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DEPOLYMERIZATION OF HEPARIN WITH DIAZOMETHANE. STRUCTURE OF *N,O*-METHYLATED, EVEN-NUMBERED OLIGOSACCHARIDES PRODUCED BY β-ELIMINATIVE CLEAVAGE OF THE 2-AMINO-2-DEOXYGLYCOSYLIC LINKAGE

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ABSTRACT

The long-period reaction of heparin with excess diazomethane at 20° resulted in cleavage at the β-position of the uronic acid carboxyl group to give a mixture of methyl α- and β-glycosides of *N,O*-methylated di-, tetra-, and hexa-saccharides having a 4,5-unsaturated uronic acid, nonreducing end-group. The major disaccharides obtained were methyl *O*-(4-deoxy-3-*O*-methyl-α-L-*threo*-hex-4-enopyranosyluronic acid 2-sulfate)-(1→4)-2-deoxy-3-*O*-methyl-2-(*N*-methylsulfoamino)-α- and -β-D-glucopyranoside. The reaction of heparin at 4° yielded a mixture of methylated, higher-molecular-weight oligosaccharides, which retained some affinity for antithrombin III–Sepharose.

INTRODUCTION

In 1974, Kiss[1] described the depolymerization of the quarternary salt of heparin by β-elimination with diazoalkane to give di- and tetra-saccharides having a 4,5-unsaturated uronic acid, nonreducing end-group. As far as we are aware, no original paper on this topic has been published. Pectin, a polyuronide, had been reported to be also depolymerized by treatment with diazomethane under mild conditions[2]. In this paper, we describe the depolymerization of heparin with diazomethane on the basis of isolation and characterization of methylated disaccharide disulfates and disaccharide trisulfates, together with evidence on the structure of other oligosaccharide products.

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RESULTS AND DISCUSSION

A solution of the sodium salt of heparin in a small volume of water was treated with an excess solution of diazomethane in diethyl ether with vigorous stirring for a long period at 20°. Gel-filtration of the reaction product on a column of Sephadex G-25 indicated extensive depolymerization of heparin (Fig. 1). Almost identical patterns of elution were observed for ten samples treated on the same scale, except for some variation in the amount of Fr. IV. The tributylammonium salt of the polysaccharide is soluble in ethanol or dimethyl sulfoxide to give a homogeneous solution. When this was treated with an excess solution of diazomethane in diethyl ether, with or without tributylamine as a base, little depolymerization of heparin occurred in either case.

Fractions III and IV (see Fig. 1) were separately collected and chromatographed on a column of Ecteola-cellulose (HCO_3^-) (Figs. 2a and 3). The main subfractions of Fraction III, Fractions III-6 and -7, were rechromatographed on Ecteola-cellulose column, and then converted into sodium salts. Each compound recovered from Fractions III-6 and -7 migrated slightly faster than heparin as a single, sharp band on the electrophorogram (inset of Fig. 2a). The materials of Fractions III-3 and -4, which gave broad bands matching with each other in migrating distance and weakly stained with Alcian Blue dye on the electrophorogram (inset of Fig. 2a), were separately chromatographed on a column of Ecteola-cellulose

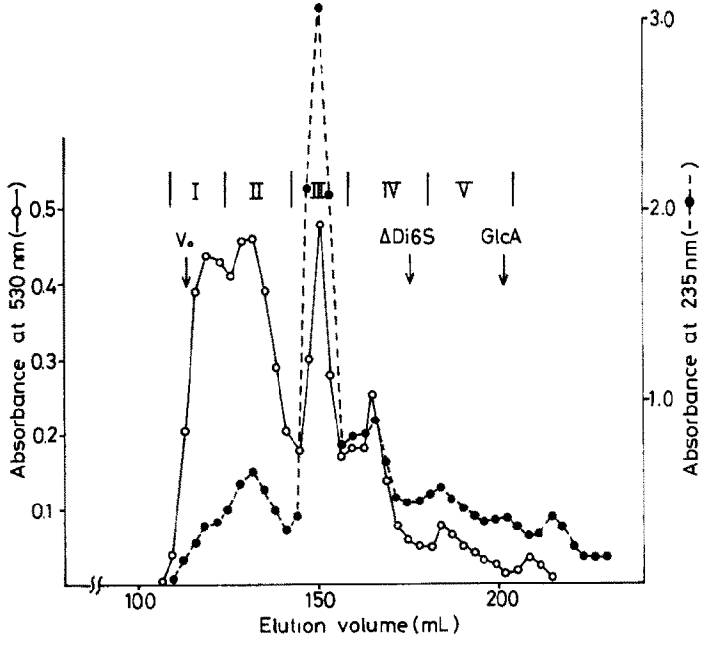


Fig. 1. Gel-filtration, on Sephadex G-25 (superfine), of the reaction products of heparin treated with diazomethane at 20°. The arrows indicate the positions of elution of standard 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate (ΔDi6S) and D-glucuronic acid (GlcA).

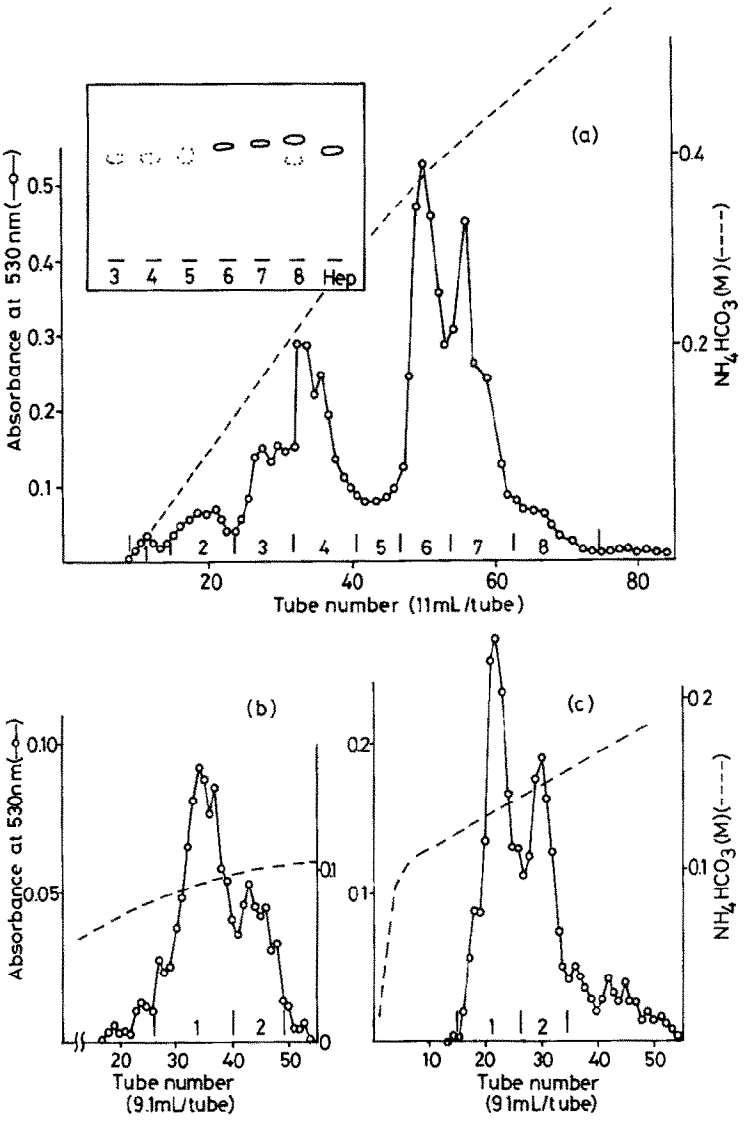


Fig. 2. Anion-exchange chromatography, on Ecteola-cellulose (HCO_3^-), of (a) Fraction III (from Fig. 1). (b) Fraction III-3 (from Fig. 2a), and (c) Fraction III-4 (from Fig. 2a). Electrophoretic patterns of Fractions III-3–III-8 and heparin are shown in the inset.

(HCO_3^-) (Figs. 2b and 2c). Each of the Fractions III-3-1, -3-2, -4-1, and -4-2 was isolated as ammonium salt. Fractions I and II (Fig. 1) were rechromatographed on a column of Sephadex G-25 in order to obtain preparations that were homogeneous in molecular size. Yields and analytical data of all the materials isolated are shown in Table I.

The ^{1}H-n.m.r. spectrum of Fraction III-6 (Fig. 4a and Table II) showed three

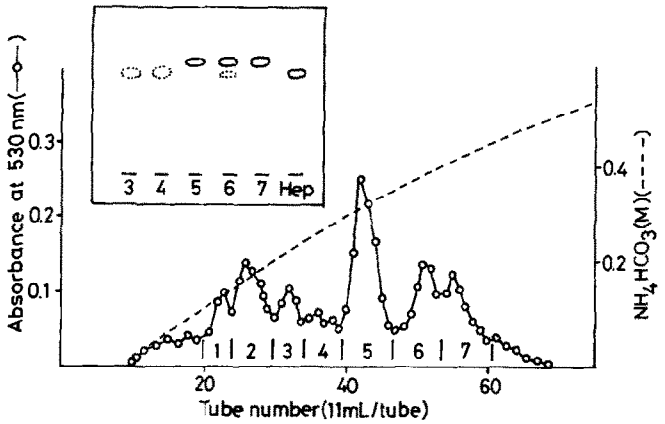


Fig. 3. Anion-exchange chromatography, on Ecteola-cellulose (HCO_3^-), of Fraction IV (from Fig. 1). Electrophoretic patterns of Fractions IV-3–IV-7 and heparin are shown in the inset.

TABLE I

YIELDS AND ANALYTICAL DATA OF THE FRACTIONS OBTAINED BY DEPOLYMERIZATION AND PERMETHYLATION OF HEPARIN WITH DIAZOMETHANE

Fraction	*Yield*[a] *(mg)*	$[\alpha]_D^{28}$ *(degrees)*	*O.r.d.* $[\phi]$		*Uronic acid (%)*	*Sulfate (%)*
			λ_{max} *223 nm*	λ_{min} *247 nm*		
Heparin					39.37	35.74
I	90[b]				28.22	29.35
II	91[b]				23.33	25.70
III-2	4.7[c]				11.05	19.28
III-3-1	4.2[c]				15.96[d]	28.44[d]
III-4-1	6.1[c]	−16.1	+25940	−7580	18.01[d]	28.27[d]
III-4-2	4.1[c]	+8.8	+20820	−6750	17.77[d]	28.33[d]
III-6	20.9[b]	−18.0	+20850	−8690	19.11[e]	39.69[e]
III-7	22.9[b]	+18.1	+29620	−4550	19.16[e]	39.72[e]
IV-5	11.1[b]	−50.4			19.00[f]	30.61[f]

[a]Obtained from 500 mg of heparin. [b]Sodium salt. [c]Ammonium salt. [d]Values calc. for 3 (ammonium salt): uronic acid, 30.42; sulfate 30.42. [e]Values calc. for 1 (sodium salt): uronic acid, 26.33; sulfate, 39.50. [f]Values calc. for 5 (sodium salt): uronic acid, 30.66; sulfate, 30.67.

signals for methoxyl groups at δ ~3.5 and a signal due to the *N*-methyl linked to the sulfoamino group at δ 2.8, but no signal for a methyl ester group was observed at δ ~3.7. Since the uronic acid carboxyl group is effectively esterified with diazomethane for a short period at low temperature[3], the methyl ester group formed might have been hydrolyzed under the slightly basic conditions, as the pH of the aqueous layer was ~9.0 at the end of the reaction. The intensities of the H-1 signal at δ 4.71 and H-1′ signal at δ 5.50 were equal to that of the olefinic H-4′ signal at δ 6.22. The large coupling constant for H-1 and the negative optical rotation

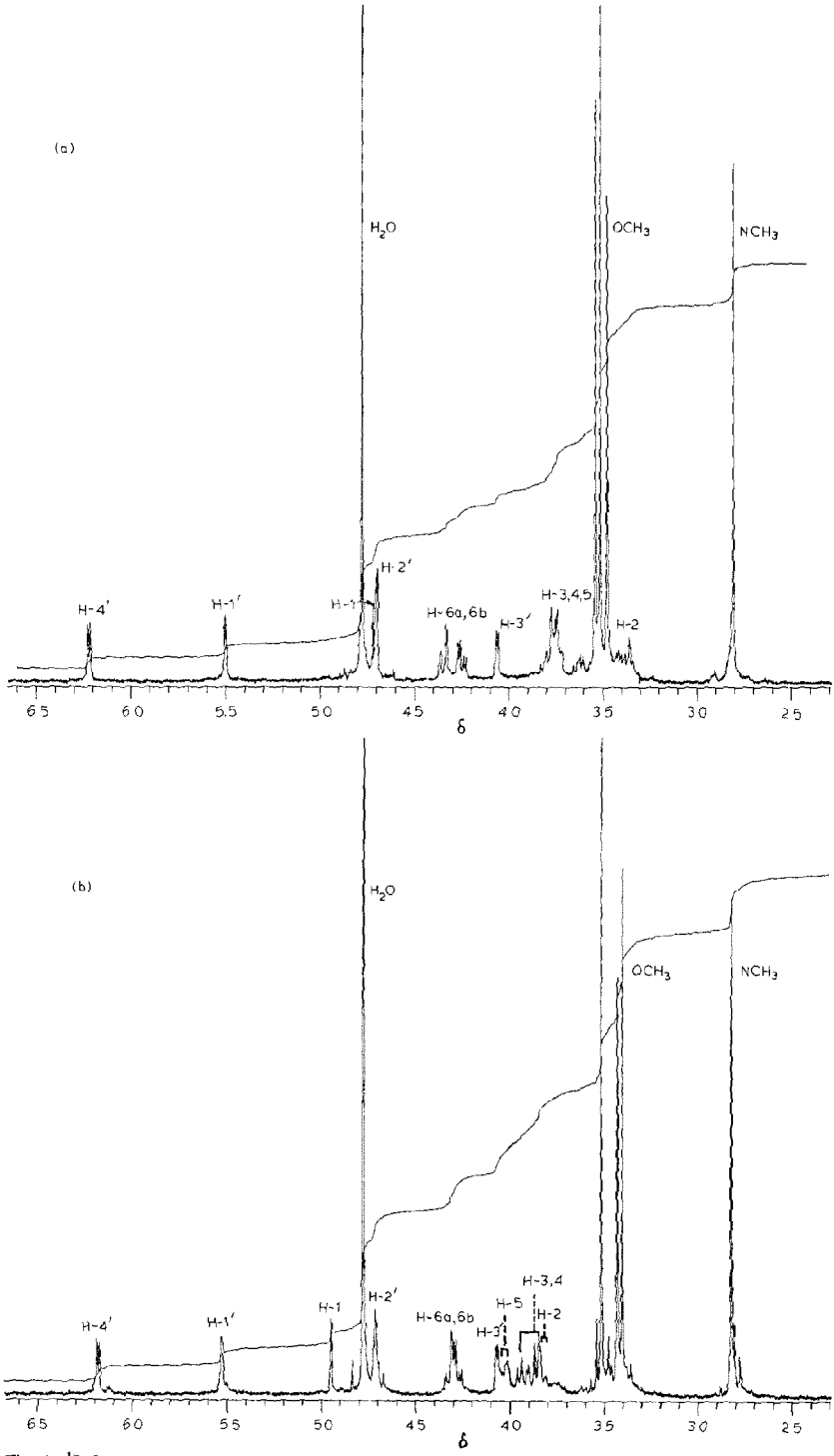


Fig. 4. ^1H-N.m.r. spectra at 360 MHz for solutions in deuterium oxide, of (a) Fraction III-6 (**1**) and (b) Fraction III-7 (**2**). Chemical shifts (δ) and coupling constants are given in Table II.

TABLE II

^{1}H-N M R DATA (δ) FOR FRACTIONS III-6 (**1**) AND -7 (**2**)[a]

Proton	*Fraction*	
	III-6 (Compound **1***)*	*III-7 (Compound* **2***)*
H-1	4.71 ($J_{1,2}$ 8.5)	4.95 ($J_{1,2}$ 2.8)
H-2	3.36 ($J_{2,3}$ 9.5)	3.83–3.84 ($J_{2,3}$ 10.0)
H-3	}	}
H-4	} 3.71–3.83	} 3.85–3.96
H-5	}	4.02 ($J \sim 10$)
H-6a	4.25 ($J_{6a,6b}$ −11.2)	4.28 ($J_{6a,6b}$ −11.2)
H-6b	4.35 ($J_{5,6a}$ 4.5)	4.32 ($J_{5,6a}$ 4.5)
	($J_{5,6b}$ 1.5)	($J_{5,6b}$ 2.2)
H-1′	5.50 ($J_{1',2'}$ 1.7)	5.53 ($J_{1',2'}$ 2.3)
H-2′	4.70 ($J_{2',3'}$ 1.6)	4.72 ($J_{2',3'}$ 1.8)
H-3′	4.06 ($J_{3',4'}$ 5.0)	4.07 ($J_{3',4'}$ 4.8)
H-4′	6.22 ($J_{2',4'}$ 1.2)	6.18 ($J_{2',4'}$ 1.0)
NCH_3	2.81	2.83
OCH_3	3.48	3.41
	3.51	3.43
	3.54	3.52

[a]Coupling constants in Hz.

were consistent with the β-configuration of the 2-amino-2-deoxy-D-hexosyl residue. The u.v. spectrum of Fraction III-6 showed a maximum absorption at 234 nm, and its optical rotatory dispersion exhibited a negative Cotton effect (Table I). The spectral data and sulfate analysis of Fraction III-6 (Table I) indicated that the compound was the methyl β-D-glycoside of a *N,O*-methyl disaccharide trisulfate containing a 4,5-unsaturated uronic acid as the nonreducing end-group (**1**). The conformation of the uronic acid group was supported by the long-range coupling ($J_{2,4}$ 1.2 Hz) of the signal for H-4′ (double doublet) with the signal for H-2′ as reported by Perlin and Mackie[4].

In addition to some spectral characteristics (Fig. 4b and Table II) and the sulfate analysis (Table I), the small coupling constant for H-1 of Fraction III-7 as compared to that of Fraction III-6 and the positive optical rotation suggested that Fraction III-7 was the α anomer of Fraction III-6. Since the chemical shift for H-1 of Fraction III-7 was higher than that previously reported[5] for H-1 of the 2-amino-2-deoxy-D-glucopyranose residue of heparin (δ 5.40), the ^{1}H-n.m.r. spectra of authentic methyl 2-deoxy-2-sulfoamino-α- (**7**) and -β-D-glucopyranoside (**10**) were examined (Table III). The signals for H-1 of **7** and **10** appeared at 0.45 and 0.29 p.p.m. lower than those of the respective parent 2-deoxy-2-sulfoamino-D-glucopyranoses (**6** and **9**). The proton H-1 of Fraction III-7 showed a chemical shift close to that of an authentic α-D-glycoside and that of Fraction III-6 exhibited a δ value lower than that of an authentic β-D-glycoside, but the coupling constants ($J_{1,2}$ and

TABLE III

^{1}H-N M R DATA (δ) FOR 2-DEOXY-2-SULFOAMINO-D-GLUCOPYRANOSE DERIVATIVES (SODIUM SALTS)[a]

Compound	*H-1*	*H-2*	*Reference*
6	5.42 ($J_{1,2}$ 3.5)	3.20 ($J_{2,3}$ 10.0)	6
7	4.97 ($J_{1,2}$ 3.6)	3.19 ($J_{2,3}$ 10 2)	
8	5.45	3.27	4
9	4.70 ($J_{1,2}$ 8.0)	2.98 ($J_{2,3}$ 10.0)	6
10	4.41 ($J_{1,2}$ 8.2)	2.95 ($J_{2,3}$ 9.3)	
11	4.75	3.05	4

[a]Coupling constants in Hz.

$J_{2,3}$) for Fractions III-6 and -7 were similar to those for the $^4C_1$ (D) conformation of authentic β- and α-D-glycosides, respectively. These spectral data support structure **2** for Fraction III-7. Compounds **1** and **2** correspond, respectively, to the *N,O*-methyl derivative of the β- and α-D-glycoside of the disaccharide that was obtained by digestion of heparin with bacterial heparinase[4]. Fractions III-6 and -7 were found to contain a small proportion of partially methylated disaccharides. This was deduced from the reduced intensities of the methoxyl signals in the ^{1}H-n.m.r. spectra.

The deshielding effect of the sulfate group on geminal and vicinal protons has been reported[7]. By comparing the chemical shifts for H-2 of **7** and **10** (Table III) with those of **1** and **2** (Fractions III-6 and -7, Table II), it was concluded that the *N*-methylsulfoamino group deshields more strongly the geminal proton of the α- than that of the β-glycoside.

The uronic acid contents of Fractions III-6 and -7, as determined with D-glucurono-6,3-lactone as a standard, gave only ~73% of the value calculated for **1** and **2**. This may be explained by the presence of the double bond, as 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate (ΔDi6S) gives only ~85% of the calculated value for uronic acid, and the visible spectra of the color substances obtained by treatment of Fractions III-6 and -7 with carbazole were different from that of D-glucurono-6,3-lactone; the ratios of the absorbance at $\lambda_{1\ max}$ 524 nm to that at $\lambda_{2\ max}$ 388 nm for Fractions III-6 and -7 were 2.08 and 2.14:1, respectively, whereas the ratio of the absorbance at $\lambda_{1\ max}$ 524 nm to that at $\lambda_{2\ max}$ 392 nm for D-glucurono-6,3-lactone was 4.31:1. The observed uronic acid content of other disaccharides, as reported in Table I, was also lower than the calculated value. Therefore, the elution diagram obtained by measuring the absorbance at 530 nm (Fig. 1) does not represent the real amounts of oligosaccharide fractions. The oligosaccharide fractions having smaller molecular weight probably exist in a proportion higher than that calculated from the elution diagram.

Although Fractions III-4-1 and -4-2 were not sufficiently purified because of the small quantities available, comparison of their ^{1}H-n.m.r. data (Table IV) with those of **1** and **2** (Fractions III-6 and -7, Table II) showed a significant upfield shift

TABLE IV

^{1}H-N.M R. DATA FOR FRACTIONS III-4-1 (**3**) AND -4-2 (**4**)[a]

Proton	*Fraction*	
	III-4-1 (Compound 3)	*III-4-2 (Compound 4)*
H-1	4.69 ($J_{1,2}$ 8.5)	4.93 ($J_{1,2}$ 3.0)
H-2	3.27	
H-3		
H-4	3.7–3.8	3.7–4.0
H-5		
H-6	Overlap with methoxyl peak	
H-1′	5.43 ($J_{1',2'}$ 1.6)	5.45 ($J_{1',2'}$ 1.6)
H-2′	4.64 ($J_{2',3'}$ 1.1)	4.67 ($J_{2',3'}$ 1.2)
H-3′	3.97 ($J_{3',4'}$ 4.9, $J_{1',3'}$ 1.1)	3.99 ($J_{3',4'}$ 4.9, $J_{1',3'}$ 1.2)
H-4′	6.09 ($J_{2',4'}$ 1.5)	6.09 ($J_{2',4'}$ 1.4)
NCH_3	2.81	2.82
OCH_3	3.435	3.39
	3.444	3.41
	3.50	3.45
	3.53	3.51

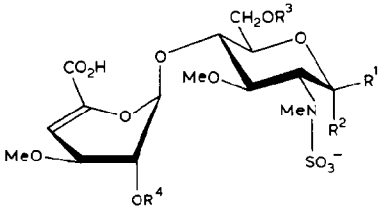
[a]Coupling constants in Hz.

of the H-6a,6b signals and an additional methoxyl signal for the former compounds. Otherwise, the ^{1}H-n.m.r. spectra of Fractions III-4-1 and -4-2 were similar to those of **1** and **2**, respectively. The sulfate contents of Fractions III-4-1 and -4-2 (Table I) was somewhat lower than the value calculated for an *N,O*-methylated disaccharide disulfate (**3**). The spectral and analytical data suggested structures **3** and **4** for Fractions III-4-1 and -4-2, respectively.

The sulfate content and ^{1}H-n.m.r. spectrum (the latter not shown) of Fraction III-3-1 suggested that it is a mixture of methylated disaccharide disulfates.

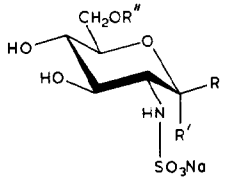
Fraction IV-5 (Fig. 3 and Table I) had a reducing power detectable by the Park–Johnson method (data not shown), but did not show any mutarotation. The sulfate content of the fraction was close to the value calculated for an *N,O*-methyl disaccharide disulfate (see footnote to Table I). Its ^{1}H-n.m.r. spectrum at 90 MHz showed an H-4′ signal (1 H) at δ 6.10, H-1′ and H-1*e* signals (~1.5 H) at δ 5.64, an H-1*a* signal at δ 4.86 (not determined owing to overlapping with the signal of water), an H-6 signal (2 H) at δ 4.37, methoxyl signals (7.8–8.3 H) at δ 3.4–3.5, and *N*-methyl signals (3 H) at δ 2.78 and 2.68. These data suggested that Fraction IV-5 is a mixture of α and β anomers of reducing disaccharide disulfates, as illustrated by **5**.

From the reducing power of other subfractions of Fractions III and IV (data not shown), it appeared that Fraction III preponderantly contained nonreducing disaccharides, whereas Fraction IV mainly contained the reducing disaccharides.



1 $R^1 = OMe, R^2 = H, R^3 = SO_3^-, R^4 = SO_3^-, Na$ salt
2 $R^1 = H, R^2 = OMe, R^3 = SO_3^-, R^4 = SO_3^-, Na$ salt
3 $R^1 = OMe, R^2 = H, R^3 = Me, R^4 = SO_3^-, NH_4$ salt
4 $R^1 = H, R^2 = OMe, R^3 = Me, R^4 = SO_3^-, NH_4$ salt
5* $R^1, R^2 = H, OH, R^3 = SO_3^-, R^4 = Me$, Na salt

*The conformation of **5** is unknown



6 $R = R'' = H, R' = OH$
7 $R = R'' = H, R' = OMe$
8 $R = H, R' = OH, R'' = SO_3Na$
9 $R = OH, R' = R'' = H$
10 $R = OMe, R' = R'' = H$
11 $R = OH, R' = H, R'' = SO_3Na$

As the proportion of Fraction IV (Fig. 1) varied slightly with each preparation, its formation was probably due to an incomplete methylation of the disaccharide disulfates released. The different behaviors of the reducing disaccharide disulfates and of their methyl glycosides in gel-filtration on Sephadex G-25 (Fig. 1) and in anion-exchange chromatography (Figs. 2a and 3) were clearly evident.

The ^{1}H-n.m.r. spectra of Fractions I and II at 90 MHz (Table V) showed signals for ~5.05 and ~3.10 H-1 protons located on the nonreducing group and internal residues, respectively, indicating that Fractions I and II were predominantly composed of hexa- and tetra-saccharides, respectively. The signal at δ 4.99 (~0.5 H) in both spectra was attributed to H-1*e* of the residue bearing a methyl aglycon group in α configuration. The signals at δ 4.67 and 4.72 in the spectra of Fractions I and II, respectively, which are due to H-1*a* of the residue bearing a methyl aglycon group in β configuration, could not be determined quantitatively because of overlapping with the signal of water. These data indicated that Fraction I contains also a mixture of methyl α- and β-glycosides of hexasaccharides, and Fraction II a similar mixture of glycosides of tetrasaccharides. The ^{1}H-n.m.r. spectra of Frac-

TABLE V

^{1}H-N M R DATA (δ) FOR FRACTIONS I AND II

Protons	*Fraction*	
	I	*II*
Olefinic	6.12 (1)	6.11 (1)
Anomeric	4.99 (0.53)[a]	4.99 (0.54)[a]
	4.67	4.72
	5.26, 5.47 (5.05)	5.31, 5.44, 5.53 (3.10)
Methoxyl group	3.44	3.46
	3.56	3.56
	3.64	3.65
N-Methyl group	2.83, 2.91 (9.2)	2.83, 2.90 (6.3)
Acetyl group	2.09 (2.9)[b]	2.10 (0.8)[b]
	(0.33 mol/GlcN)	(0.27 mol/GlcN)

[a]H-1*e* of reducing terminal 2-amino-2-deoxy-D-glucose residue. [b]*N*-Acetyl content of starting heparin: 0.15 mol/GlcN.

tions I and II showed signals for acetamido groups corresponding to 0.33 and 0.27 group per disaccharide unit, respectively. These contents are higher than that of the original heparin (0.15 mol/disaccharide unit). On the other hand, the intensity of the acetamido group signal in the ^{1}H-n.m.r. spectrum of an unfractionated sample of Fraction III indicated a content of 0.10 mol/disaccharide unit. Therefore, the 2-acetamido-2-deoxy-D-glucopyranosyl linkage in heparin seems to be more resistant to cleavage by diazomethane than the 2-deoxy-2-sulfoamino-D-glucopyranosyl linkage. The ratio of the intensity of the H-1 signals to that of the acetamido group signal, in the ^{1}H-n.m.r. spectrum of the unfractionated reaction mixture, was close to that in the ^{1}H-n.m.r. spectrum of the starting heparin. Moreover, the spectrum of the reaction mixture showed no signal due to the *N*-methylamino or *N,N*-dimethylamino group, which might have been formed by methylation of a by-product possessing a free amino group. These results indicate that neither *N*-acetyl nor *N*-sulfate group of heparin was removed during the reaction with diazomethane. On the other hand, both acetamido and sulfoamino groups seem to be susceptible to methylation with diazomethane, as judged from the ratio of the intensity of the *N*-methyl signal to that of the olefinic proton signal (Table V). The ^{1}H-n.m.r. spectra of Fractions I and II showed several intense signals for methoxyl groups at δ 3.40–3.65, but they were not quantitatively estimated owing to overlap with the signals due to the ring protons.

When Fractions I and II were again treated with excess diazomethane, they were slightly depolymerized (elution diagrams not shown). The treatment of dimeric *N*-acetylchondrosine with excess diazomethane for a long period at 25° gave methylated disaccharides in ~35% yield as determined by the absorbance at 530

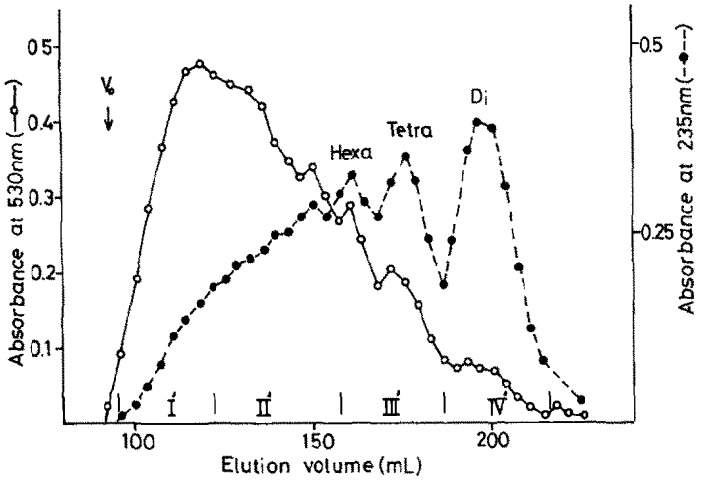


Fig. 5. Gel-filtration, on Sephadex G-50 (superfine), of the reaction products of heparin treated with diazomethane at 4°.

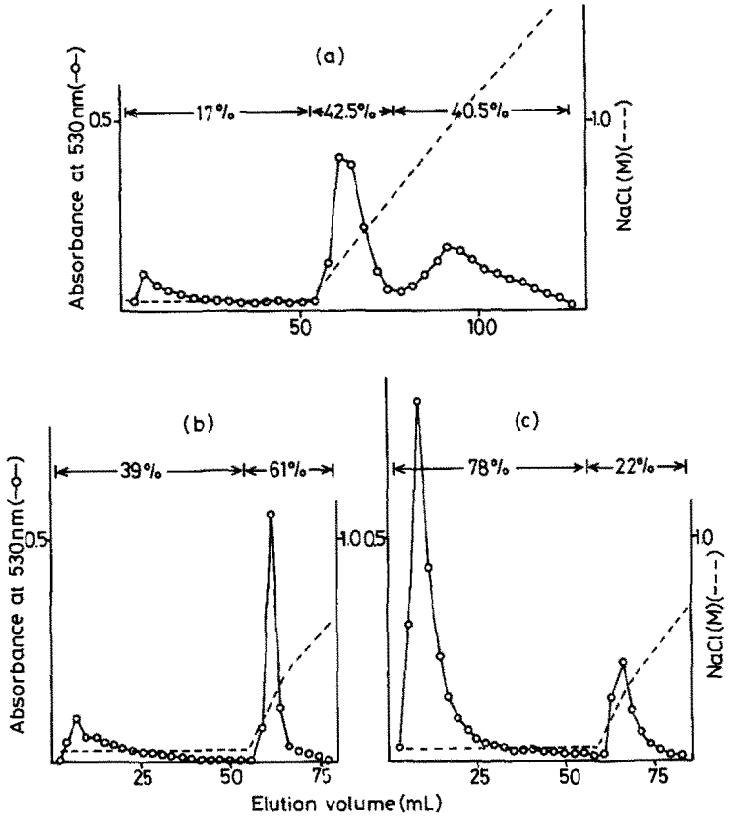


Fig. 6. Affinity-chromatography, on antithrombin III-Sepharose, of (a) heparin, (b) Fraction I', and (c) Fraction II'.

nm of the product of the carbazole reaction. The recovered tetrasaccharide, which had been almost completely *O*-methylated and partially *N*-methylated, was further treated with excess diazomethane to give methylated disaccharides in ~18% yield. These results suggested that methylation of hydroxyl groups stabilizes the 2-amino-2-deoxy-D-hexopyranosyl linkage against β-elimination by diazomethane treatment.

In conclusion, in the reaction with excess diazomethane for a long period at 20°, (a) heparin was cleaved at the β-position of the uronic acid carboxyl group to give a mixture of methyl α- and β-glycosides of *N,O*-methyl di-, tetra-, and hexasaccharides having a 4,5-unsaturated uronic acid, nonreducing end-group; (b) the *N*- and *O*-sulfate, and acetamido groups were stable during this reaction; (c) the presence of some reducing oligosaccharides in the products was probably due to incomplete methylation; and (d) the resulting *N,O*-methyl tetra- and hexa-saccharides remained almost unchanged upon further treatment with diazomethane.

When heparin, dissolved in a small amount of water, was treated for 18 h at 4° with an excess solution of diazomethane in diethyl ether and the reaction product fractionated by gel-filtration on Sephadex G-50 (Fig. 5), the degree of depolymerization was much lower than at 20° (Fig. 1). Fractions I′ and II′ approximately corresponding to octa- to tetradeca-saccharides and higher-molecular-weight oligosaccharides, respectively, were separately applied to an antithrombin III–Sepharose column[8]. The elution patterns (Fig. 6) showed that 61% of Fraction I′ and 22% of Fraction II′ had low affinity for antithrombin III–Sepharose, but neither fraction had high affinity for the gel.

Other glycosaminoglycuronans, such as chondroitin 6-sulfate and dermatan sulfate, were also depolymerized under similar conditions to give, as main products, even-numbered oligosaccharides composed of tetra- and hexa-saccharides having a 4,5-unsaturated uronic acid, nonreducing end-group. This demonstrates the usefulness of diazomethane treatment as a method for cleaving the 2-amino-2-deoxy-D-hexopyranosyl linkages of glycosaminoglycuronans.

EXPERIMENTAL

Materials. — Commercial hog-mucosal heparin (anticoagulant activity, 159 units/mg) was obtained from Sigma Chemical Co. (St. Louis, MO 63178), chondroitin 6-sulfate, pig-skin dermatan sulfate, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate (ΔDi6S), and Serva Ecteola-cellulose from Seikagaku Kogyo Co. (Tokyo). Diazomethane in diethyl ether was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide[9], obtained from Tokyo Kasei Kogyo Co. (Tokyo). Purified bovine antithrombin III was prepared as described by Damus and Rosenberg[10], and the protein was coupled to cyanogen bromide-activated Sepharose CL-4B according to the procedure of Cuatrecasas[11]. Analysis of the antithrombin III-substituted Sepharose indicated a protein content of 8.6 mg/mL of gel. Sodium salts of methyl 2-deoxy-2-sulfoamino-α- and -β-D-glucopyranoside were prepared by the procedure previously reported[12];

the latter compound was amorphous, m.p. 196–198°, $[\alpha]_D^{28}$ −34.1° (*c* 0.476, water).

Anal. Calc. for $C_7H_{14}NNaO_8S \cdot 0.5\ H_2O$: C, 27.63; H, 4.97; N, 4.60; SO_4^{2-}, 31.57. Found: C, 27.80; H, 5.21; N, 4.61; SO_4^{2-}, 31.39.

Dimeric *N*-acetylchondrosine was prepared by the method previously reported[13].

Methods. — Uronic acid content was determined by the method of Bitter and Muir[14], modified[15] by increasing the borate concentration to 0.2M; sulfur content by the method of Terho and Hartiala[16]; and reducing value by the method of Park and Johnson[17]. Optical rotatory dispersion was measured with a Jasco J-20 automatic recording spectropolarimeter. ^{1}H-N.m.r. spectra were recorded with a Nicolet NT-360 spectrometer at 24° for Fractions III-4-1, -4-2, -6, and -7 (HOD peak, δ 4.77), and with a Varian EM-90 spectrometer at 80° for Fractions I and II, and at 22° for Fraction IV-5 and sodium salts of methyl 2-deoxy-2-sulfoamino-α- and -β-D-glucopyranoside (HOD peak, δ 4.68), for solutions in deuterium oxide (99.95%); chemical shifts are expressed downfield from the signal of sodium 4,4-dimethyl-4-silapentane-1-sulfonate; samples were preliminarily treated with deuterium oxide (99.75%). Cellulose acetate membrane-electrophoresis was performed on Separax strips (Fuji Photo-Film Co., Tokyo) in 0.1M phosphate buffer (pH 8.0), with a current of 1.0 mA/cm for 35 min. The strips were stained with 0.5% Alcian Blue in 3% acetic acid.

Depolymerization and permethylation of heparin with diazomethane at 20°. — To a solution of heparin (50 mg) in water (0.5 mL) was added dropwise, a solution of diazomethane in diethyl ether (~60 mL) freshly prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (10 g), and the solution was vigorously stirred for 24 h at 20° with a mechanical stirrer. Diethyl ether was evaporated, and the residue treated again with the diazomethane solution for the same period. After evaporation of diethyl ether, the residue was dissolved in water, and the solution filtered, concentrated to a small volume *in vacuo,* and applied to a column (1.5 × 150 cm) of Sephadex G-25 (superfine). The column was eluted with 0.2M ammonium hydrogencarbonate. The eluate was collected in 3.0-mL fractions, and each fraction analyzed for uronic acid content and for the absorbance at 235 nm, and pooled as indicated in Fig. 1. Each of the Fractions I–V (obtained from ten experiments on the scale just described) was separately pooled.

(a) Rechromatography of Fractions I and II on Sephadex G-25. Each of the pooled Fractions I and II was lyophilized to remove ammonium hydrogencarbonate, and the residue rechromatographed on a column (1.5 × 150 cm) of Sephadex G-25 (superfine) in 0.2M ammonium hydrogencarbonate. The fractions having a homogeneous molecular size (elution diagram not shown) were pooled and lyophilized to give an ammonium salt that was converted into the sodium salt by passing through a column of Dowex 50W-X2 (Na^+, 50–100 mesh). The inorganic salt was removed by passing a solution of the sodium salt in 1:10 (v/v) ethanol–water through a column (1.5 × 90 cm) of Sephadex G-15. The fractions giving a positive carbazole reaction were combined and lyophilized to afford the Fractions I or II as a white powder.

(b) Fractionation of Fraction III on Ecteola-cellulose. Pooled Fraction III was lyophilized. A solution of half of the residue in water (1 mL) ws applied to a column (2.7 × 23 cm) of Ecteola-cellulose (HCO_3^-, 200–400 mesh) anion exchanger, and the column eluted with a linear gradient of 20mM–1.0M ammonium hydrogencarbonate (total volume 1 L). The eluate was collected in 11-mL fractions, each of which was analyzed for uronic acid content and pooled as indicated in Fig. 2a. The other half of the residue was fractionated in the aforementioned manner, and pooled Fractions III-6 and -7 were rechromatographed, separately, on a column (1.6 × 31 cm) of Ecteola-cellulose (HCO_3^-, 200–400 mesh) anion exchanger. Elution with a linear gradient of 0.2–0.8M ammonium hydrogencarbonate (total volume 0.6 L) gave an ammonium salt that was converted into the sodium salt, and then desalted as described under (*a*). The fractions giving a positive carbazole reaction were collected and lyophilized to afford Fraction III-6 or -7 as a pale-yellow powder.

Pooled Fraction III-4 was lyophilized, and the residue applied to a column (1.4 × 40 cm) of Ecteola-cellulose (HCO_3^-, 200–400 mesh) anion exchanger that was eluted with a linear gradient of 0.1–0.4M ammonium hydrogencarbonate (total volume 0.6 L). The eluate was collected in 9.1-mL fractions, and each fraction analyzed for uronic acid content and pooled as indicated in Fig. 2c. The fractions of tube number 16–26 and 27–34 were combined and lyophilized separately. Each of the residues was desalted through Sephadex G-15, and the fractions giving a positive carbazole reaction were collected and lyophilized to give Fraction III-4-1 or -4-2 as a pale-yellow powder.

Fraction III-3 was fractionated by a procedure similar to that described for Fraction III-4, except that the elution was performed with a linear gradient of 50mM–0.3M ammonium hydrogencarbonate (Fig. 2b).

(c) Fractionation of Fraction IV on Ecteola-cellulose. Pooled Fraction IV was lyophilized, and the residue fractionated on Ecteola-cellulose by a procedure similar to that described for Fraction III. The pooled fractions corresponding to Fraction IV-5 were lyophilized, and the material isolated as ammonium salt was converted into the sodium salt, and purified by passing through Sephadex G-15.

The yields and analytical data of the materials isolated are listed in Table I.

Depolymerization and permethylation of heparin with diazomethane at 4°. — A solution of heparin (60 mg) in water (1.0 mL) was vigorously stirred for 18 h at 4° with a solution of diazomethane in diethyl ether (~30 mL), freshly prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (5 g). After evaporation of diethyl ether, the residue was dissolved in a small volume of water. The solution was filtered, concentrated to a small volume, and applied to a column (1.5 × 150 cm) of Sephadex G-50 (superfine) which was eluted with 0.2M ammonium hydrogencarbonate. The eluate was collected in 3.6-mL fractions, each of which was analyzed for uronic acid content and for absorbance at 235 nm, and pooled as shown in Fig. 5. Each of the pooled fractions (I′–IV′) was lyophilized and the material isolated as ammonium salt was converted into the sodium salt by passing through a column of

Dowex 50W-X2 (Na^+). After lyophilization, Fractions I′ (16.4 mg), II′ (21.5 mg), III′ (13.1 mg), and IV′ (6.6 mg) were obtained as a white powder.

Treatment of Fractions I and II with diazomethane. — A solution of diazomethane in diethyl ether (~30 mL) freshly prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (5 g) was added to a solution of Fraction I (9.8 mg) or II (9.2 mg) in water (0.1 mL each). The mixture was vigorously stirred for 2 days at 25°. Diethyl ether was evaporated, the residue dissolved in water (5 mL), and the solution filtered. The filtrate was concentrated to a small volume, and applied onto a column (1.5 × 150 cm) of Sephadex G-25 (superfine), which was eluted with 0.2M ammonium hydrogencarbonate (elution diagrams not shown).

Reaction of dimeric N*-acetylchondrosine with diazomethane.* — A solution of dimeric *N*-acetylchondrosine (12.4 mg) in water (0.3 mL) was treated with a solution of diazomethane in diethyl ether (~90 mL) freshly prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (15 g) with vigorous stirring for 3 days at 25°. Diethyl ether was evaporated, and the residue chromatographed on a column (1.5 × 150 cm) of Sephadex G-25 (superfine), prepared in 0.2M ammonium hydrogencarbonate, by a procedure similar to that described in the preceding paragraph. The fractions corresponding to di- and tetra-saccharides were separately collected and lyophilized. After being examined by ^{1}H-n.m.r. spectroscopy, the tetrasaccharide fraction (6.55 mg) was recovered and treated again with a solution of diazomethane in diethyl ether (~60 mL) freshly prepared (from 10 g of the nitrosamide). The mixture was evaporated and the residue obtained chromatographed on Sephadex G-25 for estimation of the degree of depolymerization.

Affinity chromatography of methylated heparin oligosaccharides on antithrombin III–Sepharose. — The procedure applied was essentially that described by Laurent *et al.*[8]. A sample of heparin or of the methylated heparin oligosaccharides (Fractions I′ and II′, Fig. 5) (each ~2.5 mg) was applied to a column (7.6 mL; 1.8 × 3 cm) of antithrombin III–Sepharose in 50mM Tris · hydrochloric acid buffer, pH 7.4–50mM sodium chloride at 4°. The column was washed with the same buffer (50 mL) and eluted with a linear gradient of 50mM–3M sodium chloride in Tris · hydrochloric acid buffer, pH 7.4 (100 mL). The eluate was collected in 3.0-mL fractions, and each fraction analyzed for uronic acid content (Fig. 6).

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