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Structural studies on heparan sulphate from human lung fibroblasts

Characterization of oligosaccharides obtained by selective periodate oxidation of D-glucuronic acid residues followed by scission in alkali

Ingrid SJÖBERG and Lars-Åke FRANSSON
Department of Physiological Chemistry 2, University of Lund, S-220 07 Lund, Sweden

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1. ^{3}H- and ^{35}S-labelled heparan sulphate was isolated from monolayers of human lung fibroblasts and subjected to degradations by (*a*) deaminative cleavage and (*b*) periodate oxidation/alkaline elimination. Fragments were resolved by gel- and ion-exchange-chromatography. 2. Deaminative cleavage of the radioactive glycan afforded mainly disaccharides with a low content of ester-sulphate and free sulphate, indicating that a large part (approx. 80%) of the repeating units consisted of uronosyl-glucosamine-*N*-sulphate. Blocks of non-sulphated [glucuronosyl-*N*-acetyl glucosamine] repeats (3–4 consecutive units) accounted for the remainder of the chains. 3. By selective oxidation of glucuronic acid residues associated with *N*-acetylglucosamine, followed by scission in alkali, the radioactive glycan was degraded into a series of fragments. The glucuronosyl-*N*-acetylglucosamine-containing block regions yielded a compound *N*-acetylglucosamine–R, where R is the remnant of an oxidized and degraded glucuronic acid. Periodate-insensitive uronic acid residues were recovered in saccharides of the general structure glucosamine–(uronic acid–glucosamine)$_n$–R. 4. Further degradations of these saccharides via deaminative cleavage and re-oxidations with periodate revealed that iduronic acid may be located in sequences such as glucosamine-*N*-sulphate → iduronic acid → *N*-acetylglucosamine. Occasionally the iduronic acid was sulphated. Blocks of iduronic acid-containing repeats may contain up to five consecutive units. Alternating arrangements of iduronic acid- and glucuronic acid-containing repeats were also observed. 5. ^{3}H- and ^{35}S-labelled heparan sulphates from sequential extracts of fibroblasts (medium, EDTA, trypsin digest, dithiothreitol extract, cell-soluble and cell-insoluble material) afforded similar profiles after both periodate oxidation/alkaline elimination and deaminative cleavage.

In recent years the synthesis and secretion of proteoglycans (glycosaminoglycans) by cultured cells of different origin have been studied in a number of laboratories. Although the composition, as well as the quantities, of glycosaminoglycans vary considerably, one glycan, heparan sulphate, seems to be a ubiquitous component of cell surfaces (Dietrich & Montes de Oca, 1978). The release of heparan sulphate chains from their proteoglycans is usually accomplished by trypsin digestion. The heparan sulphate isolated from ascites hepatoma cells by this procedure was highly heterogeneous with regard to sulphate content (Nakamura *et al.*, 1978). The cell surface heparan sulphate produced by primary cultures of mouse embryo cells consisted of two forms that differed in sulphate content (Keller *et al.*, 1978). One form was similar to the heparan sulphate produced by cell lines that exhibit contact inhibition of growth. The other form was akin to heparan sulphate derived from virus-transformed cell lines that lack growth control. Although heparan sulphate has been implicated in several specific cellular functions such as growth control, cell–cell contact and binding of lipoproteins and lipoprotein lipase (see also Olivecrona *et al.*, 1977), its overall biological function remains unknown. Accordingly, the structural variability of this glycan has never been rationalized.

The carbohydrate backbone of heparan sulphate

Abbreviations used: GlcN, α-D-glucosamine with an unspecified amino group; $GlcNH_3^+$, α-D-glucosamine with free amino group; GlcNAc and $GlcNSO_3$, *N*-acetylated and *N*-sulphated glucosamine; OseA, glycuronic acid; GlcA, β-D-glucuronic acid; IdoA, α-L-iduronic acid; $-SO_4$, ester sulphate group; aMan, anhydro-D-mannose.

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is identical with that of heparin, i.e. alternating glucosamine and uronic acid residues joined via 1→4 linkages (Lindahl, 1976). The amino sugar moieties are either *N*-acetylglucosamine, *N*-sulphamidoglucosamine or *N*-sulphamidoglucosamine with -SO_4 at C-6 (all α-D). The uronic acid residues are either glucuronic acid (β-D) or iduronic acid (α-L) with the latter residues carrying -SO_4 at C-2 to a variable extent. Therefore considerable structural heterogeneity may be envisaged.

The methods available to determine the structural heterogeneity of heparan sulphate involve scission of bonds between glucosamine and uronic acid residues either by deaminative cleavage with HNO_2 ($GlcNSO_3$→OseA linkages) (Cifonelli, 1968) or by enzymic degradation using various specifically adapted heparitinases (Linker & Hovingh, 1975; Silva *et al.*, 1976). These methods have mainly been applied to so-called heparin byproducts from bovine lung and, in the case of cell-surface heparan sulphate, the heparitinases have only been used to distinguish the material from chondroitin sulphate, dermatan sulphate or heparin (Kleinman *et al.*, 1975).

Work from this laboratory has demonstrated that glucuronic acid residues associated with *N*-acetylglucosamine can be selectively oxidized by periodate (Fransson, 1978). By treatment with alkali, oxyheparan sulphate is fragmented into a series of oligosaccharides of the general structure GlcN-$[OseA–GlcN]_n$–R, where R is the remnant of an oxidized and degraded glucuronic acid residue (Fransson *et al.*, 1980*a*). As these oligosaccharide fragments comprise the *N*-sulphated and iduronic acid-rich segments of heparan sulphate, they can be further characterized by deaminative cleavage and re-oxidations with periodate (Fransson *et al.*, 1980*a*).

In the present study ^{3}H- and ^{35}S-labelled heparan sulphate isolated from human embryonic lung fibroblasts and fractions thereof have been fragmented by periodate oxidation/alkaline elimination. The various fragments have been fractionated by gel- and ion-exchange-chromatography and characterized by re-oxidations with periodate and deaminative cleavage.

Experimental

Materials

Glycosaminoglycan standards (hyaluronate, dermatan sulphate, chondroitin sulphate and heparan sulphate), chondroitin sulphate oligosaccharides (hyaluronidase) and enzymes [chondroitinase-ABC (EC 3.2.1.35), papain and trypsin] were obtained from sources listed previously (Malmström *et al.*, 1975; Sjöberg & Fransson, 1977; Fransson, 1978). Carrier-free $Na_2{}^{35}SO_4$ (124Ci/mol) and D-[1-^{3}H]glucosamine (3.2Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Materials used for cell cultures (Earle's minimal essential medium, calf serum and antibiotics) were the same as described earlier (Malmström *et al.*, 1975). Insta-gel and Omnifluor were bought from Packard AB, Bandhagen, Sweden. Sephadex gels and Blue Dextran 1000 were products of Pharmacia Fine Chemicals (Uppsala, Sweden). Microgranular DEAE-cellulose (Whatman type DE-32) was used for ion-exchange chromatography.

Radioactivity was measured with a Packard 2450 liquid-scintillation counter. The scintillation mixture used was Instagel (5ml of liquid mixed with 0.5ml of sample).

Preparation of radioactively labelled glycosaminoglycans

^{3}H- and ^{35}S-labelled heparan sulphate was prepared by methods that have been described in detail elsewhere (Malmström *et al.*, 1975; Sjöberg & Fransson, 1977). The procedure includes the following steps. Fibroblasts (human embryonic lung), grown in monolayer, were maintained on sulphate-poor medium before the addition of radioactivity (5μCi of $Na_2{}^{35}SO_4$ and 1μCi of D-[1-^{3}H]glucosamine/ml of medium). After incorporation of radioactivity for 12h, medium and cells were collected separately. The cell layer was detached by scraping.

In another preparation the cell culture was subjected to sequential extraction (Sjöberg *et al.*, 1979). The following fractions were obtained: medium, an EDTA-extract of the monolayer, a trypsin digest of the detached cells, a dithiothreitol extract of the trypsin-treated cells, material subsequently solubilized from the cells by freeze-thawing and treatment with trichloroacetic acid and finally an insoluble cell fraction.

After addition of carrier heparan sulphate and dermatan sulphate (0.5mg of each) each fraction or extract was dialysed extensively against 0.1M-$(NH_4)_2SO_4$ and then against water, freeze-dried and digested with papain. The radioactively labelled glycans were fractionated by ion-exchange chromatography, either by gradient elution (see below) or in a step-wise manner (Sjöberg *et al.*, 1979).

Degradative methods

Digestion with chondroitinase-ABC was carried out in 0.5M-Tris/HCl, pH8.0, at 37°C overnight. The reaction mixture contained (per ml) 0.2 unit of enzyme, 0.1mg of bovine serum albumin, radioactive substrate and 1mg of carrier (heparan sulphate and dermatan sulphate).

Deaminative cleavage of bonds between *N*-sulphamidoglucosamine and uronic acid was performed

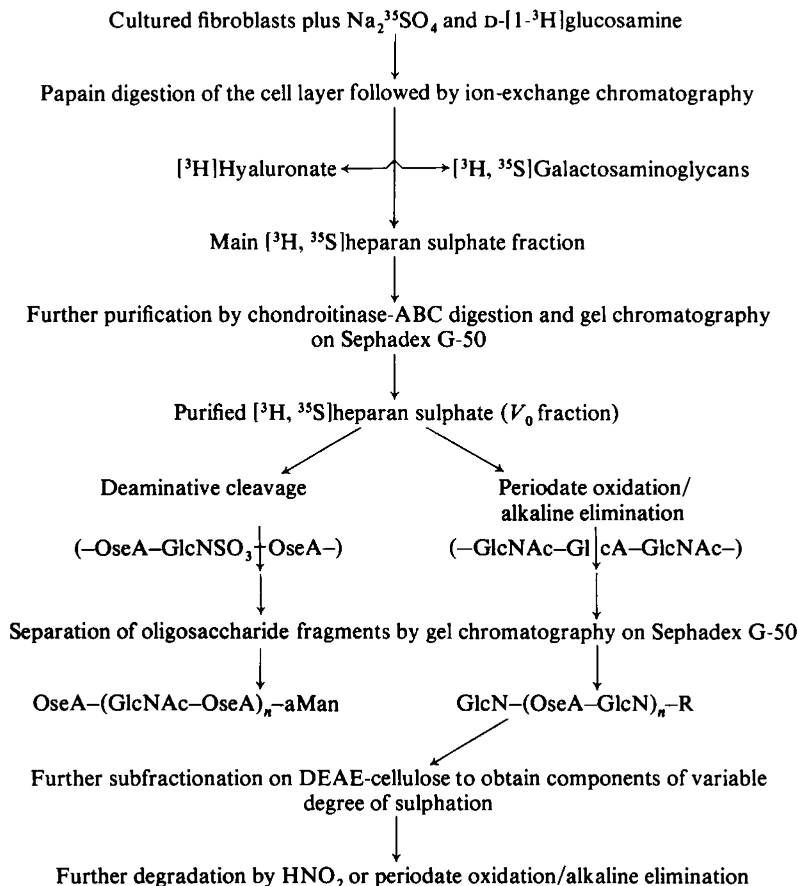
with the pH 1.5/HNO_2 method (Shively & Conrad, 1976*a*). Periodate oxidations were conducted with 0.02M-sodium metaperiodate/0.05M-sodium formate (pH 3.0) at 4°C for 24h in the dark (Fransson, 1978; Fransson *et al.*, 1980*a,b,c*). Reactions were terminated by the addition of 0.1 vol. of 10% (w/v) D-mannitol. After dialysis against distilled water, oxidized products were cleaved by alkaline elimination at pH 12.0 for 30min at room temperature. The products of the above degradations were generally resolved by gel chromatography (for details, see legends to the appropriate Figures).

Release of glucosamine from heparan sulphate was carried out with 6M-HCl at 100°C for 8h. Hydrolysates were then subjected to descending paper chromatography on Whatman 3MM paper in butan-1-ol/pyridine/0.1M-HCl (5:3:2, by vol.) for 28h. Papers were cut into strips (1 cm) which were immersed in scintillation liquid (Omnifluor in toluene, 4g/litre; 10ml/strip of 2 cm^2) and subjected to radioactivity measurements.

Results

Isolation of ^3H- and ^{35}S-labelled heparan sulphate (Scheme 1)

Fibroblasts in culture were given $Na_2{}^{35}SO_4$ and D-[1-^{3}H]glucosamine. ^{3}H- and ^{35}S-labelled glycosaminoglycans were isolated from the cell monolayer after papain digestion (see above). This material was separated into five fractions by ion-exchange chromatography (Fig. 1). Fraction 1, which did not bind to the resin, contained ^{3}H but not ^{35}S. It is assumed to represent glycopeptides derived from cell-surface glycoproteins. Fraction 2, which also contained ^{3}H but no ^{35}S, emerged in the position of standard



Scheme 1. *Flow diagram for the isolation and degradation of ^{3}H- and ^{35}S-labelled heparan sulphate*
The general formulae of the degradation products are given below. R, remnant of an oxidized and degraded glucuronic acid residue.

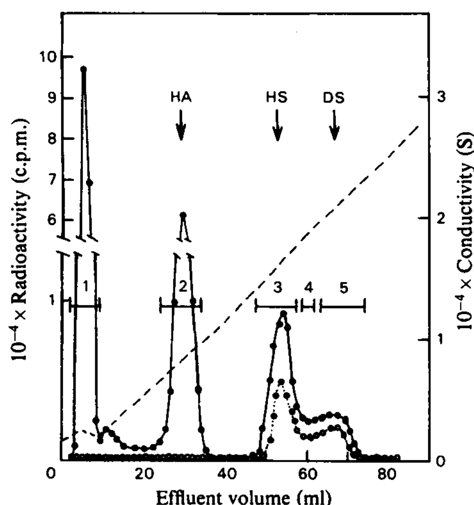


Fig. 1. *Ion-exchange chromatography of* ^3H- *and* ^{35}S-*labelled glycosaminoglycans isolated from human lung fibroblasts (monolayer)*

Radioactive polysaccharides were isolated from the cell monolayer as described in the Experimental section. The column was DE-32 DEAE-cellulose (6mm x 140mm) equilibrated with 0.1 M-sodium acetate, pH 5.0. Elution was with a linear gradient (0.1–2.5 M-sodium acetate, pH 5.0; total elution volume, 100 ml) at a rate of 3 ml/h. The shape of the gradient was determined by conductivity measurements. The points of elution of hyaluronate (HA), heparan sulphate (HS) and dermatan sulphate (DS) are indicated. Material (1–5) was pooled as indicated by horizontal bars, dialysed against water and freeze-dried. ●, ^3H; O, ^{35}S.

hyaluronic acid. The glycans of fractions 3–5 were all labelled with both ^3H and ^{35}S. Fraction 3, which had the same elution position as a heparan sulphate standard, was further purified by digestion with chondroitinase-ABC (to degrade galactosaminoglycans) followed by gel chromatography on Sephadex G-50 (Fig. 2*a*). One portion of the material, emerging with the void volume, was subsequently subjected to deaminative cleavage and re-chromatographed on the same column (Fig. 2*b*). Approx. 80% of the $^{35}SO_4{}^{2-}$ that was incorporated into heparan sulphate was released upon HNO_2 treatment. The remainder was associated with fraction I (OseA–aMan). The other degradation products included tetrasaccharide (II) and one sharply eluted oligosaccharide component (III) of hexa- or octasaccharide size. Neither of these components contained sulphate. After hydrolysis of the ^3H- and ^{35}S-labelled heparan sulphate, all the ^3H radioactivity migrated as glucosamine upon paper chromatography.

Periodate oxidation/alkaline elimination of heparan sulphate (Scheme 1)

The purified ^3H- and ^{35}S-labelled heparan sulphate (Fig. 2*a*) was oxidized with periodate at pH 3.0 and 4°C, followed by scission in alkali. The degradation products were separated on Sephadex G-50 into four fractions (Fig. 2*c*). Fraction I contained free sulphate and small saccharide fragments, whereas fractions II–IV should contain oligosaccharides of the general structure GlcN–[OseA–GlcN]$_n$–R with n varying from 1 to 6. Larger fragments had a higher $^{35}S/^3H$ ratio than did smaller ones.

Fraction I was further purified by ion-exchange chromatography (Fig. 3*a*). The major portion of the material (^3H-labelled) did not bind to the resin (peak 0). This material, which had an R_{GlcN} of 0.4 in paper chromatography, yielded glucosamine upon acid hydrolysis. It is concluded that this material was GlcNAc–R, where R is –O–C(CHO)=CH_2 (the remnant of an oxidized and degraded GlcA) as described earlier (Fransson *et al.*, 1980*c*). A portion of fraction I was retarded on the DEAE-cellulose column. After desalting on Sephadex G-25, subfraction I:1 yielded two components, one a non-sulphated tri- or tetrasaccharide (I:1a) and one a sulphated monosaccharide (I:1b). The former is proposed to be GlcN–OseA–GlcN–R, and the latter could be a sulphated GlcNAc–R. Fraction I:2 contained free sulphate (I:2b) and, presumably, a sulphated version of GlcN–OseA–GlcN–R. Oligosaccharide fraction II (Fig. 2*c*) was separated into four fractions by ion-exchange chromatography (Fig. 3*b*). Subfractions II:1, II:2 and II:3 contained saccharides of the general structure GlcN–OseA–GlcN–R as indicated by gel chromatography (Table 1). The first component (II:1), which was non-sulphated, was, presumably, identical or similar to I:1a. The second and third components (II:2 and II:3) seemed to differ in sulphate content and emerged in different positions upon ion-exchange chromatography (Fig. 3*b*). To ascertain the position of sulphate groups (*N*- and *O*-SO_3) the saccharides were subjected to deaminative cleavage or re-oxidations with periodate. As shown in Table 1, component II:1 was unaffected by HNO_2 but was degraded by periodate oxidation/alkaline eliminations ($K_{av.}$ 0.45→0.54) in keeping with a structure GlcNAc–GlcA–GlcN–R. The next component (II:2) was entirely desulphated by HNO_2. However, only partial depolymerization took place (two components with $K_{av.}$ 0.43 and 0.54 were obtained). It is inferred that this saccharide suffered *N*-desulphation followed by a ring contraction reaction, rather than the usual cleavage of the adjacent glycosidic bond (Shively & Conrad, 1976*b*). Therefore, the main ^3H-containing component ($K_{av.}$ 0.43) should contain the desulph-

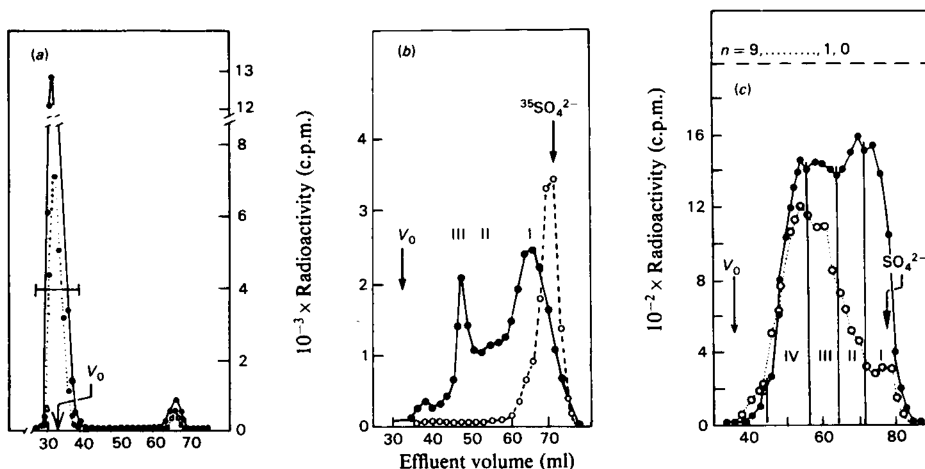


Fig. 2. *Gel chromatography on Sephadex G-50 of (a) ^3H- and ^{35}S-labelled glycosaminoglycan fraction 3 (Fig. 1) after digestion with chondroitinase-ABC, and purified ^3H- and ^{35}S-labelled heparan sulphate after (b) deaminative cleavage and (c) periodate oxidation/alkaline elimination*

The ^{3}H- and ^{35}S-labelled glycosaminoglycan fraction 3 was digested with chondroitinase-ABC as described elsewhere (Sjöberg & Fransson, 1977) and applied to the column (in *a*). Column size, 8mm × 1500mm; eluent, 0.2M-pyridine acetate, pH 5.0; elution rate, 6ml/h; V_0, elution volume of Blue Dextran. The material emerging with the void volume [horizontal bar in (*a*)] was pooled and freeze-dried. One portion was treated with HNO_2 (Shively & Conrad, 1976*a*) and re-chromatographed on the same column (*b*). Another portion was subjected to periodate oxidation/alkaline elimination as described in the Experimental section and re-chromatographed on the same column (*c*). SO_4^{2-}, elution volume of $^{35}SO_4^{2-}$. Fractions were pooled as indicated by vertical lines. ●, ^{3}H; O, ^{35}S.

Table 1. *Results of deaminative cleavage and periodate oxidation/alkaline elimination of oligosaccharide subfractions derived from ^3H- and ^{35}S-labelled heparan sulphate*

The purified ^{3}H- and ^{35}S-labelled heparan sulphate was degraded by periodate oxidation/alkaline elimination and the products were separated on Sephadex G-50 into fractions I, II, III and IV (Fig. 2*c*). Subsequently, fractions II, III and IV were resolved into subfractions by ion-exchange chromatography (Fig. 3). The numbering of the subfractions is given below. The various fractions were desalted on a column (9mm × 1400mm) of Sephadex G-50 that was eluted with 0.2M-pyridine acetate, pH 5.0, at a rate of 10ml/h; V_0 (elution volume of Blue Dextran), 35ml; V_t (elution volume of ^3H_2O), 100ml. The treatments were deaminative cleavage with HNO_2 or periodate oxidation/alkaline elimination (IO_4^-/OH^-) followed by gel chromatography on the same column. The $K_{av.}$ values were recorded in all cases and the presence or absence of $^{35}SO_4^{2-}$ in the various components is indicated below. *Main components. $K_{av.}$ for $^{35}SO_4^{2-}$, 0.55. For *n* see Scheme 1.

Fraction		Size	Treatment ...	None		HNO_2		IO_4^-/OH^-	
G-50	DEAE	*n*		$K_{av.}$	$^{35}SO_4^{2-}$	$K_{av.}$	$^{35}SO_4^{2-}$	$K_{av.}$	$^{35}SO_4^{2-}$
II	1	1		0.45	–	0.46	–	0.54	–
II	2	1		0.45	+	0.43	–	0.49	(+)
						0.54	+	0.54	+
II	3	1		0.45	+	0.43	+	0.43	+
						0.53	+	0.53	+
III	4–6	3		0.40*	+	0.40	(+)	0.40	+
						0.48	+	0.45	+
						0.54	++	0.55	+
III	7–9	4		0.31*	+	0.40	(+)	0.40	++
						0.50	+	0.43	+
						0.56	++	0.56	+
IV	10	5		0.18	+	–		0.21	++
						0.50	+	0.43	(+)
						0.55	++	0.55	+

ated saccharide 2-aldehydo-*O*-pentofuranoside–OseA–GlcN–R and the split product OseA–GlcN–R, whereas the retarded peak should comprise free anhydromannose plus sulphate. This was confirmed by ion-exchange chromatography (as in Fig. 3). Three ^{3}H-labelled components were observed, one of which was not bound to the resin (i.e. anhydromannose). As the other two components seemed to carry a net negative charge, their amino-sugar residues must be *N*-acetylated. It is proposed that saccharide II:2 carried an *N*-sulphate group on the non-reducing terminal amino-sugar residue. The major portion of the uronic acid residues in this saccharide were susceptible to periodate oxidation/alkaline elimination with concomitant release of free sulphate. This suggests that these residues could be either glucuronic acid or iduronic acid (Fransson *et al.*, 1980*a*) leading to a structure GlcNSO$_3$–GlcA/IdoA–GlcNAc–R. As shown in Table 1, saccharide II:3 lost half of its sulphate groups during deamination, in keeping with the presence of equal proportions of *N*-SO$_3$ and *O*-SO$_3$ groups. The finding that half of saccharide II:3 was resistant to periodate oxidation/alkaline elimination indicates that ester-sulphate was located on iduronic acid residues. An iduronic acid(-SO$_4$)-containing saccharide must also contain *N*-sulphamidoglucosamine, as the C-5 epimerase only recognizes glucuronic acid associated with *N*-sulphamidoglucosamine (Lindahl *et al.*, 1976). Thus, a saccharide sequence GlcNSO$_3$–IdoA(-SO$_4$)–GlcNAc–R is proposed for a portion of II:3. The periodate-sensitive saccharides in II:3 may comprise structures like GlcNSO$_3$–GlcA/IdoA–GlcNAc–R. with the additional -SO$_4$ on either amino sugar. The oligosaccharide fraction II:4–6 comprised saccharides of the general structure GlcN–OseA–GlcN–OseA–GlcN–R. Further degradations with HNO$_2$ and by re-oxidation with periodate (results not shown) suggested that their general features were similar to those of I:1–3, i.e. they contained both *N*-acetylglucosamine and *N*-sulphamidoglucosamine as well as glucuronic acid and iduronic acid.

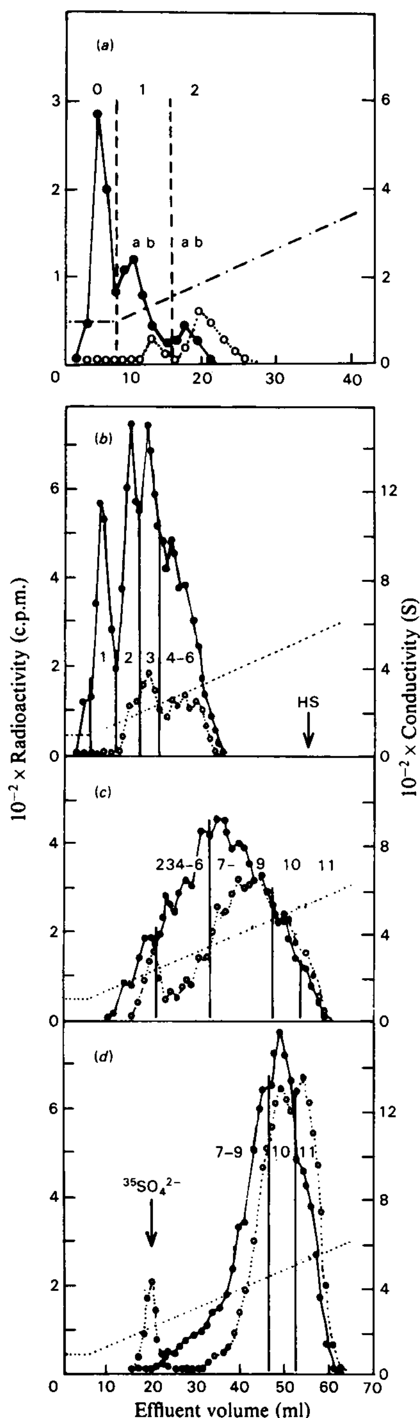


Fig. 3. *Ion-exchange chromatography of oligosaccharide fragments obtained by periodate oxidation/alkaline elimination of ^{3}H- and ^{35}S-labelled heparan sulphate*

The ^{3}H- and ^{35}S-labelled heparan sulphate was purified by ion-exchange chromatography (Fig. 1; fraction 3) and gel chromatography after chondroitinase-ABC digestion (Fig. 2*a*; void volume fraction). This material was subjected to periodate oxidation/alkaline elimination and separated on Sephadex G-50 (Fig. 2*c*) into four oligosaccharide fractions. These fractions (I–IV) were further purified by ion-exchange chromatography; the fractions are I (*a*), II (*b*), III (*c*) and IV (*d*). For column and technical details see the legend to Fig. 1. HS, the elution volume of the starting material. Fractions were pooled as indicated by vertical lines and desalted by gel chromatography (Table 1). ●, ^{3}H; O, ^{35}S.

The oligosaccharide fractions III:4–6, III:7–9 and IV:10 were desalted on Sephadex G-50. The saccharides were still heterogeneous in size, but three components corresponding to saccharides with $n = 3$, 4 and 5 (see Scheme 1) were isolated (Table 1). When these materials were subjected to HNO_2 treatments, the saccharides of III:4–6 and of III:7–9 were extensively *N*-desulphated, yet only partially depolymerized. The larger oligosaccharides (fraction IV:10) were both desulphated and depolymerized. Reoxidation of saccharide III:4–6, which had the general structure GlcN–OseA–GlcN–OseA–GlcN–OseA–GlcN–R, followed by scission in alkali resulted in extensive degradation and release of free sulphate. One component ($K_{av.}$ 0.45) had the same elution volume as II:1–3. Hence, this component should correspond to a fragment GlcN–OseA–GlcN–R, implying that the original saccharide III:4–6 comprised sequences containing alternating glucuronic acid and iduronic acid-bearing repeats. The glucuronic acid residues are susceptible to reoxidation, whereas iduronic acid residues are not (Fransson *et al.*, 1980*c*). In fraction III:7–9 ($n = 4$) a large part of the material was completely resistant to periodate oxidation. This is indicative of the presence of four consecutive IdoA–$GlcNSO_3$ repeats in these fragments. The saccharide III:7–9 ($n = 4$) also contained alternating iduronic acid- and glucuronic acid-containing repeats as a fragment GlcN–OseA–GlcN–R ($K_{av.}$ 0.43) was produced. Similar results were also obtained with the higher oligosaccharides (IV:10).

Degradation of ^{3}H- and ^{35}S-labelled heparan sulphate from various cell extracts

Radioactively labelled heparan sulphates isolated from various cell culture fractions were fragmented by periodate oxidation/alkaline elimination. Only minor differences in the extent of degradation could be discerned. The ^{3}H- and ^{35}S-labelled heparan sulphates isolated from the various fractions were also subjected to deaminative cleavage. These profiles were almost identical.

Discussion

The cell-associated ^{3}H- and ^{35}S-labelled heparan sulphate isolated from human lung fibroblasts showed a relatively sharp elution profile on DEAE-cellulose. The charge density was approx. equal to that of a standard with one sulphate/hexosamine moiety. The material contained a large proportion (approx. 80%) of OseA–$GlcNSO_3$ repeats. The bulk of the GlcA–GlcNAc repeats were present in a block sequence of approx. 3–4 units. Alternating arrangements, i.e. OseA–GlcNAc–OseA–$GlcNSO_3$, were also present to a limited extent. The proportion of ester sulphate was generally low. By fragmentation of the radioactive glycan via periodate oxidation/alkaline elimination information regarding the uronic acid composition of *N*-sulphated block regions could be gained. In this procedure the GlcA–GlcNAc repeats of the glycan were oxidized and degraded. The compound GlcNAc–R was derived from blocks of such repeats. Fragments of the structure GlcN–GlcA–GlcN–R with no sulphate (I:1a and II:1) accounted for 10–15% of the total ^{3}H-radioactivity. As glucuronic acid flanked by two *N*-acetylglucosamine residues is generally sensitive to periodate whereas glucuronic acid associated with *N*-sulphamidoglucosamine or glucosamine with a free amino group may be resistant to oxidation (Fransson *et al.*, 1980*c*) it is possible that saccharides I:1a and II:1 were derived from sequences like –GlcA–GlcNAc–GlcA–$GlcNSO_3$/$GlcNH_3^+$–GlcA– where the internal glucuronic acid residue might survive oxidation. A fragment GlcNAc–GlcA–$GlcNH_3^+$–R produced from this sequence (with or without release of sulphate) would be expected to carry no net negative charge. However, the results of ion-exchange chromatography indicated that the fragment was negatively charged. It is possible that the amino group of the terminal glucosamine residue of this fragment was joined to the –CHO of the R group (Fransson *et al.*, 1980*c*) which is sterically feasible. Formation of an aldimine could conceivably take place during the alkaline elimination reaction.

The sulphated small saccharides II:2 and II:3 had the general structure $GlcNSO_3$–OseA–GlcNAc–R with an additional *O*-sulphate group in the latter case. The uronic acid position was either glucuronic acid, iduronic acid or iduronic acid-SO_4. The presence of sequences such as $GlcNSO_3$–IdoA–GlcNAc is in accordance with the results of other investigations. Deaminative cleavage of intermediates in the biosynthesis of heparin (Höök *et al.*, 1974) yielded a fragment IdoA–GlcNAc–OseA–aMan where the iduronic acid was originally joined to an *N*-sulphamidoglucosamine moiety. Although the extent of sulphation of iduronic acid has not been directly assessed in the present study, the results suggested that a large part of the -SO_4 groups were located in iduronic acid making IdoA(-SO_4)–$GlcNSO_3$ a characteristic repeating unit, in contrast with the situation in heparin where most of these repeats also carry -SO_4 at C-6 of the amino-sugar. Block regions of IdoA–$GlcNSO_3$ (with or without sulphate on iduronic acid) may comprise up to five consecutive units in the present polymer. The higher saccharides (the III and IV-series) included fragments with a composition that allowed for alternating arrangements of IdoA–$GlcNSO_3$ and GlcA–$GlcNSO_3$ (GlcNAc) repeating units. This appears to be another conspicuous feature of heparan sulphate (Fransson *et al.*, 1980*a*). In particular, aggregating

forms of heparan sulphate (Fransson *et al.*, 1980*b*) contain alternating sequences.

In a separate comparative study radioactive heparan sulphates were isolated from sequential extracts of the cell culture. Each species was subjected to periodate oxidation/alkaline elimination and to deaminative cleavage. The profiles obtained with both degradation procedures were remarkably similar, suggesting that there is no selective secretion (or endocytosis) of certain heparan sulphate species. This is in contrast to results obtained with galactosaminoglycans from these extracts (Malmström *et al.*, 1975; Sjöberg & Fransson, 1977; Sjöberg *et al.*, 1979). Dermatan sulphate–chondroitin sulphate copolymers of different tissue culture fractions exhibit considerable variations in their iduronic acid/glucuronic acid ratios.

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