Molecular organization of heparan sulphate from human skin fibroblasts

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The molecular structure of human skin fibroblast heparan sulphate was examined by specific chemical or enzymic depolymerization and high-resolution separation of the resulting oligosaccharides and disaccharides. Important features of the molecular organization, disaccharide composition and *O*-sulphate disposition of this heparan sulphate were identified. Analysis of the products of HNO₂ hydrolysis revealed a polymer in which 53% of disaccharide units were *N*-acetylated and 47% *N*-sulphated, with an *N*-/*O*-sulphate ratio of 1.8:1. These two types of disaccharide unit were mainly located in separate domains. Heparitinase and heparinase scission indicated that the iduronate residues (37% of total hexuronate) were largely present in contiguous disaccharide sequences of variable size that also contained the majority of the *N*-sulphate groups. Most of the iduronate residues (approx. 70%) were non-sulphated. About 8–10% of disaccharide units were cleaved by heparinase, but only a minority of these originated from contiguous sequences in the intact polymer. Trisulphated disaccharide units [α -*N*-sulpho-6-sulphoglucosaminyl-(1→4)-iduronate 2-sulphate], which are the major structural units in heparin, made up only 3% of the disaccharide units in heparan sulphate. *O*-Sulphate groups (approx. 26 per 100 disaccharide units) were distributed almost evenly among C-6 of *N*-acetylglucosamine, C-2 of iduronate and C-6 of *N*-sulphated glucosamine residues. The results indicate that the sulphated regions of heparan sulphate have distinctive and potentially variable structural characteristics. The high content of non-sulphated iduronate in this heparan sulphate species suggests a conformational versatility that could have important implications for the biological properties of the polymer.

INTRODUCTION

The heparan sulphates are complex linear polysaccharides found almost ubiquitously on the cell surface and in the extracellular matrix of mammalian cells. Their strategic location makes them candidates for the regulation of interactions between cells and their micro-environment, and roles for heparan sulphate in a wide range of biological functions including growth control, cell adhesion and anticoagulation have been indicated by various studies (for reviews see Höök *et al.*, 1984; Gallagher *et al.*, 1986; Rapraeger *et al.*, 1987; Gallagher & Lyon, 1989). Some of these proposed functions are likely to depend on specific sequences in the polysaccharide structure, a view supported by the elucidation of the high-affinity pentasaccharide-binding site for antithrombin III in heparin (Lindahl *et al.*, 1984).

The widely documented structural heterogeneity of heparan sulphates arises from a stepwise series of polymer-level modifications of the non-sulphated precursor, *N*-acetylheparosan, composed of alternating GlcNAc and GlcA residues (Riesenfeld *et al.*, 1982; Silbert & Baldwin, 1984). The first modification step is the conversion of GlcNAc into GlcNSO₃. Only about 50% of the hexosamine residues are converted into the

N-sulphated derivative, the conversion sites tending to be in clusters rather than distributed evenly along the polysaccharide chain (Fransson *et al.*, 1980; Winterbourne & Mora, 1981; Gallagher & Walker, 1985). *N*-Sulphation occupies a key position in determining the overall sulphation pattern of the final biosynthetic product (Gallagher *et al.*, 1986; Lindahl *et al.*, 1986), and this is supported by recent studies showing the production of undersulphated heparan sulphate in a Chinese-hamster ovary cell mutant defective in *N*-sulphotransferase activity (Bame & Esko, 1989). Further modifications occur largely within or adjacent to the *N*-sulphated domains. A specific epimerase converts GlcA into IdoA and the polymer is then further modified by a complex pattern of substitution by ester (*O*-)sulphate groups (Gallagher *et al.*, 1986; Lindahl & Kjellén, 1987). Only a fraction of potential substrates are utilized by the polymer-modifying enzymes, and both qualitative and quantitative differences in *O*-sulphation have been identified in heparan sulphates from different cells, and even in the polysaccharides produced by a single cell type (Hampson *et al.*, 1984; Gallagher & Walker, 1985; Fedarko & Conrad, 1986). *O*-Sulphation is diminished in heparan sulphates produced by transformed and malignant cells (Winterbourne & Mora, 1981; Pejler *et al.*,

Abbreviations used: d.p., degree of polymerization (i.e. for a disaccharide d.p. = 2 etc.); UA, uronic acid; GlcA, glucuronic acid; GlcA(2S), glucuronic acid 2-sulphate; IdoA, iduronic acid; IdoA(2S), iduronic acid 2-sulphate; GlcNAc, *N*-acetylglucosamine; GlcNSO₃, *N*-sulphated glucosamine; GlcNSO₃(6S), *N*-sulphated glucosamine 6-sulphate; GlcNSO₃(3S), *N*-sulphated glucosamine 3-sulphate; ΔGlcA and ΔIdoA, unsaturated glucuronic acid and iduronic acid residues; since these are products of specific polysaccharide lyase cleavage it is possible to designate the structure of the hexuronate residue as it would have occurred in the intact polymer.

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1987; Pejler & David, 1987). More detailed analysis of the fine structure and domain organization of heparan sulphate is required in order to elucidate the functional significance of this variability.

Recently we described a new electrophoretic method for the separation of oligosaccharides derived by scission of complex glycosaminoglycans (Turnbull & Gallagher, 1988). This technique provides high resolution of these molecules and yields 'oligosaccharide maps' of the intact polymer that reveal important structural features and demonstrate the distinctive nature of different heparan sulphates. In the present paper we describe further investigation of the structure of a skin fibroblast heparan sulphate. Use of gradient PAGE, gel filtration and ion-exchange-chromatography techniques, combined with specific reagents for chain scission, has enabled the identification of different classes of structural domain present in heparan sulphate, defining their size range, end groups and to an extent their likely internal sequences. Furthermore we have assessed the overall disaccharide composition and the approximate disposition of the *O*-sulphate groups; taken together the data reveal important new features of the molecular organization of heparan sulphate.

EXPERIMENTAL

Materials

D-[1-³H]Glucosamine (sp. radioactivity 27 mCi/mg) and Na₂³⁵SO₄ (sp. radioactivity 25–40 Ci/mg) were obtained from Amersham International. Heparinase and heparinase II were obtained from Sigma Chemical Co., and heparitinase I and chondroitin ABC lyase were obtained from Seikagaku Kogyo Co., Tokyo, Japan. Cell-culture media were supplied by Gibco, with the exception of newborn-calf serum obtained from Flow Laboratories. Bio-Gel P-6 and Bio-Gel P-2 (200–400 mesh) were from Bio-Rad Laboratories. A 32 cm vertical-slab-gel unit (SE620) was supplied by Hoefer Scientific Instruments (San Francisco, CA, U.S.A.), and the Trans-blot system was obtained from Bio-Rad Laboratories. Biotrace RP nylon membrane was supplied by Gelman Sciences, En³Hance spray surface autoradiography enhancer was obtained from NEN Research Products, DuPont (U.K.) Ltd. Autoradiography cassettes were supplied by Genetic Research Ltd. X-Omat AR X-ray film and development chemicals were supplied by Kodak. An f.p.l.c. system and Mono-Q columns were obtained from Pharmacia-LKB. Tris, heparin (from pig intestinal mucosa; ammonium salt) and heparan sulphate (from bovine kidney) were all supplied by Sigma Chemical Co. All other reagents and chemicals used were of AnalaR or AristaR grade from BDH Chemicals.

Cell culture, radiolabelling and preparation of intact heparan sulphate chains

Confluent cultures of adult human skin fibroblasts were maintained at 37 °C (5% CO₂ in air) in Eagle's Minimal Essential Medium supplemented with 15% (v/v) donor-calf serum, 2 mM-glutamine, 1 mM-sodium pyruvate, non-essential amino acids, penicillin (100 units/ml) and streptomycin (100 µg/ml).

Cultures were incubated for 72 h with Na₂³⁵SO₄ (10 µCi/ml) and [³H]glucosamine (10 µCi/ml). The medium was removed and the cell layers were carefully washed twice with warm (37 °C) Dulbecco's phosphate-

buffered saline A. The combined medium and rinsings were centrifuged (200 g for 10 min) and the supernatant was stored at –20 °C.

The medium proteoglycans were subjected to initial purification by ion-exchange chromatography. Samples were applied to a DEAE-Sephacel column (1 cm × 5 cm) and washed through with 0.3 M-NaCl in 20 mM-sodium phosphate buffer, pH 6.8, to elute contaminating proteins and hyaluronic acid. Remaining proteoglycans (principally heparan sulphate and chondroitin sulphate/dermatan sulphate) were then eluted with a linear gradient of 0.3–1 M-NaCl in 20 mM-phosphate buffer. Fractions corresponding to heparan sulphate proteoglycan (eluted at approx. 0.53 M) were collected and desalted on a Sephadex G-25 column (2.5 cm × 40 cm) and freeze-dried. Traces of chondroitin sulphate and dermatan sulphate were removed by treatment with 1 unit of chondroitin ABC lyase for 3 h at 37 °C. The proteoglycans were then digested with Pronase (5 mg/ml) for 4 h at 37 °C. Heparan sulphate chains were recovered by step elution with 1 M-NaCl from a 2 ml DEAE-Sephacel column after washing with 0.3 M-NaCl to elute chondroitin sulphate/dermatan sulphate oligosaccharides and peptide fragments. After dialysis against distilled water the heparan sulphate chains were freeze-dried in preparation for further analysis.

Depolymerization of heparan sulphate glycosaminoglycans

Heparitinase (heparitinase I from Seikagaku Kogyo Co.) and heparinase II (from Sigma Chemical Co.) were used at a concentration of 20 munits/ml in 100 mM-sodium acetate buffer, pH 7.0, containing 0.2 mM-calcium acetate and 1 mg of bovine kidney heparan sulphate/ml as carrier. Samples were incubated at 43 °C for 16 h. Heparinase was used at a concentration of 20 munits/ml in the same buffer as heparitinase but containing 1 mg of heparin/ml as carrier. Samples were incubated at 30 °C for 16 h. In both cases digestion was stopped by heating at 100 °C for 2 min, and parallel control digests, containing unlabelled heparan sulphate or heparin only, were carried out to monitor the progress of the reaction. Increase in absorbance at 232 nm was measured to ensure that the end point of depolymerization was reached with each batch of digests. HNO₂ deaminative cleavage was carried out by using the low-pH method of Shively & Conrad (1976). Samples were dried down by centrifugal evaporation, reconstituted in 10 µl of 1 M-HNO₂ solution and incubated for 15 min at 20 °C. The reaction was stopped by addition of 2 µl of 1 M-Na₂CO₃.

Gel chromatography

Gel chromatography of intact chains and glycosaminoglycan oligosaccharide fragments was performed on Sepharose CL-6B, Bio-Gel P-6 and Bio-Gel P-2 columns (120 cm × 1 cm) in 0.5 M-NH₄HCO₃ eluted at a flow rate of 4 ml/h. In each case 1 ml fractions were collected and small portions were taken for scintillation counting of radioactivity. Fractions corresponding to disaccharides were pooled and freeze-dried in preparation for further analysis.

Oligosaccharide mapping by gradient PAGE

Radiolabelled heparan sulphate scissioned with various reagents was mapped by gradient PAGE as described by Turnbull & Gallagher (1988), with some

modifications. Briefly, 20–30 % polyacrylamide-gradient gels (32 cm × 16 cm × 0.75 mm) were prepared and samples were electrophoresed as described previously. After equilibration of the gel in low-ionic-strength transfer buffer (10 mM-Tris/acetate buffer, pH 7.9, containing 0.5 mM-EDTA) for 10 min, oligosaccharides were transferred on to positively charged nylon membrane (Biotrace RP) in a Trans-blot tank by using the same buffer. Transfer was carried out at 10 V for 4 h at 4 °C. The oligosaccharides were detected by fluorography of the membrane by using En³Hance surface autoradiography enhancer and Kodak X-Omat AR X-ray film as described previously.

Low-pH ion-exchange chromatography with the use of Mono-Q f.p.l.c.

Disaccharides from gel-filtration chromatography were fractionated at low pH (pH 2.0) by NaCl gradient elution from a Mono-Q anion-exchange column. At pH 2.0 the charge contribution from the uronic acid carboxy groups is minimal and the disaccharides are resolved on the basis of their sulphate content. Samples in 1 ml of equilibration solution (10 mM-HCl, pH 2.0) were loaded on to the Mono-Q column (0.5 cm × 5 cm) by means of Pharmacia f.p.l.c. pumps, followed by a 4.5 ml wash with the same solution. A linear gradient of 0–1 M-NaCl (15 ml in 10 mM-HCl, pH 2.0) was then run through the column, followed by a 1.5 ml wash with the original solution. A flow rate of 1 ml/min was used, and 0.3 ml fractions were collected, covering the entire run (70 fractions). Portions were taken from each fraction for scintillation counting of radioactivity.

The system was calibrated with disaccharides of known sulphate content. The elution positions were as follows: hyaluronate disaccharide (unsulphated), fractions 4–10; chondroitin sulphate monosulphated disaccharide, fractions 22–26; chondroitin sulphate disulphated disaccharide, fractions 40–45; heparin trisulphated disaccharide [Δ IdoA(2S) α 1-4GlcNSO₃(6S)], fractions 58–62. Free ³⁵SO₄²⁻ was eluted as a sharp peak at fractions 30–31, and was clearly resolved from O-sulphated disaccharides (see Fig. 3).

Scintillation counting of radioactivity

Samples (maximum volume of 0.3 ml) were mixed with 3 ml of Ready-Value scintillant (Beckman Instruments) and radioactivity was determined in a Betatrac 6895 counter (Tracor Analytic). Counting efficiencies were determined for both ³⁵S and ³H by using suitable channel-window settings in order to allow calculations of radioactivity (d.p.m.) to be made on the dual-labelled samples.

RESULTS

Gel chromatography of heparan sulphate oligosaccharides

The heparan sulphate chains prepared from the proteoglycan secreted by human skin fibroblasts (see the Experimental section) were eluted after alkali treatment from Sepharose CL-6B as a single peak, K_{av} 0.34 (result not shown), corresponding to an M_r of approx. 45000 (Wasteson, 1971). The heparan sulphate chains were eluted as a narrow symmetrical peak when subjected to gradient ion-exchange chromatography on Mono-Q (result not shown). In order to investigate further the domain structure of this heparan sulphate, three different specific scission techniques were employed. These were low-pH HNO₂ and the enzymes heparinase and heparitinase I; the glycosidic linkages that are susceptible to these reagents are shown in Table 1. Intact heparan sulphate chains were depolymerized by using these reagents and subjected to fractionation by Bio-Gel P-6 chromatography. The results are shown in Fig. 1.

Low-pH HNO₂ causes specific and near-quantitative release of N-sulphate groups, with concomitant cleavage of the adjacent hexosaminidic bond (Shively & Conrad, 1976). The resulting gel-filtration profile (Fig. 1a) was characteristic of heparan sulphate (Gallagher & Walker, 1985). As described previously, the ³H-radioactivity profile can be used to calculate the proportions of N-acetylated and N-sulphated glucosamine residues (Winterbourne & Mora, 1981; Hampson *et al.*, 1983). Since each HNO₂-generated saccharide product contains a single reducing-end anhydromannose residue (orig-

Table 1. Susceptible glycosidic linkages for reagents used to depolymerize heparan sulphate

The general formulae for resistant oligosaccharide sequences represent the sequences in the original polymer that would give rise to oligosaccharides containing internal linkages resistant to scission (e.g. $n = 1$ gives tetrasaccharides, $n = 2$ hexasaccharides etc.); R = NAc or NSO₃.

Reagent	Susceptible linkage	General formula for resistant oligosaccharide sequence
Low-pH HNO ₂	GlcNSO ₃ (±6S) α 1-4UA(±2S)	UA-[GlcNAc-GlcA] _n -GlcNSO ₃
Heparinase	GlcNSO ₃ α 1-4IdoA(2S)*	IdoA(2S)-[GlcNR-UA] _n -GlcNSO ₃
Heparitinase	GlcNR α 1-4GlcA†	GlcA-[GlcNSO ₃ -IdoA] _n -GlcNR

* Heparinase scissions linkages in highly sulphated disaccharide units of the type shown, but can also scission where GlcNSO₃ is 6-(O)-sulphated and/or 3-(O)-sulphated (Linker & Hovingh, 1977; Rice & Linhardt, 1989). Resistant linkages occur whenever IdoA(2S) is not present.

† Heparitinase shows specificity for GlcA-containing disaccharide units irrespective of the glucosamine moiety (Linhardt *et al.*, 1990). The enzyme can tolerate 6-(O)-sulphation of GlcNAc, and can also scission where disulphated disaccharide units of the type GlcNSO₃(6S) α 1-4GlcA occur. Resistant linkages are of the type GlcNSO₃(±6S) α 1-4IdoA(±2S), and will thus have a high O-sulphate content.

Fig. 1. Gel filtration on Bio-Gel P-6 of oligosaccharides produced by HNO₂, heparinase and heparitinase depolymerization of heparan sulphate

Heparan sulphate from skin fibroblasts prepared as described in the Experimental section was treated with low-pH HNO₂ (a), heparinase (b) or heparitinase (c), and fractionated on a Bio-Gel P-6 column (1 cm × 120 cm). The column was eluted with 0.5 M-NH₄HCO₃ at 4 ml/h and 1 ml fractions were collected for scintillation counting (—, ³H radioactivity; ·····, ³⁵S radioactivity). In each case a series of oligosaccharides ranging from disaccharides (Di) and tetrasaccharides (Tetra) to larger oligosaccharides [hexasaccharides (Hexa) and above] was observed, depending on the proportion of susceptible linkages and their distribution in the intact molecule. Oligosaccharides (d.p. 4 and larger) are generated when the susceptible linkages are separated by disaccharide units containing resistant linkages (for structural formulae see Table 1). Fractions corresponding to disaccharides were pooled, freeze-dried and analysed further by ion-exchange fractionation on a Mono-Q f.p.l.c. column (see Fig. 3). In the case of the heparitinase-derived oligosaccharides the peak was collected as two fractions (designated 2 and 2') as indicated. The inset in panel (b) shows the heparinase scission profile with an expanded scale in order to reveal the proportions of low-*M_r* products.

inating from a GlcNSO₃ moiety in the intact chain), the percentage of these residues in a particular peak is given by A_n/n , where A_n is the percentage of total ³H radioactivity in that peak and n is the number of disaccharide

repeat units in these oligosaccharides as determined by the elution position. This results in a value of 47% for *N*-sulphation, and hence 53% for *N*-acetylation, giving an *N*-acetyl/*N*-sulphate ratio of 1.13:1, in close agreement with previous analyses of fibroblast heparan sulphate (reviewed by Gallagher & Walker, 1985). Analysis of the HNO₂-hydrolysis profile (Fig. 1a) showed that about 50% of the *N*-sulphate groups were present in uninterrupted sequences (generating disaccharide products) and 30% were in alternating sequence with GlcNAc-containing disaccharide units (generating tetrasaccharide products). The remainder of the *N*-sulphated glucosamine residues were separated by sequences of *N*-acetylated disaccharide units in the intact polymer, giving products ranging from hexasaccharides to oligosaccharides about nine or ten disaccharide units in length (for structural formulae of resistant sequences see Table 1). Most of the ³⁵S label (87%) was eluted with the disaccharide fraction (including the free ³⁵SO₄²⁻ released from *N*-sulphamino groups), 10% was present in tetrasaccharides and the remainder (3%) was in the larger oligosaccharides.

Scission with heparinase results in an entirely different profile (Fig. 1b) from that obtained with HNO₂. Only small amounts of di- and tetra-saccharides were produced by heparinase, and larger oligosaccharides containing resistant internal sequences (see Table 1) were predominant. Very few intermediate-sized oligosaccharides (d.p. 6–12) were observed (see Fig. 1b inset), with the majority being d.p. ≥ 14 in size. Comparison of the ³⁵S/³H ratios in these fragments indicated higher sulphation in the di- and tetra-saccharides than the intermediate and higher oligosaccharides (Fig. 1b). From the depolymerization profile we calculated that approx. 8–10% of disaccharide units in heparan sulphate contain linkages susceptible to heparinase scission [i.e. GlcNSO₃(±6S)α1-4IdoA(2S)].

Scission with heparitinase, which cleaves linkages between hexosamine and glucuronate residues, gave the distinctive profile shown in Fig. 1(c). The major products were disaccharides, equivalent to 52% of the total repeating disaccharide units in the heparan sulphate chain. Of the oligosaccharides that contain resistant linkages [i.e. GlcNSO₃(±6S)α1-4IdoA(±2S); see Table 1], only a small proportion were tetrasaccharides, the remainder ranging in size from d.p. 6 to d.p. approx. 16–18. From these data it was calculated that 63% of disaccharide units in heparan sulphate contain linkages susceptible to heparitinase, i.e. GlcNRα1-4GlcA (R=Nac or NSO₃; see Table 1). The remaining 37% contain IdoA or IdoA(2S). Heparinase scission data (Fig. 1b) indicated that only about 8–10% of total disaccharide units contain the IdoA(2S) derivative, and we therefore conclude that the majority of the iduronate residues (approx. 75%) were non-sulphated. The degree of sulphation, as reflected in the ³⁵S/³H ratios of the resolved oligosaccharides in the heparitinase scission profile (Fig. 1c), increased with increasing fragment size, although some of the disaccharides in both the partially resolved peaks (designated 2 and 2' in Fig. 1c) were clearly sulphated and were subjected to further analysis by Mono-Q ion-exchange chromatography (see below).

Table 2 shows a summary of the data on the proportion of linkages susceptible to the three reagents used and their arrangement in the intact polymer. The latter can be deduced from the different size classes of oligosaccharide (d.p. 2, d.p. 4, d.p. > 6) generated by

Table 2. Proportion and distribution of linkages susceptible to HNO₂, heparinase and heparitinase in skin fibroblast heparan sulphate

Depolymerization reagent	Proportion of linkages susceptible (%)	Arrangement of disaccharides units with susceptible linkages (%)		
		Contiguous	Alternating*	Spaced†
Low-pH HNO ₂	47	49	30	21
Heparinase	10	27	16	57
Heparitinase	63	82	4	14

* These disaccharide units occur in alternating sequence with a single disaccharide unit containing a linkage resistant to the particular scission technique (see Table 1), thereby generating tetrasaccharides upon depolymerization (see Fig. 1).

† These disaccharide units are spaced apart in the intact chain by sequences of two or more disaccharide units containing linkages resistant to the particular scission technique, thereby generating oligosaccharides d.p. 6 and larger upon depolymerization (see Fig. 1).

depolymerization (e.g. disaccharides are produced wherever disaccharide units containing the susceptible linkages occur in contiguous sequence). The data show that 49% and 82% of disaccharide units cleaved by HNO₂ and heparitinase respectively occur in contiguous sequences, whereas the corresponding value for heparinase is only 27%. The majority of the heparinase-sensitive disaccharide units were separated by large resistant sequences in the intact polymer.

Oligosaccharide mapping of heparan sulphate

Heparan sulphate oligosaccharides produced by scission with low-pH HNO₂, heparinase and heparitinase were resolved by gradient PAGE as described in the Experimental section. The resulting oligosaccharide maps are shown in Fig. 2, and confirm the differences in depolymerization patterns observed by gel filtration, since the range and proportions of oligosaccharide products were broadly comparable. However, the superior resolution obtained with gradient PAGE clearly resolves multiple species in each of the size classes (d.p. 2, d.p. 4 etc.), which are generally observed only as single peaks by gel filtration. This is particularly evident for the larger heparitinase-derived oligosaccharides (Fig. 2, track 3), which have relatively high ³⁵S/³H ratios (see Fig. 1c) and therefore contain high proportions of *N*- and *O*-sulphated disaccharides (see also Table 1). In contrast, the larger oligosaccharides generated by HNO₂ scission (d.p. > 6) were less complex (Fig. 2, track 1), reflecting their low sulphate content (Fig. 1a). Heparinase maps revealed few oligosaccharides of intermediate size (d.p. 6–12; Fig. 2, track 2), consistent with the limited breakdown of heparan sulphate by this enzyme (Fig. 1b).

Low-pH anion-exchange chromatography with the use of Mono-Q f.p.l.c.

In order to investigate the structure of this heparan sulphate species further, an ion-exchange system was developed to separate di- and tetra-saccharides on the basis of their sulphate group content. Using a Mono-Q column at pH 2 we found that it was possible to resolve

Fig. 2. Oligosaccharide mapping of heparan sulphate by depolymerization with low-pH HNO₂, heparinase and heparitinase and fractionation by gradient PAGE

Heparan sulphate from skin fibroblasts prepared as described in the Experimental section was treated with low-pH HNO₂ (track 1), heparinase (track 2) or heparitinase (track 3), and the resulting oligosaccharides were separated by gradient PAGE, transferred to nylon membrane and detected by fluorography. Only the bottom 19 cm of the gel is shown, corresponding approximately to the 23–30% portion of the gradient, which is the principal resolving region of this gel. A substantial amount of the heparinase-treated sample (track 2) was observed in the upper portion of the gel, representing unresolved oligosaccharides resistant to heparinase treatment (greater than d.p. approx. 30 in size). The migration positions of Bromophenol Blue (BB), Phenol Red (PR), disaccharides (d.p. 2) and tetrasaccharides (d.p. 4) were as indicated, and oligosaccharides d.p. approx. 12–14 in size migrated to a similar position to that of Bromophenol Blue.

sulphated disaccharides rapidly with a linear gradient of NaCl (see the Experimental section). In addition, free ³⁵SO₄²⁻ was separated as a discrete peak, which was valuable for determining the *N*-/*O*-sulphate ratio of heparan sulphate and for detecting sulphatase contamination in enzyme preparations. No such contamination was observed in the present study.

The disaccharide fraction from HNO₂-treated heparan sulphate (Fig. 1a) was separated by Mono-Q f.p.l.c. into non-, mono- and di-*O*-sulphated species in the proportions 4.2:3.9:1 (Fig. 3a). Free ³⁵SO₄²⁻, corresponding to *N*-sulphate groups, accounted for 74% of the label in the disaccharide peak; taking account of the

Fig. 3. Ion-exchange chromatography of disaccharides produced by HNO_2 , heparinase and heparitinase depolymerization of heparan sulphate

Disaccharides produced by the three scission methods were prepared by Bio-Gel P-6 chromatography (Fig. 1), freeze-dried and analysed by low-pH ion-exchange chromatography (----, 0–1 M-NaCl gradient) on a Mono-Q column (—, ^3H radioactivity; ·····, ^{35}S radioactivity). Standards were used to calibrate the system (see the Experimental section) and showed that non-, mono-, di- and tri-sulphated disaccharides were eluted in the positions indicated (Non-S, Mono-S, Di-S and Tri-S respectively). Disaccharides were derived by (a) HNO_2 treatment, (b) heparinase scission, (c) heparitinase scission (peak 2) and (d) heparitinase scission (peak 2').

sulphated disaccharide units, corresponding to trisulphated disaccharide units in the intact polymer, accounted for 10.8% of disaccharides in this peak, and hence comprised only approx. 2.5% of the total disaccharide units. The original structure was probably $\text{IdoA}(2\text{S})\alpha\text{l-4GlcNSO}_3(6\text{S})$.

The disaccharide products from heparinase depolymerization (Fig. 1b) were resolved by ion-exchange into di- and tri-sulphated species (Fig. 3b), in an approximate ratio of 2.3:1. The lack of monosulphated and non-sulphated disaccharides was expected in view of the specificity of heparinase, for which GlcNSO_3 and $\text{IdoA}(2\text{S})$ are fundamental requirements (Table 1). The number of trisulphated disaccharides is low, and this is consistent with the low frequency of disulphated disaccharides in the HNO_2 hydrolysates (Fig. 3a).

Disaccharides in the heparitinase digest were partially resolved on Bio-Gel P-6, and the two peaks (designated 2 and 2', Fig. 1c), were collected separately and analysed by ion-exchange chromatography. Both peaks contained only non-sulphated and monosulphated disaccharides (Figs. 3c and 3d). Non-sulphated components comprised 91% of peak 2, but only 27% of peak 2'. The structure of the non-sulphated disaccharide units would have been $\text{GlcA}\beta\text{l-4GlcNAc}$ in the intact polymer, since heparitinase has specificity for GlcA residues. The monosulphated disaccharide in peak 2 was resistant to HNO_2 treatment (result not shown); the *O*-sulphate group is likely to be located at C-6 of the GlcNAc residue (Table 1). In contrast, the monosulphated disaccharides in peak 2' (8% of total disaccharides) were HNO_2 -sensitive (result not shown). Taking account of the substrate specificity of heparitinase (Table 1), the structure of the monosulphated disaccharide units in peak 2' would have been $\text{GlcA}\beta\text{l-4GlcNSO}_3$ in the intact polymer. In the total heparitinase-derived disaccharide peaks (2 and 2') the ratio of non- to mono-sulphated disaccharides was 3.5:1.

Combined enzymic depolymerization of heparan sulphate to disaccharides

Heparan sulphate was subjected to depolymerization with a mixture of heparinase, heparitinase I and heparitinase II. Heparinase II has a broad substrate-specificity and will cleave a high proportion of the hexosaminidic linkages in heparin/heparan sulphate, including unsulphated IdoA (Linhart *et al.*, 1990). The resulting oligosaccharides produced by the enzyme 'cocktail' were separated on a Bio-Gel P-2 column (Fig. 4). The yield of disaccharides was 97%, with the remaining 3% in tetrasaccharides. The disaccharides were resolved into two peaks (2 and 2'), with most of the ^{35}S radioactivity (88%) being eluted with the 2' peak. Treatment with HNO_2 demonstrated that the disaccharides in peak 2 were *N*-acetylated and those in peak 2' were *N*-sulphated (results not shown). These peaks were analysed further by Mono-Q f.p.l.c. (Fig. 5). Peak 2 was a mixture of non-sulphated ($\Delta\text{GlcA}\beta\text{l-4GlcNAc}$) and monosulphated disaccharides ($\Delta\text{GlcA}\beta\text{l-4GlcNAc}(6\text{S})$] (17%), whereas peak 2' contained mono-, di- and tri-sulphated disaccharides. The approximate disaccharide composition of heparan sulphate can be calculated from these analyses by comparing analytical data for the combined enzymic depolymerization (Figs. 4 and 5) with breakdown patterns obtained with individual enzymes (Figs. 1 and 3). Details are given in Table 3. Non-

remaining *O*- ^{35}S sulphate located in the tetrasaccharide and larger oligosaccharide peaks (Fig. 1a), a value of 1.8:1 was calculated for the *N*-/*O*-sulphate ratio. Di-*O*-

Fig. 4. Gel filtration on Bio-Gel P-2 of oligosaccharides produced by scission of heparan sulphate with a combination of heparinase, heparinase II and heparitinase I

Heparan sulphate was subjected to scissioning with a combination of heparinase, heparinase II and heparitinase I to depolymerize the molecule completely to disaccharides. The resulting mixture was fractionated on Bio-Gel P-2 (—, ^3H radioactivity; ·····, ^{35}S radioactivity). Peaks were collected as indicated for further analysis by ion-exchange chromatography on Mono-Q (Fig. 5).

sulphated disaccharide units accounted for 46% of total disaccharide units. In contrast, only about 3% of disaccharide units were trisulphated, with the probable structure $\Delta\text{IdoA}(2\text{S})\alpha\text{1-4GlcNS}(6\text{S})$. This value is in agreement with that predicted from HNO_2 hydrolysis (Fig. 3a). The data indicated a ratio of approx. 2:1 for disulphated/trisulphated disaccharides of the type susceptible to heparinase (see Table 3), and this was supported by a similar ratio of these disaccharides observed in the disaccharide peak resulting from heparinase scission alone (Fig. 3b). Most of the sulphated disaccharides contained only one sulphate group (34% of disaccharides). They were predominantly *N*-sulphated ($\Delta\text{UA}\alpha\text{1-4GlcNSO}_3$, 25% of disaccharides) but 9% were *N*-acetylated [$\Delta\text{GlcA}\beta\text{1-4GlcNAc}(6\text{S})$].

An unsuspected feature of the peak 2 profile in Fig. 5 was that the first disaccharide peak to be eluted from the column (peak 2) apparently contained only *N*-acetylated disaccharides, whereas the later-eluted peak (2') contained all the *N*-sulphated components, including the trisulphated derivatives, which have a significantly higher M_r than non-sulphated units. A small amount of tetrasaccharide was produced by the action of the combination of depolymerization enzymes (Fig. 4), indicating that some linkages resistant to all three enzymes may exist. The tetrasaccharide peak had a high $^{35}\text{S}/^3\text{H}$ ratio and is therefore likely to contain *N*-sulphated hexosamine. If this is so, then comparison of the amount of ^3H label in the disaccharide peak 2 with the ^3H label in the combined disaccharide peak 2' and tetrasaccharide peaks gives an *N*-acetyl/*N*-sulphate ratio of 1.22:1, in reasonable agreement with the ratio calculated from HNO_2 hydrolysis (1.13:1; Fig. 1a).

DISCUSSION

The structural analysis of complex polysaccharides

Fig. 5. Ion-exchange chromatography of disaccharides produced by near-complete depolymerization of heparan sulphate (Fig. 4)

Disaccharides produced by the near-complete depolymerization of heparan sulphate by using a combination of heparinase, heparinase II and heparitinase I were partially resolved by Bio-Gel P-2 chromatography (Fig. 4), freeze-dried and further separated by low-pH ion-exchange chromatography on a Mono-Q column (—, ^3H radioactivity; ·····, ^{35}S radioactivity). (a) Peak 2; (b) peak 2'.

such as heparan sulphate poses a major analytical problem. The principal variables in the molecular fine structure of heparan sulphate are the composition of the hexuronate residues and the content and arrangement of the *O*-sulphate groups (see the Introduction). In the present study we have combined specific chemical and enzymic scission and high-resolution separation of the oligosaccharides and disaccharides to elucidate important new characteristics of the heparan sulphate from human skin fibroblasts. The results also raise important questions in relation to polymer biosynthesis and function.

The well-recognized domain structure of heparan sulphate, exhibited by the presence of clusters of *N*-acetylated or *N*-sulphated disaccharide sequences, was confirmed by the HNO_2 and heparitinase depolymerization profiles (Figs. 1a and 1c). The *N*-acetylated sequences varied in size from d.p. 4 to approx. d.p. 20; some of the larger extended *N*-acetylated sequences probably occur directly adjacent to the protein-linkage region of the chain, as observed previously in fibroblast heparan sulphate (Lyon *et al.*, 1987). The heparitinase profile also

Table 3. Disaccharide composition of skin fibroblast heparan sulphate

These compositions are based on the disaccharide content of peak 2 (*N*-acetylated disaccharides) and peak 2' (*N*-sulphated disaccharides) resolved on Bio-Gel P-2 after depolymerization of heparan sulphate with polysaccharide lyases (Fig. 4) and analysed by ion-exchange chromatography with the use of Mono-Q f.p.l.c. (Fig. 5). The disaccharide units shown represent the structures as found in the intact polymer. The structural assignments are the closest approximations possible within the limitations of the techniques used.

Disaccharide unit type	Composition (% of total disaccharide units)	<i>N</i> -Acetylated disaccharides		<i>N</i> -Sulphated disaccharides	
		Composition (%)	Structure*	Composition (%)	Structure†
Non-sulphated	46	46	GlcA β 1-4GlcNAc	—	—
Monosulphated	34	9	GlcA β 1-4GlcNAc(6S)	10	GlcA β 1-4GlcNSO ₃
				15	IdoA α 1-4GlcNSO ₃
Disulphated	14	—	—	7	IdoA α 1-4GlcNSO ₃ (6S)
				7	IdoA(2S) α 1-4GlcNSO ₃
Trisulphated	3	—	—	3	IdoA(2S) α 1-4GlcNSO ₃ (6S)
Total	97	55		42	

* The estimate of the content of GlcA β 1-4GlcNAc(6S) is supported by the presence of 37 % of the total *O*-sulphate ³⁵S label in tetrasaccharides and larger oligosaccharides after HNO₂ hydrolysis (Fig. 1a). Since most of this *O*-sulphate is likely to be GlcNAc(6S) (Sanderson *et al.*, 1984), this corresponds to about nine or ten *O*-sulphate groups.

† Data from heparitinase scission indicated that 10 % of disaccharides are GlcA β 1-4GlcNSO₃, so the remaining 15 % of monosulphated disaccharides in the *N*-sulphated peak (Fig. 5b) must therefore be IdoA α 1-4GlcNSO₃. Since the total percentage of linkages cleaved by heparitinase was approx 10 % (Fig. 1b) and 3 % of the required IdoA(2S) is in the trisulphated fraction, about 7 % of the disulphated fraction will be IdoA(2S) α 1-4GlcNSO₃. The remaining disulphated disaccharides are likely to contain 6-*O*-sulphated GlcNSO₃ linked to IdoA.

demonstrated the close association of IdoA with the sulphate-rich regions of heparan sulphate (i.e. the *N*-sulphated domains), and of GlcA with GlcNAc in regions where the level of sulphate substitution is very low. The association of IdoA with GlcNSO₃ would be predicted from the specificity of the hexuronate epimerase (Lindahl & Kjellén, 1987).

The relatively low C-2 *O*-sulphation of IdoA residues (only about 25 % were sulphated) was an interesting finding in view of the close coupling of epimerization and C-2 *O*-sulphation in the biosynthesis of heparin (Lindahl & Kjellén, 1987). The heparitinase scission profile (Fig. 1c) indicated the existence of domains composed of contiguous sequences of IdoA-containing disaccharide units. Since IdoA is always adjacent to GlcNSO₃, these sequences, which vary in length from two to eight disaccharide units, represent the minimum size of the *N*-sulphated domains. Short sequences of disaccharide units containing non-sulphated IdoA have been detected previously in human lung fibroblasts on the basis of their resistance to mild periodate oxidation (Sjöberg & Fransson, 1980). Similar sequences were not identified in a recent study of heparan sulphate from cultured endothelial cells (Nader *et al.*, 1987). The non-sulphated IdoA residue has considerable conformational versatility (Sanderson *et al.*, 1987; Casu *et al.*, 1988), and the IdoA-rich sequences in skin fibroblast heparan sulphate are likely to be flexible domains that could be very important for the binding properties of the polysaccharide at the cell surface.

Only 8–10 % of disaccharide units were susceptible to heparitinase scission (Fig. 1b), and the majority of these were separated by large resistant sequences in the intact polymer (Table 2). Thus heparin-like domains consisting of multiple sequences of sulphated IdoA-containing

disaccharide units are rare in heparan sulphate, confirming earlier observations on other cell types (Cifonelli & King, 1977; Nader *et al.*, 1987). The ratio of di- to tri-sulphated heparitinase-cleavage disaccharide units (see Fig. 3b and Table 3) indicated that the former species, GlcNSO₃ α 1-4IdoA(2S), is the predominant cleavage site for this enzyme in the skin fibroblast heparan sulphate. Trisulphated disaccharide units of structure IdoA(2S) α 1-4GlcNSO₃(6S) are minor constituents, comprising only 3 % of disaccharide units (Figs. 4 and 5). Yoshida *et al.* (1989) also found a low concentration of this trisulphated disaccharide unit in bovine kidney heparan sulphate. However, a relatively high proportion of these disaccharide units (approx. 12 %) has been observed in heparan sulphate from a neuroblastoma cell line (Hampson *et al.*, 1983, 1984), indicating that this is a variable structural feature. The content of trisulphated units in heparan sulphate chains from cell cultures is significantly lower than that in a 'heparan sulphate-like' fraction obtained as a by-product of heparin preparation (Guo & Conrad, 1989).

Analysis of the disaccharides produced by heparitinase revealed an important difference in the control of sulphation of GlcNAc and GlcNSO₃. Heparitinase produces disaccharides from sequences of the type GlcA β 1-4GlcNR α 1-4GlcA (R = NAc or NSO₃) irrespective of the presence of *O*-sulphate at C-6 of the hexosamine moiety (Linhardt *et al.*, 1990; see Table 1). Some of the *N*-acetylated disaccharides derived from these sequences were *O*-sulphated (Figs. 1c and 3c), but when the amino sugar was GlcNSO₃ *O*-sulphation was not detected. This was deduced from the lack of disulphated components in the *N*-sulphated disaccharide peak (peak 2' in Fig. 1c) analysed by Mono-Q f.p.l.c. (Fig. 3d). The results suggest that different enzymes may catalyse the *O*-sulphation of

Scheme 1. Schematic representation of the principal modifications presumed to be involved in the biosynthesis of skin fibroblast heparan sulphate

The disaccharide GlcNAc α 1-4GlcA represents the basic repeating unit of the initial polymerization product, *N*-acetylheparosan. The scheme illustrates the steps likely to be involved in the conversion of this polymer by the principal modifications required to form the trisulphated disaccharide GlcNSO₃(6S) α 1-4IdoA(2S). It assumes a similar sequence of biosynthetic reactions as those elucidated for heparin (Lindahl *et al.*, 1986; Lindahl & Kjellén, 1987). *O*-Sulphation of GlcNAc at C-6 is also included in the scheme. Based on the data described in this paper, the proportion of disaccharide units in *N*-acetylheparosan undergoing conversion at each stage is shown adjacent to the arrows, and indicates clearly the incomplete nature of these modifications in heparan sulphate. The scheme accounts for about 20–22 of an estimated 26 *O*-sulphate groups in the final product. The disaccharides indicated with an asterisk (*) are the most likely potential sites for further modification by virtue of C-6 *O*-sulphation, and would account for the majority of the remaining *O*-sulphate groups (see the Discussion section).

GlcNAc and GlcNSO₃, and this would provide a mechanism for independent regulation of the modification of the two types of hexosamine residue. An IdoA residue is likely to be a requirement for the *O*-sulphotransferase that acts on GlcNSO₃, whereas a basic requirement for a GlcNAc *O*-sulphotransferase is predicted to be an adjacent *N*-sulphated disaccharide. This is because sulphated GlcNAc residues have only been detected to date in sequences of GlcNSO₃ α 1-4UA α 1-4GlcNAc(6S) (Sanderson *et al.*, 1984). The small number of *O*-sulphate groups in the extended *N*-acetylated regions (Fig. 1a) are likely to be located at the ends of these sequences, adjacent to *N*-sulphated disaccharide units in the intact polymer.

Data from HNO₂ hydrolysis showed that the *N*-/*O*-sulphate ratio of this heparan sulphate was 1.8:1 (Figs. 1a and 3a). Since 47% of the disaccharide units were *N*-sulphated we calculated that there were approx. 26 *O*-sulphate groups per 100 disaccharide units. An estimate can also be made of the distribution of these *O*-sulphate groups: eight to ten are present on IdoA(2S) (from heparinase scission data), about nine are in the form of GlcNAc(6S) (Figs. 4 and 5) and the remainder (seven to

nine) are probably attached to C-6 of GlcNSO₃. Three of the GlcNSO₃(6S) constituents will be linked to IdoA(2S) to form the 3% of disaccharide units that are trisulphated (Figs. 4 and 5). It is noteworthy that C-6 *O*-sulphation of GlcNAc is a major site of *O*-sulphation, being approximately equal to C-2 sulphation of IdoA and probably greater than that of GlcNSO₃ at C-6. This is in marked contrast with heparin, where the latter two types of *O*-sulphate substitution are predominant. These assignments are the closest approximations that we can make to the actual sites of *O*-sulphation. Based on these data, Scheme 1 shows a representation of the principal modifications presumed to be involved in the biosynthesis of skin fibroblast heparan sulphate. It illustrates clearly the incomplete conversion of disaccharides at each potential modification step. Lindahl *et al.* (1986) proposed a speculative model for heparin biosynthesis whereby the proteoglycan traverses several extended linear arrays of membrane-bound modification enzymes arranged in the order in which the reactions proceed. A similar biosynthetic mechanism for heparan sulphate in which the individual polymer-modifying enzyme complexes are arranged in discrete clusters rather than continuous arrays could potentially generate the domain organization that we have described in the present paper. The availability of cell mutants defective in different biosynthetic enzymes (Bame & Esko, 1989) should assist the investigation of the validity of this model for heparan sulphate biosynthesis.

The oligosaccharide maps (Fig. 2) indicated that the sulphation patterns in the intact polymer were complex, and the sequence analysis of heparan sulphate still presents a major challenge. Our separation procedures did not enable us to identify the rare GlcA(2S) (Bienkowski & Conrad, 1985) and GlcNSO₃(3S) residues, the latter being an essential component of the antithrombin III-binding sequences in heparin and heparan sulphate (Lindahl *et al.*, 1984; Pejler *et al.*, 1987). However, our present techniques have revealed new insights into the molecular basis of the structural polymorphism of heparan sulphate, and should be valuable for comparing the fine structural characteristics of different heparan sulphate species.

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