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SOME CHEMICAL, ENZYMIC, AND PHYSICAL PROPERTIES OF DISACCHARIDES FROM BEEF-LUNG HEPARIN*

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ABSTRACT

A study is reported of the reactivities of the disaccharides isolated after deamination of beef-lung heparin and reduction of the products by sodium borotritide: 2,5-anhydro-*O*-(α-L-idopyranosyluronic acid sulfate)-D-mannitol sulfate, SIMS; 2,5-anhydro-*O*-(α-L-idopyranosyluronic acid)-D-mannitol sulfate, IMS; 2,5-anhydro-*O*-(α-L-idopyranosyluronic acid sulfate)-D-mannitol, SIM; and 2,5-anhydro-*O*-(β-D-glucopyranosyluronic acid)-D-mannitol sulfate, GMS. Results for the non-sulfated disaccharides IM and GM, prepared by desulfation of SIMS and GMS, are also reported. SIMS and SIM were inert to purified α-L-iduronidase, showed unexpected resistance to periodate oxidation, and lost sulfate rapidly in 50mM hydrochloric acid at 100°. Hydrolysis of IM and of IMS was catalyzed by α-L-iduronidase, and of GM and GMS by β-D-glucuronidase; the radioactive products were identified as 2,5-anhydro-D-mannitol (aM) and its sulfate (aMS). The products SIM and IMS obtained by deamination of heparin and desulfation of SIMS (the major deamination product) are apparently identical. In heparin partially desulfated by methanolic hydrogen chloride, residual sulfate groups were mostly linked to L-iduronic acid residues. Chemical, chromatographic, and electrophoretic methods that are valuable for separation and characterization of the disaccharides are described.

INTRODUCTION

Studies of heparin and heparan sulfates, the subject of so many reports and reviews in past years, have established the structures of these important polysaccharides in broad outline[2,3]. Numerous details of the fine structure of these ramified macromolecules remain to be elucidated, however. We report herein, and in the accompanying report[4], the characterization of some oligosaccharides isolated from degradation products of beef-lung heparin. The intention in undertaking this work was to provide defined reference compounds for the study of the fine structure of

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heparin by degradative techniques, and for use as models for study of chemical and enzymic reactions. This report is concerned with the disaccharides chemically derived from heparins by nitrous acid degradation[5-7], followed by sodium borotritide reduction[8,9]. This family of radioactively-labeled disaccharides ostensibly consists[7] of: 2,5-anhydro-*O*-(α-L-idopyranosyluronic acid sulfate)-D-mannitol sulfate (SIMS); 2,5-anhydro-*O*-(α-L-idopyranosyluronic acid)-D-mannitol sulfate (IMS); 2,5-anhydro-*O*-(α-L-idopyranosyluronic acid sulfate)-D-mannitol (SIM); 2,5-anhydro-*O*-(α-L-idopyranosyluronic acid)-D-mannitol (IM); 2,5-anhydro-*O*-(β-D-glucopyranosyluronic acid)-D-mannitol sulfate (GMS); and 2,5-anhydro-*O*-(β-D-glucopyranosyluronic acid)-D-mannitol (GM). Since the preliminary report of our own results, independent reports have been published regarding isolation of this series of disaccharides[10,11], and individual members thereof[12-14]. The corresponding family of disaccharides having a 2,5-anhydro-D-mannose reducing-group[15], and some carboxyl-reduced disaccharides[8,9], have also been studied. Nevertheless, we consider it desirable to describe our own work on heparin disaccharides, because (i) it confirms and supplements the published findings of others, (ii) the techniques and information developed are the specific basis for work on larger heparin oligosaccharides, and (iii) new observations have been made. The last mentioned include: useful chromatographic and electrophoretic techniques: studies of susceptibility of the disaccharides

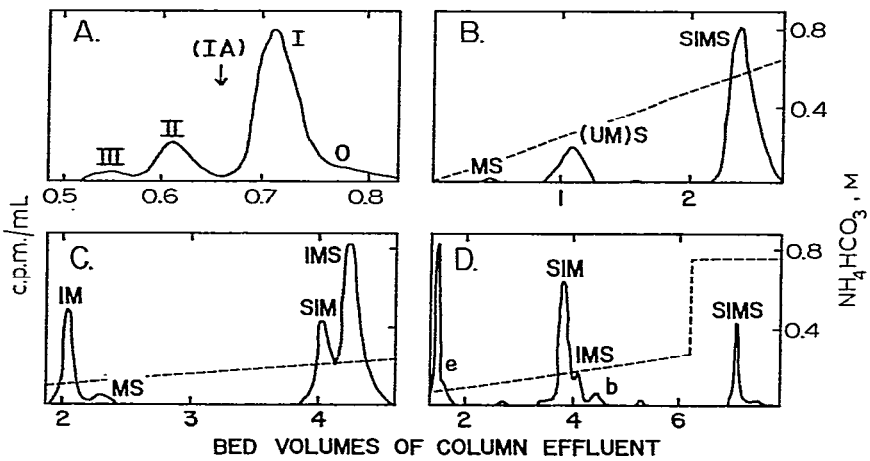


Fig. 1. Column chromatography of disaccharides from beef-lung heparin: (A) Mixture from reaction of heparin with butyl nitrite, chromatographed on Sephadex G-25 Superfine, with M sodium acetate as developer. Peak I contained the disaccharides. Peaks for inorganic sulfate and chloride, not shown, appeared at 0.75 and 0.96 bed volume. (B) Disaccharide fraction from A, chromatographed on ECTEOLA-cellulose developed with ammonium hydrogencarbonate in a gradient (dashed line), after preliminary development with 20mM ammonium hydrogencarbonate. (C) Reaction mixture from partial desulfation of SIMS, on ECTEOLA-cellulose, and ammonium hydrogencarbonate gradient from start of development. Residual SIMS, not shown, was eluted with 0.75M ammonium hydrogencarbonate. (D) Reaction mixture from deamination of desulfated heparin, on ECTEOLA-cellulose, and ammonium hydrogencarbonate gradient from start. Peak b remains unidentified, and Peak e contains a mixture, of which GM is one component. About 20% of the radioactivity applied was not eluted.

to attack by enzymes, dilute acid, and periodate; studies of the product pattern with these reagents; and localization of the sulfate ester groups in "desulfated" heparin.

RESULTS AND DISCUSSION

Preparation of disaccharides by deaminative degradation. — Butyl nitrite deamination[6] of a beef-lung preparation, at low temperature and pH, reduction[8] of the 2,5-anhydro-D-mannose end groups to 2,5-anhydro-D-[^{3}H-1]mannitol with excess sodium borotritide, and chromatography[7] on Sephadex G-25 gave chiefly a disaccharide fraction (I), as illustrated in Fig. 1A and Table I. Under these conditions, the amounts of hexasaccharide (III) and tetrasaccharide (II) produced exceeded those expected from the known, relatively minor *N*-acetyl content of beef-lung heparin[6]. This result apparently represented incomplete cleavage, caused by the rearrangement of 2-amino-2-deoxy sugar residues to 2-aldehydopentosides, a side reaction reported for deaminative cleavage[8,16]. The butyl nitrite reagent was, nevertheless, adopted as a suitable compromise for the present work. Some other reagents in common use[8,17] promoted more complete cleavage (Table I), but generated more fragments approximating tri- and mono-saccharide in size, as judged by elution from gel columns (IA and O, Fig. 1A). The nature of these species has been only incompletely studied. Examination of the "monosaccharide fraction" (Fig. 1A, O) from a butyl nitrite deamination mixture by paper electrophoresis and paper chromatography revealed numerous radioactive spots. These products, both neutral and acidic, corresponded only in minor proportion to any reduced monosaccharides anticipated, and were thought to arise mainly from labelling of smaller fragments produced by oxidation reactions. Such secondary fragmentations, where prominent, tend to make degradative deamination patterns difficult to interpret.

TABLE I

PRODUCTS OF DEAMINATION OF A BEEF-LUNG HEPARIN SPECIMEN BY THREE REAGENTS[a]

Product[b]	*Reagent*		
	BuONO[c] *at −18°*	*HONO*[d] *(low pH)*	*$NaNO_2$*[e] *and AcOH*
Peak 0 ("monosaccharides")	8	11	26
Peak I (disaccharides)	71	77	65
Peak IA ("trisaccharides")	0	8	0
Peak II (tetrasaccharides)	17	4	8
Peak III (hexasaccharides)	4		1

[a]Numerical values represent percent of total radioactivity of the reduced deamination products, based on gel filtration data. [b]The major products of deamination are known to contain even number of monosaccharide residues (*cf.* ref. 7). Peak IA, intermediate between I and II, when present, is presumed to contain trisaccharides merely from its position on columns. [c]Cifonelli and King[6]. [d]Shively and Conrad[8]. [e]Lagunoff and Warren[17].

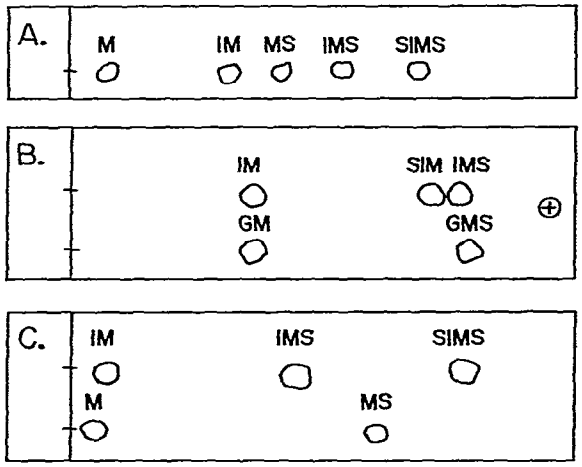


Fig. 2. Electrophoresis of sample mixtures: (A) On t.l.c. strips at pH 5.3; (B) on t.l.c. strips at pH 2.9; and (C) on Whatman 3MM paper at pH 1.7. At pH 5.3 or 1.7, SIM or GMS (not shown) are not resolved from IMS.

As seen in Fig. 1B, the disaccharide fraction (I) from gel filtration was readily separated by charge class on ECTEOLA-cellulose columns[7], yielding a homogeneous disulfate fraction, designated SIMS, and a mixed monosulfate fraction, (UM)S. Although produced during deamination of lung heparan sulfate[7,18], nonsulfated disaccharides are not obtained from beef-lung heparin. As described by others[11], GMS was readily separated from the mixed disaccharide monosulfate fraction by preparative paper chromatography. The residual mixture of IMS and SIM was resolved by chromatography on ECTEOLA-cellulose using a shallow gradient of developer concentration, as illustrated in Fig. 1C. Elimination of the intermediate fractions gave two, homogeneous monosulfate preparations, designated IMS_1 and SIM_1*. Mixtures of SIM and IMS were more recently resolved by electrophoresis at pH 2.9, as illustrated in Fig. 2. This last separation, which was found to be rapid and useful for analytical purposes, was shown, by electrophoretic experiments at a variety of pH values, to depend on a small difference in pK_2, which is ~3.4 for SIM and ~3.1 for IMS. (Although, at pH 2.9, GMS migrates slightly more rapidly than IMS, these two species were not resolved.) As also described[11], IMS and SIM were separable by a second paper-chromatography step.

Disaccharide composition of heparin. — These three separation routes for monosulfates gave similar analytical results for the composition of disaccharide mixtures. The disaccharides from butyl nitrite deamination of beef-lung heparin typically had the composition: SIMS, 83%; GMS, 6.5%; SIM, 5.9%; and IMS, 4.3%. If it is assumed that the excessive fragmentation observed in deamination by the Lagunoff

*The subscripts indicate the origin of these specific preparations, comparison of which with others will be described. Where different preparations have been shown to be interchangeable, the subscript is omitted.

and Warren[17], or Shively and Conrad[8], methods is not selective for regions of heparin that are rich in *N*-acetyl groups, the yields of tetra- and tri-saccharides by these methods (Table I) correspond to a maximum content of some 0.10 acetyl group per disaccharide unit. Furthermore, assuming the composition just cited for a disaccharide fraction to be representative of non-*N*-acetylated regions of the polymer (including those lost to side reactions), the overall analytical data of this portion of the polymer may be reconstructed by summing the radioactivities of appropriate species and calculating their ratios. On this basis, the ratio of D-glucuronic acid to total uronic acid residues in these regions of the specimen examined was 0.07 : 1, and the molar ratio of total sulfate groups to uronic acid residues was 2.83 : 1.

Indirect routes for preparation of disaccharides. — A convenient route to IMS and SIM was the partial desulfation of the more abundant, and glucuronic-free, SIMS under mild conditions (15mM hydrogen chloride in methanol, 18 h, 4°). The products were separated by chromatography on ECTEOLA-cellulose, as shown in Fig. 1C. The resulting preparations were IM_2 (16% of recovered radioactivity), IMS_2 (43%), SIM_2 (21%), and $SIMS_2$ (20%). The preparations of GM and IM_1 used were obtained by desulfation of GMS and SIMS, respectively, in methanolic hydrogen chloride, and chromatographic purification of the products on ECTEOLA-cellulose.

Composition of disaccharides. — The results of analyses of representative disaccharide preparations for uronic acid and sulfate were in satisfactory agreement with their postulated structures, as shown in Table II. Assignment of the identity of the uronic acid residues and their anomeric configuration is largely based on the experiments with partially purified rat-liver α-L-iduronidase and rat preputial gland β-D-glucuronidase that are shown in Table III. Each of these enzyme preparations was devoid of the activity of the other, as shown in tests with synthetic substrates and confirmed with the disaccharides (Table III). α-L-Iduronidase has been shown previously to have no action on β-D-glucopyranosyluronic[19], α-D-glucopyranosyl-

TABLE II

ANALYSES OF DISACCHARIDE PREPARATIONS[a]

Preparation	*Uronic acid*	*Sulfate*
$SIMS_2$	1.02	1.97
IMS_2	1.05	0.91
SIM_2	0.99	0.96
GMS	0.95	0.86
IM	0.94	
GM	0.92	

[a]Analytical values are expressed as molar ratios of uronic acid or total sulfate to radioactive 2,5-anhydro-D-mannitol end-group (8.16 Ci/mol). The uronic acid analyses for SIMS, IMS, SIM, and IM are based on an L-iduronic, and the analyses for GMS and GM on a D-glucuronic acid color standard.

TABLE III

ACTION OF β-D-GLUCURONIDASE AND α-L-IDURONIDASE ON DISACCHARIDES

Substrate	*β-D-Glucuronidase*		*α-L-Iduronidase*	
	Hydrolysis (%)	*Radioactive product*	*Hydrolysis (%)*	*Radioactive product*
SIMS	<5		<5	
IMS_2	<5		88	aMS
SIM_2	<5		<5	
IM_2	<5[a]		61	aM
GMS	84	aMS	<5	
GM	81[a]	aM	<5	

[a] β-D-Glucuronidase, 550 U/L in digest; all others 55 U/L. α-L-Iduronidase digests contained 13 U/L of this enzyme.

uronic, or β-L-idopyranosyluronic acids[20]. β-D-Glucuronidase is also known to be highly specific[20,21]. The enzymic data show that GM and GMS contain β-D-glucopyranosyluronic, and that IM_2 and IMS_2 contain α-L-idopyranosyluronic acid residues. Because IM_2, IMS_2, and SIM_2 all derived from SIMS by *O*-desulfation, susceptibility of IM_2 and IMS_2 to α-L-iduronidase showed the parent substance, SIMS, and the remaining product, SIM_2, to contain an α-L-idopyranosyluronic acid residue as well (IMS_1 is also a substrate). The radioactive products of the reactions listed in Table III showed, in paper electrophoresis at pH 5.3 and in paper chromatography (solvents *A* and *D* for aM, and solvents *A* and *B* for aMS), mobilities identical with those of authentic specimens. Isolation of aMS from enzymic hydrolysis products obviously locates the position of the sulfate group in the 2,5-anhydro-D-mannitol residue of IMS and GMS. The action of α-L-iduronidase on SIM and SIMS is presumably blocked by sulfate substitution on the α-L-idopyranosyluronic acid residue[22]. These assignments for sulfate substitution are supported by the periodate oxidation studies considered next.

Periodate oxidation of disaccharides. — The action of sodium metaperiodate at pH ~5 (the pH of the preparations), 25°, and low ionic strength, is illustrated in Fig. 3. IM and GM were oxidized rapidly, IMS and GMS at a moderate rate. As summarized in Table IV, mild acid-treatment of the oxidized products yielded 2,5-anhydro-D-mannitol (aM) from IM or GM, and 2,5-anhydro-D-mannitol sulfate (aMS) from IMS or GMS. This was the behavior expected for disaccharides having a uronic acid residue susceptible to oxidation and glycosidically linked to C-4 of a 2,5-anhydro-D-mannitol residue. Moreover, the isolation of aMS from the oxidation products of IMS and GMS verifies the placement of the sulfate ester group on the 2,5-anhydro-D-mannitol residue in these compounds. That SIM and SIMS were extremely resistant to attack by periodate oxidation (Fig. 3) was an unexpected finding, as the 2-sulfate substitution on the uronic acid residue of heparin (long the accepted

TABLE IV

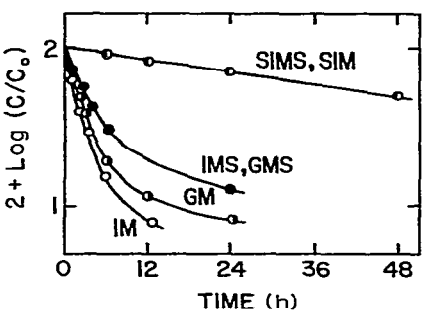
RADIOACTIVE PRODUCTS FORMED INITIALLY IN PERIODATE OXIDATION OF DISACCHARIDES, WITH SUBSEQUENT MILD-ACID TREATMENT

Substrate	*Time (h)*	*Products (%)*				
		SIMS	*(UM)S*[a]	*UM*[a]	*aMS*	*aM*
SIMS	6	92	2		6	
IMS	1		77	1	22	
SIM	6		85	3	4	8
IM	1			60		40
GMS	1		79	3	18	
GM	1			67		33

[a]UM designates a nonsulfated disaccharide with unspecified uronic acid and (UM)S a disaccharide monosulfate, with unspecified location of the sulfate ester group.

view[3]) is expected to leave a 3,4-diol grouping open to periodate attack in these disaccharides. Paradoxically, the most cogent evidence for 2-sulfate substitution came from the early observation of Foster *et al.*[5] that "all the uronic acid had been destroyed" after periodate treatment (under conditions apparently quite similar) of the products of deamination of beef-lung heparin by nitrous acid. The discrepancy between this earlier study and the present one may be accounted for by a difference in pH, a factor shown to be critical for polymeric heparin[23]. In any case, 2-sulfate substitution need not necessarily be incompatible with the periodate resistance now observed, as some such factor as electrostatic repulsion of periodate ion by a local high density of negative charge may be operating[23].

Acid hydrolysis studies. — The lability of the disaccharides to action of dilute mineral acids was studied, and the results are summarized in Fig. 4 and Table V. The long-known resistance to acid hydrolysis of β-D-glucosyluronic acid residues is exemplified here by the results for GM, and the lesser stability of the α-L-idosyl-



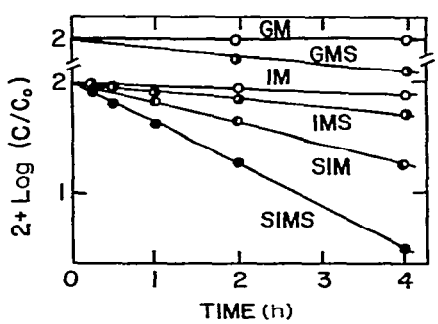


Fig. 3. Kinetic study of oxidation of disaccharides (2mM) by 8mM sodium periodate at 25°, low ionic-strength, and pH ~5.

Fig. 4. Kinetic study of hydrolysis of disaccharides by 50mM hydrochloric acid at 100°.

TABLE V

RADIOACTIVE PRODUCTS FORMED INITIALLY ON HEATING DISACCHARIDES AT 100° WITH 50mM HYDROCHLORIC ACID

Substrate	*Time (min)*	*Products (%)*				
		SIMS	*(UM)S*[a]	*UM*[a]	*aMS*	*aM*
SIMS	30	42	50	4	3	1
IMS	60		78	12	10	
SIM	30		81	18		1
IM	240			74		26
GMS	60		84	16		
GM	240			98		2

[a]For abbreviations, see footnote to Table IV.

uronic acid residues[25] is reflected in the appreciable, if still slow, hydrolysis of IM. The initial hydrolysis of IMS_2 and GMS (aMS, not shown, behaved similarly), representing largely the loss of ester sulfate (Table V), was somewhat more rapid. The moderate rate was consistent with recorded observations for the usual sugar sulfates. On the other hand, hydrolysis of SIM_2 and SIMS, representing largely the loss of sulfate groups from iduronic acid residues, was strikingly rapid. Such behavior had been observed previously for the carboxyl-reduced analog of SIMS[8,9], an (idopyranosyluronic acid) sulfate, and it may be general for hexopyranoside 2-sulfate[26]. The analogy is incomplete, because, unlike the examples cited, there is little concomitant glycoside hydrolysis for SIMS (although some is seen in SIM; see Table V).

The selectivity of partial desulfation by methanolic hydrogen chloride was qualitatively similar, in the case of SIMS, to that with mineral acid. In both cases, approximately twice as much IMS as SIM was produced. It was of interest to examine also the relative lability of various sulfate groups in polymeric heparin for comparison. It had been reported by several laboratories that heparin exhaustively desulfated by action of methanolic hydrogen chloride[17] retained almost one resistant sulfate ester group per disaccharide unit[28,29]; the present work agreed with these reports. The deamination of this sulfate-poor product from heparin (Lagunoff and Warren procedure)[17] was studied. An ECTEOLA-cellulose chromatogram of the reaction mixture (Fig. 1D) showed the disaccharides produced (about 80% of total radioactivity) to have the composition: IM, 25%; SIM, 37%; IMS, 7%; SIMS, 13%; and miscellaneous, 18% (the identity of the peaks was checked by electrophoresis and paper chromatography). The preponderance of SIM over IMS was in agreement with an earlier report on the deamination of "desulfated" heparin, which gave a disaccharide characterized as *O*-[sodium (methyl α-L-idopyranosyluronate) 2-sulfate]-(1→4)-2,5-anhydro-D-mannose by i.r. data and by sulfate hydrolysis with tissue extracts[13]. These differences between polymer and disaccharide models in differential lability

of the two types of sulfate ester group are, of course, striking. A point of difference between disaccharide and polymer is the presence in the polymer of overall conformational restraints[30], and of positively charged ammonium groups in the polymer (formed from the original sulfoamino groups early in the reaction). The usefulness of either factor in rationalizing the findings is not at once apparent. A study of the desulfation of heparin by aqueous acid and by dimethyl sulfoxide–pyridine has been reported recently[31].

Homogeneity and identity of various disaccharide preparations. — The conformity to first-order kinetics in acid hydrolysis of SIMS was excellent, even to the point of 97% reaction (Fig. 4). This was taken to be convincing evidence against gross heterogeneity of the glycosidic linkage and sulfate substitution in this preparation. By extrapolation, IMS_2 and SIM_2, both derived from SIMS, were also homogeneous. A comparison was undertaken of these preparations with IMS_1 and SIM_1, both available directly from deamination of the polymer. The two pairs of preparations coincided in their relative elution positions in ECTEOLA-cellulose column chromatography, and in their migration in electrophoresis at pH 2.9. The mobilities in paper chromatography (solvent *A*) of the IM specimens produced by desulfation of SIMS, IMS_1, or SIM_1 were also identical. Moreover, the rates of acid hydrolysis and the formation of hydrolysis products for SIM_1 and SIM_2, and for IMS_1 and IMS_2, agreed within experimental error. It was concluded that, at least, gross variations in glycosyluronic linkages or position of sulfate groups were unlikely in that portion of the original beef-lung heparin represented by the nitrous acid disaccharides studied; for butyl nitrite deaminations, these accounted directly for at least 55% of the mass of the polymer (~20% was represented by oligosaccharides containing *N*-acetyl groups, 18% by oligosaccharides resulting from deamination without cleavage, and 3% by "monosaccharide" fragments).

Welcome as such semblance of regularity may be, continued experience with these systems suggests caution. Throughout this and related studies of heparin degradation, by whatever method, improved separation techniques have regularly revealed additional, unidentified minor products, many of them amounting to 0.5–5% of the mixtures. Limitations of time and material often precluded effective study of such minor components. It is in no case clear whether they represent irregularity or inhomogeneity of the polymeric preparation, which might be physiologically meaningful, or merely the incomplete predictability of the degradative reactions applied.

EXPERIMENTAL

Materials. — A single preparation (17 g) of beef-lung heparin was used throughout. It had been purified[32] from the sodium salt of heparin of pharmaceutical grade, a generous gift from Dr. L. L. Coleman of The Upjohn Co. (Kalamazoo, MI 49001). 1,2-*O*-Isopropylidene-L-idurono-6,3-lactone, prepared by Dr. Robert Friedman, and D-glucurono-6,3-lactone, obtained from Pfanstiehl Labs. (Waukegan, IL 60085), were used as standards for uronic acid colorimetry. Phenyl α-L-idopyranosiduronic

acid[33] and 2,5-anhydro-D-[1-^{3}H]mannitol[34] were prepared as described. For the latter preparation, sodium borotritide was used at pH 8.6 in the reduction step. 2,5-Anhydro-D-mannitol 1-[^{35}S]sulfate (aMS, same as 6-sulfate) was isolated as a crystalline brucine salt after reaction of 2,5-anhydro-D-mannitol with [^{35}S]sulfur trioxide–trimethylamine in *N*,*N*-dimethylformamide[35]. Sodium borotritide was obtained from New England Nuclear (Boston, MA 02118) (typically, 150 C/mol), and D-[U-^{14}C]glucose and D-[U-^{14}C]glucitol from Amersham Corporation (Arlington Hgts, IL 60005). Regularly received batches of butyl nitrite (Eastman Organic Chemicals, Rochester, NY 14650) were distilled, and the middle fractions were preserved at −18°. To remove peroxides, 1,2-dimethoxyethane was dried over molecular sieves (Type 4A, Fisher Scientific Co. Pittsburgh, PA 15219) and distilled from a small amount of Vitride (Eastman). Potassium dihydrogenborate was formed in solution from boric acid and standardized potassium hydroxide. Dowex 1-X8 and Dowex 50-X4 (both 200–400 mesh) were purchased from J. T. Baker Co. (Phillipsburgh, NJ 08865).

Analytical methods. — Uronic acid was estimated colorimetrically[36] using an L-iduronic acid standard, except for GM and GMS, for which a D-glucuronic acid standard was used. Ester sulfate was determined colorimetrically[37], after heating the sample in 6M hydrochloric acid for 16 h at 100° in a sealed tube, evaporation *in vacuo* in the presence of sodium hydroxide, treatment of the dry residue with excess ammonium hydroxide, and evaporation to dryness. Radioactivity was measured with a Packard 3310 scintillation spectrometer using standard [^{3}H]toluene and [^{14}C]toluene for calibration, and a "cocktail" containing PPO (4 g/L) and POPOP (0.05 g/L) in 1:1 (v/v) 2-methoxyethanol–toluene for aqueous samples, and in toluene for samples on paper, ion-exchange cellulose paper, and t.l.c. film.

De-ionization of saccharides. — Experiments were generally designed so that acetate was the anion in any electrolytes used. Where sulfate ion was introduced by deaminations or desulfations, the first step in de-ionization was addition of excess barium acetate and removal of barium sulfate by centrifugation. Cations were removed by rapid passage through Dowex 50 (H^+) columns. An amount of sodium acetate slightly in excess of the amount required to convert acidic carbohydrate groups to sodium salts was added to the effluent. When the borate ion was present in the original solution, as after borohydride reductions, boric acid was removed at this point by repeated codistillation *in vacuo* with methanol. The acetic acid present, and the azide ion present in the original solution, were finally removed by repeated codistillation *in vacuo* with water.

Paper chromatography and electrophoresis. — Paper chromatograms were developed by downward irrigation. Whatman No. 1 paper was used for analytical purposes. For preparative purposes, Whatman 3MM paper was used, and zones of interest were extracted with aid of "spin-thimbles" (Reeve–Angel). Solvent *A*, 57:14:29 (v/v) 1-butanol–acetic acid–water, was particularly useful in separating GMS (9.2 cm migration on Whatman No. 1 paper in 64 h) from IMS (11.3 cm) plus SIM (12.2 cm). Solvent *B*, 2:2:1 (v/v) 1-butanol–acetic acid–12.5% ammonia was

more effective in resolving IMS and SIM[11]. Solvent *C*, 7:6:7 (v/v) 1-butanol–pyridine–water, used as a developer for papers that had been impregnated with 50mM potassium tetraborate and dried before spotting[38], readily separated D-glucitol from D-glucose. Solvent *D* was 18:3:1:4 (v/v) ethyl acetate–acetic acid–formic acid–water.

Spots were located by autoradiography. Paper chromatograms or electropherograms (or guide strips cut from these, for preparative applications) were passed through a solution of 0.7% of PPO in ether, and dried[39]. Kodak SB-5 X-ray film was maintained in close contact with the papers for 1–7 days at −78°, then developed, through courtesy of the Department of Radiology, in a diagnostic, rapid-development system. Spots (5 μL) showing 2×10^5 d.p.m. of tritium were detectable, after chromatography, by overnight exposure. Generally, individual components constituting 3% of mixtures tested were detected. For quantitative purposes, spots localized by radioautography were excised, immersed in scintillation cocktail, and counted.

High-voltage electrophoresis at ~40 V/cm was performed on Whatman 3MM paper or cellulose-coated t.l.c. strips (Eastman Chromagram 13255, or J. T. Baker Bakerflex). The paper was used in a tank with horizontal bed[38], and the t.l.c. strips were used in a Savant TLE-20 tank of apex design. For paper, the available migration-path (exclusive of wicks) was ~30 cm, and for t.l.c. strips, 15 cm. Resolution was somewhat sharper with paper, but the t.l.c. sheets required less material and shorter exposure in radioautography. At pH 1.7, the buffer was 1.6M formic acid[7]; at pH 5.3, 80mM pyridine in 50mM acetic acid[7]; and at pH 2.9, 25mM pyridine in 0.15M formic acid.

The pK_2 of IMS and SIM, were determined by electrophoresis in pyridine–formic acid buffers. The distances from a standard spot of 2,5-anhydro-D-mannitol (aM) to the spot for its sulfate (aMS), and to the spot for the disaccharide were measured at pH values between pH 2.7 and 3.9. A plot of the ratio of these distances as a function of pH gave pK_2 as the point of inflection.

Deamination and reduction reactions. — The procedures illustrated are those used generally. Heparin (75 mg, ~125 μmol of uronic acid) was treated with butyl nitrite at low temperature and pH, and the reaction mixture was processed under precisely the conditions and proportions reported[6]. The product, at a final volume of 2.5 mL, was adjusted to pH ~8.5 (paper) by addition of 0.5M potassium dihydrogenborate (0.25 mL) and 0.5M boric acid (0.05 mL). The solution was chilled, and treated with sodium borotritide (0.1 mmol) of calibrated specific activity, added as a solution in a small volume of 50mM sodium hydroxide. The mixture was stored for 1 h at 0°, and 1 h at 25°, and then tested for residual borohydride (see below). If none remained, addition of sodium borotritide under the conditions just described was repeated. When reduction was complete, the excess of borohydride was removed by addition of acetone. Acetone (0.2 mmol) and 2-propanol (1 mL) were added, and the volatile radioactivity was distilled *in vacuo* into a dry-ice trap. The residual solution was de-ionized as described. When the deamination conditions of Lagunoff and Warren[17], or Shively and Conrad[8], were used, the procedure for reduction at pH 8.6, after removal of the excess of nitrous acid (and acetic acid) by repeated co-

distillation *in vacuo* with methanol, and water, was the same. The procedures described here were scaled down as required for application to amounts as small as 50 nmol, with some increase in the proportion of reaction volumes and of reagents.

For standardization, sodium borotritide as received was dissolved in 2-aminopropane, and supplemented, when desired, with a nonisotopic compound. Specific activities of 10–120 Ci/mol were used. Aliquots of the solution were evaporated in a vacuum desiccator. One aliquot was used to calibrate the specific activity. For this purpose, D-[^{14}C]glucose (160 Ci/mol) was purified by preparative paper chromatography with solvent *A*, counted, and added to a known quantity of nonisotopic D-glucose for a final, specific activity of 0.369 Ci/mol. A 50-μCi portion of the borohydride was allowed to react with 0.5 μmol of the standard D-[^{14}C]glucose in 30mM potassium borate (35 μL) (pH 8.6) as described. After addition of acetone (10 μL), portions of the reaction mixture and of a control (acetone added before D-[^{14}C]glucose) were subjected to paper chromatography in solvent *C* (3 days). The D-glucitol spots were excised and extracted with water, and the specific activity of the sodium borotritide was calculated from measurements of ^{14}C and ^{3}H radioactivity in the extract, after correction for the small control values.

A sensitive test for borohydride, apparently novel, was useful. A minute droplet of test solution was placed on filter paper that had been freshly moistened with a solution prepared from 0.1M silver nitrate and excess ammonia (Tollens' reagent). Presence of borohydride caused darkening of the paper instantaneously or within 30 s, depending on concentration.

Column chromatography. — For gel chromatography, a column (115 × 1.6 cm) of Sephadex G-25 Superfine (Pharmacia Fine Chemicals, Piscataway, NJ 08854), was developed by upward flow with M sodium acetate buffer (pH 6) containing 0.02% of sodium azide by use of a Pharmacia P-3 peristaltic pump. The load was typically 25–100 mg of carbohydrate in 1 mL of solution. About two fractions of 3 mL/h were collected and assayed for radioactivity. Appropriate fractions were pooled and de-ionized.

For chromatography on ECTEOLA-cellulose[7] (Whatman ET-11, Whatman Paper Div. Clifton, NJ 07014), this medium was freed of finest particles by repeated decantation with water, then copiously washed in a column with 0.1M hydrochloric acid, 0.1M sodium hydroxide, 0.5M acetic acid, water, M ammonium hydrogencarbonate, and starting buffer; the column was then re-poured. In a typical application, a column (26 × 2.5 cm) was loaded with a solution in water of ~40 mg of a heparin disaccharide fraction. The column was washed with water (185 mL); it was then eluted with ammonium hydrogencarbonate solution, the concentration of which was varied in a linear gradient from 0.02 to 1.0M over a volume of 700 mL, by use of a multichannel pump (Pharmacia P-3). Fractions of 10 mL were collected at the rate of 3 per h, and assayed for radioactivity. Appropriately pooled fractions were concentrated *in vacuo* and lyophilized. Column-bed volumes were adjusted to the load, with proportionate change in developer volume. A shallower gradient of

ammonium hydrogencarbonate 3–300mM in 6.25 column-bed volumes, was used to resolve IMS and SIM (*e.g.*, Figs. 1C and 1D).

Periodate oxidation of disaccharides. — In periodate oxidation studies, reaction mixtures containing only 8mM sodium periodate, and 2mM disaccharides, weakly buffered (from mode of preparation) at pH ~5, were kept at room temperature in the dark. At desired time-intervals, aliquots (10 μL) were withdrawn into 6 × 50-mm tubes, and the reaction was terminated by addition of 50mM 1,2-ethanediol (5 μL) solution. For each specimen, a control mixture was prepared, in which addition of 1,2-ethanediol preceded addition of disaccharide. At the end of the experiment, 0.2M hydrochloric acid (5 μL) was added to each tube. The tubes were sealed and heated for 15 min at 80°. After cooling and neutralization of the tube contents with 0.2M sodium acetate (5 μL), aliquots of the acid-treated reaction mixtures and controls were subjected to paper electrophoresis at pH 5.3. After radioautography, the papers were sectioned as shown by the spots made visible or by marker spots in reference lanes, and the d.p.m. counted. The proportion of each species present, based on total radioactivity found in the entire lane, was calculated for each species and its control. The relatively small control-values for each product were subtracted from the corresponding test values and added to the value for starting material. (This procedure gave satisfactory correction for action of acid alone where the extent of oxidation was moderate, but such a blank correction was obviously excessive late in the oxidation).

Graded acid hydrolysis and desulfation. — Aliquots of a 2mM solution of disaccharide in 50mM hydrochloric acid (10 μL) were sealed in small tubes and heated in a steam bath for various times. Then, the contents of each tube were neutralized with 0.10M sodium acetate (5 μL), and an aliquot was subjected to electrophoresis at pH 5.3, with quantitative determination as described in the preceding section.

For desulfation of heparin, the polymer (102 mg) was shaken for 24 h at room temperature successively with three fresh 15-mL portions of 0.1M hydrogen chloride in dry methanol, as described for cartilage chondroitin sulfate[27]. The residual solid, recovered by centrifugation and washed once with methanol, was dissolved in 0.2M aqueous triethylamine (32 mL) and stored for 18 h at 4°, to saponify the new carboxylic ester groups. After concentration to a small volume (*in vacuo*), the solution was desalted by chromatography on a Sephadex G-25 column eluted with sodium acetate, followed by the de-ionization procedure described earlier. As revealed by uronic acid colorimetry, the product emerged from the gel column at the void volume. The deamination of this preparation of "desulfated heparin" was accomplished by the Lagunoff and Warren[17] procedure. For comprehensive desulfation of disaccharides, their solutions in 0.1M hydrogen chloride in methanol were stored overnight at room temperature. For partial desulfation of SIMS, 15mM hydrogen chloride in methanol for 18 h at 4° was used. For the disaccharides, as for the polymer, saponification with aqueous triethylamine was applied to the desulfated products, which were separated and purified on ECTEOLA-cellulose columns.

Enzyme experiments. — β-D-Glucuronidase was assayed with phenolphthalyl

β-D-gluco pyranosyluronic acid[21] and α-L-iduronidase with phenyl α-L-idopyranosyluronic acid[20] as described. The unit of activity in both cases was μmol of aglycon liberated/min. β-D-Glucuronidase of specific activity 4 U/mg was prepared from a homogenate of female rat, preputial glands by autolysis and ammonium sulfate fractionation[21]; it contained no detectable α-L-iduronidase. A partially purified α-L-iduronidase of specific activity 8 mU/mg was prepared from a Triton X-100 extract of rat-liver lysosomes[20] by ammonium sulfate fractionation, acid treatment, (carboxymethyl)cellulose chromatography, and gel chromatography[41].

Digests (5 μL) of disaccharides (5mM concentration) with enzymes in 50mM sodium acetate buffer (pH 4.0), containing 0.15M sodium chloride, 3mM sodium azide, and 0.1 mg/mL of bovine serum albumin, were stored for 3 days at 25°. After dilution, analysis was made for product and residual substrate by electrophoresis at pH 5.3, as described for periodate oxidations. Similar digests of IMS and IM with α-L-iduronidase were also subjected to paper chromatography, to confirm the identity of the radioactive enzymic products.

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