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STRUCTURAL DIFFERENCES BETWEEN BEEF-LUNG HEPARAN SULPHATES WITH SPECIFIC SELF-ASSOCIATIONS

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ABSTRACT

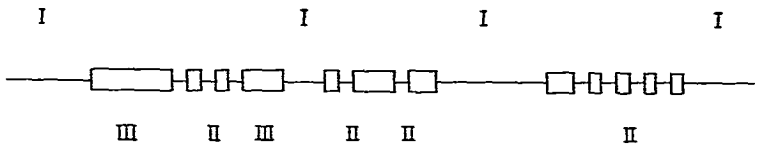
Three, specifically self-associating variants of heparan sulphate (HS2-A, HS3-A, and HS4-A) from beef lung were subjected to (*a*) deaminative cleavage of bonds between 2-deoxy-2-sulphoaminoglucose and uronic acid and (*b*) periodate oxidation of glucuronic acid residues in fully *N*-acetylated block-regions. In addition, the periodate-oxidised and alkali-cleaved chains were re-oxidised with periodate to identify the glucuronic acid residues in the *N*-sulphate-containing regions. The results showed that HS2-A was distinguished by much longer $(GlcA–GlcNAc)_n$-segments than HS3-A and HS4-A. The latter two species were characterised by the structure of the variously *N*-acetyl- and *N*-sulphate-containing regions. In HS3-A, there was a significant contribution from segments composed of both *N*-acetylated and *N*-sulphated 2-amino-2-deoxyglucose residues. The *N*-sulphate-rich regions contained chiefly iduronic acid. In contrast, HS4-A had mixed or alternating arrangements of the two epimeric uronic acids in the *N*-sulphate-rich regions. These differences may be the basis for specific self-associations between heparan sulphate chains.

INTRODUCTION

Heparan sulphate comprises a family of heparin-related glycans that, like heparin, are based on a backbone of alternating, (1→4)-linked β-D(or α-L)-hexuronic acid (HexA) and 2-amino-2-deoxy-α-D-glucose (GlcN) residues[1]. Heparan sulphates that generally occur on cell surfaces, as proteoglycan[2,3], encompass species of considerable structural variability. The origin and purpose of this chemical heterogeneity is obscure, but may be related to their postulated role in cell-recognition phenomena[4]. The ability of heparan sulphates to self-associate in a specific manner, which has been demonstrated for a series of variants from beef lung (see refs. 5–7), raises the possibility that these polysaccharides participate in cell–cell adhesion.

The chemical variability of heparan sulphate arises during the biosynthetic processing of the original $(GlcA–GlcNAc)_n$-polymer. There are two principal polymer-modification steps. In the first one, an *N*-deacetylase forms a number of GlcN residues with free amino groups[8], which are then substrates for an *N*-sulphotransferase[9]. The extent of *N*-deacetylation/*N*-sulphation and the location of modified

residues play a vital role for the ensuing modifications (see below). It may be anticipated that the nature of the deacetylase varies from one cell to another[10]. In the second modification step, the polymer composed of both GlcA–GlcNAc and GlcA–$GlcNSO_3$ repeats is a substrate for a C-5 uronosyl epimerase[11] that converts GlcA residues in $-GlcNSO_3$–GlcA–$GlcNSO_3$ or $GlcNSO_3$–GlcA–GlcNAc sequences into IdoA (see also ref. 12). This recognition requirement results in an arrangement of IdoA and GlcA residues that corresponds to the distribution of GlcNAc and $GlcNSO_3$ residues. In addition, block segments of HexA–$GlcNSO_3$ repeats may give rise to sequences composed of alternating or mixed IdoA–$GlcNSO_3$ and GlcA–$GlcNSO_3$ due to the timing of ester-sulphation[12]. The final product thus comprises "constant" (Type I in Scheme 1) and "variable" regions. The latter occur in two principal arrangements, *i.e.*, Type II with alternating or mixed GlcNAc and $GlcNSO_3$ residues, and Type III, which are largely HexA–$GlcNSO_3$-block regions. As our previous studies have shown[5–7] that the ability to self-associate is correlated with the presence of alternating or mixed repeats of IdoA–GlcN and GlcA–GlcN, it was of interest to investigate whether the specificity of the interaction is related to the arrangement of constant and variable regions in the heparan sulphate chains. Therefore, we have examined the structural features of different, association-prone, beef-lung heparan sulphates that display specificity in their chain–chain association[7].



I = $(GlcA-GlcNAc)_n$; II = $-GlcNSO_3-IdoA-GlcNAc-GlcA-GlcNSO_3-IdoA-$; III = $(IdoA/GlcA-GlcNSO_3)_n$

Scheme 1. Model of a heparan sulphate chain. The backbone $-(HexA-GlcN)_{\sim 30}-$ consists of "constant" (Type I) and "variable" (Types II and III) regions. The constant regions, which may vary in size from n = 1–10, constitute the unmodified portions of the chain. The Type II regions are variations on the theme $-(HexA-GlcNAc)_m-(HexA-GlcNSO_3)_n$ with m and n = 1–3. Owing to the specificity of the C-5 epimerase[12], IdoA only appears in the sequence $-GlcNSO_3$–IdoA–GlcNAc–. The Type III regions are generally longer (n = 3–10) and may contain only IdoA–$GlcNSO_3$ repeats. However, a complex relationship exists between the C-5 epimerase and the *O*-sulphotransferases, which may result in a significant retention of the D-*gluco* configuration. Exactly how this is achieved is not known. The positions of ester sulphate groups have been omitted in this model.

EXPERIMENTAL

Materials. — Heparin from pig mucosa and heparin by-products from beef lung were supplied by Dr. W. E. Lewis, Glaxo, Runcorn, U.K. The methods used for purifying and fractionating the heparan sulphates have been described in detail elsewhere[13]. In this procedure, series of fractions (HS1, HS2, HS3, HS4, and HS5) are obtained that have a progressively increasing content of sulphate and IdoA. Each of these fractions was further separated into more (A-fractions) or less associ-

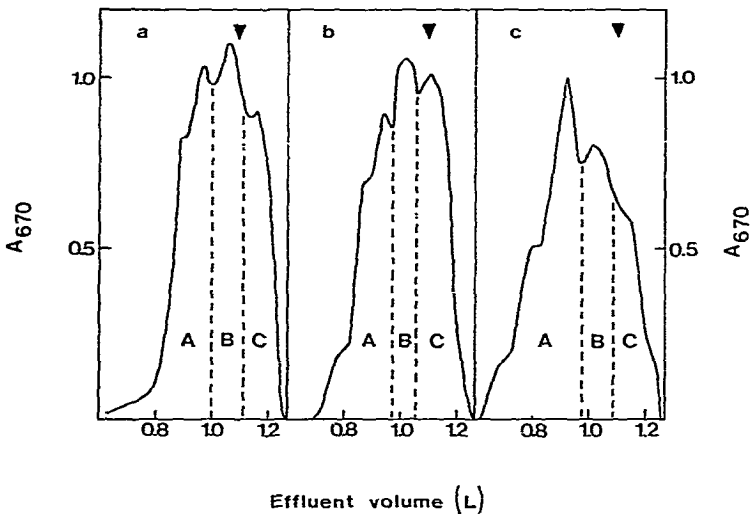


Fig. 1. Preparative gel chromatography of heparan sulphate fractions (a) HS2, (b) HS3, and (c) HS4 on Sepharose CL-6B. Samples (1–2 g) were dissolved in 20 ml of 0.5M sodium acetate (pH 7.0) and applied to a column (35 × 1600 mm) of Sepharose CL-6B. Elution was performed with the same solvent (20 mL/h). The effluent was analysed for uronate by the orcinol method (A_{670}), and fractions (A–C) were combined as indicated by dashed, vertical lines. Material was recovered by ethanol precipitation, followed by drying with absolute ethanol and ether. The elution position of commercial heparin is indicated by an arrow.

TABLE I

ANALYSES OF HEPARAN SULPHATE SUB-FRACTIONS[a]

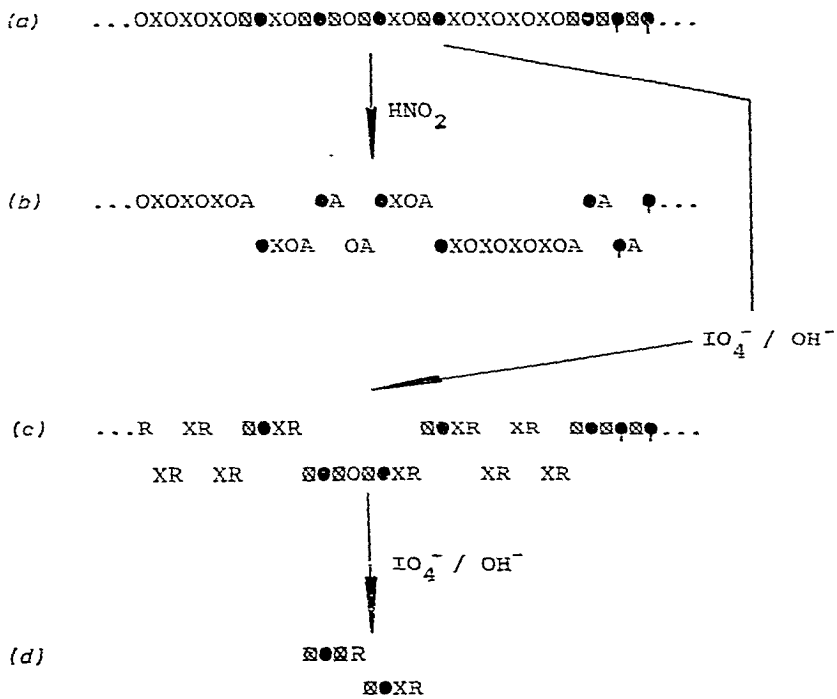
Component	*Sample*					
	HS2-A	*HS2-C*	*HS3-A*	*HS3-C*	*HS4-A*	*HS4-C*
N-SO_3/GlcN	0.20	0.30	0.35	0.60	0.40	0.60
O-SO_3/GlcN	0.25	0.40	0.35	0.80	0.40	0.85
IdoA	20	15	20	5	20	5
IdoA-OSO_3	5	15	15	35	20	35
GlcA	75	70	65	60	60	60

[a]Heparan sulphate was purified from heparin by-products from bovine lung, fractionated according to charge density (HS1–HS4); each fraction was subsequently separated into more or less association-prone variants (A and C) by gel chromatography under associative conditions. Total sulphate (O/N-SO_3) was determined after hydrolysis in 6M HCl at 100° for 8 h and *N*-sulphate (N-SO_3) after hydrolysis in 0.04M HCl at 100° for 1.5 h. Ester sulphate (O-SO_3) = O/N-SO_3 minus N-SO_3. Total uronic acid was determined by the carbazole and orcinol methods. Quantification of IdoA (sulphated and non-sulphated) and GlcA was based on carbazole-to-orcinol ratios. Sulphated IdoA (IdoA-OSO_3) was determined after periodate oxidation at pH 7.0 and 37° for 24 h. Residual carbazole-positive material was considered to represent IdoA-OSO_3. The values are expressed as the percentages of total uronic acid.

ation-prone (C-fractions) variants by gel chromatography[5] (see also Fig. 1). Analyses for the A- and C-fractions are given in Table I.

Disulphated, monosulphated, and non-sulphated disaccharides [type HexA–anManOH (2,5-anhydro-D-mannitol)] from heparin were prepared as described by Jacobsson *et al.*[14].

Analytical methods. — The quantities of hexosamine, sulphate, and hexuronate were determined by published methods[5,6,13]. The proportions of IdoA (with or without $-OSO_3$) and GlcA were determined from the carbazole-to-orcinol ratios[13]. Periodate oxidation (pH 7.0, 37°, 24 h) of heparan sulphate was conducted to determine the amount of IdoA-OSO_3[13,15]. The extent of oxidation was quantified by carbazole measurements[16]. Residual carbazole-positive material was considered to represent IdoA-OSO_3. Radioactivity was assayed with a Packard 2650 liquid scintilla-



Scheme 2. Degradation of copolymeric heparan sulphate. (*a*) A portion of a copolymeric chain that is composed of several different repeating-units where GlcA (○) may be associated with GlcNAc (X) or GlcNSO$_3$ (⊗), whereas IdoA (●) and IdoA-OSO$_3$ (♦) are largely found in *N*-sulphated regions. The GlcNSO$_3$ residues may also be 6-sulphated to a variable degree (not shown). Deaminative cleavage of bonds between GlcNSO$_3$ (⊗) and HexA (○, ●, or ♦) results in the fragmentation shown in (*b*): A = 2,5-anhydro-D-mannose. Selective periodate-oxidation of GlcA (○) in *N*-acetylated regions followed by alkaline elimination releases the *N*-sulphated regions in the form of large oligosaccharides (*c*). The GlcA residues (○) of the latter saccharides are sensitive to re-oxidation with periodate under the same conditions (*d*); R = remnant of oxidised and degraded GlcA. Experiments with model polymers have shown that GlcA associated with GlcNSO$_3$ resists periodate oxidation under the present conditions[15]. However, when present in oligosaccharides such as those obtained in (*c*), most of the residual GlcA residues are cleaved. This has been ascribed to insufficient co-operativity among the hydrogen bonds that create the secondary structure of the carbohydrate chain.

tion counter using Insta-gel (Packard, 0.5 mL of sample mixed with 5 mL of liquid) as scintillator.

Degradation methods. — Deaminative cleavage of the bonds between $GlcNSO_3$ and HexA in heparan sulphate was performed with the pH-1.5 method of Shively and Conrad[17]. The products were sometimes reduced with $Na[^3H]BH_4$ (100 mCi/mg), essentially as described by Jacobsson *et al.*[14].

Periodate oxidation of GlcA in $(GlcA–GlcNAc)_n$-block regions of heparan sulphate[15,16] was carried out in 0.02M $NaIO_4$–0.05M sodium formate (pH 3.0) at 4°. Reactions were terminated by the addition of mannitol, and oxyglycans were recovered after dialysis and freeze-drying. Scission of oxyheparan sulphates was accomplished by treatment with alkali (pH 12, 20°, 30 min). The two degradation pathways are outlined in Scheme 2.

RESULTS AND DISCUSSION

Gross chemical composition. — The heparan sulphate fractions studied here (HS2, HS3, and HS4 in Table I) vary in sulphate content from 0.45 to 1.45 mol/mol of hexosamine. Simultaneously, the total content of IdoA increases from 25 to 40% of total HexA. In the present work, more or less association-prone chains were prepared from HS2, HS3, and HS4 by gel chromatography under associative conditions (Fig. 1). As shown previously[5], the A-fractions comprise the most association-prone variants. The C-fractions, which were eluted in the position of commercial heparin, contained relatively more $IdoA\text{-}OSO_3$ residues than did the A-fractions (Table I). It should also be noted that GlcA–GlcNAc repeats may account for ~80%, 65%, and 55% of the disaccharides in HS2-A, HS3-A, and HS4-A, respectively.

Deaminative cleavage. — As shown in Scheme 2 (a→b), the bonds between $GlcNSO_3$ and HexA may be cleaved by HNO_2 treatment. The *N*-sulphated block-regions are extensively fragmented to disaccharides and higher oligomers. The former include $IdoA(\text{-}OSO_3)$–$anMan\text{-}OSO_3$* (the major repeating-unit of heparin), three mono-sulphated disaccharides [*i.e.*, $IdoA(\text{-}OSO_3)$–anMan, IdoA–$anMan(\text{-}OSO_3)$, and Glc–$anMan(\text{-}OSO_3)$], and two non-sulphated ones [*i.e.*, IdoA–anMan and GlcA–anMan] (see ref. 14). The tetrasaccharides should have the general carbohydrate sequence HexA–GlcNAc–GlcA–anMan, where HexA may be $IdoA\text{-}OSO_3$, IdoA, or GlcA, and further heterogeneity is introduced by a variation in the degree of C-6 sulphation of the hexosamine (including anMan in the product) residues. The $(GlcA–GlcNAc)_n$-segments of the original structure are released in oligosaccharide form on deaminative cleavage.

The nature of the degradation products obtained from the various heparan sulphate fractions was assessed by gel chromatography on Sephadex G-25 and on G-50. In the former case (Fig. 2), the di- and tetra-saccharide species may be resolved. Using ^3H-reduction, the two groups of fragments were identified by their ^3H-to-

*anMan = 2,5-anhydro-D-mannose.

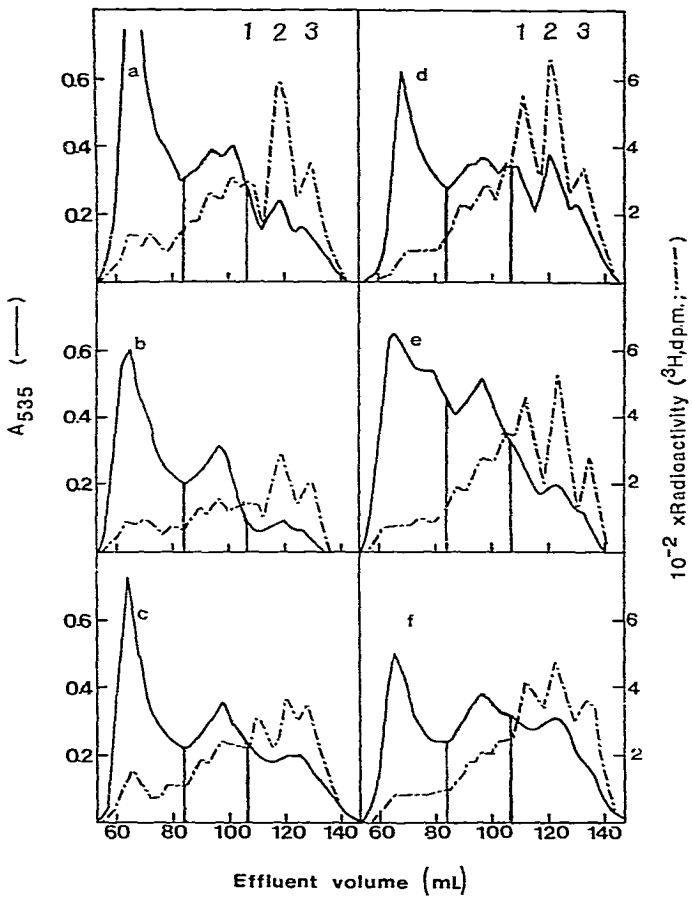


Fig. 2. Gel chromatography of deaminative cleavage products from (a–c) aggregating (A-fractions) and (d–f) non-aggregating (C-fractions) variants of heparan sulphate on Sephadex G-25. More (A-fractions) or less association-prone (C-fractions) variants (20 mg of each) of heparan sulphate fractions HS2 (a and d), HS3 (b and e), and HS4 (c and f) were treated with HNO_2, and the degradation products were reduced with B^3H_4 and chromatographed on a column (12 × 1800 mm) of Sephadex G-25, which was eluted with 0.2M pyridine acetate (pH 5.0) at 10 mL/h. The effluent was analysed for uronate (A_{535} ———) and radioactivity (^3H —·—·—·). The regions in which disaccharides, tetrasaccharides, and higher oligomers are eluted are indicated by vertical lines. Peaks 1–3 are the elution positions of IdoA(-OSO_3)-anManOH-OSO_3, HexA-anManOH(-OSO_3), and HexA-anManOH, respectively; anManOH = 2,5-anhydro-D-mannitol.

uronate ratios. As seen in Fig. 2, the disaccharides were eluted between V_e 105 and 140 mL, and there was a separation between disulphated, monosulphated, and non-sulphated products. Analysis of a commercial heparin preparation in this way yielded a peak at V_e 110–120 mL as the major product (Peak I). The latter peak was particularly prominent in the less association-prone (C-fractions) variants of heparan sulphate (Fig. 2d–f), indicating a higher proportion of IdoA-OSO_3 in these molecules. Among the more association-prone (A fractions) chains (Fig. 2a–c), there was a

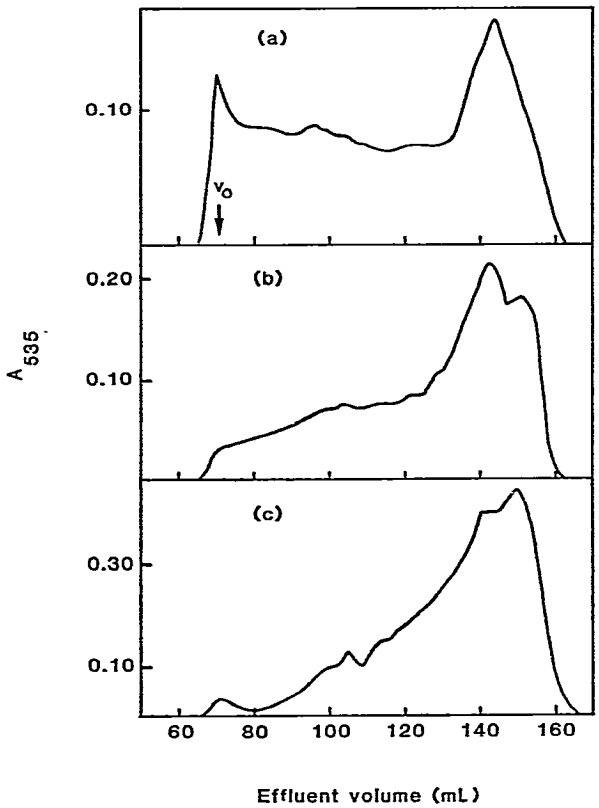


Fig. 3. Gel chromatography of deaminative cleavage products from aggregating variants of heparan sulphate fractions (a) HS2, (b) HS3, and (c) HS4 on Sephadex G-50. The materials were treated as described in the legend to Fig. 2, except that ^{3}H-reduction was omitted. The column (12 × 2000 mm) was eluted and analysed for uronate as in Fig. 2.

progressive, relative increase in the amount of non-sulphated disaccharide (Peak 3; corresponding to IdoA–GlcNSO$_3$ or GlcA–GlcNSO$_3$ in the chain) when going from HS2-A to HS3-A to HS4-A. The shapes of the tetrasaccharide profiles (middle range, V_e 85–105 mL) were similar in most cases. It was noted that HS3-A (Fig. 2b) contained a relatively large proportion of the tetrasaccharides, indicating a high incidence of alternating HexA–GlcNAc and HexA–GlcNSO$_3$ repeats.

As hexasaccharides and higher oligomers are partially or totally excluded from Sephadex G-25, the deaminative cleavage products from HS2-A, HS3-A, and HS4-A were also chromatographed on Sephadex G-50 (Fig. 3). In this case, the resolution in the di- and tetra-saccharide range is poor (V_e 130–160 mL), but the size range of the (GlcA–GlcNAc)$_n$ oligosaccharides may now be assessed. The results show that the fragments obtained from HS2-A were very long, and the elution profile extended from the void volume (larger than icosasaccharide, *i.e.*, $n > 10$) down to the hexasaccharide position (Fig. 3a). The (GlcA–GlcNAc)$_n$-segments of HS3-A and HS4-A were much shorter and also more similar in size distribution (Fig. 3b and c).

Periodate oxidation–alkaline elimination. — Periodate oxidation of heparan sulphate at pH 3.0 and 4° destroys GlcA in $(GlcA–GlcNAc)_n$-regions of the polymer[15,16]. After alkali-catalysed scission of the chain at the oxidised sites, the *N*-sulphate-rich regions may be isolated in oligosaccharide form (Scheme 2a→c). These regions comprise all of the IdoA(-OSO_3)–$GlcNSO_3$, IdoA–$GlcNSO_3$, and GlcA–$GlcNSO_3$ repeating-disaccharides of the intact chain. Experiments with model compounds have shown that GlcA is susceptible to oxidation when flanked by GlcNAc, but resistant when adjacent residues are $GlcNSO_3$ (see ref. 15). In situations like –$GlcNSO_3$–GlcA–GlcNAc or GlcNAc–GlcA–$GlcNSO_3$, the outcome is uncertain. A segment –HexA–$GlcNSO_3$–HexA–GlcNAc–HexA–$GlcNSO_3$–HexA–Glc-NSO_3– gives rise to HexA–GlcNAc–HexA–anMan after HNO_2 treatment, and quantification of the tetrasaccharides gives a measure of the incidence of such arrangements. If either of the HexA residues surrounding the GlcNAc were periodate-

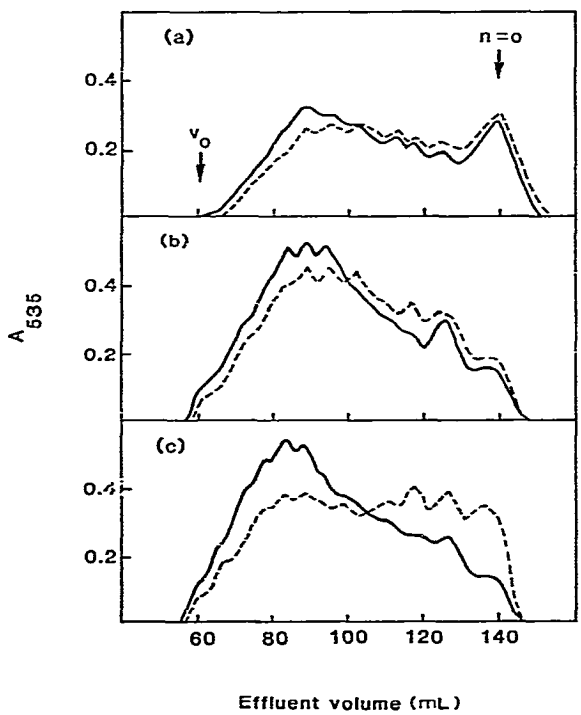


Fig. 4. Gel chromatography of periodate oxidation–alkaline elimination products from aggregating variants of heparan sulphate fractions (a) HS2, (b) HS3, and (c) HS4 on Sephadex G-50. The materials (10–20 mg) were oxidised with periodate at pH 3.0 and 4° for 24 h (see EXPERIMENTAL), dialysed, treated with alkali (pH 12, 20°, 30 min), and applied to a column (12 × 1800 mm) of Sephadex G-50, which was eluted and analysed as in Fig. 2. The result is indicated by ———. In another experiment, the same amount of sample was oxidised with periodate at low pH and temperature, cleaved with alkali, desalted on a column (10 × 1500 mm) of Sephadex G-10, and re-oxidised with periodate under the same conditions followed by alkaline elimination (– – – –); n = 0 is the position of the smallest fragment GlcN-R, where R is the remnant of an oxidised and degraded GlcA residue.

insensitive, the fragments –HexA–$GlcNSO_3$–HexA–GlcNAc–R or GlcNAc–HexA–$GlcNSO_3$–HexA–$GlcNSO_3$– would be obtained. As the yield of the latter fragments was lower[5] than that of the tetrasaccharides obtained *via* deaminative cleavage (above), some of the GlcA residues that are combined with both GlcNAc and $GlcNSO_3$ must be sensitive to oxidation. The GlcA residues flanked by two $GlcNSO_3$ residues are generally resistant to periodate oxidation. However, after cleavage of the oxidised chain in alkali, these GlcA residues also become susceptible to re-oxidation with periodate under the same conditions[5,15]. As depicted in Scheme 2 (c→d), re-oxidation with periodate followed by alkaline cleavage affords further fragmentation which permits the localisation of such GlcA residues.

The results obtained with HS2-A, HS3-A, and HS4-A are shown in Fig. 4. The fragments have the general structure GlcN–$(HexA–GlcNSO_3)_{n-1}$–HexA–GlcN–R. Those derived from HS2-A ranged from $n = 1$ to $n = 7$, with a considerable contribution from the lower range. Thus, the relatively large yields of (*a*) the tetrasaccharides HexA–GlcNAc–GlcA–anMan (Fig. 2a) after HNO_2 treatment and (*b*) the fragments GlcN–$(HexA–GlcNSO_3)_{0-3}$–HexA–GlcN–R after periodate oxidation (Fig. 4a) suggest that such a sequence as –[IdoA/GlcA–GlcNAc–GlcA–$GlcNSO_3$–$(IdoA/GlcA–GlcNSO_3)_n$–IdoA/GlcA–GlcNAc]–, with $n = 0$–3, may be quite common in HS2-A. The initial periodate-oxidation products of HS2-A were poorly affected by re-oxidation (--- in Fig. 4a), which suggests that GlcA residues are rare in the central position of this sequence. The latter may only occur a few times, on average, in each chain, as judged from the size distribution (Fig. 3a) and relative abundance (Table I) of $(GlcA–GlcNAc)_n$-regions in HS2-A.

The periodate-oxidation fragments derived from HS3-A and HS4-A had a similar size distribution, with a large proportion of deca-, dodeca- and tetradecasaccharides (Fig. 4b and c). However, the sensitivity of these fragments to re-oxidation with periodate was different (------), which indicates that such sequences as –$GlcNSO_3$–IdoA/GlcA–$GlcNSO_3$–IdoA/GlcA–$GlcNSO_3$–are more common in HS4-A than in the others. As a low degree of ester-sulphation may favour retention of the D-*gluco* configuration during biosynthesis[12], the relatively high yield of non-sulphated disaccharides (HexA–anMan) after HNO_2 treatment of this material (Fig. 2c) is in keeping with this conclusion.

CONCLUSION

As there is no evidence for a template-regulated biosynthesis of copolymeric glycosaminoglycans, the current notion has been that the processing of these polymers involves a considerable element of randomness. However, it seems biologically unattractive to develop a process in which an ordered sequence as in $–(GlcA–GlcNAc)_n$ is randomly disorganised to produce a heterogeneous mixture of heparan sulphate chains at the expense of considerable amounts of energy.

The results of the present investigation show that specifically self-associating variants of heparan sulphate may be distinguished by, at least, three structural criteria:

i.e. (*a*) the size distribution and location of $(GlcA–GlcNAc)_n$ regions, (*b*) such alternating or mixed segments as $–[GlcNSO_3–IdoA]_m–GlcNAc–[GlcA–GlcNSO_3]_n–$ (*m* and *n* = 1–3), and (*c*) variation of IdoA and GlcA in $(HexA–GlcNSO_3)_n$-segments. The present results also confirm the previous observations[5] that IdoA is generally non-sulphated in the aggregatable chains.

As low-sulphated and hence $(GlcA–GlcNAc)_n$-rich heparan sulphates aggregate poorly[5] and as *N*-desulphation and *N*-acetylation of heparan sulphate abolishes self-interaction[7], we may assume that the *N*-sulphate-rich segments (b and c above) are the contact zones (see also ref. 18). Furthermore, the size of the intervening $(GlcA–GlcNAc)_n$-segments may also play a role by placing the contact zones in their proper register. The heparan sulphate subfraction HS2-A differed from the other two largely with regard to the size and distribution of the intervening segments. Subfractions HS3-A and HS4-A were similar in this respect, but differed in the structure of the putative contact zones. In HS3-A, there was a large contribution of segments with alternating or mixed HexA–GlcNAc and $HexA–GlcNSO_3$ repeats. Consequently, most of the alternating or mixed arrangements of IdoA and GlcA may occur in these segments. The $(HexA–GlcNSO_3)_n$-segments contained mostly IdoA. In HS4-A, however, the variation in IdoA and GlcA distribution resided mainly in the $(HexA–GlcNSO_3)_n$-segments.

Heparan sulphates from lung fibroblasts bind almost exclusively to HS4-A-agarose, but not to the other forms[19]. Interestingly, lung-fibroblast heparan sulphate is composed of $(HexA–GlcNSO_3)_{1-5}$-regions that are separated by short $(GlcA–GlcNAc)_{3-4}$-segments[20], *i.e.*, a structure which resembles that of HS4-A. All of these observations point to an element of complementarity in the heparan sulphate–heparan sulphate interaction.

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