Location of N-Unsubstituted Glucosamine Residues in Heparan Sulfate*

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Functional properties of heparan sulfate (HS) are generally ascribed to the sulfation pattern of the polysaccharide. However, recently reported functional implications of rare N-unsubstituted glucosamine (GlcNH₂) residues in native HS prompted our structural characterization of sequences around such residues. HS preparations were cleaved with nitrous acid at either N-sulfated or N-unsubstituted glucosamine units followed by reduction with NaBH₄. The labeled products were characterized following complementary deamination steps. The proportion of GlcNH₂ units varied from 0.7–4% of total glucosamine in different HS preparations. The GlcNH₂ units occurred largely clustered at the polysaccharide-protein linkage region in intestinal HS, also more peripherally in aortic HS. They were preferentially located within N-acetylated domains, or in transition sequences between N-acetylated and N-sulfated domains, only 20–30% of the adjacent upstream and downstream disaccharide units being N-sulfated. The nearest downstream (toward the polysaccharide-protein linkage) hexuronic acid was invariably GlcUA, whereas the upstream neighbor could be either GlcUA or IdoUA. The highly sulfated but N-unsubstituted disaccharide unit, -IdoUA2S-GlcNH₂6S-, was detected in human renal and porcine intestinal HS, but not in HS from human aorta. These results are interpreted in terms of a biosynthetic mechanism, whereby GlcNH₂ residues are formed through regulated, incomplete action of a N-deacetylase/N-sulfotransferase enzyme.

Many biological processes depend on interactions between heparan sulfate proteoglycans (HSPGs) and proteins, such as enzymes, cytokines, growth factors, extracellular matrix proteins, and proteins produced by microbial pathogens (1–6). HSPGs are widely expressed on cell surfaces and in the extracellular matrices of most tissues. Their biological functions generally involve the carbohydrate moieties, i.e. one or more HS chains. These linear, sulfate-substituted glycosaminoglycans associate with basic amino acid residues in target proteins, sometimes through highly specific sequence motifs in the HS chain (see also reviews in Refs. 7 and 8). The proteinase inhibitor antithrombin thus binds to a unique pentasaccharide sequence that contains a rare 3-O-sulfated α-glucosamine (GlcN) unit (9). Other such rare constituents are 2-O-sulfated α-glucuronic acid (GlcUA) and N-unsubstituted GlcN residues (2).

HS and the structurally related heparin are both synthesized through a non-sulfated precursor structure composed of alternating GlcUA and N-acetylated GlcN (GlcNAc) units (2, 4, 10, 11). This precursor is modified through a series of enzymatic reactions, initiated by N-deacetylation and N-sulfation of GlcNAc residues. The resultant N-sulfated GlcN (GlcNS) residues are prerequisite to subsequent modification, involving C5-epimerization of GlcUA to i-iduronic acid (IdoUA), O-sulfation at C2 of the hexuronic acid (HexUA, i.e. GlcUA or IdoUA) and O-sulfation at C6 of GlcNS or GlcNAc units, or, less common, at C3 of GlcNS. Heparin is more extensively modified, resulting in a saccharide structure highly enriched in IdoUA2S-GlcNS6S disaccharide units, whereas the modification of HS chains is more restrained. The HS structure is typically heterogeneous, with blocks of N-sulfated sequences (NS-domains) interspersed between unmodified, N-acetylated regions (NA-domains) and mixed NA/NS domains that consist of alternating N-acetylated and N-sulfated disaccharide units (12). O-Sulfate groups and IdoUA units occur in the NS- and NA/NS- but not in the NA-domains. The domain distribution, length, and modification pattern vary considerably between different HS species, depending on tissue of origin, developmental stage, and pathophysiological condition (12–16) (see also reviews in Refs. 4, 8, and 11).

Due to their varied structure HS molecules interact in distinct fashion with different proteins. The rare structural components presumably contribute to selective protein binding. The occurrence of N-unsubstituted GlcN (GlcNH₂) units was early recognized but largely ignored, since it was believed to reflect artificial loss of N-sulfate groups during handling of HS samples (17). However, findings of recent years not only revealed GlcNH₂ residues in native HS structures, but also implicated such components with important cell-biological and pathophysiological phenomena. A monoclonal antibody that recognized GlcNH₂ units in HS thus bound in selective fashion to extracellular tissue components in fresh-frozen rat kidney (18). The presence of GlcNH₂ residues was found to correlate with the ability of bovine and human endothelial HS to bind L-selectin (although binding was not critically dependent on the GlcNH₂ residues) (19). Moreover, GlcNH₂ units were identified as targets for a 3-O-sulfotransferase isoform (3-OST-3A) that introduces a sulfate substituent at C3 (20, 21) and thereby

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1 The abbreviations used are: HSPG, heparan sulfate proteoglycan; aManh, 2,5-anhydromannitol; GlcNH₂, N-unsubstituted glucosamine; GlcNS, N-sulf-0-glucosamine; HexUA, hexuronic acid; IdoUA, l-iduronic acid; NA, N-acetylated; NS, N-sulfated; 3-OST-3A, 3-O-sulfotransferase 3A; HS, heparan sulfate.

2 O-Sulfate groups are designated 2S and 6S to indicate HexUA 2-O-sulfate and GlcNAc/S 6-O-sulfate groups, respectively.

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generates a binding site in cell-surface HS for the herpes simplex virus 1 glycoprotein D, a key player in viral invasion (22). Another monoclonal antibody, claimed to bind a GlcNH₂-containing epitope in HS (23), recognizes early scrapie lesions in murine brain (24). Finally, it was proposed that GlcNH₂ residues provide cleavage sites in HS chains for endogenous NO-derived nitrite and thus contribute to recycling of glypican-I (25).

The overall contents of GlcNH₂ residues, generally low, vary between HS species. Values ranging from 1.2 to 7.5% of total GlcN were calculated for porcine and bovine HS preparations, based on reaction with o-phthalaldehyde (26). The location of these units in relation to the various structural domains of HS chains has not been established. While some studies place the polymer (18, 26, 27), the target GlcNH₂ unit for GlcNH₂ residues in transition zones between modified (largely sulfated) and unmodified (largely N-acetylated) regions of the polymer (18, 26, 27), the target GlcNH₂ unit for the NS-domains (12). We here report a comprehensive structural analysis of GlcNH₂-containing sequences in HS from human aorta and kidney and from porcine intestine.

**EXPERIMENTAL PROCEDURES**

**Materials**—HS preparations from human aorta and human kidney were isolated as described (13, 15). HS from porcine intestinal mucosa was a gift from G. van Dedem (Diosynth, Oss, The Netherlands). Bovine lung heparin (The Upjohn Co.) was purified as described (28).

**Deaminative Cleavage of Heparan Sulfate with Nitrous Acid and Radio-labeling of Products**—Stock solutions of HS in water were prepared and concentrations (−3 mg of saccharide/ml) were determined by the meta-hydroxypHENyl method (29). Before HNO₂ treatment, the HS chains were reduced with unlabeled NaBH₄ to ascertain that subsequent radio-labeling was exclusively restricted to deamination products. The HS (−2 mg/ml) was incubated with NaBH₄ (−20 mg/ml) for 3 h at room temperature in a total volume of 200 μl of water (pH adjusted to 8–9 by NaOH). The reaction mixture was acidified with acetic acid to pH 4 and then immediately neutralized with NaOH.

Deamination of saccharide chains with nitrous acid was done at either pH 3.9 or pH 1.5, to effect cleavage at N-unsubstituted or N-sulfated GlcN residues, respectively, in strictly selective fashion (30). In both reactions the freshly prepared reagent was added to the dry samples of HS in 0.2 M NH₄HCO₃ at a flow rate of 12 ml/h. Deamination of HS (reduced with unlabeled NaBH₄) was treated with the NHO₂-pH 3.9 reagent, and the reaction products were reduced with unlabeled NaBH₄, or analyzed by gel chromatography in non-reduced form, as indicated (see Refs. 31 and 32 for methods). Radio-labeling was performed by adding 0.5 mCi of NaB₃H₄ (64 Ci/mmol, Amersham Biosciences) to the reaction mixture (containing ≤150 μg of HS), which was then incubated overnight at room temperature. The reaction was interrupted by addition of acetic acid to pH 4 (in a fume hood), followed by neutralization with NaOH (to pH 7–8). The radiolabeled HS oligosaccharides (≥2-mers) were then generally separated from non-incorporated radio-label on a 1 × 55 cm gel filtration column of Sephadex G-15 (Amersham Biosciences) in 0.2 M NH₄HCO₃, at a flow rate of 12 ml/h.

To determine the content of GlcNH₂ residues in the different HS species, 150 μg of HS (reduced with unlabeled NaBH₄) was treated with 500 μl of HNO₂-pH 3.9 reagent, and the reaction products were reduced with NaB₃H₄ as described above. The resultant radiolabeled oligosaccharides were recovered and quantified by scintillation counting, the ³H incorporated indicating GlcNH₂ units in the HS starting material. Conversion of radioactivity into molar terms was achieved through use of known amounts of bovine lung heparin as a standard that was deaminated at pH 1.5 to achieve essentially complete degradation into disaccharides. The amount of labeled disaccharides formed upon reduction with NaB₃H₄ provided a measure of specific activity, cpm of ³H/mmol disaccharide that was used to estimate the molar amounts of GlcNH₂ in HS samples.

³ The nomenclature of Conrad (3) is adopted, “upstream” and “downstream” structures being located toward the nonreducing and reducing termini, respectively, relative to a reference point.

**RESULTS**

**Contents of N-Unsubstituted Glucosamine Residues in Heparan Sulfate Preparations**

HS preparations from three sources were investigated, i.e. human aorta, human kidney, and porcine intestine. To deter-
mine the contents of GlcNH$_2$ units, each HS species was treated with nitrous acid at pH 3.9 to specifically convert these units to terminal 2,5-anhydromannose residues. Reduction with NaB$_3$H$_4$ afforded $[^3]$H$_2$ManR residues, the incorporated radioactivity indicating the GlcNH$_2$ contents of the original HS samples. The molar proportions of GlcNH$_2$ residues were calculated based on the radioactivity incorporated into heparin standard disaccharides that had been reduced with the same batch of NaB$_3$H$_4$. The GlcNH$_2$ content varied between the HS preparations and was highest (4% of total disaccharide units) in human aortic HS, intermediate (2%) in human renal HS, and lowest (0.7%) in porcine intestinal HS. Approximate estimates of molecular size by gel chromatography gave peak values for the intact intestinal and aortic HSs of ~30 kDa and ~45 kDa, respectively, both preparations being quite polydisperse (Fig. 1). These data allowed us to estimate the average number of GlcNH$_2$ units per polysaccharide chain, ~0.5 for intestinal HS and ~4 for aortic HS.

Gel chromatography (Superose 6) of intestinal HS following HNO$_2$-pH 3.9 treatment (GlcNH$_2$ target residues) showed only minimal change compared with untreated control (Fig. 1A), suggesting that any GlcNH$_2$ unit(s) present would be located upstream. The inset defines the symbols used throughout the paper; square, GlcN unit (filled, NA, N-acetylated; open, NS, N-sulfated; half-filled, NH$_2$, N-unsubstituted); triangle, aMan$_R$ unit (half-filled to indicate a GlcNH$_2$ origin; see Fig. 5); diamond, HexUA (GlcUA or IdoUA) unit. The corresponding carbohydrate structures are shown in the inset.

FIG. 1. Effect of HNO$_2$-pH 3.9 treatment of HS from porcine intestine (A) and human aorta (B). Samples were subjected to gel chromatography (Superose 6) before (solid line) or after (broken line) deamination at pH 3.9; fractions were analyzed for HexUA by the meta-hydroxydiphenyl method. Radiolabeled deamination products, ≥6-mers, recovered after reduction with NaB$_3$H$_4$ (shown for aortic HS in Fig. 3) were separated on the same column; effluent fractions were analyzed for radioactivity (open diamonds). Arrows indicate the peak elution positions of glycosaminoglycan standards.

TABLE I

| HS sample          | GlcNH$_2$-containing disaccharide units$^a$ | Labeled oligosaccharides formed on HNO$_2$-pH 3.9/NaB$_3$H$_4$ treatment of HS samples | Labeled products formed on HNO$_2$-pH 1.5 treatment of 4-mer ≥6-mer 4-mer ≥6-mer 4-mer ≥6-mer 2-mer |
|--------------------|---------------------------------------------|------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|
|                    | %                                           | 2-mer 4-mer ≥6-mer 4-mer ≥6-mer 4-mer ≥6-mer 2-mer | % | % | % | % | % |
| Human aorta        | 4 9.0 16 75 78 22 47 33 20 |                                             |                                             |                                             |                                             |                                             |                                             |                                             |
| Human kidney       | 2 20 19 61 83 17 48 30 22 |                                             |                                             |                                             |                                             |                                             |                                             |                                             |
| Porcine intestine  | 0.7 28 24 48                             |                                             |                                             |                                             |                                             |                                             |                                             |                                             |

$^a$ Percent of total disaccharide units.
naB3H4 treatment (data not shown).

chain of aortic HS contained on average /H11011
HNO2 aorta was cleaved at GlcNH2 units and the products were radiolabeled /H11011
products obtained by reduction with NaB 3H4 contained 20

–acetylation of

acetyl or an

N-acetylated (Table I; note

designation to distinguish products from fragments generated in the initial HNO2-pH 3.9 deamination). The corresponding intact sequences in aortic HS are illustrated by structures

B

the tetrasaccharides were derived from a (-GlcNH2-

HexUA-GlcNR-HexUA-GlcNH2- structure (where R is an

N-substituent pattern of se-

FIG.3 . Gel chromatography of 3H-labeled saccharides ob-

A

By contrast, gel chromatography of aortic HS following

HNO2-pH 3.9 treatment showed polydisperse products ranging from ~40 kDa to <10 kDa in size, only partly overlapping the intact untreated polysaccharide (Fig. 1B). Notably, these elution patterns were based on colorimetric HexUA analysis, and the deamination products observed thus were derived from regions upstream 3 as well as downstream of any GlcNH2 units in the chains. End-group-labeled deamination products, di- and tetrasaccharides, points
to the presence of two or more clustered GlcNH2 residues in a small proportion of the chains, alternatively a single GlcNH2 residue located very close to the non-reducing chain terminus.

Characterization of Saccharide Sequences Upstream of

N-Unsubstituted Glucosamine Residues

N-Substituent Pattern—The N-substituent pattern of sequences upstream of GlcNH2 was assessed by characterization close to either chain terminus. End-group-labeled deamination products obtained by reduction with NaB3H4 contained 20–30% each of di- and tetrasaccharides, the remaining ~50% being larger than 4-mers (gel chromatography data not shown; Table I). The size distribution of the latter components approximated a number-average representation of the intact chains (Fig. 1A), suggesting a GlcNH2 residue located toward the carbohydrate-protein linkage region. The formation also of smaller deamination products, di- and tetrasaccharides, points towards the presence of two or more clustered GlcNH2 residues in a small proportion of the chains, alternatively a single GlcNH2 residue located very close to the non-reducing chain terminus.

The three HS samples all yielded labeled 2-mer, 4-mer, and ≥6-mer, although in somewhat variable proportions (Table I; gel chromatogram shown for aortic HS in Fig. 3A). The disaccharides would represent a sequence of two adjacent GlcNH2 units with an intervening HexUA residue (sequence a in Fig. 9), whereas the tetrasaccharides were derived from a (-GlcNH2-

HexUA-GlcNR-HexUA-GlcNH2- structure (where R is an N-acetyl or an N-sulfate group). 4 To identify the N-substituents of the internal GlcNR units, the tetrasaccharides were reacted with HNO2 at pH 1.5 (cleavage at GlcNS residues), and the amounts of labeled disaccharide released were determined by gel chromatography on Sephadex G-15 (not shown). Thus 12–22% of the tetrasaccharides were found to contain an internal GlcNS unit, the remainder being N-acetylated (Table I; note

1 designation to distinguish products from fragments generated in the initial HNO2-pH 3.9 deamination). The corresponding intact sequences in aortic HS are illustrated by structures b and c in Fig. 9.

The major fractions of labeled ≥6-mers recovered after HNO2-pH 3.9/NaB3H4 treatment represent sequences of ≥5 monosaccharide units interspersed between GlcNH2 residues in the intact HS chain. Deamination at pH 1.5 of the entire ≥6-mer fractions resulted in strikingly similar degradation patterns (not shown) for the three HS species, with increasing amounts of 2-mer, 4-mer, and residual ≥6-mer, in the order

4 The 3H-labeled oligosaccharides generated by HNO2-pH 3.9 treatment would represent either sequences located between two GlcNH2 residues or the nonreducing termini of HS chains released through cleavage of a single internal GlcNH2 unit. Since the aortic HS contains on average ~4 GlcNH2 residues per chain, the latter alternative will apply to only a minor proportion of the structures deduced.
Characterization of domains upstream of N-unsubstituted glucosamine residues in aortic heparan sulfate

\[ ^{3}H \] Oligosaccharides obtained by HNO2-pH 3.9/NaBH4 treatment of aortic HS were fractionated by gel chromatography on Sephadex G-15 (Fig. 3A), and fractions corresponding to ≥6-mers were separated further by gel chromatography on BioGel P-10 (Fig. 3B). Fractions were pooled and deaminated with HNO2 at pH 1.5, and the products were separately analyzed by gel chromatography (Fig. 4). The proportions of labeled components remaining at the elution position of the corresponding intact oligosaccharide were calculated and assumed to indicate the amounts of fully N-acetylated sequence. The degradation products derived from oligomers containing one or more N-sulfated GlcN units in various positions were quantified and used to calculate the proportion of partially N-sulfated sequence.

| Table II  |
|-----------|
| N-Unsubstituted Glucosamine Residues in Heparan Sulfate |
| [^{3}H]Oligosaccharides obtained by HNO2-pH 3.9/NaBH4 treatment of aortic HS were fractionated by gel chromatography on Sephadex G-15 (Fig. 3A), and fractions corresponding to ≥6-mers were separated further by gel chromatography on BioGel P-10 (Fig. 3B). Fractions were pooled and deaminated with HNO2 at pH 1.5, and the products were separately analyzed by gel chromatography (Fig. 4). The proportions of labeled components remaining at the elution position of the corresponding intact oligosaccharide were calculated and assumed to indicate the amounts of fully N-acetylated sequence. The degradation products derived from oligomers containing one or more N-sulfated GlcN units in various positions were quantified and used to calculate the proportion of partially N-sulfated sequence. |
| % | % | % |
| 6-mer | 12 | 13 | 15 | 11 | 49 | n.d.* |
| 8-mer | 13 | 14 | 15 | 14 | 5.8 | 6.2 |
| 10-mer | 15 | 15 | 15 | 15 | 3.6 | 9.4 |
| 12-mer | 11 | 11 | 15 | 15 | 2.3 | 9.4 |
| ≥14-mer | 49 | N.D.* | 4.9* | 44* |
| Σ18 | Σ82 |
| ^* N.D., none determined. Oligosaccharides ≥14-mers were poorly resolved (Fig. 3B), and were therefore not subjected to deamination at pH 1.5. The proportions of fully N-acetylated and partially N-sulfated sequence were instead calculated assuming that 10% of these fragments resisted deamination. |

Composition of ['GlcNS']-HexUA-GlcNH2-derived disaccharides

[^{3}H]Oligosaccharides obtained by HNO2-pH 3.9/NaBH4 treatment of aortic HS were fractionated by gel chromatography on Sephadex G-15 (Fig. 3A), and fractions corresponding to ≥6-mers were pooled and further deaminated at pH 1.5. The labeled disaccharide products were isolated and analyzed by anion-exchange HPLC and paper chromatography (see “Experimental Procedures”).

| Table III  |
|-----------|
| Composition of ['GlcNS']-HexUA-GlcNH2-derived disaccharides |
| [^{3}H]Oligosaccharides obtained by HNO2-pH 3.9/NaBH4 treatment of aortic HS were fractionated by gel chromatography on Sephadex G-15 (Fig. 3A), and fractions corresponding to ≥6-mers were pooled and further deaminated at pH 1.5. The labeled disaccharide products were isolated and analyzed by anion-exchange HPLC and paper chromatography (see “Experimental Procedures”). |
| HS sample | GlcUA | IdClUA | GlcUA | IdClUA | GlcUA | IdClUA | GlcUA | IdClUA |
|-----------|-------|-------|-------|-------|-------|-------|-------|-------|
| Human aorta | 45 | 30 | 16 | 6 | 3 | n.d.* |
| Human kidney | 42 | 10 | 27 | 5 | 10 | 6 |
| Porcine intestine | N.D.* | N.D. | 12 | 9 | 31 | 14 |
| ^* N.D., none detected. |

Due to shortage of material the composition of the HexUA-[^{3}H]aManR pool (34% of total disaccharides) was not determined (N.D.).

The yield of disaccharide defines the proportion of sequence d in Fig. 9, whereas the tetrasaccharides account for part of sequence e, about 25% of the GlcNH2 residues in aortic HS are linked to an upstream -GlcNS-HexUA- structure. To obtain information about more remote upstream regions, the initial ≥6-mer fraction of aorta HS (HNO2-pH 3.9 cleavage) was separated further by gel chromatography on BioGel P-10 (Fig. 3B). A series of poorly resolved components was obtained, including an appreciable proportion of material excluded from the gel (≥10 kDa; see also Fig. 1B). The parent HS chains thus contained regions with closely adjacent GlcNH2 residues as well as extended regions lacking such residues. The clearly discernible 6- to 12-mer fractions were recovered and separately subjected to HNO2-pH 1.5 treatment (Fig. 4, A–D). A major portion of each end-labeled species was fragmented into smaller, even-numbered oligomers, indicative of N-sulfate groups. The oligosaccharide patterns locate GlcNS units to all potential positions upstream of GlcNH2 residues. Nevertheless, a fraction of each labeled oligosaccharide retained the elution position of the initial HNO2-pH 3.9 deamination product, suggesting that two GlcNH2 units may be connected by fully N-acetylated -[HexUA-GlcNAc]-HexUA- stretches of variable length. The proportions of such deamination-resistant label were determined for each oligosaccharide in Fig. 4 and used to calculate the ratio between sequences fully N-acetylated (~20%) and those containing one or more N-sulfate groups (~80%) (Table II; sequences f and e, respectively, Fig. 9).

Hexaromic Acid and O-Sulfate Residues—Substrate recognition by the GlcUA C5-epimerase that converts GlcUA to IdoUA residues requires that the adjacent upstream GlcN unit be N-sulfated, whereas the adjacent downstream GlcN unit may be either N-acetylated or N-sulfated (35). The HexUA in -GlcNac-HexUA-GlcNac- or -GlcNac-HexUA-GlcN- sequences thus is invariably GlcUA, whereas that in -GlcNac-HexUA-GlcNac- or -GlcNac-HexUA-GlcN- may be either GlcUA or IdoUA. The influence of an N-unsubstituted GlcN residue on the C5-configuration of nearby HexUA units has not been in-

\[ \text{Scheme of selective cleavage and radiolabeling procedures to define sequences downstream of GlcNH2 in HS.} \]

Full-length HS was deaminated with HNO2 at pH 1.5 to cleave chains at GlcNS units. Reduction of the resultant terminal 2,5-anhydromannose residues with NaB3H4 yielded labeled (asterisk) [1-^{3}H]aManR units. Radiolabeled oligomers were then deaminated at pH 3.9 to induce cleavage at the nearest upstream GlcNH2 residue. Identity of HexUA and occurrence of O-sulfate groups are ignored in the scheme. The symbols are as in Fig. 2, except that the [1-^{3}H]aManR triangle is open to indicate a GlcNS origin.
vestigated. We therefore identified the labeled disaccharide released by HNO₂-pH 3.9/NaB₃H₄ treatment of aortic HS, and found GlcUA-[³H]aMan₉ as the only identifiable component (by anion-exchange HPLC and paper chromatography; data not shown), corresponding to a (-GlcNH₂-GlcUA-GlcNH₂-) sequence (α in Fig. 9) in the intact polymer.

The ≥6-mers obtained by HNO₂-pH 3.9/NaB₃H₄ treatment were deaminated at pH 1.5, and the resultant labeled 2'-mers were identified to provide information regarding the native -GlcNS-HexUA-GlcNH₂- structures (Fig. 9d). Appreciable variability was observed (Table III), with both GlcUA and IdoUA residues immediately upstream of the GlcNH₂ unit, and O-sulfate groups both at C2 of IdoUA and at C6 of GlcNH₂. Notably, the relative amounts of the different disaccharides differed considerably between the HS species. Analysis of [³H]disaccharides from sequence b in aortic HS gave a pattern (not shown) essentially similar to the corresponding disaccharide relating to sequence d (Table III). Labeled 4'-mers and ≥6'-mers ([HexUA-GlcNAc]₁-HexUA-[³H]aMan₉ oligosaccharides) obtained after HNO₂-pH 1.5 treatment were analyzed to identify the radiolabeled reducing-terminal disaccharide unit. Cleavage of the oligosaccharides by HNO₂-pH 3.9 treatment following hydrazinolysis yielded GlcUA-[³H]aMan₉ exclusively (data not shown). The corresponding native sequence thus, as expected, is identified as -GlcNAC-GlcUA-GlcNH₂- (Fig. 9, e and f).

**Characterization of Saccharide Sequences Downstream of N-Unsubstituted Glucosamine Residues**

**N-Substituent Pattern**—The N-substituent pattern of sequences downstream of GlcNH₂ in HS preparations was assessed by characterization of the labeled oligomers obtained after HNO₂-pH 1.5/NaB₃H₄ treatment of polysaccharide (procedure outlined in Fig. 5). Of the total [³H]label incorporated into deamination products from aortic and renal HS, 42 and 55%, respectively, appeared in disaccharides. Products ≥4-mer (58 and 45%, respectively, of the label) were separated by gel chromatography (BioGel P-10) into a series of distinct fractions, with a dominant tetrasaccharide peak (shown for aortic HS in Fig. 6). No significant change in elution pattern was observed following repeated HNO₂-pH 1.5 treatment of each product and analysis by Superdex 30 gel chromatography (data not shown), indicating that cleavage at GlcNS units had been quantitative. Each fraction (4- to 14-mers) was separately treated with HNO₂ at pH 3.9 and subjected to Superdex 30 chromatography (Fig. 7, A–G). Distinct patterns of minor labeled degradation products were obtained, again with predominant 4-mer components. To ascertain that release of these products was indeed due to cleavage at GlcNH₂ units, labeled 10-mer was subjected to N-acetylation (treatment with acetic anhydride; see “Experimental Procedures”) before reaction with HNO₂-pH 3.9. No formation of smaller degradation products was observed (Fig. 7H).

The proportions of labeled oligosaccharides obtained upon
HNO₂-pH 3.9 treatment was used to assess N-substituent patterns of sequences downstream of GlcNH₂ units in the intact HS chains (Table IV). The results for aortic and renal HS were highly similar. The results for aortic HS are illustrated in Fig. 9 (where sequences a*, c*, and f*, not labeled in this approach, were deduced from the analysis of upstream structures a, c, and f, respectively). While about one-third of the adjacent downstream GlcN neighbor residues were N-sulfated (Fig. 9, sequence g), most of the structures showed a GlcNAc unit in this position (sequences c*, h, i, f*). A major proportion of the following adjacent downstream GlcN residues were N-sulfated (sequence h), although smaller proportions of consecutive N-acetylated disaccharide units were also observed (i and f*).

Hexuronic Acid Residues—Labeled di- and tetrasaccharides released by deamination at pH 3.9 from the aortic HS 8-mer were analyzed in more detail to define the HexUA residues as well as any O-sulfate groups downstream the GlcNH₂ units. The disaccharide (corresponding to sequence g) was identified as GlcUA-[³H]aMan₉ by anion-exchange HPLC and paper chromatography. No O-sulfate groups were detected (data not shown). Digestion with β-D-glucuronidase resulted in quantitative conversion of the tetrasaccharide (sequence h) into trisaccharide, thus identifying the terminal HexUA as GlcUA (Fig. 8). We conclude from these results that the HexUA immediately downstream of a GlcNH₂ residue is exclusively GlcUA and not IdoUA.

**DISCUSSION**

The aim of the present study was to provide comprehensive information regarding the location of GlcNH₂ residues in HS, of particular importance in view of recent reports on functional implications of such units (19–23). A variety of techniques have been used in previous studies of GlcNH₂ residues in HS, and the conclusions have been diverse. Two studies located such units to transition zones between modified (N-sulfated) and unmodified (N-acetylated) saccharide regions (26, 27). Moreover, in their study of glypican-1-linked HS produced by cultured endothelial cells Ding et al. (27) proposed that GlcNH₂ residues would be preferentially located close to the polysaccharide-protein linkage region. A GlcNH₂ unit was identified in a completely nonsulfated oligosaccharide epitope recognized by the monoclonal antibody 10E4 (23). By contrast, the -IdoUA25GlcNH₂- sequence identified as target for the 3-OST-3A sulfo-

![FIG. 8. Digestion with β-glucuronidase of tetrasaccharide representing sequence immediately downstream of GlcNH₂ unit in aortic HS. The labeled 8-mer generated by HNO₂-pH 1.5/NaB₃H₄ treatment of aortic HS was deaminated at pH 3.9, and the products were separated by gel chromatography as shown in Fig. 7. The tetrasaccharide fraction was isolated and digested with β-glucuronidase, and the digest was analyzed by Superdex 30 gel chromatography (broken line); undigested control (solid line). The peak elution positions of diand tetrasaccharides from heparin are indicated in the figure.](image_url)
larger fragments. Notably, the proportions of labeled 2- and 4-mers were inversely related to the proportion of GlcNH2 units, and thus accounted for half of the total \(^3\text{H}\) incorporated into intestinal HS, \((-0.5\ \text{GlcNH2 residue per chain})\), but only for 25\% of the labeled fragments obtained from aortic HS \((-4\ \text{GlcNH2 residues per chain})\) (Table I and Fig. 9). Moreover, the generation of small labeled oligosaccharides was not accompanied by any significant depolymerization of intestinal HS, as evidenced by gel chromatography of unlabeled deamination products (Fig. 1A). The labeled \(\geq 6\)-mers largely covered the range of unlabeled deamination products (Fig. 1A; number-average representation of the labeled species), suggesting the incorporation of \(^3\text{H}\) at the reducing end of oligosaccharides similar in size to the initial HS chains. These findings have been rationalized in a model showing two (or more) GlcNH2 units located close to the polysaccharide-protein linkage region of the intestinal HS chain (Fig. 10A), thus in agreement with the model suggested by Ding et al. (27). The labeled 2-4-mers would be derived from saccharide residues located between the GlcNH2 units, whereas the larger \(^3\text{H}\) oligomers represent the residual, major non-reducing portion of the initial chain. Release of small oligosaccharides from the nonreducing end of the HS chain cannot be excluded, but appears unlikely since it would not account for the formation of the large labeled products seen (Fig. 1A). Since the intestinal HS contains on average only one GlcNH2 unit for every two chains, clustering of such units as proposed in Fig. 10A \(\text{(upper sequence)}\) implies a majority of chains without any GlcNH2 residues \(\text{(lower sequence)}\). By contrast, most or all of the aortic HS chains contain GlcNH2 residues. Whereas the short labeled oligosaccharides observed (Table I and Fig. 3A) may again be attributable to clustered GlcNH2 units close to the polysaccharide-protein linkage region, the marked depolymerization of aortic HS upon HNO2-pH 3.9 treatment \(\text{(Fig. 1B)}\) clearly points to the occurrence of GlcNH2 also in more peripheral portions of the chains (Fig. 10B). The highly polydisperse size distribution of labeled \(\geq 6\)-mers \(\text{(Fig. 3B)}\) indicates variable length of saccharide sequences interspersed between adjacent GlcNH2 residues. Notably, a minor fraction of the radiolabeled fragments would represent the nonreducing termini of the HS chains.

The structures identified upstream and downstream of GlcNH2 residues in the aortic HS are highly diverse (Fig. 9). While the analytical data do not enable us to link upstream and downstream sequences around a given GlcNH2 residue, we note that most of the GlcNH2 residues are flanked on both sides by one or more N-acetylated disaccharide units. About 20–30\% of the adjacent downstream as well as upstream disaccharide units are \(\text{N-sulfated}\). Similar proportions, \(-30\%\) of the upstream and downstream sequences, contain a single \(\text{N-acetylated disaccharide unit}\) between the GlcNH2 reference point and the nearest \(\text{N-sulfated unit}\). About 10\% of the GlcNH2 residues in aortic HS occur immediately adjacent to each other whereas \(-25\%\) are joined by fully \(\text{N-acetylated saccharide of variable length}\). Apart from a higher proportion of consecutive
N-unsubstituted disaccharide units, the data for renal HS appears similar to that of aortic HS, with a predominance of N-acetylated disaccharide units flanking the GlcNH₂ residues on both sides (see Tables I and IV). Altogether, these findings indicate that GlcNH₂ units are preferentially located in NA- or NA/NS-domains but are scarce in NS-domains. Part of these residues thus may occur in transition zones between modified (N-sulfated) regions and unmodified (N-acetylated) saccharide regions as proposed (26, 27). On the other hand, our results do not support the notion (26) that GlcNH₂ units are generally restricted, and one of these, NDST-3, has much higher activity (H11022N) found neither in a Chinese hamster ovary cell mutant deficient (IdoUA-rich) and upstream of less modified domains. Interestingly, the IdoUA2S-GlcNH₂ sequence implicated as acceptor in the 3-OST-3A sulfotransferase reaction (20, 22) could be identified in our study (as part of sequence d in Fig. 9, Table III), though in minute quantity only.

The mechanism of GlcNH₂ formation during HS biosynthesis remains unclear. Several possibilities may be envisaged. A fraction of the GlcNAc residues initially incorporated into the precursor polysaccharide could be left N-unsubstituted in the concerted N-deacetylation/N-sulfation process. Residual N-acetyl groups could be selectively removed at a later stage, even after completed biosynthetic modification of the HS chain. Finally, N-sulfate groups could be selectively eliminated by an endo-sulfamidase, during or after the later stages of polymer modification. While there is presently no way of excluding any of these alternatives, we note that no endo-sulfamidase has yet been described (however, see Ref. 37). On the other hand, the two reactions of the N-deacetylase/N-sulfotransferase process are readily segregated in experimental systems involving either purified N-deacetylase/N-sulfotransferase enzyme (GlcUA-GlcNAc)-, polysaccharide substrate (38), or microsomal preparations incubated with sugar nucleotides in the absence of sulfate donor (PAPS) (39). Moreover, four distinct N-deacetylase/N-sulfotransferase isoforms have been described, and one of these, NDST-3, has much higher N-deacetylation/GlcNH₂-sulfate process. Residual N-unsubstituted Glucosamine Residues in Heparan Sulfate

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