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REACTIVITY TOWARD CHEMICAL SULFATION OF HYDROXYL GROUPS OF HEPARIN

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ABSTRACT

The pyridinium salt of hog-mucosal heparin was desulfated by heating in 3:6:1 (v/v) 1,4-dioxane–dimethyl sulfoxide–methanol at 90° for 72 h, with the intermittent addition of pyridinium chloride (5 mol/mol of disaccharide). After *N*-resulfation, the desulfated polysaccharide (tributylammonium salt) was treated with 5–20 mol of pyridine–sulfur trioxide in *N,N*-dimethylformamide per mol equiv. of hydroxyl group at −10, 0, or 20° for 1 h to undergo *O*-sulfation. The sulfated products were quantitatively analyzed for 6-sulfate and 2-sulfate esters in 2-amino-2-deoxy-D-glucose and L-idosuronic acid units, respectively, by ^{13}C- and ^{1}H-n.m.r. spectroscopy and for total sulfate content by a chemical method. The new procedure gave 97% of desulfation of heparin, *i.e.*, 100% *N*- and 6-desulfation of 2-amino-2-deoxy-D-glucose and 91.5% 2-desulfation of L-idosiduronic acid units. The *O*-resulfation proceeded according to the order of reactivity of the hydroxyl groups: HO-6 in GlcN ≫ HO-2 in IdoA > other available hydroxyl groups (HO-3 in GlcN, HO-3 in IdoA, and HO-2 or HO-3 in GlcA).

INTRODUCTION

Previously, we reported[1] that chemical sulfation of whale heparin, known to have incomplete 6-sulfation in the 2-deoxy-2-amino-D-glucose unit, could markedly increase the anticoagulant properties. In order to establish the order of sulfation of the hydroxyl groups in heparin, we desulfated heparin, then *N*-resulfated and progressively *O*-sulfated it, and finally analyzed both the degree and position of sulfate substitution.

RESULTS AND DISCUSSION

Presently, desulfation is performed mainly by methanolysis, which is faster than hydrolysis[2,3], together with a cooperative effect of 1,4-dioxane and pyridinium

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TABLE I

ANALYTICAL DATA OF DESULFATED HEPARIN

Preparation	*Uronic acid*[a]		*Hexosamine*[a] (%)	*Total S* (mol)	*GlcNS/(GlcNS+GlcNAc)* (mol)	*GlcN6S/(GlcNS+GlcNAc)* (mol)	*IdoA2S/(IdoA+IdoA2S+GlcA)* (mol)
	A[b]	*B*[c]					
Starting heparin	41.0 (0.82)	50.2	23.2	2.23	0.829[e]	0.733[e]	0.686[d]
Desulfated heparin							
Ayotte and Perlin's method[g]	55.5 (0.76)	73.0	36.6	0.128	0	0	0.116 (83.1%)[f]
The present method							
1 day[g]	51.0 (0.76)	67.3	31.4	0.201	0	0	0.183 (73.3%)[f]
3 days	54.6 (0.76)	72.2	35.2	0.065	0	0	0.058 (91.5%)[f]

[a]The values for desulfated heparins were determined on their *N*-acetylated derivatives. In parentheses, ratio A:B. [b]Determined by the method of Bitter and Muir[10]. [c]Determined by the method of Kosakai and Yosizawa[11]. [d]Calculated from the n.m.r. spectroscopic data. [e]This material was prepared by the present authors according to the procedure described by Ayotte and Perlin[4]. [f]Degree of 2-desulfation of IdoA2S. [g]Pyridinium chloride added 1 h after the reaction started.

chloride. As previously observed for the *N,O*-desulfation of heparin[2], the free amino groups liberated by prior *N*-desulfation stabilize the residual *O*-sulfate groups by salt formation. Consequently, a high concentration of pyridinium chloride was used to accelerate the *O*-desulfation of heparin prior to *N*-desulfation (see Table I). The chemically determined, total S contents of the desulfated heparins approximately agreed with those calculated from the degree of 2-*O*-sulfation of L-iduronic acid units, which could be estimated by n.m.r. spectroscopy. The quantitative determination of uronic acid and hexosamine indicated that such treatment of heparin had no effect on the sugar components.

The *N*-acetylated and *N*-resulfated derivatives of the desulfated heparin, the starting material, and desulfated, *N*-acetylated heparin obtained by the procedure of Ayotte and Perlin[4] were chromatographed on Sephadex G-100. The gel-filtration patterns indicated that the solvolytic conditions did not fragment the heparin chain to any great extent (Fig. 1). The ^{1}H-n.m.r. spectra of the desulfated heparin and its *N*-acetylated or *N*-resulfated derivative did not show any signal due to methoxyl groups at δ ~3.5, indicating no deglycosidation due to methanolysis (data not shown). The desulfated, *N*-acetylated heparin obtained by the present method showed a tendency to partial molecular association during gel-filtration, in contrast to its *N*-resulfated derivative, which suggested some conformational change of the heparin chain induced by the lack of bound sulfate groups.

The hydroxyl groups of desulfated, *N*-resulfated heparin were sulfated by the method employed for the chemical sulfation of chondroitin 4- and 6-sulfates, and dermatan sulfate[5]. The total S content of the desulfated, *N*-resulfated heparin agree

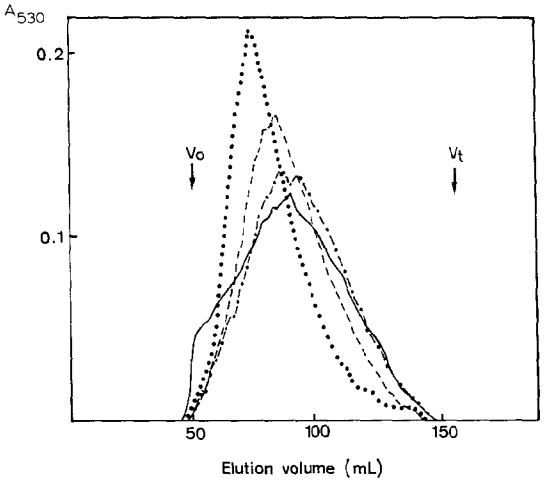

Fig. 1. Elution diagrams of *N*-acetylated or *N*-sulfated products of desulfated heparins by gel-filtration on Sephadex G-100. Gel-filtration of the samples was performed by the procedure given in the Experimental section. Starting heparin (· · · ·); *N*-acetylated product of the heparin desulfated by the method of Ayotte and Perlin (----); and *N*-acetylated product (——) and *N*-sulfated product (-·-·-·-) of the heparin desulfated by the present method.

TABLE II

ANALYTICAL DATA OF *O*-SULFATED PRODUCTS OF DESULFATED, *N*-RESULFATED HEPARIN[a]

Preparation	*Temp. (degree)*	*Reagent*[c]	*Total S*	*(A) GlcNS/(GlcNS+GlcNAc)*	*(B) GlcN6S/(GlcNS+GlcNAc)*	*(C) IdoA2S/(IdoA+IdoA2S+GlcA)*	*A+B+C*	*Δ*[b]
Desulfated, *N*-resulfated heparin			0.95	0.875	0	0.058	0.93	0.02
O-Sulfated product								
	−10	5	1.30	0.875	0.265	0.095	1.24	0.06
		10	1.46	0.885	0.400	0.142	1.43	0.03
		20	2.09	0.895	0.917	0.208	2.02	0.07
	0	5	2.46	0.895	1.00	0.418	2.31	0.15
		10	2.68	0.895	1.00	0.521	2.42	0.26
	20	10	3.40	0.895	1.00	0.705	2.60	0.80

[a]In mol. [b]Total S minus A + B + C. [c]Mol of pyridine–sulfur trioxide per mol equiv. of available hydroxyl group.

with that calculated from the degrees of *N*-sulfation of aminodeoxyhexose units and 2-*O*-sulfation of L-iduronic acid units (IdoA2*S*), both of which could be estimated by n.m.r. spectroscopy. This result indicated the reliability of the method used for determining sulfate substitution in the heparin chain (Table II). The data of progressive *O*-sulfation indicated that the HO-6 groups of aminodeoxyhexose units are highly reactive for sulfation, as expected from our previous report[5], and the HO-2 group of L-iduronic acid units also is moderately reactive. The data also indicated that sulfation at −10° took place almost exclusively on the HO-6 group of aminodeoxyhexose units and on the HO-2 group of L-iduronic acid units according to the order of reactivity of these hydroxyl groups. The discrepancy between the total chemically determined and the n.m.r.-spectroscopically determined S contents, which is due to sulfation at the hydroxyl groups, except the HO-6 in aminodeoxyhexose units and HO-2 in L-iduronic acid units, increased rapidly as the reaction temperature rose. Sulfation of HO-3 of 2-deoxy-2-sulfoamino-D-glucose 6-sulfate (GlcN*S*6*S*) units may be determined by ^{1}H-n.m.r. spectroscopy[19], provided that the 3-sulfated residue is located at the proper position in the antithrombin-binding oligosaccharide sequence with a natural sulfation pattern. The lower-sulfated products obtained by the sulfation at −10° scarcely contained other sulfate group substitution (including 3-sulfate esters of GlcN), in GlcN*S*, GlcN6*S*, and IdoA2*S* units, as judged from their Δ values (Table II). In contrast, the higher-sulfated products obtained at 0° and 20°, clearly contained sulfate group substitution, possibly 3-sulfate esters of GlcN, other than GlcN*S*, GlcN6*S*, and IdoA2*S* units, as indicated by their Δ values. Although we tried some n.m.r. spectroscopic studies of these higher-sulfated products, no confirmative information about 3-sulfation of GlcN units was obtained.

We have reported[1] that the sulfation of whale heparin having a high affinity for antithrombin (anticoagulant activity, 154 USP units/mg) in *N*,*N*-dimethylformamide with 5 mol pyridine–sulfur trioxide at −10° resulted in the prominent enhancement of anticoagulant activity (217 USP units/mg). The data of Table II also imply that this phenomenon is due to preferential 6-sulfation in the aminodeoxyhexose units of whale heparin.

EXPERIMENTAL

Materials. — Hog mucosal heparin was purchased from V.G.F. Corporation (New York, NY 10170), and purified by the method of Rodén *et al.*[6] to remove contaminating dermatan sulfate. Pyridine–sulfur trioxide was prepared as described[7], and stored in a desiccator. Trimethylamine–SO_3 was obtained from Aldrich Chem. Co. Inc. (Milwaukee, WI 53201). Dimethyl sulfoxide and *N*,*N*-dimethylformamide, which were reagent grade, were dried over CaH_2 and molecular sieves (Type 4A), respectively, and redistilled under reduced pressure. Pyridinium chloride (1:1 salt, m.p. 87°) was prepared by freeze-drying of an aqueous solution containing equimolar amounts of pyridine and hydrogen chloride.

Analytical methods. — ^{1}H-N.m.r. spectra were recorded with a Varian XL-400 spectrometer on samples, pre-exchanged with D_2O, at a concentration of ~10 mg/mL of D_2O, at 80°. Chemical shifts are referenced to the signal of internal 1,4-dioxane (δ 3.7) in D_2O at 80°. ^{13}C-N.m.r. spectra were recorded with a Varian XL-400 spectrometer, at a sample concentration of ~60 mg/mL of D_2O, at 80°. Chemical shifts are referenced to the signal of internal 1,4-dioxane (δ 67.4) in D_2O at 80°.

The S content of the starting heparin and resulfated products of desulfated heparins was determined by a modification of the turbidimetric procedure[8], after hydrolysis with M HCl for 5 h at 110°. The S content of the desulfated heparin was determined by the l.c. method as described[9]. The uronic acid content was determined by the method of Bitter and Muir[10], and by a modification of this procedure[11] that differs by increasing the BO_3^{3-} concentration to 0.2M. The hexosamine content was determined by a modification of the Blix–Gardell procedure[12], after hydrolysis with 3M HCl for 15 h at 100°.

Electrophoresis was performed on cellulose acetate strips (Separax) in 0.1M LiCl–10mM HCl (pH 2.0) as described by Breen *et al.*[13]. The desulfated heparin samples were *N*-acetylated[14] prior to the electrophoresis, and run at 1 mA/cm for 15 min. The strips were stained with 0.5% Alcian Blue in 3% acetic acid. Sodium hyaluronate was used as reference compound. Analytical gel-filtration on Sephadex G-100, of the *N*-acetylated or *N*-sulfated products of desulfated heparin, was carried out according to the following procedure. The samples (1.5 mg), dissolved in 0.2M NaCl (1 mL), were loaded on a column (1.6 × 88 cm) of Sephadex G-100 and eluted with the same solvent at a flow rate of 12 mL/h at room temperature. Fractions (2.0 mL) were collected, and each of these was analyzed for uronic acid[10].

Analysis of sulfate substitution in the heparin chain. — The residual S content in desulfated heparin preparations was determined by the l.c. method[9], and the total S content of the starting heparin and resulfated products of the desulfated heparin was determined by the conventional chemical method[8], as described above. Both the degree and the position of sulfate substitution in the heparin chain of all the samples were determined, particularly the *N*-sulfation and 6-sulfation of aminodeoxyhexose units, and the 2-sulfation of L-iduronic acid units. As described by Casu *et al.*[16], and Gatti *et al.*[17], the degree of *N*-sulfation of GlcN units was estimated from the signal intensities of GlcN*S* (C-2) at δ 59.0 and GlcNAc (C-2) at δ 55.0, as well as that of 6-sulfation of GlcN units from the signal intensities of GlcN6*S* (C-6) at δ 67.5 and GlcN (C-6) at δ 61.0. The degree of 2-sulfation of IdoA units was estimated by a comparison of the signal intensities of IdoA2*S* (H-1) at δ 5.2 and IdoA (H-5) at δ 4.7, as established by Ayotte and Perlin[4], and by Mulloy and Johnson[18].

Desulfation of heparin. — The sodium salt of heparin (1 g) was passed through a column of Dowex 50W-X4 (H^+, 50–100 mesh) cation-exchange resin at 4°, and the effluent was neutralized with pyridine and lyophilized to give a white powder (~980 mg). To this pyridinium salt (900 mg), moistened with methanol (10

mL), were added dimethyl sulfoxide (60 mL) and 1,4-dioxane (30 mL), successively, and the resultant solution was heated at 90° with stirring. To the mixture was added a solution of pyridinium chloride (823 mg, 5 mol/mol of disaccharide unit) in 3:6:1 (v/v) 1,4-dioxane–dimethyl sulfoxide–methanol (4 mL), 24 h after the reaction started, and the mixture was kept at 90° for an additional 48 h. The reaction was terminated by the addition of cold water (300 mL) and the pH of the solution was adjusted to 9.5 with M NaOH. The solution was dialyzed against distilled water (3 × 20 L) for 24 h and the dialyzate (pH 6.5) lyophilized to give the desulfated product (406 mg) as a pale-yellow powder.

N-*Resulfation of desulfated heparin.* — The *N*-resulfation was carried out by the procedure of Lloyd *et al.*[15]. To a solution of desulfated heparin (400 mg) in water (64 mL) were added Na_2CO_3 (400 mg) and trimethylamine–sulfur trioxide (400 mg), successively, and the mixture was stirred for 24 h at 55°. The mixture was diluted with cold water (50 mL) and the solution (pH 9.5) was dialyzed against water (3 × 20 L) for 24 h. The dialyzate was lyophilized to give the sodium salt of the desulfated, *N*-resulfated heparin (~430 mg) as a white powder.

Sulfation of desulfated, N-*resulfated heparin.* — A solution of the sodium salt of desulfated, *N*-resulfated heparin (400 mg) in water (15 mL) was passed through a column (1.5 × 20 cm) of Dowex 50W-X4 (H^+, 50–100 mesh) cation-exchange resin at 0°, and the pH of the effluent was adjusted to 5.5 by the addition of 10% tributylamine in ethanol. The solution was extracted three times with diethyl ether (each 80 mL) and lyophilized to give the tributylammonium salt (~490 mg) as a white powder. To a solution of this salt (100 mg) in *N,N*-dimethylformamide (6 mL), kept at −12°*, was added a solution of pyridine–sulfur trioxide (5–20 mol/mol equiv. of available hydroxyl group) in *N,N*-dimethylformamide (10 mL) kept at −12°*, and the mixture was stirred for 1 h at −10°. After the temperature of the cooling bath had been lowered to −20°, the reaction was terminated by the addition of cold water (16 mL) and the pH of the solution was adjusted to 10.0 with 0.1M NaOH. The solution was diluted with ethanol (3 vol.) saturated with anhydrous sodium acetate, and kept for 2 h at 0° to give a white precipitate. The precipitate was collected by centrifugation at 1500*g* for 15 min and dissolved in water (20 mL). The solution was dialyzed against distilled water (3 × 20 L) for 24 h and the dialyzate (pH 6.5) lyophilized. The residue was dissolved in water (3 mL) and the solution was desalted on a column (2.5 × 92 cm) of Sephadex G-15 in 10% ethanol. The effluent, freed from inorganic salts, was collected and lyophilized to give the sodium salt of the sulfated product (65–78 mg) as a white powder.

*In the case of the reaction at 0°, each of the *N,N*-dimethylformamide solutions were cooled to −2° before the reaction started.

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