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STRUCTURAL FEATURES OF HEPARIN VARIANTS HAVING HIGH ANTI-X_a CLOTTING-ACTIVITY

LARS-ÅKE FRANSSON, BIRGITTA HAVSMARK,
Department of Physiological Chemistry 2, University of Lund, P.O. Box 750, S-220 07 Lund 7 (Sweden)
IAN A. NIEDUSZYNSKI*, THOMAS N. HUCKERBY**,
Departments of Biological Sciences and Chemistry**, University of Lancaster, Lancaster LA1 4YQ (Great Britain)*
AND WALTER E. LEWIS
Glaxo Operations UK Ltd., Westfield Road, Runcorn WA7 4TW (Great Britain)

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ABSTRACT

Two heparin-related preparations from beef lung and pig mucosa are able to inhibit the enzymic activity of the clotting factor X_a. These preparations were subjected to deaminative cleavage and periodate oxidation–alkaline elimination. The following structural features were observed: (*a*) *N*-acetylated and glucuronate-rich regions are short and frequently intercalated between *N*-sulphated and iduronate-rich segments of deca- to hexadeca-saccharide size; (*b*) in the latter segments, sulphated iduronate occurs together with non-sulphated iduronate and glucuronate in a random fashion. These characteristics are distinctly different from those of regular heparan sulphate and of archetypal heparin.

INTRODUCTION

The coagulation factors (zymogens) of blood plasma are able to bind to the surface of aggregated platelets in the presence of Ca^{2+}. The zymogens are sequentially activated *via* limited endopeptidase action. In the final step, active factor X (X_a) cleaves prothrombin into diffusible thrombin and an anchoring peptide. Thrombin may then convert soluble fibrinogen into insoluble fibrin. A large part of the thrombin that is released from the surface of the platelet plug is bound to, and inactivated by, antithrombin III, an important plasma anti-protease which also inactivates factor X_a in case it is released from the platelet surface. Free factor X_a is potentially more thrombogenic than thrombin[1], and the possibility of selectively inhibiting X_a-activity in plasma is being explored in many laboratories.

We have reported[2] that certain heparan sulphate/heparin variants both from pig mucosa and beef lung are able to inhibit the activity of factor X_a measured both in amidase and in prothrombin→thrombin conversion assays. The present study is

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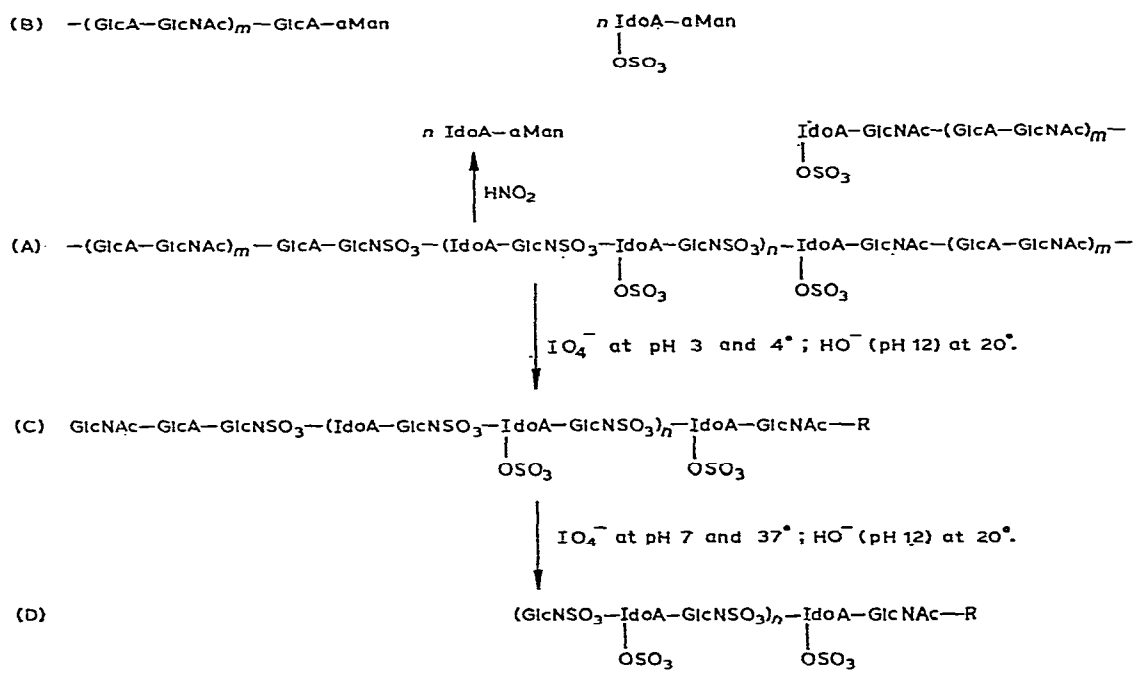
concerned with a chemical characterisation of these variants in terms of the proportions and arrangements of the principal, repeating disaccharide-units, *i.e.*, GlcA–GalNAc, GlcA–$GlcNSO_3$, IdoA–$GlcNSO_3$, and IdoA(-OSO_3)–$GlcNSO_3$; the last three units may also carry -OSO_3 groups at C-6 of $GlcNSO_3$ to a variable degree.

EXPERIMENTAL

Materials. — The pig-mucosa heparin sub-fractions 26S and 26P were described elsewhere[2]. The uronic acid composition of 26S was GlcA, 35%; IdoA, 15%; and IdoA-OSO_3, 50% (in % of total HexA); and the anticoagulant activity was 100 (US Pharmacopaeia), 65 (activated partial thromboplastin time, APTT), and 130 (anti-X_a) units/mg. The beef-lung heparan sulphate/heparin sub-fractions HS1–HS6 were obtained as outlined previously[3]. Sub-fraction HS5, which had appreciable, direct anti-X_a activity[2], had the following chemical analyses: GlcA, 50%; IdoA, 5%; and IdoA-OSO_3, 45%.

Methods. — Carbon n.m.r. spectra were obtained at 25.05 MHz, as described elsewhere[4]. Deaminative cleavage of bonds between $GlcNSO_3$ (C-1) and HexA residues was performed at pH 1.5 by the method of Shively and Conrad[5]. After evapora-



Scheme 1. *Degradation of a heparan sulphate chain.* A hypothetical sequence (A) may be subjected to deaminative cleavage of $GlcNSO_3$–HexA bonds, to give the products shown in (B). Selective periodate oxidation of GlcA associated with GlcNAc in the original chain (A), followed by scission in alkali, produces the fragment depicted in (C). Complete oxidation of all non-sulphated HexA residues, followed by alkaline elimination, yields the segment shown in (D).

tion of excess of HNO_2 with methanol, degradation products were resolved by gel chromatography (see legend to the appropriate Fig.).

Periodate oxidation was performed under two different conditions. Solutions of glycans (2 mg/ml) were treated with sodium metaperiodate (20mM) either in 50mM sodium formate buffer (pH 3.0) at 4° (oxidation of GlcA associated with GlcNAc residues) or in 50mM sodium phosphate buffer (pH 7.0) at 37° (oxidation of all non-sulphated HexA residues) both in the dark for 0–24 h. Aliquots were treated with a molar excess of mannitol and analysed for uronic acid as described earlier[6]. In preparative-scale oxidations, the reactions were terminated as described above, the solutions were desalted by dialysis and freeze-dried, and the residues were dissolved (5 mg/ml) in 0.01M NaOH (pH 12), kept at room temperature for 30 min, neutralised, and subjected to gel chromatography (also see refs. 3 and 4).

The various degradation methods used in this study are outlined in Scheme 1 (also see ref. 7).

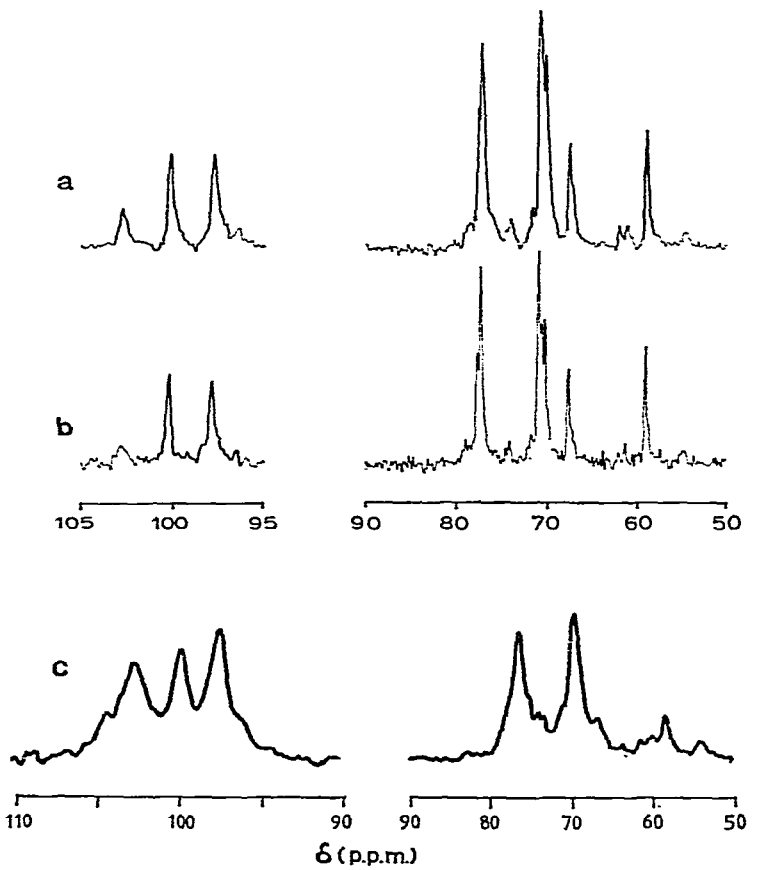


Fig. 1. ^{13}C-N.m.r. spectra for heparin and heparan sulphate preparations 26S (*a*), 26P (*b*), and HS5 (*c*). The left-hand column illustrates the anomeric region, and the right-hand column shows resonances from other ring carbons. The bands corresponding to carboxyl and methyl signals are omitted.

RESULTS

Gross chemical composition. — The two heparin-related variants (26S and HS5) that had direct anti-X_a activity were both relatively rich in IdoA-OSO_3 (50 and 45%, respectively) and in GlcA (35 and 50%, respectively). The latter residue may be associated with either GlcNAc or $GlcNSO_3$. The ^{13}C-n.m.r. analyses indicate that $GlcNSO_3$ is more common than GlcNAc, especially in 26S and 26P (Figs. 1a and 1b; *cf.* δ 58–59 and 54–55 which are the C-2 resonances[4] of the respective sugars). The C-1 resonances of GlcA joined to $GlcNSO_3$ or GlcNAc appear at δ 102–103, with the former slightly downfield. Hence, the broadness of this envelope suggests that several components are present, including GlcA–(GlcNAc), GlcA–($GlcNSO_3$), and IdoA–($GlcNSO_3$/GlcNAc). Other signals that are well represented in the antithrombin-binding sequence (δ 56.5, C-2 of $GlcNSO_3$ with -OSO_3 at C-3; and δ 95,

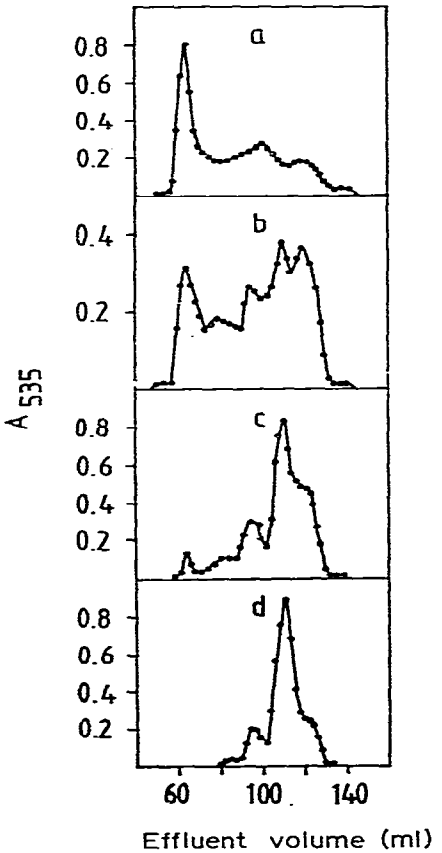


Fig. 2. Gel chromatography of deamination products of heparan sulphate and heparin. The heparan sulphate fractions HS2 (*a*) and HS5 (*b*) and the heparin fractions 26S (*c*) and 26P (*d*) were treated with HNO_2 at pH 1.5, and the products were subjected to chromatography on Sephadex G-25 (superfine): column size, 12 mm × 1800 mm; eluant, 0.2M pyridine acetate (pH 5.0), 10 ml/h; analysis, uronic acid (carbazole–borate); void volume, 65 ml.

C-1 of GlcNAc) were not particularly prominent[8,9] in 26S and HS5. However, the two signals around δ 60–62 were significant in both 26S and HS5, but very low in 26P (the material of low anti-X_a activity); one of these signals has previously been assigned[4] to C-6 of 2-amino-2-deoxyglucose.

Deaminative cleavage. — Cleavage of bonds between $GlcNSO_3$ and HexA residues in the various heparin-related glycans was performed with HNO_2, and the results are shown in Fig. 2. Heparan sulphate HS2 (Fig. 2a), which lacks both anti-X_a and anti-thrombin activity, yielded mainly large fragments of the general structure $(GlcA–GlcNAc)_n$. In HS5 (Fig. 2b), the major products were tetra- and di-saccharides; the latter was obtained as a double peak (V_e = 105–130 ml). The most-retarded component should comprise non-sulphated disaccharides, *i.e.*, IdoA–aMan* and GlcA–aMan. The high anti-X_a heparin sub-fraction 26S (Fig. 2c) afforded even larger quantities of di- and tetra-saccharide, again with a significant proportion of low- or non-sulphated disaccharides. The latter repeats were less common in the low anti-X_a material 26P (Fig. 2d), in which $IdoA(\text{-}OSO_3)–GlcNSO_3(\text{-}OSO_3)$ must be the major repeating-unit (see also Fig. 1b, where the C-1 resonances of the respective sugars are dominant). Oligomers of $(GlcA–GlcNAc)_n$ repeats in the tetra- to hexa-saccharide range were also obtained from the two materials having high anti-X_a activity (Figs. 2b and 2c).

Periodate oxidation. — As shown elsewhere[7], periodate oxidation of heparin-related glycans at pH 3.0 and 4° selectively destroys GlcA in $(GlcA–GlcNAc)_n$-segments. As shown in Fig. 3a, heparin preparation 26S (high anti-X_a variant) lost ~25% of its uronate content under these conditions. The corresponding figure[3] for HS5 is 35%; this is in keeping with a higher content of $(GlcA–GlcNAc)_n$-segments in the latter material, which was indicated by the n.m.r. analyses. This is also illustrated in Fig. 2b, where these segments were released in oligosaccharide form by HNO_2 treatment and eluted primarily in the void volume of Sephadex G-25. In both cases, the amount of GlcA that was susceptible to periodate oxidation under the above conditions was lower (by 10 and 15% for 26S and HS5, respectively) than the estimated total content of GlcA. This would imply that a corresponding proportion of these residues were located in $GlcA–GlcNSO_3$ repeats. In this combination, GlcA is resistant to oxidation at low pH and temperature[7].

Periodate oxidation at pH 7.0 and 37° destroys all non-sulphated HexA residues in heparin-related glycans[7]. Treatment of heparin preparation 26S (Fig. 3a) in this way destroyed an additional 25% of the uronate content, suggesting that both $GlcA–GlcNSO_3$ and $IdoA–GlcNSO_3$ repeats were present in this material (10 and 15%, respectively). The corresponding figure[3] for HS5 is 20%, which suggests that $GlcA–GlcNSO_3$ was the more common (15 and 5%, respectively). In the heparin material 26P (Fig. 3b) of low anti-X_a activity, repeating disaccharides of the type $HexA–GlcNSO_3$ with non-sulphated uronate must be very rare, as the consumption of HexA by periodate was approximately the same under both conditions.

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*aMan connotes 2,5-anhydro-D-mannose.

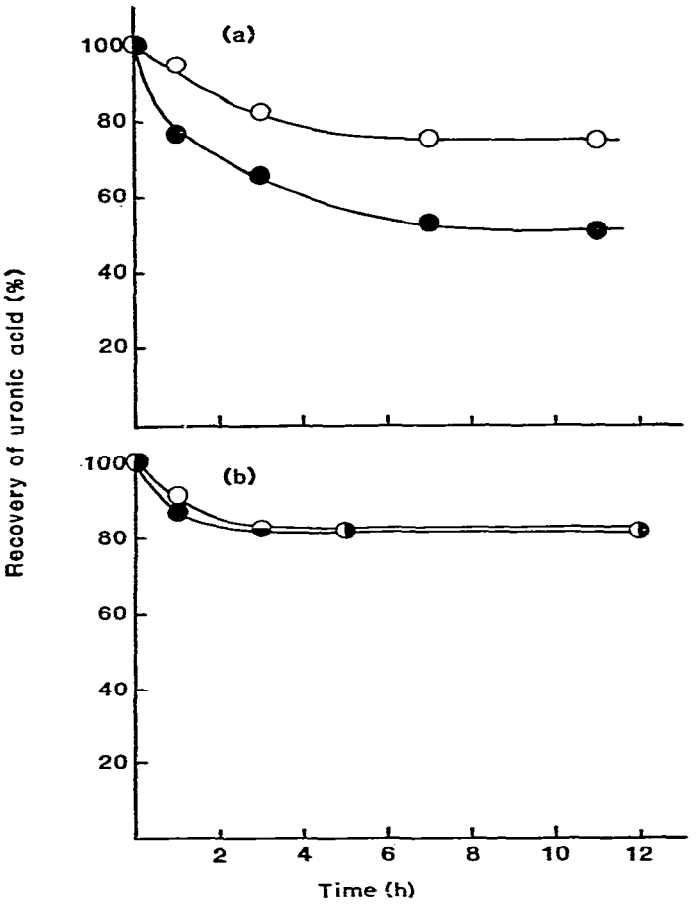

Fig. 3. Periodate oxidation of heparin preparations 26S (*a*) and 26P (*b*) at pH 3.0 and 4° (—○—) and at pH 7.0 and 37° (—●—). The extent of oxidation was monitored by carbazole measurements. The colour yield at $t = 0$ was set at 100%.

To obtain information about the size distribution of $(HexA–GlcNSO_3)_n$-segments, the oxyheparins were cleaved under alkaline conditions. Cleavage of GlcA in $(GlcA–GlcNAc)_n$-block regions of heparan sulphate HS2, which has no anti-coagulant activity, afforded the fragmentation profile shown in Fig. 4a. The oligomers produced were mainly $GlcN–(HexA–GlcN)_{1-4}$-R, where R is the remnant of an oxidised and degraded GlcA. Oxidation and cleavage of all non-sulphated HexA residues yielded $GlcN–[IdoA(-OSO_3)–GlcN]_{1-2}$-R. The two heparin-related glycans that displayed direct X_a-inhibition (HS5 and 26S in Figs. 4b and 4c, respectively) yielded a similar spectrum of oligomers with sulphated IdoA, *i.e.*, $GlcN–[IdoA-(-OSO_3)–GlcN]_n$-R of $n = 1–10$ with a maximum around 5–6. However, the $HexA–GlcNSO_3$ repeats were arranged differently in the two preparations, as indicated by the results obtained after selective periodate oxidation of GlcA residues in GlcA–

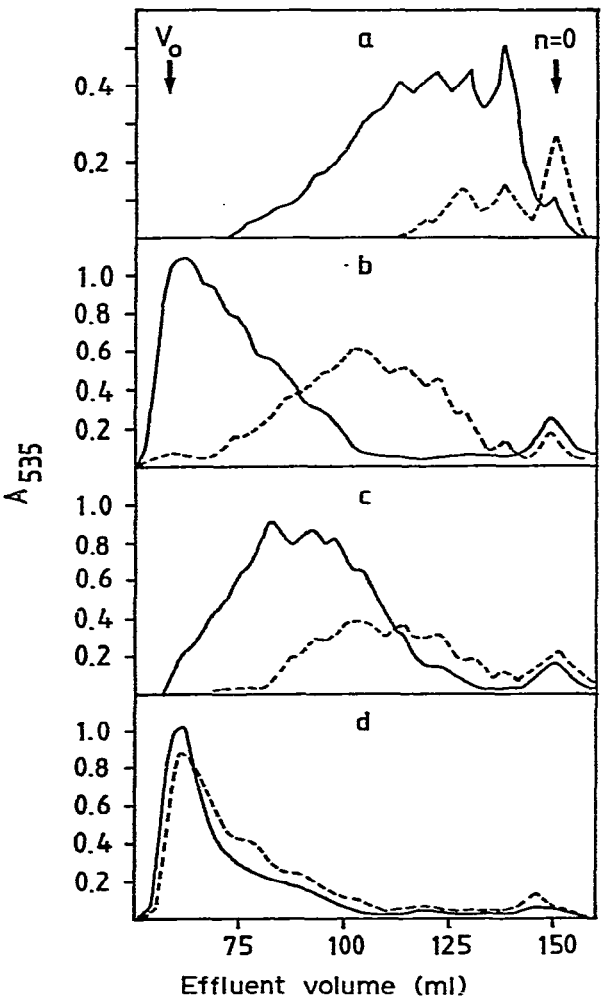

Fig. 4. Gel chromatography of heparan sulphate fractions HS2 (*a*) and HS5 (*b*) and heparin fractions 26S (*c*) and 26P (*d*) after periodate oxidation at pH 3 and 4° (——) or pH 7 and 37° (– – –) for 24 h, both followed by alkaline elimination: column, Sephadex G-50 (superfine); size, 12 mm × 1800 mm; eluant, 0.2M pyridine acetate (pH 5.0), 10 ml/h; analysis, uronic acid (carbazole–borate); n = 0, the elution position of GlcNAc-R where R is the remnant of an oxidised and degraded uronic acid residue; void volume, 58 ml.

GlcNAc regions. In HS5 (Fig. 4b), the segments GlcN–(HexA–GlcN)$_n$-R had $n > 6$, whereas n was 5–8 in 26S. Finally, heparin with low anti-X_a potency (26P) yielded very large fragments after both types of oxidation, indicating that it consists of [IdoA(-OSO_3)–$GlcNSO_3$]$_n$ repeats where $n \gg 7$.

DISCUSSION

Heparin-related glycans[2] that are able to inhibit clotting factor X_a in the ab-

sence of antithrombin III exhibit structural features that set them apart from both regular heparan sulphate and archetypal heparin. These features may be summarised as follows: (*a*) approximately half of the repeating units are IdoA(-OSO_3)–$GlcNSO_3$ largely present in short segments of 3–6 repeats, (*b*) these segments are part of larger regions (containing 5–8 repeats) that incorporate IdoA–$GlcNSO_3$ and GlcA–$GlcNSO_3$ units. The latter account for ~25% of all repeating disaccharides. Although the extent of 6-sulphation of $GlcNSO_3$ has not been estimated quantitatively, it seems fair to propose that a significant proportion of these $GlcNSO_3$ residues have HO-6 free. This may be a secondary feature related to the unusually high content of GlcA–$GlcNSO_3$ repeats. Retention of the D-*gluco* configuration may be favoured by undersulphation[10].

The *N*-sulphated segments that comprise IdoA-OSO_3, IdoA, and GlcA residues should span ~25% of the chain-length, assuming a molecular weight of 12000–15000. As these segments account for ~67% of the material by weight, they must occur two or three times in each chain, on average. These regions are joined *via* $(GlcA–GlcNAc)_n$-segments, some of which are very short. The latter segments are essential for X_a-inhibitory activity, as brief periodate oxidation of these GlcA residues abolished the activity[2]. As discussed elsewhere[2], the anti-X_a activity of certain heparin species is unrelated to the antithrombin III-binding sequence. The structural features described here accord with this statement. It has also been demonstrated[11] that dermatan sulphates are able to exert anticoagulant activity which is independent of antithrombin-III. Although dermatan sulphate and heparin are based on different carbohydrate backbones, they share certain structural features, namely, the presence of mixed or alternating regions containing IdoA(-OSO_3), IdoA, and GlcA.

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