Carbohydrate Research, 127 (1984) 75–94
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURAL STUDIES ON HEPARIN. TETRASACCHARIDES OBTAINED BY HEPARINASE DEGRADATION*

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(Received January 31st, 1983; accepted for publication in revised form, July 11th, 1983)

ABSTRACT

Three tetrasaccharides representing major structural sequences of heparin were isolated in good yield and characterized after degradation of heparin by purified flavobacterial heparinase. *N*-Desulfation was necessary to achieve good separation of these closely related compounds from each other. One of the tetrasaccharides was shown to be derived from the fully sulfated repeating segments; to contain L-iduronic acid and six sulfate groups, and have the structure $\Delta_{4,5}$-Hex*p*A-(2-SO_4)-(1→4)-α-D-Glc*p*N-(*N*-SO_4)-(6-SO_4)-(1→4)-α-L-Ido*p*A-(2-SO_4)-(1→4)-D-GlcN-(*N*-SO_4)-(6-SO_4). The second contained a D-glucuronic acid unit that was nonsulfated instead of the L-iduronic acid, and the third, obtained in a fairly low yield, contained five sulfate groups, three of which being located on the disaccharide at the nonreducing end, and having the structure $\Delta_{4,5}$-Hex*p*A-(2-SO_4)-(1→4)-α-D-Glc*p*N-(*N*-SO_4)-(6-SO_4)-(1→4)-α-L-Ido*p*A-(2-SO_4)-(1→4)-D-GlcN-(*N*-SO_4). All tetrasaccharides had a sulfated, unsaturated uronic acid unit at the nonreducing end, confirming that the heparinase requires sulfated L-iduronic acid units for activity.

INTRODUCTION

The overall structure of heparin has been determined by chemical and enzymic methods over an extended time period and is by now well established. Owing to the many possible structural variants in terms of uronic acid composition, *N*- and *O*-sulfation, and *N*-acetylation, the detailed sequences of substituents have remained somewhat elusive. As heparin is widely used as an important pharmacological agent, a great deal of effort has been devoted to elucidating the precise structural requirements for anticoagulant activity. Ingenious chemical approaches have been used to determine the "active site" responsible for interaction with antithrombin[1,2], and some "active" fragments have also been prepared by enzymic degrada-

*This work was supported by the Veterans Administration and by the National Institute of Arthritis, Metabolic and Digestive Diseases, United States Public Health Service (Grant AM-13 412).

0008-6215/84/$ 03.00

tion[3,4]. However, somewhat different structural features seem to be required for thrombin or factor Xa inactivation, different tests for anticoagulant activity give divergent results, and size-to-activity relationship indicates the necessity for auxiliary sites[5]. Most of all, it should be stressed that the emphasis on anticoagulation may be somewhat misplaced at least in biological terms. The biological role of heparin is essentially unknown and very likely does not involve anticoagulation. It has been shown to play a role in platelet activation[6,7], angiogenesis[8], lipoprotein-lipase liberation[9], fibronectin binding[10], and in some other biological systems. Therefore, detailed information about the entire structure of this group of polysaccharide chains is necessary to encompass an understanding of biological function. Some chemical studies toward this goal have been carried out[11–13] and have been very useful. In the work presented herein, we have carried out structural analysis of tetrasaccharides obtained by heparinase degradation of the polymer. In conjunction with earlier studies on disaccharides[14,15], the data reported herein give important detailed information about a substantial portion of the structure of this heterogeneous polysaccharide. In addition, new information about the specificity of the *Flavobacterium* heparinase has been obtained. The major advantages of the enzymic approach to structure determination are the good yields of breakdown products and the specificity toward linkages broken, which complement the chemical methods. In addition, the compounds described herein are useful models for testing chemcal reactions, such as periodate and nitrous acid degradation, which show some unexpected reactions for heparin-like compounds.

EXPERIMENTAL

Materials. — Beef liver heparin was obtained from ICN (Irvine, CA). Crude and purified heparinases were prepared from extracts of *Flavobacteria* as described previously[16]. Trisulfated and disulfated disaccharides containing 4,5-unsaturated uronic acid were obtained by heparinase degradation of heparin[14,17].

Methods. — Uronic acid was determined by a carbazole[18] and an orcinol[19] method, sulfate by a colorimetric procedure[20], and hexosamine by a modified Elson–Morgan procedure[21] using Ehrlich's reagent. Periodate degradation was carried out by the method of Fransson *et al.*[12], and nitrous acid degradation by the low pH (for *N*-sulfated hexosamine) and high pH (for free-amino hexosamine) procedure of Shively and Conrad[22]. Reduction with borotritide was carried out with sodium borotritide[26] obtained from Research Products International Corp. (Mount Prospect, IL 60056). For paper chromatography, Whatman No. 1 paper was used with either 10:3:7 (v/v) butanol–acetic acid–water, or 4:6:3 (v/v) butanol–acetic acid–M ammonium hydroxide as solvents. Electrophoresis was carried out on Whatman No. 1 paper in pyridine–formic acid buffer[23] at pH 3.0, or in 0.1M hydrochloric acid[24] for 30 min at 1000 V in a flat-plate system. Compounds were located either with a short wave-length lamp or by an alkaline silver nitrate reagent[25]. Column chromatography will be described in detail in the text or figures when approp-

riate. ^{1}H-N.m.r. spectra were recorded at 300 MHz with a Varian SC-300 instrument. Chemical shifts were measured with reference to external sodium 4,4-dimethyl-4-silapentane-1-sulfonate (Stohler Isotope Chemicals, Inc., Waltham, MA; Cat No. D461, Lot No. D294). Samples were dissolved at concentrations of either 10 or 100 mg/mL of deuterium oxide (99.96 atom %; Merck Sharp and Dohme Ltd., Dorval, Quebec, Canada).

Ninhydrin degradation. — The tetrasaccharides (10 mg each) were dissolved in a solution composed of 4% ninhydrin (0.1 mL), 4% pyridine (0.1 mL), and distilled water (0.2 mL). The solution was heated for 30 min at 100°, placed on a small column of AG 50 (H^+), and eluted with water. The eluate was made neutral with sodium acetate. The solution was extracted with chloroform and butanol to remove the ninhydrin, and evaporated in a vacuum desiccator.

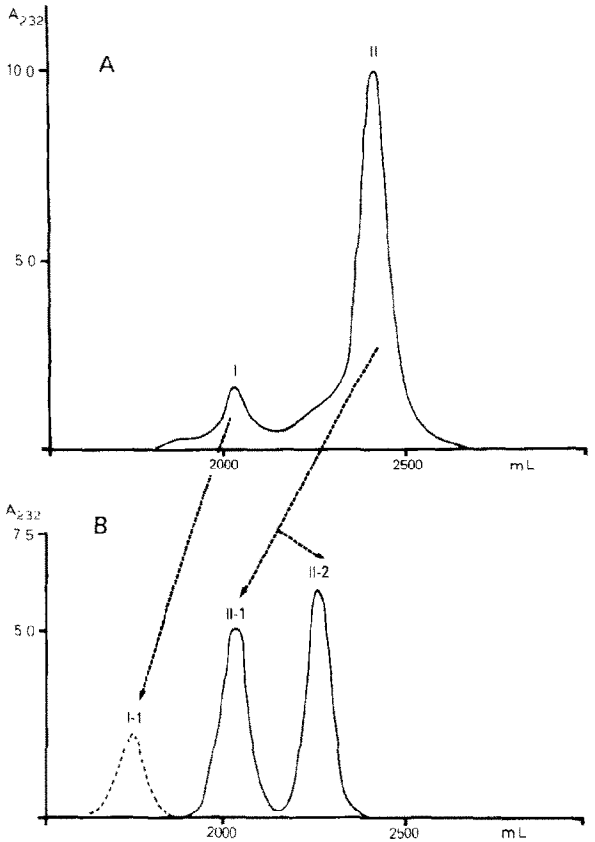


Fig. 1. Ion-exchange chromatography of tetrasaccharide fractions: (*A*) Fractionation of the tetrasaccharide peak obtained from a Sephadex G-25 column (see text for conditions). (*B*) Fractionation of tetrasaccharides obtained from chromatography (*A*) after *N*-desulfation. Material in Peak I (*A*) was applied separately and eluted in a single peak (I-1) (a small amount of material was eluted later and not examined). Material in Peak II (*A*) was applied and now eluted into two peaks (II-1 and II-2) (see text for conditions).

RESULTS

Isolation of tetrasaccharides. — Heparin (5 g in 200 mL of 50mM sodium acetate, pH 7.0, mM in calcium acetate) was degraded with purified heparinase (17 mg) for 48 h at room temperature. Additional enzyme (10 mg) was then added and incubation continued for 72 h. The solution was concentrated in a flash evaporator to 10 mL and added to a Sephadex G-50 column (200 × 3.1 cm). The column was eluted with 0.2M sodium chloride in 10% ethanol, 8 mL was collected per tube, and elution was monitored by absorption at 230 nm. Fractions containing generally di-, tetra-, hexa-, and higher mol. wt. oligo-saccharide peaks[17] were each combined. This column separated high mol. wt. oligosaccharides well from low ones, but did not resolve the lower mol. wt. compounds very well. Therefore, the peak fractions obtained were further subfractionated on a column (20 × 1.1 cm) of AG 1-X2 (Bio-Rad, 200–400 mesh), and eluted with a gradient generated from 750 mL of 0.2M and 750 mL of 3 5M sodium chloride. The fractions consisting mainly of di-, tetra-, hexa-, and higher mol. wt. oligosaccharides[17] were collected. As even at this point some overlap between low-sulfated hexasaccharides and highly sulfated tetrasaccharides may occur; the fractions were further fractionated on a column of Sephadex G-25 (10 × 2.1 cm) by elution with 0.2M sodium chloride in 10% ethanol and collecting 8-mL fractions. Materials in the major peaks corresponding to di-, tetra-, hexa-, and higher oligosaccharides were monitored by paper chromatography, combined, and desalted on a column of Sephadex G-10 (100 × 2.1 cm). Total recovery was 3900 mg (78%). In this fractionation, small amounts of di- and hexa-saccharide contaminating the tetrasaccharide fraction were removed. Individual yields were as follows: di-, 800 mg; tetra-, 1000 mg; hexa-, 775 mg; and

TABLE I

ANALYTICAL DATA FOR *N*-DESULFATED TETRASACCHARIDES

Physical and chemical properties	*Tetrasaccharides*		
	IU-6SN	*IU-5SN*	*GU-5SN*
A_{232}/mg	5.2	5.1	4.7
Uronic acid	L-Iduronic	L-Iduronic	D-Glucuronic
Uronic acid (%)			
Carbazole	32.8	32.8	34.4
Orcinol	7.5	7.5	6.8
Sulfate (%)	28.8	21.0	17.4
Hexosamine(%)	24.6	25.3	21.9
Free amino group (TNBS)* (%)	9.0	8.6	11.8

*This method using 2,4,6-trinitrobenzenesulfonic acid[29] gives satisfactory results for free aminodeoxy sugars, but is not quantitative for polymers, unless the *N*-desulfated polymer itself is used as a standard.

oligosaccharides, 1336 mg. The tetrasaccharide fraction was resistant to further hydrolysis by the heparinase, whereas the oligosaccharides could be degraded somewhat further by the enzyme if re-incubated. The tetrasaccharide fraction contained both D-glucuronic and L-iduronic acid[27]. As the uronic acid residue at the non-reducing end is unsaturated, this observation indicated that mixtures of tetrasaccharides were present. Therefore, the tetrasaccharide fraction was further purified by ion-exchange chromatography by use of 400-mg portions of tetrasaccharides in two successive experiments. A column (117 × 2.1 cm) of Bio-Rad AG 1-X2, 200–400 mesh was eluted with a linear gradient generated from 0.5M (2000 mL) and 3.5M (2000 mL) sodium chloride. As shown in Fig. 1A, two major peaks were obtained. Material in Peaks I and II was isolated by desalting on a Sephadex G-10 column and lyophilization. Analyses[27] still showed the presence of both D-glucuronic and L-iduronic acid in both peaks. To facilitate further fractionation of these closely related compounds, the fractions were *N*-desulfated by the procedure of Inoue and Nagasawa[28]. The method was modified slightly for these low-molecular-weight materials. Instead of dialysis at alkaline pH, the samples were treated with Bio-Rad AG 50-X4 (H^+) and neutralized with sodium acetate. They were then placed individually on the same AG 1-X2 column (117 × 2.1 cm) described earlier and eluted with the same gradient. As shown in Fig. 1B, material from Peak I still gave one peak, and uronic acid analysis showed only L-iduronic acid. Material from Peak II, then, gave two well resolved peaks. Peak II-1 contained D-glucuronic acid only, and Peak II-2 L-iduronic acid only (see Table I). The fractions within peaks were combined, desalted on Sephadex G-10 columns, and lyophilized. Yields from 800 mg of starting material were as follows: Peak I-1 (Tetrasaccharide IU-5SN)*, 51 mg; Peak II-1 (Tetrasaccharide GU-5SN)*, 213 mg; and Peak II-2 (Tetrasaccharide IU-6SN)* 244 mg. When chromatographed, material from the shoulder in peak II (Fig. 1) consisted of a mixture (43 mg). Analyses of these *N*-desulfated tetrasaccharides are shown in Table I. The analyses are consistent with tetrasaccharide structures as based on the u.v. absorbance indicating a 4,5-unsaturated disaccharide repeating unit[14]. The sulfate content of Tetrasaccharide IU-6SN indicates that it contains four *O*-sulfate groups, *i.e.*, it is derived from a "fully" sulfated tetrasaccharide. Tetrasaccharides IU-5SN and GU-5SN appear to contain one sulfate unit less**.

N-Resulfation. — A portion of each tetrasaccharide was *N*-resulfated with pyridine–sulfur trioxide[30] in order to obtain the corresponding original product. After sulfation, the solutions of the samples were passed through columns of AG 50-X8, made neutral with sodium acetate, desalted on columns of Sephadex G-10, and purified on columns of AG 1-X2 (200–400 mesh). For Tetrasaccharides IU-6S

*Abbreviations used: S, sulfate; GlcNS, 2-deoxy-2 sulfoamino-D-glucose. N denotes a *N*-desulfated compound (*e.g.*, IU-5SN), N being omitted when the parent tetrasaccharide is referred to. For a *N*-acetylated tetrasaccharide, the usual abbreviation NAc is used (*e.g.*, IU-5SNAc).

**Sulfate analysis *per se* cannot be relied on to distinguish between three and four sulfate groups.

TABLE II

ANALYTICAL DATA FOR TETRA- AND DI-SACCHARIDES[a]

Physical chemical properties	*Tetrasaccharides*			*Disaccharides*	
	IU-5S (1)	*GU-5S* (2)	*IU-6S* (3)	*ΔTSD*[b] (4)	*ΔDSD* (5)
A_{232}/mg	4.3	4.4	4.3	8.6	10.1
Uronic acid (%)					
Carbazole	27.0	27.0	35.0	33.0	28.7
	(1.3)	(1.4)	(1.8)	(1.9)	(1.2)
Orcinol	6.6	7.3	6.0	7.1	9.1
	(0.3)	(0.4)	(0.3)	(0.4)	(0.4)
Carbazole-to-orcinol ratio	4.1:1	3.8:1	6.0:1	4.8:1	3.2:1
Sulfate (%)	27.3	27.8	32.1	35.2	28.4
	(2.7)	(2.9)	(3.0)	(4.0)	(2.4)
Hexosamine (%)	18.6	18.1	18.1	16.2	21.6
	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)

[a]Molar ratios to hexosamine are given in parentheses. The tetrasaccharides were obtained after *N*-resulfation of tetrasaccharides separated after *N*-desulfation. [b]Trisulfated disaccharide repeating unit of heparin[14].

and GU-5S a column (117 × 2.1 cm) eluted as described earlier, and for Tetrasaccharide IU-5S a column (54 × 1.1 cm) eluted with a linear gradient generated from 0.5M (250 mL) and 3.0M (250 mL) sodium chloride were used. The major amount of *N*-resulfated compounds was eluted at the same salt concentration as the parent material (before *N*-desulfation), but some lower sulfated material was also present. Material within the major peaks was combined, desalted, and lyophilized. Yields were ~56% from the *N*-desulfated compounds. Analyses are shown in Table II. They are consistent with those in Table I, indicating that Tetrasaccharide IU-6SN (from Peak II-2) is more highly sulfated than Tetrasaccharide IU-5SN (from Peak I-1) and GU-5SN (from Peak II-1).

N-Acetyl and arabinose derivatives. — *N*-Acetyl derivative were prepared in order to evaluate the effect, if any, on analytical values, of substituents of the amino sugar unit. Tetrasaccharides IU-6SN and GU-5SN were *N*-acetylated according to the procedure of Levvy and McAllen[31]. The samples were desalted on a column of Sephadex G-10 and lyophilized. The yield was 80% on a weight basis, and analyses are shown in Table III. To be noted is the higher sulfate content of Tetrasaccharide IU-6SNAc and the differences, in the ratio of the carbazole-to-orcinol color values, for the *N*-acetyl, free amino (Table I), and *N*-sulfated compound (Table II)*.

*Color reactions are frequently used not only for quantitative determination, but also to determine uronic acid composition. It is, therefore, important to be aware of the effects of substituent groups on these reactions.

TABLE III

ANALYTICAL DATA FOR D-*arabino* AND *N*-ACETYLATED DERIVATIVES OF *N*-DESULFATED TETRASACCHARIDES

Derivatives	A_{232}/*mg*	*Uronic acid (%)*		*Sulfate (%)*	*Hexosamine (%)*
		Carbazole	*Orcinol*		
From IU-6S (3)					
Free amino group	5.0	37.3	7.5	26.5	24.4
D-*arabino*	5.5	38.1	33.2	28.4	12.6
N-Acetylated	5.9	33.1	15.7	29.5	27.2
From GU-5S (2)					
Free amino group	4.4	30.5	10.1	17.5	20.3
D-*arabino*	5.7	38.1	28.9	20.7	11.6
N-Acetylated	5.1	33.1	14.1	20.2	23.4

TABLE IV

ANALYTICAL DATA FOR PRODUCTS OBTAINED FROM DI- AND TETRA-SACCHARIDES BY TREATMENT WITH PERIODATE (pH 7.0), FOLLOWED BY ALKALI, AND PURIFICATION ON A SEPHADEX G-25 COLUMN

Products from	*Molar ratio of uronic acid (carbazole) to* A_{232}
ΔTSD (4)[a]	0.80
Tetrasaccharide IU-5S (1)	1.5
Tetrasaccharide GU-5S (2)	0.60
Tetrasaccharide IU-6S (3)	1.8

[a]This disaccharide was resistant to periodate but was treated by the same procedure as a reference.

In order to verify the structure and also to study the effect on analytical values, the two tetrasaccharides were treated with ninhydrin to give compounds having an L-arabinose unit at the reducing end[32]. The yield was 59% on a weight basis. Analyses are shown in Table III. The low value for hexosamine and the high value for the orcinol reaction are appropriate for a compound containing a pentose unit in place of one of the glucosamine units. The sulfate value for the derivative of Tetrasaccharide IU-6S again was higher than that for Tetrasaccharide GU-5S.

The following experiments were carried out to determine details of the structures of the tetrasaccharides.

Periodate oxidation to establish the presence of a sulfate ester on the internal residue of uronic acid. — Under the conditions of Fransson *et al.*[12], at pH 7.0, Tetrasaccharide GU-5S was completely oxidized by periodate as shown by Sephadex G-50 chromatography and loss of uronic acid (ref. 18 and Table IV). Tetrasaccharides IU-6S and IU-5S were not degraded by the reagent* (Table IV).

*Under the conditions used, any nonsulfated uronic acid would have been degraded regardless of sulfate ester substitution on a neighboring monosaccharide unit.

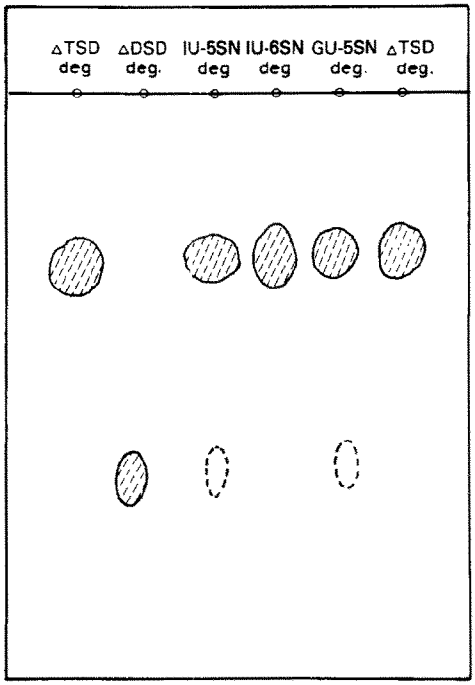


Fig. 2. Tracing of paper chromatogram, in 10:3:7 (v/v) butanol–acetic acid–water with downward irrigation for 40 h, of disaccharides obtained by nitrous acid degradation of tetrasaccharides, and standard disaccharides. Cross-hatched spots were detected by short-wave u.v. light or the silver nitrate reagent, and dashed-line spots by the silver nitrate reagent only: ΔTSD deg., degradation product of **4**; ΔDSD deg., degradation product of **5**; IU-5SN deg., degradation product of 1; IU-6SN deg., degradation product of 3; and GU-5SN deg., degradation product of 2.

Nitrous acid degradation for identifying the constituent disaccharides. — The free amino tetrasaccharides (~100–200 μg each) were treated with nitrous acid under the conditions of Lagunoff *et al.*[33], and the products were chromatographed directly on paper in butanol–acetic acid–water. The disaccharides derived from the nonreducing end (detectable by u.v. absorbance as well as the silver nitrate reagent) from all three tetrasaccharides migrated at the same rate as the trisulfated disaccharide standard, also treated with nitrous acid (Fig. 2). This indicated that the nonreducing disaccharide unit on all tetrasaccharides was the same and fully sulfated. The differences between the tetrasaccharides, therefore, had to be present on the disaccharide unit of the reducing end. As they were difficult to detect by the silver nitrate reagent, the tetrasaccharides were again treated with nitrous acid as just described, and the resulting disaccharides were reduced with sodium borotritide*. These products were examined by electrophoresis on Whatman No. 1

*When the tetrasaccharides were reduced prior to nitrous acid treatment, and then treated, some radioactive label was lost, and a variety of products was obtained, which makes this procedure unsuitable. Reduction after nitrous acid treatment is know to lead to an anhydromannitol unit as a major product[13].

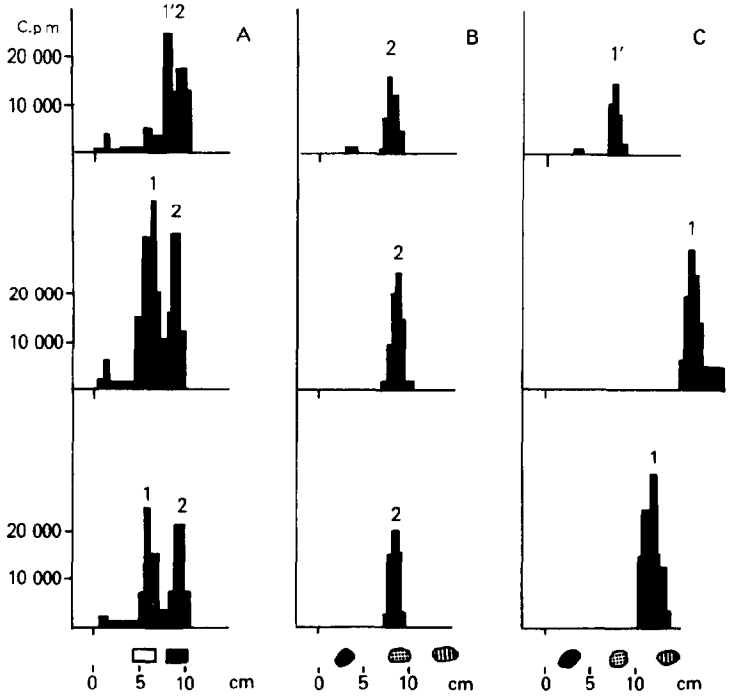


Fig. 3. Paper electrophoresis and paper chromatography of disaccharides obtained by nitrous acid degradation of tetrasaccharides. Top row (across): products of Tetrasaccharide IU-6SN; middle row: Tetrasaccharide IU-5SN; and bottom row: Tetrasaccharide GU-5SN. Column A; paper electrophoresis patterns of labeled disaccharides (the major peak in the top figure was labeled 1'and 2 as it was not clear whether this was a double peak (see results of the paper chromatography, columns B and C); □, ΔDSD (after nitrous acid and reduction); 2,5-anhydro-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2-sulfate)-D-mannitol (**6**); and ■, ΔTSD (after nitrous acid and reduction); 2,5-anhydro-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2-sulfate)-D-mannitol 6-sulfate (**7**). Column B: paper chromatography, in 4:6:3 (v/v) butanol–acetic acid–M ammonium hydroxide with downward irrigation for 24 h, of material eluted from peak 2 of column A; standards: solid circle, ΔTSD (**4**); dotted circles, ΔDSD (**5**); and hatched circles, monosulfated disaccharides [Δ4,5-HexA-(1→4)-GlcNS]. Column C; paper chromatography of material from Peak 1 (1') of Column A; solvent and standards as in Column B. The material in 1' from Tetrasaccharide IU-6S (**3**) (top) moved at the same rate as that of 2 in column B, *i.e.*, disulfated disaccharide, and material in Peaks 1 from Tetrasaccharides IU-5S (**1**) and GU-5S (**2**) moved at about the same level as the monosulfated disaccharide standard. The difference in movement between the disaccharide from Tetrasaccharide IU-5S (**1**) and that from Tetrasaccharide GU-5S (**2**) is probably due to structural differences.

paper in pyridine–formic acid, pH 3.0. The paper was cut into 0.5-cm strips which were suspended in scintillation fluid and counted in the scintillation counter. As can be seen in Fig. 3A, two radioactively labeled areas were found in the products from Tetrasaccharides IU-5SN and GU-5SN, and one (or two) in the products of Tetrasaccharide IU-6SN. The slower moving components correspond to monosulfated disaccharide standard $\Delta_{4,5}$-HexA(2-S)→Manol, and the faster to disulfated disaccharide $\Delta_{4,5}$-HexA(2-S)→Manol(6-S). In order to verify that the separated peaks represented different compounds and to determine the significance, if any,

of the double peak for Tetrasaccharide IU-6SN, the radioactively labeled areas were eluted individually and chromatographed on Whatman No. 1 paper in butanol–acetic acid–M ammonium hydroxide. As can be seen in Figs. 3B and C, the material corresponding to a monosulfated disaccharide on the electrophoresis strip migrated more rapidly on paper chromatography than material corresponding to a disulfated disaccharide, and both migrated essentially like the appropriate standard disaccharides. The products of nitrous acid degradation contain the disaccharides from the nonreducing, as well as the reducing end and, as pointed out earlier, the nonreducing disaccharide contains three sulfate groups (originally). Therefore, the

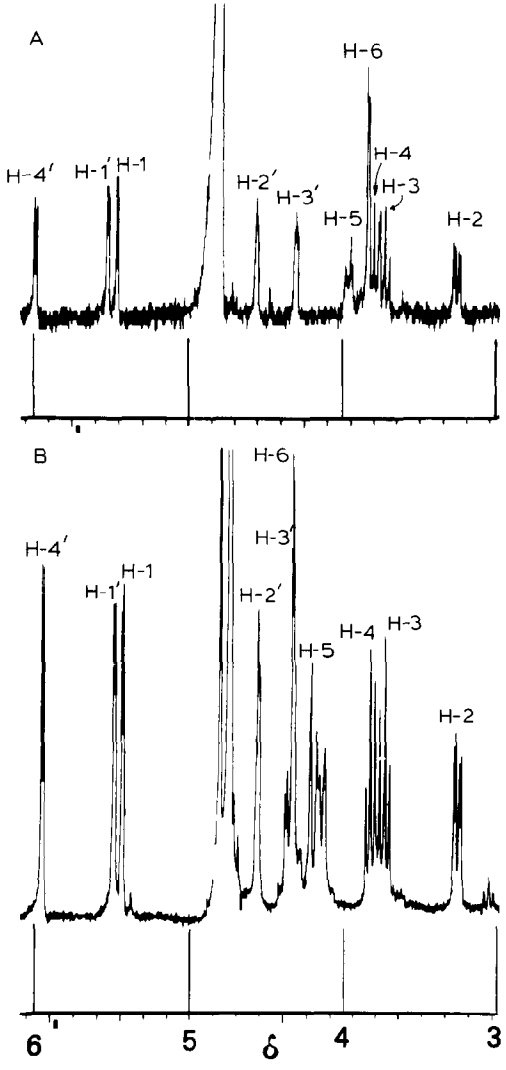


Fig. 4. ^{1}H-N.m.r. spectra of standard disaccharides: (A) disulfated unsaturated disaccharide (**5**); and (B), trisulfated unsaturated disaccharide (**4**).

data indicate that the monosulfated disaccharides obtained from Tetrasaccharides I-1(IU-5SN) and II-1(GU-5SN) had derived from the reducing end of the tetrasaccharides. This result shows that the tetrasaccharides had contained only two sulfate groups before nitrous acid treatment, which is consistent with the analytical data reported in Table I. The disaccharide obtained from the reducing end of Tetrasaccharide II-2(IU-6SN) contained two sulfate groups, *i.e.*, three sulfate groups in the original tetrasaccharide*. All available evidence indicates that the D-glucuronic acid moiety in heparin is nonsulfated[1]. In addition, when the disaccharide obtained from Tetrasaccharide GU-5S was treated with crude β-D-glucuronidase, paper chromatography showed that D-glucuronic acid was liberated. No activity could be detected when the disaccharides obtained from the other tetrasaccharides were treated with the crude enzyme, though it does also contain an α-L-iduronidase. Therefore, the two sulfate groups of the reducing-end disaccharide of Tetrasaccharide GU-5S are linked to the hexosamine unit [*i.e.*, GlcA→GlcNS(6-S)]. On the other hand, it was not clear where the second sulfate group on Tetrasaccharide IU-5S was located, though resistance to periodate oxidation suggested a linkage to the L-iduronic acid unit, *i.e.* IdoA(2-S)->GlcNS. In order to define this structure more adequately, an ^{1}H-n.m.r. study was carried out.

^{1}H-N.m.r. study. — Two well characterized disaccharides were used as reference compounds: a 4,5-unsaturated, trisulfated disaccharide from heparin[14] and a 4,5-unsaturated, disulfated disaccharide from heparan sulfate[14]**. In the ^{1}H-n.m.r. spectra of these two compounds (Fig. 4, Tables V and VI), that of the disulfated disaccharide shows several shifts in signals, as compared to that of the trisulfated compound. The signal for H-3 (uronic acid) is shifted upfield from δ 4.31 in the trisulfated to 4.287 in the disulfated disaccharide spectrum. The signal for H-5 (2-amino-2-deoxy-D-glucose) is shifted upfield from 4.15 to 3.938, and the signals for H-6a and -6b (2-amino-2-deoxy-D-glucose) upfield from δ 4.32 in the trisulfated to 3.83 in the disulfated (which does not have a 6-sulfate in the hexosamine unit) disaccharide spectrum.

The spectrum of Tetrasaccharide IU-6S (six sulfate groups) (Fig. 5, and Tables V and VI) shows, in general, signals due to the unsaturated trisulfated disaccharide at the nonreducing end, and additional signals derived from the internal L-iduronic acid 2-sulfate unit (Table VII). Assignment of the signals agrees with those of heparin[34].

The spectrum of Tetrasaccharide IU-5S was compared to those of the disulfated disaccharide and Tetrasaccharide IU-6S (Fig. 6, and Tables VI, VII, and VIII). For the signals of the 2-amino-2-deoxy-D-glucose unit(s), one of those for H-2 was shifted downfield by comparison to those of either the trisulfated disaccharide or Tetrasaccharide IU-6S. The signals in the H-3 region were more closely

*Nitrous acid treatment removes the *N*-sulfate group in the reaction leading to 2,5-anhydromannose.
**This disaccharide is composed of 2-sulfoamino-2-deoxy-D-glucose and an unsaturated uronic acid 2-sulfate [ΔHexA(2-OS)→GlcNS).

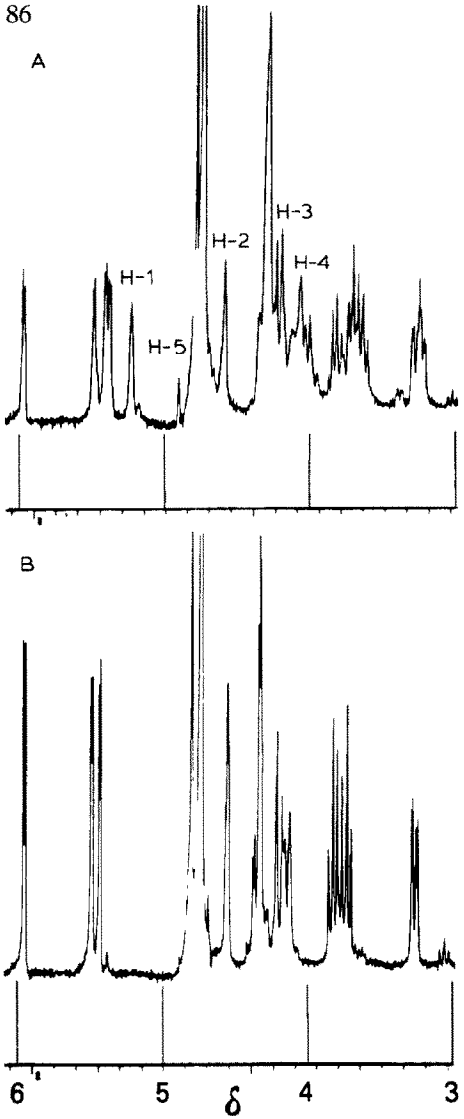


Fig. 5. ^{1}H-N.m.r. spectra of: (A) Tetrasaccharide IU-6S (**3**); and (B) Disaccharide ΔTSD (**4**). Only the signals due to the L-iduronic acid unit are identified.

TABLE V

^{1}H-N M.R. DATA FOR THE UNSATURATED URONIC ACID UNIT OF DI- AND TETRA-SACCHARIDES

Compounds	*Chemical shifts (δ)*				*Coupling constants (Hz)*			
	H-1	*H-2*	*H-3*	*H-4*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{2,4}$
ΔTSD (**4**)	5.472	4.540	4.31	5.934	3.60	2.95	4.37	1.04
ΔDSD (**5**)	5.51	4.54	4.28	5.97	3.19		4.67	
Tetrasaccharide IU-6S (**3**)	5.48	4.58	4.34	5.96	2.55		4.64	
Tetrasaccharide IU-5S (**1**)	5.52	4.64	4.3	6.0				
Tetrasaccharide GU-5S (**2**)	5.45	4.56	4.28	5.93	2.24		4.75	

TABLE VI

^{1}H-N.M R DATA FOR THE HEXOSAMINE UNIT OF DI- AND TETRA-SACCHARIDES

Compounds	*Chemical shifts (δ)*						
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6a*	*H-6b*
ΔTSD (**4**)	5.417	3.242	3.714	3.809	4.15	4.35	4.33
ΔDSD (**5**)	5.45	3.25	3.72	3.79	3.94	(3.82)	(3.82)
Tetrasaccharide IU-6S (**3**) (internal unit)	5.38	3.26	(3.84–3.62)[a]	(3.84–3.62)	4.1	(4.28–4.35)[b]	
Tetrasaccharide IU-5S (**1**) (reducing end)	5.41	3.25	(3.84–3.62)	(3.84–3.62)[a]	4.1	(4.28–4.35)[b]	
Tetrasaccharide IU-5S (**2**) (reducing-end and internal unit)	5.40–5.46	3 22–3.32	3.62–3.76	3.82–3.88	4.16 and 3.94	4.3 and 3.88	
Heparin[34]	5.401	3.298	3.673	3.771	4.035	4.407	4.278
	Coupling constants (Hz)						
	$J_{1,2}$	$J_{1,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6a}$ $J_{5,6b}$		$J_{6a,6b}$
4	3.34	10.23	9.00	9.49	3.66 and 3.26		−11.52
5							
3	3.63	9.97					
1	3.30	10.54					
2							
Heparin[34]	3.66	9.98	9.09	9.23	2.92 and 2.15		−11.23

[a]H-3 and H-4 signals of internal and reducing units were not distinguishable. [b]H-6a and H-6b signals of internal and reducing-end units were not distinguishable. [c]Signals of internal and external units were not distinguished because of small amount of samples.

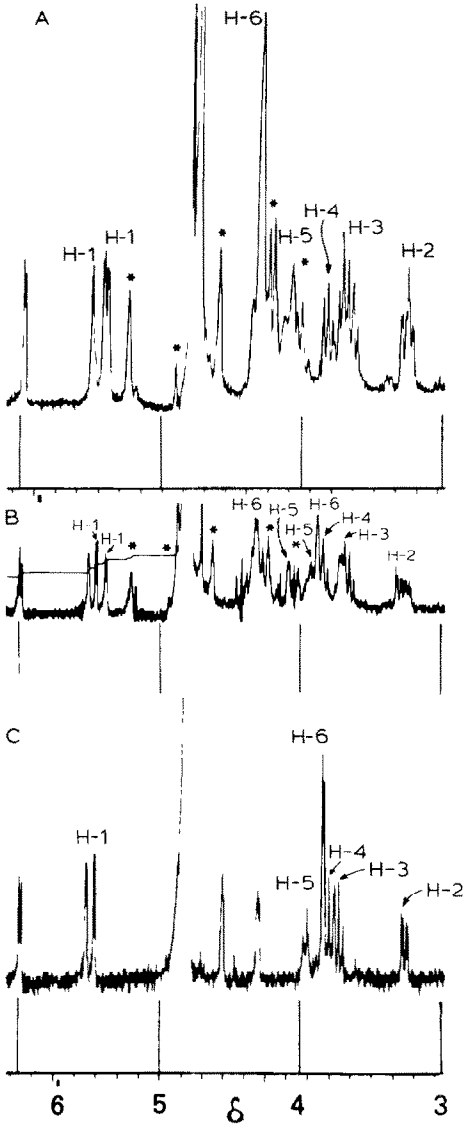


Fig. 6. ^{1}H-N.m.r. spectra of: (A) Tetrasaccharides IU-6S (**3**), (B) IU-5S (**1**), and (C) Disaccharide ΔDSD (**5**). Only the signals due to the 2-amino-2-deoxy-D-glucose unit(s) are identified. The signals due to the L-iduronic acid unit are marked with an asterisk. The spectrum of Tetrasaccharide IU-5S (**1**) shows smaller peaks because less material was available.

grouped together than those of Tetrasaccharide IU-5S, and those for H-4 shifted downfield. The H-5 and H-6a,b signals of the disulfated disaccharide and the trisulfated disaccharide were present in the spectrum of Tetrasaccharide IU-5S, indicating the absence of a sulfate group from one of the six positions of the two hexosamine units. As the disaccharide at the nonreducing end contains three sul-

TABLE VII

^{1}H-N M R. DATA FOR THE L-IDURONIC ACID 2-SULFATE UNIT OF TETRASACCHARIDES AND HEPARIN

Compounds	*Chemical shifts (δ)*					*Coupling constants (Hz)*			
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$
Tetrasaccharide IU-6S (**3**)	5.23	4.34	4.23	4.01	4.85[a]	2.55			2.95
Tetrasaccharide IU-5S (**2**)	5.22	4.3	4.28	4.0	4.85[a]				
Heparin[34]	5.22	4.35	4.20	4.11	4.82	2.64	5.90	3.44	3.09

[a]The signal for H-5 at δ 4.85 may be due to a small contamination with a tetrasaccharide containing a nonsulfated L-iduronic acid unit[35].

TABLE VIII

^{1}H-N M.R DATA FOR THE D-GLUCURONIC ACID UNIT OF TETRASACCHARIDE GU-5S (**2**)

Compounds	*Chemical shifts (δ)*					*Coupling constants (Hz)*			
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$
Tetrasaccharide GU-5S (**2**)	4.552	3.331	3.801	3.704	3.937	8.05	8.75	4.35	9.91
Hyaluronic acid[36]	4.457	3.339	3.575	3.737	3.720	7.7	8.5	8.5	9.0
Sodium D-glucuronate[37 a]	4.31	2.91	3.1	3.1	3.24	9.0	9.0		10.0
(Methyl 4-*O*-methyl-α-D-glucopyranosyl)uronic acid[37 a]	4.46	?	?	3.09	3.46	4.0		10.0	10.0

[a]For a solution in di($^2H_6$)methyl sulfoxide.

fate groups, it is concluded that the 6-sulfate ester is missing on the 2-amino-2-deoxy-D-glucose unit at the reducing end. The signals of H-1 and -3 of the L-iduronic acid unit were also present in the spectrum of Tetrasaccharide IU-5S, indicating the presence of a sulfated L-iduronic acid unit. The H-2 (uronic) signal was shifted downfield in the spectrum of Tetrasaccharide IU-5S, and upfield in the spectrum of the disulfated disaccharide as compared to that of Tetrasaccharide IU-6S. The signal for H-1 (hexosamine) was shifted downfield in the spectrum of Tetrasaccharide as IU-5S in comparison to the signal of Tetrasaccharide IU-6S, but was the same as that of H-1 of the disaccharide.

As expected, the spectrum of the D-glucuronic acid-containing tetrasaccharide (Fig. 7, and Tables V and VIII) differed considerably from that of the fully sulfated, L-iduronic acid-containing compound (Tetrasaccharide IU-6S) owing to the presence of D-glucuronic acid instead of a L-iduronic acid 2-sulfate unit. Assignments of signals are in part from literature values (see Table VIII) for D-glucuronic acid and hyaluronic acid, and from the signals of the spectra of the trisulfated disaccharide and Tetrasaccharide IU-6S. The coupling constant of the H-1 signal of the D-glucuronic acid unit in the tetrasaccharide is lower than the corresponding one of sodium D-glucuronate[37].

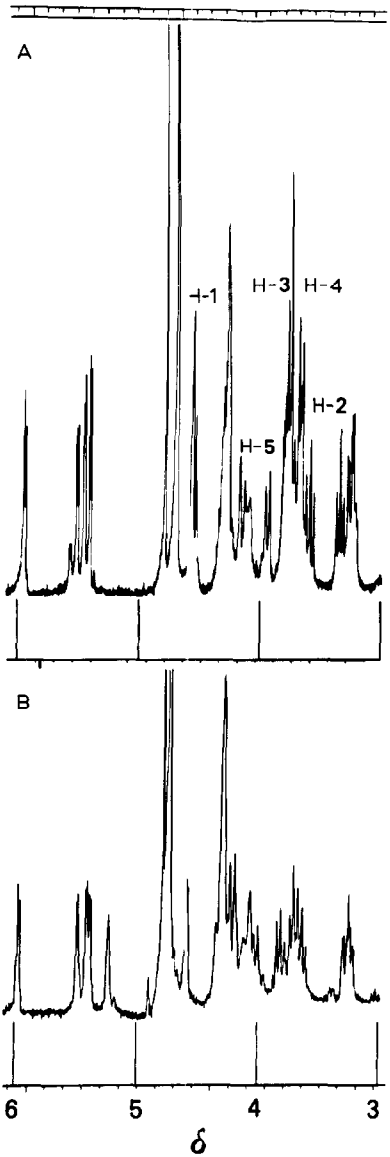
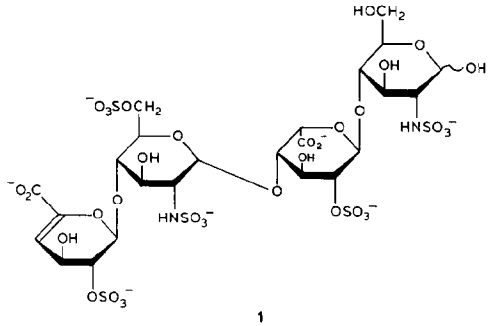
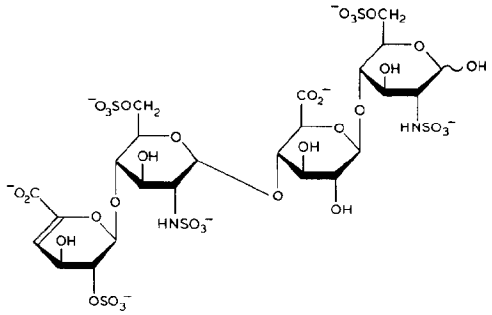


Fig. 7. ^{1}H-N.m.r. spectra of: (A) Tetrasaccharides GU-5S (**2**), and (B) IU-6S (**3**). Only the signals due to the D-glucuronic acid unit are identified.

From these data, it can be concluded that Tetrasaccharide IU-5S consists of a trisulfated, unsaturated disaccharide unit at the nonreducing end, like the other tetrasaccharides, and of a disaccharide composed of L-iduronic acid 2-sulfate and 2-deoxy-2-sulfoamino-D-glucose at the reducing end (**1**).



1



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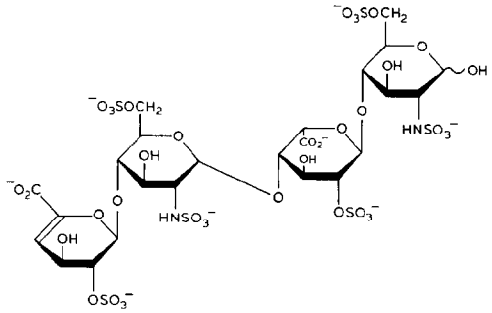

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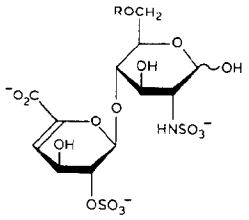
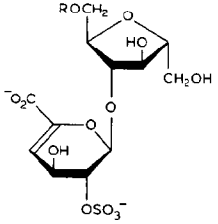


4 R = SO_3^-
5 R = H



6 R = H
7 R = SO_3^-

DISCUSSION

Detailed structural arrangements of the heparin chains is of considerable importance because of the very important role of the polysaccharide as a pharmacological agent which seems involved in a variety of biological interactions, some real, some perhaps artifactual. It is also likely that some of the biological roles ascribed to heparin may be due to heparan sulfate, which contains heparin-like chain segments[38,39]. The demonstration that the anticoagulant activity of heparin is dependent on a fairly definite structural arrangement of subunits[1,2] constituting an "active site" has led to a new appreciation of the need for precise information on structures of glycosaminoglycans which, until then, were thought to contain mainly random variations. The emphasis on heparin structure has been concerned with its anticoagulant properties. However, in view of its other roles, which are biologically more relevant, more information on total subunit arrangement needs to be obtained. The overall chemical features of commercially available heparin, which is quite pure, are fairly well defined. The polysaccharide is heterogeneous in size and somewhat in composition, though the proportions of L-iduronic to D-glucuronic acid, *N*-acetyl *vs.* *N*-sulfate content, and the overall degree of sulfation fall into a sufficiently narrow range to allow a characterization of heparins somewhat independent of anticoagulant activity. There may be some overlap with the heparan sulfates[40], but this is not critical. It should also be stressed that the heterogeneity observed may not be quite as pronounced within an individual polymer chain. In addition, this heterogeneity may really be functional rather than strictly random or due to "errors" in synthesis.

The enzymic approach to structural analysis used here is complementary to purely chemical methods, and in some ways superior. The overall yield of disaccharides *vs.* oligosaccharides gives information about the proportion of uniform, fully sulfated segments (*i.e.*, three sulfate groups per repeating unit) to the variable areas. The structure of the oligosaccharides gives information about the variable, and perhaps more biologically relevant segments. The main advantage over chemical degradation methods is the high yield of products. Tetrasaccharides were obtained in a total yield of about 20% with a 10% yield of purified final products, whereas chemical methods producing active fragments, though ingenious, gave[1] a yield of 0.15%. In this respect, the low yield of Tetrasaccharide IU-5S is noticeable (**1**); it is evidently a minor component and, considering overall yields and the molecular size of the heparin used, it may be present in only one chain out of five. The much higher yields of Tetrasaccharide GU-5S (**2**), which contains D-glucuronic acid, indicated that it may be present in each polymer chain. This does not reflect total D-glucuronic acid content, as it also occurs in hexa- and higher oligo-saccharides[1,41], but reflects the areas where D-glucuronic alternates with L-iduronic acid sulfate units.

The structural determinations using periodate and nitrous acid degradation gave fairly straightforward results for Tetrasaccharides IU-6S (**3**) and GU-5S (**2**),

but were more difficult to interpret for Tetrasaccharide IU-5S (**1**). This compound appeared similar to Tetrasaccharide IU-6S (**3**) but had a slightly lower sulfate content and was eluted earlier from ion-exchange resins; first, it was assumed to lack the sulfate group of the L-iduronic acid unit[42], but resistance to periodate oxidation indicated that this unit was sulfated. The reducing-end disaccharide obtained by nitrous acid degradation had only one sulfate group, but its location was uncertain. However, the ^{1}H-n.m.r. spectra of the tetrasaccharides did show that Tetrasaccharide IU-5S (**1**) lacked a 6-sulfate group at one of the hexosamine units and that both uronic acid residues were sulfated. Therefore, Tetrasaccharide IU-5S (**1**) appears to be the same as Tetrasaccharide IU-6S (**3**) except that the 6-sulfate group is missing from the 2-amino-2-deoxy-D-glucose reducing end. Another interesting observation obtained from the ^{1}H-n.m.r. spectra is that the coupling constants $J_{1,2}$ and $J_{3,4}$ of the unsaturated uronic acid residue can be affected by the adjacent monosaccharide unit and even by a unit once removed [see Table V; cf. spectrum of ΔTSD (**4**) with that of DSD (**5**), and that of Tetrasaccharide GU-5S (**1**) with that of IU-6S (**3**)].

All the component disaccharides of the tetrasaccharides **1–3** have been shown previously to be present in heparin[15]. These structures do not contain the "active site" for antithrombin binding [1–3,42]. This site apparently would have to be present in the higher oligosaccharides that do contain D-glucuronic acid and *N*-acetyl groups. The relationship between the compounds described herein and a tetrasaccharide, also obtained by heparinase action and which seems to have anticoagulant activity[44], is not clear.

The data reported herein are consistent with and confirm the specificity of the heparinase proposed previously[42], namely that an L-iduronic acid 2-sulfate unit has to be present at the point of cleavage. All the tetrasaccharides have a sulfated unsaturated uronic acid at the nonreducing end. The resistance to cleavage of the internal linkage of Tetrasaccharides IU-5S (**1**) or IU-6S (**3**), though it contains the required L-uronic acid unit, is not clear. Size specificity would appear to be the factor involved, *i.e.*, once formed a tetrasaccharide is not degraded. A further structural implication of enzyme specificity is that an L-iduronic acid 2-sulfate unit has to be linked to the reducing group of the tetrasaccharides in order for that linkage to be cleaved, and an *N*-sulfated hexosamine unit has to be linked to the unsaturated uronic unit at the nonreducing end. Thus, the presence of a particular hexasaccharide sequence may be deduced from the present data. Though a particular sequence, such as that of Tetrasaccharide IU-5S (**1**), may be rare and therefore not present in each chain, this sequence still represents a structural feature that must be taken into account in terms of potential biological function. In that respect, the "active site" for antithrombin binding occurs in only one chain out of three. After application to higher oligosaccharides, the method described will be applicable to the determination of detailed structural sequences in heparins from various sources.

REFERENCES

1 L. THUNBERG, G BACKSTROM, AND U. LINDAHL, *Carbohydr. Res.*, 100 (1982) 393–410.
2 G. M. OOSTA, W. T. GARDNER, D. L. BEELER, AND R. D. ROSENBERG, *Proc. Natl. Acad. Sci. U.S.A.*, 78 (1981) 829–833.
3 N. OTOTANI, M. KIKUCHI, AND Z. YOSIZAWA, *Biochem. J.*, 205 (1982) 23–30.
4 B. CASU, P. ORESTE, G. TORRI, G. ZOPPETTI, J. CHOAY, J. LORMEAU, M. PETITOU, AND P. SINAY, *Biochem. J.*, 197 (1981) 599–609.
5 S. RADOFF AND I DANISHEFSKY, *Arch. Biochem. Biophys.*, 215 (1982) 163–170.
6 E. W. SALZMAN, R. D. ROSENBERG, M. H. SMITH, J. N. LINDON, AND L. FAVREAU, *J. Clin. Invest.*, 65 (1980) 64–73.
7 S. F. MOHAMMAD, W. H. ANDERSON, J. B. SMITH, H. Y. K. CHUANG, AND R. G. MASON, *Am. J. Pathol.*, 104 (1981) 132–141.
8 S. TAYLOR AND J. FOLKMAN, *Nature (London)*, 297 (1982) 307–312.
9 E. D. KORN, *J. Biol. Chem.*, 215 (1955) 1–14.
10 D. E. SMITH AND L. T. FURCH, *J. Biol. Chem.*, 257 (1982) 6518–6523.
11 B. WEISSMANN AND H. CHAO, *Carbohydr. Res.*, 92 (1981) 255–268.
12 L.-Å. FRANSSON, A. MALMSTROM, AND I. SJOBERG, *Carbohydr. Res.*, 80 (1980) 131–145.
13 J. E. SHIVELY AND H. E. CONRAD, *Biochem.*, 15 (1976) 3943–3950.
14 P. HOVINGH AND A. LINKER, *Carbohydr. Res.*, 37 (1974) 181–192.
15 I. JACOBSSON, M. HOOK, I. PETTERSSON, U. LINDAHL, O. LARM, E. WIRIN, AND K. VON FIGURA, *Biochem. J.*, 179 (1979) 77–87.
16 A. LINKER AND P. HOVINGH, *Methods Enzymol.*, 28 (1972) 902–911.
17 A. LINKER AND P. HOVINGH, *Biochemistry*, 11 (1972) 563–568.
18 Z. DISCHE, *J. Biol. Chem.*, 167 (1947) 189–198.
19 A. H. BROWN, *Arch. Biochem.*, 11 (1946) 269–278.
20 T. T. TERHO AND K. HARTIALA, *Anal. Biochem.*, 41 (1971) 471–476.
21 D. A. SWANN AND E. A. BALAZS, *Biochim. Biophys. Acta*, 130 (1966) 112–129.
22 J. E. SHIVELY AND H. E. CONRAD, *Biochemistry*, 15 (1976) 3932–3942.
23 M. B. MATHEWS, *Biochim. Biophys. Acta*, 48 (1961) 402–403.
24 E. WESSLER, *Anal. Biochem.*, 41 (1971) 67–69.
25 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1950) 444–445.
26 T. W. LIM, I. G. LEDER, G. BACH, AND E. F. NEUFELD, *Carbohydr. Res.*, 37 (1974) 103–109.
27 M. HÖÖK, U. LINDAHL, AND P.-H. IVERIUS, *Biochem. J.*, 137 (1974) 33–43.
28 Y. INOUE AND K. NAGASAWA, *Carbohydr. Res.*, 46 (1976) 87–95.
29 J. T. GALAMBOS AND R. SHAPIRA, *Anal. Biochem.*, 15 (1966) 334–340.
30 L. LEVY AND F. J PETRACEK, *Proc. Soc. Exp. Biol. Med.*, 109 (1962) 901–905.
31 G. A. LEVVY AND A. MCALLEN, *Biochem. J.*, 73 (1959) 127–133.
32 E. A. DAVIDSON AND K. MEYER, *J. Am. Chem. Soc.*, 77 (1955) 4796–4798.
33 D. LAGUNOFF AND G. WARREN, *Arch. Biochem. Biophys.*, 99 (1962) 396–400.
34 G. GATTI, B. CASU, AND A. S. PERLIN, *Biochem. Biophys. Res. Commun.*, 85 (1978) 14–20.
35 L. AYOTTE, E. MUSKAYAKARARA, AND A. S. PERLIN, *Carbohydr. Res.*, 87 (1980) 297–301.
36 D. WELTI, D. A. REES, AND E. J. WELSH, *Eur. J. Biochem.*, 94 (1979) 505–514.
37 F. HEATLEY, J. E. SCOTT, AND B. CASU, *Carbohydr. Res.*, 72 (1979) 13–23.
38 A. LINKER AND P HOVINGH, *Biochim. Biophys. Acta*, 385 (1975) 324–333.
39 L.-Å. FRANSSON, I. SJOBERG, AND B. HAVSMARK, *Eur. J. Biochem.*, 106 (1980) 59–69.
40 P. HOVINGH AND A. LINKER, *J. Biol. Chem.*, 257 (1982) 9840–9844.
41 A. LINKER AND P. HOVINGH, unpublished results.
42 A. LINKER AND P. HOVINGH, in N. M. MCDUFFY (Ed.), *Heparin, Structure, Cellular Functions and Clinical Applications*, Academic Press, New York, 1979, pp. 3–24.
43 N. OTOTANI AND Z. YOSIZAWA, *J. Biochem. (Tokyo)*, 90 (1981) 1553–1556.
44 R. J. LINHARDT, A. GRANT, C. L. COONEY, AND R. LANGER, *J. Biol. Chem.*, 257 (1982) 7310–7313.