Carbohydrate Research, 62 (1978) 235–244

PERIODATE OXIDATION OF THE D-GLUCURONIC ACID RESIDUES IN HEPARAN SULPHATE AND HEPARIN

LARS-ÅKE FRANSSON

Department of Physiological Chemistry 2, University of Lund, S-22007 Lund (Sweden)

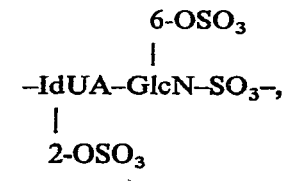
(Received March 29th, 1977; accepted for publication May 9th, 1977)

ABSTRACT

On oxidation with periodate at pH 7.0 and 37°, the uronic acid residues of heparan sulphate preparations were almost completely destroyed, whereas only 20% of those in heparin were susceptible to oxidation. At pH 3.0 and 4°, 30–40% of the uronic acid residues in heparan sulphates were destroyed, but very few in heparin. In all cases at pH 3.0 and 4°, the L-iduronic acid residues were resistant to oxidation, whereas a large proportion of the D-glucuronic acid residues were affected. However, a small but significant proportion of the D-glucuronic acid residues resisted oxidation. The initially periodate-resistant D-glucuronic acid residues were destroyed when alkali-treated oxyheparan sulphate was treated with periodate. When heparan sulphate and heparin derivatives containing mainly *N*-acetylated 2-amino-2-deoxy-D-glucose residues were treated with periodate at pH 3.0 and 4°, most of the D-glucuronic acid residues were destroyed, whereas the L-iduronic acid residues were resistant.

INTRODUCTION

Heparan sulphate and heparin are composed of alternating 2-amino-2-deoxy-glucose and uronic acid residues joined *via* α-D-1,4 and β-D(or α-L)-1,4 linkages, respectively[1]. The amino sugar moieties are either GlcNAc, GlcN-SO_3, or GlcN-SO_3 with -OSO_3 at C-6. The uronic acid residues are either L-iduronic acid (IdUA) or D-glucuronic acid (GlcUA), with the former occasionally 2-*O*-sulphated. Heparin is primarily composed of the repeating unit



whereas heparan sulphate displays considerable structural heterogeneity, notably variations in the ratios of GlcNAc to GlcN-SO_3, and IdUA to GlcUA.

Structural studies on these polymers have been performed[1] by using both

chemical and enzymic degradations. Deaminative cleavage of the GlcN-SO_3 to uronic acid (UA) bonds has provided information concerning the distribution of *N*-sulphate groups[2]. After degradation of heparan sulphate by bacterial eliminases, five different disaccharide repeats (type ΔUA–GlcN) have been identified[3]; ΔUA denotes 4,5-unsaturated glycuronic acid. Since the configurational difference at C-5 is eliminated by these enzymes, the distribution of IdUA and GlcUA could not be assessed. Estimations of the uronic acid composition of heparan sulphates and heparin have shown that IdUA residues are associated with *N*-sulphated regions[4,5]. Furthermore, the proportion of IdUA increases with increasing content of ester sulphate[5].

In a previous report from this laboratory, a procedure for the controlled degradation of copolymeric galactosaminoglycans was described[6]. The degradation was initiated by selective periodate oxidation of IdUA residues at low pH and temperature. In the present work, heparan sulphate and heparin have been subjected to periodate oxidation under the same conditions. In contrast to the previous results, the IdUA residues of the two glucosaminoglycans were resistant to periodate oxidation, while most of the GlcUA residues were oxidized.

EXPERIMENTAL

Materials. — Dermatan sulphate and hyaluronic acid were prepared from pig skin[7]. Aortic heparan sulphate was a gift from Professor S. Gardell (0.3M $MgCl_2$ fraction in Table I in Ref. 8). Mucosal heparan sulphate was prepared by Dr. J. A. Cifonelli (fraction 1,25:2 in Table VIII in Ref. 9). Heparin (stage 14) from pig intestinal mucosa was kindly provided by Professor U. Lindahl, (prepared according to Ref. 10). Another heparin preparation (Inolex) and an *O*/*N*-desulphated and re-*N*-acetylated derivative thereof were obtained from Professor L. Rodén. Analytical data for these preparations are given in Refs. 8–11.

L-Iduronic acid was isolated by preparative ion-exchange chromatography (see below) of hydrolysed (2M trifluoroacetic acid, 100°, 4 h) pig-skin dermatan sulphate.

Analytical and chromatographic methods. — Uronic acid was determined by an automated version of the carbazole–borate method[12].

Ion-exchange chromatography of glycosaminoglycans was performed on a column (6 × 140 mm) of microgranular Whatman DE-32 DEAE-cellulose. The ion exchanger was precycled according to the recommendation of the manufacturers and finally equilibrated with 0.10M sodium acetate buffer (pH 5.0; starting buffer). The samples (1–2 mg) were dissolved in the starting buffer and applied to the column. Elution was performed with a linear gradient (0.10–2.50M sodium acetate, pH 5.0; total elution volume, 100 ml) at a rate of 3 ml/h. The shape of the gradient was determined by conductivity measurements.

Gel chromatography of degradation products was carried out on a column

(12 × 1800 mm) of Sephadex G-25 (superfine, Pharmacia) eluted with 0.2M pyridine–acetate (pH 5.0) at a rate of 10 ml/h.

Ion-exchange chromatography of free hexuronic acids was performed on a column (6 × 1400 mm) of AGl-X4 (formate) resin (0–400 mesh, Bio-Rad), which was eluted with 0.3M formic acid at a rate of 20 ml/h at 28°. The effluent was analysed for uronic acid by the automated carbazole method. For further details, see Ref. 13.

Degradation and modification procedures. — *N*-Desulphoheparan sulphate was prepared by heating the material in 0.04M HCl at 100° for 90 min, followed by dialysis and freeze-drying. *N*-Acetylheparan sulphate was obtained by treatment of *N*-desulphoheparan sulphate with acetic anhydride[14].

Periodate oxidations were performed as follows. Solutions of polysaccharides (2 mg/ml) in 20mM sodium periodate in 50mM buffers of sodium citrate (pH 3.0) or sodium phosphate (pH 7.0) were incubated at 4° or 37° in the dark. In the case of heparin, oxidations were performed in the presence of 0.2M $NaClO_4$ to minimize periodate–polyanion repulsion. Aliquots were treated with a molar excess of D-mannitol and analyzed for uronic acid by the Dische carbazole method[15]. For preparative-scale oxidations, the terminated reaction mixture was dialysed and lyophilized.

Oxyheparan sulphate was reduced with potassium borohydride (2 mg per mg of polysaccharide 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 polysaccharide was recovered after dialysis and lyophilisation.

Another portion of oxyheparan sulphate was degraded in alkaline solution (pH 12, 5 mg/ml) at room temperature for 30 min. The degradation products were re-isolated by chromatography on Sephadex G-25 (see above).

Deaminative cleavage of GlcN-SO_3 to UA bonds was achieved with nitrous acid at low pH by the procedure of Shively and Conrad[16]. Excess of nitrous acid was removed by repeated evaporations with methanol. Degradation products were subsequently fractionated by gel chromatography, and appropriate fractions were combined and lyophilized.

Free hexuronic acids were released from heparan sulphate or heparin (2–5 mg) by the procedure of Höök *et al.*[11]. This procedure includes acid hydrolysis with trifluoroacetic acid, deaminative cleavage with nitrous acid, and repeated acid hydrolysis. The final hydrolysate was kept in 1 ml of 0.1M ammonia at room temperature overnight for conversion of lactones into free acids. The sample was then evaporated to dryness prior to ion-exchange chromatography (see above).

RESULTS

Characterization of heparan sulphate and heparin. — The heparan sulphate and heparin preparations used in this study had been analysed by other workers[8–11]. In the present study, the samples were subjected to ion-exchange chromatography (Fig. 1). The points of elution of aortic and mucosal heparan sulphate and of heparin

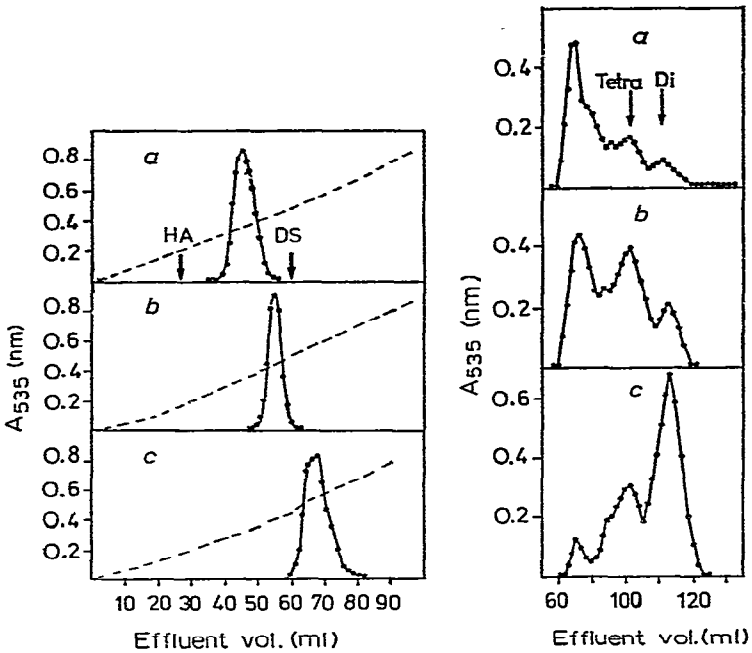


Fig. 1. Ion exchange-chromatography on DE-32 DEAE-cellulose (gradient elution) of (*a*) aortic heparan sulphate, (*b*) mucosal heparan sulphate, and (*c*) heparin: ●—●, carbazole; ——, conductivity. The points of elution of hyaluronate (HA) and pig-skin dermatan sulphate (DS) are indicated in the upper graph.

Fig. 2. Gel chromatography on Sephadex G-25 of products obtained on deamination of (*a*) aortic heparan sulphate, (*b*) mucosal heparan sulphate, and (*c*) heparin: ●—●, carbazole; V_0, 70 ml.

are in accordance with the recorded SO_4/disaccharide molar ratios of these preparations, *i.e.*, ~0.5, 1.0, and 2–3, respectively.

In order to assess the amount and distribution of *N*-sulphate groups, the three samples were treated with nitrous acid, followed by gel chromatography (Fig. 2). In agreement with previous observations[11], aortic heparan sulphate has the lowest, and heparin the highest, content of GlcN-SO_3 groups. Thus, aortic heparan sulphate contains large segments of UA–GlcNAc units, whereas heparin contains blocks of UA–GlcN-SO_3 periods. Mucosal heparan sulphate, which contains equal amounts of *N*-sulphate and *N*-acetyl groups[9], was degraded to tetrasaccharide (carbohydrate sequence UA–GlcNAc–UA–aMan) to a considerable extent[17]; aMan denotes 2,5-anhydro-D-mannose. This result is consistent with the presence of alternating units containing GlcNAc and GlcN-SO_3.

Periodate oxidation of heparan sulphate and heparin. — The rate of oxidation of heparan sulphate and heparin was studied at pH 3.0 and 4°, and at pH 7.0 and 37°. As shown in Fig. 3, the destruction of UA residues in the heparan sulphates (*a* and *b*) was almost complete at pH 7.0 and 37°, whereas only 20% of the UA residues in

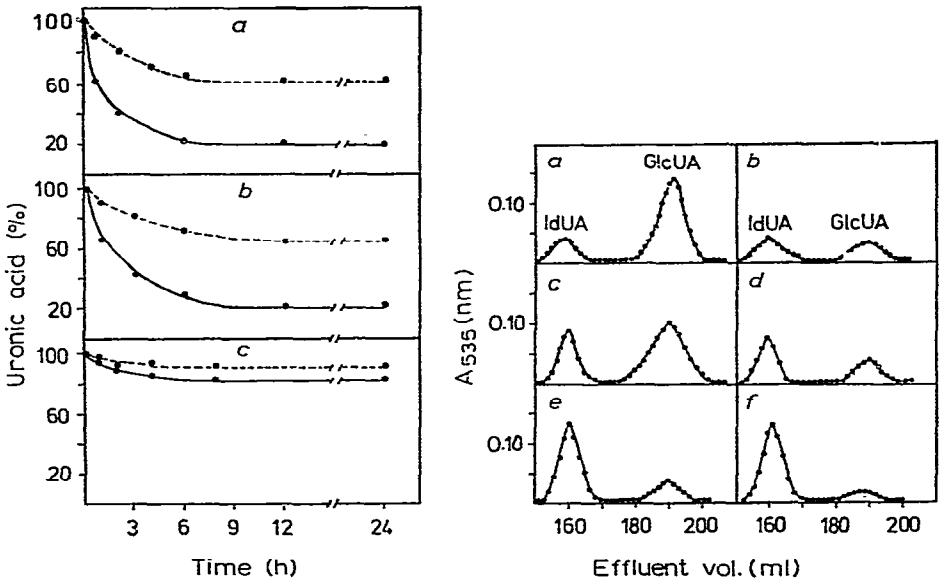


Fig. 3. Effect of pH and temperature on the rate of oxidation of (*a*) aortic heparan sulphate, (*b*) mucosal heparan sulphate, and (*c*) heparin. Oxidations were performed at pH 3.0 and 4° (●—●), and at pH 7.0 and 37° (●—●).

Fig. 4. Ion-exchange chromatography (AG1-X4) of uronic acids from aortic heparan sulphate (*a–b*), mucosal heparan sulphate (*c–d*), and heparin (*e–f*) before (*a*, *c*, and *e*) and after (*b*, *d*, and *f*) exhaustive periodate oxidation at pH 3.0 and 4°. The GlcUA peak was invariably broad and asymmetrical (see also Fig. 6). It has been observed that, after the final hydrolysis step, a small amount of UA-aMan still remains. These disaccharides are eluted in almost the same position as GlcUA (L. Rodén, personal communication).

heparin (*c*) were susceptible to oxidation.* This is in accordance with the current view that the IdUA residues of heparan sulphate are largely nonsulphated, while those of heparin are almost entirely 2-*O*-sulphated[1].

Periodate oxidation at pH 3.0 and 4° yielded limited destruction of the UA residues in the two heparan sulphate preparations (~40% and 30%, respectively; whereas < 10% of the UA residues in heparin were oxidized). The UA composition of the three samples was determined before and after exhaustive (24-h) periodate oxidation at pH 3.0 and 4°. As shown in Fig. 4, the IdUA content of the polymers remained unchanged, whereas the GlcUA content was markedly diminished. The destruction of GlcUA was largest in aortic heparan sulphate and lowest in heparin. It should be noted that a small but significant proportion of the GlcUA residues in all samples was resistant to periodate oxidation at low pH and temperature.

*It should be noted that the destruction of UA residues was monitored by the original Dische method, and that the data refer to the absorbances at t = 1, 2, 3 . . . h, as percentages of the absorbance at t = 0.

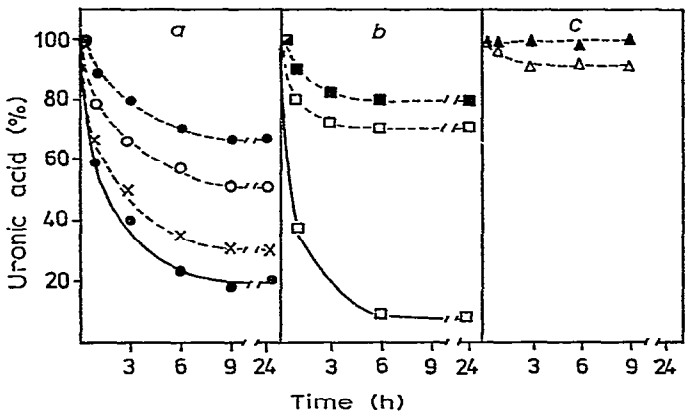


Fig. 5. Periodate oxidation of (*a*) mucosal heparan sulphate before (●) and after *N*-desulphation and re-*N*-acetylation (○), (*b*) heparin before (■) and after *O*/*N*-desulphation and re-*N*-acetylation (□), and (*c*) oxyheparan sulphate after reduction (▲) and after treatment with alkali (△); ----, pH 3.0 and 4.0; —, pH 7.0 and 37°. A sample of the saccharides UA–GlcNAc–(GlcUA–GlcNAc)$_n$–UA–aMan, with $n > 2$ (Fig. 2b; void volume peak), was also oxidized (x).

Periodate oxidation of derivatives of heparan sulphate and heparin. — As shown in Fig. 5a, *N*-desulphated and re-*N*-acetylated heparan sulphate was oxidized to a larger extent (~50%) at pH 3.0 and 4° than was the starting material (~30%). An oligosaccharide fraction (x) containing the saccharides UA–GlcNAc–(GlcUA–GlcNAc)$_n$–UA–aMan was extensively oxidized under the same conditions, almost to the same extent as was the starting material at pH 7.0 and 37°*. These results suggest that GlcUA associated with GlcNAc moieties is oxidized completely by periodate, whereas GlcUA associated with GlcN-SO_3 may be resistant to oxidation.

Similar results were obtained with heparin (Fig. 5b). In intact heparin, ~20% of the UA residues were oxidized at low pH and temperature, whereas ~30% of the UA residues were oxidized in *O*/*N*-desulphated, re-*N*-acetylated heparin under the same conditions. The UA residues of the latter preparation were extensively destroyed at pH 7 and 37°. This indicates that, despite the presence of GlcNAc residues, non-sulphated IdUA residues must remain resistant to oxidation.

In order to investigate whether inter-residue interactions could interfere with the extent of oxidation, oxyheparan sulphate was reduced and reoxidized. As shown in Fig. 5c, no further oxidation was observed, indicating that cross-links of the hemiacetal-type were not formed[18]. However, scission of oxyheparan sulphate by alkali resulted in the appearance of a small but significant number of periodate-sensitive UA residues (Fig. 5c).

The uronic acid composition of the various heparan sulphate and heparin derivatives was determined before and after periodate oxidation at pH 3.0 and 4°

*It should be pointed out that the saccharide UA–GlcNAc–(GlcUA–GlcNAc)$_n$–UA–aMan contains a few IdUA residues in the nonreducing terminal and reducing terminal disaccharide repeats, respectively[5].

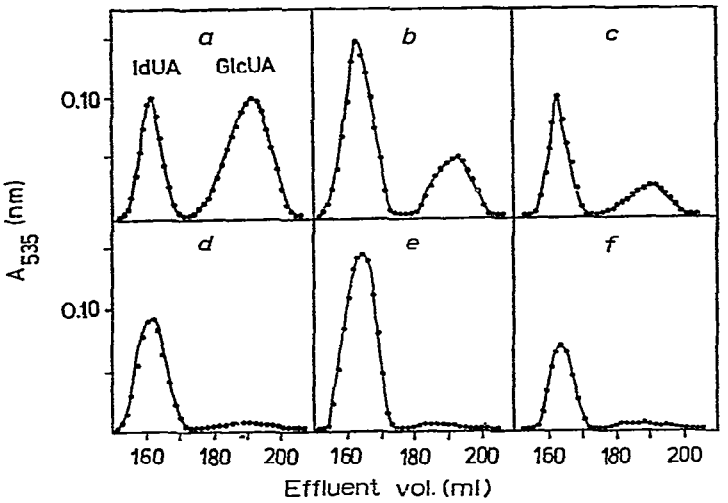


Fig. 6. Ion-exchange chromatography of uronic acids from *N*-desulphated and re-*N*-acetylated heparan sulphate (*a* and *d*), *O*/*N*-desulphated and re-*N*-acetylated heparin (*b* and *e*), and alkali-treated oxyheparan sulphate (*c* and *f*) before (*a–c*) and after (*d–f*) exhaustive periodate oxidation at pH 3.0 and 4°.

(Fig. 6). In *N*-desulphated and re-*N*-acetylated heparan sulphate (*a* and *d*) and in *O*/*N*-desulphated and re-*N*-acetylated heparin (*b* and *e*), most of the GlcUA residues were oxidized by periodate. Similarly, in the fragments formed by periodate oxidation–alkaline elimination of heparan sulphate (*c* and *f*), most of the GlcUA residues were destroyed upon reoxidation with periodate. In the latter case, the possibility that a small proportion of the IdUA residues were also rendered susceptible to oxidation cannot be excluded.

DISCUSSION

The results of the present study show that nonsulphated IdUA residues in heparan sulphate and heparin are resistant to periodate oxidation at pH 3.0 and 4°. This is in contrast to results obtained with dermatan sulphate[18], where IdUA residues were readily oxidized under a variety of conditions. When considering the structural differences between heparan sulphate and dermatan sulphate, as well as conditions which affect periodate oxidation, the present results may be explained as follows.

Oxidative cleavage between C-2 and C-3 of the uronic acid residues in glycosaminoglycans is prevented or inhibited if (*1*) HO-2 and HO-3 have a diaxial configuration, (*2*) HO-2 or HO-3 are sulphated, or (*3*) HO-2 and HO-3 are involved in hydrogen bonding[18].

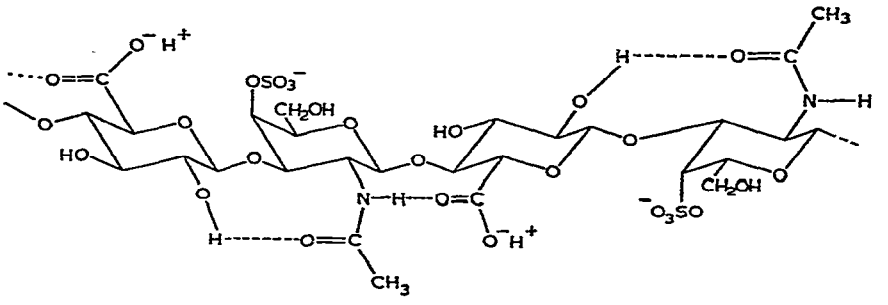
It is currently accepted[18,19] that the IdUA (*i.e.*, nonsulphated) residues of dermatan sulphate are in the $^4C_1$ conformation, whereas the IdUA residues of heparin (largely 2-*O*-sulphated) adopt the $^1C_4$ conformation[19]. This difference in conformation is likely to be a function of sulphation of IdUA, as sulphate ester groups are

generally axially disposed (in the $^1C_4$ form, HO-2 and HO-3 are both axial). Therefore, nonsulphated IdUA residues in heparin-related molecules (*i.e.*, desulphated heparin and various forms of heparan sulphate) may be expected to occupy the $^4C_1$ form. Results of X-ray diffraction studies seem to support this notion[19]. Sodium salts of heparan sulphate exhibit an axial periodicity that eliminates the participation of $^1C_4$ forms. However, with calcium salts of heparan sulphate, a new X-ray fibre pattern emerges identical with that obtained from calcium heparin[19]. Because segments of heparan sulphate chains contain repeating units that are identical to those of heparin[1], it has been proposed that oriented preparations are mixtures of a "heparan-sulphate-like" phase and a "heparin-like" phase, and that the former crystallizes preferentially in the sodium-salt form[20].

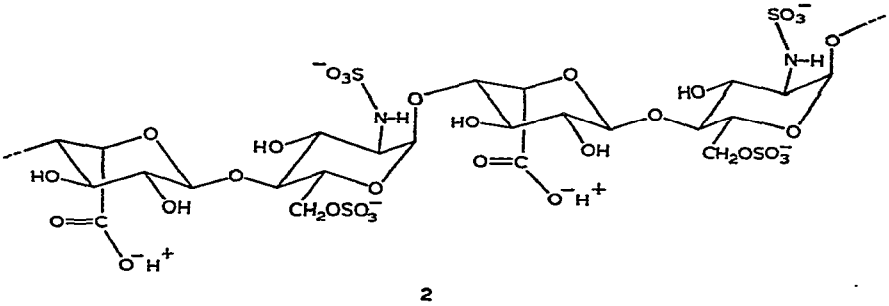
The results presented in this study confirm that non-sulphated IdUA residues in heparin-related molecules adopt the $^4C_1$ conformation, because the uronic acid residues were readily oxidized by periodate at pH 7.0 and 37°. Although the two chair forms of IdUA are energetically almost equivalent[19], the activation energy should be considerable. In contrast, non-sulphated IdUA residues of heparan sulphate and desulphated heparin were resistant to periodate oxidation at pH 3.0 and 4°. These results must be interpreted in terms of a hydrogen-bonding scheme.

In the case of chondroitin sulphate, an array of hydrogen bonds, as shown in **1**, has been proposed by Scott and Tigwell[21]. This arrangement would undoubtedly be stabilized by low pH and temperature. Presumably, IdUA residues having axial COOH groups do not participate in this type of secondary structure. However, galactosaminoglycans contain β-D-hexosaminidic bonds, whereas heparin and heparan sulphate contain α-linked GlcN residues (see **2**). This difference in geometry might favour hydrogen bonding between protonated IdUA-carboxyl and other groups, *e.g.*, >N-H in sulfoamino or acetamido groups. Cooperative effects can also be achieved by concomitant hydrogen-bonding between HO-3 of IdUA and other neighbouring oxygens, creating periodate-resistant structures. Presumably, COOH groups of GlcUA residues in heparan sulphate attain an unfavourable position in this respect. In keeping with this view, the present results show that a large proportion of the GlcUA residues of heparan sulphate are oxidized by periodate at low pH and temperature. This is in agreement with the results of Scott and Tigwell[21], who reported that more than 70% of the theoretical consumption of periodate by heparan sulphate occurred at a rapid rate. The sample used (aortic heparan sulphate) had a GlcUA content of 81% of the total uronic acid.

In the present study, it was observed that the extent of oxidation of GlcUA was greatest in aortic heparan sulphate. In mucosal heparan sulphate, which contains more GlcN-SO_3 moieties (see Fig. 2), the oxidation of GlcUA was not quite as extensive. Furthermore, periodate oxidation of various heparan sulphate derivatives indicated that GlcUA associated with GlcNAc residues is completely oxidized, whereas GlcUA in combination with GlcN-SO_3 may be resistant. The periodate-resistant GlcUA residues were rendered susceptible to oxidation either by exchanging *N*-sulphate for *N*-acetyl or by scission of oxyheparan sulphate in alkaline medium.



1



2

Since the protection against periodate oxidation exerted by the presence of *N*-sulphate groups can be abolished by chain cleavage, cooperative inter-residue hydrogen-bonding may be involved. Further studies of the periodate oxidation of heparan sulphate are required in order to clarify these points. In particular, characterization of the oligosaccharides obtained after periodate oxidation–alkaline elimination of heparan sulphate ought to be worthwhile.

ACKNOWLEDGMENTS

The author thanks Drs. I. Carlstedt, L. Cöster, A. Malmström, M. Paulsson, and I. Sjöberg for many stimulating discussions, Professor U. Lindahl for valuable suggestions, and Mrs. Birgitta Havsmark for expert technical assistance. The work was supported by grants from the Swedish Medical Research Council (B76-13X-00139-12C), Gustaf V: s 80-årsfond, "Greta och Johan Kocks Stiftelser", Trelleborg, Sweden, and the Medical Faculty, University of Lund.

REFERENCES

1 U. Lindahl, *Int. Rev. Sci., Org. Chem. Ser. 2*, 7 (1976) 283–312.
2 J. A. Cifonelli, *Carbohydr. Res.*, 8 (1968) 233–242.
3 P. Hovingh and A. Linker, *Carbohydr. Res.*, 37 (1974) 181–192.
4 R. L. Taylor, J. E. Shively, H. E. Conrad, and J. A. Cifonelli, *Biochemistry*, 12 (1973) 3633–3637.
5 M. Höök, U. Lindahl, and P. H. Iverius, *Biochem. J.*, 137 (1974) 33–43.
6 L.-Å. Fransson and I. Carlstedt, *Carbohydr. Res.*, 36 (1974) 349–358.

7 L.-Å. FRANSSON AND L. RODEN, *J. Biol. Chem.*, 242 (1967) 4161–4169.
8 C. A. ANTONOPOULOS, S. GARDELL, AND B. HAMNSTRÖM, *J. Atheroscler. Res.*, 5 (1969) 9–15.
9 L. RODEN, J. BAKER, J. A. CIFONELLI, AND M. B. MATHEWS, *Methods Enzymol.*, 28 (1972) 73–140.
10 U. LINDAHL, J. A. CIFONELLI, B. LINDAHL, AND L. RODEN, *J. Biol. Chem.*, 240 (1965) 2817–2820.
11 M. HÖÖK, U. LINDAHL, AND P.-H. IVERIUS, *Biochem. J.*, 137 (1974) 33–43.
12 D. HEINEGARD, *Chem. Scripta*, 4 (1973) 199–201.
13 L.-Å. FRANSSON, L. RODEN, AND M. L. SPACH, *Anal. Biochem.*, 23 (1968) 317–330.
14 G. A. LEVVY AND A. MCALLAN, *Biochem. J.*, 73 (1959) 127–133.
15 Z. DISCHE, *J. Biol. Chem.*, 167 (1949) 189–198.
16 J. E. SHIVELY AND H. E. CONRAD, *Biochemistry*, 15 (1976) 3932–3942.
17 J. A. CIFONELLI, *Carbohydr. Res.*, 8 (1968) 233–242.
18 L.-Å. FRANSSON, *Carbohydr. Res.*, 36 (1974) 339–348.
19 I. A. NIEDUSZYNSKI AND E. D. T. ATKINS, *Proc. Symp. Colston Res. Soc.*, 26 (1975) 323–334.
20 H. F. ELLOWAY AND E. D. T. ATKINS, *Biochem. J.*, 161 (1977) 495–498.
21 J. E. SCOTT AND M. J. TIGWELL, *Biochem. Soc. Trans.*, 3 (1975) 662–664.