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ISOLATION AND CHARACTERIZATION OF HEPARIN DISACCHARIDES

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ABSTRACT

Purified porcine heparin was hydrolyzed with 0.5M hydrochloric acid for 20 h at 80°. The neutralized hydrolyzate was subjected to gel filtration through Sephadex G-10. Fractions containing products of higher molecular weight than disaccharide were treated repeatedly by the foregoing procedure. The disaccharide-containing fractions thus obtained were purified by preparative, high-voltage paper electrophoresis at pH 1.9, and two disaccharides were then separated by multiple, preparative paper-chromatography. The yields of the disaccharides (P-4 and P-5) were 13.1 and 3.8 mg, respectively, in terms of D-glucuronic acid, from 300 mg of the starting heparin. As P-4 was not homogeneous, it was further purified by preparative, high-voltage paper electrophoresis at pH 2.7. Analytical data of P-4 and P-5 and of their *N*-acetyl derivatives (P-4A and P-5A) before and after reduction with sodium borohydride indicated that P-4 and P-5 consisted of equimolar amounts 2-amino-2-deoxy-D-glucose and hexuronic acid, having hexuronic acid at the reducing end. The hexuronic acid in P-4A and P-5A was determined by g.l.c. to be L-iduronic acid and D-glucuronic acid, respectively. After reduction of the products obtained by Smith degradation of the reduced P-4A and P-5A, threitol and erythritol, respectively, and also glycerol were detected by g.l.c. The α-anomeric configuration of the 2-acetamido-2-deoxy-D-glucopyranosyl linkages of P-4A and P-5A was suggested by i.r. and p.m.r. spectra and by the high dextrorotations; $[\alpha]_D^{27}$ values of P-4A and P-5A were +91.6° (in water) and +114.9° (in water), respectively. The foregoing observations indicated that P-4 and P-5 were *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-L-idopyranuronic acid and *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-D-glucopyranuronic acid, respectively.

INTRODUCTION

It had been considered that heparin is a polymer consisting of a sulfated derivative of a repeating *O*-(2-sulfoamino-2-deoxy-α-D-glucopyranosyl)-(1→4)-α-D-glucopyranosyluronic acid (see ref. 1). In a previous paper[2] we reported that L-iduronic acid was the major hexuronic acid in the deamination product of heparin, Also, this uronic acid was detected in the deamination product of a disaccharide fraction obtained by acid hydrolysis[2]. Therefore, we assumed previously[2] that deaminative cleavage of the glycosidic linkage between C-4 of D-glucuronic acid and C-1 of 2-amino-2-deoxy-D-glucose resulted in the concomitant epimerization of D-glucuronic acid to L-iduronic acid. However, L-iduronic acid has been shown to be the predominant uronic acid of heparin[3-8]. Moreover, it has been shown recently that L-iduronic acid residues are formed by epimerization, at the polymer level, at C-5 of D-glucuronic acid residues[9,10]. In addition, Lindahl and Axelsson[6] stated that the new uronic acid in our disaccharide should be L-iduronic acid, because they obtained the corresponding disaccharide consisting of L-iduronic acid and 2-amino-2-deoxy-D-glucose after acid hydrolysis of heparin.

In order to reinvestigate the previously described disaccharide, we isolated disaccharides from an acid hydrolyzate of heparin. This paper reports the isolation and characterization of two disaccharides from porcine heparin. In addition, a correction of the previous assumption was made.

EXPERIMENTAL

Materials. — Purified porcine heparin was purchased from Taiyo Fishery Co. Ltd., Yokosuka. Threitol and erythritol were prepared by the reduction of threose and erythrose obtained by the method of Perlin[11]. D-Glucuronic acid was purchased from Nakarai Chemicals Ltd., Tokyo. L-Iduronic acid was the material reported previously[12]. Other materials were from commercial sources.

Preparation of crude disaccharides. — Purified porcine heparin (300 mg) was treated with 0.5M hydrochloric acid (30 ml) in a glass-stoppered tube for 4 h at 40°, followed by heating for 20 h at 80°. The hydrolyzate was cooled in an ice-bath, and then neutralized with M sodium hydroxide. The neutralized solution was concentrated to low volume *in vacuo*, and then passed through a column (146 × 2.65 cm) of Sephadex G-10, collecting 5-ml fractions. As disaccharides were eluted between fractions 69 and 87, these fractions were combined. The fractions eluted prior to fraction 69, which contained products of higher molecular weight than disaccharide, were pooled and then evaporated under diminished pressure. The residue was hydrolyzed with 30 ml of 0.5M hydrochloric acid for 20 h at 80°. The hydrolyzate was then treated by the foregoing procedure. The procedure was repeated 9 times altogether. The resulting disaccharide fractions were combined and then concentrated to low volume, and the concentrate was desalted by gel filtration through a column (146 × 2.65 cm) of Sephadex G-10. The desalted solution was then concentrated and lyophilized.

High-voltage paper electrophoresis. — High-voltage paper electrophoresis was carried out on a Toyo HPE-V apparatus at 5–20°, with Toyo filter paper No. 50. The electrophoresis was performed in formic acid (98%)–acetic acid–water (176:100:724, by volume; pH 1.9) at a potential gradient of 50 V/cm for 70 min and/or in pH 2.7 buffer[12] that had been prepared by the addition of 98% formic acid to pyridine–acetic acid–water (1:10:89, by volume), at a potential gradient of 100 V/cm for 110 min. Compounds were located by staining with alkaline silver reagent.

Paper chromatography. — Paper chromatography was carried out on Toyo filter paper No. 50, by one of the following methods: (*a*) multiple ascending development with ethyl acetate–acetic acid–water (100:34:40, by volume), ethyl acetate–acetic acid–water (100:37:50, by volume), and ethyl acetate–acetic acid–water (100:40:60, by volume), in succession (each development was conducted for 15 h at 28° after drying the paper); (*b*) descending development in pyridine–ethyl acetate–water–acetic acid (5:5:3:1, by volume) for 15 h at 20°; and (*c*) descending development in 1-butanol–pyridine–water (6:4:3, by volume) for 24 h at 20°. Compounds were located by staining with alkaline silver reagent.

N-*Acetylation of disaccharides.* — Fractions P-4 and P-5 were *N*-acetylated by the method of Danishefsky and Steiner[13], and the resulting products were designated as P-4A and P-5A, respectively.

Deamination of disaccharides. — Deamination of disaccharides was performed with sodium nitrite (10 moles/mole) in 10% acetic acid overnight at 2–4°. The mixture was applied to Toyo filter paper No. 50, and subjected to high-voltage paper electrophoresis at pH 1.9 as already described. The product remaining at the origin was eluted with water from the paper. The solution was concentrated and then lyophilized. The deamination products thus obtained were then methanolyzed with 3% methanolic hydrogen chloride for 8 h at 100°, and then analyzed by g.l.c. G.l.c. of the trimethylsilyl ethers of the foregoing products was conducted by the methods of Sweeley *et al.*[14] and Yamakawa and Ueta[15], using a 3-m column packed with 5% Ucon LB 550 on Chromosorb W (AW-HMDS) at 165°.

Reduction of disaccharides. — A portion (2 mg) of the *N*-acetylated disaccharide (P-4A or P-5A) was dissolved in 1.0 ml of 0.1M borate buffer (pH 7.3) and then 1.0 ml of 0.1M sodium borohydride was added to the solution under cooling in an ice-bath. The mixture was kept overnight at 4° and then acidified with dilute hydrochloric acid to decompose the excess of reductant, and lyophilized. The residue was dissolved in a small volume of water, and then desalted by gel filtration through a column (140 × 1.4 cm) of Sephadex G-10. The fractions containing the reduced disaccharide were combined and then concentrated and lyophilized.

Smith degradation of the reduced disaccharides. — Smith degradation was carried out by the method of Yamaguchi and Kuriyama[16]. A portion (1 mg) of the reduced disaccharide prepared by the foregoing method was oxidized with 3 ml of 0.015M sodium metaperiodate for 30 h at 1–2° in dark. When oxidation was effected for 60 min at 0°, part of the hexuronic acid was oxidized, but most of the 2-acetamido-2-deoxy-D-glucose residues remained intact. Therefore, we conducted the procedure for 30 h at 1–2° to oxidize 1,2-glycol groups completely. The oxidized products thus obtained were then reduced with sodium borohydride overnight at 1–2°. The mixture was acidified with acetic acid to decompose the excess of reductant, with subsequent addition of 2 drops of saturated, aqueous lead acetate. The mixture was then cooled in an ice-bath, kept for a while, and then filtered through a Toyo membrane filter

TM-2. The filtrate was passed through a column (10 × 1 cm) of Dowex 50 resin (H^+ form), and boric acid was removed from the effluent by repeated evaporation to dryness with methanol under diminished pressure. The residue was hydrolyzed with 1.0 ml of 0.5M hydrochloric acid for 4 h at 100°. Hydrochloric acid was removed from the hydrolyzate by repeated evaporation to dryness with methanol *in vacuo*. The residue was then dissolved in 1.0 ml of water, treated with 10 mg of sodium borohydride under cooling in an ice-bath, and then kept overnight at 4°. The excess of reductant was decomposed with acetic acid, and the mixture was passed through a column (5 × 1 cm) of Dowex 50 resin (H^+ form). Boric acid was removed by the same procedure as before. The residue was heated again with 0.5M hydrochloric acid for 2 h at 100°, followed by the same treatment as before. By these treatments, most of the tetronic acid was converted into tetritol. The products were then trifluoroacetylated by the method of Imanari *et al.*[17], and analyzed by g.l.c. G.l.c. was conducted on a Shimadzu GC-4B gas chromatograph, using a 3-m column packed with 3% SE-30 on Chromosorb W (AW-HMDS) at 70°.

Infrared spectra. — I.r. spectra were recorded on 1-mg samples of the compound in pellets (200 mg) of potassium bromide, using a Hitachi Model EPI-G2 i.r. spectrophotometer.

Proton magnetic resonance (p.m.r.) spectra. — P.m.r. spectra were recorded at 100 MHz with a JNM-PS-100 spectrometer, using sodium 4,4-dimethyl-4-silapentane-1-sulfonate as the internal standard in deuterium oxide.

Determination of constituents. — Hexuronic acid was determined by the carbazole method of Dische[18] and by the carbazole-borate method of Bitter and Muir[19]. 2-Amino-2-deoxy-D-glucose was determined by the indole method of Dische and Borenfreund[20], and by the Elson–Morgan procedure of Gardell[21] after hydrolysis with 4M hydrochloric acid for 12 h at 100°. Hexuronic acid was also determined by g.l.c. as follows: *N*-acetylated disaccharide was methanolyzed with 3% methanolic hydrogen chloride for 15 h at 100°, and then the solution was evaporated to dryness under diminished pressure. G.l.c. of trimethylsilyl derivatives of the methyl hexosiduronates was conducted as described for the analysis of the deamination products.

RESULTS

Preparation of the disaccharide fraction. — Purified porcine heparin (300 mg) was hydrolyzed with 0.5M hydrochloric acid as described in the experimental section, followed by gel filtration through a column of Sephadex G-10 (Fig. 1). As analytical data indicated that the fractions between 69 and 87 contained disaccharides, these fractions were combined. The fractions eluted prior to fraction 69 contained oligosaccharides of higher molecular weight than disaccharide. These fractions were then pooled and subjected to rehydrolysis to obtain disaccharides as described in the experimental section.

The disaccharide fractions thus obtained from nine such treatments were

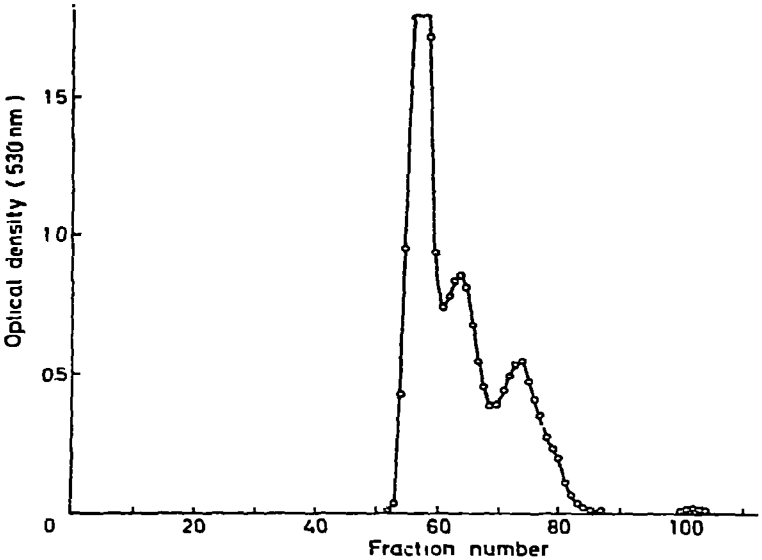

Fig. 1. Gel filtration of the acid hydrolyzate of heparin. The content of uronic acid in each fraction was determined by the method of Bitter and Muir[19].

combined and then treated as described in the experimental section. The resulting, lyophilized products were then subjected to preparative, high-voltage paper electrophoresis, using the pH 1.9 buffer as described in the experimental section. Five spots were observed by staining with alkaline silver reagent (Fig. 2). The substances on the paper, designated 1–5, were eluted with water from the paper. Analytical data for these substances indicated that disaccharides were present in the eluate from 3, which was designated E-3. The yield of E-3 was 29% of the weight of the starting heparin, in terms of D-glucuronic acid as determined by the method of Bitter and Muir[19].

Isolation of two disaccharides. — The foregoing component (E-3) was purified by preparative paper-chromatography, applying multiple, ascending development as described in paper-chromatographic method (*a*) (Fig. 3). Six spots (1–6) were detected by staining with alkaline silver reagent The product in each spot was eluted from the paper with water. Analytical data indicated that the products from spots 4 and 5 were disaccharides, denoted P-4 and P-5, respectively. Disaccharides P-4 and P-5 were purified by rechromatography twice and three times, respectively, by the same preparative, paper-chromatographic procedure. The yields of P-4 and P-5 were 13.1 and 3.8 mg, respectively, in terms of D-glucuronic acid as determined by the method of Bitter and Muir[19]. Of the disaccharides, P-5 was shown to be homogeneous by paper chromatography in the three systems used and by high-voltage paper electrophoresis in the two buffers described, whereas P-4 was not homogeneous. Therefore, P-4 was further purified by preparative, high-voltage paper electrophoresis using the pH 2.7 buffer After elution from the major portion of the spot on the paper, the resulting preparation of P-4 was homogeneous in the foregoing criteria.

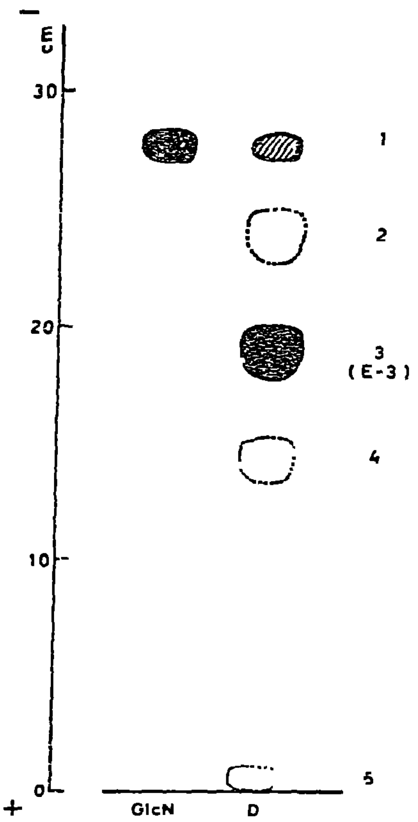

Fig. 2. Tracing of a high-voltage, paper electrophoretogram of the disaccharide fraction in formic acid-acetic acid-water (176 100:724, by volume, pH 1.9) buffer at potential gradient of 50 V/cm for 70 min. Products were located by staining with alkaline silver reagent. GlcN, 2-amino-2-deoxy-D-glucose; D, disaccharide fraction.

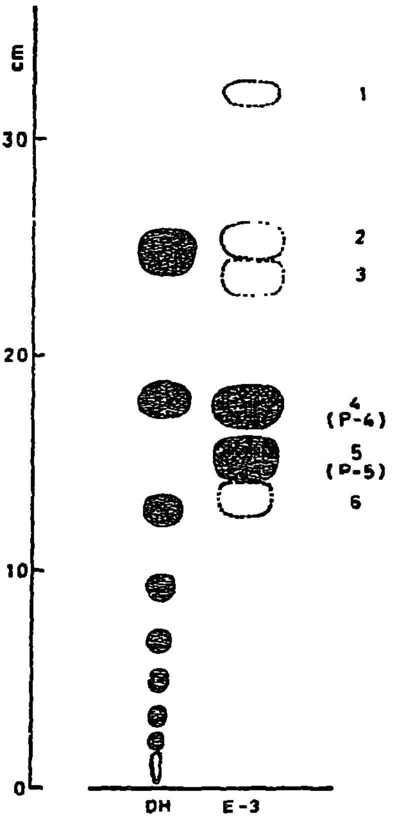

Fig. 3. Tracing of a paper chromatogram of E-3. Paper chromatography was performed by multiple, ascending development with ethyl acetate-acetic acid-water (100·34:40, by volume), ethyl acetate-acetic acid-water (100.37:50, by volume), and ethyl acetate-acetic acid-water (100·40:60, by volume), in succession Each development was carried out for 15 h at 28 after drying the paper. Products were located by staining with alkaline silver reagent. DH is a partial hydrolyzate of dextran (0.5M sulfuric acid 25 min, 100°).

Characterization of disaccharides. — Analytical data for the purified preparation of P-4 and P-5 and their *N*-acetyl derivatives (P-4A and P-5A) are listed in Table I. The data showed that these compounds consisted of equimolar amounts of hexuronic acid and 2-amino-2-deoxy-D-glucose. The reduction products from P-4A and P-5A contained 0 and 2%, respectively, of hexuronic acid in terms of D-glucuronic acid as compared with the starting materials. These observations indicated that P-4 and P-5 were disaccharides, having hexuronic acid at the reducing end. The hexuronic acid values obtained by the carbazole method of Dische were high, as was that for the starting heparin.

TABLE I

SUGAR COMPOSITION[a] OF DISACCHARIDES AND THEIR DERIVATIVES

Fraction	*Uronic acid*		*2-Amino-2-deoxy-D-glucose*	
	D[b]	*B-M*[c]	*D-B*[d]	*G*[e]
P-4	1.50	1.00	0.75	0.85
P-4A	1.25	1.00		0.88
P-4AR[f]		0.00	1.00	
P-5	1.53	1.00	0.80	0.93
P-5A	1.35	1.00		0.87
P-5AR[g]		0.02	1.00	

[a] Sugar composition is expressed as the molar ratio to uronic acid value, as determined by the method of Bitter and Muir[19] for P-4, P-4A, P-5, and P-5A. For their reduction products (P-4AR and P-5AR), it is expressed as molar ratio to 2-amino-2-deoxy-D-glucose, as determined by the method of Dische and Borenfreund[20]. [b] Determined by the method of Dische[18]. [c] Determined by the method of Bitter and Muir[19]. [d] Determined by the method of Dische and Borenfreund[20]. [e] Determined by the method of Gardell[21]. [f] Reduction product of P-4A. [g] Reduction product of P-5A.

Paper chromatograms of the acid hydrolyzate (4M hydrochloric acid, 12 h, 100°) showed the presence of 2-amino-2-deoxy-D-glucose as the sole 2-amino-2-deoxy-D-hexose. G.l.c. of the trimethylsilyl ethers of the methyl glycosides of hexuronic acid showed the presence of L-iduronic acid and D-glucuronic acid in P-4A and P-5A, respectively (Fig. 4). Moreover, g.l.c. of the deamination products of P-4 and P-5 showed the presence of the same hexuronic acid as just noted, in addition to 2,5-anhydro-D-mannose; the latter gave a major peak (emergence time 25 min), and two minor peaks (emergence times, 22 and 30 min). These data indicated that P-4 and P-5 were *O*-(2-amino-2-deoxy-D-glucopyranosyl)-L-idopyranuronic acid and *O*-(2-amino-2-deoxy-D-glucopyranosyl)-D-glucopyranuronic acid, respectively.

The reduction products from P-4A and P-5A were subjected to Smith degradation. The products were then reduced with sodium borohydride as described in the experimental section. The results of g.l.c. of the products showed the presence of threitol and erythritol, respectively, as well as glycerol; these compounds gave peaks having emergence times of 12 min (glycerol), 23 min (erythritol), and 30 min (threitol). Glycerol was not detected in an early stage of oxidation, but tetritols were always detected. The reduced P-4A and P-5A each consumed 3 moles per mole of periodate.

These observations indicated that O-4 of the hexopyranuronic acid of P-4 and P-5 was substituted by 2-amino-2-deoxy-D-glucopyranose.

The optical rotations, $[\alpha]_D^{25}$, of sodium salts of P-4A and P-5A were +91.6° (in water) and +114.9° (in water), respectively. The high dextrorotation of these compounds suggested the α-anomeric linkage in the 2-acetamido-2-deoxy-D-glucopyranoside component.

I.r. spectra of sodium salts of P-4A and P-5A showed weak absorptions at 835 cm^{-1} for P-4A and at 840 cm^{-1} for P-5A, suggesting the presence of α-glycosidic linkages in these compounds.

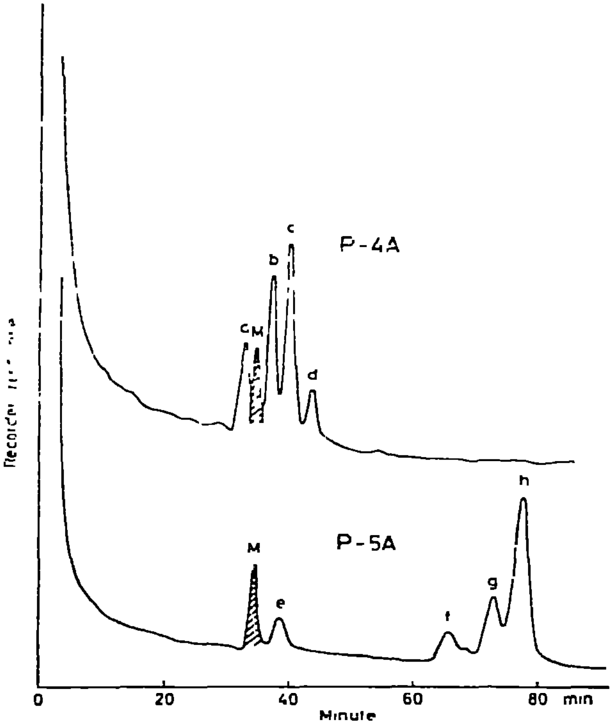

Fig. 4. Gas chromatograms of uronic acids of P-4A and P-5A. G.l.c. of trimethylsilyl derivatives of methyl hexosiduronates was performed with a 3-m column packed with 5% Ucon LB 550, on Chromosorb W at 165 as described in the text. M, mannitol; a, b, c, and d, L-iduronic acid; e, f, g, and h, D-glucuronic acid. As gas chromatograms of authentic samples of L-iduronic acid and D-glucuronic acid were identical with those of P-4A and P-5A, respectively, the chromatograms of the authentic uronic acids are not shown in this figure. M, internal standard.

P.m.r. spectra of sodium salts of P-4A and P-5A are shown in Fig. 5. In the spectra, isolated signals, **b** to **e** from P-4A and **f** to **g** from P-5A, were observed. Signals of **e** at δ 2.00 and **j** at δ 2.03 can be ascribed to the acetate methyl group of 2-acetamido-2-deoxy-D-glucopyranosyl residues in the $^4C_1$ conformation[22].

In the spectrum of P-4A, signal **a** (δ 5.11–5.04) may be the overlapped signals of the anomeric protons of the two consituent sugars. After reduction of P-4A, signal **a** changed to a narrow-spaced doublet (δ 5.20, $J = 3$ Hz). This signal may be ascribed to H-1 of the α-anomer of a 2-acetamido-2-deoxy-D-glucopyranosyl residue in the $^4C_1$ conformation. In addition, the signals that disappeared after the reduction of signal **a** may be assigned to α- and β-anomeric protons of L-idopyranuronic acid. The two doublets **b** (δ 4.63) and **c** (δ 4.40), having spacings of 3.0 and 2.1 Hz, respectively, may be ascribed to H-5 of the α- and β-anomers of an L-idopyranuronic acid residue in the $^1C_4$ conformation, based on the assignment of Perlin *et al.*[23]. Also, signal **d** (δ 4.19), having a small coupling-constant ($J = 3.1$ Hz), may be ascribed to H-3 of the L-idopyranuronic acid residue, as assigned by Perlin *et al.*[23].

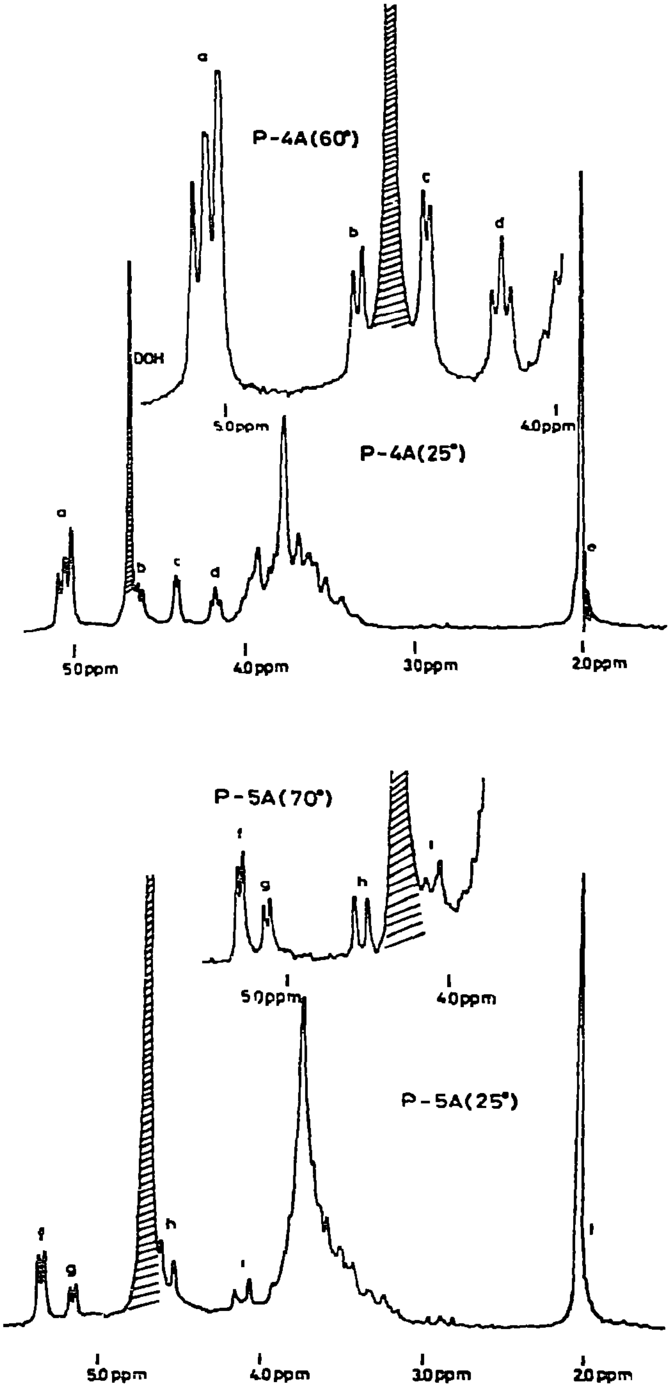

Fig. 5. P.m.r. spectra of sodium salts of P-4A and P-5A. P.m.r. spectra were recorded at 25 and 60 for P-4A, and at 25 and 70° for P-5A.

In the spectrum of P-5A, signal **f** (δ 5.36) may be attributed to the α-anomeric proton of the 2-acetamido-2-deoxy-D-glucopyranosyl residue, because it is a one-proton doublet of small spacing ($J = 3.0$ Hz). Judging from the chemical shift and large coupling constant ($J = 8.0$ Hz) of signal **h** (δ 4.58), it may be ascribed to H-5 of a D-glucopyranuronic acid residue in the $^4C_1$ conformation. A doublet signal **g** (δ 5.18) exhibiting narrow spacing ($J = 3.5$ Hz), and signal **i** (δ 4.13) having a large coupling-constant ($J = 9.0$ Hz), may be attributed to the anomeric protons of the α- and β-anomers, respectively, of the D-glucopyranuronic acid residue.

DISCUSSION

In the previous paper[2], we found L-iduronic acid in the deamination products of heparin. In addition, a disaccharide fraction consisting of hexuronic acid and 2-amino-2-deoxy-D-glucose was obtained from an acid hydrolyzate of heparin[2]. As hexuronic acid values determined by the carbazole method were very high, the hexuronic acid was assumed to be D-glucuronic acid[2], because it had been thought that D-glucuronic acid was the major hexuronic acid in heparin. However, L-iduronic acid was the only detectable uronic acid in the deamination product of the disaccharide[2]. We assumed, therefore, that L-iduronic acid might be a product of epimerization from D-glucuronic acid, which was produced by deaminative cleavage of the α-D-(1→4) linkage of 2-amino-2-deoxy-D-glucopyranoside[2]. However, L-iduronic acid has been found to be a major hexuronic acid in heparin, together with D-glucuronic acid[3-8]. Also, Lindahl and Axelsson[6] isolated a disaccharide component of heparin, 2-amino-2-deoxy-D-glucosyl-L-iduronic acid, from an acid hydrolyzate of heparin, although it was not characterized definitively.

In the present paper, we isolated two disaccharides (P-4 and P-5), from an acid hydrolyzate of porcine heparin by gel filtration, preparative, high-voltage paper electrophoresis, and preparative, paper chromatography. The data of the characterization, described in the Results section, of P-4 and P-5 and their *N*-acetyl derivatives (P-4A and P-5A) indicate that P-4 and P-5 are *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-L-idopyranuronic acid, and *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-D-glucopyranuronic acid, respectively. As the ratio of the yields of these compounds was approximately 3:1, the disaccharide reported previously[2] might be mainly P-4. Moreover, D-glucuronic acid in P-5 did not produce L-iduronic acid by deamination. Therefore, the L-iduronic acid found previously[2] in the deamination products of the disaccharide and heparin was not the epimerization product of D-glucuronic acid, but a major constituent of these compounds. This conclusion coincides with the report of Lindahl and Axelsson[6].

The (1→4) glycosidic linkages of 2-amino-2-deoxy-D-glucopyranose in heparin have been reported to be α-D (see ref. 1). Specifically, Wolfrom *et al.*[24] reported the isolation and characterization of *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-α-D-glucopyranose from a graded acid hydrolyzate of partially *O*-acetylated, partially desulfated, completely carboxyl-reduced, and *N*-acetylated heparin. Furthermore, a disaccharide identical to P-5 (and P-5A) was isolated and characterized by Danishefsky and Steiner[13] from an acid hydrolyzate of heparin. The present study on P-5 (and P-5A) has confirmed their results.

On the other hand, the presence of an *O*-(2-amino-2-deoxy-α-D-glucopyranosyl)-(1→4)-α-L-idopyranosyluronic acid unit in heparin was suggested by n.m.r.-spectral studies at the polymer level[5,25], as well as by the susceptibility to heparinase[8,26,27]. This was also suggested from biosynthetic studies on heparin[9,10]. Rigorous evidence of this structure was provided directly in the present study through the isolation and characterization of P-4 from an acid hydrolyzate of heparin.

In the present study, two kinds of disaccharide units from an acid hydrolyzate of heparin were isolated at the same time and then characterized. The results should be valuable for the direct elucidation of the structure of heparin. It should be noted, however, that these disaccharides give no information about the sulfate groups in heparin, an important structural aspect that cannot be studied by the approach used here.

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