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HEPARIN TRISACCHARIDES WITH NONREDUCING 2-AMINO-2-DEOXY-α-D-GLUCOPYRANOSYL END-GROUPS SUITABLE AS SUBSTRATES FOR CATABOLIC ENZYMES*

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ABSTRACT

Heparin trisaccharides having the sequence *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-*O*-α-L-idopyranosyluronic acid-(1→4)-2,5-anhydro-D-[1-^{3}H]mannitol have been prepared, as substrate models for studying sulfatases of heparan sulfate catabolism, by α-L-iduronidase cleavage of previously reported heparin tetrasaccharides, with additional chemical and enzymic modification as required. Three series are described, including isomeric sulfate esters of that trisaccharide with no *N*-substituent, with *N*-acetyl substitution, and with *N*-sulfate substitution. New features of the substrate specificity of the hydrolases used, including iduronate sulfatase, α-L-iduronidase, glucosamine 6-sulfate sulfatase, and heparin sulfamidase, were observed, and simple procedures for partial purification of these hydrolases are reported. The structures assigned to the trisaccharides are supported by the mode of preparation, reactions, regularities in electrophoretic behavior, and identities of the products of deamination.

INTRODUCTION

Particularly from considerations of pathology, the normal function of lysosomal exo-enzymes in the stepwise catabolism of sulfated glycosaminoglycans, including heparan sulfate, has been of interest (see refs. 1 and 2 for reviews). Studies involving sulfatases[3] have required model oligosaccharide substrates of defined structure, derived from heparan sulfate or heparin, with terminal nonreducing glucosamine residues, and specified sulfate substitution. Some oligosaccharide substrates matching this description have been reported[4–7]. The ready availability of defined di- and tetra-saccharides from the degradative deamination of heparin[8–10] has afforded a useful source of starting materials and reference compounds for flexible preparation of additional, needed trisaccharides of this substrate class. Some

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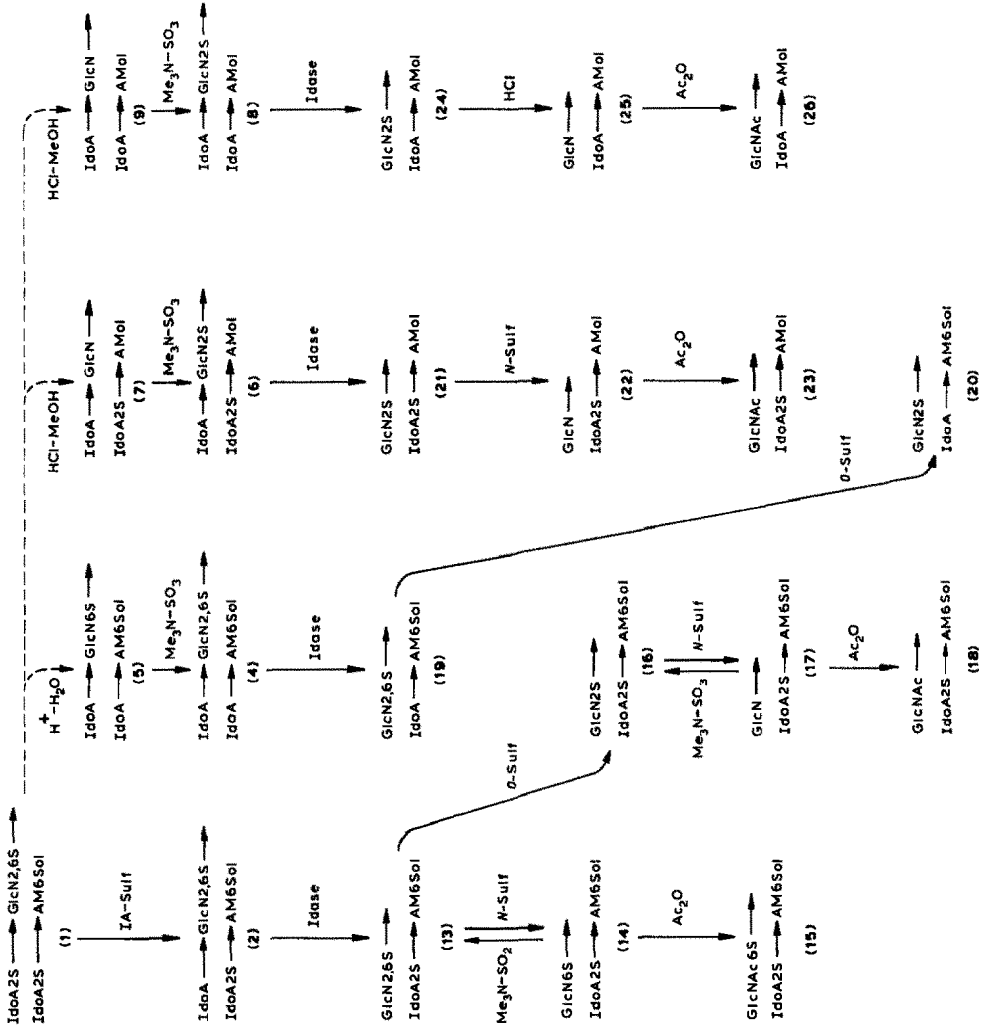
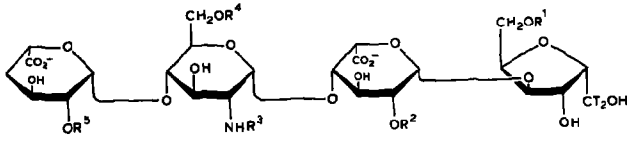
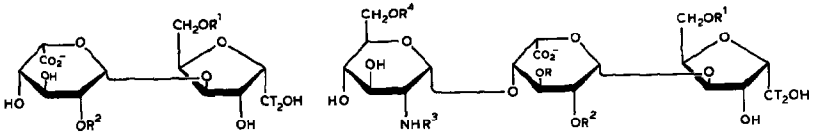


Scheme 1. Summary of the reactions used to prepare heparin trisaccharides. Reactions described previously are indicated by dashed lines. The enzymes indicated are: IA-Sulf, L-iduronate sulfatase; Idase, α-L- iduronidase; O-Sulf, glucosamine 6-sulfate sulfatase; and N-Sulf, heparin sulfamidase. The reagents indicated are: $Me_3N–SO_3$, alkaline aqueous trimethylamine–sulfur trioxide at 55°; Ac_2O, cold alkaline aqueous acetic anhydride; and HCl, 50mM hydrochloric acid at 80°.



	R^1	R^2	R^3	R^4	R^5
1	SO_3^-	SO_3^-	SO_3^-	SO_3^-	SO_3^-
2	SO_3^-	SO_3^-	SO_3^-	SO_3^-	H
3	SO_3^-	SO_3^-	H^+	SO_3^-	H
4	H	H	SO_3^-	SO_3^-	SO_3^-
5	H	H	H^+	SO_3^-	SO_3^-
6	H	SO_3^-	SO_3^-	H	H
7	H	SO_3^-	H^+	H	H
8	H	H	SO_3^-	H	H
9	H	H	H^+	H	H



	R^1	R^2
10	SO_3^-	SO_3^-
11	SO_3^-	H
12	H	SO_3^-

	R^1	R^2	R^3	R^4
13	SO_3^-	SO_3^-	SO_3^-	SO_3^-
14	SO_3^-	SO_3^-	H	SO_3^-
15	SO_3^-	SO_3^-	Ac	SO_3^-
16	SO_3^-	SO_3^-	SO_3^-	H
17	SO_3^-	SO_3^-	H	H
18	SO_3^-	SO_3^-	Ac	H
19	SO_3^-	H	SO_3^-	SO_3^-
20	SO_3^-	H	SO_3^-	H
21	H	SO_3^-	SO_3^-	H
22	H	SO_3^-	H^+	H
23	H	SO_3^-	Ac	H
24	H	H	SO_3^-	H
25	H	H	H^+	H
26	H	H	Ac	H

preliminary findings of such preparative experiments and their application to the study of enzymes have been reported[3].

The heparin trisaccharides **13–26** described herein, all sharing the sequence *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-*O*-α-L-idopyranosyluronic acid-(1→4)-2,5-anhydro-D-[1-^{3}H]mannitol, were prepared as shown in Scheme 1. It will be noted that all of the trisaccharides are derived, directly or indirectly, from the same precursor, the heparin tetrasaccharide **1** preparation previously designated[10] II-4NS.

RESULTS AND DISCUSSION

Preparation of trisaccharide tri-O-sulfates. — The tetrasaccharide IdoA2S→GlcN2,6S→IdoA2S→AM6Sol (**1**), presumably because of interference by sulfate ester substitution on the terminal, nonreducing L-idopyranosyluronic acid group, was not a substrate for purified α-L-iduronidase, as anticipated from results reported for a disaccharide analog (see ref. 11). Rather unexpectedly, the tetrasaccharide tri-*O*-sulfate preparation previously designated[10] II-3NHb was also resistant to α-L-iduronidase, apparently because of interference by the unsubstituted amino group of this tetrasaccharide. This preparation, a mixture of tri-*O*-sulfates in which the isomer **3** having a nonsulfated, nonreducing terminal idopyranosyluronic acid group was the preponderant compound (71%), had been derived[10] from **1** by mild acid hydrolysis and chromatographic separation. After chemical *N*-sulfation of **3**, the purified product **2** was now a substrate for purified α-L-iduronidase. A preparative digest of **2** with this enzyme preparation was incubated for 5 days (5mM substrate; fresh enzyme added after 2 days). Based on the specificity of α-L-iduronidase, the trisaccharide sequence GlcN2,6S→IdoA2S→AM6Sol (**13**) could be assigned to the product, a new compound of increased electrophoretic mobility (Fig. 1, lanes 2 and 3). In chromatography of the digest on DEAE-Sephadex acetate, **13** seemed well resolved from the earlier eluted residual substrate and other trace components. Nevertheless, this preparation (Prepn. A) contained some 10–15% of a contaminant having a lower electrophoretic mobility (Fig. 1, lane 4), which interfered with its sensitivity as a sulfatase substrate[3].

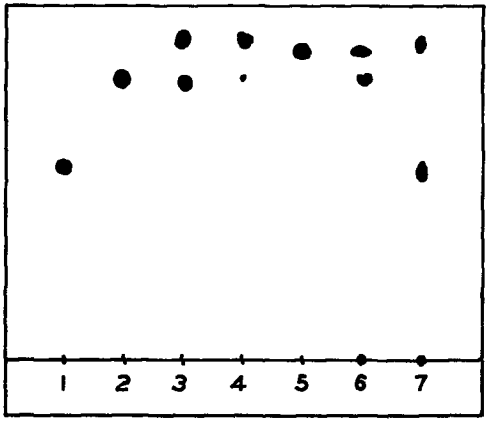


Fig. 1. Electropherograms on cellulose-coated t.l.c. film at pH 1.7: (1) IdoA-GlcN6S→IdoA2S→AM6Sol (**3**); (2) the *N*-sulfation product **2**; (3) formation of **13** by digestion of **2** with α-L-iduronidase; (4) purified **13**; (5) tetrasaccharide **1**; (6) formation of **2** by digestion of **1** with L-iduronate sulfatase; and (7) formation of **14** by digestion of **13** (lane 4) with heparin sulfamidase.

In the final version of this preparation, a modified route exploited the selectivity of two additional hydrolases. Action of a partially purified iduronate sulfatase on **1** (*cf.*, ref. 12), in a pilot experiment, selectively removed the 2-sulfate group from the terminal idopyranosyluronic acid group (as subsequently verified) to give **2** (Fig. 1, lanes 5 and 6), unencumbered by isomeric tri-*O*-sulfates. Trials also showed that the *N*-sulfate group in trisaccharide **13** (the iduronidase product) could be hydrolyzed by heparin sulfamidase[13] (Fig. 1, lanes 4 and 7). At values near pH 7, the product of this reaction (**14**) differed in charge by two units from its precursor, a circumstance greatly facilitating chromatographic purification. Identification of the unsubstituted amino group was, in part, supported by acetylation and sulfation under mild aqueous conditions of samples of **14** analogously prepared, as described later.

On the basis of the trials described, the tetrasaccharide **1** was incubated with L-iduronate sulfatase (9 days; fresh enzyme added after 6 days). Electrophoresis showed a 60% conversion to **2**. Without isolation of the product, crude iduronidase, a preparation rich in heparin sulfamidase, was added and allowed to act for 3 days more. Because, in a preliminary trial, difficulties with insoluble carbohydrate–protein complexes had been encountered, the final digest was treated with Pronase. Electrophoresis on DEAE-cellulose paper (see Fig. 2A) now showed that 78% of the total radioactivity resided in GlcN6S→IdoA2S→AM6Sol (**14**). This trisaccharide was isolated by chromatography, as illustrated in Fig. 2B. A portion of the product was chemically *N*-sulfated to give Prepn. C of **13**. Electropherograms of these preparations of **13** and **14**, illustrated in Fig. 2C, showed the presence in each of minor unidentified contaminants, detectable as slightly slower-moving satellite spots, whose radioactivity in both cases was some 2% of the total lane radioactivity. The corresponding *N*-acetyl derivative (**15**), produced by *N*-

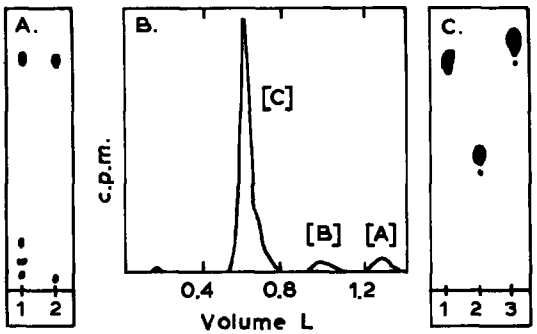


Fig. 2. Digest of **1** with a mixture of L-iduronate sulfatase and crude L-iduronidase containing heparin sulfamidase. (A) Electropherogram on DEAE-cellulose paper at pH 5.9 showing: (1) Standards (from bottom): **1**, **2**, **13**, and **14**; (2) the digest, used as a load for the column in B. (B) Chromatography of the digest (4.4 μmol of radioactive saccharides) on an ECTEOLA-cellulose column of 90-mL bed volume, developed on a linear gradient formed from 100 mL each of 0.1 and 1.3M $(NH_4)HCO_3$. In a column loaded with standards, the position of peak [A] was occupied by **1**, of peak [B] by **2** plus **13**, and of peak [C] by **14**. (C) Electropherogram at pH 1.7 showing completed preparations of: (1) **1**, (2) **14**, and (3) **13**.

acetylation of a similar preparation of **14**, appeared homogeneous on electropherograms.

Preparation of trisaccharide di-O-sulfates. — The trisaccharide **13**, (GlcN2,6S→IdoA2S→AM6Sol) was digested for 2 h with partially purified glucosamine 6-sulfate sulfatase. Only one spot, migrating more slowly than the substrate, was detectable on electropherograms of the digest. The structure GlcN2S→IdoA2S→AM6Sol (**16**) was assigned to the purified product (DEAE-Sephadex) on the basis of a previous study of the specificity of the hydrolase used[3].

In a similar approach, **13** was digested for 3 days with a mixture of glucosamine 6-sulfate sulfatase and crude α-L-iduronidase, here used as a source of heparin sulfamidase. The two products seen on electropherograms were separated and purified by chromatography on ECTEOLA-cellulose. The minor product was identical with **14**, whose generation from **13** by action of heparin sulfamidase has already been indicated (Fig. 1, lane 7). This product accumulated in the digest because of its extremely slow hydrolysis by glucosamine 6-sulfate sulfatase[3]. The major product, GlcN→IdoA2S→AM6Sol (**17**), apparently resulted from the primary action of glucosamine 6-sulfate sulfatase, followed by a hydrolysis of the *N*-sulfate group catalyzed by heparin sulfamidase[3]. The intermediate **16** was not present in the preparative digest, but could be demonstrated in trial digests when heparin sulfamidase action was suppressed by inclusion of 5mM sodium sulfite, known to be an inhibitor of that sulfatase[13]. In other experiments, *N*-sulfation of **17** gave a product having the electrophoretic mobility of **16**, whose more direct preparation is indicated above. *N*-Acetylation of **17** gave **18**.

An attempt to secure a homogeneous specimen of an isomeric trisaccharide di-*O*-sulfate, GlcN2,6S→IdoA→AM6Sol (**19**) was only partially successful. The tetrasaccharide di-*O*-sulfate preparation that had previously been designated II-2NHc and shown[10] to contain some 72% of the IdoA→GlcN6S→IdoA→AM6Sol (**5**) was *N*-sulfated. The resulting product **4** was incubated for 3 days with crude α-L-iduronidase containing 5mM sodium sulfite to inhibit heparin sulfamidase. The digest contained a minor component having the electrophoretic mobility of the substrate, presumably tetrasaccharide isomers with a sulfate substitution at O-2 of the terminal L-idopyranosyluronic acid group. Some 80% of the radioactivity in the digest was contained in a single, faster spot on electropherograms, and was attributed to a product having the sequence GlcN2,6S→IdoA→AM6Sol (**19**) based on the previous deamination analysis[10] of **5**. Chromatography on ECTEOLA-cellulose columns failed to resolve the mixture, and impure **19** was used to generate a homogeneous monosulfate preparation, as described later.

Preparation of trisaccharide monosulfates. — Impure preparation **19** was digested for 3 days with glucosamine 6-sulfate sulfatase and the digest was applied to an ECTEOLA-cellulose column. The eluates contained a major peak (81% of total radioactivity), followed by three small peaks that were not further investigated. Electrophoresis of the material recovered from the major peak gave a single spot having the mobility anticipated for a trisaccharide *N,O*-disulfate. To this prep-

aration was assigned the sequence GlcN2S→IdoA→AM6Sol (**20**). As in the case of its isomer, whose preparation is described later, the sequence assigned was supported by deamination experiments, described in a subsequent section, and by the previously established sequence of its tetrasaccharide precursor.

The tetrasaccharide *O*-monosulfate **7**, designated II-1NH in the earlier report[10], had been produced by controlled methanolysis of **1**. It was *N*-sulfated and the resulting product was estimated, on the basis of analysis before *N*-sulfation[10], to contain 83% of the isomer of sequence IdoA→GlcN2S→IdoA2S→AMol (**6**). Electropherograms of a digest of **6** with crude α-L-iduronidase (3 days) showed a spot migrating faster than substrate, and another spot at the origin. The two substances were readily separated on an ECTEOLA-cellulose column. The preparation isolated from the more retarded peak, to which the sequence GlcN2S→IdoA2S→AMol (**21**) was assigned, had the electrophoretic mobility anticipated for a trisaccharide *N,O*-disulfate. The trisaccharide preparation isolated in about equal amount from the early peak, to which was assigned the sequence **22**, had apparently resulted from secondary action on **21** of the heparin sulfamidase present in the crude enzyme preparation used; *N*-acetylation of **22** gave **23**.

Preparation of nonesterified trisaccharides. — The nonsulfated tetrasaccharide **9**, previously designated[10] II-ONH, was *N*-sulfated and the product purified (DEAE-Sephadex). The resulting preparation of **8** was digested for 3 days with crude α-L-iduronidase. Electrophoresis showed a single spot migrating somewhat

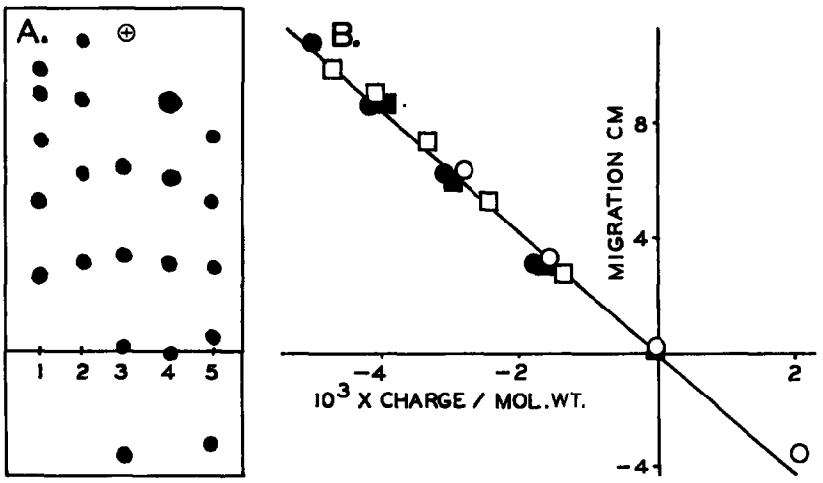


Fig. 3. Electrophoretic comparison of series of heparin oligosaccharides at pH 1.7. (A) Electropherograms of synthetic mixtures of previously isolated, electrophoretically homogeneous preparations: Lane 1, IdoA→GlcN2S→IdoA→AMol (**8**) and sulfate esters **5**, **4**, **2**, and **1**; lane 2, GlcN2S→IdoA→AMol (**24**) and sulfate esters **18**, **16**, and **13**; lane 3, GlcN→IdoA→AMol (**25**) and sulfate esters **22**, **17**, and **13**; lane 4, GlcNAc→IdoA→AMol (**26**) and sulfate esters **20**, **18**, and **15**; and lane 5, IdoA→GlcN→IdoA←AMol (**9**), sulfate esters **7**, **5**, **3**, and IdoA2S→GlcN→IdoA2S→AM6Sol. (B) For the same data, migration toward the cathode is plotted as a function of charge *vs.* molecular ratio for the compounds of lane 1 (□); lane 2 (●); lane 3 (○); and lane 4 (■).

faster than the substrate. (No evidence of secondary heparin sulfamidase action was detectable.) Purification (ECTEOLA-cellulose) gave the trisaccharide preparation **24**. Mild acid hydrolysis of a portion of **24** (50mM hydrochloric acid, 80°, 3 h) caused partial removal of *N*-sulfate groups. The hydrolyzate was made neutral and applied to an ECTEOLA-cellulose column. (This step was subsequently judged superfluous.) The percolate, which contained the nonsulfated compounds, was applied to a small Dowex 50 (H^+) column. After this column had been washed with water, the now electrophoretically homogeneous nonsulfated trisaccharide **25** was eluted with dilute aqueous ammonia. *N*-Acetylation gave **26**.

Electrophoretic relationships. — Thin-film electrophoresis at pH 1.7 was the technique chiefly used for detecting the results of chemical and enzymic transformations, for quantitative estimation of the compounds produced, and for judging homogeneity of the preparations. Fig. 3A illustrates the data for the three trisaccharide series described and for the tetrasaccharide *N*-sulfate series, some members of which have only now been prepared. For comparison, data are included for **9** and its sulfate esters, all available from earlier work[10].

Comparisons of the electrophoretic mobilities of successive members of each series give an impression of regularity that is reinforced by the plot of Fig. 3B. This agreement with expectation may be taken as excellent confirming evidence for the correctness of the net charge and molecular weight attributed to each preparation.

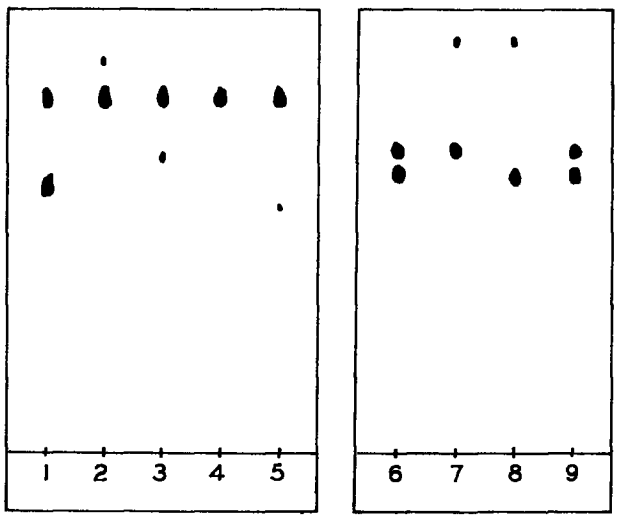


Fig. 4. Electrophoresis at pH 5.3 (lanes 1–5) and pH 2.9 (lanes 6–9) of the radioactive products from deamination of trisaccharide preparations. Lane 1: disaccharide disulfate **10** (faster spot) and monosulfate **11** standards. Lanes 2–5 show deamination products from: (2) **13**; (3) **14**; (4) **16**; and (5) **17**. Lanes 6 and 9: disaccharide monosulfate **11** (faster spot) and **12** standards. Lanes 7 and 8 show deamination products from: (7) **20** and (8) **21**. The disaccharide monosulfates are not resolved at pH 5.3. The minor spots seen in lanes 2, 3, 5, 7, and 8 correspond in mobility with those for the untreated trisaccharides, whose electrophoresis at these pH values is not shown. Components corresponding to 3–5% of the total lane radioactivity gave visible spots under the conditions used.

It must be emphasized that no distinction could be made by the electrophoretic criterion between isomers that differ only in sulfation pattern, which are not resolved by this technique.

Deamination experiments. — The structures reported in Scheme I are based on the structure previously established[10] for the common tetrasaccharide precursor **1**. This was ultimately dependent on chemical findings[14] for the structure of heparin, reinforcement for which has been obtained from more recent n.m.r. data (see ref. 15).

To verify the sulfation patterns of the trisaccharide compounds, the radioactive disaccharides produced by nitrous acid treatment were examined. Deamination of **13, 14, 16,** and **17** gave the readioactive compound IdoA2S→AM6Sol (**10**), as demonstrated by electrophoresis (see Fig. 4). This result showed that the sulfate ester groups of the L-iduronic acid and 2,5-anhydro-D-mannitol residues were intact and, by extension, that these internal sulfate groups were unaffected by glucosamine 6-sulfate sulfatase, used in the preparation of **16** and **17**. Deamination produced IdoA→AM6Sol (**11**) as the radioactive product from **20** and IdoA2S-AMol (**12**) from **21** (Fig. 4), as shown by electrophoresis[9] at pH 2.9. These findings were consistent with the sulfation patterns attributed to these trisaccharides and to the tetrasaccharides[10] used as precursors. Deamination data are unavailable for the impure trisaccharide **19**. However, the sequence assigned to the major component is supported by deamination analysis of its tetrasaccharide precursor[10] **5**, and its product of enzymic hydrolysis (**20**), and by the high yields obtained in the transformations used.

EXPERIMENTAL

Materials. — Radioactive heparin tetrasaccharides[10] and disaccharides[9] had a specific activity of 1.45 MBq/μmol. [N-$^{35}SO_4$]Heparin (740 KBq/mg) was obtained from Amersham–Searle Corp. DEAE-Sephadex A-50, Sephadex G-100, and Sepharose 4B were products of Pharmacia Fine Chemicals. Whatman ET-11 ECTEOLA-cellulose was from the Reeve Angel Co., DEAE-Biogel A from Bio-Rad Laboratories, hydroxylapatite (Hypatite C) from Clarkson Chemical Co., and Pronase from Calbiochem Corp. Heparin, used for affinity chromatography, bovine serum albumin, and the remaining biochemicals were purchased from Sigma Chemical Co. Heparin–Sepharose was prepared as described[16]. Eastman Kodak SB X-ray film was used for fluorograms of electrophoresis patterns.

General methods. — Details of most of the methods used have been described[9,10]. Thin-film electrophoresis was the analytical method generally used, with detection of spots and quantitation of radioactivity performed as previously described. Cellulose-coated t.l.c. sheet (Bakerflex from J. T. Baker Chemical Co.) was moistened with buffer and blotted before application of 2-μL spots. Except for the separations of disaccharides shown in Fig. 4, where pH values of 2.9 and 5.3 were used[9], the buffer was 1.6M formic acid (pH 1.7). In some cases, particularly where excessive protein concentration in digests caused complexing of saccharides

at the origin of electropherograms, the ion-exchange–electrophoresis technique[10] was useful. *N*-Acetylation of oligosaccharides was effected at 0° with a solution of 1% aqueous acetic anhydride in 0.4M $KHCO_3$ solution. Following *N*-acetylation, *N*-sulfation[10], or buffered nitrous acid deamination[10], the products were generally purified by chromatography on ECTEOLA-cellulose or DEAE-Sephadex columns, and the solutions de-ionized by treatment with Dowex 50 (H^+) and evaporated *in vacuo*. Proteolysis with Pronase (0.25 mg/mL) was conducted for 24 h at pH 7 in the presence of 50μM $CaCl_2$. Protein was estimated colorimetrically[17] with a bovine serum albumin standard.

Column chromatography. — Chromatography on ECTEOLA-cellulose columns[9], prewashed and developed with $(NH_4)HCO_3$ solutions, was the technique generally used for fractionation and purification of saccharides, as illustrated in Fig. 2. Shallower gradients were used for compounds of lesser charge. Preliminary data suggest that radically different concentrations of developer are required with adsorbent from diverse sources. When specified, DEAE-Sephadex acetate columns were used[10], at pH 9.2, with developer concentrations adjusted to the charges of the compounds to be separated. Continued experience with this system confirms its outstanding resolving power, but suggested advisability of caution in interpreting some results. Repeated efforts to duplicate the reported resolution of tetrasaccharide fractions II-3NHa and II-3NHb (ref. 10, Fig. 3) have been unsuccessful and no distinction was possible between the substances in the two peaks on basis of components, sequence, or electrophoretic behavior. A further disadvantage of the DEAE-Sephadex columns is the limited load accommodated, a circumstance tending to increase the contamination of products by extractives from the separation medium. The ECTEOLA-cellulose columns tolerated far higher loading, but their resolving power is considerably poorer. For example, mixtures of isomeric sulfated tetrasaccharides form a single sharp peak on these columns.

Enzyme assays. — All assays are reported in units (U) corresponding to a substrate consumption of 1 μmol per min. α-L-Iduronidase was assayed with 4-methylumbelliferyl α-L-idopyranosiduronic acid[18], heparin sulfamidase with (N-$^{35}SO_4$)heparin[11]; L-iduronate sulfatase[11] with **10**; and glucosamine 6-sulfate sulfatase[3] with **13** from the present work.

Enzyme digests of oligosaccharides. — Generally, digests contained 0.1–0.3mM substrate, enzyme, buffer, 3mM NaN_3, and sufficient dialyzed, bovine serum albumin supplement to maintain a minimum protein concentration of 0.2 mg/mL. They were incubated for 1–4 h at 37° or 1–9 days at 23°. With L-iduronate sulfatase (0.1 mU/mL), the buffer was 50mM sodium acetate (pH 4.5), and with α-L-iduronidase (0.5 mU/mL purified, 1 mg/mL crude) or glucosamine 6-sulfate sulfatase (2 mU/mL), 50mM sodium formate (pH 4.0).

Enzyme preparations. — α-L-Iduronidase was partially purified from beef liver. An homogenate of frozen tissue (150 g) in water was treated at 0° with 4M acetic acid (24 mL), made neutral after 1 h, and centrifuged. Fractionation[19] of the

extract from 1 kg of tissue with heparin–Sepharose, Sephadex G-100, and hydroxylapatite gave 2.0 mg of protein with a specific activity of 1.21 U/mg. This preparation, referred to as purified α-L-iduronidase, was devoid of detectable β-D-glucuronidase, L-iduronate sulfatase, glucosamine 6-sulfate sulfatase, or heparin sulfamidase; it lost activity almost completely in 10 months at −18°. The crude α-L-iduronidase, prepared as described with omission of the hydroxylapatite step, had a specific activity of 12 mU/mg, contained abundant heparin sulfamidase, and lost about 40% of its initial activity in 3 years at −18°.

For the preparation of partially purified heparin sulfamidase from beef spleen, an homogenate of fresh tissue (150 g) with cold water (4 vol.) was subjected to three cycles of freezing and thawing, and centrifuged. A 0.3–0.4 satd. $(NH_4)_2SO_4$ fraction of the extract was dialyzed against 50mM Tris · HCl (pH 7.0) (Buffer A), containing 50mM NaCl, and then applied to a heparin–Sepharose column (150-mL bed volume), previously equilibrated with Buffer A–50mM NaCl solution. The column was washed with Buffer A–0.2M NaCl (500 mL), and the bulk of the enzymic activity was eluted with Buffer A–0.4M NaCl (300 mL). This eluate was brought to 0.6 satn. with solid $(NH_4)_2SO_4$ and the precipitate was dialyzed against Buffer A–50mM NaCl. The resulting solution, which contained 100 mg of protein, was stored at −18° for use in the present work. The preparation liberated 42 pmol of $^{35}SO_4^-$/min/mg of protein in assays with [N-$^{35}SO_4$]heparin and its activity under typical digest conditions with **16** as the substrate was 0.44 mU/mg. Glucosamine 6-sulfate sulfatase was not detectable. The α-L-iduronidase activity was 0.43 mU/mg.

L-Iduronate sulfatase was partially purified from a human plasma fraction[20]. A dialyzed solution of the protein was subjected to chromatography on DEAE-Biogel A as described[20]. The protein in the most active fractions was precipitated with $(NH_4)_2SO_4$, added to 0.70 saturation. A dialyzed solution of the precipitate (specific activity 0.08 mU/mg) was stored at −18° for use in the present work.

Glucosamine 6-sulfate sulfatase was prepn. S-II, partially purified from beef kidney[3]. The specific activity of this preparation, assayed with **13**, erroneously reported as 28.5 mU/mg, was 7.1 mU/mg.

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