Note

Sulfation of dextran with piperidine-*N*-sulfonic acid*

KINZO NAGASAWA, HARUE HARADA, SUEKO HAYASHI, AND TOSHIKO MISAWA
School of Pharmaceutical Sciences, Kitasato University, Shirokane 5-9-1, Minato-ku, Tokyo 108 (Japan)
(Received July 19th, 1971; accepted for publication October 18th, 1971)

Since the discovery of heparin and its biological activities[1–3], a number of polysaccharide sulfates have been synthesized and their biological activities examined, among them, most extensively, dextran sulfate[4,5].

A variety of sulfating reagents and reaction media have been used: Sulfuric acid causes extensive degradation, even when employed under controlled conditions[6–8], as do chlorosulfonic acid and sulfur trioxide when applied alone[9,10]. When combined with Lewis bases, these two reagents cause less degradation, and they have been widely used[9,11]. Recently, several types of sulfur trioxide complexes have been synthesized and used for the preparation of polysaccharide sulfates[12–14]. Both hydrolytic and alcoholytic cleavages of sulfamic acid and its *N*-substituted derivatives are catalyzed by polar solvents, and the alcoholytic reaction was applied to the preparation of some biologically-related sulfate esters[15,16]. The present report describes the non-degrading sulfation of dextran with piperidine-*N*-sulfonic acid, in a homogeneous solution of dimethyl sulfoxide.

RESULTS AND DISCUSSION

Sulfation in a homogeneous solution is essential for obtaining a synthetically sulfated polysaccharide having a homogeneous distribution of the sulfate groups. A preliminary experiment showed that dimethyl sulfoxide, which is one of the effective catalysts for the alcoholytic sulfation with sulfamic acid compounds[16], can dissolve, at a temperature above 40°, dextrans having a wide range of molecular weight. The reactivity and solubility properties of piperidine-*N*-sulfonic acid were found to be better than those of sulfamic acid and other sulfamic acid derivatives.

It was found that maximum sulfation had taken place at 80° and no improvement was observed at higher temperatures (Table I). At 80°, both the highest sulfur content and highest yield were observed after a 1-h reaction (Table II). Sulfation of dextrans ($\overline{M}_w$ 39,500 and 3,400) at 80° for 1 h with various concentrations of pip-

*This work has been presented in part at the 24th Annual Meeting of the Pharmaceutical Society of Japan, Kyoto, April 7–10, 1967.

eridine-*N*-sulfonic acid gave nearly quantitative yields, and the sulfur content increased with the concentration of the reagent (Table III).

The observed molecular weights of two products having different sulfate contents were close to those calculated from the degree of polymerization of the starting dextran ($\bar{M}_w$ 39,500, D.P. 244) and from the sulfate content (Expts. 2 and 4,

TABLE I

EFFECT OF TEMPERATURE ON SULFATION OF DEXTRAN[a]

Reaction temperature (degrees)	*Sulfur content (%)*	*Yield (g)*
40	0.0	0.98[b]
60	1.11	1.00
70	8.22	1.50
80	15.81	2.16
90	13.77	2.06
100	13.25	1.83

[a]Dextran (1 g, $\bar{M}_w$ 39,500) was treated with piperidine-*N*-sulfonic acid (6.12 g) for 1 h. [b]Nonsulfated dextran was recovered.

TABLE II

EFFECT OF REACTION TIME ON SULFATION OF DEXTRAN[a]

Reaction time (min)	*Sulfur content (%)*	*Yield (g)*
15	8.38	1.08
30	13.63	1.60
60	15.81	2.16
90	15.20	1.72
120	14.54	1.78
180	14.28	1.80

[a]Dextran (1 g, $\bar{M}_w$ 39,500) was treated with piperidine-*N*-sulfonic acid (6.12 g) at 80°.

Table III). In view of the sulfur content of each product (Tables I and II), it is possible that the variations of the yield of reaction products are not caused by the degradation of the polysaccharide. These results suggest that no appreciable depolymerization of the polysaccharide occurred during the sulfation at 80° and that no substantial difference in the degree of sulfation exists between high-molecular and low-molecular dextrans.

The intermolecular uniformity of sulfate group distribution was examined by fractional precipitation of a 1% aqueous solution of dextran sulfate (sulfated product of high-molecular dextran; D.S. 1.31, [η] 0.237) with ethanol, each fraction being analyzed for its amount and sulfur content (Fig. 1): 91.5% of the dextran sulfate was

TABLE III

EFFECT OF PIPERIDINE-*N*-SULFONIC ACID CONCENTRATION ON PROPERTIES OF THE DEXTRAN SULFATE FORMED[a]

Sample (Exp. No.)	*Concentration of piperidine-N-sulfonic acid (moles of reagent: glucose unit)*	*Yield (g)*	*Sulfur (%)*	*Sulfate*[b]	$\bar{M}_w$		*Intrinsic viscosity* [η]
					Found	*Calc.*[c]	
High-molecular dextran ($\bar{M}_w$ 39,500)							
1	1:1	1.17	2.12	0.12			0.172
2	2:1	1.41	9.41	0.69	51,000	56,730	0.216
3	3:1	1.80	12.89	1.12			0.224
4	6:1	2.30	14.94	1.46	83,000	76,230	0.229
Low-molecular dextran ($\bar{M}_w$ 3,400)							
5	1:1	1.07	4.05	0.24			0.0444
6	3:1	1.66	11.22	0.89			0.0455
7	6:1	1.28	14.90	1.45			0.0430

[a]Dextrans (1 g, $\bar{M}_w$ 39,500 and 3,400) were treated with various concentrations of piperidine-*N*-sulfonic acid for 1 h at 80°. [b]Ratio of sulfate group to glucose unit. [c]The calc. $\bar{M}_w$ was based on both the D.P. of starting dextran and the sulfur content.

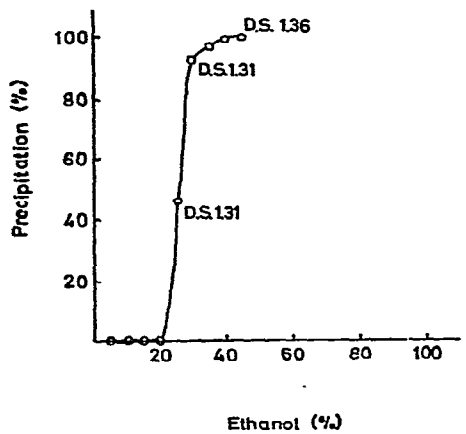

Fig. 1. Yield and degree of sulfation (D.S., ratio of sulfate group to glucose unit) of dextran sulfate fractionated with ethanol. A solution of dextran sulfate (2 g, sulfated product of high-molecular dextran: S, 14.43%, D.S. 1.31, [η] 0.237) in 0.9% sodium chloride (200 ml) was precipitated by gradual addition of absolute ethanol.

precipitated at an ethanol concentration of 30%, and the precipitated fractions had a rather uniform sulfate ester content.

The reactivity of sulfamic acid was similar to that of the *N*-substituted derivatives only at a lower reagent concentration, *i.e.* 1:1 or 1:3, but, at a higher reagent concentration, the reactivity of the former reagent was less than the reactivities of the latter reagents which were nearly equal to each other (Fig. 2). Of the two *N*-substi-

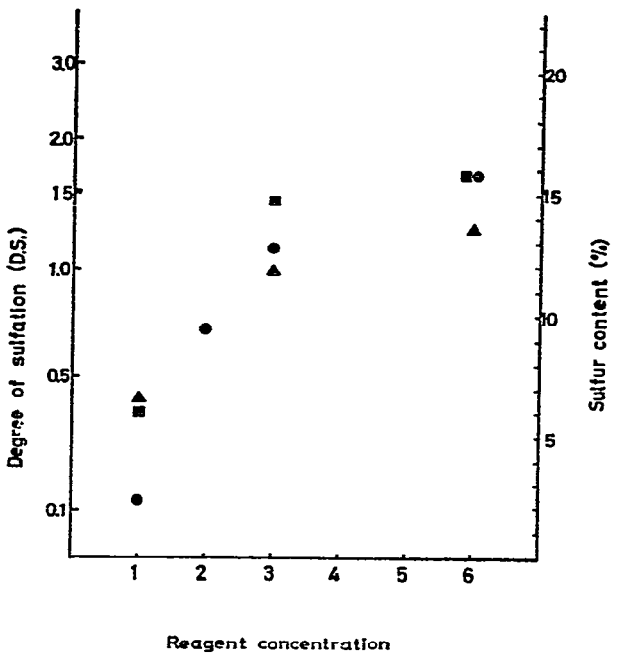

Fig. 2. Sulfur content and degree of sulfation of dextrans sulfated with various sulfamic acid derivatives in various molar concentrations, expressed in ratio of mole of reagent to glucose unit. The dextran (1 g, $\bar{M}_w$ 39,500) was treated with each reagent for 1 h at 80°: ●, Piperidine-*N*-sulfonic acid; ■, *N*-cyclohexylsulfamic acid; ▲, sulfamic acid.

tuted derivatives, piperidine-*N*-sulfonic acid was preferred because of its solubility in dimethyl sulfoxide and its limited side reactions.

The blood-anticoagulant activity of some of the dextran sulfates prepared was generally much lower than that of heparin (Table IV), although a highly sulfated

TABLE IV

ANTICOAGULANT AND LIPEMIA CLEARING ACTIVITIES OF DEXTRAN SULFATES

Sample (Exp. No.)	$\bar{M}_w$		*Sulfur (%)*	*Sulfate*[a]	*Anticoagulant activity (unit/mg)*	*Lipemia clearing activity (Difference in absorbancy at 640 nm)*
	Found	*Calcd.*				
2	51,000	56,730	9.41	0.69	0.04	0.095
3		67,430	12.89	1.12	2.96	0.140
4	83,000	76,230	14.94	1.46	13.12	0.260
5		3,920	4.05	0.24	[b]	[b]
6		5,320	11.22	0.89	0.18	[b]
7		6,550	14.90	1.45	3.82	0.285
Heparin					125.8	0.670
Dextran sulfate[c]	12,300		14.36	1.35	8.36	0.583

[a]Ratio of sulfate group to glucose unit. [b]No detectable activity observed. [c]This sample was prepared from dextran ($\bar{M}_w$ 39,500) by a concomitant depolymerization and sulfation in cold conc. sulfuric acid[8].

dextran having a high-molecular weight (Expt. 4), showed about one-tenth of the anticoagulant activity of heparin. The difference in molecular weight had no influence on the relationship between the clearing activity and the sulfur content of these dextran sulfates (Expts. 4 and 7), but a marked difference was observed when different methods of sulfation (Expts. 2–7) and a dextran sulfate prepared by another method[8] were used. This difference may be attributable to some factors other than molecular weight or degree of sulfate substitution, and it is probably related to the position of sulfate groups in the glucose unit.

EXPERIMENTAL

Materials. — Dextrans [Lot No. 8687, $\overline{M}_w$ 39,500 (by light-scattering determination), [η] 0.189 and Lot No. 1504, $\overline{M}_w$ 3,400, [η] 0.0469] from *Leuconostoc mesenteroides* B512 were obtained from Pharmacia, Uppsala, Sweden. Dimethyl sulfoxide was dehydrated over calcium hydride and distilled under reduced pressure. Piperidine-*N*-sulfonic acid was synthesized from chlorosulfonic acid and piperidine[15], and *N*-cyclohexylsulfamic acid was prepared from commercially available sodium cyclamate[15]. Heparin sodium having a potency of 126 units (U.S.P.)/mg was obtained from Daiichi Pure Chemicals Co. Ltd., Tokyo Japan. Type 36/32 of Visking tube (Visking Co. Ltd., U. S. A.) was used for dialysis.

Paper electrophoresis. — Paper electrophoresis was carried out on Toyo Roshi No. 50 filter paper (25 × 9 cm) at 22 V/cm for 30–60 min, using a pyridine acetate buffer (5:1:5:250, v/v pyridine–acetic acid–butyl alcohol–water, pH 5.8). The samples were applied at 4.5 cm from the center of the paper. After electrophoresis, spots of the anions, such as dextran sulfate, and inorganic sulfate were detected as dark areas under u.v. light, and then stained by a 1% (w/v) ethanolic solution of Toluidine Blue.

Analytical procedures. — The sulfur content was determined with Dodgson's turbidimetric method[17], and the degree of substitution (D.S.) was calculated from the following equation: $\text{D.S.} = \text{S}(\%) \times 162 \times [3200 - \text{S}(\%) \times 103]^{-1}$. The weight-average molecular weight ($\overline{M}_w$) was determined by the light-scattering method using a Shimadzu light-scattering photometer; measurement was carried out at five concentrations of a sample in 0.1M sodium chloride and at ten angles (30–135°) using the wavelength of 436.1 nm. A modified Ubbelhode viscometer[18] was used for the determination of the intrinsic viscosity at 25 ± 0.1° in 0.9% (w/v) aqueous sodium chloride[4].

Sulfation procedure for high-molecular dextran. — The finely powdered dextran ($\overline{M}_w$ 39,500, dried over phosphorus pentoxide at 80–85°, *in vacuo* for 2 h) (1 g) was dissolved in dimethyl sulfoxide (28 ml) at 40°. Piperdine-*N*-sulfonic acid was added to the solution which was heated for 1 h at 80° under stirring. The reaction mixture was diluted with water (70 ml) and dialyzed against tap water for 48 h. After the absence of inorganic sulfate had been tested, the dialyzed solution was passed through a column of Dowex 50W (X2, Na^+, 50–100 mesh). The effluent and washings were combined, concentrated to *ca.* 20 ml *in vacuo*, and filtered to remove

a small amount of impurities. The clear concentrate was freeze-dried to give a powdery product which was dried over phosphorus pentoxide *in vacuo* for 3 h at 80°.

Sulfation procedure for low-molecular dextran. — The sulfation of low-molecular dextran ($\overline{M}_w$ 3,400) was performed in the same manner as just described. The reaction mixture was diluted with water (70 ml) and 10% (w/v) barium acetate was added to remove the inorganic sulfate. After centrifugation, the supernatant was passed through a column of Dowex 50W (X2, Na^+, 50–100 mesh). The effluent and washings were combined, evaporated *in vacuo* to *ca.* 20 ml, and filtered. Ethanol (10 vol.) was added to the clear concentrate, and the precipitate formed was washed successively with ethanol and ether, and dried by exposure to air. The resulting powdery product was dried over phosphorus pentoxide *in vacuo* for 3 h at 80°.

Fractional precipitation. — A procedure analogous to that of Whistler *et al.*[14] was used. Sodium dextran sulfate (2 g) (S, 14.43%, D.S. 1.31, [η] 0.237) was dissolved in 0.9% (w/v) aqueous sodium chloride (200 ml). The solution was precipitated at 0–3° with abs. ethanol added in 5% increments. After each addition, the solution was kept for 20 h at this temperature, and the precipitate was centrifuged off, dissolved in a small amount of water, and the solution dialyzed for 48 h against tap water. It was freeze-dried and the resulting product dried *in vacuo* for 3 h at 80°. The yield and sulfate content of each fraction were determined.

Determination of blood-anticoagulant activity and lipemia clearing activity. — The U.S.P. method of anticoagulant activity assay[19] was used (Table IV). In this method, rabbit plasma is recalcified and the minimum sample concentration yielding fluidity of serum for 30 min is compared with that of standard heparin. The volume of each additive was scaled down to one-quarter, except the volume of 1% calcium chloride which was reduced to one-half. The lipemia-clearing activity was determined by the method based on the determination of the decrease of turbidity of a synthetic lipoprotein substrate[20] (Table IV).

ACKNOWLEDGMENTS

The authors thank Dr. T. Kamata of the Government Chemical Industrial Research Institute, Tokyo, for his assistance in the measurement of molecular weight by the light-scattering method and Mrs. N. Tanoura for her technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research of the Ministry of Education.

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