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Reaction between Carbohydrates and Sulfuric Acid. III. Depolymerization and Sulfation of Chitosan by Sulfuric Acid[1]

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Transformation of chitosan in conc. H_2SO_4 was investigated. Neither reaction temperature (0, 10, and 30°) nor time (1—10 hr) affected the degree of sulfation of chitosan, but it was markedly influenced by temperature of the ether used to separate sulfated products. It was proved that depolymerization of chitosan, which had been observed to be extremely stable to conc. H_2SO_4, was influenced by the remaining N-acetyl content in chitosan. As expected, chitosan was greatly depolymerized by treatment with hydrated conc. H_2SO_4 (80 and 90% of sulfuric acid by weight), and sulfated in a lesser extent than that of conc. H_2SO_4.

A hydrolysis constant, K_1 7.0×10^{-2} min^{-1}, of the acid-labile sulfate in sulfated chitosan indicated a reliable formation of the N-sulfate bound to the amino group in sulfated chitosan.

Since discovery of heparin and its biological activities,[3-5] a number of polysaccharide sulfates have been synthesized and their biological activities examined.[6-9] Especially, in relation to the role of N-sulfate in heparin structure, many works have been reported on the preparation and biological activities of sulfated products of deacetylated mucopolysaccharides[10,11] or synthetically β-aminoethylated polysaccharides.[8,12,13]

The preceding papers of this series[1,14] reported on the sulfation and depolymerization of naturally occurring polysaccharides, particularly chitin and chondroitin 6-sulfate, by treatment with concentrated sulfuric acid. The present report deals with the transformation –sulfation and depolymerization– of chitosan with concentrated sulfuric acid.

Experimental

Materials——Commercial chitin was purified by the method of Hackman.[15] Chitosan (N-Ac 1.71%, corresponding to 92% deacetylation and $M\bar{w}$ $1.2-5\times10^5$) was prepared by treatment of the purified chitin with 40% NaOH at 115° for 6 hr,[10,16] and this chitosan was used throughout this investigation. Partially deacetylated chitin samples with different N-Ac content (2.68, 6.63, and 7.90%, corresponding to 83, 55, and 45% deacetylation, respectively) were prepared from the same chitin by treatment with 40% NaOH at 115°, 20% NaOH at 110°, and 10% NaOH at 104° for 2 hr, respectively. Concentrated sulfuric acid (conc.

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H_2SO_4) was a reagent grade containing 96% of sulfuric acid by weight. Organic solvents and reagents, which are all special reagent grade, were used without further purification. Type 36/32 of Visking tube was used for dialysis.

Methods——Paper electrophoresis was performed by the procedure described in the preceding reports.[1,14] The number-average molecular weight ($M\bar{n}$) was determined by the hypoiodite method,[6] and the weight-average molecular weight ($M\bar{w}$) was determined by the light-scattering method as described in the previous reports.[1,14] The determination of $M\bar{w}$ of chitosan was carried out on a solution in 0.5% HCOOH.

Sulfur and N-acetyl content were analyzed by the Dodgson's turbidimetric method[17] and the method of Kuhn and Roth,[18] respectively. The determination of N-sulfate groups (N-S) and of inorganic sulfate were carried out by using both the procedures of Foster, *et al.*[19] and Kawai, *et al.*[20] as described in the previous paper.[14]

Sephadex-gel filtration of sulfated chitosan was performed on a column (2.5 × 90.5 cm) of Sephadex G-150. To 2 ml of a 0.2% Blue dextran in 0.1 M NaCl, 8.13 mg of a sample (sample No. 965, $M\bar{w}$ 31,000, total-S 17.29%, N-S 6.92%) of sulfated chitosan and 13.09 mg of potassium D-glucose 6-sulfate were added, and the solution was applied on the column, eluted with 0.1 M NaCl at 25°. Effluent was collected in 5 ml fractions at a flow rate of 33 ml/hr. Sulfated chitosan was assayed by the Toluidine blue binding technique,[21] and Blue dextran and D-glucose 6-sulfate were analyzed by the determination of optical density at 660 nm and by the 3,6-dinitrophthalic acid method,[22] respectively.

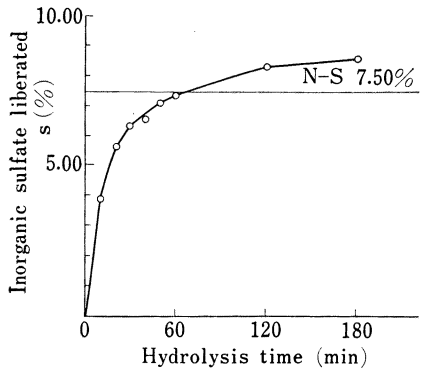

Fig. 1. Hydrolysis Curve of Sulfated Chitosan in 0.1N HCl at 99.5°

Determination of Hydrolysis Constant of Sulfated Chitosan——A Sample (sample No. 987, total-S 15.20%, N-S 7.50%, $M\bar{n}$ 21800, 22.81 mg) of sulfated chitosan was dissolved in 10 ml of 0.1N HCl. A 2-ml aliquot of this solution was heated in a sealed tube at 99.5° for the indicated time, and the inorganic sulfate liberated was determined by the method described above. From the hydrolysis data shown in Fig. 1, a hydrolysis constant (K_1) of N-sulfate in the sulfated chitosan was calculated.

Treatment of Chitosan with conc. H_2SO_4——Finely powdered chitosan was dried *in vacuo* over P_2O_5 for 3 hr at 75°. Two grams of the dried chitosan was added in small portions during 15 min to 40 ml of conc. H_2SO_4 with vigorous stirring at the temperatures indicated until in homogeneous gelatinous state. The reaction mixture was stirred for a certain period and then poured into 400 ml of ether kept at a constant temperature. The preciptate formed was collected on a sintered glass filter, then washed with cold ether. The precipitate was immediately dissolved in ice-water, and neutralized with cold 30% NaOH. The neutralized solution was dialyzed against tap water for 40 hr. After confirmation of the absence of inorganic sulfate, the dialyzed solution was concentrated to *ca.* 10 ml at a temperature below 40° *in vacuo*, and centrifuged. Ten volumes of ethanol saturated with sodium acetate was added and the precipitate formed was filtered off, washed successively with ethanol and ether, and dried *in vacuo* over P_2O_5 for 2 hr at 80°.

Treatment of Partially Deacetylated Chitin with conc. H_2SO_4——One of the partially deacetylated chitin samples was finely powdered and dried *in vacuo* over P_2O_5 for 3 hr at 75°. Two grams of the dried material was treated with 40 ml of conc. H_2SO_4 for 2 hr at 0°, and the reaction product was precipitated by ether at −30°. The precipiate was washed with ether and dissolved in ice-water, immediately neutralized with cold 30% NaOH, then dialyzed five times against distilled water (800 ml). The nondialyzable fraction was operated in a similar fashion as described above, and the powdery product obtained was dried over P_2O_5 for 2 hr at 80°. The pooled dialyzable fractions were concentrated to *ca.* 100 ml. A solution of 10% $Ba(OAc)_2$ was added to remove inorganic sulfate, after centrifugation, the supernatant solution was applied to a column of Dowex 50 (W × 2, 50—100 mesh, Na^+) to remove the barium ions. The effluent and washings were combined, evaporated *in vacuo* to 10—20 ml, and then centrifuged. The dialyzable product was precipitated and dried as described for the nondialyzable product.

Treatment of Chitosan with Hydrated conc. H_2SO_4——Finely powdered chitosan (2 g) was added in small portions during 15 min to 40 ml of the hydrated conc. H_2SO_4 (90% or 80% of sulfuric acid by weight)

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with vigorous stirring at the indicated temperature for 3 hr. The reaction mixture was more sticky than that treated with conc. H_2SO_4. Ether (400 ml) was added to the reaction mixture at −30°, and the vitreous precipitate was separated by decantation from the etherish supernatant. The precipitate was immediately added to crashed ice and neutralized by a solution of 30% NaOH. The solution neutralized was dialyzed against tap water for 40 hr. After confirmation of the absence of inorganic sulfate, the dialyzed solution was concentrated to *ca.* 10 ml *in vacuo* and centrifuged. Ten volumes of ethanol saturated with NaOAc was added and the precipitate was filtered off, washed and dried as the usual way described. The product thus obtained was weighed and analyzed for total−S% and $\bar{M}n$.

Result and Discussion

Because depolymerization and sulfation occur in this reaction as observed on the cases of other polysaccharides,[1,14] it is important to determine the molecular weight of the reaction products. The weight average molecular weight ($\bar{M}w$) and number average molecular weight ($\bar{M}n$) of sulfated chitosan were determined by the light-scattering method and reducing end-group analysis, respectively. As shown in Table I, the $\bar{M}w$ and $\bar{M}n$ values of a sulfated chitosan are nearly identical and the fact suggests that the degree of depolymerization can be discussed from the $\bar{M}n$ values.

Table I. Properties of Sulfated Chitosan

Sulfur content (%)		Molecular weight		$[\eta]^{a)}$	$[\alpha]_D^{22b)}$
Total-S	N-S	$\bar{M}w$	$\bar{M}n$		
17.29	6.92	31000	33800	0.313	−10.74°

Chitosan (2 g) was treated with conc. H_2SO_4 at 0° for 2 hr, and the sulfated product was precipitated by ether at −20°. The yield of nondialyzable fraction (samlpe No. 965) was 0.87 g.
a) determined in water at 25±0.05° *b*) determined in water at c=0.93

In order to see the effect of reaction temperature on the products, the reaction with conc. H_2SO_4 was carried out at 0°, 10°, and 30° for 2 hr, and each reaction mixture was poured into ether at −30°. It is noticeable that although the relatively higher reaction temperature was used, all the products of these reactions were recovered in nondialyzable fractions. The results in Fig. 2a and 2b showed that the higher the reaction temperature was, depolymerization was greater, but the approximate sulfur content to each other resulted. This phenomenon is consistent with the result obtained in the case of chondroitin 6-sulfate except that the progress in depolymerization of chitosan is by far smaller than that of chondroitin 6-sulfate.

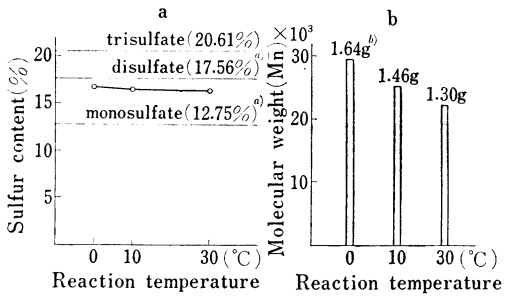

Fig. 2. Effect of Reaction Temperature on the Sulfation and Depolymerization of Chitosan with conc. H_2SO_4

The reaction was carried out at the indicated temperature for 2 hr, and the sulfated product was separated by ether at −30°.
a) Sulfur content was calculated on the sulfated chitosan supposed to have an equimolar amount of O- and N-sulfate.
b) The figures on top of empty bars represent the weight of nondialyzable product obtained from 1 g of starting chitosan.

The effect of reaction time on the sulfation of chitosan was examined. Chitosan was treated with conc. H_2SO_4 at 0°, and an aliquot of the reaction mixture was taken at 1 hr intervals during 10 hr and poured into ether at −30°. As can be seen in Fig. 3, the reaction time had substantially no effect on both the total-S and N-S content of the products, and all of the product obtained by 10 hr reaction was recovered in nondialyzable fraction.

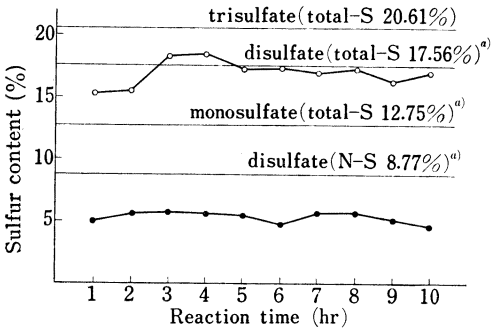

Fig. 3. Effect of Reaction Time on the Sulfation of Chitosan with conc. H_2SO_4

Chitosan was treated with conc. H_2SO_4 at 0° for 1—10 hr, and the sulfated product was separated by ether at −30°.
—○—: total-S (%), —●—: N-S (%)
a) Sulfur content was calculated on the sulfated chitosan supposed to have an equimolar amount of O- and N-sulfate.

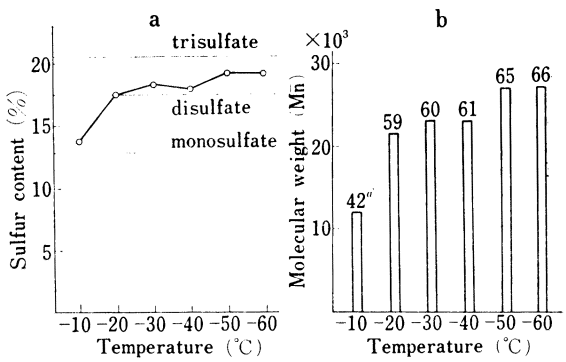

Fig. 4. Effect of Temperature of Ether on Sulfated Chitosan

The reaction was carried out at 0° for 2 hr, and the sulfated chitosan was precipitated by ether at the indicated temperature.
a) The figures on top of bars represent the degree of polymerization of unit D-glucosamine.

As described in the preceding paper,[1] the degree of sulfation in chondroitin 6-sulfate was markedly influenced by temperature of the ether which was used for precipitation of the reaction product. In order to clarify the effect of temperature of ether on sulfated chitosan, chitosan was treated with conc. H_2SO_4 for 2 hr at 0°, and the reaction mixture was evenly divided into six portions. Each the reaction mixture was poured into ether at the indicated temperature. As shown in Fig. 4a, the data on the products indicate that the sulfur content of the products was lower when the temperature of ether was higher, and among the reaction conditions tested on sulfation of chitosan, the temperature of ether was the most effective one. Moreover the temperature of ether seems to slightly affect the degree of depolymerization (Fig. 4b).

As reported in the first paper of this series,[14] a remarkable depolymerization occurred when chitin was treated with conc. H_2SO_4 at −5° for 2 hr, and resulted in yielding the nondialyzable (56%) and dialyzable (44%) fractions. On the other hand, none of the sulfated chitosan was found in the dialyzable fraction even after treatment at 0° for 10 hr (Fig. 3). As pointed out previously,[14] this extreme stability to depolymerization was attributed to the stabilizing effect of the free amino group in the chitosan molecule. In order to clarify a relationship between the residual N-acetyl content and depolymerization of chitosan, partially deacetylated chitin samples were treated with conc. H_2SO_4. The data in Table II show that the larger the increase in residual N-acetyl content is, the greater the degree of depoly-

Table II. Effect of Remaining N-Acetyl Group in the Chitosan on Sulfation and Depolymerization of Chitosan with conc. H_2SO_4

Percentage of deacetylation (%)	Reaction conditions			Products					
				Nondialyzable fraction			Dialyzable fraction		
	Temp. (°C)	Time (hr)	Temp. of ether (°C)	Yield[a] (g)	Total-S (%)	$\bar{M}n$	Yield[a] (g)	Total-S (%)	$\bar{M}n$
83	0	2	−30	2.56	13.82	22200	0.01	—[b]	—[b]
55	0	2	−30	1.77	12.63	12900	0.27	12.27	—[b]
45	0	2	−30	1.61	13.25	11200	0.72	13.08	—[b]

a) weight obtained from 2 g of starting material *b*) not determined

merization results. This fact seems to account for the role of N-acetyl groups on the depolymerization of chitosan molecules. Formation of the products with a lower sulfur content is probably due to the existence of not a little amount of N-acetyl group.

To examine the effect of hydration of conc. H_2SO_4 on the sulfated products, chitosan was treated with hydrated sulfuric acid at different temperatures. The data in Table III, as expected, show that the larger the increase in hydration of sulfuric acid is, the greater both the progress of depolymerization and the suppression in sulfation result. In this way, a treatment of 2 g of chitosan with 80% sulfuric acid at 20° for 3 hr formed 1.11 g of the nondialyzable product with total-S 10.77% and $\bar{M}n$ 5300.

TABLE III. Depolymerization and Sulfation of Chitosan with Hydrated Sulfuric Acid

Reaction conditions			Nondialyzable product		
Temp. (°C)	Time (hr)	Temp. of ether (°C)	Yield[a] (g)	Total-S (%)	$\bar{M}n$
90% sulfuric acid					
0	3	−30	2.34	14.83	24400
10	3	−30	2.42	14.67	11100
20	3	−30	2.37	13.53	8700
80% sulfuric acid					
0	3	−30	0.68[b]	9.58	7400
10	3	−30	0.29[b]	9.57	6400
20	3	−30	1.11	10.77	5300

a) Weight obtained from 2 g of starting material.
b) These low yields are due to experimental error.

Each sulfated chitosan migrated uniformly as a homogenous single spot on paper electrophoresis. Sephadex-gel filtration was performed on the sulfated chitosan obtained by the reaction at °0 for 2 hr. As can be seen in Fig. 5, the elution diagram showed a high degree of polydispersity in molecular size, and elution volume of the sample was much smaller than expected from its molecular weight. This latter phenomenon seems to be common to a high-molecular polyanion such as heparin[23] and chondroitin polysulfate.[1]

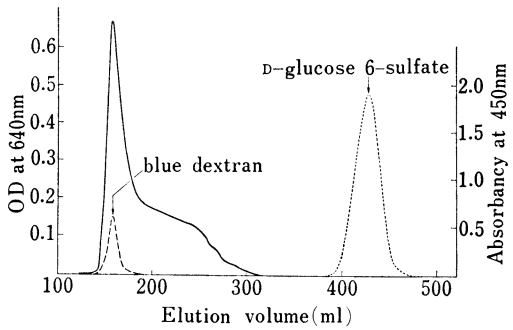

Fig. 5. Elution Diaram of Sulfated Chitosan Chromatographed on Sephadex G-150 Column

A sample of sulfated chitosan (sample No. 965, $\bar{M}w$ 31000, total-S 17.29%) was chromatographed on Sephadex G-150 column (2.5×90.5 cm) by elution with 0.1 M NaCl.
——: sulfated chitosan, ——: Blue dextran,
……: D-glucose 6-sulfate

In order to obtain a further information on the acid-labile sulfate, termed as "N-sulfate," in sulfated chitosan, a hydrolysis constant as to "N-sulfate" was determined. A hydrolysis constant, K_1 7.0×10^{-2} min^{-1}, obtained by the hydrolysis of a sample (sample No. 987, total-S 15.20%, N-S 7.50%, $\bar{M}n$ 21800) of sulfated chitosan in 0.1N HCl at 99.5°, was very close to those reported on heparin (K_1 6.2×10^{-2} min^{-1}, in 0.1N HCl at 99.5°)[24] and D-glucosamine N-sulfate (K_1 6.24×10^{-2} min^{-1}, in 0.1N HCl at 100°).[25]

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This result indicates that "N-sulfate" fairly exactly corresponds to the amount of the sulfate bound to the amino group in sulfated chitosan molecule.

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