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Structure and Activity of a Unique Heparin-derived Hexasaccharide*

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A hexasaccharide representing a major sequence in porcine mucosal heparin has been enzymatically prepared from heparin. Its structure was determined by an integrated approach using chemical, enzymatic, and spectroscopic methods. Two-dimensional ^{1}H homonuclear COSY, C-H correlation NMR, and selective irradiation were used to assign many of the NMR resonances. In addition, new techniques including sulfate determination by ion chromatography and Fourier transform IR and californium plasma desorption mass spectroscopy have been applied, resulting in an unambiguous structural assignment of ΔIdoAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp(1→4)-α-D-GlcNAcp6S-(1→4)-β-D-GlcAp(1→4)-α-D-GlcNp2S3S6S (where ΔIdoA represents 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid, p represents pyranose, and GlcA and IdoA represent glucuronic and iduronic acid). This hexasaccharide contains a portion of the antithrombin III-binding site and has a K_d of 4 × 10^{-5} M. Unlike other small heparin oligosaccharides, which are specific for coagulation factor Xa, it inhibits both factors IIa and Xa equally through antithrombin III. This hexasaccharide may have the unique capacity to act primarily through heparin cofactor II to inhibit thrombin (factor IIa) and shows over half of heparin's heparin cofactor II-mediated anti-factor IIa activity. These studies suggest the occurrence of contiguous binding sites on heparin for Xa, antithrombin III, and heparin cofactor II.

Heparin is a polydisperse sulfated copolymer of 1→4-linked glucosamine and uronic acid residues and has been used over the last half-century as an anticoagulant. Despite a yearly use of over 50 million doses in the United States alone (1), heparin's exact chemical structure and the precise nature of its anticoagulant and antithrombotic activities remain unclear.

Heparin's anticoagulant activity is due to the presence of specific sequences, or protein-binding sites, in the heparin chain (2, 3). Heparin's role in anticoagulation involves the regulation of the coagulation cascade primarily through the serine protease inhibitor ATIII[1] (4). Recently, a second serine protease inhibitor, HCII, has been purified and characterized (5, 6). Unlike ATIII, HCII acts specifically on factor IIa, and its activity can be potentiated with either heparin or dermatan sulfate (7). Certain heparinoids, which are believed to act through HCII, have demonstrated promise as antithrombotics with reduced hemorrhagic complications (8).

Although the structure-activity relationship of the heparin-ATIII interaction has been explored in detail (9, 10), little is known about the structural requirements for heparin-potentiated inhibition by HCII. We report the isolation of a major heparin-derived hexasaccharide which may act through HCII and has a high anticoagulant activity. We have determined its structure using an integrated approach involving enzymatic, chemical, and spectroscopic methods. This hexasaccharide contains a portion of the ATIII-binding site, suggesting that ATIII- and HCII-binding sites may be contiguous.

EXPERIMENTAL PROCEDURES

Materials

The sodium salt of heparin from porcine intestinal mucosa (167 USP units/mg) was obtained from Hepar Industries. Sephadex G-50 superfine, Sephadex G-10, and cyanogen bromide-activated Sepharose 4B were from Pharmacia P-L Biochemicals. HPLC gel-permeation chromatography was done on Toyo Soda TSK-Gel G3000SW 0.75 × 50-cm and G2000SW 0.75 × 50-cm columns (in series) with a 0.75 × 10-cm guard column. Ion-exchange chromatography was on a Whatman Partisil M9 10/50 SAX (semipreparative) column and a Spherisorb 5-μm particle size 0.46 × 25-cm SAX (analytical) column with 0.46 × 5-cm guard columns obtained from Phase Separations. Indole and sodium periodate were from Fischer. Spectrapor dialysis tubing (M_r cutoff = 1,000 and 50,000) was purchased from Spectrum Medical. Concentration was performed in a stirred ultrafiltration cell using a YC05 (M_r cutoff = 300) membrane from Amicon Corp. Barium nitrite was from Mallinckrodt Chemical Works. Heparitinase (EC 4.2.2.8) having a specific activity of 250 IU/mg was obtained from Miles Laboratories Inc. DSS and ^2H_2O (99.996 atom %) were from Aldrich. Coomassie Blue G-250, carbazole, 2-thiobarbituric acid, dermatan sulfate, and thrombin assay substrate Chromozym TH were purchased from Sigma. Activated Thrombofax Reagent Optimized (for aPTT) was obtained from Ortho Diagnostics Systems Inc. Factor Xa amidolytic assay kit was from Hellena Laboratories. Pure bovine thrombin and raw, unbleached porcine mucosal heparin were gifts from Dr. Whyte Owen of the Mayo Clinic. Pure human HCII was generously provided by Dr. Michael Griffith of Hyland Therapeutics. Human plasma was obtained from the University of Iowa Hospital Blood Bank. Other chemicals and reagents were reagent grade.

Methods

Heparin Depolymerization—Heparin (8.3 mg/ml) was depolymerized at 30 °C with 0.03 IU/ml (11) heparinase (heparin lyase, EC 4.2.2.7), having a specific activity of 5 IU/mg, in a solution of 250 mM sodium acetate, 2.5 mM calcium acetate at pH 7.0. The reaction was monitored by removing aliquots and measuring the absorbance at 232 nm after a 1:41 dilution into 0.03 N hydrochloric acid (12). After a constant absorbance was achieved (~8 h), the sample was frozen, freeze-dried, and stored at −70 °C.

Low-pressure GPC of Heparin-derived Oligosaccharides—The freeze-dried oligosaccharide mixture was reconstituted with distilled water to a concentration of 83 mg/ml. One ml of this solution was applied to a 1.5 × 240-cm column packed with Sephadex G-50

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[1] The abbreviations used are: ATIII, antithrombin III; HCII, heparin cofactor II; p, pyranose; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt; GPC, gel-permeation chromatography; SAX, strong-anion exchange; HPLC, high-pressure liquid chromatography; aPTT, activated partial thromboplastin time.

superfine. The column was eluted at 10 ml/h with 200 mM sodium chloride, and the eluant was measured continuously at 232 nm while 2-ml fractions were collected. The combined fractions (totaling 20 ml) corresponding to the hexasaccharide components were concentrated by pressure filtration to a volume of 1 ml, frozen, and stored at −70 °C.

Preparative HPLC of Hexasaccharides—The hexasaccharide mixture (30 mg/0.3 ml) was loaded onto a semipreparative SAX column (13) equilibrated with 0.2 M sodium chloride, pH 3.5. The column was eluted with a 400-ml linear gradient (concentration (y in M) at any time (x, in s) = $0.0006\,x + 0.2$) of sodium chloride, pH 3.5, at 6 ml/min. The elution profile was measured at 2 absorbance units fullscale at 232 nm; and each peak was collected, desalted by dialysis in M_r cutoff = 1000 bags against 100 volumes of distilled water, and freeze-dried. The peak corresponding to the major hexasaccharide component was rechromatographed, collected, desalted by dialysis followed by GPC on a 2.5 × 50-cm Sephadex G-10 column eluted with distilled water, frozen, freeze-dried, and stored at −70 °C.

Analytical GPC and SAX HPLC—GPC-HPLC on coupled TSK gel columns (14) was performed on sample (3 μg/20 μl) by eluting with 1.5 M sodium chloride at a flow rate of 0.5 ml/min. The detector was set at 0.02 absorbance unit full scale, and retention times were measured to ±0.1 s. Analytical SAX-HPLC (14) was performed on a 5-μm Spherisorb column equilibrated with 0.2 M sodium chloride at pH 3.5. Sample (2 μg/20 μl) was applied to this column and eluted using a 90-ml linear gradient (concentration (y in M) at any time (x, in s) = $0.004\,x + 0.2$) of sodium chloride, pH 3.5, at 1.5 ml/min. The column was monitored at 232 nm at 0.02 absorbance unit full scale.

Chemical Assays—Uronic acid was determined by the carbazole method (15). Periodate oxidation was performed using 5 mM sodium periodate in 50 mM sodium phosphate buffer, pH 7.0, at 37 °C for 24 h (16). After periodate oxidation, the samples were dialyzed (M_r cutoff = 1000 membrane) against 100 volumes of distilled water. The uronic acid was determined both before and after periodate oxidation (13). The sensitivity of the unsaturated sugar at the nonreducing end to periodate oxidation was determined using 2-thiobarbituric acid (17). Protein was determined using Bradford reagent (18).

Degradative Methods—Nitrous acid degradation was performed at low pH (19), after which the samples were neutralized with ammonium sulfamate and immediately analyzed by electrophoresis, made visible by indole spray (13), and by analytical SAX-HPLC with detection at 232 nm. Heparitinase degradation was performed in 250 mM sodium acetate, 2.5 mM calcium acetate at pH 7.0. The sample (6.3 mg/ml) was treated with heparitinase (17 IU/ml, specific activity 250 IU/mg) for 7 h at 43 °C and monitored by GPC-HPLC and SAX-HPLC with detection at 232 nm. Sulfate analysis was performed by pyrolysis and was quantitated by conductance using ion chromatography (14).

Spectroscopic Methods—The molar absorptivity was determined at 232 nm by weighing salt-free sample and dissolving in 0.03 N hydrochloric acid. Circular dichroism was measured at 1 mg/ml in distilled water at pH 7 on a JASCO J-500A CD spectropolarimeter. IR spectroscopy was performed on 200 μg of sample in 20 mg of KBr by diffuse reflectance on a Nicolet Fourier transform infrared spectrometer. NMR was performed on two spectrometers: a Bruker WM360 (^{1}H at 360 MHz and ^{13}C at 90.56 MHz) and an IBM NR80 (^{1}H at 80 MHz and ^{13}C at 20.15 MHz). Samples were prepared at suitable concentrations, after exchange, in D_2O (13). The spectra were obtained at 25 °C. In cases where it was necessary to view signals close to the HOD signal, a spectra was also obtained at 70 °C. The Bruker resolution enhancement package was used to sharpen signals, and a symmetry operation was used to reduce the intensity of the HOD signal in the proton spectrum. The COSY spectrum was obtained using the Bruker two-dimensional COSY program with a sine bell window on 16 scans by 256 experiments with sweep width = 1199.041, time domain = 512 words, spectrometer frequency = 360.132, Hertz/point = 2.342, delay 1 = 2, and delay 0 = 3×10^{-6}. The CHORTLE C-H correlation was performed on the 6000 scan sine and cosine spectra collected at delay times of 0.4, 1.0, 2.4, and 3.2 ms. The CHORTLE C-H correlation was used to make assignments only when the signal-to-noise ratio was greater than 3. Californium plasma desorption mass spectrometry was performed on the tridodecylmethyl ammonium salt of the oligosaccharide sample by Dr. Catherine McNeal, Department of Chemistry, Texas A & M University.

Activity and Binding Assays—The anticoagulant activities were measured by aPTT clotting assay (20). ATIII-mediated anti-factor IIa and anti-factor Xa activities were measured by amidolytic assays (20) using pure ATIII (21), pure factor IIa (a generous gift from Dr. Whyte Owen, Mayo Clinic), and a factor Xa amidolytic assay kit (20). HCII was purified by ammonium sulfate fractionation and heparin-Sepharose affinity chromatography (5), resulting in a preparation free of contaminating ATIII. HCII-mediated anti-factor IIa activity was measured by amidolytic assay (22). Heparin-free dermatan sulfate was prepared using nitrous acid (23). Standard curves were prepared using both heparin and dermatan sulfate. Oligosaccharide binding to ATIII was demonstrated by loading 200 μg of sample on a 4-ml ATIII-Sepharose column equilibrated with 100 mM Tris-HCl buffer at pH 7 and eluting with a 50-ml linear sodium chloride gradient (0–2 M) in the same buffer (24). Oligosaccharide binding was quantitated using equilibrium dialysis (25). A polycarbonate equilibrium dialysis chamber with a 2-ml cell was separated into two equal sides by a M_r cutoff = 50,000 dialysis membrane permitting oligosaccharide but not ATIII to pass. ATIII (3 mg) was placed on one side of the membrane and the oligosaccharide (92 μg) on the other side. Controls were run in the absence of either oligosaccharide or protein. Both compartments contained 0.15 M sodium chloride in 0.01 M Tris-HCl buffer. The cells were shaken slowly at 4 °C, and 50-μl aliquots were removed from each compartment periodically over the course of 4 days. Oligosaccharide was quantitated by uronic acid assay, and ATIII was measured using protein assay(to check for protein diffusion across the membrane). After equilibrium was reached, high ATIII-affinity heparin (650 μg) (26) was added to the ATIII side; and, after 4 more days oligosaccharide concentration on the opposite side was again measured. The K_d was determined by subtracting nonspecific binding (not displaceable by heparin) from total binding and assuming a binding stoichiometry of 1:1.

RESULTS AND DISCUSSION

Heparin lyase acts on heparin's major linkage: →4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S-(1→ (where IdoA represents iduronic acid) (Fig. 1). This results in a reproducible distribution of oligosaccharides, each containing an even number of sugar residues (32). Fractionation of this oligosaccharide mixture on the basis of size by low-pressure GPC and charge by SAX-HPLC (Fig. 2, *A* and *B*) resulted in the isolation of a major hexasaccharide component **I**. Ten mg of commercial porcine heparin contains approximately 1 mg of this major hexasaccharide component. Both commercial heparin and raw, unbleached porcine mucosal heparin (still linked to portions of the core protein) contain similar amounts of this hexasaccharide. The purity of this hexasaccharide was assessed chromatographically by GPC-HPLC and SAX-HPLC and determined to be >98%, easily sufficient for structural characterization. Samples of even higher purity for activity studies were prepared by reapplication on analytical SAX-HPLC. The chromatographic purification of hexasaccharide **I** gave some information with regards to its structure. The

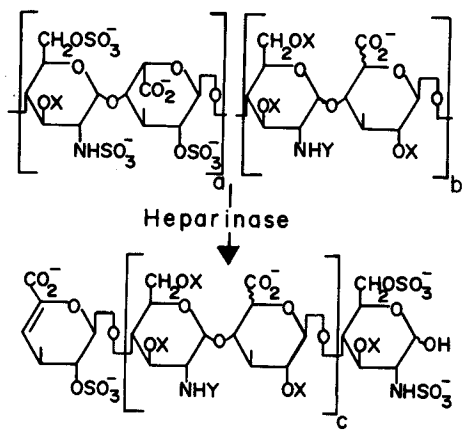
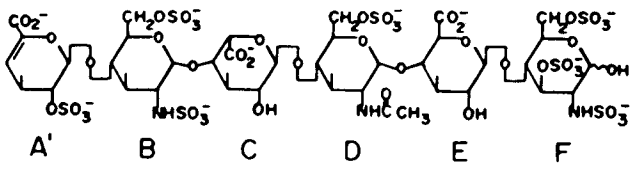
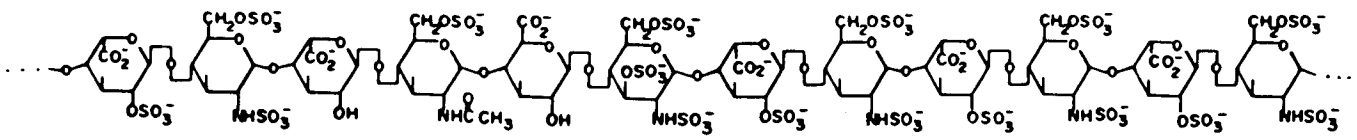


FIG. 1. **Heparin is comprised of heparinase-cleavable (*a*) and -uncleavable (*b*) glycosidic linkages.** Depolymerization results in a disaccharide, tetrasaccharides, hexasaccharides, etc. when c = 0, 1, 2 etc. Structural variability includes the stereochemistry at the 5-position in the uronic acid residue and in substitution where X = H or SO_3^- and $Y = CH_3CO$ or SO_3^-.



I



II

STRUCTURES I AND II

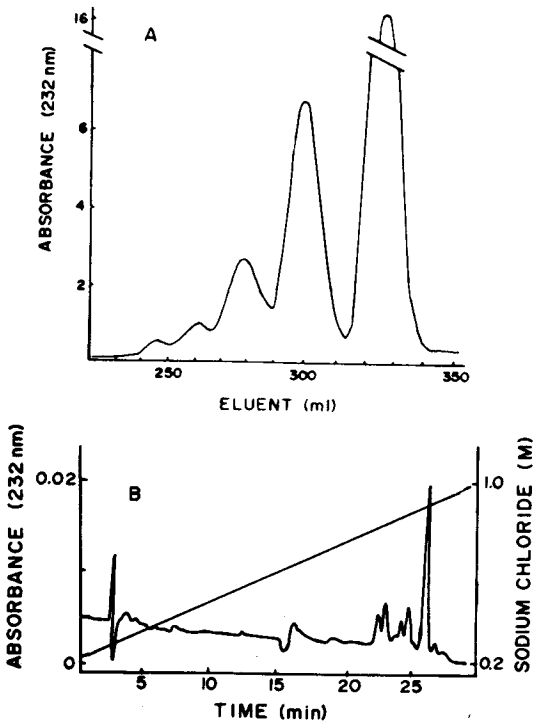


FIG. 2. **Preparation of hexasaccharide I from the oligosaccharide mixture (Fig. 1).** *A*, low-pressure GPC of oligosaccharide on a 1.5 × 240-cm Sephadex G-50 superfine column eluted with 0.2 M sodium chloride at 10 ml/h. The peaks at 247, 262, 279, 300, and 328 ml correspond to decasaccharide, octasaccharide, hexasaccharide, tetrasaccharide, and disaccharide fractions. *B*, SAX-HPLC of the hexasaccharide fraction (270–290 ml from *A*) eluted with a linear sodium chloride gradient (0.2–1.0 M) with the major peak eluting at 26.5 min corresponding to hexasaccharide **I**.

K_{av} obtained for the sample on GPC-HPLC, when compared to oligosaccharide standards (14), gave a calculated molecular weight of 1943. The hexasaccharide's late elution time on SAX-HPLC was consistent with the presence of from six to eight sulfate groups (14). The carbazole assay demonstrated the presence of 3 mol of uronic acid residues for each mole of hexasaccharide. Pyrolysis of hexasaccharide **I** followed by the quantitative determination of sulfate using ion chromatography (14) confirmed the presence of seven sulfate groups.

The structure of hexasaccharide **I** was established using an integrated approach involving enzymatic, chemical, and spectroscopic techniques. The 6 sugar residues labeled A'-F, from the nonreducing end, were elucidated in the following manner.

The nonreducing end (residue A') is an unsaturated uronic acid afforded by the elimination mechanism and is consistent with the known specificity of heparin lyase (33). Hexasaccharide **I** had a molar absorptivity in 0.03 N hydrochloric acid of 5275 ± 7% M^{-1} cm^{-1} at 232 nm, which is identical to that reported for the major heparinase-derived disaccharide having the same chromophore (13, 34). The ^1H and ^{13}C NMR assignments (Table I) for A'(H-1) and A'(C-1) show the presence of an unsaturated uronic acid. The A'(H-4) and A'(C-4) signals are consistent with the site of unsaturation in the nonreducing end uronic acid residue between carbons 4 and 5 (13). The insensitivity of this residue to periodate oxidation, established by the failure to detect the expected formyl pyruvate using thiobarbiturate assay (17), indicates the presence of a sulfate group at either the 2- or 3-position. The presence of sulfate at the 2-position was confirmed by the downfield shift of A'(H-2) and A'(C-2) to 4.61 and 78.16 ppm, respectively (13, 35). The characteristic shift of the A'(H-1) signal shows the anomeric configuration of the unsaturated uronic acid to be α-L (13, 35, 36).

Quantitative carbazole assay demonstrates the presence of two additional uronic acid residues (C and E). The sensitivity of both residues to periodate oxidation indicated that neither was sulfated. The stereochemistry at C-5 determines whether these residues are iduronic or glucuronic acid. The H-1, C-1, and H-5 signals (Table I) are consistent with both nonsulfated iduronic and glucuronic residues being present (13, 37, 38). A commercial heparitinase[2] (EC 4.2.2.8), which cleaves specifically at the →4)-α-D-GlcNAcp6(S or OH)(1→4)-β-D-GlcAp(1→ linkage (where GlcA represents glucuronic acid) (27), was used to determine whether residue C or E was a glucuronic acid residue. The resulting fragments were iden-

[2] Commerical heparitinase is sold as a mixture of heparitinases 1 and 2, each having different specificities (33); however, a recent study (27) shows that only heparitinase 1 is present.

TABLE I

^1H and ^{13}C NMR assignments of hexasaccharide I

Chemical shifts assigned against DDS. Signals at 23.80 and 176.13 ppm are for ^{13}C NMR and at 2.05 ppm for ^1H NMR of the *N*-acetyl group.

	A′	B	C	D	E	F
^1H (360 MHz)						
H-1	5.54[a]	5.37[a,b]	5.05[a]	5.36[a,b,c]	4.60[a,b]	5.46[b,c]
H-2	4.61[a,d]	3.29[a,b,c]	3.58[c]	3.41[a,b,c]	3.38[a,b]	3.44[b,c]
H-3	4.31[b]	3.69[b]	3.72[e]	3.61[a]	3.85[a,b]	4.44[b,c]
H-4	6.10[a,b,c]	3.83[a,c]	4.01[e]	3.83[a]	3.84[a]	3.97[b]
H-5		4.10[a]	4.85[a]	4.07[a]	3.90[a]	4.15[b]
H-6,6′		4.2, 4.35[a]		4.2–4.35[d]		4.21–4.37[b]
^{13}C (90.5 MHz)						
C-1	99.26[a]	98.87[a]	103.26[a]	97.95[a]	103.97[a]	93.17[a]
C-2	78.16[a]	59.45[a,d]	75.84[a]	55.77[a]	75.36[a]	58.92[c,d]
C-3	65.16[a]	71.47[e]	[f]	70.88[e]	70.88[e]	77.59[d]
C-4	109.54[a,c]	79.65[a]	76.67[a]	78.87[a]	76.49[a]	79.53[a]
C-5	147.1[a]	71.25[e]	[f]	70.27[e]	71.05[c]	71.05[e]
C-6	175.46[a]	68.30[a,c]	176.84[a,e]	69.17[a,c]	176.65[a,e]	68.07[c]

[a] Assigned using model compounds (13, 35, 40).
[b] Assigned by two-dimensional ^1H homonuclear COSY.
[c] Assigned by CHORTLE C-H correlation (28).
[d] Assigned by selective irradiation.
[e] Tentative assignment.
[f] Signals difficult to assign.

tified as a disaccharide and a tetrasaccharide by monitoring the enzymatic reaction with GPC-HPLC (Fig. 3, *A–C*). SAX-HPLC and sulfate analysis by ion chromatography established the fragments to be a trisulfated disaccharide and a tetrasulfated tetrasaccharide (Fig. 3, *D–F*). This result confirms our assignment of residues C and E as iduronic and glucuronic acid, respectively.

The presence of three glucosamine residues (B, D, and F) was established by characteristic ^1H and ^{13}C signals (Table I). All three residues (B, D, and F) are 6-sulfated as revealed by the downfield position of the C-6 and H-6 signals at 68.69 and 4.2–4.35 ppm, respectively (13). Integration of the ^1H NMR signal at 2.05 ppm and the presence of a signal at 55.77 ppm demonstrates that 1 of the glucosamine residues is *N*-acetylated. Two of the glucosamine residues are *N*-sulfated, as shown by the downfield shift of their C-2 signals as compared to an *N*-acetylated glucosamine residue. Hexasaccharide **I** was degraded chemically using nitrous acid and enzymatically using heparitinase 1 to localize the *N*-acetyl group. Nitrous acid treatment resulted in an unsaturated disulfated disaccharide (by GPC-HPLC and SAX-HPLC), consistent with cleavage between residues B and C with *N*-desulfation and ring contraction of glucosamine residue B. Heparitinase degradation of hexasaccharide **I** resulted in an unsaturated trisulfated disaccharide and tetrasulfated tetrasaccharide produced by eliminative cleavage of the glycosidic linkage be-

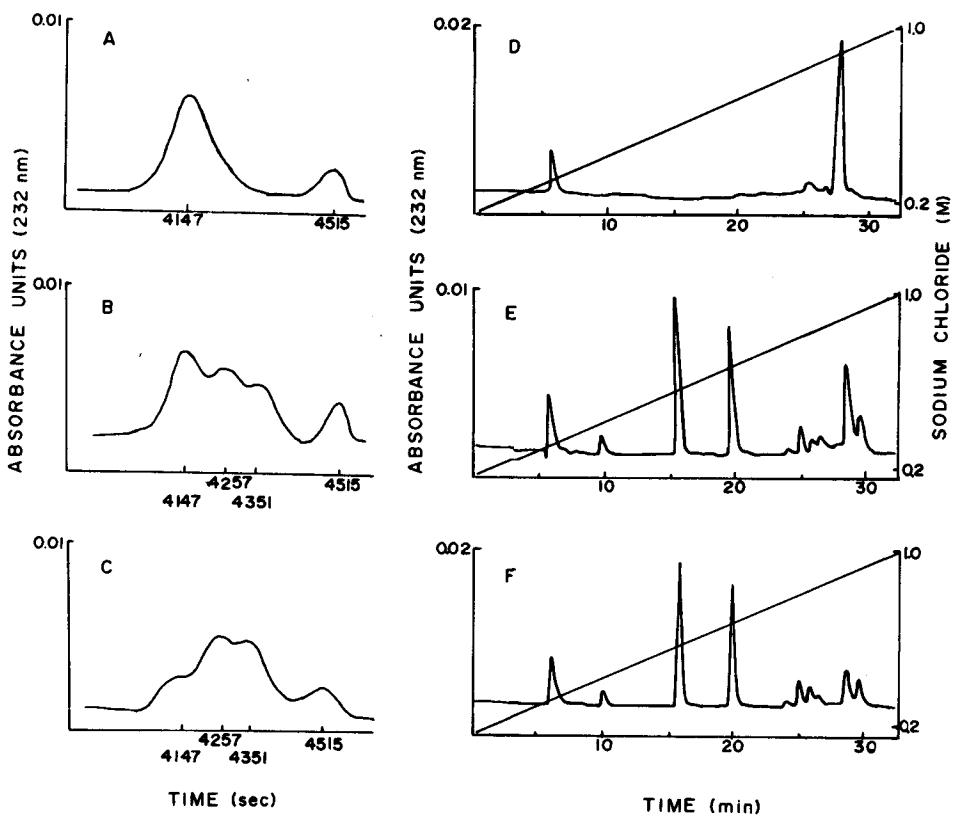


FIG. 3. **Treatment of hexasaccharide I with heparitinase 1 at 43°C and analysis using GPC-HPLC (*A–C*) and SAX-HPLC (*D–F*).** When subjected to GPC prior to the addition of enzyme (*A*), hexasaccharide **I** and acetate (internal standard) could be seen as peaks at 4147 and 4515 s, respectively. Similarly, on SAX-HPLC (*D*), acetate and hexasaccharide **I** gave peaks at 6 and 28 min, respectively. After addition of heparitinase 1, samples were taken out at 4 h (*B* and *E*) and 6 h (*C* and *F*), representing 60 and 85% reaction completion, respectively. As the hexasaccharide peak disappeared, two new peaks were detected by GPC (*B* and *C*) at 4257 and 4351 s, respectively, corresponding to the tetrasaccharide and disaccharide fragments. Similarly, the disappearance of hexasaccharide **I** corresponded to the appearance of peaks on SAX-HPLC (*E* and *F*) identified as the trisulfated disaccharide at 16.5 min and the tetrasulfated tetrasaccharide at 20 min, respectively. The additional minor peaks observed are believed to be associated with the commercial enzyme preparation used.

tween residues D and E, consistent with the known specificity of heparitinase 1. The heparitinase-derived tetrasaccharide was demonstrated to be *N*-acetylated by the presence of a peak at 2.2 ppm in the 80 MHz ^{1}H NMR. These data clearly establish that residue B is *N*-sulfated and residue D is *N*-acetylated.

The structural assignment made, as detailed above, still requires the placement of one additional sulfate group to be consistent with the seven sulfates determined present by ion chromatography. Californium plasma desorption mass spectroscopy showed an intense molecular ion at 7510 mass units in the positive-ion spectra of the tridodecylmethyl ammoniun salt. This corresponds to a molecular weight of 1833 for the neutral sodium salt, which is identical to the molecular weight calculated from the structure of hexasaccharide **I**, confirming the presence of one acetyl and seven sulfate groups. In addition, nitrous acid treatment of hexasaccharide **I** followed by paper electrophoresis and visualization with indole spray reagent resulted in only a single spot corresponding to a disulfated fragment. This indicates the failure of the reducing end glucosamine residue to de-aminate, ring-contract, and form in an anhydromannose residue which could be detected with indole reagent. The resistance of GlcN2S3S6S to nitrous acid treatment has been reported (29, 39) and suggests that the seventh sulfate is present on the 3-position of the reducing terminus, residue F. Two-dimensional homonuclear ^{1}H COSY (correlated spectroscopy) NMR (Fig. 4) was required to conclusively establish the 3-*O*-sulfation of this residue. Off-diagonal peak connectivities clearly demonstrate the coupling of FH2,1, FH2,3, FH4,3, and FH4,5 and is similar to that seen in a two-dimensional ^{1}H COSY NMR spectra of a pentasaccharide having a 3-*O*-sulfated glucosamine residue (40). The structure of the sugar residue at the reducing end of hexasaccharide **I** confirms a previous report (3, 25) that the specificity of heparinase permits a sulfate at the 3-position of the glucosamine residue at which heparinase acts.

The α-D-configuration at the anomeric center was characterized by the H-1 and C-1 chemical shifts (36). The ultraviolet circular dichroism of hexasaccharide **I** gave a maximum positive molar ellipticity at 192 nm of 320 degrees/M-cm and a negative band at 232 nm (1 g/liter in distilled water at pH 7 and 25 °C, 0.1-cm path length). The characteristic NMR signals and the positive CD band at 192 nm (41) are consistent with alternating (1→4)-α- and -β-glycosidic linkages. Fourier transform infrared spectroscopy of hexasaccharide **I** showed the characteristic C-O-S bands at 800–850 cm^{-1} (42) and had a distinct fingerprint region. Fourier transform infrared spectroscopy was useful in rapidly identifying hexasaccharide **I** prepared at different times without requiring the complete recharacterization of its structure.

The primary structure of hexasaccharide **I**, particularly the adjacency of *N*-acetylglucosamine and glucuronic acid residues (D and E), is consistent with the current understanding of heparin's biosynthesis (43). This hexasaccharide is contained within a known heparin-derived dodecasaccharide **II**, and it contains the nonreducing portion of the ATIII-binding site (9, 29).

The anticoagulant activity of hexasaccharide **I** was examined by aPTT clotting assay, and its amidolytic activity was measured using ATIII-mediated anti-factor IIa and anti-factor Xa and HCII-mediated anti-factor IIa assays. The results are given in Table II. Hexasaccharide **I** exhibits an aPTT activity of 68 units/mg, higher than any heparin oligosaccharide smaller than a hexadecasaccharide (degree of polymeri-

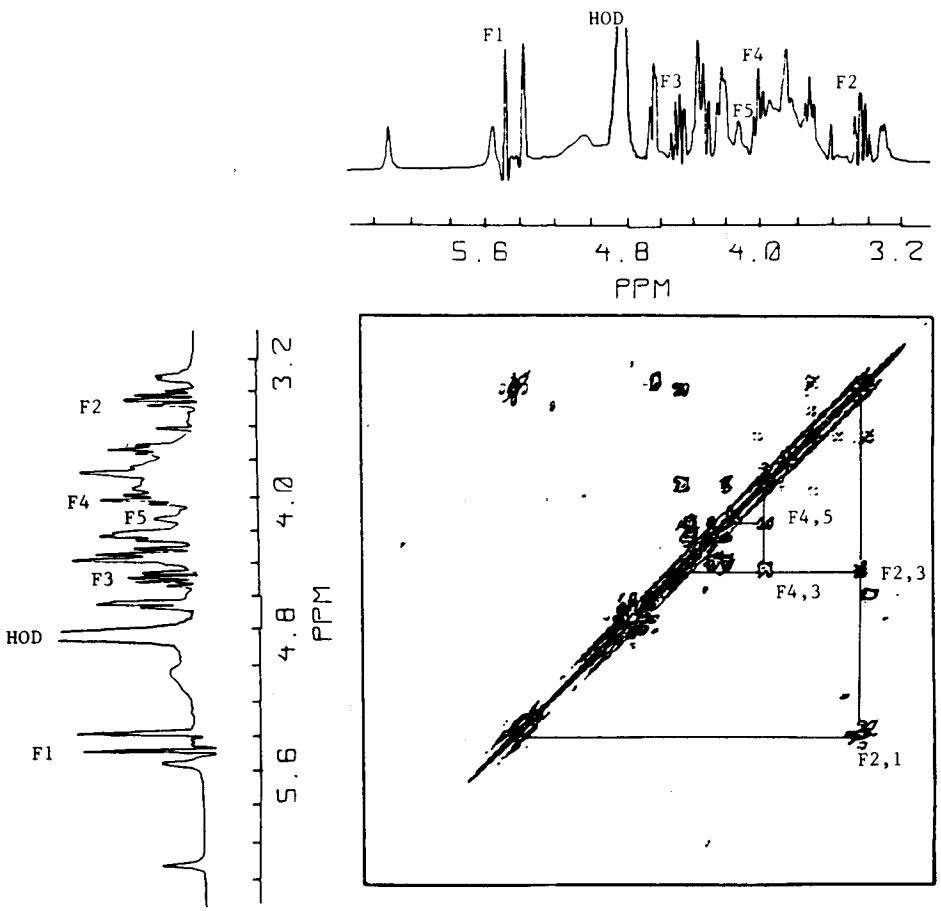


FIG. 4. **Two-dimensional ^{1}H homonuclear COSY NMR of hexasaccharide I.** The spectra was obtained on 12 mg of hexasaccharide **I** sodium salt in 200 μl of D_2O at pH 4.2. Assignments of specific peaks in the ^{1}H spectra are shown (see Table I for additional assignments). Off-diagonal peak connectivities used to demonstrate the presence of a 3-sulfated glucosamine residue at the reducing terminus of hexasaccharide **I** are shown.

TABLE II

Anticoagulant activity of hexasaccharide I and dermatan sulfate

Sample	Assay	units/mg[a]	wt % of heparin[a]
Hexasaccharide I			
	aPTT	68	40
	ATIII-anti-Xa	6	4
	ATIII-anti-IIa	6	4
	HCII-anti-IIa	84,[b] 138[c]	50,[b] 83[c]
Dermatan sulfate			
	aPTT	15	10
	ATIII-anti-Xa	<0.2	<0.1
	ATIII-anti-IIa	<0.2	<0.1
	HCII-anti-IIa	3,[b] 1[c]	2,[b] 0.6[c]

[a] Heparin (167 USP units/mg) was assigned an arbitrary value of 167 units/mg in each assay, and the activities of hexasaccharide I and heparin-free dermatan sulfate were determined from a heparin standard curve.

[b] Assay was performed using plasma as a source of HCII.

[c] Assay was performed using purified HCII free of contaminating ATIII.

zation = 16) (25, 29, 44–46). ATIII-mediated inhibition of factor IIa is generally believed to require co-binding of both proteins and hence requires large heparin oligosaccharides (degree of polymerization > 14). The ATIII-mediated anti-factor IIa activity of hexasaccharide **I**, measured using an amidolytic assay, shows this compound to have only 4 weight % of heparin's activity. Initial binding studies were performed using ATIII-Sepharose. Only half of the 0.1 μmol of hexasaccharide **I** sample loaded onto an ATIII-Sepharose column (having a capacity to bind 0.5 μmol of heparin) bound, and all of this material could be eluted by applying a 2 M sodium chloride wash. On reapplication of the binding portion, again only half bound and it eluted at the same ionic strength as previously. In both cases, the bound and unbound portions exhibited identical activity by coagulation assays. Our failure to reproducibly bind hexasaccharide **I** onto ATIII-Sepharose and the absence of a full ATIII-binding site in this compound (9) suggested that it had a weak ATIII binding affinity. A dissociation constant of 4×10^{-5} M for hexasaccharide **I** binding to ATIII, measured by equilibrium dialysis, was consistent with our expectations of a weak interaction based on the reports of other partial binding sites (Table III). The ATIII-bound hexasaccharide **I** could be quantitatively displaced using high ATIII-affinity heparin ($K_d = 2 \times 10^{-8}$ M) (29). These data suggested that it was unlikely that hexasaccharide **I** was acting on factor IIa through ATIII. Also unlikely was its action on factor Xa through ATIII as supported by a low ATIII-mediated anti-factor Xa activity (by amidolytic assay). The unlikely possibility that the high aPTT activity of hexasaccharide **I** was due to the presence of a higher oligosaccharide impurity could be ruled out on the basis of two arguments. First, the high activity (68 units/mg) and high purity (>98%) would require the impurity to have an absurdly high activity of over 3000 units/mg. Second, the disappearance of hexasaccharide **I** on heparitinase treatment (Fig. 3, *A–F*) corresponded to its loss of aPTT activity. These results show that hexasaccharide **I** has anticoagulant activity and suggest that it might be acting through an alternative ATIII-independent pathway.

Recently, HCII-mediated inhibition of factor IIa has been established (5). HCII acts specifically on factor IIa, and its action can be potentiated by either heparin or dermatan sulfate (7). An amidolytic assay specific for HCII inhibition of factor IIa (22) showed hexasaccharide **I** to have 83 weight % of heparin's activity and 100 times the activity measured for an equivalent weight of dermatan sulfate. Heparin-free dermatan sulfate has about 10% of heparin's activity on the aPTT assay, suggesting that this assay is sensitive to HCII inhibition. It is also interesting to note that the major trisaccharide sequence in dermatan sulfate (→4)-α-L-IdoAp-(1→3)-β-D-GalAp4S-(1→4)-β-D-GlcAp(1 → (where GalA represents galacturonic acid)) resembles residues C, D, and E in hexasaccharide **I**.

Our results suggest that HCII inhibition of factor IIa may be similar to ATIII inhibition of factor Xa in that co-binding is either not required or occurs at a contiguous site. Furthermore, our data suggest that an HCII-binding site might reside in the hexasaccharide A'BCDEF (Table III). Finally, the lower aPTT activity of dodecasaccharide **II**, which contains hexasaccharide residues A–F, must be rationalized. We suggest this is largely due to competition for binding to dodecasaccharide **II** by ATIII and HCII. ATIII binding to heparin is known to be stronger than HCII binding to heparin (6, 7). The aPTT assay uses plasma containing both ATIII and HCII. The higher concentration of tight-binding ATIII might completely tie up dodecasaccharide **II** in an ATIII complex, preventing its action through HCII. The lower ATIII-binding affinity of hexasaccharide **I** might permit HCII to effectively compete with ATIII, resulting in HCII-mediated inactivation factor of IIa.

The hexasaccharide sample was also examined for other biological activities. Hexasaccharide **I** showed <30 weight % of heparin's capacity to inhibit complement activation (30) and did not promote endothelial tissue cell growth as measured by [^{3}H]thymidine uptake (31).

In conclusion, the successful structural characterization of a complex molecule like hexasaccharide **I** within 3 months of its isolation indicates the value of an integrated approach to

TABLE III

Dodecasaccharide II

Sample[a]	Size	ATIII binding K_d	aPTT % of heparin	IIa·AT/Xa·AT[b]	Ref.
		M			
Heparin	dp (average) = 34[c]	2×10^{-8}	100	1.0	29
A BCDEFGHIJKL'	Dodeca	3×10^{-8}	12	0.1	29, 46
DEFGH	Penta	2×10^{-7}	<3	<0.1	44, 45
A'BCDEF- - -	Hexa	4×10^{-5}	40	1.0	[d]
-EFGHIJ'	Hexa	2×10^{-4}			25
CDEF- - -	Tetra	1×10^{-3}			25

[a] A' is an unsaturated residue formed by the action of a lyase (33). L' and J' are anhydromannose sugars formed by the action of nitrous acid (19).

[b] Ratio of ATIII-mediated anti-factor IIa and anti-factor Xa activities as measured by amidolytic assays.

[c] dp, degree of polymerization.

[d] See Table II.

oligosaccharide structure elucidation. The observation that hexasaccharide **I** may act on factor IIa through HCII indicates that heparin's anticoagulant activity is still not completely understood and suggests that detailed structure-activity studies on heparin potentiation of HCII are necessary.

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Note Added in Proof—Hexasaccharide **I** failed to bind to pure HCII immobilized to concanavalin A-Sepharose under conditions which bound heparin.

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