Carbohydrate Research, 58 (1977) 47–55

SOLVOLYTIC DESULFATION OF GLYCOSAMINOGLYCURONAN SULFATES WITH DIMETHYL SULFOXIDE CONTAINING WATER OR METHANOL

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(Received October 5th, 1976; accepted for publication, December 13th, 1976)

ABSTRACT

A solvolytic desulfation of glycosaminoglycuronan sulfates was developed by treatment of their pyridinium salts with dimethyl sulfoxide containing 10% of water or methanol at 80–100°. Chemical and physical studies showed that the solvolytic desulfation is a useful method applicable to all the known glycosaminoglycuronan sulfates without producing depolymerization or unfavorable chemical changes in the polysaccharide molecules. An almost completely desulfated, *N*-acetylated heparin (S: 0.12%) was obtained by treatment of an *N*-desulfated and *N*-acetylated heparin with dimethyl sulfoxide containing 10% of methanol for 2 h at 100°.

INTRODUCTION

For the effective hydrolysis of bound sulfate ester groups from glycosaminoglycuronan sulfates, a method that does not affect the glycosidic linkages or other chemical structures would be important for the study of the chemical structure of polysaccharides and for the elucidation of their biosynthesis, especially for that of heparin.

The methods of desulfation used until now are mainly based on methanolysis according to the procedure of Kantor and Schubert[1]. Wolfrom *et al.*[2] have reported that a repeated desulfation with 0.15M hydrogen chloride in methanol of heparin (S: 5.01%) that had been *N*-acetylated after a preliminary desulfation with 0.15M hydrogen chloride in methanol for 3 days at room temperature gave a desulfated, *N*-acetylated heparin methyl ester having only 0.2% of sulfur. When the method is applied to glycosaminoglycuronan sulfates, including heparin, methanolysis of the glycosidic linkages may take place, as pointed out by Usov *et al.*[3], but no detailed examination of the depolymerization has been reported, as far as we are aware. In 1972, Usov *et al.*[4] reported that the sulfated galactans of red seaweeds were sucess-

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fully desulfated by heating the pyridinium salts in 2% pyridine–dimethyl sulfoxide. In a previous paper[5], we reported that the *N*-sulfate groups of heparin were selectively removed by treating the pyridinium salt with dimethyl sulfoxide containing 5% of water or methanol for 90 min at 50°. The present study indicates that desulfation of all the known glycosaminoglycuronan sulfates can be accomplished successfully by heating the pyridinium salts in dimethyl sulfoxide containing 10% of water or methanol at 80–100°, without depolymerization, chemical changes in the sugar components, or *N*-deacetylation.

EXPERIMENTAL

Materials. — Hog-mucosa heparin was purchased from Cohelfred Laboratories, Chicago, Ill., and had an anticoagulant activity of 165 U.S.P. units. Chondroitin 4- and 6-sulfates, and dermatan sulfate were obtained from Seikagaku Kogyo Co., Tokyo. A keratan polysulfate fraction obtained from shark cartilage was kindly provided by Dr. T. Furuhashi of Seikagaku Kogyo Co; ~ 18.7% of the total hexosamine in the keratan polysulfate was 2-amino-2-deoxy-D-galactose, as determined with an amino acid analyzer. Whale heparin was purchased from Tokyo Kasei Co., Tokyo, and had an anti-coagulant activity of 147.3 U.S.P. units. Heparan sulfate, supplied by Upjohn Co., Kalamazoo, Mich., was purified by fractionation on a column of Dowex-1 (Cl^-) resin[6]. The fraction eluted with 1.25M sodium chloride was used in this study.

Analytical methods. — The methods for the quantitative determination of the sulfate, uronic acid, and hexosamine content have been reported previously[5]. Electrophoresis was performed on cellulose acetate strips (Separax) in 0.1M ammonium acetate buffer, pH 9.2. Samples were run at 1 mA/cm for 17 min, and the strips were stained with a 0.5% solution of Alcian Blue. The *N*-acetyl content of heparin and desulfated heparins was estimated by the n.m.r. method, as reported previously[5]. The *N*-acetyl contents of chondroitin 6-sulfate, its desulfated product, and desulfated, *N*-acetylated heparins were measured by *g l c*, after hydrolysis of the sample solutions (7.8%) in 2M hydrochloric acid for 3 h at 100°, on a glass column (0.3 × 100 cm) packed with Chromosorb 101, the column and detector temperatures being 140° and 210°, respectively, and nitrogen the carrier gas (40 ml/min); 3 µl of the hydrolyzate were injected.

Determination of molecular weight. — The weight-average molecular weight ($M\bar{w}$) was determined by the light-scattering method with a Shimadzu Light-scattering Photometer. Measurements were made on five concentrations of heparin or on one concentration of each desulfated heparin in 0.1M sodium chloride, at ten angles (30–135°), with a wavelength of 436.1 nm. The experimental data obtained were corrected for both the depolarization degree and the selective adsorption.

Gel-filtration of heparin and desulfated heparins on Sephadex G-75. — The polysaccharides (1.6 mg), dissolved in 0.1M sodium chloride (3 ml), were applied to a column (1.5 × 90 cm) of Sephadex G-75 and eluted with the same solvent at a flow

rate of 20 ml/h. The eluate was collected in 3-ml fractions, and each fraction was analyzed for uronic acid content.

Desulfation of heparin with dimethyl sulfoxide containing water or methanol. — The pyridinium salt of heparin prepared by the method previously reported[5] was desulfated with dimethyl sulfoxide containing water or methanol by a method virtually identical with that described for the *N*-desulfation of heparin[6]. Aliquots of the reaction mixture were withdrawn at regular time-intervals, and the sulfur content or electrophoretic mobility of the desulfated heparins isolated was determined.

Preparation of partially desulfated heparins. — The pyridinium salt of heparin (1 g) was dissolved in dimethyl sulfoxide containing 10% of water, and the solution was kept for 7 h at 100°. The reaction mixture was cooled, diluted with an equal volume of water, and the pH adjusted to 9.0 by the addition of 0.1M sodium hydroxide. The solution was dialyzed against running tap water for 24 h, and then against distilled water for 20 h. Lyophilization of the dialyzate gave 0.58 g of partially desulfated heparin (named "partially desulfated heparin-H_2O").

The same treatment of the pyridinium heparin (1 g) with dimethyl sulfoxide containing 10% of methanol gave 0.54 g of partially desulfated heparin (named "partially desulfated heparin-CH_3OH").

Preparation of completely desulfated, N-acetylated heparins. — *N*-Acetylation of partially desulfated heparin-H_2O was performed by the method of Danishefsky *et al.*[7]. The sodium salt of *N*-acetylated, partially desulfated heparin-H_2O was converted into the pyridinium salt as previously reported. The salt (100 mg) was desulfated with dimethyl sulfoxide containing 10% of methanol for 2 h at 100°. The reaction mixture was treated by the method just described to give 84.4 mg of completely desulfated, *N*-acetylated heparin (named "completely desulfated, *N*-acetylated heparin-I") as a white powder.

N-Acetylation of the *N*-desulfated heparin obtained by treating heparin with dimethyl sulfoxide containing 5% of water at 50° for 90 min[5] gave the sodium salt of *N*-desulfated, *N*-acetylated heparin in an almost quantitative yield. The pyridinium salt (100 mg) was desulfated by the same procedure as just described to give 63.3 mg of completely desulfated, *N*-acetylated heparin (named "completely desulfated, *N*-acetylated heparin-II").

Desulfation of glycosaminoglycuronan sulfates with dimethyl sulfoxide containing 10% of water. — The glycosaminoglycuronan sulfates shown in Table IV were converted into their pyridinium salts by a method identical with that described for heparin. Each was desulfated with dimethyl sulfoxide containing 10% of water for 5 h at 80°, and the products isolated were analyzed for sulfur and *N*-acetyl contents.

Desulfation of chondroitin 6-sulfate or heparin by other methods. — The sodium salts of chondroitin 6-sulfate and heparin were desulfated by the general procedure of Kantor and Schubert[1], which had been used for the complete desulfation of chondroitin sulfate[1] and heparin[2], respectively. The pyridinium salt of heparin was desulfated by the method of Usov *et al.*[3,4].

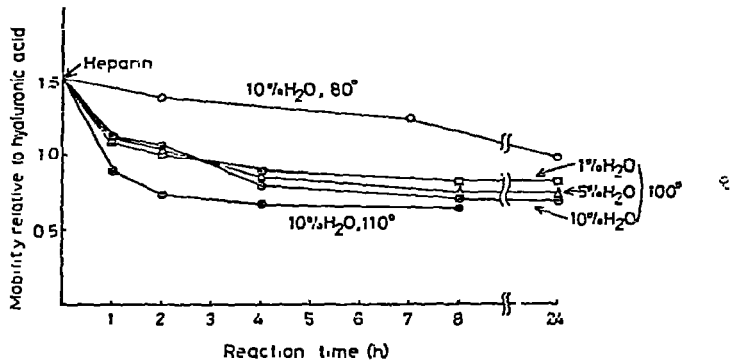

Fig. 1. Relative electrophoretic mobility of heparins desulfated with dimethyl sulfoxide containing water under various conditions.

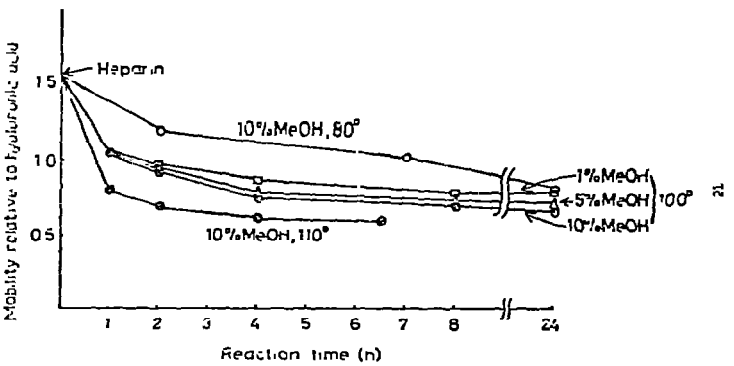

Fig. 2. Relative electrophoretic mobility of heparins desulfated with dimethyl sulfoxide containing methanol under various conditions.

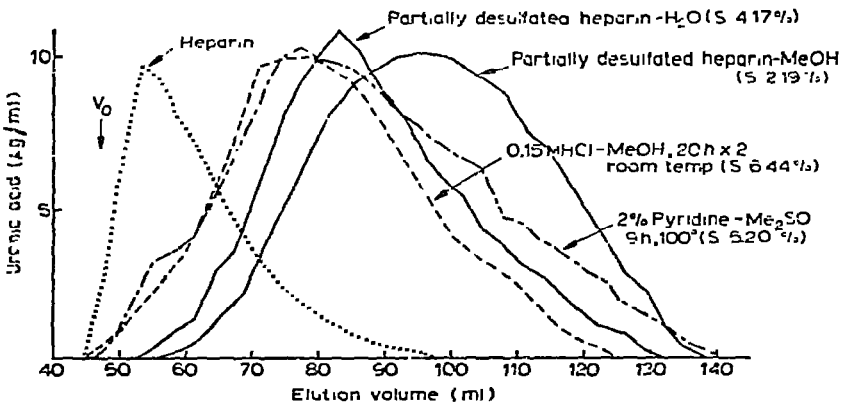

Fig. 3. Gel-filtration of heparin and desulfated heparins obtained by different methods on Sephadex G-75 in 0.1M NaCl solution.

RESULTS AND DISCUSSION

The pyridinium salt of heparin was treated, in dimethyl sulfoxide solutions, with 1, 5, and 10% of water or methanol at various temperatures, and the time courses of desulfation are shown in Figs. 1 and 2. Progress in the desulfation was followed by the electrophoretic mobility of the desulfated products relative to that of hyaluronic acid, at pH 9.2, on cellulose acetate film. The rate of desulfation at 100° increased slightly with the increasing concentration of water or methanol from 1% to

TABLE I

TIME COURSE OF THE SOLVOLYTIC DESULFATION OF HEPARIN WITH DIMETHYL SULFOXIDE CONTAINING 10% OF WATER OR METHANOL

Reaction condition		*S (%) of desulfated product*[a]	
Temp. (°)	*Time (h)*	*10% H_2O–Me_2SO*	*10% $MeOH$–Me_2SO*
	0	12.11 (2.21)	12.11 (2.21)
100	0.65		5.21 (0.71)
100	1	5.56 (0.76)	4.78 (0.64)
100	2.25	4.81 (0.64)	3.59 (0.46)
100	4.5	4.16 (0.54)	2.42 (0.30)
100	7	3.60 (0.46)	1.99 (0.24)
100	14	2.46 (0.30)	1.18 (0.14)
100	24	1.86 (0.22)	0.81 (0.09)
80	48		2.44 (0.30)
110	2		2.55 (0.32)

[a]Total sulfate content as sulfur. Molar numbers of total sulfate are given in parentheses. Molar numbers of total sulfate in heparin, partially desulfated heparins, and completely desulfated *N*-acetylated heparins in Tables I–IV were calculated according to the equation $(S \times x)100/[C_{12}H_{17-x-y}O_{10}NH_{1-y}(COCH_3)_yNa + (SO_3Na)_x] =$ Total sulfate (S%), where x is the molar number of total sulfate per mol of hexosamine and y is the molar number of *N*-acetyl group per mol of hexosamine.

TABLE II

TIME COURSE OF SOLVOLYTIC DESULFATION OF *N*-ACETYLATED PRODUCT OF PARTIALLY DESULFATED OR *N*-DESULFATED HEPARIN WITH DIMETHYL SULFOXIDE CONTAINING 10% OF METHANOL AT 100°

Reaction time (h)	N-*Acetylated product of partially desulfated heparin* *S (%)*[a]	N-*Acetylated product of* N-*desulfated heparin* *S (%)*[a]
0	3.83 (0.55)	8.07 (1.36)
0.5	0.44 (0.056)	0.59 (0.075)
2	0.13 (0.016)	0.17 (0.021)
5	0.11 (0.014)	0.19 (0.024)
8	0.14 (0.018)	0.15 (0.019)

[a]See footnote to Table I.

TABLE III

CONDITIONS OF PREPARATION AND CHEMICAL COMPOSITION OF HEPARIN, PARTIALLY DESULFATED HEPARINS, AND COMPLETELY DESULFATED HEPARINS

Starting material	*S* (%)[a]	*Condition of desulfation*			*Desulfated product*
		Solvent	*Temp.* ( )	*Time* (*h*)	
Heparin	12.11 (2.21)				
Heparin	12.11 (2.21)	10% H_2O–Me_2SO	100	7	Partially desulfated heparin–H_2O
Heparin	12.11 (2.21)	10% MeOH–Me_2SO	100	7	Partially desulfated heparin–CH_3OH
N-Acetylated product of partially desulfated heparin–H_2O[f]	3.83 (0.55)	10% MeOH–Me_2SO	100	2	Completely desulfated *N*-acetylated heparin-I
N-Acetylated product of *N*-desulfated heparin[g]	8.07 (1.36)	10% MeOH–Me_2SO	100	2	Completely desulfated *N*-acetylated heparin-II

[a]See footnote to Table I. [b]Determined by the carbazole–borate method. [c]Determined by the carbazole method. [d]Calc. from the mol. wt. of starting material and the degree of desulfation of the partially desulfated heparins. [e]Determined by the g.l.c. method. [f]Prepared by *N*-acetylation of the heparin partially desulfated with 10% H_2O–Me_2SO for 7 h at 100°. [g]Prepared by *N*-acetylation of the heparin *N*-desulfated with 5% H_2O–Me_2SO for 90 min at 50°.

5%, and 10%. The rate of desulfation in dimethyl sulfoxide containing 20% of water or methanol at the same temperature apparently slowed down, although data are not shown here. Examination of the desulfation in dimethyl sulfoxide containing water or methanol showed an acceleration with the rise in temperature. The reactions at 110° were fairly rapid, but the coloration of the reaction mixtures increased. Comparison of the results in Fig. 1 and Fig. 2 shows that the rate of desulfation in dimethyl sulfoxide containing water is lower than that in the same solvent containing methanol at any concentration.

The products of the desulfation of the pyridinium salt of heparin with dimethyl sulfoxide containing 10% of water or methanol at 100° were isolated at intervals, and their sulfur content determined (Table I). As shown in Figs. 1 and 2, a rapid desulfation takes place at first, which is mainly due to the *N*-desulfation of heparin occurring within 1 h after the start of the reaction[5], and then gradual desulfation follows.

An *N*-desulfated, *N*-acetylated heparin was prepared by *N*-acetylation of *N*-desulfated heparin (S 8.87%), obtained by treating[5] heparin with dimethyl sulfoxide containing 5% of water for 90 min at 50°. A partially desulfated, *N*-acetylated heparin was also prepared by *N*-acetylation of a partially desulfated heparin (S 4.17%, partially desulfated heparin–H_2O, see Table III), which was obtained by treating heparin with dimethyl sulfoxide containing 10% of water for 7 h at 100°. The

S (%)[a]	Uronic acid (%)			Hexosamine (%)	N-Acetyl (mole)	Mw (× 10^{-4})		Yield (%)
	(A)[b]	(B)[c]	(A)/(B)			Found	Calc.[d]	
12.11 (2.21)	38.86	48.28	0.80	24.58	0.12 (0.102)[e]	1.43		
4.17 (0.54)	49.81	71.49	0.70	37.79	0.13	1.01	1.01	96.4
2.19 (0.26)	60.80	82.29	0.74	38.55	0.12	0.98	0.94	96.1
0.12 (0.015)	46.61	57.66	0.81	39.63	1.03[e]			97.7
0.18 (0.023)	48.44	58.50	0.83	40.76	0.99[e]			93.4

pyridinium salts of the two preparations were treated with dimethyl sulfoxide containing 10% of methanol at 100° to remove the residual, bound sulfate groups. As shown in Table II, desulfation of the two compounds progressed rapidly, and the proportion of the residual sulfate groups became approximately constant 2 h after the start of the reaction*. The results reported in Tables I and II suggest that the free amino groups in heparin, liberated by prior *N*-desulfation, combine with the ester sulfate groups to stabilize the polysaccharide against further desulfation.

For a comparison of the present method with previously described methods, the sodium salt of heparin was treated by the method of Kantor and Schubert[1,2], and by the method of Usov *et al.*[3,4]. The sulfur content of a desulfated heparin obtained by the former method (0.15% hydrogen chloride in methanol, room temperature, 20 h, two treatments) was 6.44%, and that of a desulfated heparin obtained by the latter method (2% pyridine in dimethyl sulfoxide, 9 h, 100°) was 5.20%.

The conditions of preparation, chemical composition, and physical properties of partially desulfated heparin-H_2O and -CH_3OH, and completely desulfated *N*-acetylated heparin-I and -II are summarized in Table III. The yields of products

*The *N*-acetylated products of the partially desulfated heparin-H_2O and the *N*-desulfated heparin were treated with dimethyl sulfoxide containing 10% of methanol for 2 h at 100°, and the final products obtained are named "completely desulfated *N*-acetylated heparin-I and -II", respectively (Table III).

were high, and the quantitative data for uronic acid, hexosamine, and *N*-acetyl groups indicate that the treatment of heparin under these conditions had no effect on the sugar components and substituents. The molecular weights of partially desulfated heparin-H_2O and -CH_3OH respectively agree with those calculated from the degree of desulfation and the molecular weight of the starting material. Therefore, the solvolytic conditions used did not fragment the heparin chain to any great extent.

Gel chromatography on Sephadex G-75 of partially desulfated heparin-H_2O and -CH_3OH was performed together with the starting material and the desulfated heparins obtained by other methods (Fig. 2). All the elution curves of the desulfated preparations were broader than that of the starting material, and shifted markedly to the retarded areas according to the increasing degree of the desulfation. The values of the molecular weights (Table III) indicate that the polysaccharide chains of both partially desulfated heparin-H_2O and -CH_3OH had not been degraded. Therefore, the broadened elution peaks of the desulfated compounds are probably due to either a natural polydispersity in chain-length of the heparin molecule, revealed by the loss of most of the bound sulfate groups, or to inter- or intra-molecular heterogeneity in the distribution of residual, bound sulfate groups.

The solvolytic conditions established with heparin were applied to the desulfation of other glycosaminoglycuronan sulfates. These compounds were more easily desulfated than heparin with dimethyl sulfoxide containing 10% of water at 80° (see Table IV). The glycosaminoglycuronan sulfates having a 2-acetamido-2-deoxy-D-hexose component were generally more easily desulfated than various heparin preparations or heparan sulfate, in agreement with the results obtained on desulfation

TABLE IV

SOLVOLYTIC DESULFATION OF GLYCOSAMINOGLYCURONAN SULFATES WITH DIMETHYL SULFOXIDE CONTAINING 10% OF WATER FOR 5 H AT 80°

Starting material		*Desulfated product*		
	S (%)[a]	*S* (%)[a]	N-*Acetyl* (*mole*)	*Yield* (%)
Chondroitin 4-sulfate	6.51 (1.03)	0.88 (0.11)		89.5
Chondroitin 6-sulfate	6.65 (1.06)	0.45 (0.06)	0.98	94.4
Dermatan sulfate	6.67 (1.06)	1.75 (0.23)		83.9
Heparin	12.11 (2.21)	6.53 (0.92)		93.0
N-Acetylated product of partially desulfated heparin-H_2O[b]	3.83 (0.55)	1.67 (0.22)		98.0
N-Acetylated product of *N*-desulfated heparin[c]	8.07 (1.36)	1.76 (0.23)		97.0
Whale heparin	8.41	4.23		50 mg→33 mg
Heparan sulfate	5.33	2.78		50 mg→36 mg
Keratan polysulfate	7.46	1.16		50 mg→25 mg
Chondroitin 6-sulfate[c]	6.65 (1.06)	1.12 (0.14)	0.99	90.6

[a]See footnote to Table I. [b]See footnotes to Table III. [c]Desulfated by the method of Kantor and Schubert[1] (0.15M HCl in methanol, 20 h, at room temperature, two treatments).

of the partially desulfated heparins with or without prior *N*-acetylation (Tables I and II).

Chondroitin 6-sulfate seems to be more easily desulfated than its 4-sulfate isomer. Both the yield and degree of desulfation of desulfated chondroitin 6-sulfate obtained by the solvolysis procedure were slightly better than those obtained by the method of Kantor and Schubert[1]. The content of *N*-acetyl groups in the two desulfated chondroitin 6-sulfates, as determined by g.l.c., indicates that no cleavage of the *N*-acetyl group in either product occurred during these reactions.

Dermatan sulfate, partially desulfated, *N*-acetylated heparin, and *N*-desulfated *N*-acetylated heparin, all of which have L-iduronic acid as the major uronic acid component, seem to be rather resistant to solvolytic desulfation, when compared with chondroitin 6- and 4-sulfates which have only D-glucuronic acid as the uronic acid component.

The results obtained in the present study suggest that solvolytic desulfation with dimethyl sulfoxide containing 10% of water or methanol is more satisfactory for the liberation of bound sulfate groups in glycosaminoglycuronan sulfates than the previously described methods[1-4] as far as the simplicity, rapidity, and homogeneity of the reaction is concerned.

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