Carbohydrate Research, 110 (1982) 135–144
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

STRUCTURAL REQUIREMENTS FOR HEPARAN SULPHATE SELF-ASSOCIATION

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(Received March 17th, 1982; accepted for publication, April 19th, 1982)

ABSTRACT

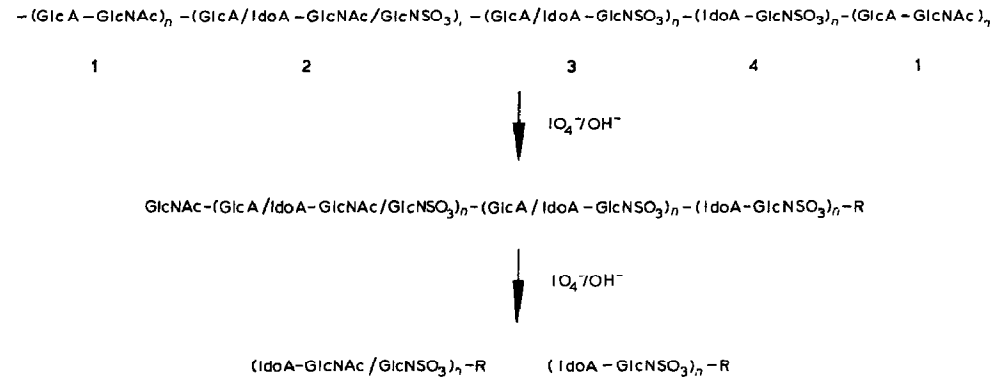
To investigate heparan sulphate self-association, various sub-fractions of beef-lung heparan sulphate have been subjected to affinity chromatography on heparan sulphate–agarose. A particular variant of heparan sulphate was chiefly bound to matrices substituted with the same or cognate heparan sulphates. *N*-desulphation and *N*-acetylation abolished the chain–chain interaction. Also, dermatan sulphates and chondroitin sulphates showed affinity for heparan sulphate–agarose. [^{3}H]Heparan sulphates that were bound to a heparan sulphate–agarose were desorbed by elution with the corresponding heparan sulphate chains and also with unrelated heparan sulphates, heparin, and the galactosaminoglycans to various degrees. However, the corresponding heparan sulphate species was the most efficient at low concentrations. Dextran sulphate was unable to desorb bound heparan sulphate. When the corresponding heparan sulphate was *N*-desulphated/*N*-acetylated, carboxyl-reduced, or periodate-oxidised (D-glucuronate), the modified polymer was unable to displace [^{3}H]heparan sulphate from heparan sulphate–agarose. The displacing ability of heparin was also destroyed by periodate oxidation. It is concluded that self-interaction between heparan sulphate chains is strongly dependent on the overall molecular conformation. The *N*-sulphate and carboxylate groups as well as the integrity of the D-glucuronate residue are all essential for maintaining the proper secondary structure.

INTRODUCTION

Copolymeric glycosaminoglycan chains, *i.e.*, heparan sulphate or dermatan sulphate, can self-associate, as demonstrated by affinity chromatography of free chains on glycosaminoglycan–agarose gels[1–5]. A series of heparan sulphate sub-fractions from bovine lung displayed specific self-interactions, inasmuch as they were chiefly bound to gels substituted with cognate heparan sulphate chains[5]. Similar results were obtained with radiolabelled heparan sulphates from different fibroblastic cell-lines[6]. Affinity gels derivatised with heparan sulphate oligomers of the general structure GlcN–(IdoA/GlcA–GlcN)$_n$–R could also bind intact heparan sulphate

chains[7]. It was concluded that segments comprising both iduronate (IdoA) and glucuronate (GlcA) residues may serve as contact zones in this interaction.

Comparisons between the three specifically self-associating variants from beef lung showed that they differ with regard to the size of the $(GlcA–GlcNAc)_n$ regions (see **1** in Scheme 1) and the nature of the $[(IdoA–GlcN)/(GlcA–GlcN)]_n$ variable regions (**2** and/or **3** in Scheme 1)[8]. Furthermore, the variable regions of heparan sulphates with high affinity for one another were markedly similar, suggesting that self-association may be due to complementary and co-operative interactions between a number of contact zones[9].



Scheme 1. Degradation of a hypothetical heparan sulphate copolymeric sequence. The numbers **1**–**4** indicate various principal types of repeating disaccharide units, *i.e.*, **1** constitutes the original non-sulphated units, and **2**–**4** those obtained by biosynthetic modifications. Type **2** are repeats with either glucuronate or iduronate and with the hexosamine residue either *N*-acetylated or *N*-sulphated; such repeats are often arranged as follows: –$GlcNSO_3$–IdoA–GlcNAc–GlcA–$GlcNSO_3$–. Type **3** repeats constitute *N*-sulphated block-regions where the GlcA residues would be flanked by two $GlcNSO_3$ moieties. Type **4** repeats are "heparin"-like and may also be heavily ester-sulphated on both sugar residues (not indicated in the structure). In type **2** and **3** repeats, the $GlcNSO_3$ residues are also largely ester-sulphated, whereas the IdoA residues are often non-sulphated.

In the present study, the structural requirements for binding to heparan sulphate–agarose have been investigated in more detail.

EXPERIMENTAL

Materials. — Beef-lung heparan sulphates were prepared as described earlier[10]. A series of fractions (HS1, HS2, HS3, HS4, and HS5) was obtained that had a progressively increasing content of sulphate and IdoA. Each of these fractions was further fractionated into more (A-fractions) or less association-prone (C-fractions) variants by gel chromatography[11]. Analytical data for these variants are published elsewhere[8]. Pig-mucosa heparin was kindly supplied by Dr. W. E. Lewis (Glaxo, Runcorn, U.K.). Dermatan sulphate from pig skin was obtained as described[12]. The present preparation was fractionated (as Cs^{2+} salts) consecutively into 18, 36,

and 50% ethanol precipitates. Analyses for these fractions have been presented elsewhere[13] and show that the two former fractions contain 75 and 60% IdoA of total uronate, respectively; hence, the variants were designated DS_{75} and DS_{60}. Chondroitin 4-sulphate was obtained from the Chicago collection. [^{3}H]Heparan sulphates were prepared after partial periodate oxidation (5 min; see below) followed by reduction with Na[^{3}H]BH_4 as described previously. Radiolabelled heparan sulphates were used within one month of preparation. Other sources of materials were as follows: dextran sulphate (mol. wt. ~500,000) and Sepharose 4B, Pharmacia Fine Chemicals; Na[^{3}H]BH_4 (100 mCi/mg), New England Nuclear; Insta-Gel, Packard; and guanidine HCl, Sigma Chemical, purified as described[5].

Affinity matrices. — The procedures for immobilising heparan sulphates onto Sepharose 4B have been outlined[5,7]. Agarose was derivatised with adipic acid dihydrazide using CNBr. Heparan sulphate chains were oxidised briefly (5 min) with periodate (~5% destruction of GlcA), and aldimines formed between the spacer and the oxyglycan were finally stabilised by reduction.

Affinity chromatography. — Studies of the binding between various glycosaminoglycans were performed at room temperature on columns (6 × 100 mm) containing Sepharose 4B substituted with various forms of heparan sulphate. The gels were equilibrated with 0.15M NaCl, and samples (100 or 200 μg of radiolabelled or unlabelled polysaccharide) dissolved in 50 or 100 μL of the same solvent were applied. Then the column was eluted (*a*) with a linear gradient of 0.15M NaCl→1.5M guanidine HCl (total volume, 100 mL); or (*b*) with 0.15M NaCl, followed by a freshly made glycosaminoglycan solution (usually 0.5 mg/mL of 0.15M NaCl) and, finally, by 2M guanidine HCl, all at a rate of ~3 mL/h. The effluents were collected in 1.1–1.2-mL fractions (LKB Redirac equipped with a drop counter) and analysed, when appropriate, for uronate by the orcinol method[14] or for ^{3}H by liquid scintillation in a Packard 2650 instrument with Insta-Gel as scintillator; see also refs. 5 and 7.

Modification and degradation of polysaccharides. — Periodate oxidation of GlcA in $(GlcA–GlcNAc)_n$-block regions of heparan sulphate was carried out with 2 mg of glycan/mL of 0.02M $NaIO_4$/0.05M sodium formate (pH 3.0) at 4° for 5 min (partial oxidation) or 24 h (complete oxidation). Reactions were stopped by the addition of mannitol, and oxyglycans were recovered after dialysis and freeze-drying. Alkali-catalysed scission of oxyglycans was conducted at pH 12 and room temperature for 30 min (see also refs. 5–11).

Carboxyl reduction was performed in the presence of carbodiimide in the following manner. A solution of heparan sulphate (210 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (Calbiochem) in water (50 mL) was maintained at pH 4.75 using 0.1M HCl. After 1–2 h, 3.78 g of $NaBH_4$ was added and the solution was kept at 50° for 15 min. Then excess of glacial acetic acid was added and the carboxyl-reduced polymer was recovered after dialysis and freeze-drying.

N-Desulphation of heparan sulphate was accomplished by the solvolytic procedure of Nagasawa *et al.*[15]. *N*-acetylation was effected with acetic anhydride[16].

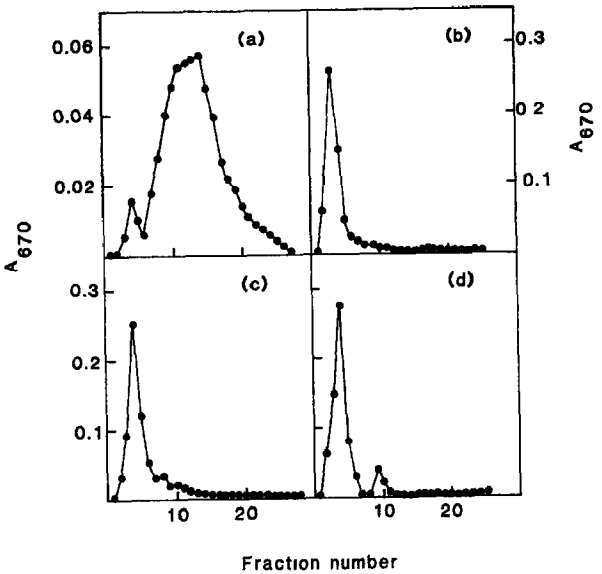


Fig. 1. Affinity chromatography of heparan sulphate HS2-A (a), HS3-A (b), HS4-A (c), and *N*-desulphated, *N*-acetylated HS2-A (d) on HS2-A-agarose. For preparation of the affinity matrix, application of sample, and operation of the column, see Experimental. Elution, linear guanidine HCl gradient (up to 1.5M); analysis, orcinol reaction for uronate.

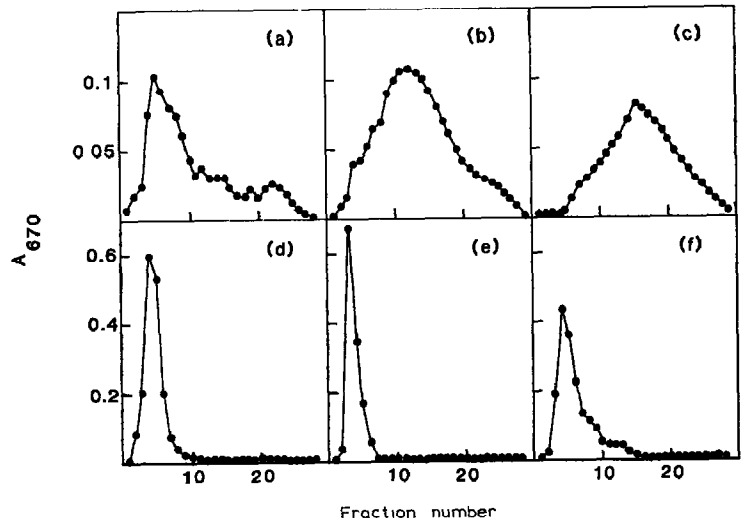


Fig. 2. Affinity chromatography of heparan sulphate HS2-A (a), HS3-A (b), HS4-A (c), HS5-A (d), heparin (e), and *N*-desulphated, *N*-acetylated HS4-A (f) on HS4-A-agarose. For details, see legend to Fig. 1 and Experimental.

RESULTS

Binding of glycosaminoglycans to heparan sulphate–agarose. — Interactions between free and immobilised heparan sulphate chains are maximised[7] when the affinity gels are charged with solutions of free chains in the range 1–2 mg/mL. Bound chains were displaced by elution with 2M guanidine HCl. Using these conditions, it was observed (Fig. 1a) that heparan sulphate sub-fraction HS2-A was effectively bound to gels substituted with the same chains. Other association-prone variants of heparan sulphate, *i.e.*, HS3-A and HS4-A, had no affinity for this matrix (Fig. 1b and c). Furthermore, *N*-desulphation and *N*-acetylation of HS2-A abolished affinity for the cognate chains (Fig. 1d). When HS4-A-agarose was used as affinity matrix, HS2-A showed little or no binding (Fig. 2a), whereas HS3-A and HS4-A were bound (Fig. 2b and c). Non-associating, heparin-related chains, *i.e.*, HS5 and commercial heparin (Fig. 2d and e), passed straight through an HS4-A–agarose. Again, *N*-desulphation and *N*-acetylation of HS4-A destroyed its affinity for intact HS4-A chains (Fig. 2f).

When various galactosaminoglycans were examined by affinity chromatography on HS4-A–agarose (Fig. 3b and c), both relatively GlcA-rich dermatan sulphates (DS_{60}) and chondroitin 4-sulphate exhibited binding that was essentially of the same strength as that of HS4-A (-----). Relatively IdoA-rich dermatan sulphate (DS_{75}) had a somewhat weaker affinity (Fig. 3a) as judged from its elution position.

Displacement of [^3H]heparan sulphate from heparan sulphate–agaroses using glycosaminoglycans. — It was shown earlier[5] that partially periodate-oxidised and radiolabelled heparan sulphates may be bound to heparan sulphate-derivatised gels and displaced by elution with a guanidine gradient (see, *e.g.*, Fig. 6 in ref. 5). In the present study, elution with glycosaminoglycan solutions (0.5 mg/mL) was attempted. As shown in Fig. 4, [^{3}H]heparan sulphate type HS2-A interacted with an HS2-A–agarose. Elution with HS1 (Fig. 4a) released no ^{3}H; the bound heparan sulphate was

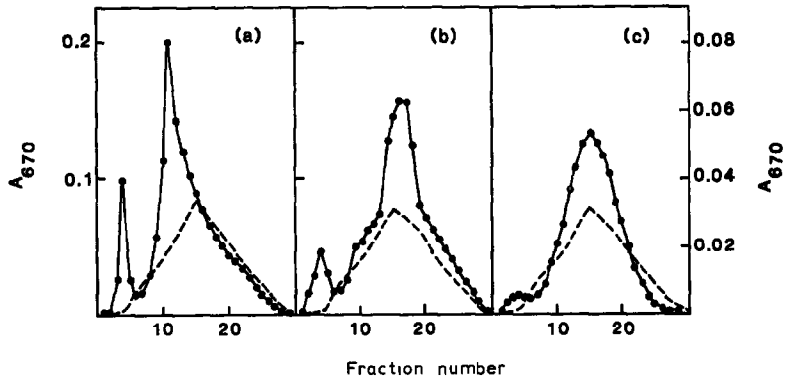


Fig. 3. Affinity chromatography of dermatan sulphate DS_{75} (a), DS_{60} (b), and chondroitin 4-sulphate (c) on HS4-A-agarose. For details, see legend to Fig. 1 and Experimental; -----, elution profile of HS4-A for comparison.

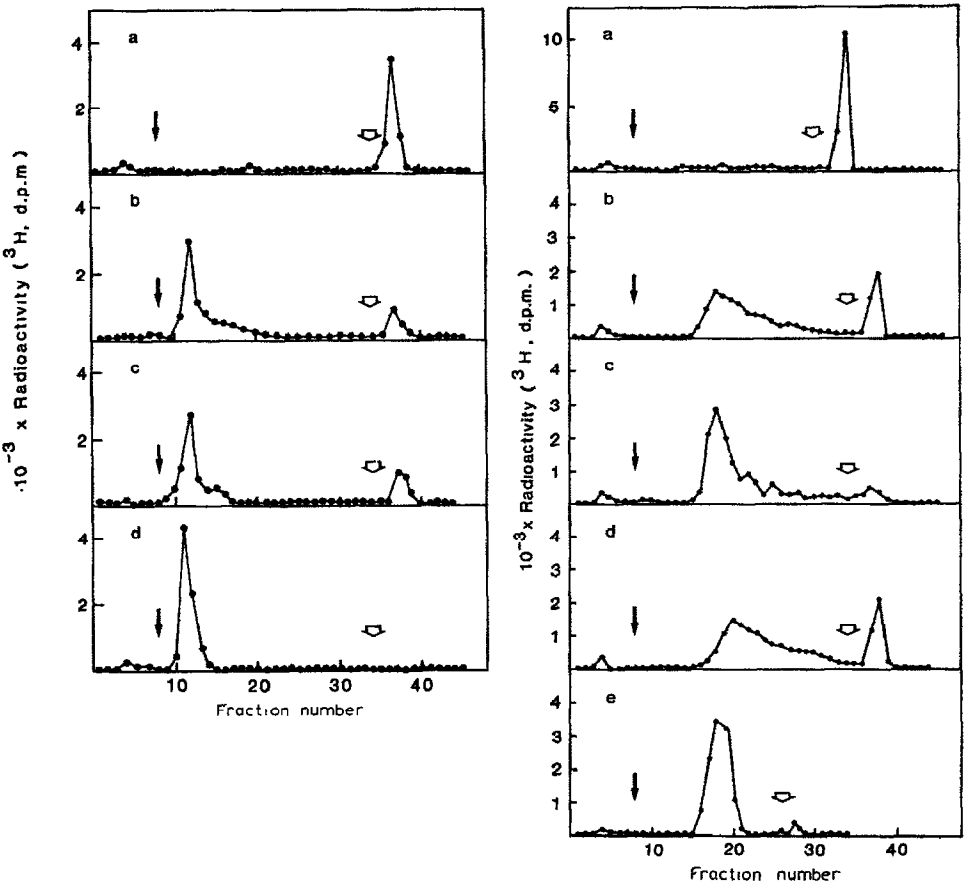


Fig. 4. Affinity chromatography of [^{3}H]heparan sulphate HS2-A on HS2-A-agarose using solutions of heparan sulphate HS1 (a), HS2-A (b), HS3-A (c), or HS4-C (d) as eluting solvents. Samples were applied in 0.15M NaCl, the columns were washed with the same solvent, and then (↓) solutions containing 0.5 mg of the indicated heparan sulphate species per mL of 0.15M NaCl were passed through the columns. Finally, the gels were washed with 2M guanidine HCl (⇓). Analysis, ^{3}H-radioactivity. For further details, see Experimental.

Fig. 5. Affinity chromatography of [^{3}H]heparan sulphate HS4-A on HS4-A-agarose using solutions of heparan sulphate HS2-A (a), HS3-A (b), HS4-A (c), HS4-C (d), or HS5 (e) as eluting solvents. For details, see legend to Fig. 4 and Experimental.

displaced by 2M guanidine HCl. However, elution with HS2-A removed almost entirely the [^{3}H]HS2-A from the affinity matrix (Fig. 4b). Although HS3-A did not interact with HS2-A–agarose (Fig. 1b), the former heparan sulphate species was able to compete with [^{3}H]HS2-A for binding to its cognate gel (Fig. 4c). Also, non-associating, heparin-related glycans, *e.g.*, HS4-C (Fig. 4d), were able to displace [^{3}H]HS2-A entirely. Similar experiments were also conducted with [^{3}H]HS4-A on HS4-A–agarose. Bound material could not be displaced with HS2-A solutions (Fig. 5a), but both HS3-A and HS4-A (Fig. 5b and c) were able to compete with

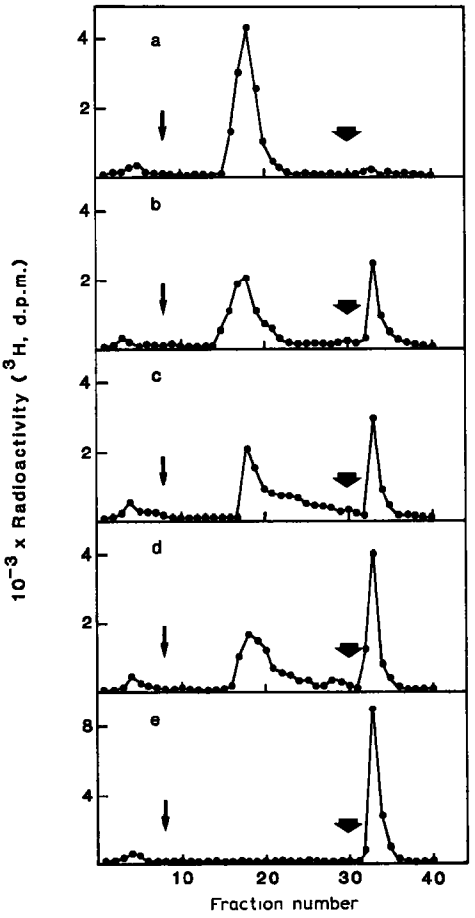


Fig. 6. Affinity chromatography of [^{3}H]heparan sulphate HS4-A on HS4-A-agarose using solutions of heparin (a), dermatan sulphate DS_{75} (b), dermatan sulphate DS_{60} (c), chondroitin 4-sulphate CS (d), or dextran sulphate (e) as eluting solvents. For details, see legend to Fig. 4 and Experimental.

HS4-A for binding. However, HS4-A was more effective than HS3-A. Non-associating variants, *e.g.*, HS4-C, were also partially successful in this respect (Fig. 5d). It should be noted that the heparin-related fraction HS5 was a very effective inhibitor of HS4-A-to-HS4-A–agarose association (Fig. 5e), despite the fact that it was not retarded on an HS4-A–agarose gel (Fig. 2d). In all of the foregoing experiments, the eluting solvents contained 0.5 mg of polysaccharide gel per mL. When HS3-A, HS4-A, or HS4-C was used at higher concentrations (up to 2 mg/mL), the elution position of [^{3}H]HS4-A (as in Fig. 5b, c, and d) was displaced to an earlier position (from fractions 18–20 to 15) and each of these variants completely eluted the bound heparan sulphate (results not shown).

To investigate the displacing ability of other polysaccharides upon the [^{3}H]-HS4-A-to-HS4-A–agarose association, the experiments shown in Fig. 6 were per-

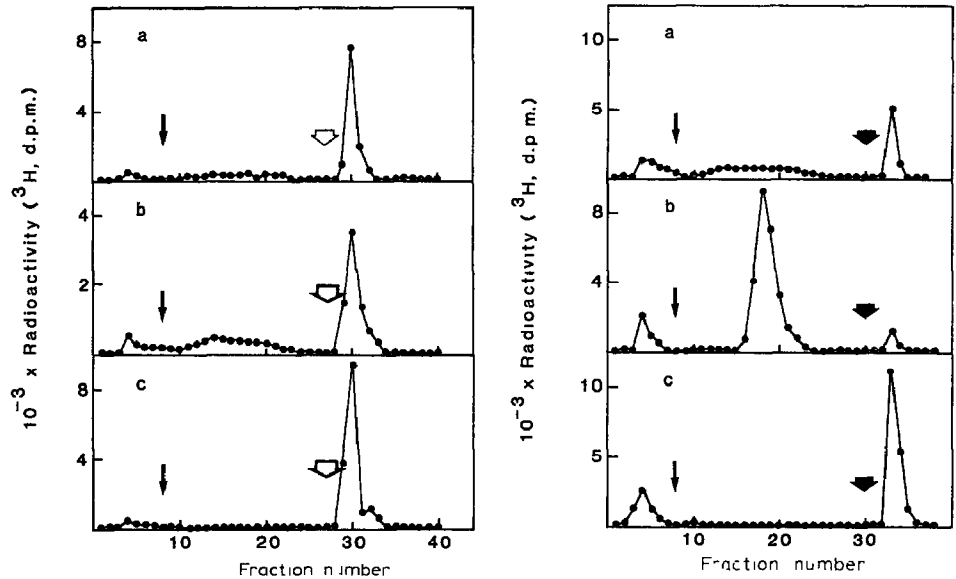


Fig. 7. Affinity chromatography of [^{3}H]heparan sulphate HS4-A on HS4-A-agarose using solutions of *N*-desulphated, *N*-acetylated HS4-A (a), carboxyl-reduced HS4-A (b), or periodate-oxidised HS4-A (c) as eluting solvents. For details, see legend to Fig. 4 and Experimental.

Fig. 8. Affinity chromatography of [^{3}H]heparan sulphate HS4-A on HS4-A-agarose using solutions of *N*-desulphated heparin (a), *N*-desulphated, *N*-acetylated heparin (b), or periodate-oxidised heparin (c) as eluting solvents. For details, see legend to Fig. 4 and Experimental.

formed. Whereas commercial heparin completely eluted [^{3}H]HS4-A from an HS4-A–agarose column (Fig. 6a), the three galactosaminoglycans DS_{75}, DS_{60}, and CS (Fig. 6b, c, and d) were only partially successful at the same concentration (0.5 mg/mL). The IdoA-rich DS_{75} was the most efficient (Fig. 6b), despite the fact that it showed a weaker affinity for the HS4-A-gel than did DS_{60} and CS (Fig. 3a). The unrelated polymer dextran sulphate was unable to desorb heparan sulphate (Fig. 6e).

To assess the chemical features required for self-association, various modifications of heparan sulphate were effected before affinity chromatography. As shown in Figs. 1d and 2f, *N*-desulphation and *N*-acetylation of HS2-A or HS4-A abolished binding to their corresponding gels. Furthermore, *N*-desulphated and *N*-acetylated HS4-A was unable to elute [^{3}H]HS4-A from an HS4-A-gel (Fig. 7a). Other modifications of HS4-A which effectively destroyed the displacing ability were carboxyl reduction (Fig. 7b) and periodate oxidation of GlcA residues (Fig. 7c) *without* subsequent scission of the chain. For heparin, the displacing ability was greatly reduced by *N*-desulphation alone (Fig. 8a), but, after subsequent *N*-acetylation, the capacity to displace [^{3}H]HS4-A from HS4-A–agarose was fully restored (*cf.* Figs. 6a and 8b). As in the case of HS4-A (Fig. 7c), periodate oxidation of GlcA residues *without* subsequent scission of the chain yielded a heparin preparation that was completely unable to inhibit the HS4-A self-association (Fig. 8c).

DISCUSSION

The heparan sulphate–heparin family of glucosaminoglycans have a common carbohydrate back-bone, *i.e.*, →4)-HexA-(1→4)-α-GlcN-(1→. As the HexA residues may be either GlcA, IdoA, or IdoA-SO_4, and the GlcN residues either GlcNAc or $GlcNSO_3$ (with or without SO_4-6), the chemical heterogeneity is large. Beef lung contains a wide variety of heparan sulphates which differ in many respects (see Introduction). Previous and present results indicate that heparan sulphates may self-associate in a specific manner which may be related to (*a*) the detailed chemistry of the contact zones and/or (*b*) the precise arrangement of the contact zones and the intervening segments, *i.e.*, $(GlcA–GlcNAc)_n$. The contact zones comprise both IdoA–GlcN and GlcA–GlcN disaccharides arranged in a mixed or alternating fashion[7]. The intervening segments are also essential inasmuch as they connect the contact zones and provide sufficient co-operativity.

The results reported in this study indicate that the general molecular shape (secondary structure) of a heparan sulphate chain must also be preserved for binding to occur. Oxidation of GlcA residues in the intervening segments evidently introduces sufficient perturbations to prevent interaction, as seen in both binding and competition studies. Reduction of the carboxyl groups or *N*-desulphation/*N*-acetylation of heparan sulphate yielded similar results. It is likely that a secondary structure is dependent on the formation of hydrogen bonds across the glycosidic linkages and both -COOH, $-NH-SO_3^-$, and -NH-CO- groups may participate in the generation of a stable structure. Alterations of these groups may thus affect the shape of the chain. It should also be pointed out that GlcA residues associated with $GlcNSO_3$ in heparan sulphate are largely resistant to periodate oxidation, whereas replacement of *N*-sulphate with *N*-acetyl renders the GlcA residues susceptible to periodate[17]. On the basis of X-ray diffraction studies on oriented fibres of heparan sulphate, Elloway and Atkins have proposed[18] a helical model that incorporates hydrogen bonds involving the HO-3 of GlcA.

As discussed above, the putative contact zones comprise both IdoA- and GlcA-containing repeats. The arrangements of these units may provide a chemical-recognition mechanism. Interestingly, dermatan sulphate, which also contains both IdoA and GlcA, is able to interact with heparan sulphate. In competition experiments, however, the homologous heparan sulphate self-association seems stronger than the heterologous interaction. The effect of chondroitin sulphate (all GlcA) on the heparan sulphate self-association was unexpected and is difficult to explain at the present stage. It is equally obscure why IdoA-rich dermatan sulphate displaces heparan sulphate from heparan sulphate–agarose better than it binds to the same gel.

It has been shown[7] that the contact zones themselves do not associate firmly, presumably because they are too short to provide sufficient co-operativity. This reasoning would predict that intact chains, or oligomers thereof, which comprise portions of the recognition sequences would compete with the chain–chain interaction even if they are not retained on heparan sulphate–agarose. The efficient

displacement of [^{3}H]HS4-A from HS4-A–agarose caused by heparin may be ascribed to the presence of such recognition sequences in this polymer. Regions that comprise both IdoA and GlcA are indeed present in some heparin chains[17,19]. It is not likely that the effect of heparin is due to its high negative-charge density, as (*a*) *N*-desulphated, *N*-acetylated heparin was active, (*b*) periodate oxidation of GlcA in heparin destroyed the displacing ability, and (*c*) dextran sulphate was ineffective. It was also noted that non-associating or associating, but unrelated, heparan sulphate variants that do not bind to HS4-A–agarose would displace [^{3}H]HS4-A therefrom, provided the concentration of chains in the eluting solvent was sufficiently high. In the following paper, the effect of various heparan sulphate oligosaccharides is described[20].

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (567), Greta och Johan Kocks Stiftelser, Gustaf V:s 80-års fond, Alfred Österlunds Stiftelse, Riksförbundet mot reumatism, and the Medical Faculty, University of Lund.

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