# Structural Diversity of N-Sulfated Heparan Sulfate Domains: Distinct Modes of Glucuronyl C5 Epimerization, Iduronic Acid 2-O-Sulfation, and Glucosamine 6-O-Sulfation†

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ABSTRACT: The N-sulfated regions (NS domains) represent the modified sequences of heparan sulfate chains and mediate interactions of the polysaccharide with proteins. We have investigated the relationship between the type/extent of polymer modification and the length of NS domains in heparan sulfate species from human aorta, bovine kidney, and cultured NMuMG and MDCK cells. C5 epimerization of D-glucuronic acid to L-iduronic acid was found to be extensive and essentially similar in all heparan sulfate species studied, regardless of domain size, whereas the subsequent 2-O-sulfation of the formed iduronic acid residues varies appreciably. In aorta heparan sulfate, up to 90% of the formed iduronate residues were 2-O-sulfated, whereas in kidney heparan sulfate 2-O-sulfation occurred only in ≤50% of the iduronate residues. The degree of 2-O-sulfation was consistently increased with increasing NS domain length, suggesting a correlation between 2-O-sulfation efficiency and length of the polymeric substrate during heparan sulfate biosynthesis. By contrast, 6-O-sulfation of glucosamine units did not correlate to domain size. 6-O-Sulfation exceeded 2-O-sulfation in NS domains from kidney heparan sulfate, but was very low in aorta heparan sulfate. Remarkably, total O-sulfation of NS domains, i.e., the sum of 2-*O*- and 6-*O*-sulfate groups, was highly similar in all heparan sulfate samples investigated. The results reveal marked tissue-specific variation in the sulfation patterns of NS domains and indicate previously unrecognized distinctions in the coordination of the three polymer modification reactions during heparan sulfate biosynthesis.

Heparan sulfate (HS)[1] proteoglycans on cell surfaces and in the extracellular matrix are implicated in various biological processes due to their interactions with multiple extracellular protein ligands. These interactions are mostly mediated via the HS components of the proteoglycans, that bind to growth factors/cytokines, matrix components, effectors and modulators of enzymatic catalysis, microbial coat proteins, etc., and thereby regulate the tissue localization and biological activities of the proteins (*1, 2*). Characterization of HS oligosaccharides with affinity to proteins such as antithrombin (*3, 4*) and peptide growth factors (*5*−*10*) has led to identification of specialized protein binding HS domains with ligand-specific structural distinctions. These functional domains derive from enzymatic modification in the Golgi apparatus of the primary polymerization product of HS/heparin biosynthesis, composed of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine units [(GlcA-GlcNAc)$_n$] (*2, 11*). The nascent polymer is first subjected to partial N-deacetylation/N-sulfation of the GlcNAc residues. The modification occurs in a regioselective fashion, giving rise to (i) consecutively N-sulfated regions (NS domains), (ii) regions of alternating N-acetylated and N-sulfated disaccharide units (NA/NS domains), and (iii) domains that escape modification and remain N-acetylated (NA domains). The further modification reactions, C5 epimerization of GlcA residues into iduronic acid (IdoA) residues and O-sulfation, all occur in the vicinity of previously incorporated *N*-sulfate groups. O-Sulfation is frequently found at C2 of IdoA units and at C6 of GlcN units. Rarely, O-sulfation occurs also at C2 of GlcA units (*12*) and at C3 of $GlcNSO_3$ residues, the latter sulfate substitution providing a hallmark for the antithrombin-binding HS/heparin sequence (*3, 13*).

In HS biosynthesis, the product of a given modification reaction generally serves as a substrate for the next reaction. Thus, the C5 epimerization of GlcA units cannot occur without preceding N-deacetylation/N-sulfation of GlcN on the nonreducing side, and the formed IdoA units are 2-O-sulfated much more efficiently than GlcA residues (*14*). IdoA 2-O-sulfation is almost exclusively confined to NS domains although (unsulfated) IdoA units are also found in the NA/NS domains (*15*), presumably because the IdoA 2-*O*-

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[1] Abbreviations: aMan$_R$, 2,5-anhydromannitol; FGF, fibroblast growth factor; GlcA, D-glucuronic acid; GlcN, glucosamine; HexA, hexuronic acid; HS, heparan sulfate; IdoA, L-iduronic acid; MDCK, Madin−Darby canine kidney; NA, N-acetylated; NMuMG, normal murine mammary gland; NS, N-sulfated.

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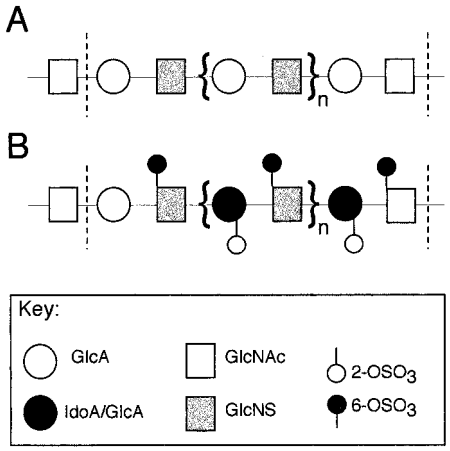


FIGURE 1: Schematic representation of the NS domain structure. (A) Unmodified NS domain structure. The NS domain (between the dashed vertical lines) encompasses an N-sulfated disaccharide unit at the nonreducing terminus (to the left), the internal domain portion (-HexA-$GlcNSO_3$-)$_n$ (where $n$ is the number of disaccharide units), and an N-acetylated disaccharide unit at the reducing terminus (to the right). (B) NS domain with maximal degree of C5 epimerization, 2-O-sulfation, and 6-O-sulfation. At the nonreducing terminus, C5 epimerization/2-O-sulfation of the GlcA residue cannot occur due to lack of a vicinal $GlcNSO_3$ unit on the nonreducing side in the native polysaccharide. The maximum number of IdoA residues and 2-*O*-sulfate groups is thus $n+1$, whereas the maximum number of GlcN 6-*O*-sulfate groups is $n+2$. The potential further modification of NS domains by $GlcNSO_3$ 3-O-sulfation is not shown.

sulfotransferase requires a contiguous N-sulfated structure for substrate recognition. Although the issue of substrate specificity thus constrains the number of possible structures occurring in HS, there is an appreciable variation in the type and extent of polymer modification between different HS species, as studied by compositional disaccharide analysis of chemically or enzymatically depolymerized HS chains (*12*, *15*−*20*). It is assumed that the modification reactions are, at least in part, processive, such that a single enzyme molecule catalyzes modification of consecutive target units. It has further been hypothesized that some of the reactions may be 'coupled', perhaps because of association of the involved enzymes into complexes (*2*, *21*). In fact, sequence analysis of HS oligosaccharides from cultured 3T3 fibroblasts indicated that the internal portions of NS domains invariably carry 2-O-sulfated IdoA residues (*21*), suggesting a coupling between GlcN N-deacetylation/N-sulfation, GlcA C5 epimerization, and IdoA 2-O-sulfation reactions.

In the present study, we have determined the degree of polymer modification in series of size-fractionated NS domains from various HS species, to better understand the relationship between NS domain length and degree of modification. We further wanted to examine the degree of variability in NS domain structure between different HS preparations. The NS domains were obtained by selective deaminative cleavage of N-deacetylated HS polymers, yielding GlcA-$GlcNSO_3$-[HexA-$GlcNSO_3$]$_n$-HexA-$aMan_R$ structures (the $aMan_R$ residues being derived from GlcNAc units; see Figure 1 and Results for further information). These domains thus contain the internal disaccharide units of NS domains and flanking disaccharide units both at the nonreducing and at the reducing ends (Figure 1), and represent most of the modified portions of the native HS polymers.

Analysis of the products demonstrates that C5 epimerization of GlcA residues to IdoA residues, IdoA 2-O-sulfation, and GlcN 6-O-sulfation are differentially related to NS domain length, 2-O-sulfation being the only modification step that is consistently enhanced with increasing domain size. Moreover, the levels of 2-*O*- and 6-*O*-sulfate substitution within the NS domains display marked variability between different HS species.

## MATERIALS AND METHODS

*HS Preparations.* Human aorta HS was prepared from an autopsy specimen of aortic wall tissue from a 70-year-old male as described previously (*20*). Bovine kidney HS was a generous gift from Dr. Keiichi Yoshida, Seikagaku Corp., Japan. Cultured normal murine mammary gland (NMuMG) and Madin−Darby Canine Kidney (MDCK) cells were maintained on plastic dishes at 37 °C in Dulbecco's Modified Eagle's Medium containing 5% heat-inactivated (30 min at 56 °C) fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 60 $\mu$g/mL penicillin G, and 50 $\mu$g/mL streptomycin sulfate (all reagents from Statens Veterinärmedicinska Anstalt, Uppsala). Cells were metabolically labeled with [$^3$H]-GlcN (10 $\mu$Ci/mL; Amersham Pharmacia Biotech) for 48 h after which the cell plates were placed on ice, the conditioned media were removed, and the cell layers were washed with ice-cold phosphate-buffered saline, pH 7.4. HS was purified from the combined conditioned media and wash buffers as described earlier (*18*, *19*). The purified HS preparations were quantitatively degraded into lower molecular weight species by treatment with $HNO_2$ at pH 1.5 (*22*) (see below), indicating that the preparations did not contain contaminating $^3$H-labeled macromolecules (data not shown).

*Preparation of NS Domains.* Samples of HS [0.2−0.5 mg of tissue-derived HS or (1.5−5) × $10^6$ dpm of [$^3$H]HS from the cell cultures] were subjected to N-deacetylation by hydrazinolysis as described earlier (*18*, *23*). Briefly, HS preparations were incubated in hydrazine hydrate containing 30% water (Fluka, Buchs, Switzerland) and 1% (w/v) hydrazine sulfate (Merck, Darmstadt, Germany) in tightly sealed glass tubes at 100 °C for 5 h. Hydrazine hydrate was removed by repeated evaporation and intermittent addition of water, after which the samples were desalted by chromatography on PD-10 columns (Amersham Pharmacia Biotech) in water. The N-deacetylated HS preparations were treated with $HNO_2$ at pH 3.9 for 10 min at room temperature, after which the pH was raised to ∼9 by addition of 2 M $Na_2CO_3$. The treatment causes deaminative cleavage of the polysaccharide at N-unsubstituted GlcN units generated by N-deacetylation, resulting in cleavage products with anhydromannose residues at the reducing termini (*22*). These were converted into 2,5-anhydromannitol ($aMan_R$) residues by reduction with $NaBH_4$ for 2 h at room temperature. To introduce radiolabel to the previously unlabeled aorta and kidney HS oligosaccharides, the cleavage products of these HS species were first treated with $NaB^3H_4$ (4 $\mu$Ci/$\mu$g of polysaccharide; Amersham Pharmacia Biotech) overnight at room temperature and further reduced with unlabeled $NaBH_4$. After reduction, excess $NaBH_4$ was destroyed by acidifying the samples to pH ∼4 by addition of acetic acid followed by neutralization with NaOH. The oligosaccharides from metabolically labeled HS were thereafter subjected to chromatography on a column of Bio-Gel P-10 (1 × 190 cm; Bio-

Rad) in 0.5 M $NH_4HCO_3$. Fractions corresponding to 4−12-mer oligosaccharides were pooled and desalted by centrifugal evaporation. The oligosaccharides labeled with $NaB^3H_4$ were first separated from unincorporated radioactivity by chromatography on a column of Sephadex G-15 (1 × 90 cm) in 0.2 M $NH_4HCO_3$ and then subjected to chromatography on Bio-Gel P-10 as described above.

*Compositional Disaccharide Analysis.* Samples of intact HS (50 $\mu$g or 2 × $10^5$ dpm) or NS domains [(1−3) × $10^5$ dpm] were subjected to deaminative cleavage by $HNO_2$ at pH 1.5 for 10 min at room temperature. At this pH, the reagent causes deaminative cleavage of the saccharide at the $GlcNSO_3$ units (*22*), resulting in depolymerization of the previously prepared NS domains into disaccharides. In the case of intact HS chains, the internal portions of NS domains yield disaccharides whereas the occurrence of one or more GlcNAc residues between the N-sulfated disaccharide units results in the formation of tetrasaccharides and longer oligosaccharides, respectively. The cleavage products from the previously unlabeled intact HS chains or from the end-$^3$H-labeled oligosaccharides were radiolabeled with $NaB^3H_4$ as described above, followed by chromatography on a column of Sephadex G-15 (1 × 190 cm) in 0.2 M $NH_4HCO_3$. Fractions corresponding to disaccharides were pooled and desalted by centrifugal evaporation. The composition of disaccharides was analyzed by anion exchange chromatography on a Partisil-10 SAX column eluted by a step gradient of $KH_2PO_4$. Fractions of 1 mL were collected and analyzed for radioactivity. The disaccharide peaks were identified by comparing their elution positions with heparin-derived standard disaccharides *(24)*.

The nonsulfated disaccharide derivatives GlcA-a$Man_R$ and IdoA-a$Man_R$ are not separated from each other on the Partisil-10 SAX chromatography. Furthermore, they coelute with $^3$H-labeled impurities generated upon radiolabeling of the samples with $NaB^3H_4$. To remove the radiolabeled impurities from nonsulfated disaccharides in $NaB^3H_4$-labeled samples, the disaccharide preparations were subjected to preparative high-voltage electrophoresis (80 V/cm) on Whatman no. 3 paper in 0.83 M pyridine/0.5 M acetic acid, pH 5.3 (*25*). The nonsulfated disaccharides were eluted from the paper with water and dried by centrifugal evaporation. The GlcA-a$Man_R$ and IdoA-a$Man_R$ derivatives were thereafter separated by descending paper chromatography on Whatman no. 1 paper in ethyl acetate/acetic acid/water (3:1:1 by volume). The paper strips were cut into 1 cm segments and eluted with water. Radioactivity in the eluates was quantified by liquid scintillation counting. The nonsulfated disaccharides from the metabolically radiolabeled HS samples were analyzed similarly except that the high-voltage electrophoresis step was omitted.

## RESULTS

We first assessed the overall composition of NS domains in the HS species included in the study by analyzing the disaccharides obtained by cleavage of the polysaccharides with $HNO_2$ at pH 1.5. These disaccharides are derived from the internal portions of NS domains. Disaccharides were separated by strong anion exchange chromatography on Partisil-10 (Figure 2A), and the data were used to assess the 2-O- and 6-O-sulfation of such sequences (Figure 2B).

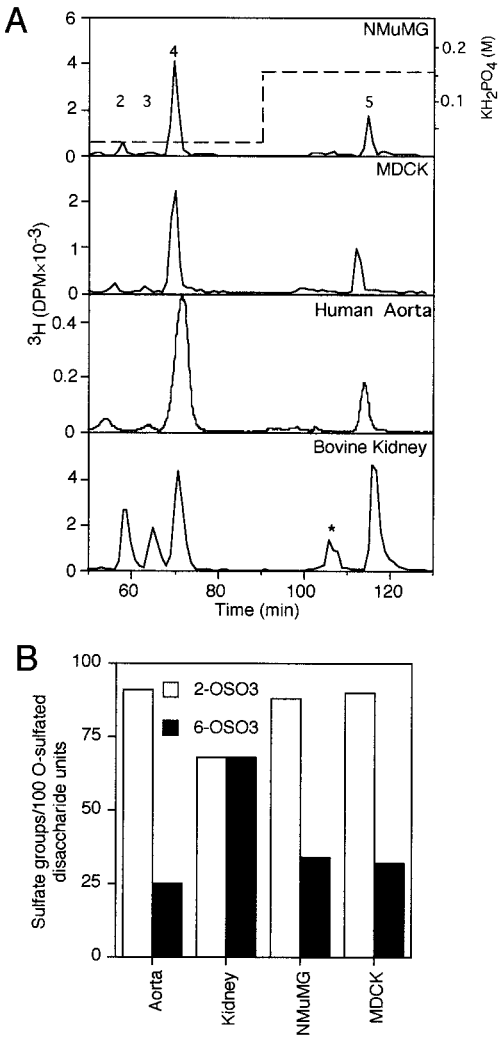


FIGURE 2: Disaccharide composition of the internal NS domain portions of the various HS species. (A) Samples of [$^3$H]HS or unlabeled HS were subjected to deaminative cleavage by $HNO_2$ (pH 1.5), and the resultant disaccharides were reduced with $NaBH_4$ or with $NaB^3H_4$, respectively. Labeled disaccharides were recovered by gel chromatography and separated on a Partisil 10 SAX anion exchange HPLC column, eluted with a step gradient of $KH_2PO_4$ (- - -) (shown in the top panel). The peaks correspond to the following disaccharide structures: (2) GlcA-a$Man_R$(6-$OSO_3$); (3) IdoA-a$Man_R$(6-$OSO_3$); (4) IdoA(2-$OSO_3$)-a$Man_R$; (5) IdoA(2-$OSO_3$)-a$Man_R$(6-$OSO_3$). The peak marked with an asterisk (*) represents tetrasaccharide contaminants, in part due to 'anomalous' ring contraction upon $HNO_2$ cleavage (*22*). (B) Diagrammatic representation of proportions of 2-*O*- and 6-*O*-sulfate groups in the NS domains.

Human aorta HS displayed abundant 2-O-sulfation but low 6-O-sulfation, whereas in bovine kidney HS the two sulfate substituents occurred in equal proportions, in agreement with our previous data on the same HS preparations (*15*, *20*). The NS domains of HS from cultured NMuMG and MDCK cells resembled those of aorta HS in disaccharide composition but had a somewhat higher degree of 6-O-sulfation (Figure 2).

We next proceeded to analyze NS domains that were separated with regard to size. To prepare NS domains, HS was N-deacetylated and subjected to deaminative cleavage at the resultant $GlcNH_3^+$ residues with $HNO_2$ at pH 3.9. The products thus obtained have the structure GlcA-$GlcNSO_3$-[HexA-$GlcNSO_3$]$_n$-HexA-a$Man_R$, the a$Man_R$ corresponding to a GlcNAc unit in the native saccharide (Figure 1). The

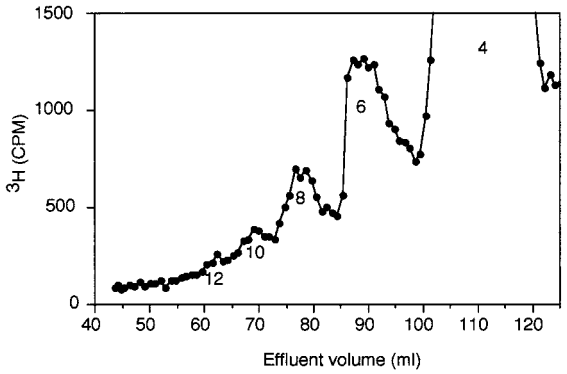


FIGURE 3: Preparation of size-defined NS domains. Bovine kidney HS was N-deacetylated and cleaved with $HNO_2$ (pH 3.9) followed by reduction of the cleavage products with $NaB^3H_4$. The resultant labeled oligosaccharides were chromatographed on a column of Bio-Gel P-10. Fractions of 1.2 mL were collected at a flow rate of 2.3 mL/h and analyzed for radioactivity. The sizes of the cleavage products are indicated (as number of monosaccharide units) below the peaks.

cleavage products were separated by chromatography on Bio-Gel P-10, as shown for the oligosaccharides from bovine kidney HS in Figure 3. The partial overlap of, in particular, the longer oligosaccharide species was presumably due to variable sulfation of the NS domains. The distribution of different NS domain sizes differed somewhat between the HS species such that aorta HS yielded the highest proportions of tetrasaccharides, whereas HS from NMuMG cells displayed the highest proportions of long ≥10-mer NS domains (Table 1). The size-fractionated NS domains were treated with $HNO_2$ at pH 1.5, and the resultant disaccharides were reduced and separated by strong anion exchange chromatography, as shown for metabolically [$^3$H]GlcN-labeled NS domains from NMuMG cells in Figure 4. Figure 5 shows corresponding separations of the O-sulfated NS domain disaccharides (labeled by reduction with $NaB^3H_4$) from aorta and kidney HS. Because the nonsulfated HexA-aMan$_R$ disaccharide derivatives (corresponding to peak 1 in Figure 4) coelute with $^3$H-labeled impurities arising upon $NaB^3H_4$ reduction, their proportions were determined separately by high-voltage paper electrophoresis and paper chromatography. The results were used to calculate the proportions of IdoA, 2-*O*-sulfate, and 6-*O*-sulfate in the various domain species, expressed as units/100 disaccharide units (Table 1). The numbers of IdoA units, 2-*O*-sulfate groups, and 6-*O*-sulfate groups/domain were calculated and plotted against domain length (Figures 6 and 7A), and were also expressed as a percentage of modified residues vs all potential substrate residues (see Figure 1) to assess the 'efficiency' of the various modification events (Figure 7B).

*Glucuronyl C5 Epimerization.* In the analyzed GlcA-GlcNSO$_3$-[HexA-GlcNSO$_3$]$_n$-HexA-GlcNAc structures, the internal and reducing terminal HexA positions potentially represent targets of C5 epimerization in the native sequence, whereas the nonreducing terminal GlcA unit would be resistant, due to the requirement for a neighboring nonreducing GlcNSO$_3$ residue (*14*) (Figure 1). Analysis of C5 epimerization indicated that in each of the HS species, the majority of potential GlcA substrates were epimerized (Figure 6). Slight differences in the efficiency of epimerization between the HS species were noted, such that in human aorta HS, the average number of nonepimerized GlcA substrate units was less than 1 per NS domain, whereas kidney HS appeared to contain >1 GlcA residue/domain in addition to that at the nonreducing terminus. Compositional analysis of the reducing terminal disaccharide units of NS

Table 1: Disaccharide Composition and Degree of O-Sulfation of NS Domains[a]

| | | | deamination product (% of total disaccharides) | | | | | | | type of O-sulfation (sulfate groups/100 disaccharide units) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | fragment length | % of total NS domains | GlcA-aMan$_R$ | IdoA-aMan$_R$ | GlcA-(2-OSO$_3$)-aMan$_R$ | GlcA-aMan$_R$-(6-OSO$_3$) | IdoA-aMan$_R$-(6-OSO$_3$) | IdoA-(2-OSO$_3$)-aMan$_R$ | Ida(2-OSO$_3$)-aManR-(6-OSO$_3$) | 2-OSO$_3$ | 6-OSO$_3$ | total-OSO$_3$ |
| NMuMG | 4-mer | 35 | 60 | 17 | 0 | 5 | 6 | 11 | 2 | 13 | 13 | 26 |
| | 6-mer | 15 | 43 | 24 | 0 | 5 | 4 | 14 | 10 | 24 | 19 | 43 |
| | 8-mer | 15 | 37 | 9 | 1 | 8 | 8 | 23 | 14 | 38 | 30 | 68 |
| | 10-mer | 13 | 26 | 11 | 0 | 8 | 5 | 32 | 17 | 49 | 30 | 79 |
| | 12-mer | 12 | 18 | 10 | 1 | 8 | 4 | 44 | 15 | 60 | 27 | 87 |
| MDCK | 4-mer | 41 | 53 | 13 | 3 | 7 | 10 | 12 | 3 | 18 | 20 | 38 |
| | 6-mer | 15 | 38 | 20 | 0 | 6 | 6 | 22 | 9 | 31 | 21 | 52 |
| | 8-mer | 18 | 29 | 12 | 0 | 6 | 11 | 30 | 12 | 42 | 29 | 71 |
| | 10-mer | 11 | 25 | 12 | 0 | 6 | 8 | 37 | 12 | 49 | 26 | 75 |
| | 12-mer | 9 | 21 | 11 | 1 | 6 | 6 | 48 | 9 | 58 | 21 | 79 |
| aorta HS | 4-mer | 66 | 62 | 11 | 2 | 4 | 1 | 16 | 4 | 22 | 9 | 31 |
| | 6-mer | 19 | 45 | 7 | 1 | 3 | 2 | 36 | 6 | 43 | 11 | 54 |
| | 8-mer | 6 | 30 | 8 | 1 | 4 | 1 | 53 | 4 | 58 | 9 | 67 |
| | 10-mer | 4 | 22 | 5 | 1 | 3 | 1 | 66 | 2 | 69 | 6 | 75 |
| | 12-mer | 3 | 19 | 6 | 1 | 3 | 2 | 66 | 3 | 70 | 8 | 78 |
| bovine kidney | 4-mer | 43 | 40 | 18 | 0 | 18 | 24 | 0 | 0 | 0 | 42 | 42 |
| | 6-mer | 22 | 24 | 12 | 0 | 23 | 29 | 6 | 6 | 12 | 58 | 70 |
| | 8-mer | 15 | 27 | 11 | 0 | 21 | 18 | 10 | 13 | 23 | 52 | 75 |
| | 10-mer | 9 | 21 | 10 | 0 | 20 | 18 | 16 | 15 | 31 | 53 | 84 |
| | 12-mer | 6 | 23 | 14 | 0 | 17 | 20 | 14 | 12 | 26 | 49 | 75 |

[a] NS domains were subjected to deaminative cleavage by $HNO_2$ at pH 1.5. The resultant disaccharides were reduced with $NaBH_4$/$NaB^3H_4$ and purified by chromatography on a column of Sephadex G-15. Disaccharides were pooled, desalted, and analyzed on a Whatman Partisil-10 SAX column. The proportions of the nonsulfated HexA-aMan$_R$ disaccharide derivatives in the $NaB^3H_4$–labeled samples were determined by high-voltage paper electrophoresis, and the proportions of GlcA-aMan$_R$ and IdoA-aMan$_R$ units in the HexA-aMan$_R$ pools were assessed by paper chromatography (see Materials and Methods).

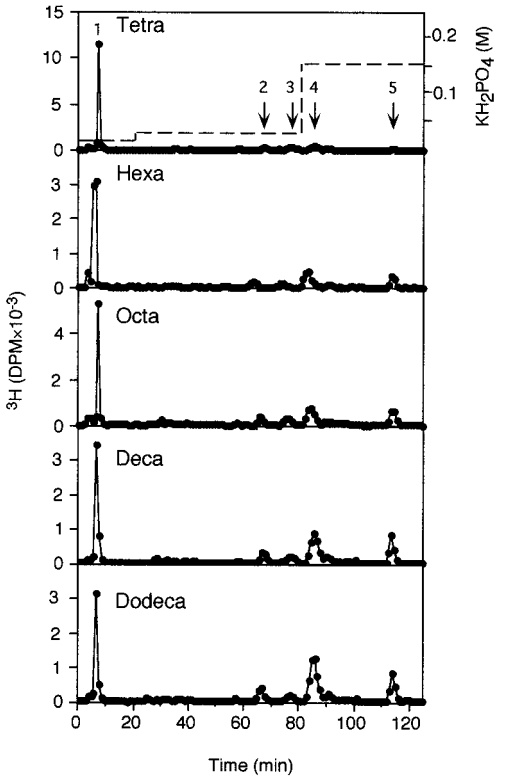


FIGURE 4: Compositional disaccharide analysis of NS domains from NMuMG cells. Aliquots of [$^3$H]NS domains from HS of NMuMG cells were subjected to deaminative cleavage by $HNO_2$ (pH 1.5). Disaccharides were recovered by gel chromatography and separated on a Partisil-10 SAX anion exchange HPLC column, eluted with a step gradient of $KH_2PO_4$ (- - -) (shown in the top panel). The peaks correspond to the following disaccharide structures: (1) HexA-aMan$_R$; (2) GlcA-aMan$_R$(6-OSO$_3$); (3) IdoA-aMan$_R$(6-OSO$_3$); (4) IdoA(2-OSO$_3$)-aMan$_R$; (5) IdoA(2-OSO$_3$)-aMan$_R$(6-OSO$_3$).

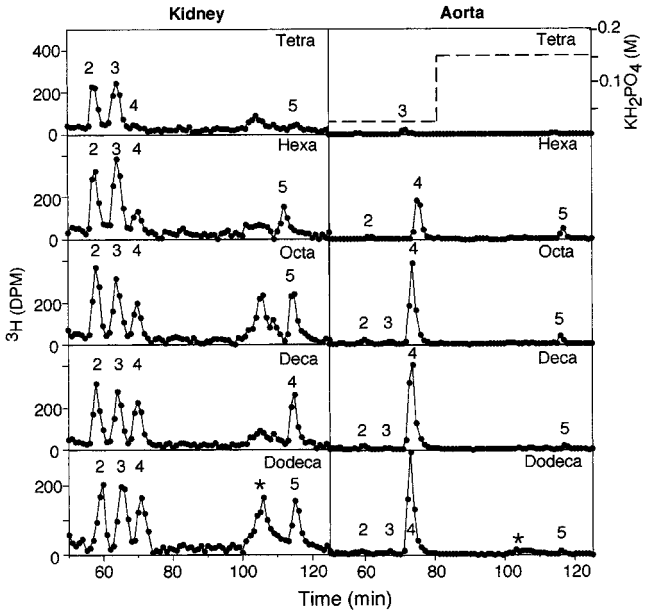


FIGURE 5: Compositional analysis of O-sulfated disaccharide species from aorta and kidney HS NS domains. Samples of NS domains were reacted with $HNO_2$ (pH 1.5) followed by reduction and radiolabeling of the resultant disaccharides with $NaB^3H_4$. Disaccharides were recovered by gel chromatography and separated on a Partisil-10 SAX anion exchange HPLC column as described under Materials and Methods and in the legend to Figure 4. For peak numbering, see legend to Figure 4. The peak marked with an asterisk (*) represents tetrasaccharide contaminants, in part due to 'anomalous' ring contraction upon $HNO_2$ cleavage (*22*).

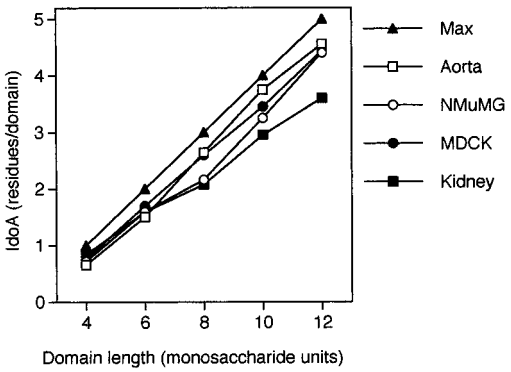


FIGURE 6: Degree of C5 epimerization in variously sized NS domains. The numbers of IdoA residues per domain were calculated as explained in the text and plotted against domain length (as monosaccharide units). "Max" represents the theoretical maximum for the number of IdoA residues per domain.

domains from kidney HS revealed appreciable proportions of IdoA (data not shown), indicating that GlcA residues were found not only at the reducing termini but also in the internal disaccharide units of the domains. In aorta HS, the efficiency of epimerization (percent of all potential substrate residues; see Figure 7B) increased somewhat with increasing NS domain length, ranging from ~70% in tetrasaccharides to ≥90% in deca-/dodecasaccharides, whereas in the other three HS species this parameter was not significantly affected by domain length. Collectively, these data indicate that although the N-deacetylation/N-sulfation of a GlcNAc unit is generally followed by IdoA formation at the vicinal position toward the reducing end, this reaction does not occur in a uniform fashion in the different HS species. The high degree of epimerization in aorta HS would largely result in NS domains containing exclusively IdoA units at the internal positions, whereas the less efficient epimerization in kidney HS yields NS domains with internal GlcA residues.

*O-Sulfation.* In NS domains from aorta HS, the degree of 2-O-sulfation varied between ~50 and ≥80% of the potential HexA positions (Figure 7B), corresponding to 2-O-sulfation of 70−90% of the IdoA residues. Notably, the 10−12-mer domains were sulfated more efficiently than shorter domains. The pattern of IdoA 2-O-sulfation followed closely that of epimerization, conforming to the concept of 'coupling' between the two reactions (Figure 7B). However, the degree of 2-O-sulfation was invariably lower than that of epimerization, indicating that a portion of IdoA units escapes sulfation. Contrary to 2-O-sulfation, the proportions of 6-O-sulfate groups in aorta HS were extremely low and did not average 1 residue/fragment at any domain size. Moreover, the degree of 6-O-sulfation did not correlate with domain length. Analysis of the disaccharide units from the reducing termini of the fragments indicated a low (10−15%) degree of O-sulfation, that was almost exclusively found at C6 of the GlcNAc residues (data not shown), indicating that the 2-*O*-sulfate groups occurred predominantly in the internal IdoA residues.

Analysis of O-sulfation of NS domains from kidney HS revealed a highly different pattern. First, the degree of 2-O-sulfation in this HS species was ≤50% of that in aorta HS, such that only a fraction, ≤50%, of the potential IdoA substrates had undergone 2-O-sulfation (Figure 7B), as

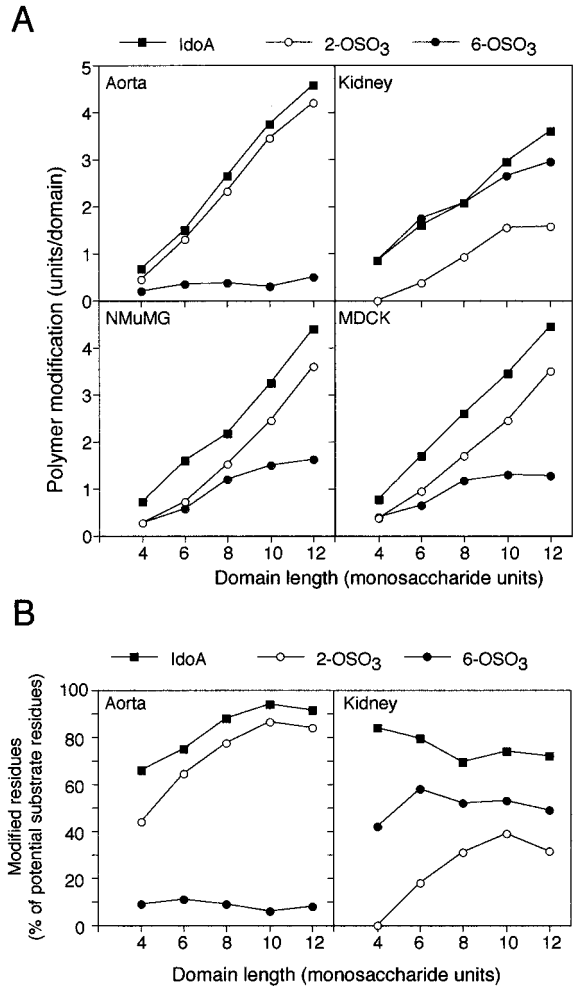


FIGURE 7: Occurrence of polymer modification events in NS domains. (A) The numbers of IdoA units, 2-*O*-sulfate groups, and 6-*O*-sulfate groups per domain were plotted against domain length. (B) The extent of polymer modification in NS domains from aorta and kidney HS is expressed as a percentage of the total potential substrate sites modified the for a given modification reaction (see Figure 1).

compared to ~90% in aorta HS. Similarly to aorta HS, however, the efficiency of 2-O-sulfation increased with increasing domain length. Second, the degree of GlcN 6-O-sulfation in kidney NS domains was particularly high, such that up to 50−60% of all GlcN residues carried 6-*O*-sulfate groups. The level of 6-O-sulfation in kidney HS thus was more than 6 times higher than in aorta HS, throughout the range of domain sizes. Although the number of 6-*O*-sulfate groups per domain increased in longer NS domains (Figure 7A), the efficiency of sulfation was proportionally similar (Figure 7B), indicating that the 2-O- and 6-O-sulfation reactions are differentially influenced by NS domain length. In kidney HS NS domains, a high proportion (>60%) of the reducing terminal disaccharides were 6-O-sulfated (data not shown), indicating that the 6-O-sulfation is not restricted to the internal N-sulfated disaccharide units but is also found in the terminal disaccharides with GlcNAc residues.

NS domains from HS isolated from cultured NMuMG and MDCK cells were relatively similar with regard to O-sulfate substitution, despite the different tissue and species origins of the cells (murine mammary gland epithelium and canine kidney epithelium, respectively). The structural properties of these HS species largely averaged those of aorta and

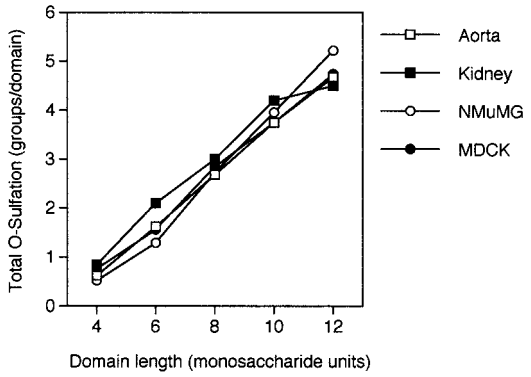


FIGURE 8: Overall O-sulfation of NS domains from the various HS species. The sum of the number of 2-*O*- and 6-*O*-sulfate substituents in a given domain has been plotted against domain length.

kidney HS. The 2-O-sulfation thus was somewhat lower than in aorta HS but clearly higher than in kidney HS, whereas 6-O-sulfation showed the opposite relationship (Figure 7A). Also in these HS species, the efficiency of 2-O-sulfation (but not 6-O-sulfation) increased with increasing domain length.

Although the patterns of 2-O- and 6-O-sulfation of NS domains from the different HS species differed appreciably, the size-matched NS domains showed remarkably similar degrees of total O-sulfate substitution (i.e., the sum of 2-O- and 6-O-sulfation) (Figure 8). The differential O-sulfation patterns thus are not directly related to the overall degree of O-sulfation.

In summary, the current data indicate three distinct patterns of polymer modification for the NS domains of HS: (i) near-maximal epimerization and 2-O-sulfation with occasional 6-O-sulfation in aorta HS; (ii) heavy 6-O-sulfation and lower 2-O-sulfation in kidney HS, accompanied by slightly down-regulated C5 epimerization; and (iii) the intermediate pattern of relatively high 2-O-sulfation with moderate 6-O-sulfation in HS from MDCK and NMuMG cells.

## DISCUSSION

The broad functional spectrum of HS in biological and pathological contexts is becoming increasingly evident. This development has encouraged attempts to elucidate the mechanisms governing HS biosynthesis as well as the structural specificity of protein binding NS domains of HS. Although data on the functional properties and substrate recognition of HS biosynthesis enzymes are emerging, little information is still available of the factors that control the assembly of the biologically active domains of HS. In the current study, we have assessed the contribution of one such factor by studying whether the polymer modification process is influenced by NS domain size, i.e., by the length of the polymeric substrate made available for the biosynthetic machinery. To understand the extent of variability in the process, we also investigated how the overall structural diversity between distinct HS species is reflected by the modification pattern of the corresponding size-fractionated NS domains.

Our results indicate that the relationship between the degree of polymer modification and NS domain size differed for the three distinct modification events, i.e., C5 epimerization of GlcA to IdoA, 2-O-sulfation of IdoA, and 6-O-sulfation of GlcN. Generally, the number of modified

residues, as expected, increased with increasing size of the NS domains. However, the efficiency of the process, i.e., the extent of modification in relation to the number of available substrate sites, varied for each reaction. The GlcA C5 epimerization thus occurred with high efficiency in all HS species. The IdoA 2-O-sulfation was the only modification reaction that was consistently more efficient with increasing domain length; this pattern was seen regardless of variations in the overall degree of 2-O-sulfation between the different HS species. By comparison, the levels of GlcN 6-O-sulfation were more equal between domains of different sizes (but varied markedly between the different HS species), indicating distinct modes of 6-O-sulfation and 2-O-sulfation along the polymeric substrate. Remarkably, the overall degrees of O-sulfation of size-matched NS domains were quite similar between the different HS species (Figure 8), despite the marked differences in the proportions of 2-*O*- and 6-*O*-sulfate groups. Although the distribution of different NS domain sizes differed somewhat between the four HS species (Table 1), there does not seem to be a clear-cut relationship between this parameter and the epimerization/O-sulfation reactions.

The present study revealed three distinct patterns of modification for HS NS domains. Whereas the NS domains from aorta and kidney HS were highly different, NS domains from the cultured NMuMG and MDCK cells were relatively similar in structure. These findings raise a question as to whether the organ-specific distinctions in HS composition remain preserved in cell lines maintained in culture over extended periods of time. Comparative analyses with HS from various cell lines, the corresponding tissues, and perhaps also from primary cell cultures would be needed to elucidate this important issue in more detail. The tissue-specific HS structures may also reflect differential expression of specific HS proteoglycan core proteins in different cell and tissue types, assuming that the core protein structure influences the assembly of the HS chains. To the best of our knowledge, however, no such influence has yet been demonstrated.

Based on the present findings, models of average NS domains of different sizes from aorta and kidney HS may be designed (Figure 9). The structures of aorta HS NS domains agree with the sequence information of NS domains from 3T3 fibroblasts (*21*) in that practically all the internal IdoA units are 2-O-sulfated, whereas little sulfation was found in the reducing terminal disaccharide units. The modification pattern of aorta HS thus would agree with the idea of 'coupling' between the C5 epimerization and IdoA 2-O-sulfation reactions (*2, 21*). However, no such coupling was seen in kidney HS, that also contained high proportions of IdoA but little 2-*O*-sulfate. Instead, kidney HS shows an unusually high degree of GlcN 6-O-sulfation. Such an inverse relation might be expected if IdoA 2-*O*-sulfotransferase and GlcN 6-*O*-sulfotransferase compete for the same -IdoA-$GlcNSO_3$- substrate during HS biosynthesis (see ref *26*), since 6-O-sulfation of this structure would preclude 2-O-sulfation. Notably, the reverse process, i.e., IdoA 2-O-sulfation followed by adjacent $GlcNSO_3$ 6-O-sulfation, is feasible and generates a trisulfated -IdoA(2-$OSO_3$)-$GlcNSO_3$(6-$OSO_3$)- unit. The GlcN 6-*O*-sulfotransferase occurs in at least three distinct isoforms, whereas only a single isoform of IdoA 2-*O*-sulfotransferase has been identified *(11)*. It is not yet known whether distinct 6-*O*-sulfotransferase isoforms are required for transfer of sulfate to non-O-sulfated and previously 2-O-sulfated -IdoA-$GlcNSO_3$- structures. Furthermore, it will be of interest to determine if 6-O-sulfation of internal and reducing terminal NS domain positions involves different sulfotransferase species. In kidney HS, appreciable 6-O-sulfation was found in the reducing terminal disaccharide units containing GlcNAc residues.

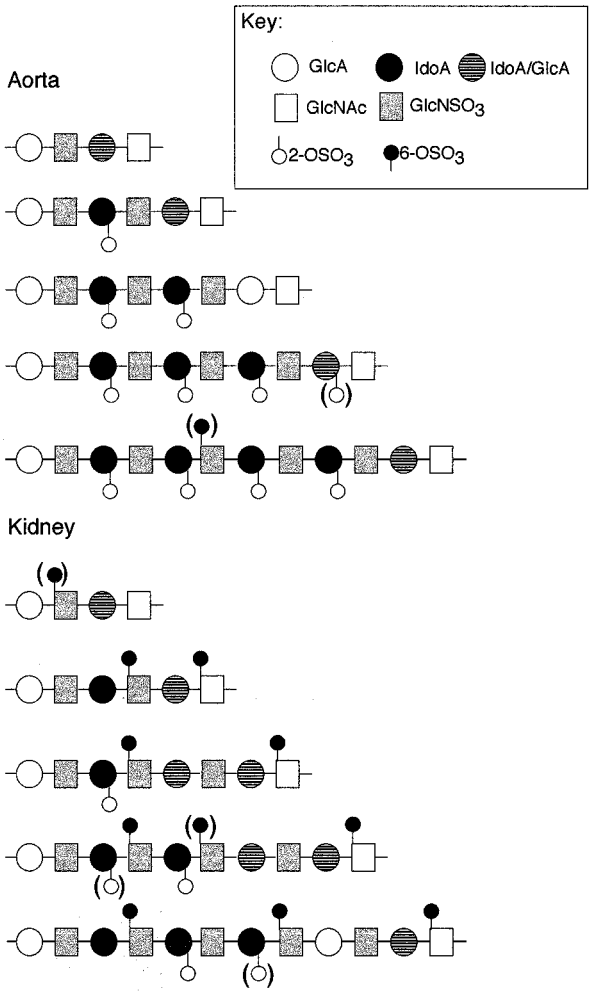


FIGURE 9: Schematic representation of NS domain structures in aorta and kidney HS. The average numbers of IdoA residues, 2-*O*-sulfate groups, and 6-*O*-sulfate groups per domain were used to construct the depicted structural models of average NS domains of different sizes. The structural information derives from the overall domain composition (Table 1 and Figure 6) and from the separate analysis of the reducing disaccharide units (see Materials and Methods). The positioning of the modified residues in tentative, particularly with regard to structures where the extent of modification is low.

Structural analyses of HS domains with affinity toward various proteins indicate that the minimal size and *O*-sulfate substitution of NS domains required for interaction differ according to the ligand. For instance, binding of HS to FGF-2 is mediated by a pentasaccharide sequence containing only a single IdoA 2-*O*-sulfate group (*5, 27*), whereas the closely related FGF-1 requires both 2-*O*- and 6-*O*-sulfate groups, preferably in the form of a trisulfated -IdoA(2-$OSO_3$)-$GlcNSO_3$(6-$OSO_3$)- disaccharide unit (*6, 8*). Such units have also been implicated in the binding of HS to the long A chain of platelet-derived growth factor (*10*) and to hepatocyte growth factor (*9*). More intricate, the activation of FGF receptors by FGF-2 is supported by 10−12-mer HS frag-

ments that contain both 2-*O*- and 6-*O*-sulfate groups (*28*, *29*). The proportions of the trisulfated -IdoA(2-$OSO_3$)-$GlcNSO_3$(6-$OSO_3$)- units were relatively low in all HS species, averaging less than 1 residue/domain. We note that the highest proportions were seen in HS from NMuMG cells, that displayed much lower overall degree of 6-O-sulfation than kidney HS, indicating that the proportions of the trisulfated units do not necessarily correlate to the overall degree of 6-O-sulfation. The formation of -IdoA(2-$OSO_3$)-$GlcNSO_3$(6-$OSO_3$)- structures thus appears to represent a critical step during HS biosynthesis, as regards the protein binding specificity of the polysaccharide product, and further studies should be designed to address the factors that commit the machinery to generate the trisulfated structure instead of mono-(2-O- or 6-O-)sulfated disaccharide species. While little information is still available on interactions primarily mediated via 6-*O*-sulfate groups, we note that kidney HS might be preferentially tailored for such interactions. A candidate protein may be vascular endothelial growth factor-165, whose binding to heparin has been suggested to require $GlcNSO_3$(6-$OSO_3$) residues but not 2-*O*-sulfate groups *(30)*.

## REFERENCES


1. Bernfield, M., Kokenyesi, R., Kato, M., Hinkes, M. T., Spring, J., Gallo, R. L., and Lose, E. J. (1992) *Annu. Rev. Cell Biol. 8*, 365−393.
2. Salmivirta, M., Lidholt, K., and Lindahl, U. (1996) *FASEB J. 10*, 1270−1279.
3. Bourin, M. C., and Lindahl, U. (1993) *Biochem. J. 289*, 313−330.
4. Lindahl, U., Thunberg, L., Bäckström, G., Riesenfeld, J., Nordling, K., and Björk, I. (1984) *J. Biol. Chem. 259*, 12368−12376.
5. Faham, S., Hileman, R. E., Fromm, J. R., Linhardt, R. J., and Rees, D. C. (1996) *Science 271*, 1116−1120.
6. DiGabriele, A., Lax, I., Chen, D. I., Svahn, C. M., Jaye, M., Schlessinger, J., and Hendrickson, W. A. (1998) *Nature 393*, 812−817.
7. Lyon, M., Deakin, J. A., Mizuno, K., Nakamura, T., and Gallagher, J. T. (1994) *J. Biol. Chem. 269*, 11216−11223.
8. Kreuger, J., Prydz, K., Pettersson, R. F., Lindahl, U., and Salmivirta, M. (1999) *Glycobiology 9*, 723−729.
9. Ashikari, S., Habuchi, H., and Kimata, K. (1995) *J. Biol. Chem. 270*, 29586−29593.
10. Feyzi, E., Lustig, F., Fager, G., Spillmann, D., Lindahl, U., and Salmivirta, M. (1997) *J. Biol. Chem. 272*, 5518−5524.
11. Lindahl, U., Kusche-Gullberg, M., and Kjellén, L. (1998) *J. Biol. Chem. 273*, 24979−24982.
12. Lindahl, B., Eriksson, L., and Lindahl, U. (1995) *Biochem. J. 306*, 177−184.
13. Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J., and Zhang, L. (1997) *J. Clin. Invest. 99*, 2062−2070.
14. Kjellén, L., and Lindahl, U. (1991) *Annu. Rev. Biochem. 60*, 443−475.
15. Maccarana, M., Sakura, Y., Tawada, A., Yoshida, K., and Lindahl, U. (1996) *J. Biol. Chem. 271*, 17804−17810.
16. Turnbull, J. E., and Gallagher, J. T. (1990) *Biochem. J. 265*, 715−724.
17. Jayson, G. C., Lyon, M., Paraskeva, C., Turnbull, J. E., Deakin, J. A., and Gallagher, J. T. (1998) *J. Biol. Chem. 273*, 51−57.
18. Salmivirta, M., Safaiyan, F., Prydz, K., Andresen, M. S., Aryan, M., and Kolset, S. (1998) *Glycobiology 8*, 1029−1036.
19. Safaiyan, F., Lindahl, U., and Salmivirta, M. (1998) *Eur. J. Biochem. 252*, 576−582.
20. Feyzi, E., Saldeen, T., Larsson, E., Lindahl, U., and Salmivirta, M. (1998) *J. Biol. Chem. 273*, 13395−13398.
21. Merry, C. L. R., Lyon, M., Deakin, J. A., Hopwood, J. J., and Gallagher, J. T. (1999) *J. Biol. Chem. 274*, 18455−18462.
22. Shively, J. E., and Conrad, H. E. (1976) *Biochemistry 15*, 3932−3942.
23. Guo, Y., and Conrad, H. E. (1989) *Anal. Biochem. 176*, 96−104.
24. Pejler, G., Bäckström, G., Lindahl, U., Paulsson, M., Dziadek, M., Fujiwara, S., and Timpl, R. (1987) *J. Biol. Chem. 262*, 5036−5043.
25. Kusche, M., Lindahl, U., Enerbäck, L., and Roden, L. (1988) *J. Biol. Chem. 263*, 15474−15484.
26. Conrad, H. E. (1998) *Heparin-Binding Proteins*, Academic Press, San Diego.
27. Maccarana, M., and Lindahl, U. (1993) *Glycobiology 3*, 271−277.
28. Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U., and Rapraeger, A. C. (1993) *J. Biol. Chem. 268*, 23906−23914.
29. Pye, D. A., Vives, R. R., Turnbull, J. E., Hyde, P., and Gallagher, J. T. (1998) *J. Biol. Chem. 273*, 22936−22942.
30. Ono, K., Hattori, H., Takeshita, S., Kurita, A., and Ishihara, M. (1999) *Glycobiology 9*, 705−711.


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