Carbohydrate Research, 100 (1982) 393–410
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

FURTHER CHARACTERIZATION OF THE ANTITHROMBIN-BINDING SEQUENCE IN HEPARIN

LENNART THUNBERG, GUDRUN BÄCKSTRÖM, AND ULF LINDAHL

Department of Medical and Physiological Chemistry, Swedish University of Agricultural Sciences, The Biomedical Center, Box 575, S-751 23 Uppsala (Sweden)

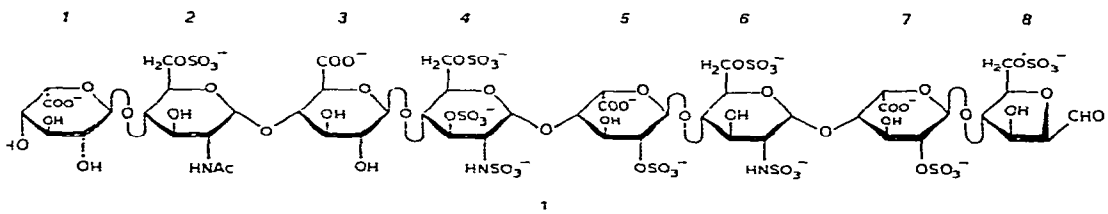
(Received August 25th, 1981; accepted for publication, September 21st, 1981)

ABSTRACT

An octasaccharide with high affinity for antithrombin, isolated after partial deaminative cleavage of heparin and previously found to have the following predominant structure (*1*) L-IdoA– (*2*) D-Glc*p*NAc-6-OSO_3^-– (*3*) D-GlcA– (*4*) D-GlcNSO_3^--3,6-di-OSO_3^-– (*5*) L-IdoA-2-OSO_3^-– (*6*) D-GlcNSO_3^--6-OSO_3^-– (*7*) L-IdoA-2-OSO_3^-– (*8*) 2,5-anhydro-D-mannose-6-OSO_3^-, has been studied further. High-voltage, paper electrophoresis of the ^3H-labelled disaccharides obtained by deamination with HNO_2 (pH 1.5) followed by reduction with Na[^3H]BH_4 showed ~25% of mono-*O*-sulfated components, in addition to L-iduronic acid(2-O-SO_3)–2,5-anhydro-D-[^3H]mannitol(6-O-SO_3). The monosulfated disaccharides were identified by high-pressure, ion-exchange chromatography as L-iduronic acid(2-O-SO_3)–2,5-anhydro-D-[^3H]mannitol, L-iduronic acid–2,5-anhydro-D-[^3H]mannitol(6-O-SO_3), and D-glucuronic acid–2,5-anhydro-D-[^3H]-mannitol(6-O-SO_3). These components originated from the reducing, terminal disaccharide residue (units *7* and *8*), as indicated by selective labelling with Na[^3H]-BH_4. The structural variability within this region suggests that it is not part of the antithrombin-binding sequence. Neither enzymic removal of the non-sulfated L-iduronic acid unit *1* nor *N*-deacetylation (by hydrazinolysis) at unit *2* had any significant effect on the affinity of the octasaccharide for antithrombin. However, removal of the disaccharide corresponding to units *1* and *2*, by selective deamination of the *N*-deacetylated octasaccharide, yielded a low-affinity hexasaccharide. In addition, a high-affinity deamination product was formed, presumably an octasaccharide containing a 6-sulfated 2-deoxy-2-C-formyl-D-pentofuranosyl unit due to ring contraction in unit *2*. These results suggest that the 6-sulfate group in unit *2* may be involved in antithrombin binding. It is concluded that the antithrombin-binding site in heparin is represented by the pentasaccharide sequence extending from unit *2* to unit *6* of the octasaccharide studied.

INTRODUCTION

The blood-anticoagulant activity of heparin is due to binding of the polysaccharide to the protease inhibitor antithrombin, resulting in accelerated inactivation of the serine proteases involved in the coagulation mechanism[1]. In commercially available preparations of heparin, more than 50% of the molecules show only low affinity for antithrombin and are essentially devoid of anticoagulant activity[2–4]. To investigate the antithrombin-binding sequence of the active heparin molecules, oligosaccharides having high affinity for antithrombin were isolated, following partial deaminative cleavage of heparin with nitrous acid[5,6]. Fractionation of such oligosaccharide mixtures by gel chromatography showed the smallest species to be the octasaccharide **1** (predominant structure; see **2** for structural variants), the most conspicuous feature of which is the 3-sulfate group on the GlcN residue *4*[7–9]. Heparin molecules with low affinity for antithrombin appeared to lack 3-sulfate groups, which were therefore assumed to be essential for high-affinity binding. Moreover, selective *N*-desulfation of **1** showed that the *N*-sulfate groups in residues *4* and *6* are both required for such interaction[10]. However, apart from these observations, little is known of the structure-function relationships for **1**. In fact, the molecular extent of the actual antithrombin-binding sequence has not been properly defined; this sequence does not necessarily span the entire octasaccharide. The purpose of the present study was to extend the functional evaluation of this molecule, with special regard to the roles of the reducing and non-reducing, terminal disaccharide residues.



1

EXPERIMENTAL

Materials. — Heparin isolated from pig intestinal mucosa was obtained from Inolex Pharmaceutical Division (Park Forest South, IL) (Stage 14 material), or from KabiVitrum AB (Stockholm) (Batch no. 22407-01). The Inolex polysaccharide was purified[11] by repeated precipitation with cetylpyridinium chloride from 1.2M NaCl.

Unlabelled, reference oligosaccharides were prepared by partial deaminative cleavage of heparin. A solution of heparin (1 g) in water (150 mL) was passed through a column (3 × 7 cm) of Dowex 50W-X8 (H^+) resin (200–400 mesh), which was subsequently washed with water (100 mL). The combined eluates were mixed with 1,2-dimethoxyethane (250 mL) and cooled to −10°. Deamination was started by adding isopentyl nitrite (10 mL). After 23 min, the reaction was interrupted by

adjusting the pH to 8.0 with 2M Tris buffer. The mixture was concentrated, and desalted by passage through a column (6 × 90 cm) of Sephadex G-15, equilibrated with 10% aqueous ethanol.

Hexosyluronic acid–2,5-anhydro-D-[^{3}H]mannitol disaccharides with *O*-sulfate groups in various positions were obtained as previously described[12].

Human antithrombin was obtained from KabiVitrum AB (Stockholm) and bound covalently to Sepharose 4B using the technique described by Höök *et al.*[3]. The resulting conjugate contained ~8 mg of protein/mL of gel.

A heparin octasaccharide with high affinity for antithrombin was isolated as follows (see also ref. 6). Heparin (1.0 g) was treated with isopentyl nitrite as described above, but for 35 min. Saturated, aqueous sodium acetate (5 mL) was added to the mixture (500 mL), which was immediately poured into 95% ethanol (5.2 litres) with stirring. The precipitated material (~250 mg of uronic acid, as determined by the carbazole reaction) was collected on a glass filter (pore size, 3–15 μm) and washed with 95% ethanol. It was then dissolved in 500 mL of 0.9M NaCl in 0.05M Tris-HCl buffer (pH 7.4), and applied to a column (5 × 27 cm) of antithrombin–Sepharose, equilibrated with the same buffer. After washing with buffered 0.9M NaCl (1500 mL), the column was eluted with 1500 mL of 3M NaCl in 0.05M Tris-HCl buffer (pH 7.4), yielding carbazole-positive material corresponding to ~10 mg of uronic acid. After desalting on a column (6 × 90 cm) of Sephadex G-15, the solution was concentrated to dryness, and the residue was dissolved in M NaCl (5 mL) and fractionated on a column (3 × 250 cm) of Sephadex G-50 (superfine grade) by elution with M NaCl at ~20 mL/h. Fractions (10 mL) were collected and analyzed for uronic acid by the carbazole reaction. The most-retarded component, an octasaccharide, was recovered and purified by re-chromatography on the same column; the final product **1** (~0.5 mg of uronic acid) contained no detectable decasaccharide.

For reduction of the 2,5-anhydro-D-mannose end-group, a solution of **1** (0.8 mg of uronic acid) in 0.1M glycine buffer (pH 7.0; 1.1 mL) was treated with 0.1 mL of 0.5M $NaBH_4$ in 0.5M KOH at room temperature for 3 h. The reaction was interrupted by adding 4M acetic acid to pH 4, followed by neutralization with 4M NaOH. The reduced octasaccharide was re-isolated by gel chromatography on Sephadex G-15 equilibrated with 0.2M H_4NHCO_3; the uronic acid-containing fractions of the eluate were combined and lyophilized.

Reduction of the octasaccharide with Na[^{3}H]BH_4, yielding a 2,5-anhydro-D-[1-^{3}H]mannitol end-group, was performed as follows. To a solution of **1** (100 μg of uronic acid) in 150 μL of 0.1M Tris buffer (pH 8.5) was added 7.5 mCi of Na-[^{3}H]BH_4 (10–15 Ci/mmol; the Radiochemical Centre, Amersham, Bucks., U.K.) in 7.5 μL of 0.5M NaOH. After 15 h at room temperature, 2 μmol of unlabelled $NaBH_4$ was added, and the mixture was left for another 4 h, acidified to pH 4 with 4M acetic acid, and neutralized with 2M NaOH. The labelled, reduced octasaccharide was isolated by chromatography on a column (1 × 170 cm) of Sephadex G-15, eluted with 0.2M H_4NHCO_3. Final purification was achieved by affinity chromatography on antithrombin–Sepharose followed by gel chromatography on Sephadex G-50, essentially

as described above. The product had a specific activity of $\sim 1.0 \times 10^5$ c.p.m. $^3H/\mu g$ of uronic acid.

^{14}C-Labelled octasaccharide was obtained by treating the *N*-deacetylated molecule with [^{14}C]acetic anhydride. Octasaccharide **1**, reduced with unlabelled $NaBH_4$, was first *N*-deacetylated by hydrazinolysis, as described below. To a sample of *N*-deacetylated octasaccharide (250 μg of uronic acid) in 0.2 mL of 0.5M Na_2CO_3–10% methanol at 0° was added 500 μCi of [^{14}C]acetic anhydride (60–120 mCi/mmol; the Radiochemical Centre, Amersham) in 10.5 μL of toluene. After 1 h at 0°, 0.3 mL of 0.5M Na_2CO_3–10% methanol and 20 μL of unlabelled acetic anhydride were added, the reaction was allowed to continue for another hour, and the pH was maintained at 7.0–7.5 by additions of saturated Na_2CO_3. After passage of the reaction products through a column (2 × 20 cm) of Sephadex G-15 equilibrated with M sodium acetate, the labelled octasaccharide was finally purified by affinity chromatography on antithrombin–Sepharose and by gel chromatography on Sephadex G-50. The final product had a specific activity of 55×10^3 c.p.m. $^{14}C/\mu g$ of uronic acid.

α-L-Iduronidase from human kidney was as described[5]. β-D-Glucuronidase from bovine liver (Grade B-10) was obtained from the Sigma Chemical Co., St. Louis, MO.

Analytical methods. — Uronic acid was determined by the carbazole reaction[13] with D-glucurono-6,3-lactone as standard. The methods used to determine radioactivity are described in ref. 14. Gel chromatography was performed with columns of Sephadex G-25 (1 × 195 cm, superfine grade, eluted with 0.2M H_4NHCO_3 at 5 mL/h) and Sephadex G-50 (1 × 200 cm, superfine grade, eluted with M NaCl at 3.6 mL/h). High-voltage paper electrophoresis (40 V/cm) was conducted on Whatman 3MM paper in 1.6M formic acid (pH 1.7).

High-pressure, ion-exchange chromatography of hexuronic acid–2,5-anhydro-D-[1-^3H]mannitol disaccharides was carried out by a slight modification of the method described by Jacobsson and Rodén[15,16]. A stainless-steel column (0.5 × 25 cm) was operated by means of two Constametric HPLC pumps equipped with a Gradient Master (Laboratory Data Control, Riviera Beach, FL) and a Rheodyne 7125 injector (Rheodyne Inc., Cotati, CA). The column was packed with Aminex A-25 anion-exchange resin (BioRad Laboratories, Richmond, CA) equilibrated with 25mM acetate buffer (pH 3.8) at 45°, and eluted at 1.0 mL/min (400–1500 p.s.i.) throughout the separation. Introduction of the sample was followed by a 20-mL wash with the acetate buffer and then by two consecutive salt gradients in the same buffer, from 0→0.5M NaCl (250 fractions of 1.0 mL) and from 0.5→2.0M NaCl (120 fractions), respectively.

Affinity chromatography on antithrombin–Sepharose was carried out with a 3-mL column of the affinity matrix, equilibrated with 0.05M NaCl in 0.05M Tris–HCl buffer (pH 7.4). Following application of the sample, the column was eluted with 25 mL of 0.05M NaCl in 0.05M Tris–HCl buffer (pH 7.4) and then with a salt gradient obtained from 40 mL of 0.05M NaCl in the mixing vessel and 36 mL of 3M NaCl in the reservoir; both salt solutions were buffered with 0.05M Tris–HCl (pH 7.4).

Generally, heparin (2 mg) was added to radiolabelled samples as an internal standard, detected by the carbazole reaction.

Degradation and modification procedures. — Treatment of saccharides with nitrous acid was performed[17] at pH 1.5 (0.33M nitrite, 10 min at room temperature) and pH 3.9 (3.9M nitrite, 10 min at room temperature). *N*-Sulfated GlcN residues are attacked at pH 1.5 but not at pH 3.9, whereas *N*-unsubstituted GlcN residues behave in the reverse manner. GlcNAc residues are resistant to either reaction. Susceptible GlcN residues are converted into 2,5-anhydromannose units, along with cleavage of the corresponding 2-amino-2-deoxyglucosidic linkages. Due to a side reaction (deaminative ring-contraction), the cleavage process is not always quantitative[17,18]. Deamination products were radio-labelled by reduction of the 2,5-anhydro-D-mannose end-groups with $Na[^3H]BH_4$. To 200 μL of deamination mixture (pH 1.5 or pH 3.9; containing 20 μg or less of uronic acid) were added 0.5 mL of M Tris buffer followed by 10 mCi of $Na[^3H]BH_4$ (10–15 Ci/mmol). After 15 h at room temperature, the pH was adjusted to 4 with 4M acetic acid and then to 7 with 4M NaOH. Labelled oligosaccharides were isolated by gel chromatography on Sephadex G-15, eluted with 0.2M H_4NHCO_3, as described above.

Deacetylation of GlcNAc residues in oligosaccharides was carried out by the method of Dmitriev *et al.*[19]. Oligosaccharide corresponding to 300 μg of uronic acid was treated with hydrazine (0.5 mL) containing hydrazine sulfate (5 mg) in a sealed glass-tube at 100° for 3 h. The reaction was interrupted by repeated addition of toluene (0.5 mL) followed by evaporation to dryness.

Oligosaccharides containing GlcN residues were *N*-sulfated with the trimethylamine–sulfur trioxide complex[20]. Ester sulfate groups were removed by treatment with dimethyl sulfoxide–10% methanol, at 100°, for the time periods indicated in the text[21].

Digestion of oligosaccharides (50 μg or less of uronic acid) with exo-glycosidases was performed in 0.25-mL reaction mixtures, containing either 60 units (2 μg of protein) of α-L-iduronidase in 0.05M acetate buffer (pH 4.3) with 0.1M NaCl, 3mM NaN_3, and 0.05% Triton X-100; or 1000 units (100 μg of protein) of β-D-glucuronidase in 0.05M acetate buffer (pH 5.0) with 0.5 mg of bovine serum albumin. The digestions were continued for 24 h at 37°.

RESULTS

The octasaccharide **1** represents the smallest deamination product of heparin that retains high affinity for antithrombin. To define the actual binding sequence within **1**, it was necessary to identify the units that are essential for antithrombin binding. This problem was approached in two ways. Firstly, it was assumed that components that are essential to binding will be invariably present in all high-affinity species; conversely, sugar residues or substituents expressing structural variability would not be directly involved in binding to antithrombin. Accordingly, identification of structural variants served to define the functional properties of the region

close to the reducing terminus of the octasaccharide. Secondly, removal or drastic modification of a unit essential to antithrombin binding would be expected to produce a low-affinity oligosaccharide, whereas manipulation of non-essential components would not affect the affinity properties. Chemical and enzymic modification of **1**, followed by affinity chromatography on immobilized antithrombin, were thus instrumental in elucidating the functional role of the non-reducing-terminal disaccharide unit.

In the following presentation, specific residues within the antithrombin-binding octasaccharide will be referred to by the numbers shown in **1**. The same mode of designation will also be applied to derivatives, including oligosaccharide fragments, containing terminal 2,5-anhydro-D-mannitol (anMan) residues. For example, the tetrasaccharide (*1–4*) is composed of D-glucuronic acid (GlcA), L-iduronic acid (IdoA), 2-acetamido-2-deoxy-α-D-glucose (GlcNAc), and anMan residues in equimolar proportions, arranged in the sequence IdoA–GlcNAc–GlcA–anMan.

The reducing-terminal disaccharide unit. — The octasaccharide was treated with nitrous acid (pH 1.5) followed by reduction with sodium [^{3}H]borohydride, thereby converting all *N*-sulfated GlcN residues ($GlcNSO_3$) (as well as the terminal anhydromannose residue *8*) into [^{3}H]anMan units. Fractionation of the products by gel chromatography yielded labelled disaccharide and tetrasaccharide in the molar proportions of ~2:1 (Fig. 1A), as expected for a heparin octasaccharide containing a single, internal GlcNAc residue that is resistant to deamination. A lower ratio, ~1:1, was observed when the octasaccharide had been reduced with unlabelled $NaBH_4$ prior to HNO_2–Na[^{3}H]BH_4 treatment (Fig. 1B), in agreement with the previous assignment[6,9,10] of an *N*-sulfate group to unit *6*. Accordingly, HNO_2 treatment of octasaccharide previously reduced with Na[^{3}H]BH_4 yielded disaccharide as the only labelled component present in significant amounts (Fig. 1C). Referring to the octasaccharide sequence, the labelled disaccharide in Fig. 1A would represent a mixture of fragments [6-^{3}H]-(*5–6*) and [8-^{3}H]-(*7–8*), whereas those in Figs. 1B and 1C would correspond to the isolated species, [6-^{3}H]-(*5–6*) and [8-^{3}H]-(*7–8*), respectively. Further characterization of the various disaccharide fractions should thus provide the information required to define the entire reducing-terminal tetrasaccharide-sequence (units *5* to *8*). Paper electrophoresis of the total, labelled disaccharide {*i.e.*, the mixture of [6-^{3}H]-(*5–6*) and [8-^{3}H]-(*7–8*)} showed a major, disulfated component along with a smaller peak of monosulfated disaccharide (Fig. 2A). Analysis of the individual fragments revealed striking differences between units *5–6* and *7–8*. Disaccharide [6-^{3}H]-(*5–6*) consisted almost exclusively of the disulfated species IdoA(2-O-SO_3)–[^{3}H]-anMan(6-O-SO_3) (Fig. 2B). This result accords with structure **1** for the octasaccharide. In contrast, the disaccharide [8-^{3}H]-(*7–8*) was heterogeneous, yielding mono- and di-sulfated components in about equal amounts (Fig. 2C). The monosulfated disaccharides were identified by high-pressure, ion-exchange chromatography, as illustrated in Fig. 3B. Separation of the entire labelled-disaccharide fraction isolated (Fig. 1A) after HNO_2–Na[^{3}H]BH_4 treatment of the octasaccharide yielded, in addition to the disulfated component, IdoA(2-O-

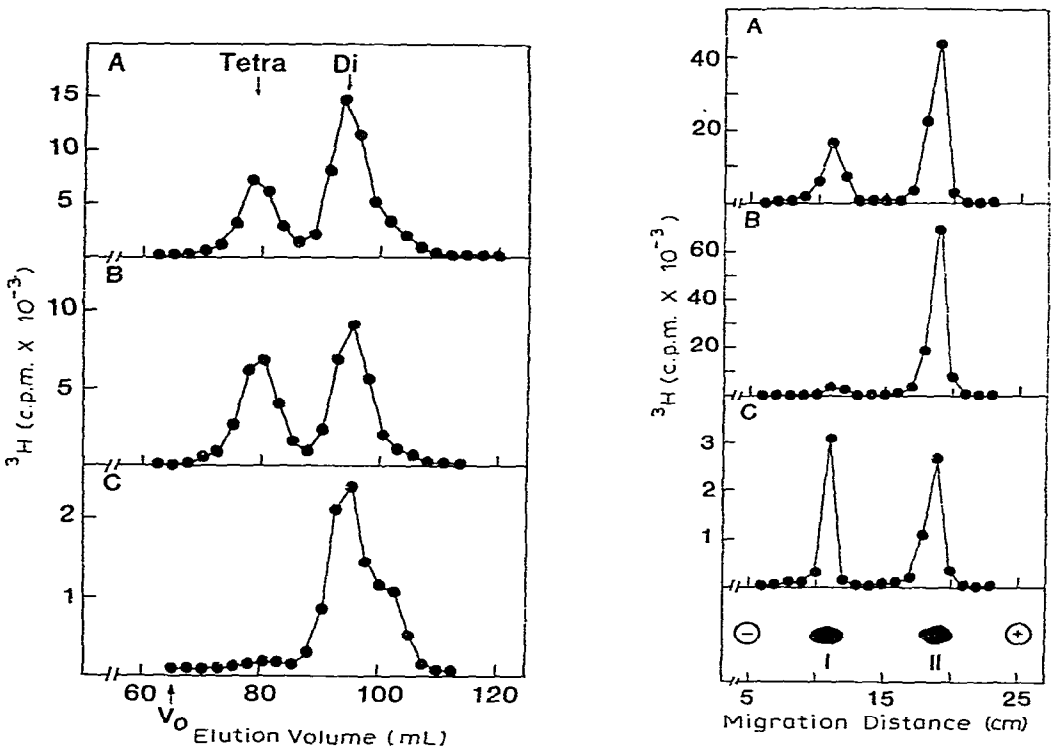


Fig. 1. Gel chromatography on Sephadex G-25 of labelled saccharides obtained A, by HNO_2(pH 1.5)–Na[^{3}H]BH_4 treatment of the antithrombin-binding octasaccharide; B, by reduction of the octasaccharide with unlabelled $NaBH_4$ followed by HNO_2(pH 1.5)–Na[^{3}H]BH_4 treatment; and C, by reduction of the octasaccharide with Na[^{3}H]BH_4 followed by deaminative cleavage with HNO_2 (pH 1.5). The arrows indicate the peak elution positions of di- and tetra-saccharide reference standards derived from heparin.

Fig. 2. High-voltage paper electrophoresis at pH 1.7 of labelled disaccharides: A, [6-^{3}H]-(*5–6*) and [8-^{3}H]-(*7–8*) (mixture); B, [6-^{3}H]-(*5–6*); and C, [8-^{3}H]-(*7–8*). The disaccharide fractions were isolated by gel chromatography, as shown in Figs. 1A–C, respectively, desalted by lyophilization, and subjected to high-voltage p.e. The standards shown below the tracings are: I, GlcA–[^{3}H]anMan-(6-O-SO_3); II, IdoA(2-O-SO_3)–[^{3}H]anMan(6-O-SO_3).

SO_3)–[^{3}H]anMan(6-O-SO_3), significant amounts of each of the three monosulfated disaccharides, GlcA–[^{3}H]anMan(6-O-SO_3), IdoA–[^{3}H]anMan(6-O-SO_3), and IdoA(2-O-SO_3)–[^{3}H]anMan. These results demonstrate that the antithrombin-binding octasaccharide has a constant structure at units *5* and *6*, but a variable structure at units *7* and *8*. The various units identified for the intact sequence are (see **2**): *5*, IdoA(2-O-SO_3); *6*, GlcNSO_3(6-O-SO_3); *7*, GlcA, IdoA, and IdoA(2-O-SO_3); and *8*, GlcNSO_3 and GlcNSO_3(6-O-SO_3). The constant occurrence of IdoA(2-O-SO_3) as unit *5* agrees with the lack of formation of labelled trisaccharide on periodate–alkali treatment of the octasaccharide [8-^{3}H]-(*1–8*)[6]; conversely, the variable sulfation pattern at unit *7* is reflected in the variable release of labelled monosacchar-

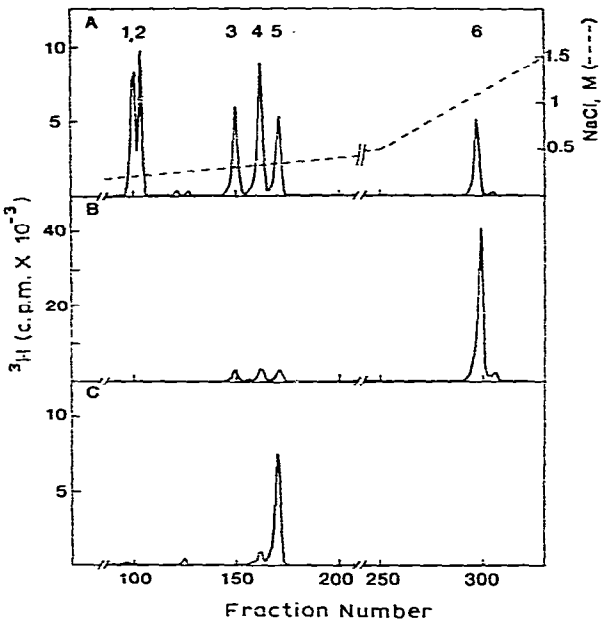
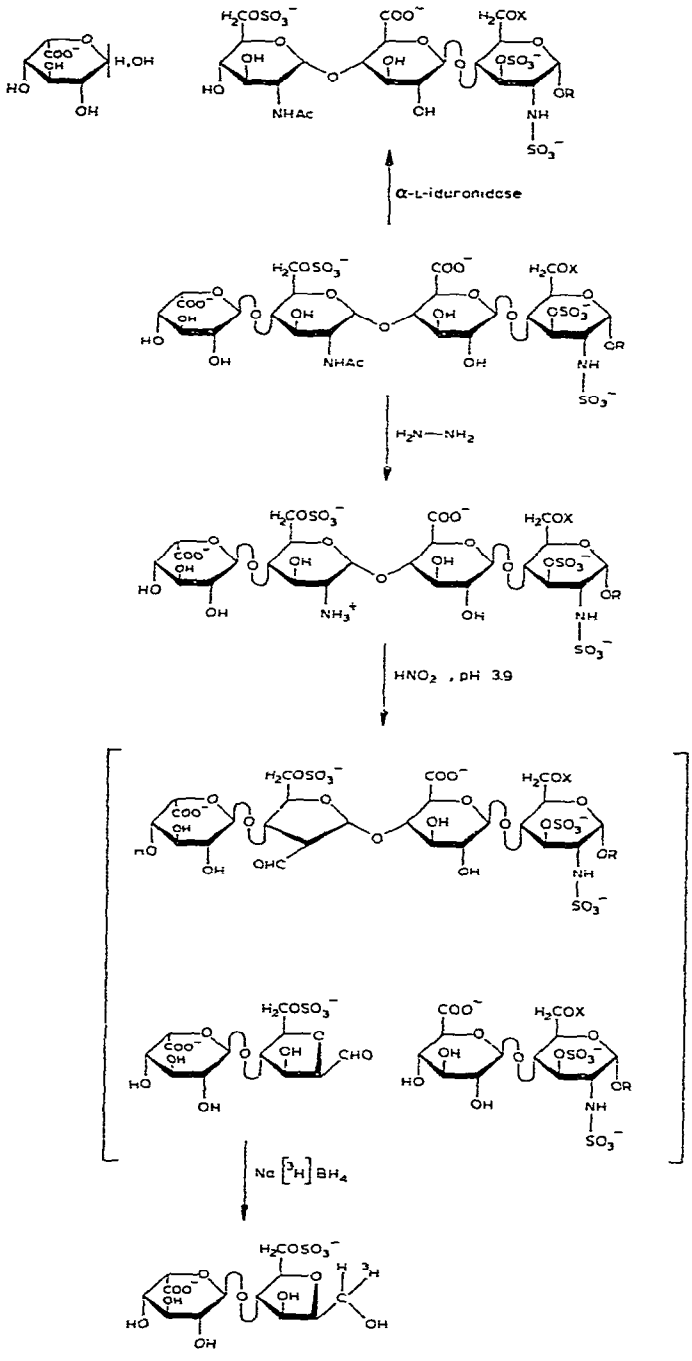


Fig. 3. High-pressure, ion-exchange chromatography on Aminex A-25 of A, disaccharide reference standards; B, a mixture of disaccharides [6-^{3}H]-(*5–6*) and [8-^{3}H]-(*7–8*); and *C*, disaccharide [2-^{3}H]-(*1–2*), derived from the antithrombin-binding octasaccharide. The reference disaccharides shown in A are 1, GlcA–[^{3}H]anMan; 2, IdoA–[^{3}H]anMan; 3, IdoA(2-O-SO_3)–[^{3}H]anMan; 4, GlcA–[^{3}H]-anMan(6-O-SO_3); 5, IdoA–[^{3}H]anMan(6-O-SO_3); and 6, IdoA(2-O-SO_3)–[^{3}H]anMan(6-O-SO_3). ——, NaCl concentration (M).

ide from different octasaccharide preparations (unpublished observation). Considering the extensive structural variability of units *7* and *8*, it appears unlikely that this portion of the octasaccharide is included in the antithrombin-binding sequence.

The non-reducing-terminal disaccharide unit. — Units *1* and *2* of the antithrombin-binding octasaccharide were modified by various enzymic or chemical methods, and the resulting products were analyzed by affinity chromatography. A schematic representation of the modification procedures is given in Scheme 1.

Octasaccharide (~ 1 μg of uronic acid) carrying a ^{3}H-label in unit *8* {[8-^{3}H]-(*1-8*)} was mixed with an excess (50 μg of uronic acid) of octasaccharide reduced with unlabelled $NaBH_4$, and the mixture was digested with α-L-iduronidase, as described in the Experimental. In order to ascertain that the non-reducing-terminal IdoA residue had indeed been removed, a sample of the product (10 μg of uronic acid) was subjected to HNO_2(pH 1.5)–Na[^{3}H]BH_4 treatment followed by gel chromatography on Sephadex G-25 (Fig. 4). A peak of ^{3}H-labelled material, presumably representing the trisaccharide [4-^{3}H]-(*2–4*), was observed, clearly retarded in relation to the tetrasaccharide [4-^{3}H]-(*1–4*) produced by a non-digested, control octasaccharide. Thus, a major fraction of the octasaccharide subjected to α-L-iduronidase digestion had been converted into a heptasaccharide. The affinity properties of



Scheme 1

Scheme 1. Modification procedures, chemical or enzymic, involving units *1* and *2* of the antithrombin-binding octasaccharide. The sugar residues are units *1* to *4*; R = the tetrasaccharide sequence extending from unit *5* to unit *8*; X = —H or $-SO_3^-$ (unit *4*). The structures enclosed by brackets represent the products expected to be formed on deamination of the *N*-deacetylated octasaccharide.

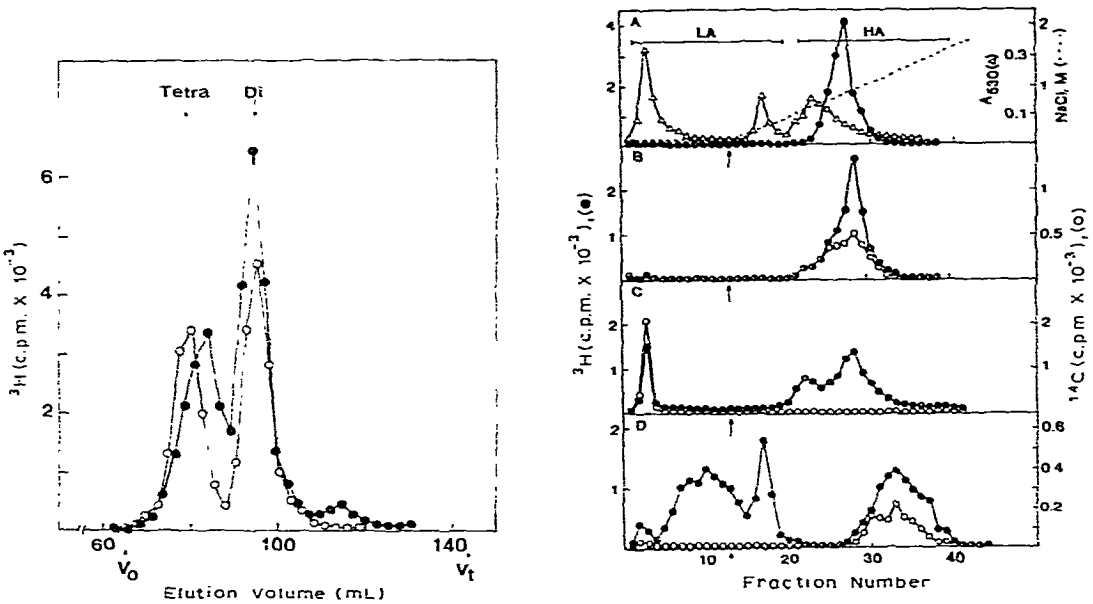


Fig. 4. Gel chromatography on Sephadex G-25 of labelled oligosaccharides obtained by HNO_2(pH 1.5)–Na[^{3}H]BH_4 treatment of antithrombin-binding octasaccharide, before (○) and after (●) digestion with α-L-iduronidase. The arrows are as in Fig. 1.

Fig. 5. Affinity chromatography on antithrombin–Sepharose of A, the ^{3}H-labelled heptasaccharide, [8-^{3}H]-(*2–8*) (●), obtained by digesting the corresponding octasaccharide with α-L-iduronidase; B, a mixture of the two labelled octasaccharides, [2-^{14}C]-(*1–8*), 10^6 c.p.m. (○), and [8-^{3}H]-(*1–8*), 2×10^6 c.p.m. (●); C, the same samples as in B, following *N*-deacetylation by hydrazinolysis; and D, a deaminated (pH 3.9) mixture of the high-affinity fraction of the *N*-deacetylated, ^{3}H-labelled octasaccharide, [8-^{3}H]-(*1–8*) (corresponding to eluate fractions 25–32 in C) and the ^{14}C-labelled octasaccharide, [2-^{14}C]-(*1–8*). The chromatograms in panels A–C all included 2 mg of heparin as internal standard; identical distributions into high- (HA) and low-affinity (LA) fractions were obtained, shown only in panel A (△, uronic acid determined by the carbazole reaction). The exceptionally retarded elution-position of the HA material in panel D is due to the absence of competitive HA-heparin. The arrows indicate the start of the NaCl-gradient; – – – –, NaCl concentration (M), shown only in panel A.

this heptasaccharide, including the labelled species [8-^{3}H]-(*2–8*), were investigated by chromatography on antithrombin–Sepharose, as shown in Fig. 5A. The labelled heptasaccharide emerged together with the more-retarded fractions of the high-affinity component of the heparin internal-standard, similar to the intact octasaccharide (*cf.* Fig. 5B). This observation suggests that the IdoA unit *1* of the octasaccharide is not directly involved in the binding to antithrombin.

The GlcNAc unit *2* was investigated as follows (see Scheme 1). Antithrombin-binding octasaccharide was *N*-deacetylated by hydrazinolysis and then re-*N*-[^{14}C]-acetylated by treatment with [^{14}C]acetic anhydride. The product, [2-^{14}C]-(*1–8*), was isolated as the high-affinity fraction following chromatography on antithrombin–Sepharose. Analysis of this product in mixture with [8-^{3}H]-(*1–8*) (*i.e.*, the native octasaccharide, labelled in unit *8* by reduction with Na[^{3}H]BH_4) showed similar

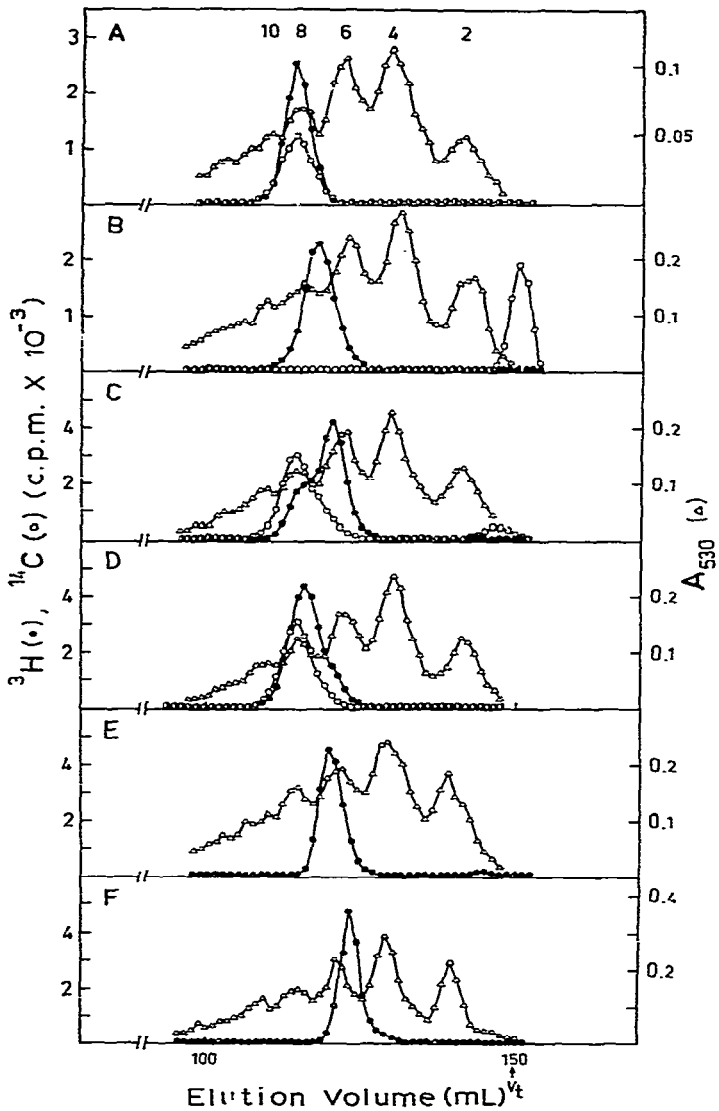


Fig. 6. Gel chromatography on Sephadex G-50 of A, a mixture of the two labelled octasaccharides, [2-^{14}C]-(*1–8*), 10^6 c.p.m. (○), and [8-^{3}H]-(*1–8*), 2 × 10^6 c.p.m. (●); B, the same sample as in A following *N*-deacetylation by hydrazinolysis; C, a mixture of [2-^{14}C]-(*1–8*) (○), and the high-affinity component (fractions 25–32 in Fig. 5C) of *N*-deacetylated [8-^{3}H]-(*1–8*) (●), both treated with nitrous acid at pH 3.9; D, material as described under C, high-affinity fraction (see Fig. 5D); E, material as described under C, low-affinity fraction (see Fig. 5D); F, material as described under E following digestion with β-D-glucuronidase. Before chromatography, each sample was mixed with reference oligosaccharides (7 mg of uronic acid) prepared by partial deamination of heparin (see Materials). Eluate fractions were analyzed for radioactivity and for uronic acid (△). The number of monosaccharide residues in the various reference oligosaccharides is indicated above the appropriate peaks.

properties for the two octasaccharides with regard to affinity properties (Fig. 5B) and molecular size (Fig. 6A). Treatment of the mixed octasaccharides {[2-^{14}C]-(*1–8*) and [8-^{3}H]-(*1–8*)} with hydrazine resulted in virtually complete *N*-deacetylation without any significant depolymerization; analysis of the products by gel chromatography on Sephadex G-50 showed a ^{14}C-labelled component (presumably [^{14}C]-acetylhydrazine) that emerged later than the disaccharide standard, while the ^{3}H-label was only slightly retarded in relation to the original octasaccharide (Fig. 6B). As expected, the ^{14}C-labelled compound no longer bound to antithrombin–Sepharose (Fig. 5C). In contrast, most of the ^{3}H-labelled material remained high-affinity with regard to antithrombin, smaller peaks of radioactivity emerging in the medium-affinity and low-affinity regions. These results suggested that loss of the *N*-acetyl group from unit *2* is compatible with high-affinity binding to antithrombin; the appearance of medium- and low-affinity fractions probably reflects limited loss of the *N*-sulfate groups from units *6* and *4*, respectively (see ref. 10). The appearance of a free amino group in unit *2* was verified by selective deamination of the GlcN residue, in the following manner. Antithrombin-binding octasaccharide, reduced with unlabelled $NaBH_4$, was subjected to hydrazinolysis as before and was then treated with HNO_2 at pH 3.9. The deamination products were reduced with Na[^{3}H]BH_4 and analyzed by gel chromatography on Sephadex G-25 (not shown). A disaccharide fraction, by prediction (see Scheme 1) largely [2-^{3}H]-(*1–2*), accounted for ~70% of the labelled deamination products. Paper electrophoresis of this fraction showed >80% mono-sulfated disaccharide, which was subsequently identified as IdoA–[^{3}H]anMan(6-O-SO_3) by ion-exchange chromatography (Fig. 3C).

Following the observation that loss of the *N*-acetyl group did not affect the affinity for antithrombin, experiments were undertaken to study the effects of *N*-sulfation of the same unit. Treatment of the *N*-deacetylated octasaccharide, [8-^{3}H]-(*1–8*), with trimethylamine–sulfur trioxide complex gave a product that was resistant to HNO_2 at pH 3.9, as shown by gel chromatography on Sephadex G-50 (not in figure). Chromatography of this product on antithrombin–Sepharose yielded an elution pattern similar to that of the native octasaccharide (not shown). Thus, an *N*-sulfate group at unit *2* does not interfere significantly with binding of the octasaccharide to antithrombin.

N-Deacetylation of the reduced octasaccharide [8-^{3}H]-(*1–8*), followed by deamination at pH 3.9, would be expected to yield the hexasaccharide [8-^{3}H]-(*3–8*), thereby providing an opportunity to extend the functional evaluation of the molecule. The high-affinity portion of *N*-deacetylated [8-^{3}H]-(*1–8*) octasaccharide (fractions 25-32 in Fig. 5C) was concentrated, desalted (by passage through a column of Sephadex G-15), mixed with a sample of intact [2-^{14}C]-(*1–8*) octasaccharide, and deaminated by treatment with HNO_2 at pH 3.9. Analytical gel-chromatography of the products on Sephadex G-50 showed that, while the *N*-acetylated, ^{14}C-labelled octasaccharide, as expected, was resistant to deamination, the *N*-deacetylated, ^{3}H-labelled species was largely converted into a smaller labelled component that chromatographed essentially with the reference hexasaccharide fraction (Fig. 6C). However,

the ^3H-pattern was clearly heterogeneous, with a shoulder projecting into the octasaccharide region. Chromatography of the same deamination products on antithrombin–Sepharose showed ^3H-labelled components with distinctly different affinity properties; a low-affinity fraction and a somewhat smaller high-affinity fraction (Fig. 5D). The latter component co-chromatographed with the ^{14}C-labelled, intact octasaccharide. Gel chromatography of the separated components showed that the low-affinity (Fig. 6E) and high-affinity (Fig. 6D) fractions corresponded to the hexasaccharide main-peak and the octasaccharide shoulder, respectively, of the unfractionated deamination products (Fig. 6C). The difference in elution position between the two components was reproducible in two separate sets of experiments.

The interpretation of these results relates to the various deamination products shown within brackets in Scheme 1. The smaller, low-affinity component would be identical to the hexasaccharide, [8-^3H]-(*3–8*), obtained by cleavage of the 2-amino-2-deoxyglucosidic linkage of unit *2*. The identity of the hexasaccharide was corroborated by digestion with β-D-glucuronidase, which produced a decrease in molecular size (Fig. 6F) compatible with conversion of [8-^3H]-(*3–8*) into a pentasaccharide [8-^3H]-(*4–8*). The slight shift in elution position of the hexasaccharide in relation to that of the reference hexasaccharide (Fig. 6E) is conceivably due to the unusual 3-sulfate group in unit *4*. The low affinity for antithrombin of this hexasaccharide suggests that some structural component(s) essential for the interaction is located at units *1–2* of the octasaccharide. This crucial component was apparently retained by the somewhat larger oligosaccharide, which showed high affinity for antithrombin. Although not yet conclusively identified, this oligosaccharide has tentatively been assigned the structure shown in Scheme 1, the 2-deoxy-2-*C*-formyl-D-pentofuranosyl unit *2* being due to deamination of a GlcN residue followed by ring contraction without cleavage of the 2-amino-2-deoxyglucosidic linkage[17,18]. Since the IdoA unit *1* does not seem to contribute to the binding of antithrombin (see above), it must be concluded that a component essential for such binding is contained within the substituted pentofuranosyl unit *2*. This conclusion strongly points to an important role for the 6-sulfate group in this unit.

If, indeed, the 6-sulfate group in unit *2* is essential for the interaction with antithrombin, it cannot be a structural variant, but must occur in all high-affinity octasaccharide species. The high yield of IdoA–[^3H]anMan(6-O-SO_3) on isolating the disaccharide [2-^3H]-(*1–2*), as described above, tends to support such a postulate. This question was also approached by determining the total number of *O*-sulfate groups in the tetrasaccharide, [4-^3H]-(*1–4*), isolated by gel chromatography (Fig. 1A) following HNO_2(pH 1.5)–Na[^3H]BH_4 treatment of the octasaccharide. High-voltage, paper electrophoresis of this tetrasaccharide (Fig. 7A) yielded two components (*a* and *b*), the more sulfated of which (*b*) migrated similarly to the di-*O*-sulfated disaccharide standard. The degree of *O*-sulfation of component *b* was determined by repeated electrophoresis of the products obtained on partial desulfation. During treatment with methanolic dimethyl sulfoxide, component *b* was converted into component *a* and, in addition, into two slower-migrating components, one of which

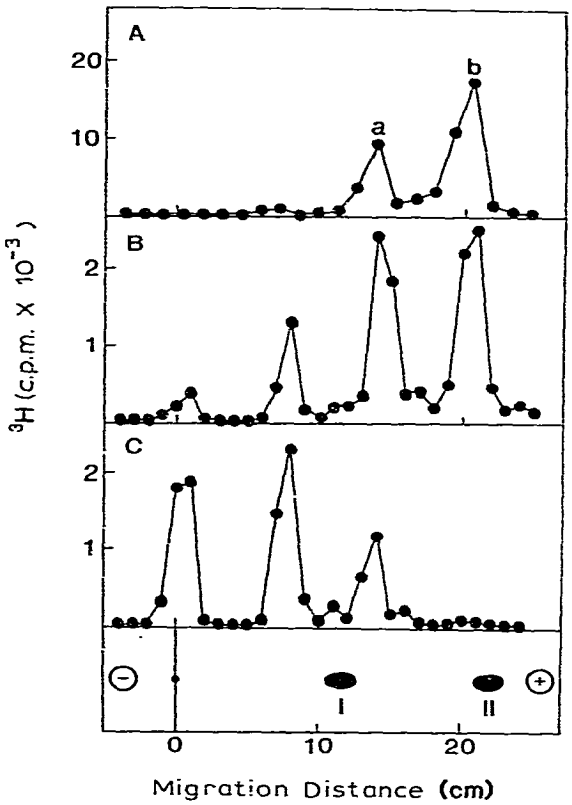


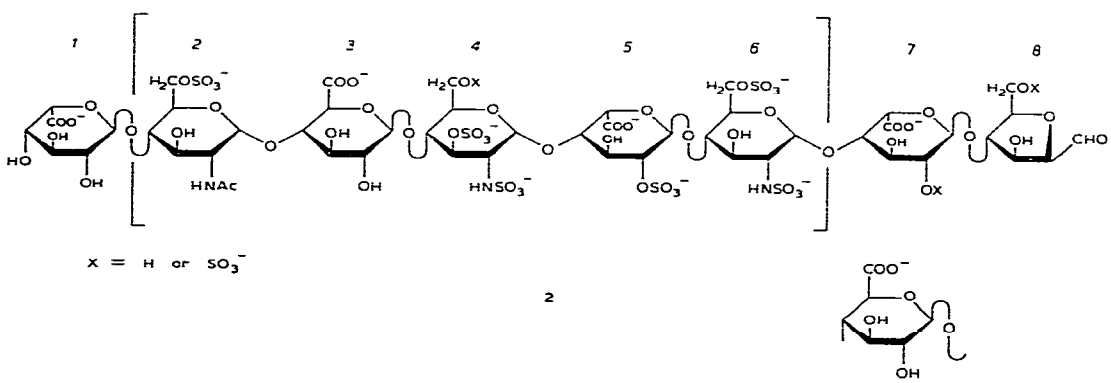
Fig. 7. High-voltage paper electrophoresis (p.e.) at pH 1.7 of A, tetrasaccharide [4-^{3}H]-(*1–4*) obtained by HNO_2(pH 1.5)–Na[^{3}H]BH_4 treatment of antithrombin-binding octasaccharide; B, component *b* following *O*-desulfation (treatment with methanolic dimethyl sulfoxide) for 15 min; and C, component *b* following *O*-desulfation for 60 min. The tetrasaccharide fraction was isolated by gel chromatography, as shown in Fig. 1A, desalted by iyophilization, and subjected to high-voltage p.e. Component *b* was isolated by preparative p.e., followed by elution from the paper. The standards of mono- (I) and di-*O*-sulfated (II) disaccharide shown below the tracings are as in Fig. 2.

was uncharged at pH 1.7 and thus non-sulfated (Figs. 7B,C). If it is assumed that each desulfated component was formed by removal of a single sulfate group from the corresponding precursor, as suggested by the relative migration distances, then the tetrasaccharides *a* and *b* should contain 2 and 3 *O*-sulfate groups, respectively, per molecule. Since the IdoA unit *1* and the GlcA unit *3* are both non-sulfated, these sulfate groups would have to be distributed between the GlcN units *2* (a 6-sulfate) and *4* (a 6-sulfate and a 3-sulfate). Thus, in the trisulfated tetrasaccharide (*b*), all potential sulfation sites would be occupied. The disulfated species (*a*) accounts for ~30% of the total tetrasaccharide (*a* + *b*). Since a similar proportion of the GlcN units *4* were shown[7] to lack a 6-sulfate substituent, tetrasaccharide *a* must have the structure IdoA–GlcNAc(6-O-SO_3)–GlcA–[^{3}H]anMan(3-O-SO_3). Lacking any significant amounts of mono-*O*-sulfated tetrasaccharide (Fig. 7A), it seems reasonable to

conclude that the GlcN unit *2* of the antithrombin-binding octasaccharide is invariably 6-sulfated.

DISCUSSION

High-affinity binding of heparin to antithrombin involves a specific structure in the polysaccharide molecule. The results of the present study indicate that this structure is a pentasaccharide sequence, corresponding to units *2* to *6* of the octasaccharide **2** isolated from heparin deamination products. The distinction between the active sequence and the surrounding structures is based primarily on a functional evaluation of the components close to the reducing and non-reducing termini of this octasaccharide. Whereas the IdoA unit *1* was found to be dispensable for the interaction with antithrombin, removal of the same unit in conjunction with the GlcNAc-(6-O-SO_3) unit *2* was accompanied by a drastic loss of binding affinity. At the other end of the molecule, the *N*-sulfate group in unit *6* was previously implicated as essential to high-affinity binding[10], whereas the present results suggest that units *7* and *8* are non-essential. These conclusions are not contradictory to the observed lack of formation of high-affinity hexasaccharide following partial depolymerization of heparin with nitrous acid[6]. Formation of a hexasaccharide by deaminative cleavage of the 2-amino-2-deoxyglucosidic linkage of unit *6* would entail loss not only of the non-essential units *7* and *8* but also of the functionally important *N*-sulfate group from unit *6*.



The molecular size of the antithrombin-binding site in heparin has been the subject of several recent investigations. The deamination products first described by Lindahl *et al.*[5] as dodeca- or tetradeca-saccharides were later resolved by gel chromatography into several components, the smallest of which was the octasaccharide investigated in the present study[6]. Rosenberg *et al.*[22] initially favored a tetrasaccharide binding-sequence (corresponding to units *1* to *4* by the present designation), but more recently recognized[23] that "it may be necessary to position elements such as trisulfated iduronosylglucosamine disaccharides on either side of the critical tetra-

saccharide sequence to attain a complete protease-inhibitor binding-site on the mucopolysaccharide[8]. In agreement with this notion, an antithrombin-binding octasaccharide, isolated after partial deamination of heparin, showed an avidity for the protein almost one order of magnitude higher than that of the homologous hexasaccharide[24] (which would lack the *N*-sulfate group corresponding to unit *6*; see above). Casu *et al.*[9] provided additional information by characterizing antithrombin-binding oligosaccharides isolated after enzymic depolymerization of heparin. The carbohydrate sequence of an octasaccharide released by a bacterial heparinase was found to be displaced by one disaccharide unit in relation to that of the analogous deamination product; the reducing terminus was a $GlcNSO_3(6\text{-}O\text{-}SO_3)$ residue corresponding to unit *6* of the latter molecule. With the data available, the affinity properties of the two octasaccharides could not be directly compared; however, the anti-X_a activities were closely similar. Moreover, an active hexasaccharide was obtained, with the same reducing terminus (and thus an intact *N*-sulfate group corresponding to unit *6*) and a Δ4,5-unsaturated hexuronic acid unit at the non-reducing end, derived (by action of the heparinase) from the non-sulfated IdoA unit *1*. The ability of this hexasaccharide to interact with antithrombin supports our notion that the non-sulfated IdoA unit *1* lies outside the binding site. The results of Casu, Choay, and co-workers thus agree with the main conclusion of the present study, that the antithrombin-binding site in heparin is represented by the pentasaccharide sequence of units *2–6* in **2**.

Although the mode of interaction of this pentasaccharide sequence and the antithrombin molecule is largely unknown, some groups of functional importance may be discerned. Such groups, presumably serving as ligands to the protein, include the *N*-sulfate groups[10] in units *4* and *6* and, as proposed in the present report, the 6-*O*-sulfate group in unit *2*. Furthermore, the 3-*O*-sulfate group in unit *4* is tacitly assumed to be essential, due to its unique distribution in the heparin molecule[7]. Conversely, two groups, the 6-*O*-sulfate group in unit *4* (a structural variant[7]) and the *N*-acetyl group in unit *2* (see Results) seem to be non-essential for the interaction with antithrombin. The functional properties of other components, including the carboxyl groups in units *3* and *5*, and the *O*-sulfate groups in units *5* and *6*, remain to be evaluated. The two latter sulfate groups appear to be invariably present in the binding sequence (see Results). However, while structural variability for a given component may be regarded as evidence against a critical role in antithrombin binding, the converse is not necessarily true for constant components; consider, for example, the IdoA unit *1*.

The functional significance of the non-sulfated IdoA residue remains unclear. The invariant occurrence of this as unit *1*, in contrast to its relative scarcity in other portions of the heparin molecule, suggests that it may be essential for the anticoagulant action of heparin. Since it does not seem to be directly involved in the binding to antithrombin, other alternatives may be considered. It is conceivable that any hexuronic acid residue other than non-sulfated IdoA [*i.e.*, GlcA or $IdoA(2\text{-}O\text{-}SO_3)$] in position *1* would interfere sterically with the binding process. Another possibility

relates to the polymer-modification reactions involved in the biosynthesis of heparin. The IdoA residues are formed by C-5-epimerization of GlcA residues at the polymer level. Since the formation of non-sulfated IdoA residues appears to be a separate and relatively early reaction step[25], these sugar residues may possibly have a regulatory, or even directory, function in the subsequent epimerization and/or *O*-sulfation reactions. Thus, the invariant occurrence of a non-sulfated IdoA residue as unit *1* could reflect its function as a biosynthetic signal associated with the formation of an essential component of the antithrombin-binding sequence. Finally, while the antithrombin-binding region is crucial for anticoagulant activity, the expression of such activity also depends on other regions of the heparin molecule[24,26,27]. Non-sulfated IdoA has been attributed unique conformational properties[28], and it is therefore possible that such a unit *1* may be required to attain the appropriate steric alignment of the various functional domains of the heparin molecule.

ACKNOWLEDGMENTS

We thank Dr. L. H. Rome (University of California, Los Angeles) for providing the α-L-iduronidase. This work was supported by grants from the Swedish Medical Research Council (2309); KabiVitrum AB, Stockholm; the National Swedish Board for Technical Development; and the Faculty of Veterinary Medicine, Swedish University of Agricultural Sciences.

REFERENCES

1 R. D. Rosenberg, *Fed. Proc.*, 36 (1977) 10–18.
2 L. H. Lam, J. E. Silbert, and R. D. Rosenberg, *Biochem. Biophys. Res. Commun.*, 69 (1976) 570–577.
3 M. Höök, I. Björk, J. Hopwood, and U. Lindahl, *FEBS Lett.*, 66 (1976) 90–93.
4 L.-O. Andersson, T. W. Barrowcliffe, E. Holmer, E. A. Johnson, and G. E. C. Sims, *Thromb. Res.*, 9 (1976) 575–583.
5 U. Lindahl, G. Bäckström, M. Höök, L. Thunberg, L.-Å. Fransson, and A. Linker, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 3198–3202.
6 L. Thunberg, G. Bäckström, H. Grundberg, J. Riesenfeld, and U. Lindahl, *FEBS Lett.*, 117 (1980) 203–206.
7 U. Lindahl, G. Bäckström, L. Thunberg, and I. G. Leder, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 6551–6555.
8 B. Meyer, L. Thunberg, U. Lindahl, O. Larm, and I. G. Leder, *Carbohydr. Res.*, 88 (1981) C1–C4.
9 B. Casu, P. Oreste, G. Torri, G. Zopetti, J. Choay, J.-C. Lormeau, M. Petitou, and P. Sinaÿ, *Biochem. J.*, 197 (1981) 599–609.
10 J. Riesenfeld, L. Thunberg, M. Höök, and U. Lindahl, *J. Biol. Chem.*, 256 (1981) 2389–2394.
11 U. Lindahl, J. A. Cifonelli, B. Lindahl, and L. Rodén, *J. Biol. Chem.*, 240 (1965) 2817–2820.
12 I. Jacobsson, M. Höök, I. Pettersson, U. Lindahl, O. Larm, E. Wirén, and K. von Figura, *Biochem. J.*, 179 (1979) 77–87.
13 T. Bitter and H. M. Muir, *Anal. Biochem.*, 4 (1962) 330–334.
14 U. Lindahl, I. Jacobsson, M. Höök, G. Bäckström, and D. S. Feingold, *Biochem. Biophys. Res. Commun.*, 70 (1976) 492–499.
15 I. Jacobsson and L. Rodén, *Fed. Proc.*, 39 (1980) 1636.
16 I. Jacobsson and L. Rodén, *Anal. Biochem.*, in press.
17 J. E. Shively and H. E. Conrad, *Biochemistry*, 15 (1976) 3932–3942.

18 B. LINDBERG, J. LÖNNGREN, AND S. SVENSSON, *Adv. Carbohydr. Chem. Biochem.*, 31 (1975) 185–240.
19 B. A. DMITRIEV, Y. A. KNIREL, AND N. K. KOCHETKOV, *Carbohydr. Res.*, 40 (1975) 365–372.
20 L. LEVY AND F. J. PETRACEK, *Proc. Soc. Exp. Biol. Med.*, 109 (1962) 901–905.
21 K. NAGASAWA, Y. INOUE, AND T. KAMATA, *Carbohydr. Res.*, 58 (1977) 47–55.
22 R. D. ROSENBERG AND L. H. LAM, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 1218–1222.
23 R. D. ROSENBERG, G. M. OOSTA, R. E. JORDAN, AND W. T. GARDNER, in R. L. LUNDBLAD, W. V. BROWN, K. G. MANN, AND H. R. ROBERTS (Eds.), *Chemistry and Biology of Heparin*, Elsevier, New York, 1981, pp. 249–269.
24 G. M. OOSTA, W. T. GARDNER, D. L. BEELER, AND R. D. ROSENBERG, *Proc. Natl. Acad. Sci. U.S.A.*, 78 (1981) 829–833.
25 I. JACOBSSON AND U. LINDAHL, *J. Biol. Chem.*, 255 (1980) 5094–5100.
26 T. C. LAURENT, A. TENGBLAD, L. THUNBERG, M. HÖÖK, AND U. LINDAHL, *Biochem. J.*, 175 (1978) 691–701.
27 E. HOLMER, G. SÖDERSTRÖM, AND L.-O. ANDERSSON, *Eur. J. Biochem.*, 93 (1979) 1–5.
28 L.-Å. FRANSSON, T. N. HUCKERBY, AND I. A. NIEDUSZYNSKI, *Biochem. J.*, 175 (1978) 299–309.