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PERIODATE OXIDATION AND ALKALINE DEGRADATION OF HEPARIN-RELATED GLYCANS

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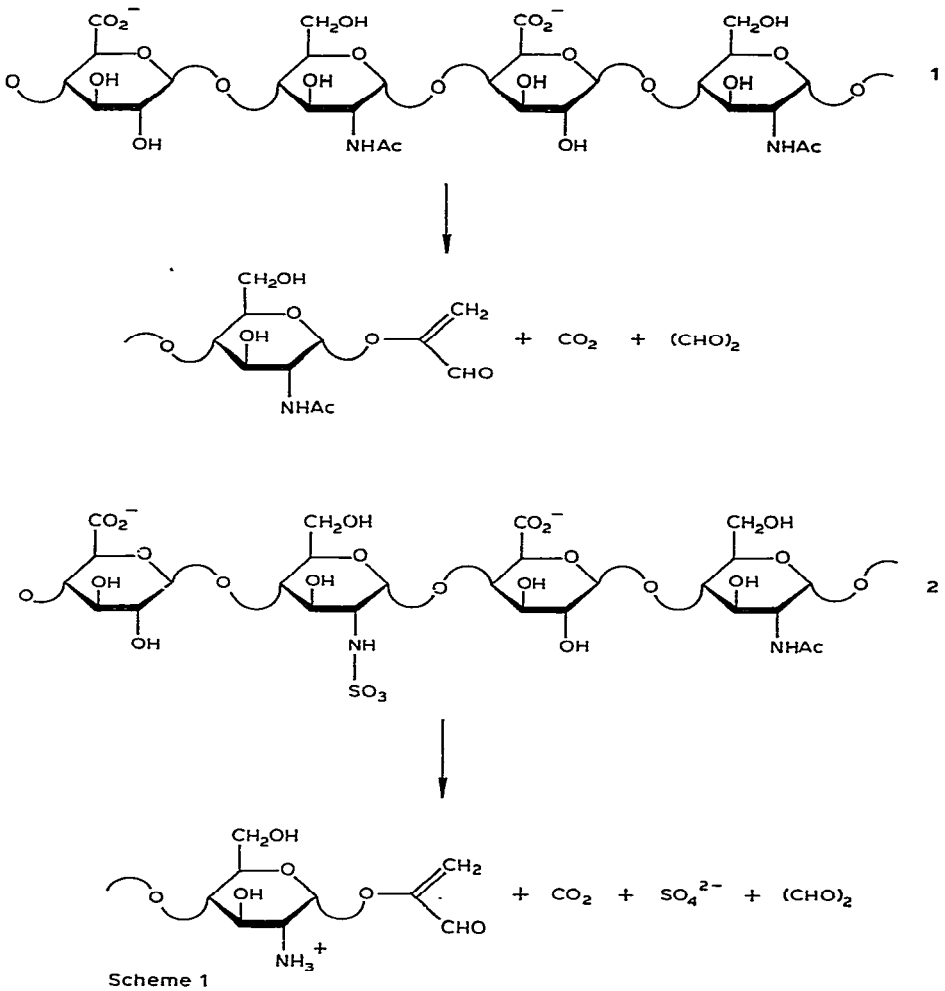
ABSTRACT

Heparin, heparan sulphate, and various derivatives thereof have been oxidised with periodate at pH 3.0 and 4° and at pH 7.0 and 37°. Whereas oxidation under the latter conditions destroys all of the nonsulphated uronic acids, treatment with periodate at low pH and temperature causes selective oxidation of uronic acid residues. The reactivity of uronic acid residues depends on the nature of neighbouring 2-amino-2-deoxyglucose residues. D-Glucuronic acid residues are susceptible to oxidation when flanked by *N*-acetylated amino sugars, but resistant when adjacent residues are either unsubstituted or *N*-sulphated. L-Iduronic acid residues in their natural environment (2-deoxy-2-sulphoamino-D-glucose) are resistant to oxidation, whereas removal of *N*-sulphate groups renders a portion of these residues periodate-sensitive. Oxidised uronic acid residues in heparin-related glycans may be cleaved by alkali, producing a series of oligosaccharide fragments. Thus, periodate oxidation–alkaline elimination provides an additional method for the controlled degradation of heparin.

INTRODUCTION

Heparin and heparan sulphate have a common carbohydrate-backbone composed of alternating, (1→4)-linked β-D (or α-L) uronic acid (UA) and 2-amino-2-deoxy-α-D-glucose (GlcN) residues. Heparin is a potent anticoagulant, and heparan sulphate has been implicated in cell–cell contact and binding of lipoproteins and lipoprotein lipase to cell surfaces[1]. The chemical structure of these glycans is quite complex, as the GlcN residues are either GlcNAc, $GlcNSO_3$, or $GlcNSO_3$ with $-OSO_3$ at C-6, and the UA residues are either L-iduronic acid (IdA) or D-glucuronic acid (GlcA) with the former occasionally 2-*O*-sulphated. Heparin is primarily composed of the repeating-unit –IdA(2-OSO_3)–$GlcNSO_3$(6-OSO_3)–, whereas heparan sulphates contain variable quantities of the repeating-units –GlcA–GlcNAc–, –GlcA–$GlcNSO_3$–, –IdA–$GlcNSO_3$–, –GlcA–$GlcNSO_3$(6-OSO_3)–, –IdA–$GlcNSO_3$(6-OSO_3)–, IdA(2-OSO_3)–$GlcNSO_3$–, and the above-mentioned, typical, heparin repeating-unit[1].

At present, two principal methods are available for the selective degradation of heparin-related glycans. Deaminative cleavage of hexosaminidic bonds between $GlcNSO_3$ and UA permits the isolation of oligosaccharide segments containing GlcNAc residues[2]. Degradation with bacterial eliminases[3,4] produces disaccharides having 4,5-unsaturated, non-reducing terminal UA-residues. By using enzymes adapted to the degradation of various heparan sulphate species, detailed information about the distribution of GlcNAc- and $GlcNSO_3$-containing units may be obtained. As cleavage of *N*-sulphated regions by eliminases produces 4,5-unsaturated UA-residues (*i.e.*, IdA and GlcA yield the same product), information about the arrangement of GlcA, IdA, and IdA-SO_4 within *N*-sulphated regions is lacking.



Scheme 1

It was recently shown[5] that, on treatment of heparan sulphate with periodate at pH 3.0 and 4°, GlcA associated with GlcNAc was oxidised. In contrast, the GlcA,

IdA, and IdA-SO_4 residues of *N*-sulphated regions were largely unaffected. We now report on the periodate-susceptibility of GlcA and IdA in combination with $GlcNH_3^+$, GlcNAc, or $GlcNSO_3$ in heparin-related molecules. Methods for scission of oxy-heparins, *i.e.*, alkaline elimination or reduction–hydrolysis, have also been evaluated.

EXPERIMENTAL

Materials. — Heparin from pig mucosa (5H 488) and heparin-by-products from beef lung were supplied by Dr. W. E. Lewis of Glaxo Operations Ltd., Runcorn, U.K. [^{35}S]-Heparan sulphate and [^{35}S]-dermatan sulphate–chondroitin sulphate copolymers were isolated from fibroblast cultures after radiosulphate incorporation, as described elsewhere[6]. [^{14}C]-Heparin precursors were obtained by incubation of mastocytoma microsomes with UDP-[^{14}C]-GlcA and UDP-GlcNAc in the absence or presence of PAPS[7]. The various precursors were isolated by ion-exchange chromatography[7].

Heparin-by-products (heparan sulphate) were fractionated according to the procedure of Rodén *et al.*[8]. Crude material (10 mg) was treated with alkaline copper sulphate to remove dermatan sulphate. The soluble material was fractionated with ethanol as the calcium salt, and glycans precipitating between 18 and 36% (v/v) of ethanol were recovered. Finally, the heparan sulphates were fractionated by stepwise precipitation as cetylpyridinium complexes from 1.2, 1.0, 0.8, 0.6, 0.4, and 0.2M NaCl.

Heparan sulphate oligosaccharides of principal sequence $(GlcA–GlcNAc)_n$ were prepared by deaminative cleavage and gel filtration[9].

Sephadex gels were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and Instagel from Packard AB, Bandhagen, Sweden. Other chemicals were of analytical grade.

Analytical methods. — Hexosamine was determined by the Elson–Morgan procedure[10,11]. Separations of 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose were performed on a Bio-Cal automatic amino acid analyzer, using a pH-6.45 buffer. The same analyzer was also used to estimate NH_3. Sulphate was quantified by the method of Terho and Hartiala[12]. Hexuronic acids were determined by the carbazole (Dische)[13] and orcinol[14] methods. The carbazole–borate method[15] was employed in an automated procedure[16] for analyses of column effluents. Free hexuronic acids were released from heparins by acid hydrolysis, deaminative cleavage, and repeated acid hydrolysis[17]. Uronic acids (IdA and GlcA) were then separated by ion-exchange chromatography[5]. Reducing power was estimated by the Park–Johnson method[18]. Anticoagulant activity was determined by the Pritchard[19] modification of the USP procedure. Radioactivity was measured with a Packard 2650 liquid scintillation counter. The scintillator used was Instagel (0.5 ml of liquid mixed with 5 ml of sample).

^{13}C-N.m.r. spectra were determined at 20 MHz with a Varian CFT-20 Fourier-transform spectrometer. Polysaccharide samples (100 mg) were dissolved in D_2O (0.8–1.0 ml) with adjustment of pH to 7–8. For further details, see ref. 9.

Degradation and modification procedures. — Solvolytic *N*-desulphation of heparin and heparan sulphate was performed by treatment of their pyridinium salts with dimethyl sulphoxide containing 5% of methanol for 1.5 h at 50°, as described by Inoue and Nagasawa[20]. *O*/*N*-Desulphation was achieved by treating corresponding material with dimethyl sulphoxide containing 10% of methanol for 4 or 24 h at 100° (ref. 21). The sulphate-to-hexosamine molar ratios after 4-h and 24-h desulphations were 0.31 and 0.12, respectively. *N*-Acetylations were accomplished by using acetic anhydride[22].

Periodate oxidation was performed under two different conditions: (*a*) at pH 3.0 and 4°, and (*b*) at pH 7.0 and 37°. Solutions of glycans (2 mg/ml) were treated with sodium metaperiodate (20mM) with or without addition of buffer (50mM). Buffers used were sodium citrate and sodium formate at pH 3.0, and sodium phosphate at pH 7.0. When buffer salts were not used, NaCl (50mM) was added, and the pH was adjusted to the desired value. Oxidations of heparin were conducted in 0.2M sodium perchlorate, in order to minimize periodate–polyanion repulsion[23]. After oxidation, samples were treated with a molar excess of D-mannitol and analysed for uronic acid[5,13]. In preparative-scale oxidations, the terminated reaction mixtures were desalted by dialysis against water, or by gel filtration, and freeze-dried. Oxyglycans were cleaved by (*a*) reduction–hydrolysis or (*b*) alkaline elimination. Reduction was performed with sodium borohydride (2 mg/mg of oxyglycan in 0.1 ml of distilled water) for 3 h at room temperature. Excess of borohydride was decomposed with glacial acetic acid. The oxidized and reduced material was recovered after dialysis by freeze-drying. Reduced oxyglycans were hydrolysed with 5mM H_2SO_4 (4 mg/ml) at 60° for 3 h. The reaction was terminated by the addition of M NaOH to pH 4–5. Alkaline elimination was accomplished by adjusting a solution of oxyheparins (5 mg/ml) with M NaOH to pH 12 and keeping it at room temperature for 30 min. The solutions were neutralized with M acetic acid. Oxygen was not excluded during alkaline treatment. Degradation products of heparin and heparan sulphate, obtained *via* the procedures described above, were fractionated by gel chromatography. Details are given below in the Fig. legends.

RESULTS

Periodate oxidation of heparin, heparan sulphates, and derivatives. — One preparation of commercial heparin and two heparan sulphate fractions (I and IV) were used (Table I). The three glucosaminoglycans differed with respect to sulphate content and UA composition. Heparan sulphate-I contained less than half a sulphate group per repeating unit, and one 2-amino-2-deoxyglucose residue out of five was *N*-sulphated. The IdA content corresponded reasonably well with the GlcN-SO_3 content. Thus, most of the GlcA was associated with GlcNAc (see also Ref. 1). Heparan sulphate-IV carried ~1 sulphate group per repeating unit, but only half of the 2-amino-2-deoxyglucose residues were *N*-sulphated. Since the IdA content was 40% of the total uronic acid, a small but significant proportion of GlcA–GlcNSO_3

TABLE I

ANALYSES[a] OF HEPARAN SULPHATE FRACTIONS AND HEPARIN

Fraction (M NaCl)	*Hexosamine (%)*	*Total sulphate/ hexosamine (mol/mol)*	N-*Sulphate/ hexosamine (mol/mol)*	*Uronic acid (carbazole) (%)*	*Uronic acid/ hexosamine (mol/mol)*	*Carbazole/ orcinol ratio*[b]	*IdA/GlcA*	*Anticoagulant activity (BP units/mg)*
I (0.2–0.4)	33.3	0.44	0.21	36.8	1.02	2.42	25:75	0
IV (0.8–1.0)	30.8	1.15	0.47	32.9	0.98	3.17	40:60	30
Heparin	20.5	2.40	0.80	30.2	1.35	4.52	75:25	157

[a]Heparin-by-products from bovine lung were freed from dermatan sulphate by copper precipitation, recovered as Ca^{2+}-salts with 36% ethanol (v/v), and finally fractionated with cetylpyridinium chloride using an increasing concentration of NaCl. In all fractions, 2-amino-2-deoxyglucose constituted >95% of the total hexosamine. Total sulphate was determined after hydrolysis in 6M HCl at 100° for 8 h, and *N*-sulphate after hydrolysis in 0.04M HCl at 100° for 1.5 h. Total uronic acid was determined both by the carbazole and orcinol methods. Quantitation of L-iduronic acid and D-glucuronic acid was accomplished by ion-exchange chromatography after acid hydrolysis, deaminative cleavage, and re-hydrolysis. Anticoagulant activity was measured by the Pritchard modification of the USP procedure. Uronic acid and hexosamine contents are expressed as percentages of dry weight. [b]The c/o ratios correlate well with IdA/GlcA (Fransson, Sjöberg, and Havsmark, *Eur. J. Biochem.*, in press).

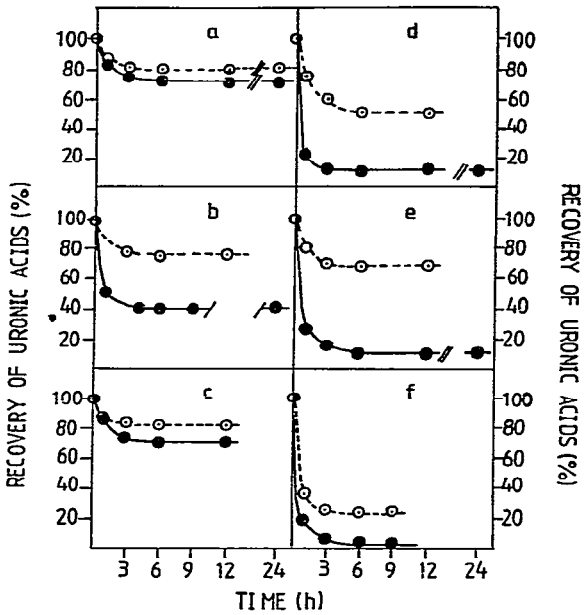


Fig. 1. Effect of pH and temperature on the rate of oxidation of (a) heparin, (b) *N*-desulphated heparin, (c) heparin that had been *N*-desulphated and then *N*-acetylated, (d) *O*/*N*-desulphated (4 h) heparin, (e) heparin that had been *O*/*N*-desulphated (4 h) and then *N*-acetylated, and (f) heparin that had been *O*/*N*-desulphated (24 h) and then *N*-acetylated. Oxidations were performed at pH 3.0 and 4° (○----○) using citrate–buffer, and at pH 7.0 and 37° (●——●) using phosphate buffer. The data refer to absorbances at $t = 1, 2, 3 \ldots$ h, as percentages of the absorbance at $t = 0$.

repeats must be present in heparan sulphate-IV. Both heparan sulphates had negligible anticoagulant activity. The analyses of the heparin preparation indicated that at least 70% of the repeating units consisted of IdA(2-OSO_3)–GlcNSO_3(6-OSO_3). Non-sulphated repeating-units, *i.e.*, GlcA–GlcNAc, accounted for 20%. Thus, the remaining 10% included GlcA–GlcNSO_3 and various undersulphated, IdA-containing units.

The rate of oxidation of heparin was studied at pH 3.0 and 4°, and at pH 7.0 and 37°. As shown in Fig. 1a, 20% of the UA residues in heparin were susceptible to oxidation at low pH and temperature. Analyses of the UA composition confirmed[5] that only GlcA was oxidized. Oxidation at pH 7.0 and 37° resulted in a recovery of 70% of the UA content, corresponding to the amount of IdA-SO_4 present in the material. After *N*-desulphation ~25% of the UA residues in heparin were destroyed by periodate at pH 3.0 and 4°, whereas as much as 60% were oxidized at pH 7.0 and 37° (Fig. 1b). *N*-Desulphation creates an additional periodate-sensitive structure in heparin (HO-3 and H_2N-2 in the GlcN moiety). Therefore, the amounts of NH_3 liberated during the two oxidations were determined. The release of NH_3 corresponded to 5% and 19% of the GlcN content at pH 3.0 and 4°, and at pH 7.0 and 37°, respectively. Thus, at pH 7.0 and 37°, significant cleavage of the C-2–C-3 bond in GlcN occurs, which may result in cleavage of the hexosaminidic bond and further

oxidation of the C-3–C-4 glycol in IdA-2-OSO_3. For heparin that had been *N*-desulphated and then *N*-acetylated, the rate and extent of oxidation under the two conditions (Fig. 1c) were indistinguishable from those of the intact material. Although these results may suggest that the extent of oxidation of GlcA and IdA residues (non-sulphated) in heparin may be largely unaffected by the presence of adjacent $GlcNH_3^+$, GlcNAc, or $GlcNSO_3$ residues, the quantities of irregular repeating-units are too small to permit a correct evaluation.

In another series of experiments, *O*/*N*-desulphated heparins were oxidized (Fig. 1d–f). In a heparin derivative that had been treated with dimethyl sulphoxide for 4 h, only 10% of the UA content (presumably residual IdA-SO_4) remained after periodate oxidation at pH 7.0 and 37° (Fig. 1d). However, oxidation at pH 3.0 and 4° decreased the UA content by ~50%. In this case, the proportions of IdA and GlcA were changed from 75:25 to 65:35. Clearly, a portion of the IdA residues had been oxidized. After *N*-acetylation of this material (Fig. 1e) ~70% of the UA content was recovered after oxidation at pH 3.0 and 4°. In this case, the proportions of IdA and GlcA were altered from 75:25 to 95:5. It is concluded that IdA residues adjacent to GlcN residues having substituted amino-groups may be resistant to periodate oxidation at low pH and temperature. Finally, heparin was exhaustively *O*/*N*-desulphated (24 h), and then *N*-acetylated and subjected to periodate oxidation (Fig. 1f). At pH 7.0 and 37°, more than 95% of the UA residues were destroyed, whereas ~75% were consumed at pH 3.0 and 4°. Thus, IdA residues adjacent to GlcNAc residues in an extensively desulphated heparin are sensitive to periodate oxidation at low pH and temperature.

The rate and extent of oxidation of various heparan sulphates were also studied under the two conditions, as shown in Fig. 2. Heparan sulphate-I, which had an IdA/GlcA ratio of 25:75, lost ~70% of its UA content upon oxidation at pH 3.0 and 4° (Fig. 2a). Analyses of the UA composition revealed that more than 90% of the GlcA residues had been consumed. Moreover, in heparan sulphate-I, the IdA residues were largely non-sulphated, as most of the uronic acids were destroyed at pH 7.0 and 37°. In heparan sulphate-IV (Fig. 2b), with a GlcA content of 60% of the total UA, only 40% of the UA residues were oxidized at low pH and temperature. The proportions of IdA and GlcA were changed from 40:60 to 65:35, and ~60% of the IdA residues in this material were estimated to be non-sulphated. Significant amounts of non-sulphated IdA and GlcA residues adjacent to $GlcNSO_3$ residues were present in heparan sulphate-IV (see also Table I). After *N*-desulphation of this material, ~40% of the UA residues were still susceptible to periodate oxidation at low pH and temperature (○----○ in Fig. 2c). Subsequent *N*-acetylation increased the amount of periodate-sensitive residues to 50% (△----△ in Fig. 2c). After the latter treatment, the GlcA content had decreased to 7% of the total UA. The experiments with *O*/*N*-desulphated (4 h) heparins (above) suggested that IdA in combination with $GlcNH_3^+$ was partially susceptible to periodate oxidation (pH 3.0 and 4°), whereas IdA–GlcNAc units may be resistant. Therefore, *N*-desulphated heparan sulphate should become more extensively oxidized than heparan sulphate that has

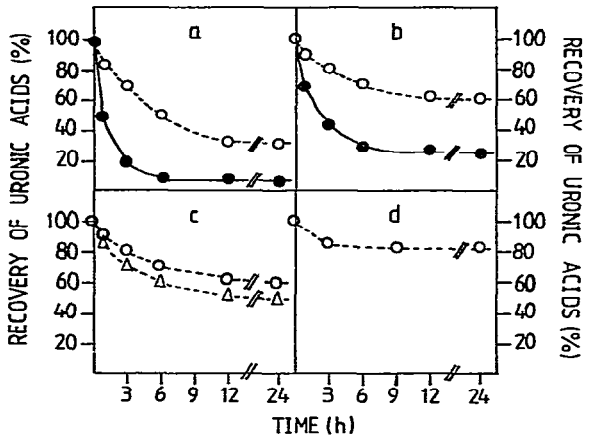
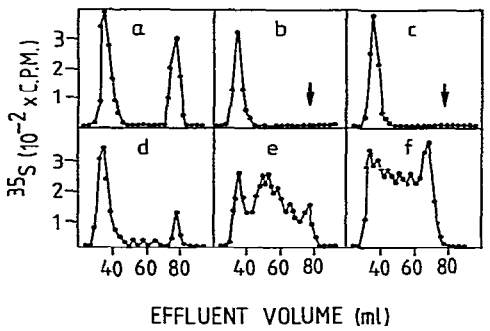


Fig. 2. Effect of pH and temperature on the rate of oxidation of (a) heparan sulphate-I, (b) heparan sulphate-IV, (c) *N*-desulphated heparan sulphate-IV before (○) and after (△) *N*-acetylation, and (d) *N*-desulphated, periodate-oxidised (pH 3.0; 4°; 24 h), alkali-treated, and *N*-acetylated heparan sulphate-IV. Oxidations were performed at pH 3.0 and 4° (○----○, △----△) and pH 7.0 and 37° (●——●) without the use of buffer salts.

been *N*-desulphated and then *N*-acetylated (Fig. 2c), unless GlcA in combination with $GlcNH_3^+$ is totally resistant to oxidation. To test this possibility, heparan sulphate-IV was *N*-desulphated, oxidized with periodate at pH 3.0 and 4°, treated with alkali to achieve chain cleavage (see below), *N*-acetylated, and finally re-oxidized with periodate (Fig. 2d). Of the UA residues in this material, ~20% were susceptible to oxidation. These residues (presumably GlcA), which were insensitive to periodate oxidation when surrounded by $GlcNH_3^+$ residues, were rendered sensitive by subsequent *N*-acetylation.

To corroborate the above findings, heparin precursors or derivatives containing large proportions of GlcA or IdA in combination with $GlcNH_3^+$, $GlcNSO_3$, or GlcNAc were subjected to periodate oxidation. Furthermore, oxidized UA-residues were cleaved, and the degradation products were fractionated by gel chromatography.

Cleavage of oxyheparins and oxyheparan sulphates. — Oxyglycans may be cleaved by reduction–hydrolysis (Smith-degradation) or alkaline elimination to fragments of the general structure $GlcN–(UA–GlcN)_n–R$, where R denotes the remnant of an oxidized and degraded UA-residue. As heparin contains labile groups (*N*-sulphate), it was considered important to test each step separately (Fig. 3). When a [^{35}S]-heparan sulphate was subjected to periodate oxidation (pH 3.0 and 4°) (Fig. 3b) or to alkali treatment (Fig. 3c) followed by gel chromatography, no release of radiosulphate was noted. However, the hydrolysis step alone (Fig. 3d) caused significant desulphation. The conditions used were those described for cleavage of oxidized and reduced dermatan sulphate[24]. This result suggested that reduction–hydrolysis was an unsuitable way to cleave oxyheparan sulphate. Periodate oxidation of [^{35}S]-heparan sulphate followed by alkaline elimination (Fig. 3e) also yielded a



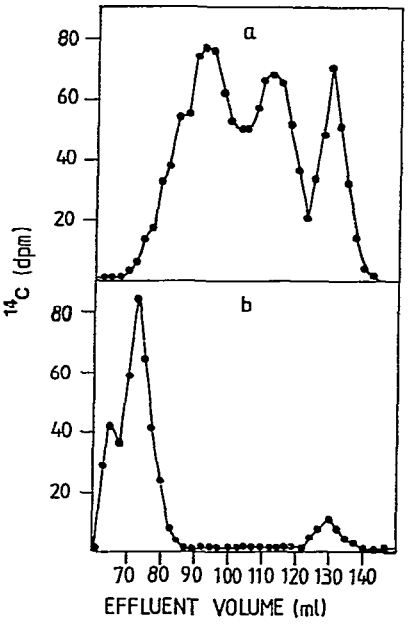


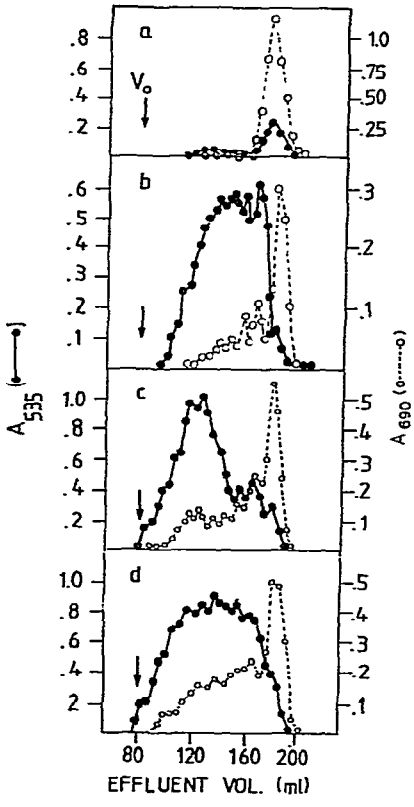
Fig. 3 (left). Gel chromatography of (a) [^{35}S]-heparan sulphate and $Na_2{}^{35}SO_4$, (b) [^{35}S]-heparan sulphate after periodate oxidation at pH 3.0 and 4° for 24 h, (c) [^{35}S]-heparan sulphate after treatment with alkali (pH 12) for 30 min at room temperature, (d) [^{35}S]-heparan sulphate after treatment with acid (5mM H_2SO_4) for 3 h at 60°, (e) [^{35}S]-heparan sulphate after oxidation with periodate as in (b) followed by alkali-treatment as in (c), and (f) ^{35}S-dermatan sulphate after periodate oxidation–alkaline elimination. Column: Sephadex G-50, superfine; size, 9 mm × 1400 mm; eluant, 0.2M pyridine acetate (pH 5.0); elution rate, 6 ml/h.

Fig. 4 (right). Gel chromatography of (a) [^{14}C]-heparin precursor I, mainly $(GlcA–GlcNH_3^+–GlcA–GlcNAc)_n$, and (b) [^{14}C]-heparin precursor III, mainly $(GlcA–GlcNSO_3)_n$, after periodate oxidation (pH 3.0; 4°)-alkaline elimination. Column: Sephadex G-25, superfine; size, 12 mm × 1800 mm; eluant, 0.2M pyridine acetate (pH 5.0); elution rate, 10 ml/h; V_0, 60 ml; V_t, 150 ml.

peak in the radiosulphate position, as well as various oligosaccharide fragments. A radiosulphate peak was not obtained with periodate-oxidized and alkali-treated [^{35}S]-dermatan/chondroitin sulphate. These results suggest that, during alkaline elimination of oxyheparan sulphate, sulphate is released from structures (presumably $GlcNSO_3$) adjacent to oxidized sites in the molecule.

Periodate oxidation–alkaline elimination of heparin-related glycans. — Selective periodate oxidation (pH 3.0 and 4°) followed by alkaline elimination was used to degrade three groups of heparin-related glycans: (*a*) [^{14}C]-heparin precursors, (*b*) derivatives of heparan sulphate, and (*c*) variously desulphated heparins.

As shown in Fig. 4, [^{14}C]-heparin precursors[7] having the dominant structures (*a*) $(GlcA–GlcNH_3^+–GlcA–GlcNAc)_n$ and (*b*) $(GlcA–GlcNSO_3)_n$ were degraded separately, and the products were fractionated on Sephadex G-25. Precursor (*a*) yielded three major components, corresponding in size to approx. tetra-, tri-, and mono-saccharides, whereas precursor (*b*) yielded one major component, which



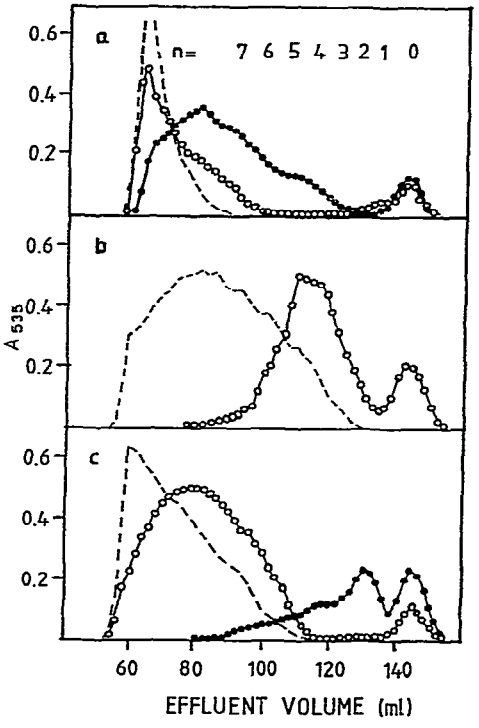


Fig. 5 (left). Gel chromatography of degradation products obtained after periodate oxidation (pH 3.0 and 4° for 24 h) and alkaline elimination of (a) heparan sulphate oligosaccharide with GlcA–GlcNAc repeating-units, (b) heparan sulphate-I, (c) heparan sulphate-IV, and (d) heparan sulphate-IV that had been *N*-desulphated and then *N*-acetylated. Column: Sephadex G-50, superfine; size, 12 mm × 2300 mm; eluant, 0.2M pyridine acetate (pH 5.0); elution rate, 10 ml/h; analysis, uronic acid (●, carbazole) and reducing power (○); V_o, elution volume of Blue Dextran; n = number of disaccharide repeats in fragments of the general formula GlcN–(UA–GlcN)$_n$–R, where R is the remnant of an oxidised and degraded uronic acid residue.

Fig. 6 (right). Gel chromatography of (a) heparin, (b) *O*/*N*-desulphated heparin, and (c) heparin that had been *O*/*N*-desulphated and then *N*-acetylated before (----) and after periodate oxidation at pH 3 and 4° (○——○) and pH 7 and 37° (●——●) for 3 h, both followed by alkaline elimination. Column: Sephadex G-50, superfine; size, 12 mm × 1800 mm; amount applied, 6 mg; eluant, 0.2M pyridine acetate (pH 5.0); elution rate, 10 ml/h; analysis, uronic acid (carbazole). The column was calibrated with degradation products of heparan sulphate (see Fig. 5b and c): general carbohydrate structure, GlcN–(UA–GlcN)$_n$–R, where R is the remnant of an oxidised and degraded uronic acid residue.

emerged close to the excluded volume. A heparan sulphate oligosaccharide of the principal structure (GlcA–GlcNAc)$_n$ was also degraded by periodate oxidation (pH 3.0; 4°)–alkaline elimination, and the products were fractionated on Sephadex G-50 (Fig. 5a). The original oligosaccharide had an elution volume of ~120 ml. After oxidation and scission, a major component in the monosaccharide region was ob-

tained. These results indicate that GlcA–GlcNAc block-regions were degraded to GlcNAc–R compounds, whereas GlcA in combination with $GlcNH_3^+$ or $GlcNSO_3$ was resistant to oxidation. Therefore, it is proposed that the non-sulphated heparin precursor (Fig. 4a) was degraded to $[GlcNH_3^+–GlcA]_2$–GlcNAc–R, $GlcNH_3^+$–GlcA–GlcNAc–R, and GlcNAc–R in almost equal proportions. The *N*-sulphated heparin precursor (*b*), which should contain ~80% of *N*-sulphate and 20% of *N*-acetyl groups[7], yielded blocks of $GlcNSO_3$–$(GlcA–GlcNSO_3)_n$–GlcNAc–R, where $n \sim 3–4$.

Further degradations of heparan sulphates confirmed that GlcA in combination with GlcNAc was sensitive to periodate oxidation, whereas GlcA adjacent to $GlcNSO_3$ was resistant. As shown in Figs. 5b and 5c, heparan sulphate-I, which contained a larger proportion of GlcA–GlcNAc units than heparan sulphate-IV (Table I), was more extensively degraded than was the other material. The most-retarded component, which had reducing power but no uronic acid, corresponded to GlcNAc–R. It may therefore be concluded that the oligosaccharides GlcNAc–$(UA–GlcN)_n$–GlcNAc–R obtained from heparan sulphate-I generally ranged from $n = 1–4$, whereas those of heparan sulphate-IV were larger ($n = 4–7$). When heparan sulphate-IV was *N*-desulphated and then *N*-acetylated prior to oxidation and scission (Fig. 5d), smaller oligosaccharide products ($n = 1–4$) were produced. This result suggests that the original GlcA–$GlcNSO_3$ repeats of heparan sulphate-IV were often interspersed between –$(IdA–GlcNSO_3)_n$– block-regions of relatively short length ($n = 1–4$).

To investigate the periodate susceptibility of IdA in combination with $GlcNSO_3$, $GlcNH_3^+$, and GlcNAc, variously desulphated heparins were examined. As shown in Fig. 6a, periodate oxidation of intact heparin at pH 3.0 and 4° followed by alkaline cleavage (○——○) afforded oligosaccharides and polysaccharide fragments that were generally larger than tetradecasaccharide ($n = 6$). In addition, a small peak of GlcNAc–R was obtained. Apparently, a major proportion of the GlcA–GlcNAc repeats of heparin (amounting to 20% of the repeating units) are present in small blocks that separate larger blocks of mainly IdA-containing units. Periodate oxidation at pH 7.0 and 37° followed by scission (●——●) resulted in a more-extensive fragmentation of the chains. The oligosaccharides obtained ranged in size from $n = 4–12$, with a peak at $n = 9$. Thus, IdA-SO_4-containing units occurred in blocks of 4–12 interrupted by single units of IdA–$GlcNSO_3$ or GlcA–$GlcNSO_3$. Periodate oxidation–alkaline elimination of heparin that had been *N*-desulphated and then *N*-acetylated gave the same elution profile on Sephadex G-50 as did the starting material (○——○ in Fig. 6a).

As shown above, IdA in combination with $GlcNH_3^+$ may be partially susceptible to periodate oxidation at pH 3.0 and 4°. Periodate oxidation–alkaline elimination of *O*/*N*-desulphated (4-h solvolysis) heparin (Fig. 6b) produced fragments in the decasaccharide region (○——○). Degradation of heparin that had been *O*/*N*-desulphated and then *N*-acetylated (Fig. 6c) afforded fragments that were only slightly smaller (○——○) than the starting material (------). This decrease in size can be entirely ascribed to oxidation and scission of GlcA–GlcNAc units. Periodate oxidation

at pH 7.0 and 37° of heparin that had been *O*/*N*-desulphated and then *N*-acetylated, followed by alkaline scission (●——●), indicated that residual IdA-OSO_3-containing units generally occurred as single units ($n = 1$). Therefore, it may be inferred that both non-sulphated and sulphated IdA residues were present within the same oligosaccharide fragments that were obtained by degradation of *O*/*N*-desulphated heparin (Fig. 6b). The sizes of the latter fragments were probably underestimated, as *O*/*N*-desulphated heparin was more retarded than heparin that had been *O*/*N*-desulphated and then *N*-acetylated (------ in Figs. 6b and c).

Structure of GlcNAc–R. — Periodate oxidation–alkaline elimination of heparan sulphate oligosaccharide $(GlcA–GlcNAc)_n$ produced a monosaccharide derivative (Fig. 5a). ^{13}C-N.m.r. spectra of this material and of a GlcNAc standard are shown in Fig. 7. The standard compound (Fig. 7a) is a mixture of the α and β anomers (δ 95.3 and 91.4, respectively, correspond to the anomeric carbons). The GlcNAc–R compound (Fig. 7b) gave one signal corresponding to an α-anomeric carbon and three more signals in this region. These are tentatively ascribed to a hydrated aldehyde, $-CH(OH)_2$, (δ 93.8), a CH_2 = group (δ 99.1), and a $=C(-)-CHO$ group (δ 108.1). The carbonyl signal at δ 175.5 was rather weak, suggesting that only >C=O of the *N*-acetyl group was present in this material. The other signal in the carbonyl region

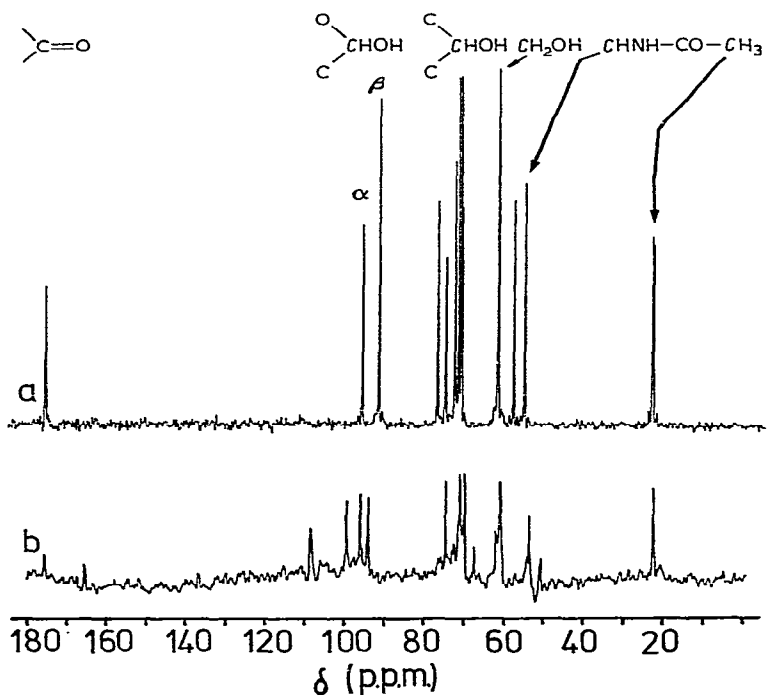


Fig. 7. ^{13}C-N.m.r. spectra of (a) 2-acetamido-2-deoxy-αβ-D-galactose and (b) component $n = 0$ in Fig. 5a.

(δ 165.1) may originate from a *C*HO carbon. Alternatively, this signal, as well as other minor peaks, may arise from products of 2,5-anhydro-D-mannose, which was located at the reducing terminus of the original oligosaccharide. These findings suggest that R in GlcNAc–R is largely a C_3-fragment of the structure -O-C(CHO)=CH_2. Furthermore, the data are consistent with a retention of the α-D configuration, as only an α-anomeric carbon signal was observed.

DISCUSSION

The GlcA and IdA residues of glycosaminoglycans have potential sites (HO-2,3) for periodate oxidation. However, the reactivity of the glycols in these uronic acids depends on the nature of neighbouring-sugar residues, as demonstrated by work in this laboratory[5,24] and by Scott and co-workers[23,25]. A GlcA residue substituted with two methyl groups at C-1 and C-4 respectively is readily oxidized by periodate[25]. In contrast, GlcA flanked by β-linked GlcNAc (as in hyaluronate) or β-linked GalNAc (as in chondroitin sulphate) is oxidized very slowly. At low pH and temperature, the extent of oxidation of such GlcA residues is negligible[24]. However, a GlcA residue flanked by α-linked GlcNAc (as in heparan sulphate) is markedly sensitive to periodate oxidation[5,25]. Scott and Tigwell[25] proposed that, in the presence of β-linked GlcNAc or GalNAc, GlcA residues participate in a hydrogen-bonding system that involves HO-2. This type of secondary structure of the glycan chain is apparently upset by changing the anomeric configuration of the hexosaminidic bond from β to α. In the present work, it was observed that GlcA in combination with α-linked $GlcNH_3^+$ or $GlcNSO_3$ is resistant to oxidation, suggesting that free amino groups and sulphamido groups may introduce an additional, or alternative, hydrogen-bonding scheme that engages the glycol grouping of GlcA. The importance of co-operative, inter-residue hydrogen-bonding is indicated by the fact that periodate-resistant GlcA residues become susceptible to oxidation after scission of the oxyglycan in alkaline medium[5].

The IdA residues of dermatan sulphate (β-hexosaminidic bonds) are readily oxidized by periodate under a variety of conditions[24,25]. In contrast, IdA residues adjacent to α-linked GlcNAc or $GlcNSO_3$ are resistant to oxidation[5,25], provided a small number of IdA-OSO_3 residues are present in the glycan. Some of the non-sulphated IdA residues are consumed when alkali-cleaved oxyheparins are re-oxidised (Fransson, unpublished observations). This finding suggests that co-operative hydrogen-bonding creates secondary structures within (IdA–$GlcNSO_3$)-containing regions of heparin and heparan sulphate, which engage the glycol groups of IdA. After removal of the *N*-sulphate groups, some of the non-sulphated IdA residues become sensitive to periodate. The periodate-insensitive IdA residues of *N*-desulphated material are located in segments that also contain IdA-OSO_3 residues. It is proposed that the secondary structures that involve the glycols of non-sulphated IdA are dependent upon the presence of an occasional IdA-OSO_3 residue. It is also possible

that non-sulphated IdA residues may adopt different chair conformations, as was proposed in a recent n.m.r. study of heparin[9].

The ring conformation of non-sulphated IdA in dermatan sulphate is a matter of dispute. X-Ray fibre diffraction data[26,27] favour the $^4C_1$ chair. However, n.m.r. data are generally interpreted in terms of the $^1C_4$ conformation[28,29]. As susceptibility to periodate oxidation under mild conditions may be regarded as an indication that HO-2 and HO-3 are diequatorially disposed, the present data favour the $^4C_1$ conformation for at least a portion of the non-sulphated IdA residues in heparan sulphate and heparin.

Selective periodate oxidation (pH 3.0 and 4°) of heparan sulphate and heparin, followed by alkaline elimination, results in cleavage of (GlcA–GlcNAc)-containing segments of the chains. The structure of the products may be generally described as GlcN–(UA–GlcN)$_n$–R. Blocks of GlcA–GlcNAc periods (**1** in Scheme 1) are degraded to GlcNAc–R, where R is the remnant of an oxidised and degraded GlcA. The remnant is probably a C_3-fragment; the loss of a CO_2H group is supported by the fact that GlcNAc–R is not bound to a DEAE-cellulose column (Fransson and Sjöberg, unpublished observations). Although GlcA flanked by $GlcNSO_3$ is resistant to oxidation, it is conceivable that, in a sequence –GlcA–$GlcNSO_3$–GlcA–GlcNAc (**2**), the latter GlcA residue is susceptible. It is suggested that sulphate which is released upon alkali treatment of oxyheparan sulphate is derived from the $GlcNSO_3$ residue adjacent to the point of cleavage.

The knowledge acquired in the present study can be used to elucidate structural features of heparin and heparan sulphate. In pig-mucosa heparin, IdA(-OSO_3)–$GlcNSO_3$(-OSO_3) repeats generally occur in clusters of 4–12 (see also ref. 22) that are interrupted by single IdA–$GlcNSO_3$ (or GlcA–$GlcNSO_3$) units. Assuming that heparin contains, on an average, 20 disaccharide-repeats (mol. weight, 12,000; disaccharide weight, 600), non-sulphated IdA (5–10% of the total uronic acid) should account for less than two residues per chain. Regions that contain sulphated as well as non-sulphated IdA residues seem to be fairly large (>7 disaccharide-repeats; *i.e.*, one-third of the molecule) and separated from each other by (GlcA–GlcNAc)-containing block-regions. The latter blocks, which account for ~20% of the chain, may thus contain, on an average, 4 repeating-units. Periodate oxidation–alkaline elimination is a useful method for characterizing intermediates in the biosynthesis of heparin. Non-sulphated-heparin precursor was split into (GlcA–GlcNAc)$_n$–GlcA–aMan (2,5-anhydro-D-mannose), with n = 1–2, and GlcA–aMan upon deaminative cleavage of bonds between $GlcNH_3^+$ and GlcA[7], indicating that GlcA–GlcNAc repeats were present as single units and occasionally as two consecutive units. Periodate oxidation–alkaline elimination of this precursor yielded $GlcNH_3^+$–R, $GlcNH_3^+$–GlcA–GlcNAc–R, and $GlcNH_3^+$–GlcA–$GlcNH_3^+$–GlcA–GlcNAc–R, indicating that GlcA–$GlcNH_3^+$ repeats were often present as two consecutive units. *N*-Sulphated-heparin precursor, which is produced from the former by *N*-deacetylation and *N*-sulphation[7], apparently contains blocks of ~4 GlcA–$GlcNSO_3$ repeats separated by single GlcA–GlcNAc units.

The structure of heparan sulphate, which is more complex and variable than that of heparin, may also be studied by periodate oxidation–alkaline elimination. A closer examination of fragments obtained from this glycan will be reported elsewhere.

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