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THE DISACCHARIDE REPEATING-UNITS OF HEPARAN SULFATE

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ABSTRACT

Five disaccharides have been isolated after degradation of heparan sulfate by heparinase (heparin lyase) and heparitinase (heparan sulfate lyase) and are suggested to represent the repeating units of the polysaccharide. They all contain a 4,5-unsaturated uronic acid residue and are: (a) A trisulfated disaccharide that is apparently identical to a disaccharide repeating-unit of heparin; (b) a disulfated disaccharide that seems unique for heparan sulfate and contains 2-deoxy-2-sulfamidoglucose and uronic acid sulfate residues; (c) a nonsulfated disaccharide containing a 2-acetamido-2-deoxyglucose residue; (d) a monosulfated disaccharide containing a 2-acetamido-2-deoxyglucose sulfate residue; and (e) a monosulfated disaccharide containing a 2-deoxy-2-sulfamidoglucose residue. Yields of these disaccharides from different heparan sulfate fractions are discussed in relation to possible arrangements of these units in the intact polymer.

INTRODUCTION

Heparan sulfate (heparitin sulfate) is a polysaccharide belonging to the group of glycosaminoglycans. It is found mainly in blood-vessel walls and is also associated with amyloid deposits and various disorders involving the metabolism of mucopolysaccharides. The polymer has a more complex composition than other glycosaminoglycans as it contains *N*-acetylglucosamine (2-acetamido-2-deoxy-D-glucose), glucosamine *N*-sulfate (2-deoxy-2-sulfamido-D-glucose), glucosamine *N,O*-disulfate (2-deoxy-2-sulfamido-D-glucose sulfate), D-glucuronic acid, L-iduronic acid[1], and probably *N*-acetylglucosamine *O*-sulfate (2-acetamido-2-deoxy-D-glucose sulfate) residues. Some structural features have been determined but the detailed structure is not known. Two main approaches for determining the arrangement of repeating units have been used: (a) Degradation by nitrous acid followed by investigation of the fragments obtained[2,3], and (b) enzymic degradation by eliminases from flavobacteria and analyses of the di- and oligo-saccharide products[4].

The isolation and purification of a heparinase (heparin lyase, EC 4.2.2.7) acting on the "heparin-like" portions of heparan sulfate and a heparitinase (heparan sulfate lyase, EC 4.2.2.8) acting on the nonsulfated and low sulfated portions[5] has

made it possible to continue structural studies by enzymic means. This paper describes the isolation and characterization of five disaccharides, which represent the structural units of heparan sulfate, obtained by degradation with heparitinase.

EXPERIMENTAL

Analytical methods. — The content of uronic acid was determined by the carbazole procedure[6], of total hexosamine by a modified Elson–Morgan method[7], of *N*-sulfated hexosamine by a nitrous acid procedure[8], and of *N*-acetylhexosamine by a modification of the Morgan–Elson[9] method. The content of sulfate group was determined by a colorimetric procedure[10], and that of acetyl groups by g.l.c.[11]. The proportion of D-glucosamine to D-galactosamine was determined by ninhydrin degradation, followed by paper chromatography[12].

Electrophoresis. — Whatman No. 1 paper strips were used in a high-voltage electrophoresis apparatus in pyridine–formic acid buffer[13], pH 3.0, at 27 V/cm. Compounds were detected by u.v. absorption with a short-wave length lamp or by spraying with the alkali–silver nitrate reagent[14].

Paper chromatography. — Compounds were chromatographed on Whatman No. 1 paper in: (I) 1-butanol–acetic acid–water (10:3:7, v/v), and (II) 1-butanol–acetic acid–M ammonium hydroxide (2:3:1, v/v). Compounds were located as described under Electrophoresis and by a ninhydrin spray reagent[15].

Materials. — Heparan sulfate fractions from lung and aorta used as starting material have been described previously[1]. The unsaturated disaccharide from chondroitin 6-sulfate (Δdi-6CS) was obtained by use of a flavobacterium chondroitinase[16] and the heparin disaccharide has been described previously[17]. The preparations of heparinase[5], heparitinase[5], and glycuronidase[18] from flavobacteria have been described. Crude β-glucuronidase (EC 3.2.1.31) was isolated from bovine testis[19]. Whale heparin was a gift from Dr. Z. Yosizawa.

Sulfated disaccharides containing a 4,5-unsaturated uronic acid residue, obtained by enzymic degradation of heparan sulfate, were prepared and isolated essentially as described previously[17]. For degradation with heparinase, 1 g of a particular heparan sulfate fraction was incubated with 500 units (0.45 mg of protein) of enzyme. For degradation with heparitinase, 2 g of heparan sulfate and 3,000 units (1.65 mg of protein) of enzyme were used. For preliminary fractionation, degradation products were placed on columns (190 cm × 3 cm) of Sephadex G-50 superfine resin and eluted with a solution of 0.2M sodium chloride in 10% ethanol[20]. Five-ml fractions were collected and the elution of the 4,5-unsaturated oligosaccharides was followed by u.v. absorption at 230 nm. Eluates within peaks were combined and placed directly on columns (33 cm × 2.1 cm) of DEAE-Sephadex A-25 ion-exchange resin, and eluted stepwise with 0.5M, 0.75M, 1.0M, 1.3M, 1.5M, and 2.0M solutions of sodium chloride; 5 ml were collected per tube. As previous experience had indicated that this separation, based mainly on charge, did not completely eliminate some overlap between di- and tetra-saccharides, fractions within elution peaks were

combined and placed on columns (120 cm × 2.1 cm) of Sephadex G-25 fine resin. Elution was achieved with 0.2M sodium chloride in 10% ethanol and 5 ml were collected per tube. The pattern was followed by u.v. absorption at 230 nm. Peak fractions were combined, desalted on a column (120 cm × 2.1 cm) of Sephadex G-10 resin, and the disaccharides obtained by lyophilization. Success of fractionation was controlled by paper chromatography and electrophoresis.

The yield of disaccharides was determined by a small-scale digestion of heparan sulfate with heparitinase. Substrate fractions were incubated at a concentration of 10 mg per ml in 0.5M sodium acetate with about 30 units of enzyme for 20 h at 43°. Aliquots containing 0.2 mg of sample (based on starting material) were chromatographed on paper in Solvent I. Spots detected by u.v. absorption were cut out and eluted with 0.03M hydrochloric acid (2.0 ml) for 1 h. Ultra-violet absorption of the solution at 230 nm was measured in a recording spectrophotometer and the amount of disaccharide was calculated from the known absorption coefficient.

Disaccharides obtained by action of heparinase. — Products obtained[1] from "heparitin 0.9", "heparitin 1.2", and "heparitin 1.4" were fractionated as just described on Sephadex resin columns. Paper chromatography indicated the presence of two compounds (Δdi-HS_a-I and -II)*[5] containing an unsaturated uronic acid residue in a peak region where disaccharides usually appear. These were separated into individual disaccharides on DEAE-Sephadex ion-exchange resin. Yields are shown in Table I for the disaccharides obtained from different heparan sulfate

TABLE I

YIELD OF DISACCHARIDE POLYSULFATES OBTAINED BY HEPARINASE DIGESTION OF VARIOUS HEPARITIN SULFATE FRACTIONS

Fractions[a]	*Sulfate content* (%)	Δ*Di-*HS_a*-I* (%)	Δ*Di-*HS_a*-II* (%)
Heparitin 0.9	9	0.6	0.6
Heparitin 1.2	13	0.7	0.4
Heparitin 1.4	17	2.4	0.1

[a]See Ref. 1.

fractions. It is apparent that yields are low, but it must be pointed out that the heparinase degrades only a very limited region of the heparan sulfate molecule[5]. Most of the degradation products were large oligosaccharides (total yield of products was 90%). Paper chromatography and electrophoresis of the disaccharides is shown in Fig. 1. It can be seen that one of the unsaturated disaccharides from heparan sulfate (Δdi-HS_a-I) has a behavior identical to that of the heparin disaccharide[17] (Δdi-He_a-I), whereas the second unsaturated disaccharide from heparan sulfate

*Disaccharides obtained from heparin are indicated by "He", from heparan sulfate by "HS", by action of heparinase by subscript "a", and by action of heparitinase by subscript "b".

(Δdi-HS_a-II) migrates more rapidly on chromatography and slower on electrophoresis than the former two first-named compounds.

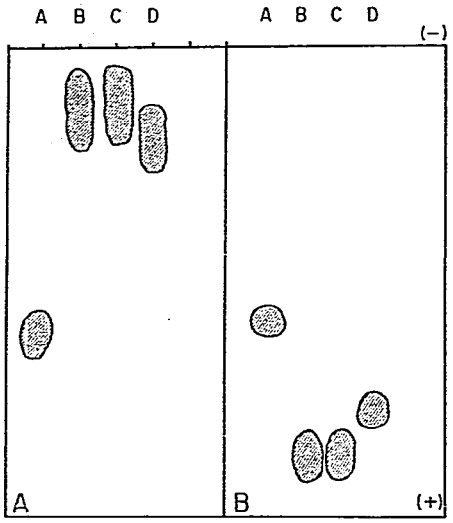

Fig. 1. Paper chromatography and electrophoresis of disaccharides obtained by heparinase action. Panel A, paper chromatography in Solvent I (the same pattern was obtained in Solvent II). Panel B, paper electrophoresis in pyridine–formic acid. A, chondroitin sulfate disaccharide; B, Δdi-He_a-I; C, Δdi-HS_a-I; D, Δdi-HS_a-II. Circled and cross-hatched spots were detected by u.v. absorption and silver nitrate reagent.

Analytical results are shown in Table II. The molar u.v. absorbance at 230 nm, as compared to those of the heparin disaccharide and other similar compounds which lie within a narrow range[16,17,21], provides evidence that the two heparinase

TABLE II

PROPERTIES OF DISACCHARIDE POLYSULFATES OBTAINED FROM HEPARAN SULFATE AND HEPARIN BY HEPARINASE DEGRADATION

Properties	*ΔDi-HS_a-I*	*ΔDi-He_a-I*	*ΔDi-HS_a-II*
Absorption at 235 nm[a]	8.2	8.6	9.6
Molar absorbance[b]	5430	5720	5400
Carbazole value (%)	28	26	29
Acetyl groups (%)	0	0	0
Sulfate groups (%)	35	36	29
Indole value for hexosamine[c] (%)	29	21	29
Ehrlich value for hexosamine[c] (%)	19	21	22
Optical rotation (degrees)[d]		+4	+4

[a]Concentration 0.1%. [b]Based on a disaccharide. (Absorbance of 5600–5900 has been reported for several similar disaccharides[17,21]). [c]Reported as anhydrohexosamine. [d]$[\alpha]_D^{25}$ (*c* 1, water).

products are disaccharides. The two disaccharides from heparan sulfate and the disaccharide from heparin have very similar analytical data, except for the lower sulfate content of Δdi-HS$_a$-II. As Δdi-HS$_a$-II also showed, on paper chromatography and electrophoresis, migration rates different from that of the other compounds, all were treated with a glycuronidase specific for unsaturated glycosyluronic acids[18] and the products examined by paper chromatography. As shown in Fig. 2, Δdi-He$_a$-I and Δdi-HS$_a$-I were degraded mainly to give α-keto acid and disulfated glucosamine residues and Δdi-HS$_a$-II to give 2-deoxy-2-sulfamidoglucose and α-keto acid residues.

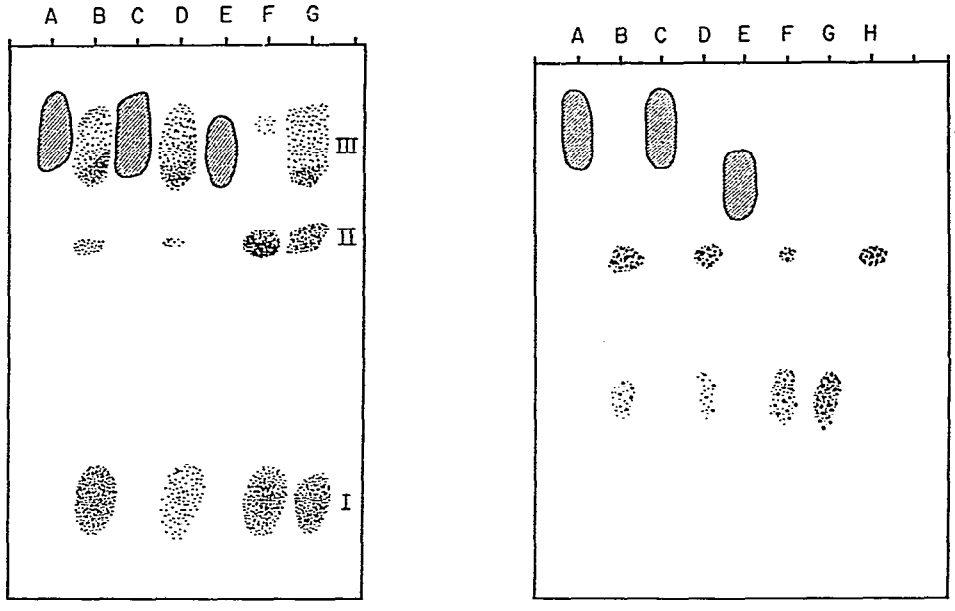

Fig. 2. Paper chromatography of glycuronidase digests of disaccharides; Solvent I, downward irrigation for 40 h. A, ΔDi-He$_a$-I; B, glycuronidase digest of Δdi-He$_a$-I; C, Δdi-HS$_a$-I; D, glycuronidase digest of Δdi-HS$_a$-I; E, Δdi-HS$_a$-II; F, glycuronidase digest of Δdi-HS$_a$-II; G, crude heparinase digest of heparin. Spots I, II and III correspond to α-keto acid, 2-deoxy-2-sulfamidoglucose, and 2-deoxy-2-sulfamidoglucose sulfate[28], respectively. Circled and cross-hatched spots were detected by u.v. absorption and silver nitrate reagent and stippled spots by silver nitrate reagent only. Note that the spot for 2-deoxy-2-sulfamidoglucose sulfate (and the enzyme product corresponding to it) overlaps with the disaccharide spots but does not give an u.v. absorption.

Fig. 3. Paper chromatography hydrolyzates of disaccharides with 0.05M trifluoroacetic acid; Solvent I, downward irrigation for 40 h. A, ΔDi-He$_a$-I; B, acid hydrolyzate of Δdi-He$_a$-I; C, Δdi-HS$_a$-I; D, acid hydrolyzate of Δdi-HS$_a$-I; E, Δdi-HS$_a$-II; F, acid hydrolyzate of Δdi-HS$_a$-II; G, glucosamine standard; H, glucosamine sulfate, obtained from 2-sulfamido-2-deoxyglucose sulfate[5] by hydrolysis with 0.04M hydrochloric acid. Circled and cross-hatched spots were detected by u.v. absorption and silver nitrate reagent and stippled spots by silver nitrate reagent only. Heavy stippling indicates spots also positive with ninhydrin.

The disaccharides were also hydrolyzed with 0.05M trifluoroacetic acid for 2 h at 100°, and the products chromatographed on paper. As shown in Fig. 3, the major product obtained from Δdi-He$_a$-I and Δdi-HS$_a$-I was glucosamine *O*-sulfate

whereas Δdi-HS_a-II yielded mainly glucosamine (none of the disaccharides did contain galactosamine). These results are due to the lability of the glycosidic linkage of the unsaturated uronic acid residue and of the *N*-sulfate group to dilute acid. The unsaturated uronic acid residue itself is apparently not stable under these conditions[22]. These data indicate that Δdi-HS_a-I is identical with Δdi-He_a-I from heparin, has a total of three sulfate groups, and contains a 2-deoxy-2-sulfamidoglucose sulfate residue, whereas Δdi-HS_a-II has a total of two sulfate groups and contains a 2-deoxy-2-sulfamidoglucose residue. In each disaccharide, one of the sulfate groups, linked to the uronic acid residue, was probably removed by a sulfatase present in the impure glycuronidase preparation, in order to account for the presence of a sulfate-free α-keto acid in the hydrolyzates.

An impure disaccharide not absorbing in the u.v. was also obtained in a yield of about 0.8%. This compound migrated on paper like Δdi-HS_a-I, and its analysis showed molar ratios of uronic acid and sulfate to glucosamine of 1.0 and 2.0, respectively. Hydrolysis of this disaccharide with crude β-glucuronidase yielded glucosamine disulfate and glucuronic acid as determined by paper chromatography. Since this compound is not unsaturated, it is probably derived from the nonreducing end residue of the heparan sulfate molecule and indicates the presence of β-D-glucosyluronic linkages.

It is of interest that, in preliminary experiments of the degradation of ω-heparin (whale heparin) by heparinase, the only disaccharide obtained appeared

TABLE III

YIELD AND PROPERTIES OF DISACCHARIDE MONOSULFATES OBTAINED FROM HEPARAN SULFATE (HEPARITIN SULFATE 1.2)[a] AND CHONDROITIN 6-SULFATE[b]

	ΔDi-HS_b-I	*ΔDi-HS_b-II*	*ΔDi-HS_b-III*	*Chondroitin 6-sulfate disaccharide*[b]
Yield (%)	20.5	5.7	7.1	
Absorption at 235 nm[c]	14.3	11.0	12.7	11.6
Molar absorbance[c]	5700	5800	5800	6100
Carbazole value (%)	42	40	41	34
Sulfate groups (%)	0	16	18	15
Acetyl groups (%)	10.8	7.8	0.2	6.9
Indole value for hexosamine[d] (%)	0	0	19	0
Ehrlich value for hexosamine[d] (%)	31	27	27	27
Galactosamine (%)	<5	<5	<5	100
Morgan–Elson value for *N*-acetylglucosamine[9]	0	0	0	70
Optical rotation (degrees)[c]	−18	+5	+9	0

[a]By action of heparitinase. [b]Disaccharide containing a 4,5-unsaturated uronic acid residue obtained from chondroitin 6-sulfate by action of a flavobacterium chondroitinase. [c]See Table II. [d]Reported as anhydrohexosamine.

identical to Δdi-HS$_a$-II. No heparin disaccharide (Δdi-He$_a$-I) could be detected in the digests.

Disaccharides obtained by the action of heparitinase. — In order to isolate the products of degradation, heparitin 1.2 was degraded by heparitinase and the digestion mixture fractionated on Sephadex and DEAE-Sephadex resins. Three distinct compounds containing an unsaturated uronic acid residue were obtained (Δdi-HS$_b$-I, -II, and -III); they were eluted from the columns in the range typical for disaccharides. Paper chromatography and electrophoresis are shown in Fig. 4, and analyses in Table III. The nonsulfated disaccharide Δdi-HS$_b$-I has been described previously[4] as "cpd I" and is included here for comparison purposes. The molar absorbance in u.v. light shows that all compounds are disaccharides[16,21]. As can be seen, Δdi-HS$_b$-II contains one sulfate group and is *N*-acetylated. ΔDi-HS$_b$-III is *N*-sulfated as shown by the indole reaction (analysis for free amino was negative[23])

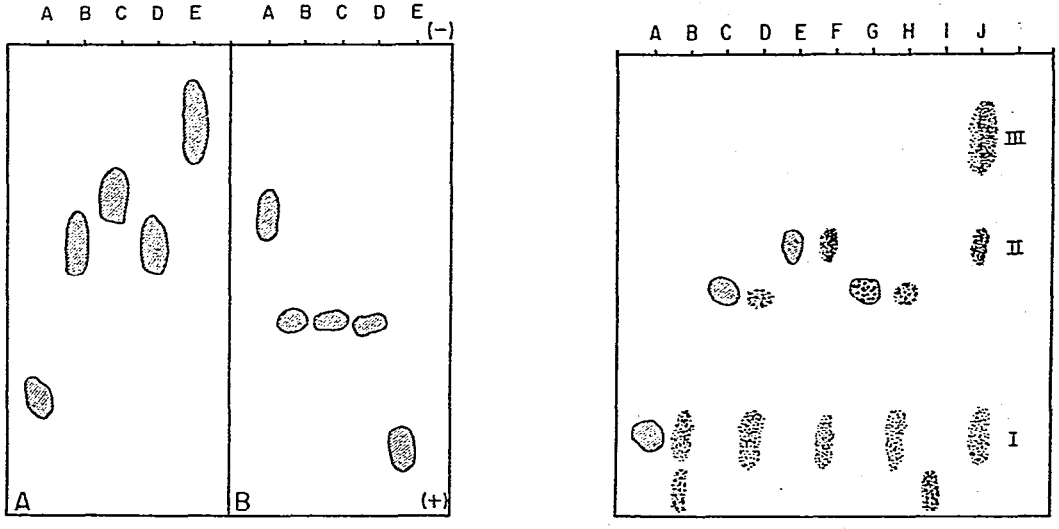

Fig. 4. Paper chromatography and electrophoresis of disaccharides obtained by heparitinase action. Panel A, paper chromatography in Solvent I (the same pattern was obtained in Solvent II). Panel B, paper electrophoresis in pyridine–formic acid. A, ΔDi-HS$_b$-I; B, Δdi-HS$_b$-II; C, Δdi-HS$_b$-III; D, chondroitin sulfate disaccharide; E, Δdi-He$_a$-I. Circled and cross-hatched spots were detected by u.v. absorption and silver nitrate reagent.

Fig. 5. Paper chromatography of glycuronidase digests of disaccharides; Solvent I, downward irrigation for 40 h. A, ΔDi-HS$_b$-I; B, enzyme digest of Δdi-HS$_b$-I; C, Δdi-HS$_b$-II; D, enzyme digest of Δdi-HS$_b$-II; E, Δdi-HS$_b$-III; F, enzyme digest of Δdi-HS$_b$-III; G, chondroitin sulfate disaccharide; H, digest of chondroitin sulfate disaccharide with induced flavobacterium enzyme containing a β-glycuronidase[16], the slower-moving spot corresponds to 2-acetamido-2-deoxygalactose 6-sulfate; I, *N*-acetylglucosamine standard; J, crude heparinase digest of heparin. Spots I, II, and III correspond to α-keto acid, 2-deoxy-2-sulfamidoglucose, and 2-deoxy-2-sulfamidoglucose sulfate[28], respectively. Circled and cross-hatched spots were detected by u.v. absorption and silver nitrate reagent and stippled spots by silver nitrate reagent only. Heavy stippling indicates spots also positive with the Ehrlich spray reagent[29]. Note that the 2-acetamido-2-deoxyhexose sulfate spot overlaps with the spots for *N*-acetyl disaccharides sulfate but does not show u.v. absorption.

and by the absence of an acetyl group. The presence of an *N*-acetyl group[4] in Δdi-HS_b-I and the chondroitin sulfate disaccharide[16] has been shown previously. The negative Morgan–Elson reaction for Δdi-HS_b-II indicates the presence of a substitution at C-4. In order to characterize the disaccharides further, they were first incubated with glycuronidase[18] and the products chromatographed on paper. As shown in Fig. 5, Δdi-HS_b-II is degraded to 2-acetamido-2-deoxyglucose sulfate (which migrates like the disaccharide itself, but gives no u.v. absorption) and an α-keto acid. ΔDi-HS_b-III is degraded to 2-deoxy-2-sulfamidoglucose and an α-keto acid. In addition, hydrolysis of the disaccharides was achieved with 0.05M trifluoroacetic acid for 2 h at 100°, or 0.04M hydrochloric acid under the same conditions, which resulted in a large increase of free amino groups for Δdi-HS-$_b$III and Δdi-He_a-I (used as reference) and a large positive value in the Morgan–Elson *N*-acetylhexosamine reaction for Δdi-HS_b-I and Δdi-HS_b-II (see Table IV). Since the *O*-sulfate group is fairly stable under these conditions of hydrolysis, the original negative Morgan–Elson reaction is due to the presence of the glycosyluronic acid linkage to C-4 of the *N*-acetylglucosamine residue[24].

TABLE IV

ACID HYDROLYSIS OF DISACCHARIDES[a]

Disaccharide	*Duration (h)*	*Free amino groups*[b]	*Morgan–Elson value*[c]
ΔDi-HS_b-I	0	0	3
	4	11	29
ΔDi-HS_b-II	0	0	3
	4	8	18
ΔDi-HS_b-III	0	0	5
	4	29	4
ΔDi-He_a-I	0	0	3
	4	20	4

[a]With 0.05M trifluoroacetic acid. [b]Percent of glucosamine residues having a free amino group[23]. [c]Analysis for *N*-acetylhexosamine by the Morgan–Elson method[9].

These results are in agreement with a structure of a disaccharide containing a 2-acetamido-2-deoxyglucose sulfate residue for Δdi-HS_b-II and with a structure of a disaccharide containing a 2-deoxy-2-sulfamidoglucose residue for Δdi-HS_b-III. Yields of these disaccharides from various heparan sulfates are shown in Table V. As can be seen, the yield of nonsulfated disaccharide decreases in proportion to the increase of sulfate content. The yields of sulfated disaccharides does not appear to vary much except for the low yield of Δdi-HS_b-III from heparitin 1.4 and the absence of Δdi-HS_b-II in the aorta heparan sulfate. The proportion of total linkages split by the heparitinase decreases as the sulfate content of the polymer increases.

TABLE V

EXTENT OF DEGRADATION AND YIELD OF DISACCHARIDES OBTAINED BY HEPARITINASE TREATMENT OF HEPARAN SULFATE FRACTIONS

Fractions	*Sulfate groups* (%)	*Proportion of linkage split*[a]	*Recovery of disaccharide*[b]	*Yield of disaccharide* (%)			
				ΔDi-HS$_b$-I	*ΔDi-HS$_b$-II*	*ΔDi-HS$_b$-III*	*Total*
Heparitin 0.5[c]	1.5	1.40	1.24	46	5	0	51
Heparitin 0.9	9	1.25	1.24	40	3	8	51
Heparitin 1.2	13	0.89	0.70	21	3	5	29
Heparitin 1.2[d]	13	1.14	0.77	21	6	7	33
Heparitin 1.4	17	0.53	0.19	3	3	3	9
Aorta heparitin[1] A-2	6.6	1.18	1.08	37	0	7	44

[a]μMoles per mg; if all linkages are split the theoretical number would be 2.5 μmoles, based on molecular weight[1] and uronic acid content. [b]μMoles per mg. [c]See Ref. 1. [d]Yields by actual isolation rather than small-scale chromatography.

RESULTS AND DISCUSSION

Recent studies have indicated that the glycosaminoglycans, with the possible exception of hyaluronic acid, have more complex structures than had originally been assumed. In terms of composition alone, heparan sulfate seems to be the most heterogeneous of all and it has been very difficult to identify subunits precisely and even more difficult to arrive at a definite pattern of arrangement of these units. Variations within a polymer chain would result mainly in a specific distribution and orientation of charged groups, and this in turn would have a significant effect on the interaction of the polysaccharide with charged molecules in its biological environment. Therefore, information about the repeating units and their pattern is quite essential for an understanding of the physical and biological properties of heparan sulfate. Data presented here on 5 different disaccharides (only one of which has been described previously) obtained by enzymic degradation of heparan sulfate give some clues about the chemical structure of the polysaccharide but have also raised some new problems.

The highly sulfated disaccharides were obtained in low yield by heparinase action (Table I), since isolated disaccharide units are not liberated by the enzyme[5], but remain as part of oligosaccharides; at least two contiguous highly sulfated disaccharides are necessary for the liberation of one. In addition, the total sulfate content of the heparan fractions themselves limits the possible amount of highly sulfated units.

Analyses, paper chromatography and electrophoresis, and hydrolysis by glycuronidase and weak acid showed that disaccharide Δdi-HS_a-I from heparan sulfate is identical with a disaccharide obtained from heparin by degradation with the same enzyme[17]. This finding supports the view that heparin-like segments are indeed present in heparan sulfate[1,2]. On the other hand, the isolation of Δdi-HS_a-II from heparan sulfate and not from heparin indicates a very significant structural difference. As Δdi-He_a-I only is obtained from heparin by the use of the same enzyme, it is not likely that Δdi-HS_a-II is derived from Δdi-HS_a-I by action of an unknown sulfatase in the purified heparinase preparation.

The sulfate analysis and electrophoretic mobility of Δdi-HS_a-I shows that it (and therefore Δdi-He_a-I) contains a total of 3 sulfate groups rather than 2 as postulated previously[17]. This finding agrees with the data published by Lindahl *et al.*[26] and Dietrich *et al.*[30] for heparin. Heparinase and heparitinase introduce 4,5-unsaturation in the uronic acid residue and, therefore, the relation of the uronic acid sulfate group to either L-iduronic or D-glucuronic acid cannot be determined. The presence of disaccharide units containing uronic acid sulfate residues in heparan is of interest with respect to a postulated[25] uronic acid sulfatase deficiency in Hunter's syndrome, a disorder leading to accumulation of dermatan as well as heparan sulfates in urine and organs of patients.

In regard to the disaccharides isolated after heparitinase action, the nonsulfated disaccharide has been described previously[4] and is included as a reference compound.

The *N*-sulfated disaccharide Δdi-HS$_b$-III appears to be one of the major repeating units of heparan sulfate, whereas it does not seem to be present in significant proportion in heparin. The isolation of Δdi-HS$_b$-II, an *N*-acetylated disaccharide *O*-sulfate similar to the disaccharides isolated from chondroitin sulfate confirms other reports[3,27] of the presence of *O*-sulfate groups in the *N*-acetylated segments of heparan sulfate. In view of the specificity of heparitinase, the yields of these disaccharides (see Table V) from different fractions of heparan sulfate give some structural information. The amount of the nonsulfated unit decreases with increasing sulfate content of the parent fraction; however, in view of the fact that heparitin 1.4 contains about 50% 2-acetamido groups, the very low yield of sulfate-free disaccharide is unexpected, in particular as the amount of Δdi-HS$_b$-II, the *N*-acetyl sulfate disaccharide, has not increased. This indicates that the relatively low sulfated heparans, such as heparitin 0.9 and aorta heparitin, have indeed fairly large blocks of repeating units containing *N*-acetyl groups, as postulated[2,4], whereas the higher sulfated polymers, especially heparitin 1.4, must have an alternating arrangement of *N*-acetyl and *N,O*-disulfate units, that makes them not amenable to degradation by the enzyme to disaccharides.— The heparitinase used in this work splits 2-acetamido-2-deoxyglycosyl linkages that have no neighboring sulfate groups or only one sulfate group linked to the glucosamine residue. It will not act when a sulfate group is linked to the uronic acid residue or two sulfate groups to the hexosamine residue—. The relatively constant yield of the sulfated disaccharides, independent of the sulfate content of the parent polymer, is also unexpected. As the higher sulfated fractions contain a higher proportion of sulfated repeating units, one must assume that the specific arrangement of heparitinase-sensitive linkages is responsible for the low yields. The low yield of highly sulfated disaccharides, after heparinase degradation, indicates that these disaccharides occur mainly in an alternating arrangement rather than in the block-type structure of the *N*-acetylated residues. As seen in Table V, the total number of linkages split by the heparitinase appears to decrease with the increasing sulfate content of the fractions.

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