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Structure of heparin-derived tetrasaccharides

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The structure of heparin was examined by characterizing a disaccharide and five of the more than a dozen tetrasaccharide components obtained by its depolymerization with flavobacterial heparinase. Enzymic depolymerization of porcine mucosal heparin results in a mixture of di-, tetra-, hexa- and higher oligo-saccharides. The di- and tetra-saccharide components represent 75mol/100mol of these heparin fragments. Ion-exchange chromatography indicates the presence of only one disaccharide, ΔIdu2S(1→4)-α-D-GlcNS6S (where Idu is iduronic acid, ΔIdu is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid, GlcN is glucosamine, GlcA is glucuronic acid and S is sulphate), but results in the isolation of five major and at least seven minor tetrasaccharide components. The structures of the disaccharide and five major tetrasaccharides were determined by chemical, enzymic, electrophoretic and spectroscopic methods, including ^{13}C, ^{1}H n.m.r. and fast atom bombardment–m.s. The structure of these five tetrasaccharides are: ΔIdu2S(1→4)-α-D-GlcNS6S(1→4)-α-L-Idu2S(1→4)-α-D-GlcNS6S; ΔIdu2S(1→4)-α-D-GlcNS6S(1→4)-β-D-GlcA(1→4)-α-D-GlcNS6S; ΔIdu2S(1→4)-α-D-GlcNS(1→4)-β-D-GlcA(1→4)-α-D-GlcNS6S; ΔIdu2S(1→4)-α-D-GlcNAc(1→4)-β-D-GlcA(1→4)-α-D-GlcNS6S; and ΔIdu2S(1→4)-α-D-GlcNAc(1→4)-α-L-Idu(1→4)-α-D-GlcNS6S. Biological activity for the disaccharide and the five major tetrasaccharides was examined, and none of them were found to possess significant anticoagulant activity.

Heparin, a polydisperse sulphated copolymer of 1→4-linked glucosamine and uronic acid residues, has been used clinically as an anticoagulant for half a century (Jaques, 1979). The major repeating unit in the heparin polymer is →4)-2-deoxy-2-sulphamino-α-D-glucopyranose 6-sulphate-(1→4)-α-L-idopyranosyluronic acid 2- sulphate-(1→ (Comper, 1981). Heparinase (heparin lyase, EC 4.2.2.7) is an eliminase that acts endolytically (Linhardt *et al.*, 1982*a*) at this major linkage, producing fragments of decreased size containing a Δ4,5 site of unsaturation in their non-reducing end (Linker & Hovingh, 1972, 1984). Only half of the linkages between glucosamine and uronic acid residue possess the structure of the major repeating unit (Linhardt *et al.*, 1982*b*; Linker & Hovingh, 1972, 1984). The fragments formed by treating porcine heparin with heparinase include a disaccharide, tetrasaccharides, hexasaccharides, as well as higher oligosaccharides. The structure of the disaccharide formed defines the linkage within heparin at which heparinase cleaves.

Heparin's major activity as an anticoagulant is thought to be derived primarily through its binding to AT III at a specific oligosaccharide sequence

Abbreviations used: DSS, 3-(trimethylsilyl)-1-propanesulphonic acid sodium salt; DMSO, dimethyl sulphoxide; f.a.b.–m.s., fast-atom-bombardment mass spectrometry; Idu, iduronic acid; ΔIdu, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; GlcA, glucuronic acid; GlcN, glucosamine; S, sulphate; Ac, acetate; l.p.l.c., low-pressure liquid chromatography; SAX, strong-anion-exchanger; g.p.c., gel-permeation chromatography; aPTT, activated partial thromboplastin time, AT III, antithrombin III; chondroitin ΔDi0S, 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-(2-acetamido-2-deoxy-α-D-galactopyranose); chondroitin ΔDi4S, 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-(2-acetamido-2-deoxy-α-D-galactopyranose-4-sulphate); chondroitin ΔDi6S, 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-(2-acetamido-2-deoxy-α-D-galactopyranose 6-sulphate); SAR, structure–activity relationship; UH4, uronic acid proton at position 4; UC4, uronic acid carbon atom at position 4; GH4, glucosamine proton at position 4 etc.

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within the heparin polymer (Lindahl *et al.*, 1979; Oosta *et al.*, 1981). Anticoagulant activity has been reported in a mixture of fragments as small as tetrasaccharides (Linhardt *et al.*, 1982*b*). Heparin displays other biological activities, including an ability to release and activate lipoprotein lipase (Olivecrona *et al.*, 1977), inhibit complement activation (Sharath *et al.*, 1984) and inhibit tumour growth (Folkman *et al.*, 1983). Little is known of heparin's structure–activity relationship (SAR) with respect to these secondary activities. Indeed the precise structure of heparin itself remains elusive.

The present study involves the fractionation and structural characterization of the disaccharide and tetrasaccharides formed on the depolymerization of heparin with heparinase. We report the structure of the disaccharide and the five major tetrasaccharides. Two of these tetrasaccharides are consistent with recently reported structures (Linker & Hovingh, 1984). The disaccharide and major tetrasaccharides represent 75 mol/100 mol of the products formed. The present study gives increased insight into the structural variability possible within heparin.

Experimental

Materials

Heparin (sodium salt) from porcine mucosa (145 units/mg) was obtained from Hepar Industries, Franklin, OH, U.S.A. Sephadex G-10, SP(sulphopropyl)-Sephadex C-50, Dowex (50W, X8, H^+ form), carbazole, Coomassie Blue G250 and sodium rhodizonate were from Sigma Chemical Co., St. Louis, MO, U.S.A. Indole was obtained from Fisher Scientific Co., Fair Lawn, NJ, U.S.A. Fractogel TSK HW40(F) and HW50(S) were from MCB Manufacturing Chemists, Gibbstown, NJ, U.S.A. Chondroitin sulphate-derived disaccharide standards were purchased from Miles Biochemicals, Elkhart, IN, U.S.A. ^2H_2O (99.996 atom %), [$^2H_6$]DMSO and DSS were from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Activated Thrombofax reagent-optimized (for aPTT) was purchased from Ortho Diagnostic Systems, Raritan, NJ, U.S.A. Factor Xa amidolytic assay kit and thrombin assay substrate S2160 were from Hellena Laboratories, Beaumont, TX, U.S.A. The pure bovine thrombin and AT III were gifts from Dr. Whyte Owen of the University of Iowa. Human plasma was obtained from the University of Iowa Hospital Blood Bank.

H.p.l.c. was performed on LDC Constametric III pumps and gradient mixer with microprocessor gradient control and data collection by using a Rheodyne 7125 injector, a Whatman Partisil M9 10/50 SAX semi-preparative column and a 5 μm-particle-size SAX analytical column (Techsphere) from Phenomenex, Rancho Palos Verdes, CA, U.S.A. (both with 5 cm × 4.6 mm guard columns), Toyo Soda TSK-Gel G2000SW 7.5 mm × 50 cm column (with 10 cm × 7.5 mm guard column), and an ISCO model 1840 variable-wavelength u.v. detector (set at 232 nm). Freeze-drying was performed on a Virtis 10-100 freeze-drier. Desalting was done using Spectra/Por dialysis tubing (1000 M_r cut-off) from Spectrum Medical, Los Angeles, CA, U.S.A. Spectrometric measurements were performed on a Pye–Unicam SP.8100 u.v. spectrophotometer, a Bruker WM 360 MHz n.m.r. spectrometer, a double-focusing Kratos-MS-50/DS-55 mass spectrometer with an extended range magnet, an Ion Tech atom-gun and a Data General Novo 4 computer. A Beckman model-R series-D paper-electrophoresis cell was used.

Methods

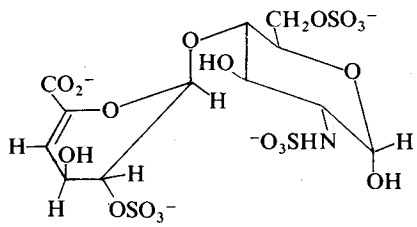
Heparinase preparation. Heparinase prepared fermentatively from *Flavobacterium heparinum* was purified by hydroxyapatite batch chromatography and chromatofocusing (Yang *et al.*, 1985; Linhardt *et al.*, 1984). The enzyme preparation had an activity of 10 units/mg of protein, a protein concentration of 5 μg/ml (Bradford, 1976) and contained no contaminating glycuronidase, sulphamidase or sulphoesterase activities (Dietrich, 1969).

Heparinase depolymerization of heparin. Heparin was depolymerized with heparinase (Grant *et al.*, 1984) and immediately frozen and stored at −70°C.

Fractionation of heparin fragments by g.p.c. The mixture of fragments was freeze-dried, re-dissolved (0.5 g in 2 ml of water) and size-fractionated (Grant *et al.*, 1984) on Fractogel TSK F50 (17 cm × 2.5 cm) and F40 (80 cm × 2.5 cm) columns run in series at 0.3 ml/min. The disaccharide and tetrasaccharide fractions were desalted by Sephadex G-10 chromatography, freeze-dried, re-applied to the TSK column and refractionated.

Fractionation of tetrasaccharides. The tetrasaccharide mixture (25 mg in 1 ml) was loaded on a semipreparative SAX column equilibrated with 0.2 M-NaCl, pH 3.5. The column was eluted with a 500 ml linear gradient [concn. (y, in M) at any time (x, in s) = $0.00056x + 0.2$] of NaCl at pH 3.5 at 6 ml/min. Each peak was collected, desalted and freeze-dried. Individual fractions were then re-chromatographed and collected.

H.p.l.c. analysis of heparin fragments. Disaccharide **I**, tetrasaccharides 1–5, and mixed fragments of various sizes, prepared by l.p.l.c.–g.p.c., were reexamined by h.p.l.c.–g.p.c. on a G-2000 column. Samples (10 μg in 20 μl) were eluted with 0.5 M-



I

NaCl, pH 3.5, at 2ml/min. Disaccharide **I** as well as each tetrasaccharide was examined for homogeneity on an analytical SAX column at 1.5ml/min by using a 180ml linear gradient of NaCl, pH 3.5 [disaccharide, y (M) $= 0.00007x$; tetrasaccharide, $y = 0.00015x + 0.2$].

Assays. Uronic acid was determined by the carbazole method (Bitter & Muir, 1962). Sulphate determination was performed on pyrolysed samples by using the barium rhodizonate method (Silvestri *et al.*, 1982). Periodate oxidation of the disaccharide and the tetrasaccharides was performed by using 5mM-sodium periodate in 50mM-sodium phosphate buffer, pH 7.0, at 37°C for 24h (Fransson *et al.*, 1978). After periodate oxidation the samples were dialysed through a 1000-M_r-cut-off membrane against 100vol. of water. The uronic acid content was determined both before and after periodate oxidation (Bitter & Muir, 1962). The loss of carbohydrate during dialysis was measured at 232nm and compensated for. Heparinase activity was assayed by the increase in the A_{232} of products (Linhardt *et al.*, 1984). The anticoagulant activity of heparin fragments was measured by aPTT and by defined chromogenic assays based on thrombin and Factor Xa (Linhardt *et al.*, 1982*b*).

Degradation with HNO_2. The chondroitin disaccharides ΔDi0S and ΔDi4S, heparin disaccharide **I** and the five major tetrasaccharides were subjected to low-pH degradation with HNO_2 (Shively & Conrad, 1976). After neutralization with ammonium sulphamate, each sample was immediately analysed by electrophoresis, made visible with indole spray, and by SAX h.p.l.c.

Electrophoresis. Electrophoresis was performed on the disaccharide and each tetrasaccharide on paper strips (Beckman no. 320046, 2.5cm × 30cm) in 0.1M-formic acid/pyridine at pH 3.0 for 50min at 800V and the components revealed with alkaline $AgNO_3$ (Trevelyan *et al.*, 1950) or indole spray reagent.

Indole spray reagent. An indole assay reagent (Lagunoff & Warren, 1962) was modified for use as a spray reagent. To 200ml of 0.5% (w/v) indole in ethanol, 15ml of 11M-HCl was added. After spraying, the paper was developed under hot air. The detection limit was 1–5 μg of sample.

^1H and ^{13}C n.m.r. Disaccharide **I** and tetrasaccharides 1–5 were freeze-dried three times from ^2H_2O. The ^1H n.m.r. (360MHz) was performed at 25 and 70°C in ^2H_2O (0.01–0.03M) using DSS as internal standard. The ^{13}C n.m.r. (90.56MHz) was performed in ^2H_2O (0.02–0.05M) with [$^2H_6$]DMSO (39.5p.p.m.) as internal standard and data was collected by using selective irradiation, coupled, broadband proton decoupled and delayed decoupled modes.

F.a.b.–m.s. The sodium salt of disaccharide **I** was prepared in thioglycerol at a concentration of 40 μg/μl and deposited on a gold-tipped direct-insertion probe. The mass spectrum was collected in the negative-ion mode by using xenon gas with the gun operating at 1.5mA tube current at an energy of 7keV.

Results

Heparin was depolymerized by using heparinase to produce a mixture of heparin fragments. These fragments were separated by low-pressure g.p.c. into di-, tetra-, hexa- and higher oligo-saccharide fractions and their average M_r values were determined by g.p.c.–h.p.l.c. (Sharath *et al.*, 1984) and reported by molar absorptivity (Linker & Hovingh, 1972).

The disaccharide fraction gave only trisulphated disaccharide **I** (99.9%), having a retention time of 3070s on SAX h.p.l.c. Disaccharide prepared with impure heparinase or that stored for prolonged periods of time contained small amounts of monosulphated and disulphated disaccharides, which were eluted at 1500s and 2010s. The tetrasaccharide fraction is more complex (Fig. 1), consisting of over a dozen components by analytical SAX h.p.l.c. The five major components were isolated and each gave a single peak on analytical SAX and g.p.c.–h.p.l.c.

The degree of sulphation of disaccharide **I**, the five tetrasaccharides and two chondroitin sulphate-derived disaccharide standards (Table 1) was determined by rhodizonate assay (Silvestri *et al.*, 1982) and electrophoresis (Weissman & Chow, 1981).

The carbazole assay demonstrates the presence of one equivalent of uronic acid in disaccharide **I** and two equivalents in all the tetrasaccharides (Table 1).

Periodate oxidation was performed on disaccharide **I** and the five major tetrasaccharides (Table 1). The oxidation was monitored by measuring the uronic acid content of the fragment. Disaccharide **I** and tetrasaccharide 1 lost no uronic acid on periodate oxidation. Tetrasaccharides 2, 3, 4 and 5 lost their internal uronic acid residues on periodate oxidation.

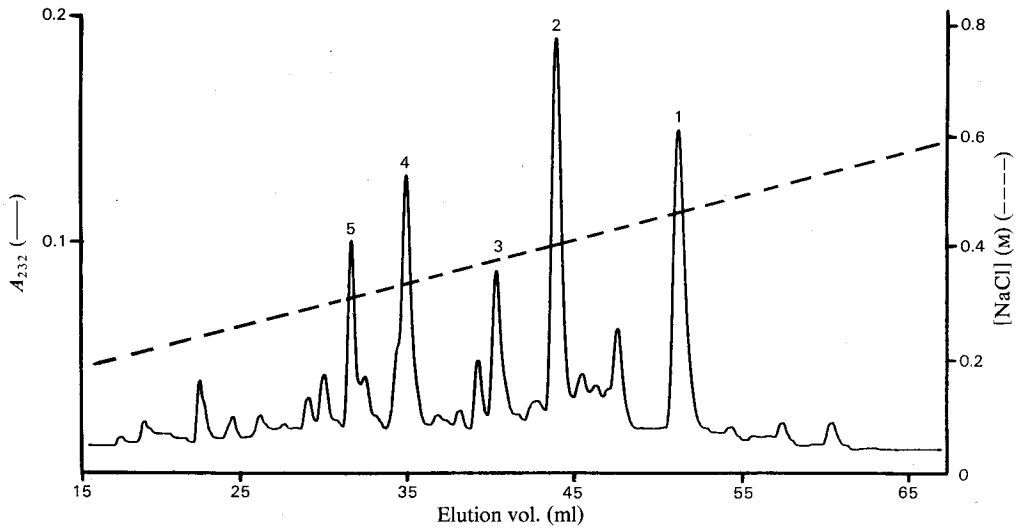


Fig. 1. *Ion-exchange chromatography of the tetrasaccharide mixture using SAX h.p.l.c. (Delaney et al., 1980)*
The tetrasaccharide sample (300 μg in 20 μl of water) was applied and the column was eluted at 1.5 ml/min with a 180 ml linear NaCl gradient ($y = 0.00015x + 0.2$), pH 3.5.

Table 1. *Sulphate and uronic acid analysis of disaccharide and tetrasaccharide fragments*
Abbreviations used: Ch, chondroitin; Di, disaccharide; Tetra, tetrasaccharide.

	Sulphate analysis			Uronic acid analysis	
Sample	Rhodizonate assay (%, w/w, of sulphur)*	$10^6 \times$ Electrophoretic mobility ($cm \cdot s^{-1} \cdot V^{-1}$)†	No. of sulphate groups	Molar ratio of uronic acid to total sugar‡	Molar ratio of uronic acid before and after periodate oxidation (*a*/*b*)§
Ch Di0S	—	1.8	0	—	—
Ch Di6S	—	2.7	1	—	—
Di **I**	14.6 (14.4)	3.8	3	1.01	1.00
Tetra 1	14.2 (14.5)	5.8	6	1.81	0.92
Tetra 2	13.2 (13.0)	5.4	5	1.82	0.52
Tetra 3	12.4 (11.3)	4.7	4	2.26	0.35
Tetra 4	9.4 (8.9)	3.8	3	2.02	0.43
Tetra 5	10.2 (8.9)	3.8	3	2.07	0.41

* Values in parentheses are theoretical values.
† Linear-regression analysis of a plot of mobility against the number of sulphate groups is linear, with a correlation of 0.996.
‡ Total sugar by u.v. (232 nm) assays; the seven minor tetrasaccharides showed a molar ratio of uronic acid to total sugar in the range 1.8–2.4.
§ *a*, the absorbance by carbazole assay after oxidation with periodate and dialysis; the compound lost on dialysis was measured by A_{232} and compensated for; *b*, the absorbance by carbazole before oxidation with periodate.

Low-pH degradation with HNO_2 cleaves *N*-sulphated glucosamine residues (Shively & Conrad, 1976). The chondroitin disaccharide standards are *N*-acetylated and show no reaction. Heparin disaccharide **I** is *N*-sulphated; on treatment with HNO_2 it yields a disulphated disaccharide having an anhydromannose reducing end. Indole spray reagent, used to reveal components after electrophoresis, reveals the presence of anhydromannose residues associated with all the HNO_2 degradation products. U.v. detection at 232 nm, used in h.p.l.c. analysis, measures only the products possessing an unsaturated uronic acid residue at the non-reducing end. By using these disaccharide standards, tetrasaccharides 1, 2 and 3 were decomposed with HNO_2 and examined by SAX h.p.l.c. and electrophoresis. Tetrasaccharides 1 and 2 show an unsaturated disulphated disaccharide and tetrasaccharide 3 a monosulphated unsaturated disaccharide by SAX h.p.l.c. By

electrophoresis tetrasaccharide 1 gives only disulphated disaccharides, tetrasaccharide 2 affords a monosulphated and a disulphated disaccharide; and tetrasaccharide 3 results in only monosulphated disaccharides. Tetrasaccharides 4 and 5 give no disaccharides but only disulphated tetrasaccharides.

The ^1H n.m.r. spectra of the disaccharide **I** and the tetrasaccharides were assigned (Table 2) on the basis of previous work involving disaccharides, oligosaccharides and heparin (Perlin *et al.*, 1971; Ototani & Yosizawa, 1974; Choay *et al.*, 1980, 1983; Gatti *et al.*, 1979) and proton-decoupling experiments. The ^{13}C n.m.r. of disaccharide **I** and the tetrasaccharides were assigned (Table 3) by using model compounds, including heparin (Gatti *et al.*, 1979), heparan (Sanderson *et al.*, 1983, 1984), oligosaccharides (Casu *et al.*, 1981) and D-glucosamine, by calculation of chemical shifts, by selective-frequency off-resonance decoupling, by broadband decoupling and by delayed-decoupling experiments. A total of 12 and 24 major peaks were observed for the disaccharide and tetrasaccharides respectively. F.a.b. mass-spectral analysis of disaccharide **I** showed a molecular ion (M) at $m/z642$ ($M-Na$).

The five major tetrasaccharides characterized in the present study are shown by the generalized structural formula **II** (1–5) with the specific substitutions described in Table 4.

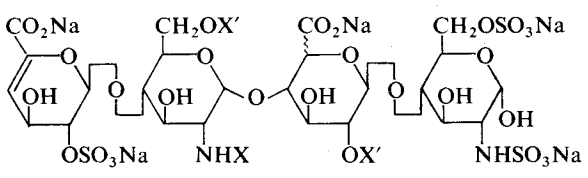
The anticoagulant activity of the disaccharide and tetrasaccharides was examined. The mixed tetrasaccharide shows a thrombin activity of 1.9 and 0.1 units/mg respectively for clotting and amidolytic assays. Neither the disaccharide nor the five individual tetrasaccharides studied showed thrombin activity. Affinity chromatography using AT III–Sepharose did not result in

Table 2. *^1H n.m.r. parameters of disaccharide and tetrasaccharides*
For abbreviations, see Table 1.

		Chemical shifts (p.p.m.) of the end sugar units									
Compound	Signal ...	UH1	UH2	UH3	UH4	GH1	GH2	GH3	GH4	GH5	GH6, GH6′
Di **I***		5.55	4.58	4.31	5.99	5.44	3.28	3.74	3.86	4.14	4.21, 4.35
		(3.2)	(2.8)	(4.4)		(3.6)	(10.3)	(9.1)	(9.6)	(2.2, 3.8)	(−11.2)
Tetra 1		5.49	4.58	4.32	5.99	5.43	3.28	3.73	3.83	4.12	4.22, 4.34
Tetra 2		5.45	4.58	4.37	6.00	5.44	3.29	3.74	3.83	4.18	4.26, 4.37
Tetra 3		5.45	4.60	4.40	5.99	5.44	3.29	3.75	3.86	4.14	4.23, 4.42
Tetra 4		5.53	4.60	4.33	6.00	5.44	3.27	3.71	3.83	4.12	4.23, 4.33
Tetra 5		5.52	4.59	4.31	6.00	5.44	3.27	3.75	3.82	4.13	4.22, 4.34

		Chemical shifts (p.p.m.) of the internal sugar units										
	Signal ...	UH1	UH2	UH3	UH4	UH5	GH1	GH2	GH3	GH4	GH5	GH6, GH6′
Heparin‡		5.22	4.35	4.20	4.11	4.82	5.40	3.28	3.67	3.77	4.03	4.28, 4.41
Heparan‡		4.50	3.39	3.71	—	—						
Tetra 1		5.24	4.32	4.22	4.01	4.81	5.37	3.30	3.67	3.82	4.09	4.28–4.35
Tetra 2		4.51	3.29	3.87	3.74	3.99	5.34	3.29	3.61	3.83	4.09	4.26–4.37
Tetra 3		4.52	3.30	3.86	3.78	4.01	5.37	3.29	3.62	†	4.09	†
Tetra 4‡		4.54	3.27	3.87	3.71	3.93	5.36	3.38	3.61	3.83	4.07	†
Tetra 5‡		5.19	†	4.22	3.99	4.80	5.36	3.45	3.62	3.82	4.09	3.86–4.00

* Coupling constants given in parentheses.
† Overlapping signals with end glucosamine moiety.
‡ Signals at 2.0 due to CH_3 of $NCOCH_3$.



II

Table 3. *^{13}C n.m.r. parameters of disaccharide and tetrasaccharides*
For abbreviations, see Table 1.

Compound	Signal ...	UC1	UC2	UC3	UC4	UC5	UC6	GC1	GC2	GC3	GC4	GC5	GC6
		Chemical shifts (p.p.m.) of the end sugar units											
Di **I***		99.7 (173.8)	77.6 (152.8)	65.9 (155.1)	109.4 (167.7)	147.3	171.8	93.9 (173.9)	60.4 (136)	72.1 (150.3)	81.2 (149)	70.7 (140.6)	69.4 (156.7)
Tetra 1		100.1	77.3	65.7	109.0	145.3	177.8	93.8	60.3	72.1	80.9	71.0	69.6
Tetra 2		99.4	77.1	64.3	109.9	149.8	178.4	92.5	59.2	70.8	79.9	70.3	69.5
Tetra 3		99.3	76.6	64.5	109.0	145.2	173.2	92.6	59.3	70.7	80.0	70.4	68.2
Tetra 4		100.9	77.5	65.9	109.0	148.2	173.7	94.3	59.3	71.9	81.8	70.9	69.4
Tetra 5		100.3	77.5	65.6	109.0	148.0	179.5	93.9	60.8	72.1	80.9	71.4	69.6
		Chemical shifts (p.p.m.) of the internal sugar units											
	Signal ...	UC1	UC2	UC3	UC4	UC5	UC6	GC1	GC2	GC3	GC4	GC5	GC6
Heparin		100.1 (172)*	76.6 (150)	70.1 (150)	77.1 (150)	70.2 (144)	175.5	97.9 (170)	59.0 (138)	70.7 (144)	77.4 (150)	70.2 (144)	67.5 (150)
Heparan		105.3	76.4					99.6	56.3				62
Tetra 1		101.9	78.1	71.2	78.9	71.5	175.8	99.8	60.6	72.3	79.9	72.2	69.6
Tetra 2		103.5	76.0	74.2	78.9	70.4	176.1	98.0	59.0	70.7	79.1	70.4	67.5
Tetra 3		103.7	76.4	‡	‡	‡	172.9	98.5	59.0	‡	79.2	‡	61.6
Tetra 4†		105.4	76.1	74.0	79.2	72.8	172.9	100.3	56.2	‡	79.5	‡	63.3
Tetra 5†		102.2	76.0	‡	79.1	‡	177.3	98.6	56.9	‡	79.8	‡	63.3

* Coupling constants given in parentheses.
† Signals at 23.5 due to CH_3 of $NCOCH_3$ and at 171.0 due to CO of $NCOCH_3$.
‡ Signals difficult to assign.

Table 4. *Structural variations of tetrasaccharides*
'OX' and 'NH-X' refer to structure **II** in the text.

Tetrasaccharide	Proportion (mol/100 mol of mixed tetrasaccharide)*	Internal glucosamine		Internal uronic acid	
		OX'	NH–X	C-5	OX'
1	16.0	SO_3Na	SO_3Na	Idu	SO_3Na
2	18.2	SO_3Na	SO_3Na	GlcA	H
3	7.5	H	SO_3Na	GlcA	H
4	14.0	H	$COCH_3$	GlcA	H
5	6.5	H	$COCH_3$	Idu	H

* As determined using analytical SAX h.p.l.c.; all remaining components were below 4.5 mol/100 mol.

the binding of disaccharide **I** or any of the five major tetrasaccharides under conditions which bind heparin (Denton *et al.*, 1981).

Discussion

Enzymic depolymerization of heparin results in a distribution of fragments that are reproducibly determined by heparinase specificity, the percentage of the linkages cleavable, and the arrangement of these linkages within the polymer (Linhardt *et al.*, 1982*a*). The specificity of heparinase has been examined by our group and others (Yang *et al.*, 1984; Linker & Hovingh, 1984; Hovingh & Linker, 1974). Heparinase cleaves the predominant α-glycosidic linkage in heparin, between the 2-sulphated iduronic acid and the 2,6-disulphated glucosamine residue. To the limits of detectability, porcine mucosal heparin is cleaved only at this linkage, thus defining the end groups of all heparinase-derived heparin fragments. Enzymic depolymerization offers certain advantages over chemical methods, including a quantitative product recovery, a well-defined site of cleavage, and a predictable distribution of products. Our approach has been to sequence heparin by determining the structure of enzymically produced fragments. Armed with their structures, the distribution of these fragments within the heparin polymer can be examined through the use of computer-simulation studies.

We confirm the structure of a single disaccharide resulting from heparin depolymerization and report the structure of five of the more than a dozen tetrasaccharides observed by SAX h.p.l.c. (Fig. 1). H.p.l.c. and l.p.l.c.–g.p.c. of disaccharide **I** and each of the five major tetrasaccharides results in a single peak eluted at a position appropriate to its M_r (Sharath *et al.*, 1984). Uronic acid analysis by the carbazole method clearly shows that all the tetrasaccharides contain 2 mol of uronic acid as contrasted with 1 mol for disaccharide **I**. The disaccharide and each tetrasaccharide showed a single component by electrophoresis (Table 1). Electrophoresis was performed at pH 3.0 to protonate the carboxylate groups while leaving the sulphate groups charged, making fragment mobility proportional to the degree of sulphation. The degree of sulphation (Table 1) was confirmed by the rhodizonate method (Silvestri *et al.*, 1982). Degradation by HNO_2 (Shively & Conrad, 1976) of tetrasaccharide 1 gave two disulphated disaccharides, showing that it possesses two *N*-sulphated glucosamine residues and that it is hexasulphated. Tetrasaccharide 2 decomposed to a disulphated disaccharide (arising from the non-reducing end) and monosulphated disaccharide, showing that it is pentasulphated. Tetrasaccharide 3 decomposed to two monosulphated disaccharides consistent with it being tetrasulphated and having two *N*-sulphated glucosamine residues with the terminal one being a 6-*O*-sulphated and the internal one possessing a 6-OH group. Tetrasaccharides 4 and 5 are not broken down by HNO_2 to disaccharides, showing that they do not possess an internal glucosamine with an *N*-sulphate group. To determine the position of the sulphate groups and the nature of the uronic acid and glucosamine residues, n.m.r. spectrometry was performed.

The ^1H and ^{13}C n.m.r. assignments for disaccharide **I** and the five major tetrasaccharides (Tables 2 and 3) were made on the basis of the following arguments. The presence of unsaturation in the non-reducing end is revealed by a UH4 signal at 6 p.p.m. (Perlin *et al.*, 1971) and a UC4 signal at 109 p.p.m. The presence of sulphate at position 2 in the non-reducing end is demonstrated by a UH2 signal near 4.6 p.p.m. and a UC2 signal at 77.6 p.p.m. (Gatti *et al.*, 1979). The 2-bond C–H and 3-bond H–C–C–H couplings show the anomeric configuration of this uronic acid to be α-L (Gatti *et al.*, 1979; Perlin *et al.*, 1972).

The reducing end sugar in the tetrasaccharides was established as *N*-sulphated glucosamine 6-sulphate by comparison with the glucosamine moiety of disaccharide **I** (Tables 2 and 3). The presence of *N*-sulphation is indicated by the GH2 and GC2 signals both downfield from the corresponding signals in glucosamine. These signals were assigned by homonuclear-decoupling and selective-heteronuclear-irradiation experiments. The presence of 6-sulphation in disaccharide **I** and in the tetrasaccharides is demonstrated by the position of the GH6 and the GC6 signals (Gatti *et al.*, 1979). Disaccharide **I** and tetrasaccharides 1, 2, 4 and 5 all exhibit this external GC6 signal at 69 p.p.m. Tetrasaccharide 3 shows signals at 68.2 p.p.m. arising from 6-sulphation at the terminal glucosamine residue, as demonstrated by the formation of only monosulphated disaccharides on degradation with HNO_2. Disaccharide **I** and all five major tetrasaccharides have identical reducing-end residues. The conformation of the glucosamine end group was determined by the 3-bond H–C–C–H couplings, and the configuration at the anomeric centre characterized by the chemical shift, the 3-bond H–C–C–H (Gatti *et al.*, 1979) and 1-bond C–H couplings (Perlin *et al.*, 1972).

The tetrasaccharides each contain an internal uronic acid and glucosamine residue. The internal uronic acid can be either iduronic or glucuronic acid, sulphated or non-sulphated. The internal uronic acid in tetrasaccharide 1 shows signals for UH1, UH5 and UC1 consistent with 2-*O*-sulphated iduronic acid (Gatti *et al.*, 1979), as confirmed by its resistance towards periodate oxidation (Table 1). Tetrasaccharide 5 shows UH1, UH5 and UC1 signals, indicating a non-sulphated internal iduronic acid residue (Ayotte *et al.*, 1980), as confirmed by periodate cleavage (Table 1). The remaining three tetrasaccharides show UH1 and UC1 signals at 4.5 p.p.m. and 103–105 p.p.m. respectively, indicating an internal glucuronic acid residue (Huckerby & Nieduszynski, 1982). Periodate oxidation of these tetrasaccharides indicates that this glucuronic acid is non-sulphated (Table 1).

The internal glucosamine residue can be a free amine, *N*-sulphated or *N*-acetylated and its 6-position can be sulphated or non-sulphated. In tetrasaccharide 1 and 2 it is 2-*N*-sulphated and 6-*O*-sulphated, as revealed by the presence of additional signals at 59–60 p.p.m. for GC2 (Gatti *et al.*, 1979) and 67–69 p.p.m. for GC6 (Gatti *et al.*, 1979; Sanderson *et al.*, 1983, 1984). Tetrasaccharide 3 has a GC2 signal at 59 p.p.m. and a GC6 signal at 62 p.p.m. indicating it is 2-*N*-sulphated and has a 6-OH (Sanderson *et al.*, 1983). Tetrasaccharides 4 and 5 both have GC2 and GC6 signals at 56–57 p.p.m. and 63 p.p.m. respectively suggesting the presence of a 2-*N*-$COCH_3$ (Casu *et al.*, 1981) and a 6-OH. Tetrasaccharides 4 and 5 both have signals at 2.0 p.p.m. in the proton (Ayotte *et al.*, 1980) and at 23.5 p.p.m. in the carbon (Casu *et al.*, 1981), confirming an *N*-$COCH_3$ group.

The disaccharide (**I**) was further characterized by f.a.b.–m.s. as having a molecular ion at 642

($M-$Na). The mass spectrum shows fragmentation due only to the loss of sodium and sulphates, with no significant interpretable ions below m/z500. We have been unable to obtain f.a.b. mass spectra of any of the five tetrasaccharides.

The structures of the five major tetrasaccharides (Table 4) are consistent with what is known about heparin biosynthesis (Comper, 1981; Lindahl, 1979). Tetrasaccharide 1, however, was unexpected, since it contains an internal α-glycosidic linkage that should be cleaved by heparinase (Linker & Hovingh, 1979, 1984). This represents the second most abundant component of the tetrasaccharide mixture and it is resistant towards cleavage, even after prolonged exposure. Tetrasaccharide 1 (0–5mM) does not inhibit the action of heparinase on heparin (0.6mM), suggesting that heparinase possesses some minimum size requirements for substrate binding.

Recently, the structure of three heparinase-derived heparin tetrasaccharides were published (Linker & Hovingh, 1984). Our work confirms the structure of two of these, our tetrasaccharides 1 and 2. However, we do not find the third, ΔIdu2S(1→4)-α-D-GlcNS6S(1→4)-α-L-Idu2S(1→4)-α-D-GlcNS, among our five major tetrasaccharides. We can speculate on several reasons for its absence. Our heparin might contain less of this tetrasaccharide and it may represent one of our minor components. Alternatively, this tetrasaccharide may be an artefact obtained when Linker & Hovingh first de-*N*-sulphate their tetrasaccharide mixture before l.p.l.c. and then re-*N*-sulphate. We observe that approx. 35mol/100mol of our mixed tetrasaccharide is *N*-acetylated, whereas Linker & Hovingh (1984) fail to report any. This may suggest the instability of the *N*-acetyl group during de-*N*-sulphation (Kenne & Lindberg, 1980).

The biological activity of disaccharide **I** and the five major tetrasaccharides was also examined. Disaccharide **I** has no anticoagulant activity. The tetrasaccharide mixture showed a low, but real, anticoagulant activity by aPTT and thrombin amidolytic assay and has been reported to have significant Factor Xa anticoagulant activity (Linhardt *et al.*, 1982*b*; Grant *et al.*, 1984). The tetrasaccharide mixture contained a small amount of 3-sulphated glucosamine as demonstrated by the presence of a characteristic shift in the ^{13}C peak in the glucosamine residue (Casu *et al.*, 1981). The 3-sulphated glucosamine residue has been established to be present in the AT III-binding site of heparin (Lindahl *et al.*, 1980). The five major tetrasaccharides do not contain a 3-sulphated glucosamine residue, and display no anticoagulant activity or binding to AT III–Sepharose.

Both heparin and heparin fragments have been shown to inhibit complement activation (Sharath *et al.*, 1984). Complement activity is observed in the tetrasaccharide mixture as well as in tetrasaccharide 1, but not in disaccharide **I** and tetrasaccharides 2, 3, 4 and 5 (Sharath *et al.*, 1984). Further studies of this and other secondary activities (Olivecrona *et al.*, 1977; Sharath *et al.*, 1984; Folkman *et al.*, 1983) as they relate to the structure of the individual heparin fragments are needed.

The methods described in the present paper represent a significant improvement in the fractionation and analysis of heparin fragments and should result in a greater insight into the structural variations within heparin.

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References

Ayotte, L., Mushayakrara, E. & Perlin, A. S. (1980) *Carbohydr. Res.* **87**, 297–301

Bitter, T. & Muir, H. M. (1962) *Anal. Biochem.* **4**, 330–334

Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254

Casu, B., Oreste, P., Toni, G., Zoppetti, G., Choay, J., Lormeau, J. C. & Petitou, R. M. (1981) *Biochem. J.* **197**, 599–609

Choay, J., Oresle, P., Torv, G. & Gatti, G. (1980) *Thromb. Res.* **18**, 573–578

Choay, J., Petitou, M., Lormeau, J. C., Sinay, P., Casu, B. & Gatti, G. (1983) *Biochem. Biophys. Res. Commun.* **116**, 492–499

Comper, W. D. (1981) in *Heparin and Related Polysaccharides Polymer Monographs* (Huglin, M. B., ed.), pp. 33–63, Gordon and Breach Science Publishers, New York

Delaney, S. R., Leger, M. & Conrad, H. E. (1980) *Anal. Biochem.* **106**, 253–261

Denton, J., Lewis, W. E. & Nieduszynski, I. A. (1981) *Anal. Biochem.* **118**, 388–391

Dietrich, C. P. (1969) *Biochem. J.* **111**, 91–95

Folkman, J., Langer, R. & Linhardt, R. J. (1983) *Science* **221**, 719–725

Fransson, L. A., Huckerby, T. N. & Nieduszynski, I. A. (1978) *Biochem. J.* **175**, 299–309

Gatti, G., Casu, B., Hamer, G. K. & Perlin, A. S. (1979) *Macromolecules* **12**, 1001–1007

Grant, A., Linhardt, R. J., Fitzgerald, G. L., Park, J. J. & Langer, R. (1984) *Anal. Biochem.* **137**, 25–32

Hovingh, P. & Linker, A. (1974) *Carbohydr. Res.* **37**, 181–192

Huckerby, T. N. & Nieduszynski, I. A. (1982) *Carbohydr. Res.* **103**, 141–145

Jacques, L. B. (1979) *Science* **206**, 528–533

Kenne, L. & Lindberg, B. (1980) *Methods Carbohydr. Chem.* **8**, 295–296