The Copolymeric Structure of Pig Skin Dermatan Sulphate

ISOLATION AND CHARACTERIZATION OF L-IDURONO-SULPHATE-CONTAINING OLIGOSACCHARIDES FROM COPOLYMERIC CHAINS

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Dermatan sulphate was degraded by testicular hyaluronidase and an oversulphated fraction was isolated by ion-exchange chromatography. This preparation, which contained fairly long segments derived from the non-reducing terminal portion of the molecule, was subjected to periodate oxidation under acidic conditions. The oxidized iduronic acid residues were cleaved by reduction-hydrolysis (Smith-degradation) (Fransson & Carlstedt, 1974) or by alkaline elimination. The oligosaccharides so obtained contained both GlcUA (glucuronic acid) and IdUA-SO₄ (sulphated iduronic acid) residues. Copolymeric oligosaccharides obtained after alkaline elimination were cleaved by chondroitinase-AC into disaccharide and higher oligosaccharides. Since the corresponding oligosaccharides obtained by Smith-degradation were unaffected by this enzyme, it was concluded that the carbohydrate sequences were GalNAc-(IdUA-GalNAc)_n-GlcUA-GalNAc. The iduronic acid-containing sequences were resistant to digestion with chondroitinase-ABC. It was demonstrated that the presence of unsulphated *N*-acetylgalactosamine residues in these sequences could be responsible for the observed effect. This information was obtained in an indirect way. Chemically desulphated dermagan sulphate was found to be a poor substrate for the chondroitinase-ABC enzyme. Moreover, digestion with chondroitinase-ABC of chondroitinase-AC-degraded dermagan sulphate released periodate-resistant iduronic acid-containing oligosaccharides. It is concluded that copolymeric sequences of the following structure are present in pig skin dermagan sulphate:

N-acetylgalactosamine moieties surrounding IdUA-SO₄ residues are unsulphated to a large extent.

In previous reports degradations of dermagan sulphate-chondroitin sulphate copolymers have been described (Fransson, 1974; Fransson & Carlstedt, 1974). The degradation procedures were initiated by periodate oxidation of unsubstituted IdUA* residues. In the preceding paper the GlcUA-containing regions of the copolymeric chains were isolated in oligosaccharide form (Fransson *et al.*, 1974). Simultaneously, oligosaccharides containing IdUA-SO₄ residues were obtained. The present paper is concerned with isolation and characterization of the latter oligosaccharides.

* Abbreviations: IdUA, L-iduronic acid; IdUA-SO₄, sulphated L-iduronic acid; GalNAcOH, *N*-acetylgalactosaminol; ΔUA, 4,5-unsaturated hexuronic acid residue; GlcUA, glucuronic acid; UA, uronic acid.

Experimental

Materials

Chondroitinases, chondro-sulphatases and carbohydrate standards were the same preparations as described in the preceding paper (Fransson *et al.*, 1974). Monosulphated trisaccharide was prepared by partial chondro-sulphatase digestion of a disulphated trisaccharide, type GalNAc-(SO₄)-GlcUA-GalNAc-SO₄ (Yamagata *et al.*, 1968).

Dermagan sulphate was obtained from pig skin after digestion with papain (preparation PS-25; Fransson, 1968a). Hyaluronidase-degraded dermagan sulphate was prepared as described previously (Fransson, 1968a). The latter material was further

fractionated by chromatography on AG-1X2 (Malmström & Fransson, 1971). Chondroitinase-AC-degraded dermatan sulphate was recovered as a 50% (v/v) ethanol precipitate after treatment with the enzyme (Malmström & Fransson, 1971). Chemically desulphated chondroitin sulphate and dermatan sulphate were prepared by methanolysis (Kantor & Schubert, 1957).

Analytical methods

The procedures employed for the determination of hexosamine, hexuronic acid, sulphate and reducing sugar have been described in preceding papers (Fransson & Carlstedt, 1974; Fransson *et al.*, 1974; Malmström & Fransson, 1971). Amino acids were determined with the use of a Bio-Cal automatic amino acid analyser after hydrolysis in 6M-HCl at 100°C for 20h. Chromatographic separation of hexuronic acids after acid hydrolysis was performed as described elsewhere (Fransson *et al.*, 1968). The same method was also used for the determination of GlcUA released by β -glucuronidase (Fransson & Rodén, 1967). Determination of viscosity-average molecular weight (M_v) was carried out as described previously (Fransson & Rodén, 1967). Assay for chondroitinase activity was by Yamagata *et al.* (1968).

Degradative and separatory methods

Dermatan sulphate fractions were oxidized by periodate at pH 3.0 and 4°C for 24h (Fransson, 1974). Oxydermatan sulphate was cleaved by reduction-

hydrolysis (Smith-degradation) or by alkaline elimination (Fransson & Carlstedt, 1974). The split products were resolved by gel chromatography on Sephadex G-50 (for further details see the legend to Fig 2). The various oligosaccharides so obtained were further degraded by chondroitinases (AC or ABC) followed by chromatography on Sephadex G-25. Final resolution of mono-, di- and trisaccharide fragments was achieved by high-voltage paper electrophoresis (Fransson *et al.*, 1974).

Results

Oversulphated dermatan sulphate fragments

Dermatan sulphate-chondroitin sulphate copolymers are degraded by testicular hyaluronidase into a number of fragments (Fransson & Rodén, 1967). In the case of pig skin dermatan sulphate approximately two-thirds of the material are obtained as high-molecular-weight products (II and IV in Scheme 1), whereas the remainder is represented by low-molecular-weight oligosaccharides (III in Scheme 1). The former material is heterogeneous with respect to size as well as charge density (Fig. 1; see also Fransson & Rodén, 1967; Malmström & Fransson, 1971). Undegraded dermatan sulphate appeared rather homogeneous (Fig. 1a), whereas hyaluronidase-degraded material displayed a considerable charge polydispersity (Fig. 1b). Analyses of the most retarded and oversulphated fraction (2.0M-NaCl) are shown in Table 1. The data indicated that in this particular fraction the

Scheme 1. Hyaluronidase degradation of copolymeric dermatan sulphate

I, Portion of a copolymeric chain; II and IV represent polymeric fragments derived from the non-reducing terminal portion or various internal portions, respectively. III, Chondroitin sulphate oligosaccharides derived from glucuronic acid-containing sections of the copolymer. It should be noted that digestion with hyaluronidase of chondroitin sulphate produces predominantly tetrasaccharides. Therefore fragments obtained after digestion of copolymeric dermatan sulphate occasionally contain a GlcUA-GalNAc-SO₄ period in the reducing end (see II). This repeating unit may be released by chondroitinase-AC (Fransson & Malmström, 1971).

Fig. 1. Ion-exchange chromatography of (a) dermatan sulphate and (b) hyaluronidase-degraded dermatan sulphate

Hyaluronidase-degraded material (5g) was subjected to chromatography on a column (4cm×50cm) of AG-1X2. The column was eluted stepwise with increasing concentrations of NaCl. Material was recovered by dialysis and freeze-drying. Yield is expressed as % of total material. The values associated with the bars represent sulphate/hexosamine molar ratios.

Table 1. Analyses of an oversulphated dermatan sulphate fraction

The material analysed corresponds to the fraction eluted with 2.0M-NaCl in Fig. 1(b).

Components	
Hexosamine (%)	24.7
Uronic acid (carbazole) (%)	14.7
Carbazole/orcinol molar ratio	0.29
Sulphate/hexosamine molar ratio	1.4
IdUA/GlcUA molar ratio	20:1
Non-reducing GlcUA*	0.05
Molecular weight (M_v)	15000
GalN/Ser molar ratio	75:1

* GlcUA liberated by β -glucuronidase; expressed as mol/mol of polysaccharide.

average number of disaccharide periods/chain was approx. 30 (disaccharide weight of approx. 500). Since the galactosamine/serine ratio was 75 it may be calculated that 2 out of 5 chains contained the linkage region to protein. Thus, the latter chains had not been cleaved by testicular hyaluronidase. By assuming that hyaluronidase-resistant chains have the same molecular-size distribution as the

entire starting material (M_v , 25000; number of disaccharide periods approx. 50), it may be concluded that the degraded chains (3 out of 5) had an average chain length of approx. 20 disaccharides. Moreover, all fragments were essentially devoid of non-reducing terminal GlcUA demonstrating that the released fragments constituted non-reducing terminal portions of the original chains. Finally, the carbazole/orcinol ratio and the hexuronic acid analysis indicated that the average number of GlcUA-containing periods was approx. 1/chain.

The above considerations serve to illustrate that oversulphated, polymeric fragments obtained after hyaluronidase-degradation of dermatan sulphate contain fairly large, distal segments of the copolymeric chain containing only a few residual GlcUA-GalNAc-SO₄ units. Frequently, the latter units are located in the reducing end of the fragment (see II in Scheme 1; see also Malmström & Fransson, 1971).

Degradation of oversulphated dermatan sulphate fragments

Hyaluronidase-degraded, oversulphated dermatan sulphate (2.0M-NaCl fraction) was oxidized by periodate and further degraded by two different procedures (reactions A and B in Scheme 2). The oligosaccharides so produced were chromatographed on Sephadex G-50. The oligosaccharide pattern obtained after Smith-degradation (reaction A) is shown in Fig. 2. (The material obtained by reaction B gave a similar pattern.) It is seen that the various uronic acid-containing fragments were resolved into, at least, four reasonably separated fractions varying in size from tetrasaccharide ($n=1$) to decasaccharide and larger ($n=4$). Analytical data for the various fractions are shown in Table 2. More than 40% of the starting material was recovered in oligosaccharide form, the deca- and hexa-saccharides being the most prominent ones. The sulphate/hexosamine molar ratios increased with increasing chain length. The smallest fragment ($n=1$) was significantly undersulphated, whereas all of the larger fragments were oversulphated.

Chondroitinase-AC digestion of periodate-resistant oligosaccharides

To determine the uronic acid composition of the periodate-resistant oligosaccharides, degradations with chondroitinase-AC were performed. Oligosaccharides produced via reactions A and B (Scheme 2) were separately treated with this enzyme. Before the results of these experiments are described some theoretical considerations should be expounded. Oligosaccharide fragments released from the reducing terminal portion in the two procedures (Scheme 2, VIII and XI) differ with respect to the character of the

Scheme 2. Scheme for the degradation of oversulphated dermatan sulphate

Both degradation procedures are initiated by periodate oxidation of unsubstituted IdUA residues in the oversulphated material (V). In reaction A aldehydes are reduced to primary alcohols and subsequent hydrolysis liberates oligosaccharide fragments VI, VII and VIII. In reaction B direct alkaline treatment produces oligosaccharides IX, X and XI. Oligosaccharides originating from internal segments contain a 4-carbon residue in the reducing end [an alcohol after reaction A, $\text{R}(\text{CH}_2\text{OH})$, or an aldehyde, $\text{R}(\text{CHO})$, after reaction B]. However, oligosaccharides released from external segments, i.e. the reducing terminal end of the chain, differ in the two procedures. Reaction A produces oligosaccharide VIII with a terminal *N*-acetyl-galactosaminitol residue (GalNAcOH), whereas in reaction B the corresponding oligosaccharide (XI) retains its GalNAc residue. It should be added that any internal, single GlcUA-GalNAc periods should be recovered in oligosaccharides of types VI and IX.

reducing terminal sugar moiety. In reaction A the oligosaccharide terminates with a GalNAcOH moiety, whereas in reaction B the analogous oligosaccharide terminates with GalNAc . In a previous study it was shown that chondroitinase digestion of a heptasaccharide containing a terminal hexosaminitol moiety afforded tetra-, di- and monosaccharide (see Fig. 5 in Fransson, 1968b). Since the tetrasaccharide contained the hexosaminitol group, it was concluded that terminal UA-GalNAcOH periods are not released by this enzyme. Consequently oligosaccharide VIII (Scheme 2) should be resistant to degradation by chondroitinase-AC. Thus, degradation with chondroitinase-AC of the various oligosaccharides produced via reactions A and B, respectively, would be expected to yield different split products. Fig. 3 shows that distinctly different degradation patterns were indeed

observed. Oligosaccharides produced via reaction A yielded monosaccharide, trisaccharide and larger oligosaccharides, whereas the B-oligosaccharides yielded, also, significant proportions of disaccharide (peaks b in Figs. 3e-3h). Further differences could be observed when the chromatograms of corresponding oligosaccharides were compared. Note that each oligosaccharide fraction should contain two types of fragments with the carbohydrate sequences, $\text{GalNAc}-(\text{UA-GalNAc})_n-\text{R}$ and $\text{GalNAc}-(\text{UA-GalNAc})_n$ (see also Scheme 2).

Oligosaccharides containing one uronosyl residue (sequences $\text{GalNAc-UA-GalNAc-R}$ and GalNAc-UA-GalNAc) produced primarily trisaccharide or higher after Smith-degradation and digestion with chondroitinase-AC (Fig. 3a, $n=1$). The larger component (Fig. 3a, d peak) had an electrophoretic mobility intermediary to those of monosulphated

Fig. 2. Gel chromatography on Sephadex G-50

Hyaluronidase-degraded, oversulphated dermatan sulphate (200mg) was subjected to degradation by reaction A in Scheme 2 (Fransson & Carlstedt, 1974). The degradation products were resolved by chromatography on a column (2.2cm $\times$ 230cm) of Sephadex G-50 (superfine) eluted with 0.2M-pyridine acetate, pH 5.0, at a rate of 25 ml/h. Effluents were assayed for uronic acid and fractions were pooled as indicated by vertical dotted lines and freeze-dried. n , Number of repeating disaccharide units in the saccharides. $n = 0$ represents the fragment GalNAc(-SO₄)-R. —, Carbazole reaction.

Table 2. Analyses of periodate-insensitive oligosaccharides

n , Number of repeating disaccharide units in the respective oligosaccharides. Yields are expressed as recovered hexosamine in percent of the hexosamine content of the starting material. The sulphate/hexosamine ratios are not corrected for loss of hexosamine in the hydrolysis procedure. Values of 1.1–1.2 are considered to be close to true equimolarity.

Oligosaccharide	Yield (%)	Sulphate/hexosamine (molar ratio)
$n = 1$	5	0.8
$n = 2$	13	1.2
$n = 3$	8	1.3
$n = 4-7$	16	1.5

tri- and di-saccharides, respectively (Fig. 4; $n = 1$; A-d). The trisaccharide peak (Fig. 3a, c peak), which was the most prominent one, contained two components, a major one with the mobility of a disulphated trisaccharide, and a minor one with the mobility of Δ UA-GalNAc(-SO₄)-R (Fig. 4; $n = 1$; A-c). Only small amounts of monosaccharide (a) were obtained. In contrast, when the corresponding oligosaccharide obtained after periodate oxidation and alkaline elimination (reaction B) was subjected to digestion with chondroitinase-AC

di- and mono-saccharides were the dominating products (Fig. 3e, $n = 1$). These components were electrophoretically identified as monosulphated disaccharide (b) and sulphated *N*-acetyl galactosamine (a), respectively. The higher oligosaccharide fraction (c) comprised both Δ UA-GalNAc(-SO₄)-R and a slow-moving component (Fig. 4; $n = 1$; B-c) similar to that obtained in the previous experiment (A-d). These data indicate that the oligosaccharide fraction (with $n = 1$) contained primarily a trisaccharide with the carbohydrate sequence GalNAc-GlcUA-GalNAcOH (reaction A) or GalNAc-GlcUA-GalNAc (reaction B) (see also compounds VIII and XI in Scheme 2). The reduced trisaccharide was resistant to chondroitinase-AC, whereas the unreduced trisaccharide was split into monosulphated disaccharide and *N*-acetylgalactosamine-sulphate. Thus, both GalNAc residues in the trisaccharide were sulphated. Also, small amounts of Δ UA-GalNAc(-SO₄)-R were obtained in both experiments (see A-c and B-c in Fig 4, $n = 1$). This compound was derived from an oligosaccharide with the sequence GalNAc-GlcUA-GalNAc-R. The non-reducing terminal GalNAc residue of this compound was recovered in fraction a in Fig. 3(a) ($n = 1$). It was identified as GalNAc-S by electrophoresis indicating that both GalNAc residues of this oligosaccharide were sulphated. The nature of the slow-moving component observed in both experiments (A-d and B-c in Fig. 4, $n = 1$) is uncertain. However, judging from the electrophoretic mobility the following carbohydrate sequence is tentatively proposed: GalNAc-IdUA-GalNAc-R. The saccharide should be substituted with one sulphate group, most likely, on the IdUA residue. It is believed that this oligosaccharide attributes to the low sulphate/hexosamine molar ratio observed for this fraction (Table 2; $n = 1$).

As shown in Fig. 3(b) digestion with chondroitinase-AC of the next higher oligosaccharide fraction ($n = 2$) obtained by Smith-degradation afforded trisaccharide (c) and penta- or hexasaccharide (d). No monosaccharide was obtained. Electrophoretic analyses of these saccharides (Fig. 4, $n = 2$) indicated that the trisaccharide (A-c) was composed of disulphated trisaccharide and Δ UA-GalNAc(-SO₄)-R, and thus was similar to the trisaccharide obtained in the previous experiment (c in Fig. 5a, $n = 1$). However, the disulphated trisaccharide obtained in the present case must contain IdUA, since continuous GlcUA-GalNAc-SO₄ periods would not survive digestion with hyaluronidase (Fransson & Rodén, 1967). When the corresponding oligosaccharide produced by periodate oxidation-alkaline elimination (reaction B) was degraded by chondroitinase-AC (Fig. 5f, $n = 2$) a significant disaccharide peak (b) was obtained, as well as a heterogeneous fraction of tri- and higher oligo-saccharides (c). The former component, which

Fig. 3. Gel chromatography on Sephadex G-25

Various periodate-resistant oligosaccharides (n varying between 1 and 4) were prepared by reaction A (a-d) or reaction B (e-h). After digestion with chondroitinase-AC (see the text) the material (5–25 mg) was subjected to gel chromatography on columns (1.2 cm $\times$ 230 cm) of Sephadex G-25 eluted with 0.2 M-pyridine acetate, pH 5.0, at a rate of 10 ml/h. —, Carbazole reaction; ----, reducing power; v_0 = 80 ml. Fractions were pooled as indicated by horizontal bars. n = 1, 2, 3 and 4 for (a)–(d) and (e)–(h) respectively.

Fig. 4. Paper electrophoresis

Various saccharides were obtained by digestion with chondroitinase-AC of fragments produced by Smith-degradation (A) or periodate oxidation-alkaline elimination (B). Standards are: Di-OS, Di-monoS and Di-diS, unsulphated, monosulphated and disulphated disaccharides respectively; Tri-monoS and Tri-diS, mono- and di-sulphated trisaccharide (type GalNAc-GlcUA-GalNAc), respectively, Compound C, Δ UA-GalNAc(SO_4)-R (Fransson *et al.*, 1974).

was electrophoretically indistinguishable from monosulphated disaccharide, must be derived from a pentasaccharide with the carbohydrate sequence GalNAc-IdUA-GalNAc-GlcUA-GalNAc (in reaction A the corresponding pentasaccharide contained a terminal alditol residue and was thus resistant to chondroitinase-AC). On electrophoretic analysis fraction c (tri- and higher oligo-saccharides) displayed a considerable heterogeneity (Fig. 4; $n=2$: B-c). It should be noted that the most highly charged component corresponded to disulphated trisaccharide. When the corresponding material obtained after Smith-degradation was analysed ($n=2$; A-d) no disulphated trisaccharide was observed. Instead a component with slightly higher mobility was found. It may therefore be tentatively concluded that the copolymeric pentasaccharide contained three sulphate groups, one of which would be located on the IdUA residue. It should be added that a significant proportion of the pentasaccharides must have the sequence GalNAc-IdUA-GalNAc-IdUA-GalNAc (with or without a reducing terminal 4-carbon fragment=R). Judging from the electrophoretic

mobilities only two sulphate groups should be present, most likely on the two IdUA residues.

Oligosaccharides containing three uronosyl residues were obtained both by reaction A and reaction B. As shown in Fig. 5(c) ($n=3$) the former oligosaccharides were degraded by chondroitinase-AC into trisaccharide (c) and higher oligosaccharides (d). The trisaccharide fraction contained only Δ UA-GalNAc(SO_4)-R (compound c) (Fig. 4; $n=3$; A-c). In contrast oligosaccharides obtained via reaction B produced both disaccharide (b) and trisaccharide (c) on digestion with chondroitinase-AC (Fig. 4; $n=3$; B-c). These data are consistent with the presence of two types of copolymeric oligosaccharides, one terminating with GalNAc and the other terminating with a C_4 residue (R). In both structures GlcUA-GalNAc periods are located in the reducing portion. The proposed sequences are: GalNAc-(IdUA-GalNAc) $_2$ -GlcUA-GalNAc-R (R is thus either a C_4 residue or H). Since the IdUA residues have survived periodate oxidation they are probably substituted with sulphate.

Fig. 5. Gel chromatography on Sephadex G-25

Oligosaccharide fractions $n=3$ produced by Smith-degradation (a) or periodate oxidation-alkaline elimination (b) were degraded by chondroitinase-AC followed by re-isolation of resistant material as shown in Fig. 3. The resistant material was subsequently digested with chondroitinase-ABC. —, Carbazole reaction; ----, reducing power. Fractions were pooled as indicated.

After digestion with chondroitinase-AC of the largest oligosaccharide fraction ($n=4$) obtained via reaction A (Fig. 3d) trisaccharide (c) and an excluded oligosaccharide peak (d) were obtained. The former component was identified as Δ UA-GalNAc(-SO₄)-R (Fig. 4; $n=4$; A-c), whereas the latter peak was heterogeneous (A-d). The analogous oligosaccharide obtained via reaction B yielded the same components plus a disaccharide (b in Fig. 3h; $n=4$). These findings indicate the presence of another pair of copolymeric oligosaccharides containing an additional IdUA-GalNAc unit. The proposed sequences are: GalNAc-(IdUA-GalNAc)₃-GlcUA-GalNAc-R/H. The uronic acid composition of the chondroitinase-AC-resistant material was confirmed by hydrolysis and ion-exchange chromatography. Analysis of fraction d in Fig. 3(h) ($n=4$) gave 99% IdUA of total hexuronic acid.

Fig. 6. Assay of chondroitinase-ABC with various substrates at pH 6.5

○, Chondroitin sulphate; ●, chemically desulphated chondroitin sulphate; △, dermatan sulphate; ▲, chemically desulphated dermatan sulphate. Incubations were performed as described by Yamagata *et al.* (1968).

Digestion with chondroitinase-ABC of periodate-resistant oligosaccharides

Detailed study of the IdUA-containing oligosaccharides was precluded by their resistance to chondroitinase-ABC. The various IdUA-containing oligosaccharides were only partially degraded by chondroitinase-ABC; one example is shown in Fig. 5. Oligosaccharides obtained via reaction A and chondroitinase-AC digestion (Fig. 5a) were completely resistant, whereas those produced by reaction B were partially degraded (Fig. 5b). The disaccharide fraction obtained in the latter case (d-1) contained both mono- and di-sulphated species (Fig. 4; $n=3$; B-d-1). In view of the fact that the reduced oligosaccharide was completely resistant it seems reasonable to conclude that the released disaccharides (B-d-1) had occupied a reducing terminal position. These results also indicate that a significant amount of oligosaccharides containing exclusively IdUA-GalNAc periods were present in this material. These sequences would be: GalNAc-(IdUA-GalNAc)_n. Information concerning the sulphate substituents of these sequences was obtained in an indirect way.

The largest, periodate-resistant oligosaccharides were significantly oversulphated (Table 2). However, with one sulphate substituent on every IdUA residue, a fair proportion of the GalNAc moieties

Fig. 7. Gel chromatography on Sephadex G-50 of chondroitinase-ABC digests of intact dermatan sulphate (a) and chondroitinase-AC-degraded dermatan sulphate (b)

Dermatan sulphate was preparation PS-25. Chondroitinase-AC-degraded dermatan sulphate was recovered by ethanol precipitation of the digest. Both preparations were treated with chondroitinase-ABC and subjected to gel chromatography as in Fig. 3. —, Carbazole reaction. Fractions were pooled as indicated by horizontal bars.

Fig. 8. Paper electrophoresis of various fractions obtained by chondroitinase-ABC digestion of intact dermatan sulphate (A series) or chondroitinase-AC-degraded material (B series)

Standards were the same as in Fig. 4.

should be unsulphated. (The highest sulphate/hexosamine molar ratio observed was 1.5.) To test the possibility that sequences containing unsulphated GalNAc moieties were poorly degraded by chondroitinase-ABC, this enzyme was assayed with polysaccharide substrates of varying degree of sulphation (Fig. 6). It is seen that in comparison with chondroitin sulphate, dermatan sulphate and desulphated chondroitin sulphate, dermatan (desulphated dermatan sulphate) was a very poor substrate. This confirms the above suggestion that polysaccharide segments containing unsulphated GalNAc moieties and IdUA with or without sulphate are poorly degraded by chondroitinase-ABC.

Oligosaccharides containing GalNAc and IdUA-SO₄ are usually not found in chondroitinase-ABC digests of intact dermatan sulphate (Yamagata *et al.*, 1968; Fransson, 1968b; Malmström & Fransson, 1971). However, in the present experiments low-molecular-weight fragments of dermatan sulphate containing these units were subjected to digestion.

Therefore, the effect of chondroitinase-ABC on intact dermatan sulphate and on partially degraded dermatan sulphate were compared. Chondroitinase-ABC degradation of intact dermatan sulphate produced a large quantity of disaccharide (Fig. 7a, peak 1) and a broad peak of material with a higher molecular weight (Fig. 7a, peak 2). When chondroitinase-AC-degraded dermatan sulphate was further degraded with chondroitinase-ABC (Fig. 7b) the yield of high-molecular-weight material was increased, and discrete peaks could be discerned (Fig. 7b, peaks 2–5). The various fractions obtained in the two experiments were examined by paper electrophoresis (Fig. 8). The disaccharide obtained from intact dermatan sulphate (A-1) contained mono- as well as di-sulphated species. The high-molecular-weight material (A-2) had a somewhat lower mobility, similar to that of linkage-region glycopeptides

(Fransson, 1968a; Fransson & Rodén, 1967). The latter material was absent in the chondroitinase-AC-degraded dermatan sulphate, owing to the fact that the linkage region is released in glycopeptide form by this enzyme (Malmström & Fransson, 1971). Instead, a series of oligosaccharides eluted in positions of tetra-, hexa-, octa- and deca-saccharides (Fig. 7b, peaks 2–5) were obtained. These oligosaccharides exhibited electrophoretic heterogeneity (Fig. 8); their mobilities were intermediary to those of mono- and di-sulphated disaccharides. Further, the disaccharide fraction obtained in this experiment (B-1) contained no disulphated component suggesting that IdUA-SO₄ residues were present in the chondroitinase-ABC-resistant oligosaccharides. This was confirmed by the finding that all of these oligosaccharides were resistant to periodate oxidation. Finally, analyses of their uronic acid composition proved IdUA to be their only hexuronic acid component. Similar results were obtained when hyaluronidase-degraded dermatan sulphate was digested with chondroitinase-ABC (L.-Å. Fransson, unpublished work).

Discussion

Oversulphated dermatan sulphate fragments were degraded by periodate oxidation in combination with reduction-hydrolysis (reaction A) or alkaline elimination (reaction B). Oligosaccharides so obtained were further degraded by chondroitinase-AC and -ABC. When the various fragments were closely examined it was found that, in addition to oligosaccharides containing only IdUA-GalNAc periods, copolymeric fragments were also present among the degradation products. The latter oligosaccharides had the following carbohydrate sequence: GalNAc-(IdUA-GalNAc)_n-GlcUA-GalNAc-R/H. (*n* varied between 1 and 3; the reducing terminal unit was either a C₄ residue, R, or a GalNAc residue.) Non-copolymeric fragments frequently terminated with GalNAc; their general sequence was GalNAc-(IdUA-GalNAc)_n with *n* varying between 2 and 4. The shortest IdUA-containing fragment had the structure GalNAc-IdUA-GalNAc-R.

Attempts to define the position of sulphate groups was hindered by the strong resistance towards chondroitinase-ABC displayed by these oligosaccharides. The sulphate/hexosamine molar ratios suggested that GalNAc residues were unsulphated. Evidence for this notion was obtained in an indirect way. Chemically desulphated dermatan sulphate was found to be an extremely poor substrate for chondroitinase-ABC, whereas desulphated chondroitin sulphate was not. This finding strongly suggested that sulphation of GalNAc was necessary for cleavage of GalNAc-IdUA bonds by this

enzyme. Moreover, the chain length of the substrate was apparently of importance. When intact dermatan sulphate was exhaustively digested with chondroitinase-ABC, mono- and di-sulphated disaccharide and linkage-region glycopeptide were the only products. In contrast, when chondroitinase-AC-degraded dermatan sulphate was further degraded by the ABC enzyme, IdUA-SO₄-containing oligosaccharides were released. The electrophoretic mobilities of these oligosaccharides suggest that a large proportion of their GalNAc residues are unsulphated. These findings agree with previous results obtained with Hunter dermatan sulphate (Sjöberg *et al.*, 1973). In this disease dermatan sulphate accumulates due to a deficiency of L-iduronosulphatase. Accordingly, the non-reducing terminal sugar moiety of the deposited polysaccharide was largely IdUA-SO₄. However, like the oligosaccharides studied in the present work the adjacent GalNAc residue was frequently unsulphated.

The present results also indicate that the two chondroitinases (AC and ABC) must be employed with some caution. The two enzymes are often used to determine the ratio between GlcUA and IdUA in copolymeric galactosaminoglycans. However, if the two enzymes are used in sequence, false ratios may be obtained.

Definitive statements concerning the role of IdUA-SO₄ units in dermatan sulphate will have to await further study. However, the existence of copolymeric sequences like GalNAc-(IdUA-GalNAc)_n-(GlcUA-GalNAc)_m raises the possibility that sulphation of IdUA is in some way involved in C-5 inversion of polymer-bound GlcUA to IdUA during biosynthesis of dermatan sulphate (Lindhahl *et al.*, 1972; A. Malmström, L.-Å. Fransson, M. Höök & U. Lindahl, unpublished work). Sulphation of IdUA could promote C-5 inversion by withdrawing the product from an unfavourable equilibrium (Lindhahl *et al.*, 1972). It is possible that IdUA-SO₄ residues thereby should be enriched in the vicinity of GlcUA-containing regions. Alternatively, the GlcUA- and IdUA-SO₄-containing sequences may have been produced by 'reverse' epimerase (Fransson *et al.*, 1973). This activity which was found in fibroblast secretions transforms polymer-bound IdUA into GlcUA in dermatan sulphate. Since GlcUA-SO₄ has never been found in this molecule, it is conceivable that IdUA-SO₄ would not be converted by 'reverse' epimerase. The copolymeric structures observed may thus have been formed as the epimerase reached a termination point.

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