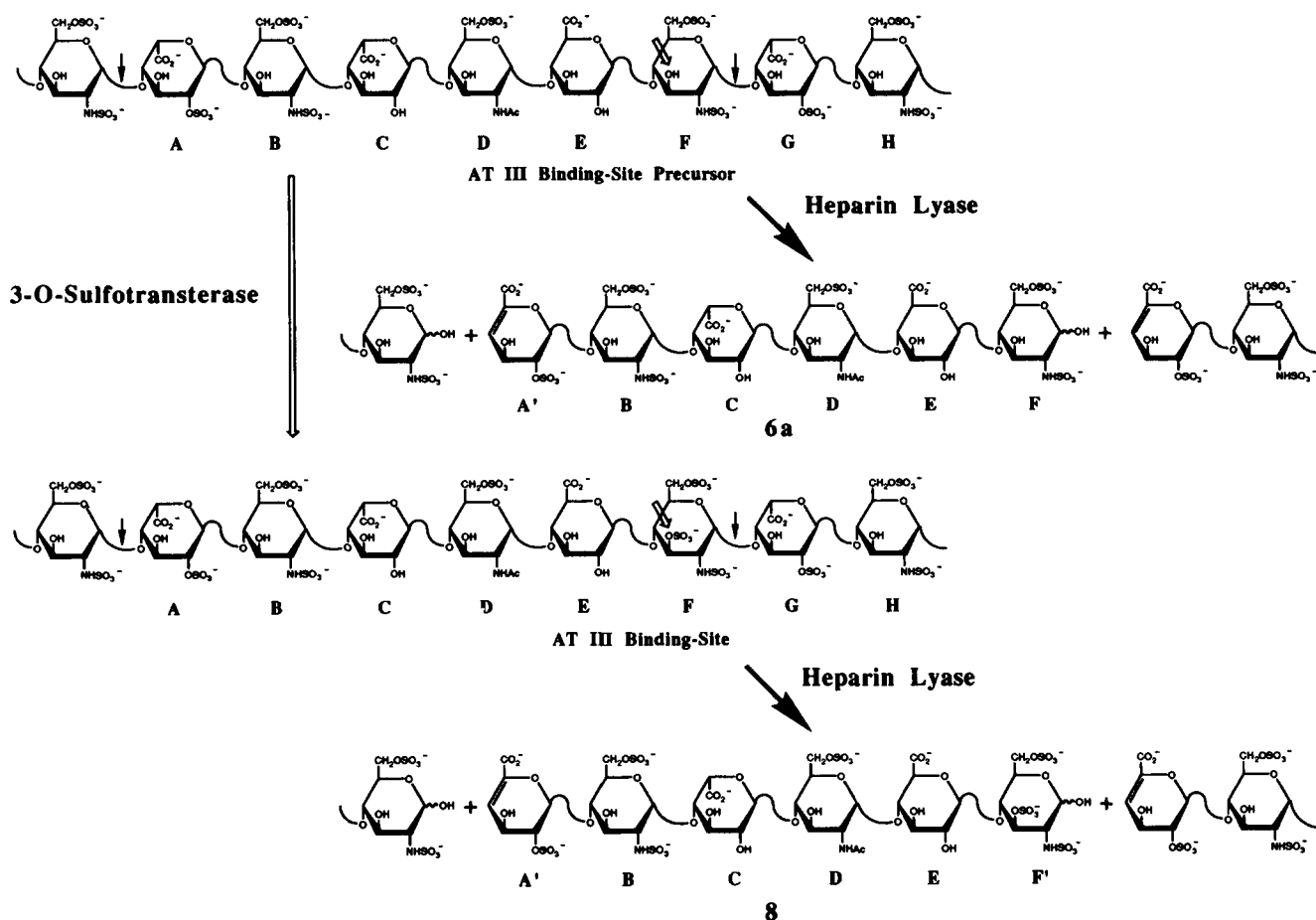


FIG. 1. A heparin chain that contains an ATIII-binding site precursor is converted through the action of 3-O-sulfotransferase to one that contains an ATIII-binding site. The products afforded on treating each of these chains with heparin lyase are shown.

primarily responsible for heparin's anticoagulant activity (Lindahl *et al.*, 1983; Atha *et al.*, 1984). However, some structural variability is possible within this site (Lindahl *et al.*, 1984; Pejler *et al.*, 1987; Guo and Conrad, 1989; Loganathan *et al.*, 1990). The primary binding site found in porcine mucosal heparin, commonly used as an anticoagulant drug, is a single well defined structure (Fig. 1). Only about one-third of chains in porcine mucosal heparin contain an ATIII-binding site and have a high affinity for ATIII (Lam *et al.*, 1976; Kim and Linhardt, 1989). The biosynthetic model has been used to explain the distribution of ATIII-binding sites (Kusche *et al.*, 1988). During the final biosynthetic step some chains are 3-O-sulfated resulting in heparin having a high affinity for ATIII while others are not and thus have a low affinity for ATIII. Further, it was suggested that all chains contain sequences appropriate for 3-O-sulfation and that selective 3-O-sulfation is primarily the result of the lack of access of certain chains to 3-O-sulfotransferase.

Thus, this biosynthetic model predicts the presence of significant quantities of ATIII-binding site precursor sites in heparin. Additionally, it suggests that these precursor sites (lacking a 3-O-sulfate) should be enriched in heparin with a low affinity for ATIII and disenriched in heparin with a high affinity for ATIII (containing a high level of 3-O-sulfate). A recent study (Kusche *et al.*, 1990) reported the isolation of significant quantities of these precursor sites from a tetrasaccharide fraction obtained through nitrous acid depolymerization of heparin. This paper uses enzyme-based oligosaccharide

mapping to test this biosynthetic model. This method permits the quantitation of the number of these precursor sites in unfractionated and affinity-fractionated heparins.

EXPERIMENTAL PROCEDURES

Materials

The sodium salt of porcine intestinal mucosa heparin (157 units/mg) was from Hepar Industries; bovine lung heparin (149 units/mg) and sulfopropyl (SP)-Sephadex were from Sigma. ATIII-agarose was from Calbiochem. Factor Xa amidolytic assay kit was from Hellen Laboratories. Heparinase, heparin lyase (EC 4.2.2.7) having a specific activity of 5 IU/mg, was prepared and purified as previously reported (Gallihier *et al.*, 1981; Yang *et al.*, 1985), or together with heparin lyase II (no EC number) and heparan sulfate lyase (EC 4.2.2.8) was obtained from Seikagaku America. Disaccharide standards derived from heparin and derivatized heparins were obtained as a kit from Grampian Enzymes (Aberdeen, Scotland). Spectropore dialysis tubing (*M*_w 1000 cut off) was purchased from Spectrum Medical. Polyacrylamide Bio-Gel P2 for desalting was from Bio-Rad. HPLC was performed using dual LDC Constametric III pumps with gradient mixer and microprocessor gradient control and data collection using a Rheodyne injector on a Phase Separation SAX column (4.6 × 250-mm analytical column and a 25 × 250-mm semipreparative column, both of 5-μm particle size) and an ISCO model 1840 variable wavelength UV detector. Gradient PAGE was performed on a Hoeffer SE 620 slab gel using a Bio-Rad 1420B power supply. High purity electrophoresis reagents were purchased from IBI, Inc. Spectrometric measurements were made on a Shimadzu UV-160 spectrophotometer, Bruker WM 360 MHz and AMX 600 MHz spectrometers, and a VG ZAB-HF mass spectrometer. TSP-d4 and ²H₂O (99.996 and 99.9 atom %) were from Aldrich Chemical Co.

Methods

Preparation of Heparin Samples—Approximately 6 g of heparin was dissolved in 120 ml of distilled water and dialyzed exhaustively, freeze-dried, and prepared at 20 mg/ml in distilled water (Linhardt *et al.*, 1988). Heparin was affinity-fractionated by loading 250 μ g in 125 μ l onto a 15 \times 55-mm ATIII-agarose column, having the capacity to bind 100 μ g of heparin, equilibrated with 20 mM Tris-hydrochloride buffer, pH 7.4, containing 0.05 M sodium chloride. The column was washed with 40 ml of 0.05 M sodium chloride (first wash), 20 ml of 0.25 M sodium chloride (second wash), and 20 ml of 1 M sodium chloride (third wash), all in 20 mM Tris-hydrochloride buffer. Affinity fractionation was repeated 20 times, and like fractions were combined and dialyzed against distilled water and freeze-dried. A total of 3.0 mg of heparin with low affinity for ATIII was obtained in the first wash, 1.7 mg of heparin obtained in the second wash was discarded, and 1.17 mg with a high affinity for ATIII was recovered in the third wash.

Preparation of Oligosaccharides—Oligosaccharides **1–8** were prepared from 5 g of porcine mucosal heparin in 250 ml of 50 mM sodium phosphate buffer at pH 7 containing 4 IU of heparinase (5 IU/mg, purified in our laboratory). The reaction mixture was incubated at 30 °C for 84 h. Heparin oligosaccharide product mixture (5 g/50 ml) was divided into 14 portions, and each was fractionated by semipreparative SAX-HPLC, and like fractions were combined. The structures of oligosaccharides **1–8** were previously reported (Rice and Linhardt, 1989; Linhardt *et al.*, 1989; Loganathan *et al.*, 1990) and are as follows: **1**, Δ UAp(1 \rightarrow 4)- α -D-GlcNp2S6S; **2**, Δ UAp2S(1 \rightarrow 4)- α -D-GlcNp2S; **3**, Δ UAp2S(1 \rightarrow 4)- α -D-GlcNp2S6S; **4**, Δ UAp2S(1 \rightarrow 4)- α -D-GlcNp2S6S(1 \rightarrow 4)- α -L-IdoAp2S(1 \rightarrow 4)- α -D-GlcNp2S; **5**, Δ UAp2S(1 \rightarrow 4)- α -D-GlcNp2S6S(1 \rightarrow 4)- β -D-GlcAp(1 \rightarrow 4)- α -D-GlcNp2S6S; **6**, Δ UAp2S(1 \rightarrow 4)- α -D-GlcNp2S6S(1 \rightarrow 4)- α -L-IdoAp2S(1 \rightarrow 4)- α -D-GlcNp2S6S; **7**, Δ UAp2S(1 \rightarrow 4)- α -D-GlcN2S6S(1 \rightarrow 4)- β -D-GlcAp(1 \rightarrow 4)- α -D-GlcNp2S3S6S; and **8**, Δ UAp2S(1 \rightarrow 4)- α -D-GlcNp2S6S(1 \rightarrow 4)- α -L-IdoAp(1 \rightarrow 4)- α -D-GlcNpAc6S(1 \rightarrow 4)- β -D-GlcAp(1 \rightarrow 4)- α -D-GlcNp2S3S6S. The structure of tetrasaccharide **4** was previously assigned as Δ UAp2S(1 \rightarrow 4)- α -D-GlcNp2S(1 \rightarrow 4)- α -L-IdoAp2S(1 \rightarrow 4)- α -D-GlcNp2S6S (Rice and Linhardt, 1989). The corrected structure reverses the position of the glucosamine residues and is based on two-dimensional COSY and ROESY NMR results (not shown). Analysis of oligosaccharide standards **1–8** by SAX-HPLC and gradient PAGE demonstrated each had a purity of >95%.

Oligosaccharide **6a** was also prepared from the same 5 g of depolymerized porcine mucosal heparin. The peak eluting from the SAX-HPLC column at 0.78–0.80 M sodium chloride was collected. The peaks from all 14 runs were pooled, freeze-dried, desalted on a 2.5 \times 35-cm Bio-Gel P2 column at 1 ml/min, and again freeze-dried. The crude oligosaccharide **6a** (8 mg, 70% pure by analytical SAX-HPLC) was again fractionated by semipreparative SAX-HPLC in a single injection (8 mg in 200 μ l) and collected, desalted, and freeze-dried as before. The resulting oligosaccharide **6a** (5.3 mg) was >95% pure by SAX-HPLC and gradient PAGE. Tetrasaccharides **3a**, **3b**, and **4a** were also prepared in the same fashion at 90% purity with the recovery of 0.6, 0.5, and 2.4 mg, respectively.

Oligosaccharide **6a** (10 μ g, >95% pure) was treated with an enzyme mixture containing 50 mIU each of heparin lyase, heparin lyase II, and heparan sulfate lyase (Linhardt *et al.*, 1990c) in 50 μ l of 5 mM sodium phosphate buffer containing 0.2 M sodium chloride at pH 7.0. After 24 h at 30 °C the mixture was diluted to 1 ml with distilled water, adjusted to pH 3.0 with hydrochloric acid, and added to a disposable polyethylene column containing 100 μ l of SP-Sephadex (also at pH 3.0) to remove protein (pI > 8). The solution containing oligosaccharide products was collected, its pH readjusted to 7.0, and it was freeze-dried. The enzyme-treated **6a** was examined by analytical SAX-HPLC, and the oligosaccharide products were identified by comparison of their retention times to those of disaccharide standards.

SAX-HPLC Analysis—To 50 μ l of each heparin sample (20 mg/ml) 450 μ l of sodium phosphate buffer (5 mM sodium phosphate, pH 7, 200 mM sodium chloride) containing 15 mIU heparinase was added. The reaction mixture was incubated at 30 °C for 8 h. At reaction completion the depolymerization mixture was frozen and stored at -70 °C until analyzed. Oligosaccharide mixture or individual oligosaccharides were analyzed by injecting 4 μ g/40 μ l onto an analytical SAX-HPLC column. The sample was eluted from the column by using a 90-ml linear gradient (0.2–1.0 M) of sodium chloride at pH 3.5 at a flow rate of 1.5 ml/min. The elution profile was monitored by absorbance at 232 nm at 0.02 absorbance unit full scale (AUFS)

and a chart speed of 15 cm/h. Retention time varies due to aging of the columns (Rice *et al.*, 1985) so that peaks were tentatively identified by coelution with an authentic sample.

Gradient PAGE Analysis—Oligosaccharide sample was analyzed by applying sample (20–40 μ g in 50 μ l) to a discontinuous PAGE gel (Rice *et al.*, 1987) using a linear acrylamide and cross-linked gradient (12–22% total acrylamide). Two gels (16 \times 32 \times 0.15 cm) were run simultaneously at 250 V for 17 h, by which time the added bromophenol blue marker dye had migrated 18 cm into the resolving gel. The gels were removed from the glass plates and stained for 30 min in Alcian Blue 0.5% (w/v) in 2% (v/v) aqueous acetic acid destained with successive washes of 2% acetic acid. Bands were identified by comigration with authentic samples.

Spectrometric Methods—Ultraviolet absorbance was determined at 232 nm by weighing a salt-free sample and dissolving in 0.03 N hydrochloric acid. One-dimensional NMR spectroscopy was performed on a Bruker WM360 (¹H at 360 MHz) and two-dimensional NMR spectroscopy on a AMX600 Bruker spectrometer (¹H at 600 MHz). Samples were prepared for ¹H NMR (500 μ l at 9.4 mM) in ²H₂O (99.996%), after exchanging three times with ²H₂O (99.9%). The spectra were obtained at 298 K. Two-dimensional ¹H COSY and ¹H ROESY NMR spectra each required overnight acquisition and used Bruker software (Loganathan *et al.*, 1990). Mass spectrometric analysis was performed by using a VG ZAB-HF mass spectrometer, equipped with an Ion Tech 11 NF saddle field atom gun (xenon at 8 kV, 1.5 mA) in the fast atom bombardment (FAB) ionization mode. Negative ion FAB spectra were obtained in both low and high resolution (for accurate measurements) modes by signal adding eight scans using the multichannel analysis scanning software of the 11–250 J data system. Samples were prepared for FAB analysis by dissolving 200 μ g of oligosaccharide in approximately 10 μ l of distilled water. Typically, 1 μ l of triethanolamine is placed on the FAB probe tip (as matrix) to which 1 μ l of the oligosaccharide solution is added (Mallis *et al.*, 1989).

Amidolytic Assay—Heparin samples were assayed for anti-factor Xa activity by the “end point” method using amidolytic substrates against a standard curve constructed by using USP heparin (Linhardt *et al.*, 1988).

RESULTS AND DISCUSSION

When porcine mucosal heparin is treated with heparin lyase, six major (each >2 mol %) oligosaccharides (**1**, **3–6**, **8**) are observed on SAX-HPLC (Fig. 2A) that account for 85–90 mol % of the oligosaccharide products (Linhardt *et al.*, 1988; Rice and Linhardt, 1989). The structures of additional minor oligosaccharides (**2** and **7**) have since been reported (Loganathan *et al.*, 1990; Linhardt *et al.*, 1990c), and many additional minor peaks corresponding to uncharacterized oligosaccharides can also be observed in the expanded SAX-HPLC chromatogram (Fig. 2B). Tetrasaccharides **4** and **6** are substrates for heparin lyase which acts at the \rightarrow 4)- α -D-GlcNp2S6X(1 \rightarrow 4)- α -L-IdoAp2S(1 \rightarrow) linkage (where X is S or OH). They are isolated intact as a result of the low catalytic efficiency V_{max}/K_m of heparin lyase toward these small oligosaccharide substrates (Rice and Linhardt, 1989).

As part of our continuing studies on heparin's structure we decided to search for a specific oligosaccharide(s) corresponding to the ATIII-binding site precursor (the sequence required for 3-O-sulfation, Fig. 1) among these minor oligosaccharides observed in Fig. 2B. A hexasulfated hexasaccharide should result from the action of heparin lyase at the ATIII-binding site precursor (Fig. 1) as predicted from the specificity of heparin lyase (Linhardt *et al.*, 1990c) and from the model for heparin biosynthesis (Kusche *et al.*, 1988). This hexasulfated hexasaccharide should elute from SAX-HPLC prior to heptasulfated hexasaccharide, **8**. In addition, the biosynthetic model predicts that this hexasaccharide should be found in molar amounts comparable or greater than its 3-O-sulfated counterpart, hexasaccharide **8**.

Based on these predictions, semipreparative SAX-HPLC (Fig. 2C) was used to obtain several minor oligosaccharides, for structural characterization, with the expectation that one

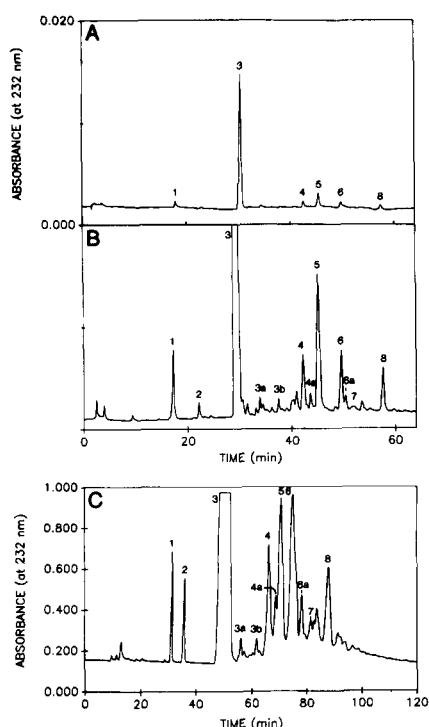


FIG. 2. SAX-HPLC chromatograms of heparin lyase-treated porcine mucosal heparin. *A* shows the analysis of 4 μ g of oligosaccharide mixture on analytical SAX-HPLC while *B* shows the analysis of 40 μ g; both are at 0.02 AUFS. *C*, a semipreparative SAX-HPLC chromatogram of 100 mg of the same oligosaccharide mixture is shown at 1 AUFS. Peaks corresponding to oligosaccharides 1–8 are indicated.

would be the desired hexasulfated hexasaccharide. These peaks identified in Fig. 2 as **3a**, **3b**, **4a**, and **6a** were first isolated from 14 semipreparative runs. After combining like fractions and desalting using a Bio-Gel P-2 column each peak was reapplied to obtain a pure oligosaccharide product. Analytical SAX-HPLC and gradient PAGE analysis confirmed the purity of each product. The molecular weight of each oligosaccharide was estimated by gradient PAGE (Linhardt *et al.*, 1990a; Rice *et al.*, 1987), and analytical SAX-HPLC gave the amount of each oligosaccharide obtained from heparin (Table I).

Gradient PAGE analysis suggested that oligosaccharides **3a**, **3b**, and **4a** were tetrasaccharides and thus did not correspond to the desired hexasulfated hexasaccharide. FAB-MS and ^1H NMR spectrometry (Table II) permitted the assignment of structures for oligosaccharides **3a**, **3b**, and **4a**. Oligosaccharide **3a** was a tetrasulfated tetrasaccharide having the structure $\Delta\text{UAp}2\text{S}(1\rightarrow4)\text{-}\alpha\text{-D-GlcNp}2\text{S}(1\rightarrow4)\text{-}\alpha\text{-L-IdoAp}2\text{S}(1\rightarrow4)\text{-}\alpha\text{-D-GlcNp}2\text{S}$. This tetrasaccharide contains a site at which heparin lyase should act, and it might also be a poor substrate for heparin lyase (as previously reported for tetrasaccharides **4** and **6** (Rice and Linhardt, 1989)). Oligosaccharide **3b** has the structure $\Delta\text{UAp}2\text{S}(1\rightarrow4)\text{-}\alpha\text{-D-GlcNp}2\text{S}(1\rightarrow4)\text{-}\beta\text{-D-GlcAp}(1\rightarrow4)\text{-}\alpha\text{-D-GlcNp}2\text{S}6\text{S}$. It had been previously reported by our group several years ago (Merchant *et al.*, 1985) and was recharacterized as no authentic oligosaccharide remained for its identification by coelution and co-migration studies. Oligosaccharide **4a**, $\Delta\text{UAp}2\text{S}(1\rightarrow4)\text{-}\alpha\text{-D-GlcNp}2\text{S}6\text{S}(1\rightarrow4)\text{-}\alpha\text{-L-IdoAp}(1\rightarrow4)\text{-}\alpha\text{-D-GlcNp}2\text{S}6\text{S}$, represents another new oligosaccharide component of heparin. Each of these minor tetrasaccharides is an expected product based on the biosynthetic model (Lindahl *et al.*, 1986; Lindahl, 1989)

TABLE I

Oligosaccharide analysis of heparins

Total mol % of all oligosaccharides is calculated by simply summing the mol % for the oligosaccharides in each column. An error of ± 0.1 mol % is possible in the measurement of each oligosaccharide. The 2.9 (LA heparin), 9.5 (HA heparin), and 6.93 (unfractionated heparin) mol %, corresponding to unknown oligosaccharides (100% – total), represents only 3.8, 3.5, and 1.3 weight % of the oligosaccharide products, respectively.

Oligosaccharide	LA heparin	HA heparin	Unfractionated heparin
	mol %	mol %	mol %
1	1.6	1.6	2.5
2	ND ^a	0.8	0.5
3	67.0	63.0	71.0
3a	0.8	0.7	0.8
3b	0.7	0.3	0.6
4	4.8	2.4	3.1
4a	0.5	0.8	0.5
5	10.0	8.5	6.9
6	9.2	4.4	3.2
6a	1.3	0.5	0.8
7	0.4	0.7	0.6
8	1.4	7.2	2.2
Total (1–8)	97.0	91.0	93.0

^a Not detected.

for heparin and the known specificity of heparin lyase (Linhardt *et al.*, 1990c).

Gradient PAGE analysis indicated that oligosaccharide **6a** had a molecular size appropriate for the desired hexasulfated hexasaccharide. Its elution position on SAX-HPLC indicated that **6a** had a slightly higher net negative charge than the hexasulfated tetrasaccharide **6** (**6** has six sulfate anions and two carboxylate anions while **6a** has six sulfate anions and three carboxylate anions). Treatment of **6a** with a mixture of heparin lyases resulted in the formation of three disaccharide products. These were identified as $\Delta\text{UAp}2\text{S}(1\rightarrow4)\text{-}\alpha\text{-D-GlcNp}2\text{S}6\text{S}$, $\Delta\text{UAp}(1\rightarrow4)\text{-}\alpha\text{-D-GlcNpAc}6\text{S}$, and $\Delta\text{UAp}(1\rightarrow4)\text{-}\alpha\text{-D-GlcNp}2\text{S}6\text{S}$ by comparison with disaccharide standards. FAB-MS analysis (Fig. 3) gave a molecular ion at 1730 corresponding to $[\text{M} - 1]^-$ (where M is the fully sodiated oligosaccharide salt) consistent with structure **6a** (Fig. 1). Low resolution FAB-MS established the sulfation of each sugar residue in hexasaccharide **6a** from the fragmentation pattern (Fig. 3). The predominant, structurally significant fragment ions at 1448, 1145, 1061, 885, and 642 correspond to $[\text{M} - \text{H}^+(\Delta\text{IdoAp}2\text{S})]^-$, $[\text{M} - \text{H}^+(\text{GlcAp}(1\rightarrow4)\text{GlcN}2\text{S}6\text{S})]^-$, $[\text{M} - \text{H}^+(\Delta\text{IdoAp}2\text{S}(1\rightarrow4)\text{GlcN}2\text{S}6\text{S})]^-$, $[\text{M} - \text{H}^+(\Delta\text{IdoAp}2\text{S}(1\rightarrow4)\text{GlcN}2\text{S}6\text{S}(1\rightarrow4)\text{IdoAp})]^-$, and $[\text{M} - \text{H}^+(\text{IdoAp}(1\rightarrow4)\text{GlcNAc}6\text{S}(1\rightarrow4)\text{GlcAp}(1\rightarrow4)\text{GlcN}2\text{S}6\text{S})]^-$.

High field (600 MHz) NMR spectroscopy was used to assign the structure of oligosaccharide **6a** (Table III). Starting with the non-reducing end of **6a**, the six sugar residues were labeled as A', B, C, D, E, and F (Fig. 1). One-dimensional 600-MHz ^1H NMR spectrum recorded in $^2\text{H}_2\text{O}$ showed eight well resolved single proton resonances in the 4.5–6.0-ppm region. Comparison of the chemical shift and coupling constant data of these signals with those reported for heparin-derived oligosaccharides (Linker and Hovingh, 1984; Merchant *et al.*, 1985; Petitou *et al.*, 1988; Loganathan *et al.*, 1990) led to the following assignment: 5.98 (A'H-4), 5.50 (A'H-1), 5.47, 5.39, 5.35 (H-1 of substituted $\alpha\text{-D-glucosamine}$ residues), 5.01 (CH-1), 4.63 (A'H-2), and 4.59 (BH-1).

The 600-MHz ^1H NMR two-dimensional COSY-45 spectrum (Fig. 4A) afforded complete spin connectivity information for each of the six sugar residues. Signals of all four protons of A' residue were easily identified by starting from the most downfield signal at 5.98 ppm (A'H-4). Its scalar

TABLE II
Spectral data for minor tetrasaccharides derived from heparin

Compound	Estimated M_r by gradient PAGE	FAB-MS [M - Na]	Fragment ions	^1H NMR signals assigned to anomeric proton			
				ΔUA	GlcNp ^a	UAp ^a	GlcNp ^b
3a ^c	1050–1150	1103	840	5.53	5.39	5.18	5.45
3b	1050–1150			5.45	5.37	4.52	5.44
4a	1150–1250	1205	945, 840, 642	5.50	5.36	5.01	5.45

^a Internal sugar residue.

^b External sugar residue.

^c No free amino group was detected using the method of Good and Bessman (1964).

FIG. 3. Negative ion FAB mass spectrum of oligosaccharide 6a.

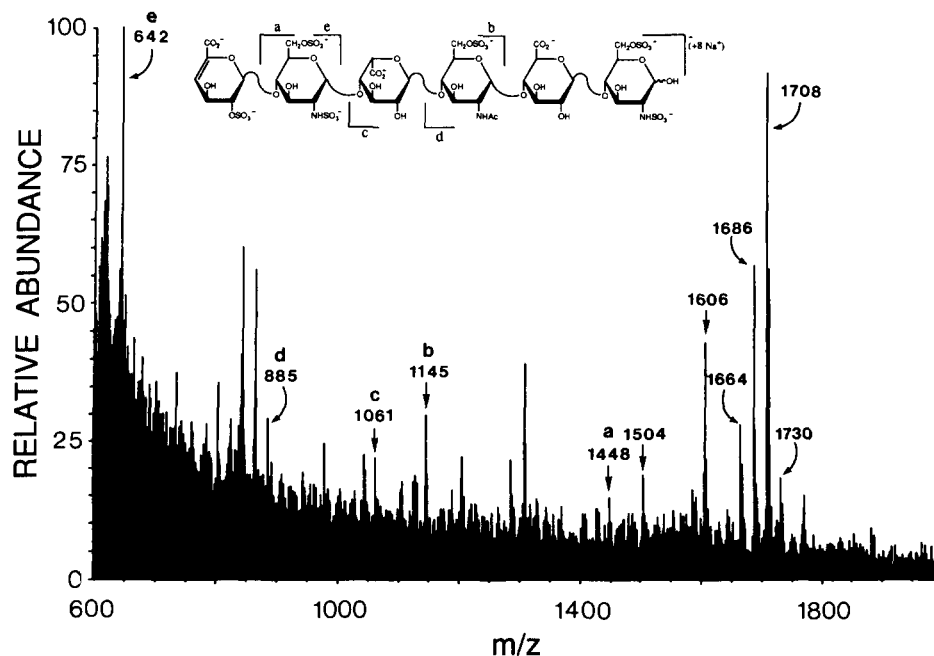


TABLE III
 ^1H NMR spectral assignments

Ring	$^2\text{H}_2\text{O/TSP-d}_4$ of oligosaccharide 6a ^a							
	H-1	H-2	H-3	H-4	H-5	H-6	H-6'	COCH ₃
A'	5.50	4.63	4.33	5.98				
B	5.35	3.27	3.64	3.83	3.98	4.21	4.36	
C	5.01	3.79	4.13	4.07	4.79			
D	5.39	3.93	3.78	3.73	4.18	4.34	4.36	2.05
E	4.59	3.35	3.71	3.75	3.83			
F	5.47	3.27	3.74	3.74	4.02	4.21	4.34	

^a Shift in ppm.

vicinal coupling to A'H-3 (4.33 ppm) as well as long range coupling to A'H-2 (4.63 ppm) are revealed by the presence of two pairs of cross-peaks. A similar spin connectivity is seen for A'H-3 with A'H-2 and A'H-1 (5.50 ppm). The chemical shift of A'H-2 is consistent with A' residue being 2-O-sulfated (Linker and Hovingh, 1984; Merchant *et al.*, 1985; Petitou *et al.*, 1988; Loganathan *et al.*, 1990). All protons of other residues B–F were also clearly identified in a similar manner by starting from their respective anomeric proton signals. The chemical shifts of 6 and 6' protons (4.2–4.36 ppm) of B, D, and E are assigned in accordance with their sulfation at C-6 (Petitou *et al.*, 1988). N-Acetylation of the D residue is revealed by the downfield shift of its H-2 signal by about 0.66 ppm relative to that of N-sulfated B residue. The most significant observation is the absence of 3-O-sulfate in F residue as shown by the upfield shift of the FH-3 signal by about 0.77 ppm compared with that of 3-O-sulfated glucosamine residue in compound 8 (Merchant *et al.*, 1985; Petitou *et al.*, 1988).

Slight upfield shifts (0.1–0.3 ppm) are also noticed in the chemical shift of FH-1, FH-2, and FH-4 due to the absence of 3-O-sulfate in F residue. Finally, the chemical shifts of CH-2 (3.79 ppm) and EH-2 (3.35 ppm) match very well with those of unsulfated α -L-idopyranosyluronic acid and β -D-glucopyranosyluronic acid residue, respectively (Merchant *et al.*, 1985; Petitou *et al.*, 1988).

Having accomplished the complete proton chemical shift assignment of all residues present in 6a, the next step was to establish their sequence. We previously demonstrated the usefulness of the two-dimensional ROESY to obtain sequence information for a heparin-derived tetrasaccharide (Loganathan *et al.*, 1990). Application of the same technique to the hexasaccharide 6a proved successful in obtaining both inter- and intraresidue NOEs. The 600-MHz ROESY spectrum of 6a is shown in Fig. 4B. The intense cross-peaks for the following sets of protons unambiguously establish the sequence of the 6 residues (A–F) in 6a: AH-1 and BH-4 (5.50, 3.83); BH-1 and CH-4 (5.35, 4.07); CH-1 and DH-4 (5.01, 3.73); DH-1 and EH-4 (5.39, 3.75); EH-1 and FH-4 (4.59, 3.74 ppm). Additional NOE cross-peaks, arising from intrasaccharide protons that are in 1,3-diaxial orientation, are also observed in the ROESY spectrum.

The amount of 6a formed from heparin lyase treatment of unfractionated heparin was first examined. The content of oligosaccharide 6a was 39% of oligosaccharide 8 on a molar basis (Table I). This is surprising, since most of the chains in porcine mucosal heparin have a low affinity for ATIII. Heparin with low and high ATIII affinity was prepared from porcine mucosal heparin. Their ATIII-mediated antifactor Xa

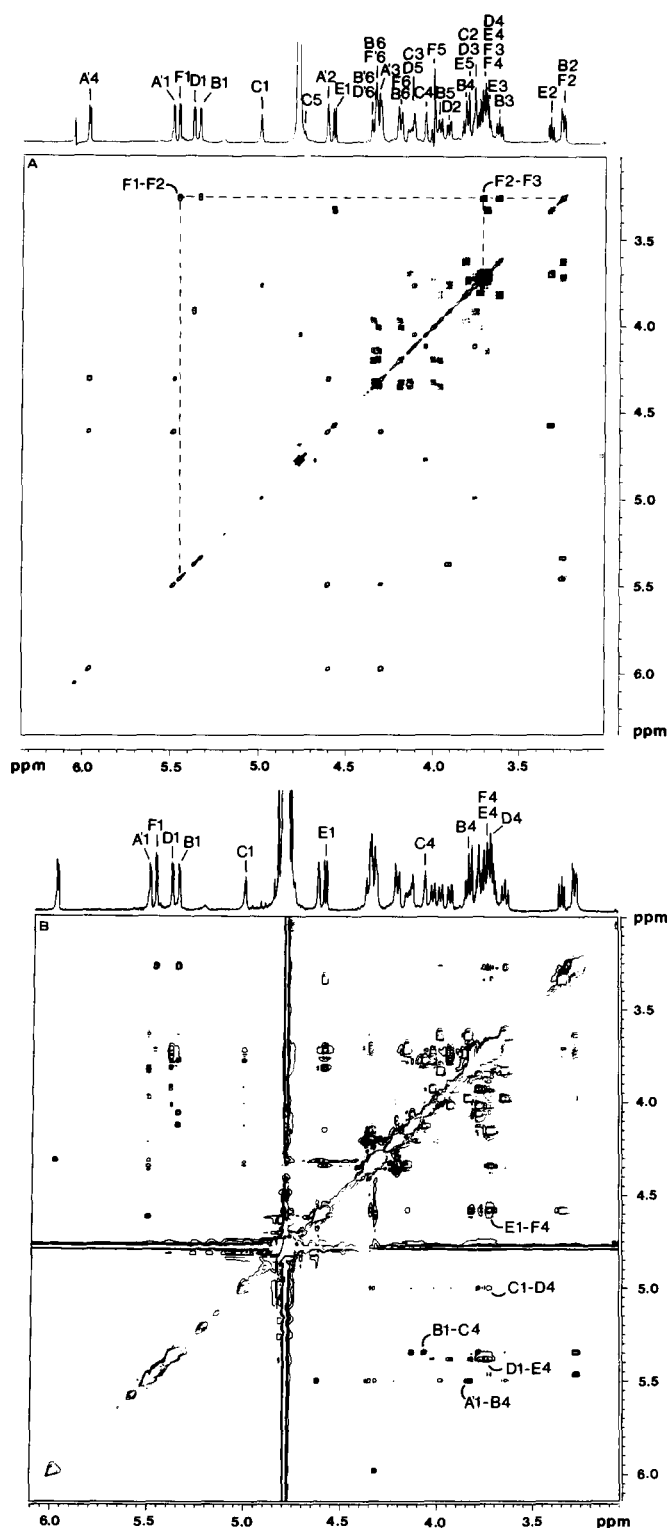


FIG. 4. Two-dimensional 600-MHz ^1H NMR spectra of **6a** ($^2\text{H}_2\text{O}$, 298 K, 9.4 mM). **A**, COSY-45 spectrum; spectral parameters include 2048 data points along F2 dimension, 512 data points along F1 dimension, 512 time increments, and 16 scans each. Following zero filling a 1024×1024 data point matrix was obtained and symmetrized. A sine window function was used in both dimensions. The spin connectivity of H-1, H-2, and H-3 protons in the F residue are shown, while all proton signals are labeled at the top. **B**, ROESY spectrum; spectral parameters include 2048 data points along F2 dimension, 256 data points along F1 dimension, 256 time increments, and 32 scans each. A mixing time of 150 ms was used. Processing of the time domain data was done in the same way as in Fig. 4A. Assignments of the NOE cross-peaks that establish the sequence in **6a** are shown.

activity was 24 and 457 units/mg, respectively. The biosynthetic model predicts that high ATIII affinity heparin should be almost completely 3-*O*-sulfated, and thus treatment with heparin lyase should afford primarily **8**, while low ATIII affinity heparin should have little or no 3-*O*-sulfation, and treatment with heparin lyase should afford primarily **6a**. These low and high affinity heparins were treated with heparin lyase, and their oligosaccharide maps are shown in Fig. 5. High ATIII affinity heparin reportedly comprises approximately one-third of the chains in porcine mucosal heparin (Lam *et al.*, 1976). The affinity fractionation in this study relied on the repetitive use of a small ATIII column. The column was underloaded, having sufficient capacity to bind 40% of the heparin sample applied. Only the non-interacting fraction (flow through at 0.05 M sodium chloride) and the tightly interacting fraction (released at >0.25 M) termed the low and high affinity fractions were examined. Thus, it is possible that some of the weakly interacting chains, not examined by oligosaccharide mapping, contained ATIII-binding sites. The high ATIII affinity heparin fraction prepared in this study still contained a small percentage of low affinity chains (and low ATIII affinity heparin probably also contained some high affinity chains). Despite these limitations in the separation and quantitation of high and low ATIII affinity heparin chains, the high and low ATIII affinity fractions analyzed were sufficiently enriched (and disenriched) to permit clear interpretation of the data obtained. A hundred heparin chains in the high affinity fraction contained 115 ATIII binding sites but only 7 ATIII binding site precursors (Table IV). The low ATIII affinity heparin fraction made up most of the porcine mucosal heparin. A hundred chains in the low affinity fraction contained 25 ATIII-binding sites and 18 ATIII-binding site precursors (Table IV). Oligosaccharide recovery was >90% for each heparin examined with very few oligosaccharides remaining unidentified in the SAX-HPLC chromatogram (Figs. 2 and 5). Thus, even if all of these precursor sites (corresponding to **6a**) were converted to ATIII-binding sites through the action of 3-*O*-sulfotransferase, low ATIII affinity heparin would not be transformed into high ATIII affinity heparin (having binding sites corresponding to **8**). A greater quantity of ATIII-binding site precursors

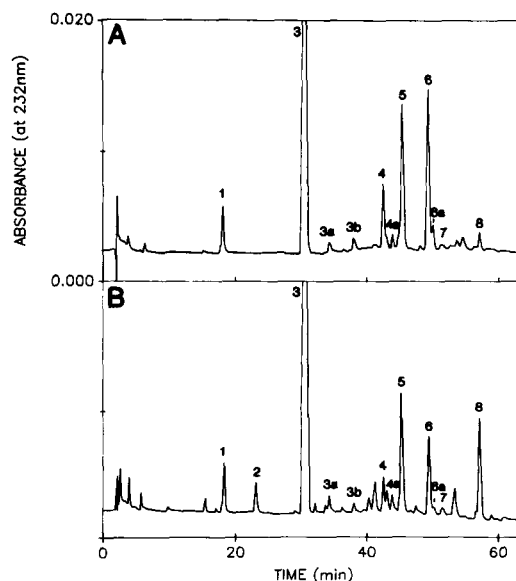


FIG. 5. SAX-HPLC chromatograms of low ATIII affinity and high ATIII affinity heparin after treatment with heparin lyase are shown in **A** and **B**, respectively. The conditions are identical to those described in Fig. 2B.

TABLE IV
Occurrence of ATIII-binding sites and binding site precursors in various heparins

	ATIII-binding sites/ heparin chain ^{a,b}	ATIII-binding site precursors/ heparin chain ^{b,c}
Low affinity heparin	0.25	0.18
High affinity heparin	1.15	0.07
Unfractionated heparin	0.43	0.13

^a Calculated by dividing the moles of oligosaccharides **7** and **8** obtained from 100 μg of heparin by 0.00833 μmol of heparin.

^b The molecular weight of heparin used in this calculation was 12,000.

^c Calculated by dividing the moles of oligosaccharide **6a** obtained from 100 μg of heparin by 0.0083 μmol of heparin.

was recently reported in a tetrasaccharide fraction obtained following nitrous acid depolymerization of ATIII affinity-fractionated heparin (Kusche *et al.*, 1990). This could be the result of some selectivity in the nitrous acid cleavage reaction. Thus, if 3-*O*-sulfation increased the stability of a glucosamine residue to deamination, then the ratio of ATIII-binding sites to binding site precursors might be much greater in higher oligosaccharide fractions. The analysis of the hexasaccharide and octasaccharide fractions was not reported nor was the yield of these higher oligosaccharides afforded on nitrous acid treatment (Kusche *et al.*, 1990).

The ATIII-binding site within heparin is known to exhibit some structural variability (Pejler *et al.*, 1987). For example, we recently described a structural variant of the ATIII-binding site with no *N*-acetylation (Loganathan *et al.*, 1990). While this is the predominant binding site found in bovine lung heparin, it represents less than one-fourth of the binding sites in porcine mucosal heparin. On treatment of porcine mucosal heparin with heparinase 0.6 mol % of oligosaccharide **7** is formed compared with 2.2 mol % of oligosaccharide **8** (both containing GlcNp2S3S6S residues characteristic of heparin's ATIII-binding site). Both oligosaccharides **7** and **8** are enriched in high ATIII affinity heparin (Kim and Linhardt, 1989). Oligosaccharide **5** has the same structure as **7** except it is missing the 3-*O*-sulfate group and thus would represent a precursor of this minor ATIII-binding site. Oligosaccharide **5** is a major product comprising 6.9 mol % of the heparinase-derived oligosaccharide mixture. The presence of multiple ATIII-binding sites (and ATIII-binding site precursors) complicates this study. The basic conclusion remains, if the formation of high affinity sequences, corresponding to oligosaccharide **8**, was limited by 3-*O*-sulfation of precursor sequence, corresponding to oligosaccharide **6a**, then **6a** should be more abundant than **8**. This, however, is not the case for oligosaccharide **7**, corresponding to an alternate but minor form of the ATIII-binding sequence. Its precursor sequence, corresponding to oligosaccharide **5**, is more abundant (Table I), suggesting that 3-*O*-sulfation of this precursor sequence may be limiting.

It is also important to point out that the biosynthetic model describes the synthesis of proteoglycan heparin, which may contain 10–12 polysaccharide chains of molecular weight 60,000–100,000 (Lindahl *et al.*, 1986). This study uses glycosaminoglycan heparin of an average molecular weight of 12,000, which is formed through the action of proteases and endoglucuronidases on proteoglycan heparin (Jacobsson and Lindahl, 1987). While there are structural differences between glycosaminoglycan heparin and the polysaccharide chains of proteoglycan heparin, these differences cannot be responsible for our failure to observe the predicted amount of precursor to the major ATIII-binding site.

The biosynthetic model places 3-*O*-sulfation of specific

precursor sites as the final step, required for the conversion of low affinity heparin to high affinity heparin. Based on this model, the fraction of chains with high affinity sites is determined either: 1) by the fraction of chains containing precursor sites, if all precursor sites are 3-*O*-sulfated; or 2) by the fraction of precursor sites that are 3-*O*-sulfated, if all chains contain these precursor sites. Insufficient precursor sites remain in porcine mucosal heparin to support the second hypothesis, that of incomplete 3-*O*-sulfation. In addition, while the major ATIII-binding site is enriched in the high affinity fraction, the precursor site is enriched to a lesser extent in the low affinity heparin fractions. Thus the selective action of 3-*O*-sulfotransferase on certain heparin chains (Lindahl *et al.*, 1986; Kusche *et al.*, 1988; Lindahl, 1989) seems an unlikely explanation for the existence of high and low affinity heparin chains. These studies suggest that the ultimate control of the fraction of high affinity chains instead resides in one of the earlier steps in heparin biosynthesis in which the precursor sites are formed. Although the presence of additional structural variants of the ATIII-binding sites and their precursors complicate this argument, they are found in smaller amounts and thus probably do not affect the conclusions that are reached.

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