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Substrate specificities of glycosyltransferases involved in formation of heparin precursor and *E. coli* K5 capsular polysaccharides †

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ABSTRACT

The *E. coli* K5 capsular polysaccharide is composed of 4)Glc*p*A(β1-4)Glc*p*NAc(α1-disaccharide units. A partially *N*-deacetylated/*N*-sulfated heptasaccharide, derived from this polymer and having a nonreducing terminal GlcNAc unit, was used as acceptor for a mastocytoma microsomal GlcA-transferase involved in heparin biosynthesis. An octasaccharide with nonreducing-terminal GlcA similarly served as acceptor for the microsomal GlcNAc-transferase. Analysis of the labeled octa- and nona-saccharides formed by transfer of monosaccharide units from UDP-[^{14}C]GlcA and UDP-[^{3}H]GlcNAc, respectively, showed that both glycosyltransferases could utilize partially *N*-sulfated acceptors. The GlcA-transferase showed a marked preference for a terminal GlcNAc-GlcA-GlcNSO$_3$-sequence, particularly when this sequence was followed by an additional *N*-sulfated disaccharide unit. Enzymes catalyzing the same GlcA and GlcNAc transfer reactions were solubilized from *E. coli* K5 membranes. The K5 capsular polysaccharide, like the heparin/heparan sulfate precursor polysaccharide, thus probably grows by stepwise, alternating addition of the two constituent monosaccharide units, from the corresponding UDP-sugars, to the nonreducing ends of the chains. Moreover, the bacterial glycosyltransferases utilized the same partially *N*-sulfated oligosaccharide substrates as the mammalian enzymes, and with similar preference for *N*-sulfate groups in certain positions.

INTRODUCTION

A polymer having the repeating structure [4)Glc*p*A(β1–4)Glc*p*NAc(α1-]$_n$ is generated as a protein-bound precursor in heparin/heparan sulfate biosynthesis. The mature glycosaminoglycans are produced by modification of this polymer, involving *N*-deacetylation and *N*-sulfation of GlcNAc units, C-5 epimerization of GlcA to IdoA units and incorporation of *O*-sulfate groups at various positions (for reviews see refs 1–4. Previous studies using microsomal enzyme preparations from mouse mastocytoma tissue[5–7] showed that formation of the [GlcA-GlcNAc-]$_n$

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sequence occurs by stepwise alternating transfer of GlcA and GlcNAc units from the corresponding UDP-sugar nucleotides to the nonreducing terminus of the nascent chain. Moreover, the rate of chain elongation was found to be markedly stimulated by concomitant enzymatic sulfation of the polymer, induced by adding the sulfate donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to the microsomal preparation[8].

The capsular polysaccharide of *E. coli* K5 has the same [GlcA-GlcNAc-]$_n$ structure as the heparin-precursor polymer[9]. Oligosaccharides derived from the K5 polymer were found to serve as substrates in the GlcA- and GlcNAc-transferase reactions involved in heparin biosynthesis. Further, experiments utilizing substrate mixtures of partially *N*-deacetylated/*N*-sulfated K5 oligosaccharides, obtained by chemical modification, suggested that saccharide sequences with *N*-sulfate groups in certain positions were preferred over the corresponding, exclusively *N*-acetylated sequences, as acceptors in the GlcA-transferase reaction[10]. We now report extended studies of the substrate specificities of the two glycosyltransferases, with regard to the *N*-substituent patterns of the acceptor structures, using more refined oligosaccharide substrates. In addition, the same substrates were applied to preliminary studies of the corresponding glycosyltransferase reactions involved in the biosynthesis of the *E. coli* K5 capsular polysaccharide. The two distinct chain elongation systems, one procaryotic, one eucaryotic, were found to be remarkably similar.

EXPERIMENTAL

Materials.—A microsomal fraction was prepared as described[11] from a transplantable mouse mastocytoma[12]. Membranes from *E. coli* wild-type O18:K5 (2980) were obtained as described[13]. UDP-[^{14}C]GlcA was prepared enzymatically from D-[^{14}C]glucose (uniformly labeled, 321 mCi/mmol; The Radiochemical Centre), as described[11]. UDP-[6-^{3}H]GlcNAc (27 Ci/mmol) was obtained from New England Nuclear. Unlabeled UDP-GlcA and UDP-GlcNAc and bovine liver β-D-glucuronidase (type B-10) were from Sigma. MBTH (3-methyl-2-benzothiazolinone hydrazone) was purchased from Aldrich Chemical Co.

Capsular polysaccharide from *E. coli* K5 was provided by Italfarmaco S.p.A., Milan, Italy, and was purified further by ion-exchange chromatography as described[10]. Oligosaccharides were generated by partial *N*-deacetylation (hydrazinolysis) of the purified K5 polysaccharide followed by deaminative cleavage at the resulting *N*-unsubstituted GlcN units (treatment with nitrous acid at pH 3.9). The resulting [GlcA-GlcNAc-]$_n$-GlcA-AnMan-ol ($n \geq 0$) oligosaccharides (AnMan-ol, 2,5-anhydro-D-mannitol product of a deaminated and reduced GlcN unit) were separated by gel chromatography[10] on Sephadex G-50, and the octasaccharide and decasaccharide fractions were recovered and desalted. Partially *N*-sulfated octasaccharide was obtained by partial *N*-deacetylation (hydrazinolysis for 2 h) followed by treatment of the product with trimethylamine–sulphur trioxide com-

plex[10]. Even-numbered ([GlcA-GlcNR]$_n$-GlcA-AnMan-ol) oligosaccharides (R = sulfonato or acetyl) were converted into the corresponding odd-numbered (GlcNR-[GlcA-GlcNR]$_{n-1}$-GlcA-AnMan-ol) species by digestion with β-D-glucuronidase[10].

Glycosyl transfer to oligosaccharides.—Enzymatic transfer of GlcNAc units was studied with detergent-solubilized enzymes, by incubating K5 octasaccharide (corresponding to 50 μg of GlcA, as determined by the carbazole reaction), 1 μCi of UDP-[^{3}H]GlcNAc, and 0.5 mg of mastocytoma microsomal protein (or 15 μg of bacterial membrane protein) in a total volume of 50 μl of 10 mM $MnCl_2$, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 1% Triton X-100, 50 mM Hepes, pH 7.2. GlcA transfer was conducted in a similar fashion, but with heptasaccharide acceptors (obtained by β-D-glucuronidase digestion of the corresponding octasaccharides) and 0.68 μCi of UDP-[^{14}C]GlcA. After incubation at 37°C for 20 min, 50 μL of 10% trichloroacetic acid was added to precipitate protein-bound endogenous saccharide acceptors, and the mixtures were centrifuged. The supernatants were neutralized with 28 μL of 1 M NaOH, mixed with 0.5 mg of carrier heparin, recentrifuged and finally applied to a HPLC column (1.6 × 60 cm) packed with a prototype gel-chromatography matrix (separation properties intermediate to those of Sephadex G-25 and G-50) developed by Pharmacia. The column was eluted with 1 M NaCl, 0.1% Triton X-100, 0.05 M Tris-HCl, pH 8.0, at a rate of 60 mL/h and fractions of ~1 mL were collected and analyzed for radioactivity. The resulting labeled oligosaccharides, separated from sugar nucleotides, were recovered and desalted by gel chromatography on Sephadex G-15. Appropriate control incubations without added oligosaccharides were done in parallel.

General methods.—Hexuronic acid was determined by the carbazole method[14] using D-glucuronolactone as a standard. Concentrations of oligosaccharides derived from the K5 polysaccharide were estimated using this reaction, assuming (as verified by actual analysis of a weighed sample of the polysaccharide) that GlcA accounted for half of the weight. 2,5-Anhydromannose was determined by a colorimetric reaction using the MBTH reagent, as described[15]. Briefly, 2 mL of sample and 0.5 mL of 0.25% (w/v) MBTH in 0.2 M HCl were incubated at 37°C for 30 min. Following the addition of 0.5 mL of 0.5% (w/v) $FeCl_3$, incubation was continued for another 5 min, and the absorbance at 650 nm was measured. Protein was estimated by the method of Lowry et al.[16]. Radioactivity was determined by liquid scintillation spectrometry using a Beckman Model LS 6000IC apparatus.

Cleavage of saccharides at *N*-sulfated or *N*-unsubstituted GlcN units was achieved by treatment with nitrous acid at pH 1.5 or 3.9, respectively[17]. The procedures of Pejler et al.[18] were followed.

Analytical gel chromatography of saccharides was performed using either the HPLC column described above or a column (1 × 190 cm) of Sephadex G-25 (superfine grade; Pharmacia), eluted with 0.2 M NaCl at a flow rate of ~3.6 mL/h. Anion-exchange HPLC of oligosaccharides was performed on a MonoQ HR 5/5 column (Pharmacia), eluted at a rate of 0.5 mL/min using a linear salt

gradient (total volume 80 mL) extending from 0 to 0.5 M NaCl in 50 mM sodium acetate, pH 4.0.

RESULTS

Strategy of the investigation.—The two glycosyltransfer reactions may be studied using acceptor saccharide sequences containing exclusively *N*-acetylated glucosamine units. GlcA and GlcNAc transfer to such hepta- and octa-saccharides, respectively, were actually demonstrated in previous experiments with microsomal glycosyltransferases from mouse mastocytoma[10]. However, these and previous studies (see Introduction) also suggested that polysaccharide formation in the intact biosynthetic system may be more complex, such that chain elongation is coupled to *N*-deacetylation/*N*-sulfation reactions. The present investigation was designed to further explore this possibility, by utilizing hepta- and octa-saccharides derived from the K5 polysaccharide, but with variable *N*-substituent patterns. To this end, an octasaccharide isolated after deaminative cleavage of partially *N*-deacetylated K5 polysaccharide was subjected to partial, presumably random, *N*-acetyl/*N*-sulfate exchange, using chemical techniques (see Materials) and used as substrate in the GlcA-transferase reaction. Removal of the nonreducing-terminal GlcA unit (1 in Fig. 1) afforded (heptasaccharide) acceptors for GlcNAc transfer.

An assembly of all possible *N*-substituent permutations, ranging from fully *N*-acetylated to tri-*N*-sulfated octa-/hepta-saccharides, is displayed in Fig. 1. To evaluate any preference, with regard to *N*-substituent recognition, of the glycosyltransferases it was important to define in quantitative terms the relative contributions of the various species. Such information was obtained by subjecting the mixtures of octasaccharides and heptasaccharides to deaminative cleavage at $GlcNSO_3$ units followed by size-separation and analysis of the resulting fragments. Hypothetical products thus generated from an arbitrarily selected di-*N*-sulfated octasaccharide and from the corresponding heptasaccharide are shown in Fig. 2 (A and C). Two colorimetric procedures, the carbazole reaction and the MBTH reaction, were used to estimate hexuronic acid and 2,5-anhydromannose units, respectively, in these products, as illustrated in the figure.

Radiolabeled oligosaccharide products obtained after incubating the heptasaccharide with UDP-[^{14}C]GlcA (yielding a ^{14}C-labeled octasaccharide), or the octasaccharide with UDP-[^{3}H]GlcNAc (yielding a ^{3}H-labeled nonasaccharide), were also cleaved by deamination of $GlcNSO_3$ units. The resulting labeled fragments, corresponding to sequences extending from the nonreducing-terminal, incorporated labeled monosaccharide units to the nearest *N*-sulfated GlcN residues (Fig. 2, B and D), were identified by gel chromatography. The preferred acceptor sequences were inferred from the patterns of such fragments.

Characterization of partially N-sulfated oligosaccharides.—Partially *N*-deacetylated/*N*-sulfated heptasaccharides, generated by digestion of the octasaccharide

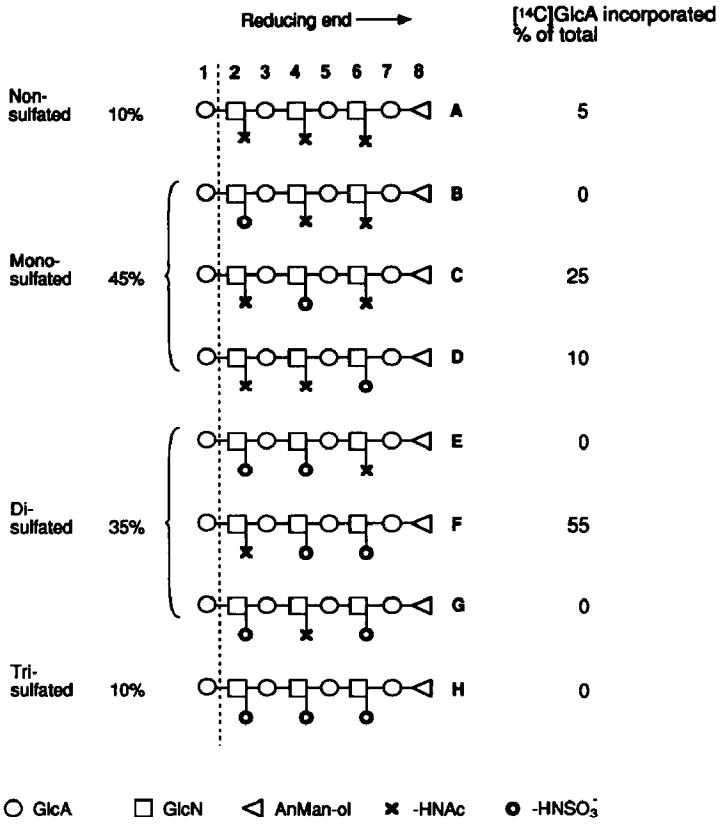

Fig. 1. Schematic representation of heptasaccharides (units **2–8**) and octasaccharides (units **1–8**) tested as acceptors in the GlcA-transferase and GlcNAc-transferase reactions, respectively. The line - - - - - - indicates the cleavage obtained by digestion with β-D-glucuronidase. The proportion of *N*-acetyl and *N*-sulfate groups was deduced from analysis of deamination products, as described in the text and illustrated in Fig. 3. It is assumed that the three species within each of the groups of mono- and di-sulfated structures occur in the same molar amounts. The column to the right indicates the distribution of incorporated [^{14}C]GlcA units between the various heptasaccharide acceptors, following incubation with the mastocytoma microsomal enzyme.

preparation with β-D-glucuronidase, were deaminated with nitrous acid. Gel chromatography of the deamination products was monitored by the MBTH reaction for aldehyde groups, thus enabling detection of the anhydromannose units formed in the procedure (see Fig. 2C). The elution pattern (Fig. 3), as expected, showed a mixture of even- and odd-numbered saccharides, the latter components originating from the nonreducing termini of parent heptasaccharides. In particular, the size of the monosaccharide peak indicated appreciable occurrence of nonreducing-terminal, *N*-sulfated GlcN unit **2** (see saccharide structures B, E, G and H in Fig. 1). Similarly, the trisaccharide would be derived from sequences (structures C and F in Fig. 1) containing *N*-acetylated unit **2** and *N*-sulfated unit **4**. The degree of *N*-sulfation of the terminal unit **2**, roughly estimated from the ratio of

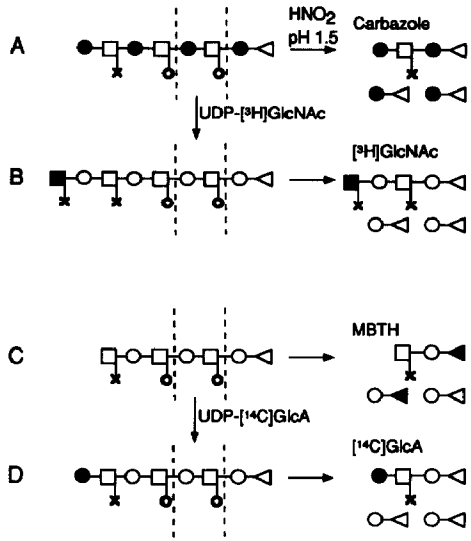

Fig. 2. Schematic representation of fragments generated by deamination of octa-(A) and hepta-saccharides (C) and of their products following incorporation of [^{3}H]GlcNAc (B) and [^{14}C]GlcA (D), respectively. The disulfated structure F (in Fig. 1) was arbitrarily selected for display. The glycosidic bonds of *N*-sulfated GlcN units are cleaved, as indicated by the vertical dashed lines, yielding the fragments shown to the right. The filled symbols indicate the target residues for the various detection methods used. The carbazole reaction detects hexuronic acid residues (that occur in the intact oligosaccharides as well as in their deamination products). The MBTH procedure was used to determine the aldehyde group of 2,5-anhydromannose residues (filled triangles) formed in the deamination reaction (the open triangles represent the terminal anhydromannitol unit, not reactive to MBTH, of the intact oligosaccharides). The products obtained after glycosyl transfer were analyzed solely by means of the incorporated radiolabel.

the monosaccharide/(monosaccharide + trisaccharide + pentasaccharide) peaks (heptasaccharide escaping detection due to prior reduction of unit **8**) was ~50%.

Similar deamination of intact octasaccharide, followed by gel chromatography and analysis of effluent fractions for hexuronic acid (carbazole reaction; see Fig. 2A) gave an elution profile (not shown) composed of ~35% disaccharide, 34% tetrasaccharide, 22% hexasaccharide, and 9% intact octasaccharide. These values were related to the predicted cleavage patterns (Fig. 2A) in order to calculate the proportions of the variously *N*-substituted parent octasaccharides. The calculations were based on simple equations (not shown) accounting for the origin of each type of fragment (for instance, the tetrasaccharide representing 100% of compound C and 50% each of compounds E, F, and G; Fig. 1), assuming random distribution of *N*-acetyl and *N*-sulfate groups. The overall octasaccharide population thus was found to contain ~10% nonsulfated molecules (component A in Fig. 1), ~45% monosulfated (components B, C, and D), ~35% disulfated (components E, F, G), and ~10% trisulfated (component H) species. Moreover, the data indicated approximately 50% *N*-sulfation of the GlcN residues, in good agreement with results obtained by the MBTH assay.

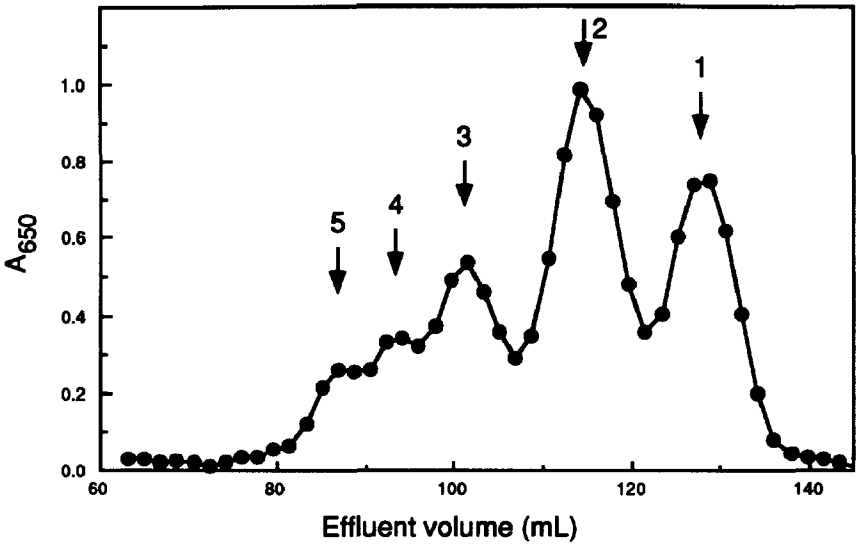

Fig. 3. Gel chromatography (Sephadex G-25) of products obtained on deamination of partially *N*-deacetylated/*N*-sulfated K5 heptasaccharides. Effluent fractions were analyzed for aldehyde groups by the MBTH procedure. The numbers above the various peaks indicate the number of monosaccharide units in the corresponding components. Peak areas were used to calculate the degree of *N*-sulfation of the parent heptasaccharide. For additional information see Fig. 2 and the text.

Microsomal GlcA transfer.—Fully *N*-acetylated, [GlcNAc-GlcA]$_3$-AnMan-ol, or partially *N*-deacetylated/*N*-sulfated K5 heptasaccharides were incubated with detergent-solubilized mastocytoma microsomal fraction and UDP-[^{14}C]GlcA (see Experimental). The resulting labeled octasaccharide products, containing a nonreducing-terminal [^{14}C]GlcA unit, were separated from excess sugar nucleotide by gel chromatography (data not shown). The yields of [^{14}C]octasaccharide generated from fully *N*-acetylated and from partially *N*-sulfated heptasaccharide (corresponding to 50 μg of hexuronic acid) were ~20000 cpm for both samples (it should be noted that the partially *N*-sulfated preparation contains appreciable proportions of species that are inactive as acceptors; see below). The labeled octasaccharides were resistant to treatment with nitrous acid at pH 3.9, as shown by gel chromatography, thus demonstrating the absence of *N*-unsubstituted GlcN units. The octasaccharide derived from the fully *N*-acetylated acceptor also resisted deamination at pH 1.5, as expected for a structure lacking *N*-sulfated GlcN residues (data not shown).

Deamination at pH 1.5 of the octasaccharide generated from partially *N*-sulfated acceptor yielded mainly labeled tetrasaccharide ([^{14}C]GlcA-GlcNAc-GlcA-AnMan-ol), along with smaller amounts of hexasaccharide and octasaccharide (Fig. 4A). The distribution of incorporated [^{14}C]GlcA units among the potential acceptor heptasaccharide structures (Fig. 1) could be roughly deduced from this pattern (see Fig. 2D), and from anion-exchange chromatography of intact, [^{14}C]GlcA-containing octasaccharide. The virtually complete absence of labeled disaccharides

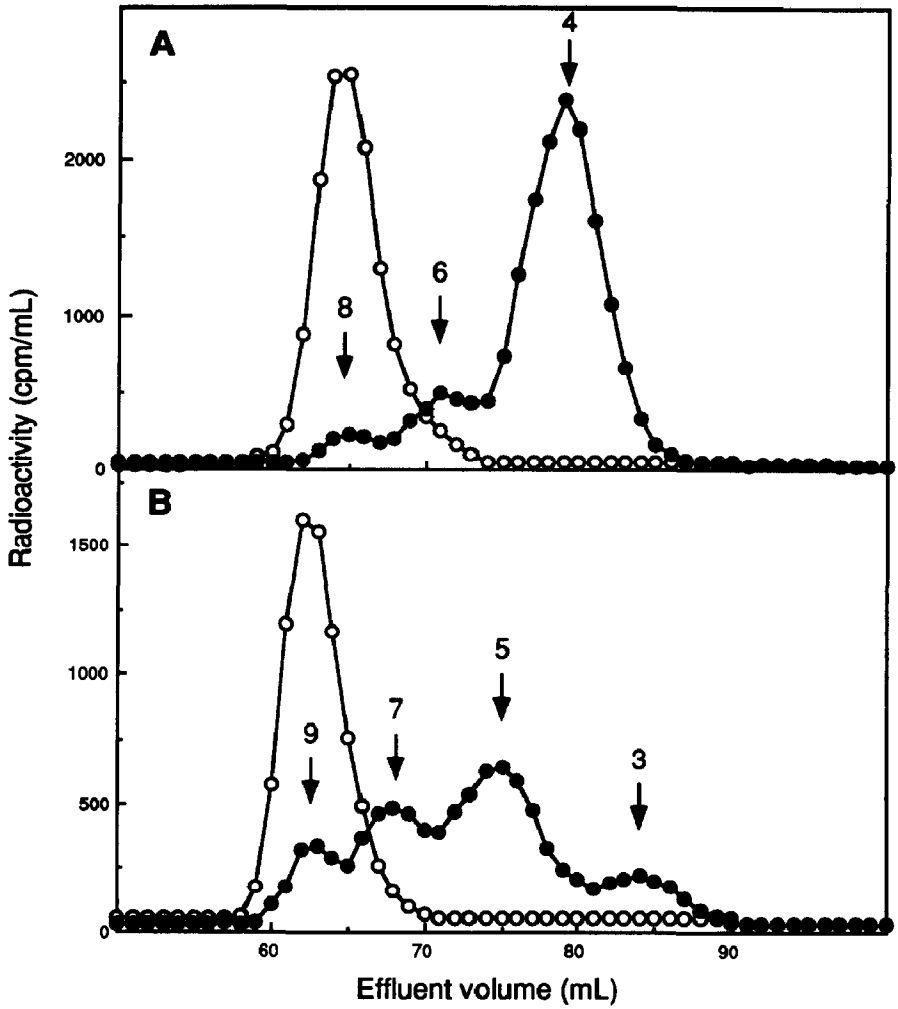

Fig. 4. Gel chromatography (HPLC) of (A) [^{14}C]GlcA-labeled octasaccharide (○); (B) [^{3}H]GlcNAc-lableled nonasaccharide (○), generated by mastocytoma glycosyltransferases from partially *N*-deacetylated/*N*-sulfated oligosaccharides, and of their deamination (pH 1.5) products (●). $V_0 = 45$ mL. Effluent fractions were analyzed for radioactivity. The numbers above the various peaks indicate the number of monosaccharide units in the corresponding components.

among the deamination products (Fig. 4A) demonstrates that GlcA transfer will occur only to acceptors with nonreducing-terminal GlcNAc rather than $GlcNSO_3$ units (position **2** in Fig. 1), thus excluding structures B, E, G, and H. The modest amounts of labeled hexasaccharide and octasaccharide observed after deamination (Fig. 4A), corresponding to ~ 10 and ~ 5% of the total incorporated ^{14}C, directly reflect the acceptor properties of heptasaccharides D and A, respectively (although these values should be considered as maximal, since hexa- and octa-saccharides could also be formed through "anomalous" ring contraction during the deamination reaction[17]). The major labeled, tetrasaccharide, deamination product has a

more complex origin. Formation of this fragment would require cleavage at an *N*-sulfated GlcN unit **4**, unit **2** being *N*-acetylated (hence resistant to deamination) and unit **1** occupied by the incorporated [^{14}C]GlcA residue (Fig. 1; see also Fig. 2D). Of the various *N*-acetylated /*N*-sulfated structural permutations conceivable, only heptasaccharides C and F would fulfil this requirement. Assuming again a random distribution of *N*-acetyl and *N*-sulfate substituents, it is calculated that these components each represent 10–15%, together ~25%, of the total pool of potential acceptor molecules. By contrast, judging from the size of the tetrasaccharide peak in Fig. 4A, compounds C and F provided >80% of the acceptor sites actually utilized by the enzyme.

Further information regarding the favored GlcA acceptor structure was obtained by anion-exchange HPLC of the intact, labeled octasaccharide generated by transfer of [^{14}C]GlcA to the partially *N*-deacetylated/*N*-sulfated heptasaccharide preparation. About 55% of this product appeared as a disulfated component, whereas ~35% was monosulfated (Fig. 5A). The disulfated compound would be derived exclusively from heptasaccharide F, the other two disulfated heptasaccharides (E and G) both being inactive as GlcA acceptors due to the occurrence of *N*-sulfated GlcN units in nonreducing-terminal position. The monosulfated fraction would be generated by transfer of [^{14}C]GlcA to heptasaccharides C and D, both of which can serve as acceptor structures (see above). The relative acceptor activities of these two species is not readily determined. However, assuming, as outlined above, that heptasaccharide D accepted ~10% of the total incorporated [^{14}C]GlcA, heptasaccharide C would account for the remaining ~25%. Structure C, which is at least as abundant as structure F in the heptasaccharide mixture (Fig. 1), thus is a less efficient acceptor of GlcA units. These findings suggest that the acceptor properties will be promoted by *N*-sulfation not only of unit **4** but also of the adjacent GlcN unit **6** (as in structure F). Clearly, more detailed information regarding substrate recognition by the GlcA-transferase may be obtained through determination of kinetic parameters using different, homogeneous acceptor structures; such oligosaccharides are not yet available.

Microsomal GlcNAc transfer.—Fully *N*-acetylated, GlcA-[GlcNAc-GlcA]$_3$-AnMan-ol, or partially *N*-deacetylated/*N*-sulfated K5 octasaccharides were incubated with detergent-solubilized mastocytoma microsomal fraction and UDP-[^{3}H]GlcNAc (see Experimental). The resulting ^{3}H-labeled nonasaccharide, as expected, emerged slightly before a [^{14}C]GlcA-containing octasaccharide on gel chromatography (Fig. 4). Under the incubation conditions employed (see Experimental), 57000 cpm and 27000 cpm of products were obtained from the fully *N*-acetylated and from partially *N*-deacetylated/*N*-sulfated octasaccharide substrate, respectively.

The latter product was subjected to deaminative cleavage at *N*-sulfated GlcN units and the resulting fragments were separated by gel chromatography. Contrary to the corresponding pattern derived from [^{14}C]GlcA-labeled octasaccharide, with its predominant tetrasaccharide peak (Fig. 4A), the [^{3}H]GlcNAc-containing deamination products yielded appreciable peaks of all odd-numbered oligosaccharides,

from trisaccharide to intact nonasaccharide (Fig. 4B). The nonasaccharide and heptasaccharide components should be derived from nonasaccharides generated through transfer of a labeled GlcNAc unit to the fully *N*-acetylated octasaccharide A and the monosulfated octasaccharide D, respectively (see Fig. 1). The major pentasaccharide peak would represent [^{3}H]GlcNAc incorporation into two acceptor compounds, C and F (see Fig. 2B), whereas the occurrence of labeled trisaccharide could reflect transfer to either of octasaccharides B, E, G, or H. Functional acceptor sequences thus may contain either *N*-acetylated or *N*-sulfated GlcN units in the penultimate, nonreducing-terminal position (unit 2).

Anion-exchange HPLC of the intact [^{3}H]GlcNAc-labeled nonasaccharides indicated, as expected, an exclusively nonsulfated product derived from the fully *N*-acetylated octasaccharide substrate (Fig. 5B). The same component was detected also among the incubation products of partially *N*-deacetylated/*N*-sulfated octasaccharide. However, most of the latter products appeared as mono- or di-sulfated components, in agreement with the notion that essentially all of the octasaccharides listed in Fig. 1 could serve as GlcNAc acceptors. The ion-exchange profile differed from that produced by the [^{14}C]GlcA-labeled octasaccharides (Fig. 5A) by being generally shifted toward less sulfated components. This difference may be rationalized by concluding that GlcA transfer is facilitated by the presence of *N*-sulfate groups in certain positions of the acceptor sequence, whereas GlcNAc transfer is largely independent of the *N*-substituent pattern.

Biosynthesis of the E. coli *K5 capsular polysaccharide.*—Despite the fact that the K5 polysaccharide is built from the same repeating disaccharide unit as the heparin/heparan sulfate precursor polysaccharide, the mechanism behind its formation has remained unclear. However, bacterial membrane preparations were shown to catalyze formation of the polysaccharide from the appropriate sugar nucleotide precursors, UDP-GlcA and UDP-GlcNAc[13]. To examine whether the bacterial glycosyltransferases operate by mechanisms similar to those involved in the mammalian system, solubilized bacterial membranes were tested for activity with exogenous K5 oligosaccharides as sugar acceptors. Incubation of GlcA-[GlcNAc-GlcA]$_4$-AnMan-ol decasaccharide with UDP-[^{3}H]GlcNAc and bacterial proteins, under conditions otherwise identical to those employed for the mastocytomal GlcNAc-transferase (see Experimental) yielded a labeled component that emerged on gel chromatography at the elution position expected for an undecasaccharide (data not shown). Conversely, the bacterial enzyme preparation catalyzed the transfer of GlcA from UDP-[^{14}C]GlcA to [GlcNAc-GlcA]$_4$-AnMan-ol nonasaccharide, forming a labeled decasaccharide. No labeled oligosaccharide products were formed on incubation of the decasaccharide with UDP-[^{14}C]GlcA or of the nonasaccharide with UDP-[^{3}H]GlcNAc (data not shown). Moreover, digestion of the [^{14}C]GlcA-labeled decasaccharide with β-D-glucuronidase resulted in quantitative release of the label as free monosaccharide (data not shown). These findings indicate that (at least under the in vitro conditions studied) the mechanism of chain elongation is the same for the K5 polysaccharide and for heparin/heparan

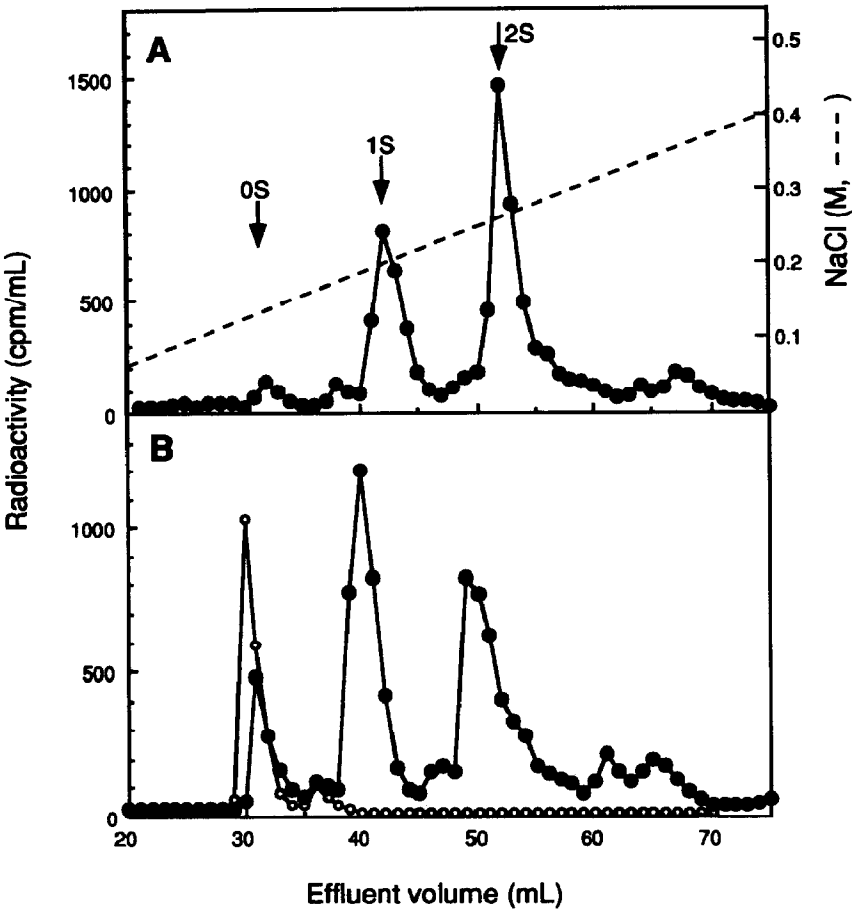

Fig. 5. Anion-exchange chromatography of (A) [^{14}C]GlcA-labeled octasaccharide; (B) [^{3}H]GlcNAc-labeled nonasaccharide, generated by mastocytoma glycosyltransferases. (○), products derived from nonsulfated octasaccharide; (●), products derived from partially *N*-deacetylated/*N*-sulfated (A) heptasaccharide, (B) octasaccharide. The designations above the various peaks indicate the number of sulfate groups (0S, no sulfate; 1S, 1 sulfate; 2S, 2 sulfate groups) per oligosaccharide. The salt gradient is indicated by the dashed line.

sulfate, involving stepwise, alternating transfer of GlcA and GlcNAc monosaccharide units from the corresponding UDP sugars to the nonreducing termini of nascent polymers.

While the K5 polysaccharide lacks sulfate substituents it was nevertheless considered of interest to test the partially *N*-deacetylated/*N*-sulfated oligosaccharides as acceptors in the bacterial glycosyltransferase reactions. Solubilized bacterial membranes were incubated with the heptasaccharide preparation and UDP-[^{14}C]GlcA, as described for the mastocytoma microsomal enzymes. Appreciable amounts of labeled octasaccharide (39000 cpm ^{14}C) were formed. Moreover, this octasaccharide was susceptible to cleavage by nitrous acid (pH 1.5), yielding a degradation pattern (Fig. 6A) highly similar to that observed after deamination of

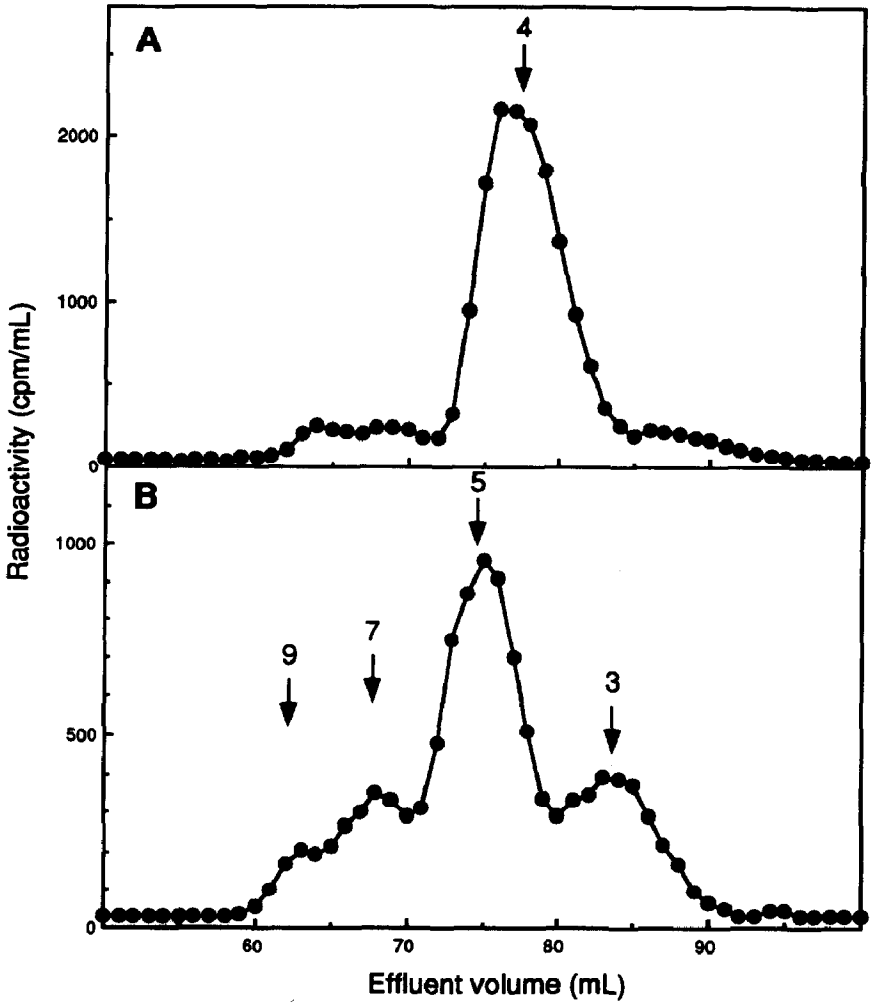

Fig. 6. Gel chromatography of deamination (pH 1.5) products of (A) [^{14}C]GlcA-labeled octasaccharide; (B) [^{3}H]GlcNAc-labeled nonasaccharide, generated by bacterial glycosyltransferases from partially *N*-deacetylated/*N*-sulfated K5 (A) heptasaccharide, (B) octasaccharide. Effluent fractions were analyzed for radioactivity.

the corresponding octasaccharide generated by the murine GlcA-transferase (Fig. 4A). The predominant formation of tetrasaccharide deamination product, along with the demonstration by anion-exchange HPLC of essentially mono- and disulfated parent octasaccharide molecules (data not shown), strongly indicated that the bacterial enzyme had preferentially recognized heptasaccharides C and F (Fig. 1) as acceptor structures. The substrate recognition properties of the bacterial and mammalian GlcA-transferases thus are strikingly similar.

Deamination of the labeled nonasaccharide generated by the bacterial GlcNAc-transferase again yielded fragments (Fig. 6B) similar (except for a somewhat more pronounced formation of pentasaccharide) to those (Fig. 4B) released

from the corresponding nonasaccharide formed by the mammalian enzyme. Odd-numbered, labeled oligosaccharides ranging from tri- to nona-saccharide were obtained, with a predominant peak of pentasaccharide. The bacterial GlcNAc-transferase, like the mammalian enzyme, thus accepts a variety of acceptor structures with different *N*-substituent patterns.

DISCUSSION

Incubation of mastocytoma microsomal enzymes with the sugar nucleotides, UDP-GlcA and UDP-GlcNAc, in the absence of the sulfate donor, PAPS, yields extended nonsulfated polysaccharide chains[5,7]. The polymerization reaction thus is not obligatorily coupled to sulfation of the chain, as is in fact apparent simply from the occurrence of the extended, *N*-acetylated block structures typically found in heparan sulfate[2,19,20]. Nevertheless, cell-free biosynthesis experiments showed that chain elongation was promoted by concomitant sulfation of the polymer[8], and this coupling was tentatively ascribed to a stimulatory effect of *N*-sulfate groups on the rate-limiting GlcA-transfer reaction[10].

In the present study this interrelationship was defined in more detail, using a mixture of variously *N*-substituted heptasaccharides as GlcA acceptors. While an exclusively *N*-acetylated $[GlcNAc\text{-}GlcA]_n$-sequence, as expected, served as an acceptor, the reaction was facilitated by the presence of *N*-sulfate groups. The distribution of such residues was found to be of critical importance. An *N*-sulfate group at the nonreducing-terminal position (unit 2 in Fig. 1) thus precludes the reaction, whereas it is tolerated or is even stimulatory at the penultimate GlcN unit 4. However, the reaction is clearly promoted by the presence of an additional *N*-sulfate residue, providing a GlcNAc-$[GlcA\text{-}GlcNSO_3\text{-}]_2$-acceptor structure. Such sequences are abundantly formed in heparin biosynthesis and occur also, albeit more sparsely, in heparan sulfate. In the latter species, *N*-acetylated and *N*-sulfated regions of variable length are interspersed in complex patterns. Typically, heparan sulfate chains appear to be initiated by an extended sequence of 8–9 consecutive *N*-acetylated disaccharide units immediately adjacent to the linkage region between the proteoglycan core protein and the polysaccharide chain proper[21]. Little is known about the regulation of *N*-deacetylation/*N*-sulfation and its interrelation with chain elongation. It seems reasonable to assume that the concerted process will involve some degree of physical interaction between the enzymes involved[10]. Interestingly, the *N*-deacetylase and *N*-sulfotransferase reactions appear to be catalyzed by the same 110 kDa enzyme protein[22]. Moreover, also the glycosyltransferase reactions seem to be associated with a single protein. Both the GlcA-transferase and the GlcNAc-transferase reactions thus were abolished due to an apparently single mutation in a CHO cell line[23]. Accordingly, recent attempts at purifying these enzymes (from bovine serum) showed that both activities could be recovered from the same gel slice (corresponding to a ~ 70 kDa component) following SDS-PAGE and renaturation of the protein[24].

The incubation system used to study the mastocytoma enzymes, based on K5 oligosaccharides and radiolabeled UDP-sugars, was also employed in attempts at defining the mechanism of elongation of the K5 polysaccharide itself. Solubilized membrane proteins from the appropriate *E. coli* strain were found to catalyze the same basic events of monosaccharide addition as previously established for the mammalian enzymes. While it is clear that data generated using artificial in vitro systems should be interpreted with care, the results nevertheless suggest that the *E. coli* K5 strain produces its capsular polysaccharide by stepwise, alternating addition of GlcA and GlcNAc units at the nonreducing terminus of the nascent chain. Probing the substrate recognition properties of the bacterial glycosyltransferases revealed further, highly unexpected, similarities with the mammalian system. Apart from the KDO group at the reducing end[13], the K5 polysaccharide carries no substituents and the GlcN units are exclusively *N*-acetylated. Still, both bacterial glycosyltransferases utilized *N*-sulfated as well as *N*-acetylated acceptors. Furthermore, the GlcA-transferase mimicked the highly elaborate substrate specificity of the corresponding mammalian enzyme, with a clear preference for acceptor structures terminating with a GlcNAc-GlcA-GlcNSO$_3^-$ sequence. Is it conceivable that the same ancient gene has been exploited in both procaryotic and eucaryotic phylogeny? Perhaps a more plausible alternative is that the microorganism has captured the gene from an infected mammalian host, and used it to generate a protective device against the environment.

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