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The Co-polymeric Structure of Pig Skin Dermatan Sulphate

DISTRIBUTION OF L-IDURONIC ACID SULPHATE RESIDUES IN CO-POLYMERIC CHAINS

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1. Pig skin dermatan sulphate was degraded by periodate oxidation followed by alkaline elimination or by chondroitinase-ABC to quantify irregular repeating units, i.e. those containing D-GlcUA (D-glucuronic acid) and L-IdUA-SO_4 (sulphated iduronic acid). 2. Previous results of periodate oxidation (Fransson, 1974) indicated repeating sequences in pig skin dermatan sulphate containing, on average, 3 D-GlcUA, 9 L-IdUA-SO_4 or 28 L-IdUA units in addition to *N*-acetylgalactosamine sulphate. However, complete digestion with chondroitinase-ABC yielded, at the most, 3–4 disulphated disaccharides/chain. Consequently, more than one-half of the L-IdUA-SO_4 residues were present in monosulphated periods, i.e. IdUA-(SO_4)-GalNAc. 3. To determine the location of L-IdUA-SO_4 residues along the copolymeric chain dermatan sulphate was digested with testicular hyaluronidase. (This enzyme cleaves GalNAc–GlcUA bonds within block regions containing D-GlcUA.) By NaB^3H_4 reduction GalNAc residues located in the reducing end of the fragments were converted into [^{3}H]GalNAcOH (*N*-acetylgalactosaminitol). Finally, the radioactive product was fragmented by periodate oxidation followed by alkaline elimination. The bulk of the radioactivity was associated with periodate-resistant oligosaccharides indicating that clusters of GlcUA-GalNAc-SO_4 periods are often adjacent to a varying number ($n = 1$–4) of L-IdUA-SO_4-containing periods. 4. To study the distribution of L-IdUA-SO_4-containing periods in relation to blocks of IdUA-GalNAc-SO_4 periods different fractions of hyaluronidase-degraded dermatan sulphate were degraded separately. In all types of fragments (mol.wts. 1500–10000) L-IdUA-SO_4-containing periods were demonstrated. In short fragments reducing terminal GalNAc-6-SO_4 (6-sulphated *N*-acetylgalactosamine) was found confirming that these sequences were joined to relatively long D-GlcUA-containing block sequences via GalNAc-6-SO_4. Moreover, low-molecular-weight oligosaccharides composed of alternating sequences were encountered. An octasaccharide derived from the carbohydrate sequence -GalNAc---GlcUA-GalNAc-IdUA-GalNAc-GlcUA-GalNAc-IdUA-GalNAc---GlcUA-GalNAc (--- indicates the position of cleavage by hyaluronidase) was identified.

Fibrous connective tissue is the major source for the acid galactosaminoglycan dermatan sulphate or chondroitin sulphate B (Meyer *et al.*, 1956). Over the past decades attempts to elucidate the chemical structure of dermatan sulphate have been made in several laboratories. The presence of both L-iduronic and D-glucuronic acid in preparations of this polysaccharide was first demonstrated by Hoffman *et al.* (1957). After degradation of pig skin dermatan sulphate with testicular hyaluronidase Fransson & Rodén (1967*a*,*b*) were able to isolate a 'hybrid' tetrasaccharide GlcUA - GalNAc(-SO_4) - IdUA - GalNAc(-SO_4)* illustrating the co-polymeric nature of this polysaccharide. The distribution of D-glucuronic acid-containing repeating units was further studied by sequential degradation with testicular hyaluronidase, β-glucuronidase and chondroitinase-AC (Fransson & Malmström, 1971). It was concluded that D-glucuronic acid-containing units occur in clusters of 1–2 block regions/chain. Moreover, single

* Abbreviations: GalNAc-SO_4, sulphated *N*-acetylgalactosamine; GalNAc-6-SO_4, 6-sulphated *N*-acetylgalactosamine; D-GlcUA, D-glucuronic acid; L-IdUA, L-iduronic acid; L-IdUA-SO_4, L-iduronic acid sulphate; UA, hexuronic acid; ΔUA, 4,5-unsaturated uronic acid; UA-GalNAc, repeat period composed of hexuronic acid and *N*-acetylgalactosamine residues; GalNAcOH, *N*-acetylgalactosaminitol.

glucuronosyl-*N*-acetylgalactosamine sulphate units are preferentially located in the vicinity of these block regions.

Previous studies have indicated that dermatan sulphate also contains sulphated hexuronosyl residues (Suzuki, 1960). By treatment of pig skin dermatan sulphate with chondroitinase-AC a glucuronic acid-free polymeric product, with a sulphate/hexosamine molar ratio of 1.54, was obtained (Malmström & Fransson, 1971). Conventional Smith degradation of this polymer afforded an iduronic acid-containing fragment with a sulphate/iduronic acid ratio of 1.8. To study the distribution of L-iduronic acid sulphate residues, methods for a selective periodate oxidation of dermatan sulphate were developed (Fransson, 1974; Fransson & Carlstedt, 1974). Oxidation of unsubstituted L-iduronosyl residues at low pH values was followed by reduction–hydrolysis or alkaline elimination to yield oligosaccharides containing D-glucuronic acid or *O*-sulpho-L-iduronic acid as hexuronic acid components (Fransson *et al.*, 1974*a*). Copolymeric oligosaccharides containing both D-glucuronic acid and *O*-sulpho-L-iduronic acid in the same chain were also demonstrated. These sequences were particularly prominent in polysaccharide fragments derived from peripheral portions of the original chain (Fransson *et al.*, 1974*b*). Further analysis of these oligosaccharides indicated that the GlcUA-GalNAc units occupied the reducing terminal end in the following manner:

$$\text{GalNAc-(IdUA-GalNAc)}_n\text{-GlcUA-GalNAc}$$
$$\qquad\qquad\quad |\qquad\qquad\qquad\qquad |$$
$$\qquad\qquad SO_4\qquad\qquad\qquad SO_4$$

Indirect evidence suggested that the hexosamine moieties of L-iduronic acid sulphate-containing sequences were unsulphated to a large extent.

The present study was undertaken to obtain more quantitative information about the distribution of L-iduronic acid sulphate residues in co-polymeric chains.

Experimental

Materials

Dermatan sulphate was obtained from pig skin by procedures described previously (Fransson, 1968). After hyaluronidase degradation of this polysaccharide the polymeric and oligomeric materials were separated by ethanol fractionation (Fransson, 1968). The polymeric material (designated PS-25-H) was further resolved by ion-exchange chromatography on AG-IX2 essentially as described by Malmström & Fransson (1971). Material eluted with 1.5M-NaCl which comprised 20% of the starting material was used in the present study (PS-25-H-1.5). Another fraction of hyaluronidase-degraded dermatan sulphate was obtained by precipitation with ethanol (preparation II: 2-25-Hyase-40 in Fransson & Rodén, 1967*b*). Finally, the oligomeric material obtained after hyaluronidase degradation of dermatan sulphate was also used in the present study. This material will henceforth be referred to as dermatan sulphate oligosaccharides ($n = 2$–8). The standards used for the calibration of the ion-exchange chromatography column have been described (Sjöberg *et al.*, 1973).

The enzymes chondroitinase-AC, chondroitinase-ABC and testicular hyaluronidase were the same preparations as described previously (Fransson & Malmström, 1971; Fransson *et al.*, 1974*a*). Both chondroitinase-AC and -ABC cleave linkages between *N*-acetylgalactosamine and D-glucuronic acid. In addition, chondroitinase-ABC cleaves *N*-acetylgalactosamine–L-iduronic acid bonds.

Analytical and chromatographic methods

Hexuronic acid was determined by the automated carbazole procedure of Heinegård (1973). Reducing power was estimated by the method of Park & Johnson (1949).

Descending paper chromatography was performed on Whatman 3MM paper in isobutyric acid–2M-NH_3 (5:3, v/v) for 30h. High-voltage paper electrophoresis was performed on the same paper in 0.1M-pyridine acetate, pH5.0 (40V/cm; 1.5h). Papers were stained with aniline hydrogen phthalate (Partridge, 1949).

Gel chromatography was carried out on columns (1.2cm×180–230cm) of Sephadex G-10, G-25 or G-50. The amount of material that was applied to the columns varied between 5 and 50mg (application vol. 1ml). The columns were eluted with 0.2M-pyridine acetate, pH5.0 (flow rate 10ml/h), and samples of the fractions (2–3ml) were assayed for uronic acid and reducing power as described above.

Ion-exchange chromatography of chondroitinase-ABC digests of dermatan sulphate were performed as follows. Material (30–40mg) was dissolved in 1ml of 0.5M-Tris–acetate, pH8.0, and digested with 0.3–0.4 unit of chondroitinase-ABC at 37°C overnight. The digests were chromatographed on Sephadex G-25 to separate linkage-region glycopeptides (eluted in the void volume) from di- and mono-saccharides. The latter material was finally resolved by ion-exchange chromatography on a column (1.2cm×40cm) of AGI-X8 (Cl^- form) eluted with a linear (0–3M) LiCl gradient. By this procedure di- and mono-saccharides are separated primarily by charge density. However, disaccharides containing 4,5-unsaturated hexuronic acid moieties are eluted later than their saturated counterparts (Fransson *et al.*, 1968).

Reduction with NaB^3H_4 and periodate oxidation

Hyaluronidase-degraded dermatan sulphate (PS-25-H, 10mg/ml of distilled water) was treated with 10mg of NaB^3H_4 (total radioactivity 0.1mCi) and left at room temperature for 1h. The reaction was terminated by the addition of acetic acid until the pH

reached 4–5. The solution was passed through a column (1cm×10cm) of AG 50W-X2 (H^+ form) which was eluted with 25ml of distilled water. The wash and effluent were combined and evaporated three times with methanol. The residue was dissolved in 5ml of 0.02M-$NaIO_4$–0.05M-sodium acetate, pH5.0, and left at 4°C for 24h in the dark. After addition of a molar excess of mannitol the oxidized material was directly subjected to alkaline treatment (pH12 by addition of 1M-NaOH) for 30min at room temperature. The solution was neutralized, concentrated to 1ml and chromatographed on Sephadex G-25. The column effluent was analysed for uronic acid and reducing power as described above. For determination of ^3H radioactivity a sample (0.5ml) was withdrawn, freeze-dried, dissolved in 0.5ml of distilled water and mixed with 5ml of Instagel (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.). Finally, radioactivity was measured in a Tri-Carb liquid-scintillation spectrometer.

Other samples of dermatan sulphate (PS-25-H-1.5; preparation II: 2-25-H-40) were directly treated with periodate (2mg of substrate/ml of 0.02M-$NaIO_4$–0.05M-sodium acetate, pH5.0 at 4°C for 24h) followed by dialysis against distilled water (3×1 litre) and treatment with alkali (pH12; 30min; 20°C). The fragments so obtained were resolved by gel chromatography (Sephadex G-25 or G-50). Further resolution of monosaccharide fragments was achieved by chromatography on Sephadex G-10.

Digestions with chondroitinases

Oligosaccharide fractions were digested with chondroitinase-AC or chondroitinase-ABC (0.1 unit of enzyme/10mg of substrate in 1ml of 0.5ml of 0.5M-Tris–acetate, pH8.0) at 37°C overnight. The products were separated by gel chromatography on Sephadex G-25.

Results

Quantitative determination of L-*iduronic acid sulphate residues*

Previous studies have shown that pig skin dermatan sulphate contains approximately 5–10% D-glucuronic acid of total hexuronic acid (Fransson & Rodén, 1967*a*,*b*). The remainder of the hexuronic acid residues have the L-ido configuration. Results of periodate oxidation indicated that 20–25% of the L-iduronic acid residues were *O*-sulphated (Fransson, 1974). Assuming that dermatan sulphate contains, on an average, 40 disaccharides/chain, it may be calculated that, on an average, 3 of these disaccharides contain D-glucuronic acid, 9 contain L-iduronic acid-sulphate, and the remainder (28) contain unsubstituted L-iduronic acid.

The presence of L-iduronic acid sulphate residues in dermatan sulphate may also be demonstrated by the

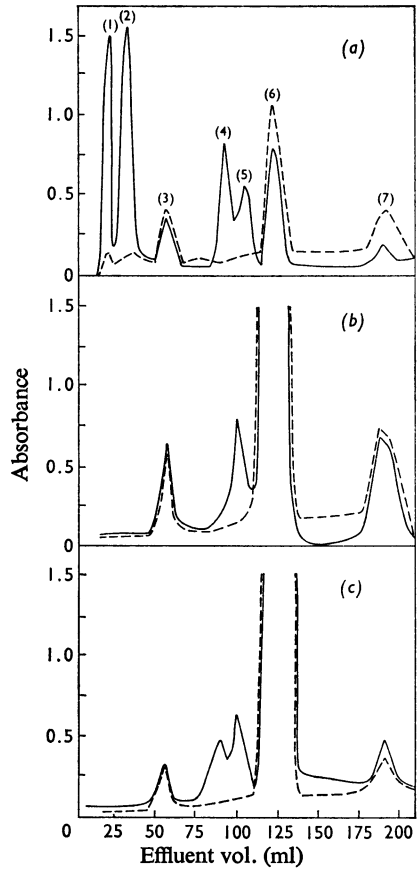


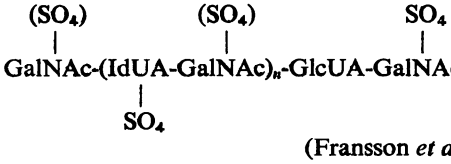
Fig. 1. *Ion-exchange chromatography (gradient elution) of di- and mono-saccharides*

(*a*) Standard chromatogram comprising (1) *N*-acetylgalactosamine, (2) *N*-acetylchondrosine (Di-OS), (3) 4,5-unsaturated, unsulphated disaccharide (ΔDi-OS), (4) monosulphated disaccharide (Di-monoS), (5) *N*-acetylgalactosamine sulphate (GalNAc-S), (6) 4,5-unsaturated, monosulphated disaccharide (ΔDi-monoS) and (7) 4,5-unsaturated, disulphated disaccharide. ——, Reducing power; ----, u.v. absorption at 232nm. (*b*) Chondroitinase-ABC digest (di- and mono-saccharides) of pig skin dermatan sulphate (PS-25). (*c*) Chondroitinase-ABC digest (di- and mono-saccharides) of hyaluronidase-degraded dermatan sulphate (PS-25-H). In the latter cases (*b* and *c* above) the chondroitinase digests were chromatographed on Sephadex G-25 to remove linkage region glycopeptides (*b*) or ABC-resistant oligosaccharides (*c*) both of which were eluted in the void volume (v_0). The included material was subsequently subjected to ion-exchange chromatography. This material comprised di- and mono-saccharides derived from the repeating periods of the digestible portions of the chains. It should be noted that hyaluronidase-degraded dermatan sulphate (*c*) also yielded a saturated disaccharide (v_e = 90ml) after digestion with chondroitinase-ABC. This disaccharide was derived from the non-reducing terminal glucuronic acid residue of hyaluronidase-degraded chains and had the following structure, GlcUA-GalNAc-SO_4.

isolation of disulphated disaccharides after chondroitinase-ABC degradation of the polymer. As shown in Fig. 1 pig skin dermatan sulphate yielded unsulphated, monosulphated and disulphated disaccharides in addition to *N*-acetylgalactosamine sulphate after enzymic degradation (Fig. 1*b*). The yields of unsulphated disaccharide and *N*-acetylgalactosamine sulphate were approximately equal, whereas the yield of disulphated disaccharide was 3–4 times larger. This value was obtained by calculating the peak areas in Fig. 1(*b*). It was also taken into account that sulphated hexosamines have lower reducing power than non-sulphated ones (approximately 70%). Since *N*-acetylgalactosamine sulphate is the principal non-reducing terminal residue of the polysaccharide chain (Sjöberg *et al.*, 1973), it may be concluded that each chain contains, on an average, 1 unsulphated and 3–4 disulphated disaccharide periods. However, results of periodate oxidation showed that the number of L-iduronic acid sulphate residues in pig skin dermatan sulphate was at least 9 (Fransson, 1974). Consequently, more than one-half of the L-iduronic acid sulphate residues must be present in monosulphated repeating units, such as IdUA(-SO_4)-GalNAc. The di- and mono-saccharides released by chondroitinase-ABC from hyaluronidase-degraded dermatan sulphate (PS-25-H) were also subjected to ion-exchange chromatography. As shown in Fig. 1(*c*) the yield of unsulphated and disulphated disaccharide was markedly decreased. The amount obtained corresponded to less than 1 repeating unit/chain. It should be added that approximately 10–15% of the repeating units in hyaluronidase-degraded dermatan sulphate were obtained in oligosaccharide form after chondroitinase-ABC digestion. On the basis of indirect evidence it was previously proposed that the chondroitinase-ABC- and periodate-insensitive oligosaccharides contained IdUA(-SO_4)-GalNAc units (Fransson *et al.*, 1974*b*). The present results further suggest that these oligosaccharides also contain IdUA(-SO_4)-GalNAc(-SO_4) and IdUA-GalNAc repeating units.

Distribution of sequences containing L-*iduronic acid sulphate residues in relation to sequences containing* D-*glucuronic acid*

In a previous study ion-exchange chromatography of hyaluronidase-degraded dermatan sulphate afforded material with sulphate/hexosamine molar ratios exceeding 1.0 (Malström & Fransson, 1971). These fractions comprised long segments derived from the non-reducing portion of the original polymer. Periodate oxidation of this material followed by reduction–hydrolysis or alkaline elimination yielded oligosaccharides containing both D-glucuronic acid and L-iduronic acid sulphate residues. It was demonstrated that the co-polymeric oligosaccharides had the following sequence:



(Fransson *et al.*, 1974*b*)

This finding indicates that repeating units containing L-iduronic acid sulphate may be intercalated between D-glucuronic acid- and L-iduronic acid-containing block regions. To investigate whether this was a common arrangement the following experiment was performed.

Hyaluronidase-degraded dermatan sulphate which accounts for two-thirds of the starting material (Fransson, 1968) was reduced with NaB^3H_4 (Scheme 1). By this treatment *N*-acetylgalactosamine residues located in the reducing end were converted into ^3H-labelled aminohexitol residues (IIa and IIb). Since

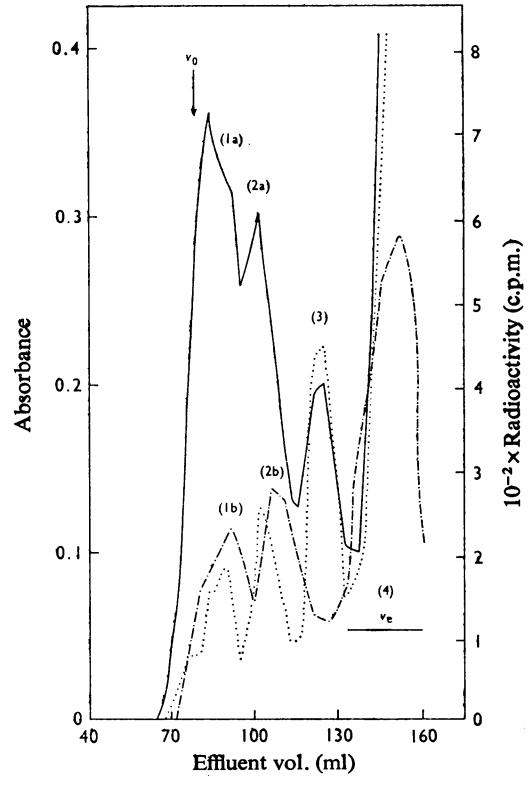
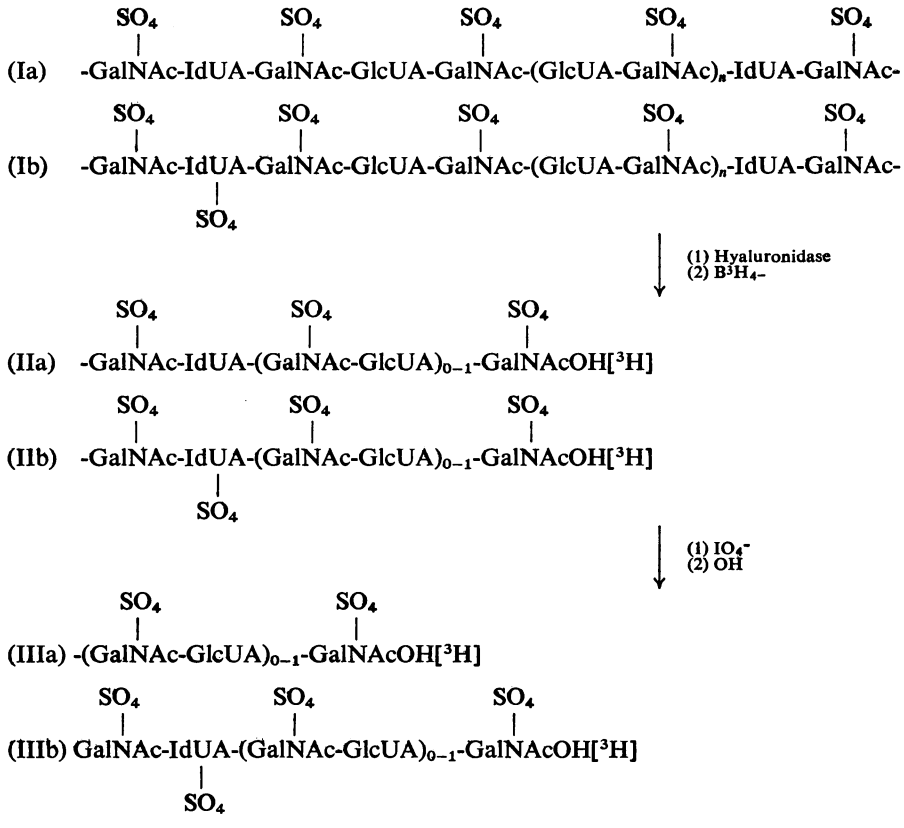


Fig. 2. *Gel chromatography on Sephadex G-25 of* ^3H-*labelled oligosaccharides*

Hyaluronidase-degraded dermatan sulphate was reduced with NaB^3H_4, oxidized with periodate, treated with alkali and subjected to gel chromatography. The column effluents were analysed for uronic acid (——), reducing power (· · · ·) and radioactivity (–·–·–·–·–). For details see the text. v_e, Elution volume; v_0, void volume.

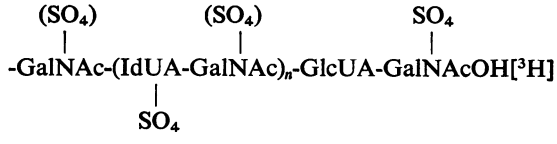


Scheme 1. *Scheme for demonstration of co-polymeric oligosaccharides containing reducing terminal GlcUA-GalNAc-SO_4 units and internal IdUA(-SO_4)-GalNAc units*

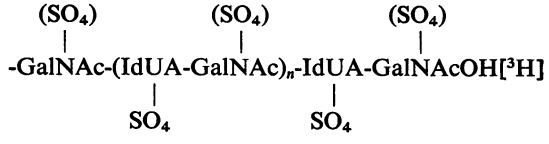
The starting material (I) is represented by two different types of sequences; (a) only GlcUA-GalNAc-SO_4 and IdUA-GalNAc-SO_4 periods are present; (b) IdUA(-SO_4)-GalNAc-SO_4 periods adjoin GlcUA-GalNAc(-SO_4) periods peripherally (to the left in this Scheme). After hyaluronidase degradation and NaB^3H_4 reduction the glucuronic acid-containing periods at the reducing end are labelled with ^3H (IIa and IIb). Finally, selective periodate oxidation of unsubstituted L-iduronic acid residues followed by alkaline elimination produces oligosaccharides (IIIa) and (IIIb).

hyaluronidase releases primarily tetrasaccharides from glucuronic acid-containing glycosaminoglycans (for references see Fransson & Rodén, 1967*a*,*b*), a few residual, reducing terminal GlcUA-GalNAc(-SO_4) periods remain in the polymer after enzymic treatment (Fransson & Malmström, 1971). Finally, the unsubstituted L-iduronic acid residues of this material (IIa) were cleaved by periodate oxidation–alkaline elimination. The oligosaccharides so obtained should include fragments derived from periods containing only residual glucuronic acid units (IIIa) and from periods containing in addition L-iduronic acid sulphate units (IIIb). It should be added that a *N*-[^3H]acetylgalactosaminitol moiety adjoining an unsubstituted L-iduronic acid residue is obtained as free ^3H-labelled *N*-acetylgalactosaminitol. The ^3H-labelled oligosaccharides obtained after the final step were subjected to gel chromatography on Sephadex G-25 as shown in Fig. 2. The carbazole-positive material was distributed in three major oligosaccharide peaks, large oligosaccharides were eluted in or near the void volume (Fig. 2, peak 1a), medium-sized oligosaccharides were eluted in the position of tetrasaccharide (Fig. 2, peak 2a) and a disaccharide fraction (Fig. 2, peak 3); material eluted with the salt (effluent volume 140ml and onwards) did not give a true carbazole colour. All of these oligosaccharide peaks showed increasing reducing power with decreasing chain length. Radioactivity was associated with long and medium-sized oligosaccharides (Fig. 2, peaks 1b and 2b). It should be noted that in the latter case (peak 2b) the radioactivity coincided neither with the reducing power nor with the carbazole reactivity. This is consistent with the fact that most of

the reducing power and the carbazole reactivity of this fraction should be attributed to a tetrasaccharide with the following carbohydrate sequence GalNAc-UA-GalNAc-R(CHO) (Fransson *et al.*, 1974*a*), whereas the radioactive compound should be a trisaccharide with the carbohydrate sequence GalNAc-UA-[^{3}H]GalNAcOH. The disaccharide fraction (peak 3 in Fig. 2) contained no radioactivity in accordance with its presumed structure, GalNAc(-SO_4)-R(CHO) (Fransson & Carlstedt, 1974). A considerable amount of radioactivity was eluted with the salt (peak 4). Attempts to desalt this material yielded only small amounts of radioactive monosaccharide which were not studied further. It should be added that the bulk of the radioactivity of this fraction emerged with the total volume (v_t) on Sephadex G-10. Thus the results of the above experiment showed that the major portion of the ^{3}H-labelled aminohexitol residues were part of oligosaccharides with the general carbohydrate sequence (GalNAc-UA)$_n$-GalNAcOH[^{3}H]. In oligosaccharides with $n = 1$ (corresponding to peak 2b in Fig. 2) the hexuronic acid component is either glucuronic acid or L-iduronic acid sulphate, whereas the larger oligosaccharides should mainly be copolymeric in the following manner:



Oligosaccharide fraction (Ib) also includes L-iduronic acid-containing non-co-polymeric sequences i.e.:



It should be added that L-iduronic acid sulphate-containing oligosaccharides may also be derived from internal segments of the polysaccharide chain. These oligosaccharides have the general carbohydrate sequence GalNAc-(IdUA-GalNAc)$_n$-R(CHO)

(Fransson *et al.*, 1974*b*).

Distribution of L-*iduronic acid sulphate residues in relation to* L-*iduronic acid-containing repeat periods*

The D-glucuronic acid-containing block regions of pig skin dermatan sulphate may be cleaved with testicular hyaluronidase (Fransson & Rodén, 1967*a*,*b*). The resulting fragments which comprise the L-iduronic acid-containing regions range from tetrasaccharide (molecular weight approximately 1000) to polysaccharides almost as long as the original polymer. In a previous study it was shown that long fragments (molecular weight 15000) derived from the non-reducing terminal portion of the chain were significantly oversulphated (Malmström & Fransson, 1971). Approximately 40% of the oversulphated material was represented by L-iduronic acid sulphate-containing oligosaccharides (Fransson *et al.*, 1974*b*). It was decided to see whether smaller fragments produced by hyaluronidase digestion also contain L-iduronic acid sulphate units. As shown in Table 1 three different preparations were used for this purpose. Preparation PS-25-H-1.5 which was obtained by ion-exchange chromatography of hyaluronidase-degraded pig skin dermatan sulphate accounted for 20% of the starting material, and had a molecular weight of 10000. Less than one-half of the chains contained the linkage region to protein, whereas approximately 60% of the chains contained non-reducing terminal D-glucuronic acid. The second polysaccharide preparation which was obtained by ethanol fractionation had a lower molecular weight (5000). Amino acid analysis indicated that only one chain out of five contained the carbohydrate–protein linkage region. Analysis of non-reducing terminal D-glucuronic acid showed somewhat less than 0.5 mol/mol of polysaccharide. These data indicate that both preparations comprise fragments derived from central as well as peripheral segments of the original polymer. The latter segments may be derived either from the non-reducing end or from the

Table 1. *Analytical data for three preparations of hyaluronidase-treated dermatan sulphate*

Data are compiled from earlier publications (Fransson & Rodén, 1967*b*; Malmström & Fransson, 1971).

Preparation	Viscosity-average molecular weight ($\bar{M}_V$)	GalN/Ser	Non-reducing GlcUA (mol/mol)	SO_4/HexN
PS-25-H-1.5	10000	31	0.61	1.01
II:2-25-H-40	5000	33	0.44	1.20
Oligosaccharides ($n = 2$–8)	1500–4000			

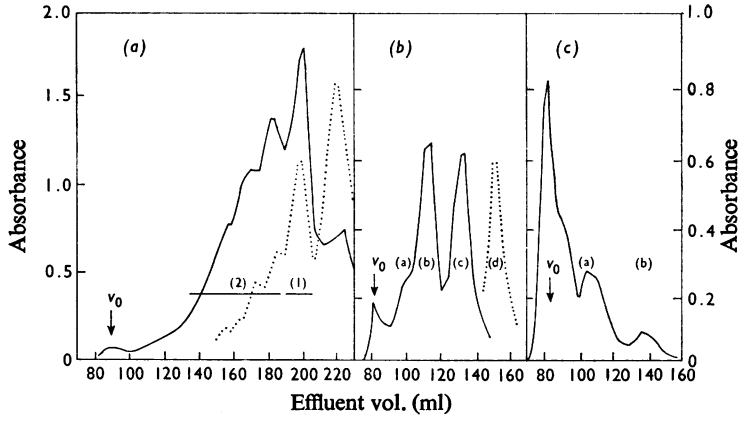


Fig. 3. *Gel chromatography of various periodate-resistant oligosaccharides derived from preparation PS-25-H-1.5*

(*a*) Gel chromatography on Sephadex G-50 of preparation PS-25-H-1.5 after periodate oxidation followed by alkaline elimination. (*b*) Gel chromatography on Sephadex G-25 of fraction (1) (see *a*) after digestion with chondroitinase-AC as described in the text. (*c*) Gel chromatography on Sephadex G-25 of fraction (2) (see *a*) after digestion with chondroitinase-ABC as described in the text. The effluents of the columns were assayed for hexuronic acid (——) and reducing power (····). v_0 ,Unspecific absorbance derived from enzyme protein. For details of peaks see the text.

carbohydrate–protein attachment region. End-group analyses of the two preparations indicated that the low-molecular-weight preparation (II: 2-25-H-40) was enriched in non-reducing terminal fragments, compared with the high-molecular-weight preparation. In agreement with the observation that non-reducing terminal fragments are generally over-sulphated (Malmström & Fransson, 1971), the sulphate/hexosamine molar ratio of preparation II: 2-25-H-40 was higher than that of the high-molecular-weight preparation. Finally, oligosaccharides ranging from disaccharide to hexadecasaccharide were also used in the present study (Fransson & Rodén, 1967*b*). This preparation represented one-third of the starting material.

The three preparations presented above were separately treated with periodate under conditions that allow selective oxidation of unsubstituted L-iduronic acid residues. The oxidation was followed by alkaline elimination and gel chromatography. The results of these experiments are shown in Figs. 3–6. When the high-molecular-weight fraction (PS-25-H-1.5) was degraded by periodate oxidation–alkaline elimination, at least five oligosaccharide peaks were obtained after chromatography on Sephadex G-50 (Fig. 3*a*). The largest peak was obtained in the position of a tetrasaccharide or trisaccharide (fraction 1; yield 10%) suggesting that this oligosaccharide had the general carbohydrate structure GalNAc-UA-GalNAc-R(CHO) or GalNAc-UA-GalNAc (see also Fransson *et al.*, 1974*a*,*b*). To determine the nature of the hexuronic acid moiety fraction (1) was digested with chondroitinase-AC and chromatographed on Sephadex G-25. As shown in Fig. 3(*b*) three major peaks were obtained. Their effluent volumes corresponded to those of trisaccharide (b), disaccharide (c) and monosaccharide (d). The latter two components were electrophoretically indistinguishable from *N*-acetylgalactosamine sulphate and monosulphated disaccharide respectively. The major portion of the trisaccharide (b) had the mobility of compound ΔUA-GalNAc(-SO_4)-R(CHO) (Fransson *et al.*, 1974*a*), whereas a minor portion migrated like disulphated trisaccharide. It may thus be concluded that fraction (1) was primarily a 1:1 mixture of trisaccharide GalNAc(-SO_4)-GlcUA-GalNAc-SO_4 and tetrasaccharide GalNAc(-SO_4)-GlcUA-GalNAc-(-SO_4)-R(CHO). Only a minor portion of the material contained L-iduronic acid, presumably in the form of trisaccharide (carbohydrate sequence GalNAc-IdUA-GalNAc) and tetrasaccharide [carbohydrate sequence GalNAc-IdUA-GalNAc-R; peak (a) in Fig. 3(*b*)]. The L-iduronic acid residues in these oligosaccharides were probably sulphated in the 2- or 3-position.

Hyaluronidase cleaves clusters of GlcUA-GalNAc periods in co-polymeric dermatan sulphate, leaving single periods undegraded. However, the hyaluronidase-degraded preparation PS-25-H-1.5 afforded a considerable amount of oligosaccharides containing two or more repeating units (fraction 2 in Fig. 3*a*; yield, 15%) after periodate oxidation–alkaline elimination. These oligosaccharides which should contain L-iduronosulphate (alone or in combination with D-glucuronic acid) were further digested with chondroitinase-ABC. As shown in Fig. 3(*c*) only moderate effect was observed. Most of the material

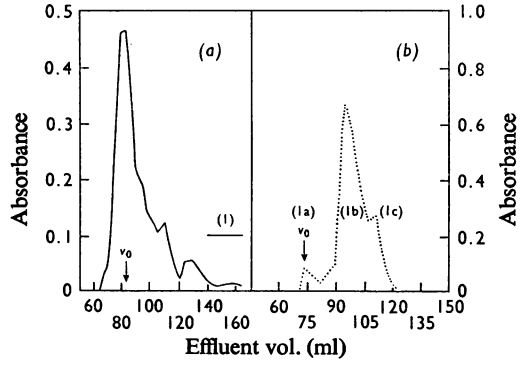


Fig. 4. *Gel chromatography of degradation products of preparation II*: 2-25-*H*-40

(*a*) Gel chromatography on Sephadex G-25 after periodate oxidation–alkaline elimination as described in the text. Assay for uronic acid was performed on the effluent (——). (*b*) Gel chromatography on Sephadex G-10 of fraction (1) (see *a*). This column was eluted with water and assayed for reducing power (····).

remained in the position of hexasaccharide or larger (v_0), whereas only small quantities of tetrasaccharide (Fig. 3*c*; peak a) and disaccharide (Fig. 3*c*; peak b) were obtained. Repeated digestion with the enzyme gave no further release of tetra- and di-saccharide. These findings are in accordance with previous observations, i.e. L-iduronic acid sulphate-containing oligosaccharides are poor substrates for chondroitinase-ABC (Fransson *et al.*, 1974*b*) most likely due to the presence of unsulphated *N*-acetylgalactosamine moieties. The fact that preparation PS-25-H-1.5 originally contained equimolar proportions of sulphate and hexosamine lends further credence to this notion.

Periodate oxidation–alkaline elimination of the medium-sized dermatan sulphate fragments (preparation II: 2-25-H-40) followed by chromatography on Sephadex G-25 gave the results shown in Fig. 4(*a*). Approximately 25% of the starting material was recovered as oligosaccharides which were excluded or partly excluded from Sephadex G-25 (v_e = 75–100ml). Material eluted in the position of monosaccharide (fraction 1) was desalted on Sephadex G-10 (Fig. 4*b*). The major component (1b) which had the elution volume of an anionic monosaccharide contained predominantly 6-sulphated *N*-acetylgalactosamine (Fig. 5A). A minor peak (1c in Fig. 4*b*) in the position of uncharged monosaccharide was also obtained. The small amounts available precluded identification of this component.

The dermatan sulphate oligosaccharides released by hyaluronidase should have the general carbohydrate sequence GlcUA-GalNAc-(IdUA-GalNAc)$_n$-GlcUA-GalNAc (Fransson & Rodén, 1967*a*,*b*). These oligosaccharides which vary in size from hexasaccharide (n = 1) to tetradecasaccharide (n = 5) were separately treated with chondroitinase-AC to remove the reducing terminal, glucuronic acid-containing disaccharide repeat. However, both tetra- and di-saccharide were released from these oligosaccharides, demonstrating that more complex sequences, like GlcUA-GalNAc-(IdUA-GalNAc)$_m$-GlcUA-GalNAc-IdUA-GalNAc, were also present in this material (one example, an octasaccharide, is shown in Fig. 6*a*). Material larger than tetrasaccharide (corresponding to fraction v_0 in Fig. 6*a*) obtained from the various oligosaccharides was pooled and further digested with chondroitinase-ABC. As shown in

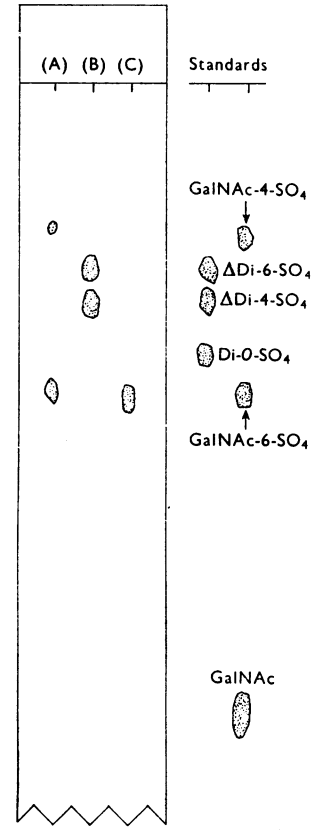


Fig. 5. *Paper chromatography of various mono- and di-saccharide fragments*

(A) Peak (1b) in Fig. 4(*b*); (B) peak (I) in Fig. 6(*b*); (C) peak (II) in Fig. 6(*c*). Standards are: GalNAc, GalNAc-4-SO$_4$ and GalNAc-6-SO$_4$, unsulphated, 4- or 6-sulphated *N*-acetylgalactosamine respectively; ΔDi-4-SO$_4$ and ΔDi-6-SO$_4$, 4- or 6-sulphated disaccharides with a 4,5-unsaturated uronic acid moiety; Di-*O*-SO$_4$, unsulphated disaccharide (*N*-acetylchondrosine).

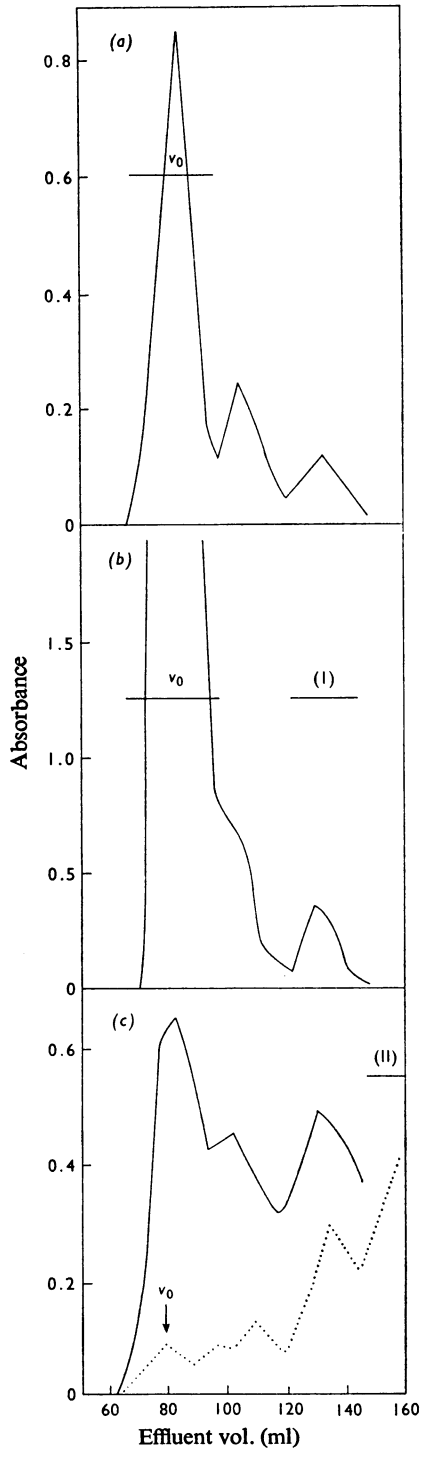


Fig. 6. *Gel chromatography on Sephadex G-25 of various dermatan sulphate oligosaccharides*

(*a*) Dermatan sulphate octasaccharide after chondroitinase-AC degradation as described in the text. (*b*) Chondroitinase-AC-resistant dermatan sulphate oligosaccharides after further digestion with chondroitinase-ABC. The chondroitinase-AC-resistant oligosaccharides correspond to peak v_0 in (*a*). (*c*) Periodate oxidation–alkaline elimination of fraction v_0 in (*b*). ——, Carbazole reaction; ····, reducing power.

Fig. 6(*b*) only small quantities of tetra- and disaccharide were obtained. The latter fraction (I in Fig. 6*b*) contained both 4- and 6-sulphated species (Fig. 5B). The chondroitinase-ABC-resistant material (v_0 in Fig. 6*b*) was finally oxidized with periodate followed by alkaline treatment. The gel chromatogram shown in Fig. 6(*c*) indicates that a considerable proportion of the material was resistant to periodate oxidation. Material eluted in the position of monosaccharide (fraction II) was desalted on Sephadex G-10 and finally subjected to paper chromatography. One spot corresponding to 6-sulphated *N*-acetylgalactosamine was obtained (Fig. 5C).

Discussion

Previous studies have indicated that pig skin dermatan sulphate contains three different types of hexuronic acid residues, i.e. L-iduronic acid, L-iduronic acid sulphate and D-glucuronic acid approximately in the ratio 9:3:1 (Fransson, 1974; Fransson *et al.*, 1974*a*). In the polysaccharide these hexuronic acid residues are linked 1,3 to *N*-acetylgalactosamine moieties forming unsulphated, monosulphated or disulphated disaccharide repeats. Unsulphated hexosamine moieties are found in relatively long oligosaccharides containing D-glucuronic acid obtained after periodate-oxidation and alkaline elimination (Fransson *et al.*, 1974*a*). These moieties occupy the non-reducing terminal indicating that they are derived from the sequence -IdUA-GalNAc-GlcUA-. The present data suggest that pig skin dermatan sulphate contains, on an average, one IdUA-GalNAc period/chain. Approximately 9 L-iduronic acid sulphate residues were present in each dermatan sulphate chain. However, the number of disulphated disaccharide periods was estimated at 3–4, on an average. It may thus be concluded that approximately 5–6 disaccharides/chain have the structure IdUA(-SO_4)-GalNAc. Consequently, monosulphated disaccharide periods (estimated at approximately 35/chain) include IdUA-GalNAc-SO_4 (26), IdUA(-SO_4)-GalNAc (5–6) and GlcUA-GalNAc-SO_4 (3).

When hyaluronidase- or chondroitinase-AC-

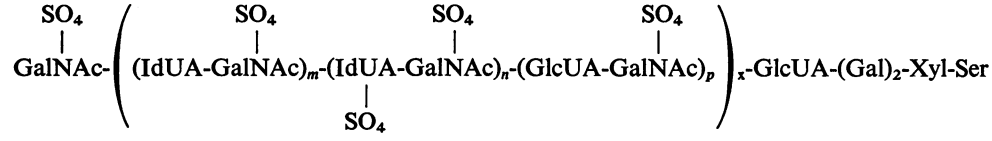


Fig. 7. *General formulae for the parent structure of pig skin dermatan sulphate*

Parentheses around certain sulphate groups indicate the possibility of sulphate gaps. The non-reducing end is to the left.

degraded dermatan sulphate is treated with chondroitinase-ABC, oligosaccharides containing L-iduronic acid sulphate are obtained (Fransson *et al.*, 1974*b*). The present study indicates that all three types of irregular repeating periods i.e. IdUA(-SO_4)-GalNAc, IdUA-GalNAc and IdUA(-SO_4)-GalNAc-SO_4, are present in these oligosaccharides.

The distribution of repeating units containing L-iduronosulphate within co-polymeric chains was also investigated in the present work. The relationship between the block regions containing D-glucuronic acid and periods containing L-iduronosulphate was elucidated by degradation of hyaluronidase-treated and reduced dermatan sulphate. By introducing ^3H label into reducing terminal *N*-acetylgalactosamine moieties disaccharide periods which were originally part of block regions containing D-glucuronic acid could be traced (see Scheme 1). Approximately one-half of the recovered radioactive oligosaccharides were GalNAc(-SO_4)-UA-GalNAc(-SO_4) where UA is GlcUA or IdUA-SO_4, whereas the remainder had the general carbohydrate sequence GalNAc-(IdUA-GalNAc)$_n$-(GlcUA-GalNAc)$_{0-1}$ with the IdUA moiety sulphated. These findings suggest that clusters of GlcUA-GalNAc-SO_4 periods are usually followed by L-iduronic acid sulphate-containing periods of varying length ($n = 1$–4 in Fig. 7).

To study the distribution of L-iduronic acid sulphate-containing periods in relation to blocks of IdUA-GalNAc-SO_4 periods (*m* in Fig. 7) three different fractions of hyaluronidase-degraded dermatan sulphate were studied. One preparation was of rather high molecular weight (10000) and contained primarily internal and reducing terminal fragments, another preparation had a lower molecular weight (5000) and contained a preponderance of non-reducing terminal fragments. Finally, the structure of oligosaccharides representing very short L-iduronic acid-containing block regions were investigated. In all fragments L-iduronosulphate-containing repeating periods were demonstrated. In the high-molecular-weight fragments these periods accounted for approximately 15% of the entire material, whereas the low-molecular-weight fragment appeared to contain even more. It was also noted that relatively short L-iduronic acid-containing fragments were linked to D-glucuronic acid-containing block regions via 6-sulphated *N*-acetylgalactosamine. This agrees with previous work which showed that 6-sulphated *N*-acetylgalactosamine moieties are preferentially associated with relatively long D-glucuronic acid-containing periods (Fransson *et al.*, 1974*a*). Moreover, the dermatan sulphate octasaccharide obtained in the present study contained a co-polymeric saccharide with the following sequence, GlcUA-GalNAc-IdUA-GalNAc -GlcUA-GalNAc-IdUA-GalNAc. Consequently, it appears that certain regions of the co-polymeric chain are composed of alternating D-glucuronic acid- and L-iduronic acid-containing repeats. The following arrangements may be typical, -(IdUA-GalNAc)-$_{1-15}$-(GlcUA-GalNAc-IdUA-GalNAc)$_{1-2}$-(GlcUA-GalNAc)$_{1-4}$-[cf. the hybrid tetrasaccharide, GlcUA-GalNAc-IdUA-GalNAc released by hyaluronidase (Fransson & Rodén, 1967*b*)]. It is proposed that the L-iduronic acid residues of these sequences are sulphated to a considerable degree, whereas the adjacent *N*-acetylgalactosamine moieties are often unsulphated (or 6-sulphated). It was found previously that unsulphated *N*-acetylgalactosamine moieties were associated with relatively long D-glucuronic acid-containing block regions (Fransson *et al.*, 1974*a*). These moieties could not be positively identified in any of the hyaluronidase produced fragments used in the present study. However, chondroitinase-ABC digestion of hyaluronidase-degraded dermatan sulphate indicated that the unsulphated disaccharide periods were recovered among the chondroitinase ABC-resistant oligosaccharides.

Any definitive statements concerning the origin and the role of L-iduronosulphate residues in dermatan sulphate cannot be made at present. Recent studies have indicated that L-iduronic acid is formed via C-5 inversion of D-glucuronic acid on the polymer level (Lindahl *et al.*, 1972; Malmström *et al.*, 1974). Although L-iduronic acid may be formed before sulphation (Malmström *et al.*, 1975), the addition of sulphate donor (3′-phosphoadenylyl sulphate) greatly enhances production of L-iduronic acid. Further, the formation of block regions containing L-iduronic acid appears to be greatly promoted by 4-sulphation of adjacent hexosamine moieties. It would thus appear that sulphation of L-iduronic acid occurs at a later stage of the biosynthetic process.

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