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ALKALINE AND SMITH DEGRADATION OF OXIDIZED DERMATAN SULPHATE–CHONDROITIN SULPHATE COPOLYMERS*

LARS-ÅKE FRANSSON AND INGEMAR CARLSTEDT

Department of Physiological Chemistry 2, Chemical Centre, University of Lund, S-220 07 Lund (Sweden)

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ABSTRACT

Unsubstituted L-iduronic acid residues in dermatan sulphate were selectively oxidized by periodate. The resulting oxydermatan sulphate was degraded by alkali to give a product mixture from which oxalacetic acid (35%) was isolated after hydrolysis and oxidation. Conditions were developed for the Smith degradation of dermatan sulphate, which gave a maximal yield of oligosaccharides combined with minimal destruction of periodate-resistant structures. Also formed was a product tentatively identified as L-threonic acid. The alkaline and Smith degradations gave similar patterns of oligosaccharide products.

INTRODUCTION

Previous reports (see ref. 1) from this laboratory have established that dermatan sulphate is a copolymer of the repeating disaccharide units D-GlcUA–GalNAc-4-SO_4 and L-IdUA–GalNAc-4-SO_4. Various enzymes have been employed in the elucidation of the carbohydrate sequence, notably testicular hyaluronidase and chondroitinase-AC, both of which cleave hexosaminidic bonds to D-glucuronic acid (D-GlcUA), and chondroitinase-ABC which, in addition, cleaves hexosaminidic bonds to L-iduronic acid (L-IdUA). No method which permits a selective cleavage of the L-IdUA–GalNAc-4-SO_4 (where GalNAc connotes 2-acetamido-2-deoxy-D-galactose) units has hitherto been available.

In the preceding paper[1], it was shown that treatment of dermatan sulphate and chondroitin sulphate with periodate at pH 3 exclusively oxidized L-IdUA residues. We now describe further degradations of the resulting oxydermatan sulphate.

EXPERIMENTAL

Materials. — The sources for chondroitin 4-sulphate, dermatan sulphate, and Sephadex were listed in the preceding paper[1]. Lactic dehydrogenase (EC 1.1.1.27)

*Periodate oxidation of dermatan sulphate and chondroitin sulphate: Part II. For Part I, see ref. 1.

(type II from rabbit muscle and) malic dehydrogenase (EC 1.1.1.37) (from pig heart) were obtained from Sigma Chemical. *N*-Acetylchondrosine [2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose, CNAc], 2-acetamido-2-deoxy-galactose sulphate (GalNAc-SO_4), and 2-acetamido-2-deoxy-3-*O*-(β-D-glucopyranosyluronic acid)-D-galactose sulphate (CNAc-SO_4) were the same preparations as described elsewhere[2]. D-*erythro*-Tetruronic acid was prepared by oxidation[3] of D-glucuronic acid with lead tetra-acetate. D-Erythruronic acid was converted into L-erythronic acid by reduction with potassium borohydride[3]. L-Threonic acid was prepared by oxidation of L-ascorbic acid[4] with permanganate–H_2SO_4.

Analytical methods. — Oxalacetic acid and pyruvic acid were assayed by following the change in absorbance at 340 nm upon incubation with malic dehydrogenase and lactic dehydrogenase, respectively[5,6]. Hexosamine was determined by the Elson–Morgan procedure[7], after hydrolysis with 6M HCl at 100° for 8 h and subsequent removal[8] of the acid in a vacuum desiccator over sodium hydroxide. Hexuronic acids were determined by the carbazole[9] and orcinol methods[10], using a boiling time of 20 min. U.v. spectra were recorded on a Beckman DK-2 spectrophotometer.

Separatory methods. — High-voltage paper electrophoresis was carried out on Whatman No. 3MM paper in 0.1M pyridine acetate (pH 5.0) at 40 volts/cm for 1 h. Detection was effected with aniline hydrogen phthalate. Glycosaminoglycan samples were analyzed by the cetylpyridinium chloride–cellulose micro-column procedure[8]. The columns were eluted with neutral solutions of magnesium chloride to yield information regarding molecular-size polydispersity[11]. The polydispersity of samples of chondroitin sulphate subjected to alkaline or acidic conditions was also assessed by gel chromatography on columns (0.9 × 142 cm) of Sephadex G-200, and elution with 0.2M pyridine acetate (pH 5.0) at 3.3 ml/h. Fractions were analyzed for uronic acid (see below).

G.l.c. was performed on a Varian Aerograph 1400 instrument equipped with a flame-ionization detector. Samples were hydrolyzed in sealed tubes with 2M trifluoroacetic acid at 100° for 4 h. The hydrolysates were concentrated at room temperature, the residues were trimethylsilylated[12], and subjected to g.l.c. on a column (0.2 × 300 cm) of 1% SE-30 on Gas-Chrom P (100–120 mesh) (Applied Sciences Lab. Inc., State College, Pennsylvania); carrier gas, nitrogen at 30 ml/min; temperature range, 60→160° at 4°/min.

Oxidation of aldehyde groups. — Samples were dissolved in a small amount of water and treated with 50 mol. of bromine in the presence of barium carbonate. After 1 h at room temperature, excess bromine was evaporated and the aqueous solution was analysed by paper electrophoresis. Some samples were hydrolyzed in sealed tubes with M trifluoroacetic acid at 100° for 1 h prior to oxidation. The acid was evaporated at room temperature.

Periodate oxidation. — Solutions of polysaccharides (2 mg/ml) in 20mM sodium metaperiodate—50mM sodium acetate (pH 5.0) or 50mM sodium citrate (pH 3.0) were incubated at 4° for 24 h in the dark. At these pH values, 95–98% of

the D-GlcUA residues resist periodate oxidation[1]. Reactions were terminated by the addition of 0.1 vol. of 10% aqueous D-mannitol, followed by dialysis against distilled water (2 × 3 l) and lyophilisation.

Smith degradation. — A solution of oxypolysaccharide (100 mg) in 10 ml of distilled water was treated with 200 mg of potassium borohydride for 3 h at room temperature. Excess borohydride was destroyed with glacial acetic acid. After dialysis against distilled water and lyophilisation, the oxidized–reduced polysaccharide was dissolved in 25 ml of 5mM H_2SO_4 and heated at 60° for 3 h. The reactions were terminated by the addition of M NaOH to pH 4–5.

Alkaline degradation of oxydermatan sulphate. — A solution of oxydermatan sulphate (5 mg/ml) was adjusted to pH 12 with M NaOH. After standing at room temperature for 30 min, the solution was neutralized with M acetic acid. Oxygen was not excluded during this alkaline treatment.

Fractionation of degradation products. — The products of Smith or alkaline degradation were eluted from columns (1.2 × 230 cm) of Sephadex G-50 (superfine) with 0.2M pyridine acetate (pH 5.0) at 12 ml/h. Fractions were analyzed for uronic acid by an automated version of the Bitter and Muir technique[13,14]. Reducing power was estimated by the method of Park and Johnson[15]. The material from the various fractions was isolated by lyophilisation and dried over phosphorus pentaoxide.

RESULTS AND DISCUSSION

Degradation of oxydermatan sulphate. Oxypolysaccharides may be cleaved after reduction by mild, acid hydrolysis[16] or directly by an alkali-catalyzed β-elimination reaction[17]. The former procedure (Smith degradation) is well known, but the mechanism of alkaline elimination is not fully understood, although little destruction of unoxidized residues in oxydermatan sulphate would be expected.

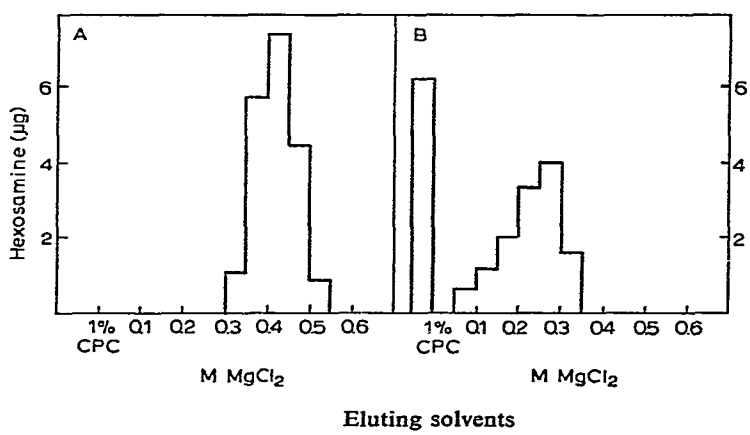


Fig. 1. Cetylpyridinium chloride–cellulose micro-column profiles of chondroitin 4-sulphate before treatment (A) and after treatment (4 mg/ml) (B) with 25mM H_2SO_4 at 80° for 1 h. The hydrolysate was neutralised and diluted to 1 mg/ml with distilled water prior to analysis.

In order to evaluate the effect of alkali or acid, chondroitin 4-sulphate was treated under a variety of conditions and the products were analyzed by the cetylpyridinium chloride–cellulose micro-column procedure. The molecular size of the polysaccharide was not appreciably altered by treatment with (*1*) 0.5M NaOH at room temperature for 48 h, (*2*) 0.25M H_2SO_4 at 25° for 8 h, (*3*) 50mM H_2SO_4 at 37° for 8 h, (*4*) 5mM H_2SO_4 at 60° for 3 h. However, extensive depolymerization occurred within 1 h on treatment with 25mM H_2SO_4 at 80° (Fig. 1). Thus, any of the conditions (*2*)–(*4*) are suitable for the Smith degradation.

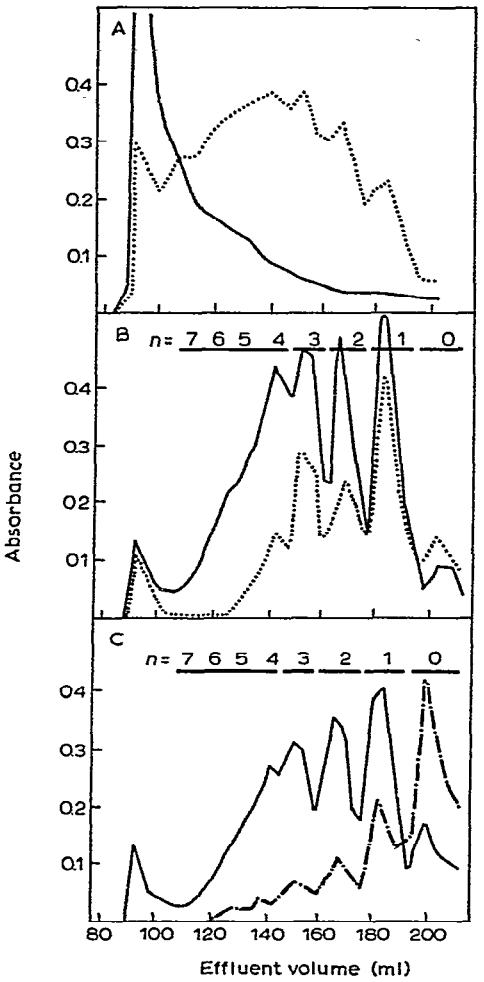
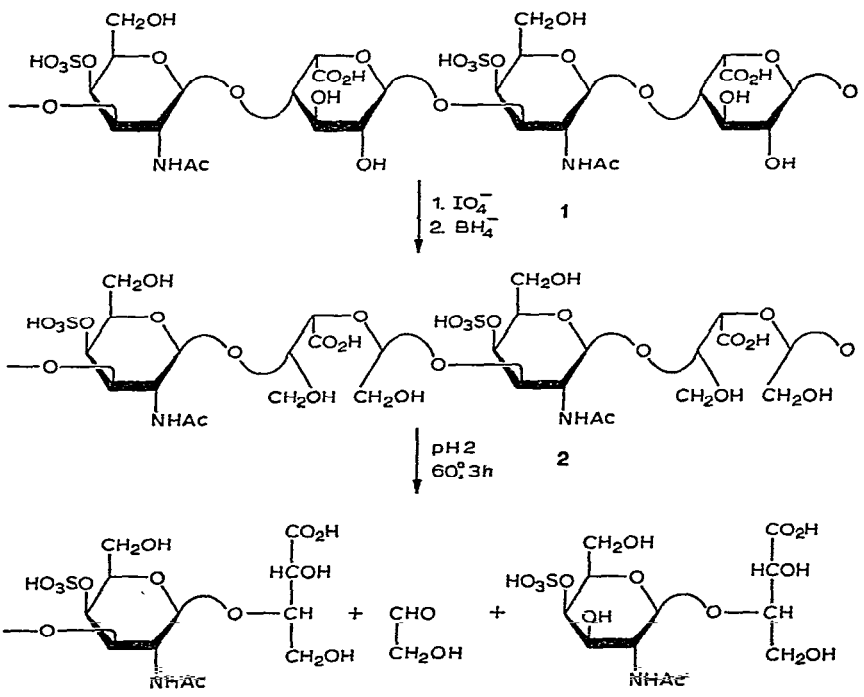


Fig. 2. Gel chromatography on Sephadex G-50 of oxydermatan sulphate (100 mg). (*1*) After reduction and treatment with sulphuric acid, and fraction analysis by the carbazole reaction: A, 0.25M, 25°, 8 h (———); 50mM, 37°, 8 h (– – –); B, 5mM, 60°, 3 h (———); 25mM, 80°, 1 h (– – –) (n = number of unoxidized uronic acid residues in the respective oligosaccharides). (*2*) After treatment with alkali (pH 12): carbazole colour (———, C), reducing power (–·–·–·–, C). Fractions were combined as indicated by the horizontal bars; $v_0 = 95$ ml.

Depolymerization by alkali or acid was also assessed by gel chromatography on Sephadex G-200. Treatment of chondroitin 4-sulphate with 10mM NaOH at room temperature for 30 min had no effect, and 5mM H_2SO_4 at 60° for 3 h. caused minor destruction.

Smith degradation of oxydermatan sulphate. Fig. 2A shows that treatment with relatively strong acid at moderate temperatures for 8 h was not sufficient to completely degrade oxidised–reduced dermatan sulphate. An optimal yield of oligosaccharide was obtained after hydrolysis in 5mM H_2SO_4 at 60° for 3 h (Fig. 2B, ———). More vigorous conditions gave a lower yield (B, — — —). Thus, treatment with acid as in Fig. 1B completely cleaves the acyclic acetals of oxidized–reduced dermatan sulphate, but the D-GlcUA-containing units of the same polymer are unaffected.

Alkali-catalyzed scission of oxydermatan sulphate. Exposure of oxydermatan sulphate to an alkali (pH 12) for 30 min, followed by gel chromatography, afforded the pattern shown in Fig. 2C. The carbazole-positive peaks (———) corresponded closely to those (Fig. 2B, ———) obtained after reduction–hydrolysis. Analyses of reducing power (Fig. 2C, — · — · —) revealed that the ratio of carbazole colour to reducing power increased with increasing chain-lengths. The most retarded components ($v_e = 200$ ml in Figs. 2B and C) both showed poor carbazole reactivity, suggesting the absence of uronic acid.



Characterization of the saccharides obtained after mild, acid hydrolysis of oxidized–reduced dermatan sulphate. The oligosaccharides resolved by gel chromatography (Fig. 2B, ———) should represent a homologous series with the carbohydrate sequence GalNAc–(UA–GalNAc)$_n$–R where UA is GlcUA or IdUA-SO_4, and R is the residue of an oxidized IdUA unit. When $n = 0$, the saccharide contains no uronic acid and the structure is GalNAc(–SO_4)–R (3). Compound 3 is derived (1→2→3) from sections of the polysaccharide composed of continuous L-IdUA–GalNAc-4-SO_4 repeating units. Thus, on Smith degradation[16], the L-iduronic acid

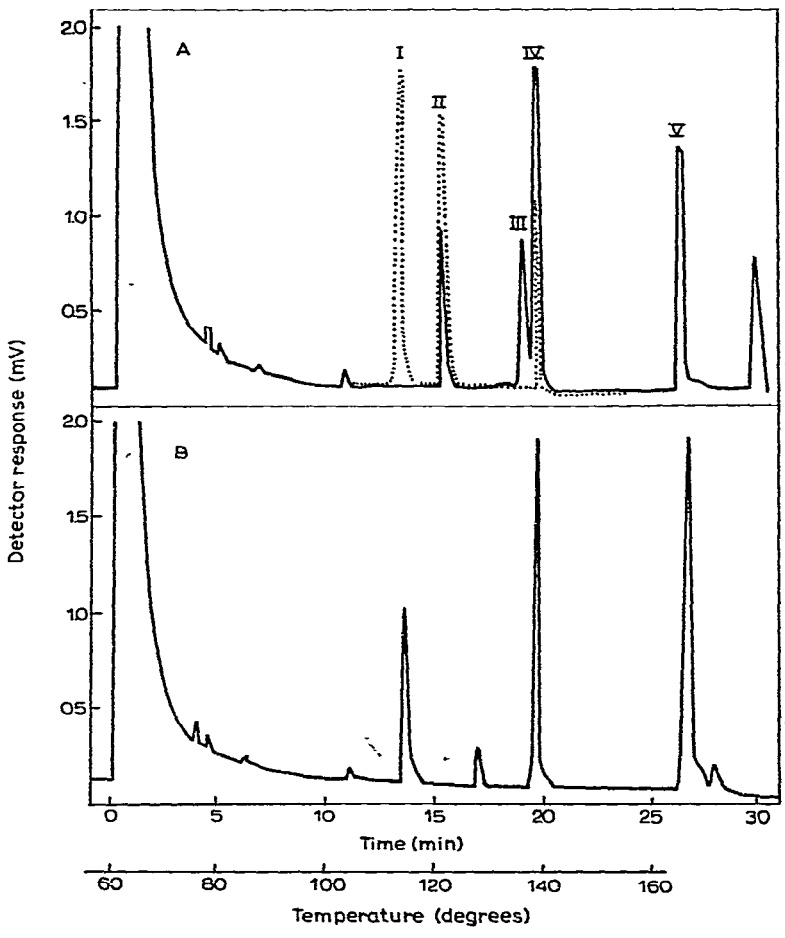
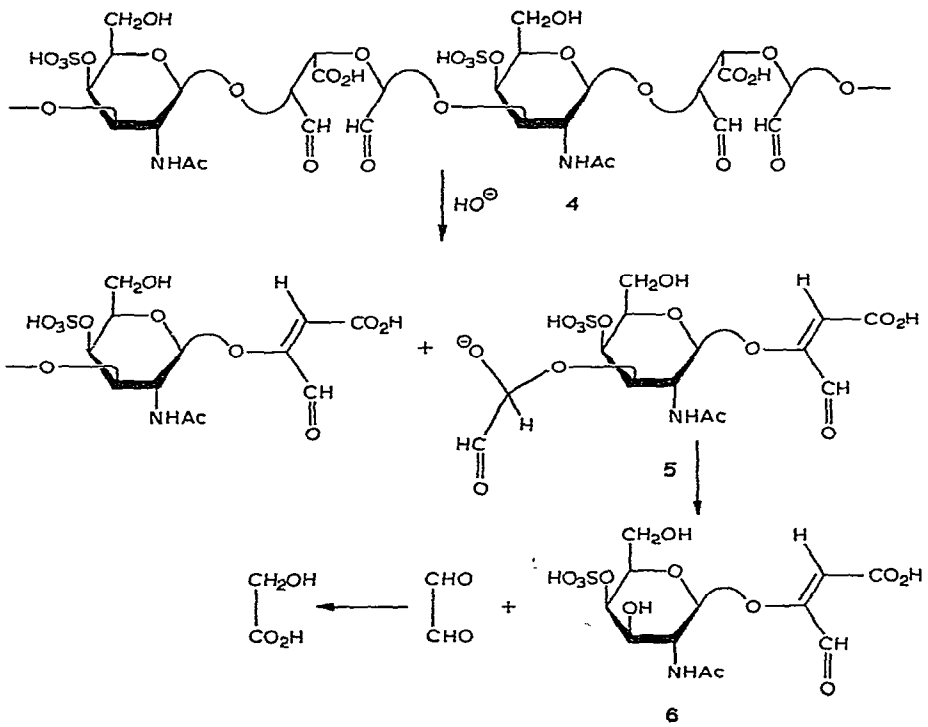


Fig. 3. G.l.c. of Me_3Si derivatives of certain sugars. A, Standard chromatogram comprising L-threono-1,4-lactone (I), L-erythrono-1,4-lactone (II), L-erythronic acid (III), L-threonic acid (IV), 2-amino-2-deoxy-D-galactose (V). The dashed line is for the L-tetronic acids after treatment with HCl (to promote lactonization). The L-erythronic acid standard invariably contained an additional component (T ~30 min) which disappeared after prolonged treatment with acid. This component may be either a polymerization product or an inadequately deformylated intermediate[3]. B, A hydrolyzate of the fraction $n = 0$ (Fig. 2B). The component with T ~17 min is unknown. The instrument was programmed for the temperature range 60–160° at 4°/min.

residues should be degraded to L-threonic acid. Hydrolysis of the fraction where $n = 0$ (Fig. 2B) yielded two compounds which were tentatively identified (g.l.c., Fig. 3) as 2-amino-2-deoxygalactose and threonic acid; D-glucuronic acid residues should yield L-erythronic acid. L-Threonic acid is lactonized rather poorly compared to L-erythronic acid.

Characterization of the oligosaccharides obtained after alkaline degradation of oxydermatan sulphate. Alkaline degradation of dialdehyde compounds obtained by periodate oxidation is thought to occur by a β-elimination reaction[18]. Thus, glycolic acid and 2,4-dihydroxybutyric acid are main products of the alkaline degradation of oxyxylan[17] and oxycellulose[19], respectively. Therefore, oxydermatan sulphate (**4**) should be degraded (**4**→**5**) by alkali into the series of oligosaccharides GalNAc-(UA–GalNAc)$_n$–R(CHO). Thus, if n = 0, **6** would be obtained.



As shown in Fig. 2C, alkaline degradation of oxydermatan sulphate afforded a series of oligosaccharides with $n = 0 \rightarrow 4$. Analyses of the various fractions are shown in Table I; two-thirds of the total hexosamine were recovered in the smallest component ($n = 0$), whereas the remainder was associated with periodate-insensitive oligosaccharides. The smallest component (**6**) contained hexosamine but no uronic acid, and displayed u.v. absorption (λ_{max} 225 nm; ε 12×10^4) indicative of an α,β-unsaturated aldehyde. After oxidation with bromine, the electrophoretic mobility was markedly increased (migration distance, 20 cm; *cf.* 18 for **6**) consistent with the con-

TABLE I

ANALYSES OF OLIGOSACCHARIDE FRACTIONS OBTAINED FROM DERMATAN SULPHATE AFTER PERIODATE OXIDATION AND ALKALINE DEGRADATION

Fraction (*n*)	*Yield*[a,b] (%)	*Absorbance at 225* nm[c]	*Hexosamine* (%)	*Uronic acid* (*carbazole*, %)	*Molar ratio of hexosamine/ uronic acid* (*carbazole*)	*Carbazole/ orcinol ratio*[d]
0	67 (68)	0.239	22.1	3.5	—	—
1	9 (10)	0.160	23.0	10.7	2.32	0.67
2	6 (6)	0.119	24.1	14.7	1.77	0.68
3	5 (6)	0.095	23.5	15.8	1.61	0.68
4–7	8 (7)	0.085	20.3	21.5	1.02	1.16

[a]Expressed as recovered hexosamine, as per cent of total hexosamine. [b]The corresponding yields of oligosaccharides obtained after Smith degradation are given in brackets. [c]Oligosaccharide concentration was 0.1 mg/ml; pH = 7.0. [d]For uronic acid determinations.

version CHO→COOH. When oxidation with bromine was preceded by hydrolysis, oxalacetic acid was formed [presumably *via* HO·C(CHO) = CH·COOH and CHO–CO–CH_2COOH], and identified by conversion into malic acid in the presence of malic dehydrogenase and reduced NAD (Table II). The oxidized hydrolysate of **6** consumed NADH corresponding to ~35% of oxalacetate. Furthermore, mild, acid hydrolysis of **6** produced GalNAc-SO_4 (paper electrophoresis). These findings confirm the structure of **6**.

TABLE II

ABSORBANCE CHANGE AT 340NM DURING INCUBATION WITH LACTIC DEHYDROGENASE (LDH) OR MALIC DEHYDROGENASE (MDH)

Sample	ΔA_{340} *with*	
	LDH	*MDH*
Pyruvic acid (114 mmoles)	0.439	
Oxalacetic acid (77 mmoles)		0.120
Unknown compound[a]	0.002	0.058

[a]Obtained after hydrolysis of **6** (100 nmoles) and oxidation of the product with bromine.

The periodate-insensitive oligosaccharides ($n = 1$–7) displayed (Table I) a decreasing absorbance at 225 nm with increasing chain-length. Moreover, their molar ratios of hexosamine to uronic acid (Dische carbazole method) were significantly higher than could be expected from oligosaccharides containing D-GlcUA as the sole hexuronic acid component. This finding, together with the low carbazole–orcinol ratios, indicated that an appreciable quantity of L-IdUA-containing oligosaccharides were present in these fractions. These L-IdUA residues most probably were sulphated at positions 2 or 3. The low colour-yield in the carbazole reaction might also be due to the presence of unsaturated moieties in these oligosaccharides. The oligosaccharides produced by the Smith degradation gave a higher absorbance in the carbazole reaction than those produced by alkaline elimination (*cf.* Figs. 2B and C, ———).

A complete characterization of D-GlcUA-containing, as well as L-IdUA-SO_4-containing, oligosaccharides will be reported elsewhere.

Thus, both Smith and alkaline degradation of periodate-oxidized dermatan sulphate may be employed for the isolation of oligosaccharides containing periodate-insensitive uronic acid residues, namely, D-GlcUA or L-IdUA-SO_4. The Smith degradation appears to be the most reliable procedure, but alkaline degradation may be useful for the production of oligosaccharides containing an aldehyde group, which could be utilised for attaching such oligosaccharides to matrices in affinity chromatography.

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REFERENCES

1 Part I, preceding paper: L.-Å. FRANSSON, *Carbohyd. Res.*, 36 (1974) 339.
2 L.-Å. FRANSSON AND B. HAVSMARK, *J. Biol. Chem.*, 245 (1970) 4770.
3 P. A. J. GORIN AND A. S. PERLIN, *Can. J. Chem.*, 34 (1956) 693.
4 R. W. HERBERT, E. L. HIRST, E. G. V. PERCIVAL, R. J. W. REYNOLDS, AND F. SMITH, *J. Chem. Soc.*, (1933) 1270.
5 R. W. VON KORFF, *Methods Enzymol.*, 13 (1969) 521.
6 J. R. WILLIAMSON AND B. E. CORKEY, *Methods Enzymol.*, 13 (1969) 468.
7 L. A. ELSON AND W. T. J. MORGAN, *Biochem. J.*, 27 (1933) 1824.
8 C. A. ANTONOPOULOS, S. GARDELL, J. A. SZIMAI, AND E. R. DE TYSSONSK, *Biochim. Biophys. Acta*, 83 (1964) 1.
9 Z. DISCHE, *J. Biol. Chem.*, 167 (1949) 189.
10 A. H. BROWN, *Arch. Biochem. Biophys.*, 11 (1946) 269.
11 L.-Å. FRANSSON, A. ANSETH, C. A. ANTONOPOULOS, AND S. GARDELL, *Carbohyd. Res.*, 15 (1970) 73.
12 C. C. SWEELEY, R. BENTLEY, M. MAKITA, AND W. W. WELLS, *J. Amer. Chem. Soc.*, 85 (1963) 2497.
13 T. BITTER AND H. MUIR, *Anal. Biochem.*, 4 (1962) 330.
14 D. HEINEGÅRD, *Chem. Scripta*, 4 (1973) 199.
15 J. T. PARK AND M. J. JOHNSON, *J. Biol. Chem.*, 181 (1949) 149.
16 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohyd. Chem.*, 5 (1965) 361.
17 R. L. WHISTLER, P. K. CHANG, AND G. N. RICHARDS, *J. Amer. Chem. Soc.*, 81 (1959) 4058.
18 D. O'MEARA AND G. N. RICHARDS, *J. Chem. Soc.*, (1958) 1204.
19 D. O'MEARA AND G. N. RICHARDS, *J. Chem. Soc.*, (1958) 4504.