REACTION BETWEEN CARBOHYDRATES AND SULFURIC ACID

PART I. DEPOLYMERIZATION AND SULFATION OF POLYSACCHARIDES BY SULFURIC ACID

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ABSTRACT

The molecular weight and sulfur content of the reaction products obtained by treatment of amylopectin, cellulose, dextran, chitin, alginic acid, chondroitin 6-sulfate, and chitosan with concentrated sulfuric acid for 2 h at 0° were determined. All the polysaccharides were sulfated and depolymerized according to their structure. Analyses of sulfated and depolymerized products of cellulose, chitin, and chondroitin 6-sulfate revealed that no structural change in the constitutional monosaccharides occurred during reaction with concentrated sulfuric acid. Drastic depolymerization and sulfation occurred when solid chitin was dissolved in concentrated sulfuric acid, followed by gradual depolymerization and sulfation; the temperature during the reaction markedly influenced the degree of depolymerization.

INTRODUCTION

Various methods which involve combinations of sulfating agents and reaction media, have been used for the sulfation of polysaccharides[1,2]. The common difficulty of the sulfation of polysaccharides is that the reaction is performed in a heterogeneous medium, because most of the polysaccharides are insoluble or only slightly soluble in the organic solvents used as the reaction medium in the conventional sulfation procedure. Consequently, it can be assumed that the constitution of the products is heterogeneous.

In 1819, Braconnot reported the dissolution of cellulose "linen" into concentrated sulfuric acid[3], and later De Carolles[4] and Fehling[5] showed that the products formed in concentrated sulfuric acid were highly degraded cellulose sulfates. Similar modifications of starch in concentrated sulfuric acid were reported by several investigators[5–7]. However, the relationship between the reaction conditions and the constitution of the products formed in the course of the reaction of polysaccharides with concentrated sulfuric acid has not been reported.

This report describes the depolymerization and sulfation of polysaccharides in the presence of concentrated sulfuric acid.

RESULTS AND DISCUSSION

It was found that dilution of the reaction mixture with cold ether was more suitable for separating the reaction products than was dilution with a large excess of ice–water and subsequent neutralization. When an excess of ether was added to the reaction mixture, the products precipitated in powdery or vitreous state. The precipitate was separated, neutralized with sodium hydroxide, and separated into dialyzable and nondialyzable fractions. The properties and yield of the sulfated products are shown in Table I. The depolymerization and sulfation under the same conditions varied considerably according to the composition and structure of the polysaccharide. In contrast to chitin, its de-*N*-acetylated product, chitosan, was very resistant to depolymerization as expected from the stabilizing effect of the free amino group[8].

For the accurate estimation of depolymerization, the weight-average molecular weight ($M\bar{w}$) of the sulfated products was determined by the light-scattering method, together with the number-average molecular weight ($M\bar{n}$), which was determined by reducing end-group analysis*. A large discrepancy in $M\bar{w}$ and $M\bar{n}$ values of the sulfated products of such neutral polysaccharides as amylopectin, cellulose, and dextran was observed (Table I). The data of the nondialyzable fraction of sulfated dextran suggest that the overestimation of $M\bar{n}$ value is probably due to the shielding, by sulfation, or to the cyclization of reducing end-groups. In contrast, the $M\bar{w}$ and $M\bar{n}$ values of the sulfated products of chitin, chitosan, alginic acid, and chondroitin 6-sulfate are

TABLE I

PHYSICAL AND CHEMICAL CONSTANTS OF THE REACTION PRODUCTS OF VARIOUS POLYSACCHARIDES WITH CONCENTRATED SULFURIC ACID

Polysaccharide	*Product*[a]						
	Nondialyzable				*Dialyzable*		
	$M\bar{w}^b$	$M\bar{n}^c$	*S*(%)	*Yield* (g)[d]	$M\bar{n}^c$	*S*(%)	*Yield* (g)[d]
Amylopectin	6,000	25,500	12.53	0.51	10,000	12.72	1.18
Cellulose	6,200	29,900	13.62	1.37	6,700	12.59	0.48
Chitin	19,900	18,000	11.49	1.66	8,400	11.86	1.30
Dextran	12,300	92,300	14.36	2.83	15,000	14.99	0.27
Alginic acid	44,600	60,900	14.87	1.37	5,700	16.99	1.89
Chondroitin 6-sulfate	9,900	11,200	12.06	1.46	3,800	11.11	0.54
Chitosan	31,000	33,800	17.29	1.74			

[a]All the polysaccharides, except chitosan, were treated for 2 h at −5°, and the reaction products were precipitated by addition of ether at −5°. Chitosan was treated for 2 h at 0°, and the product was precipitated by ether at −20°. [b]Determined by the light-scattering method. [c]Determined by reducing end-group analysis. [d]Weight obtained from 2 g of starting material

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*It has been found that treatment of D-glucose with concentrated sulfuric acid affords, in a small yield, a nondialyzable sulfated product having $M\bar{w}$ 1.600 (D.P. 4.5, by light-scattering method), $M\bar{n}$ 44 880 (end-group determination), and S 17.76%. Gel filtration on Sephadex G-25 and viscosity determination suggested that the $M\bar{w}$ value obtained by the light-scattering method was correct. Therefore, the large discrepancy between the $M\bar{w}$ and $M\bar{n}$ values can be attributed to the shielding of reducing end-groups. 2-Amino-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-glucose, and D-glucurono-3,6-lactone also formed nondialyzable, sulfated products in small yield and having reasonably small $M\bar{n}$ values (unpublished data).

nearly identical. The average molecular weight ($M\bar{w}$ and $M\bar{n}$) and yield of each fraction obtained show the extent of degradation of the polysaccharide structure by concentrated sulfuric acid (Table I). Each sulfated product migrated uniformly as a single spot on paper electrophoresis.

A sample ($M\bar{w}$ 9.900, S 12.06%) of sulfated chondroitin 6-sulfate was chromatographed on Sephadex G-100, under the conditions used for heparin[9]. The elution pattern showed a relatively high degree of polydispersity of the molecular size. Hydrolysis of a sample ($M\bar{w}$ 6.200, S 13.62%) of sulfated cellulose gave almost quantitatively D-glucose (0.97 mole) which was identified by thin-layer chromatography in three solvent systems. No cleavage of *N*-acetyl groups occurred in sulfated chitin. The molar ratio of hexosamine to uronic acid unit of the sulfated chondroitin 6-sulfate ($M\bar{n}$ 15.600, S 11.07%) was 1 to 1.02, which shows that no decomposition of the uronic acid component occurred. Partial hydrolysis of chitosan sulfate ($M\bar{w}$ 31.000, S 17.29%) having 1.93 moles of sulfate per mole of 2-amino-2-deoxy-D-glucose, under the conditions used for the de-*N*-sulfation of heparin[10], indicated the very probable formation of *N*-sulfate groups (Fig. 1).

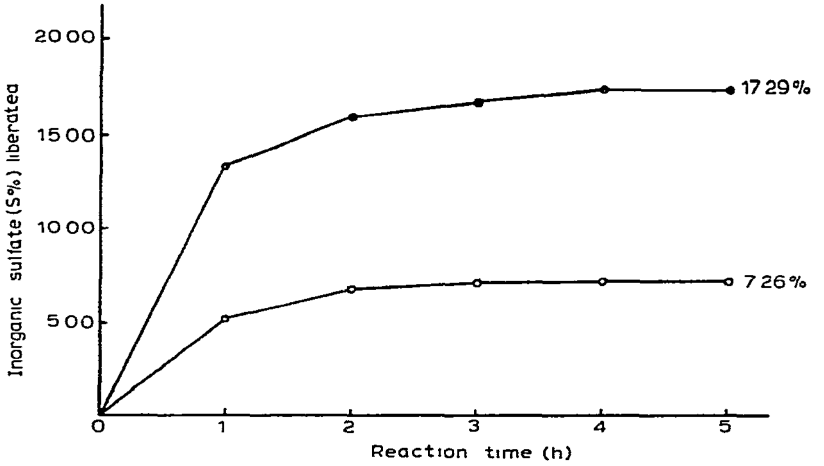

Fig. 1. Liberation of inorganic sulfate by complete and partial hydrolysis of sulfated chitosan ($M\bar{w}$ 31,000, S 17.29%). ——●——, Hydrolysis in M hydrochloric acid at 105°; and ——○——, hydrolysis in 40mM hydrochloric acid at 100°.

To investigate the influence of temperature and length of time on the sulfation of polysaccharides, chitin was treated at 5°, $-5°$, and $-20°$ for 1–6 h, and each reaction product was examined as to its number-average molecular weight ($M\bar{n}$)*, sulfur content, and yield. As shown in Fig. 2, more than half of the available hydroxyl groups of the 2-acetamido-2-deoxy-D-glucopyranosyl residues (free hydroxyls at C-3 and 6) were sulfated in the initial stage of the reaction, and additional sulfation proceeded with time. The reaction temperature affected the sulfation of chitin at both

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*As seen in Table I, the $M\bar{w}$ and $M\bar{n}$ values of chitin sulfate are very similar, and it seems reasonable to estimate the degree of depolymerization of chitin by the latter value.

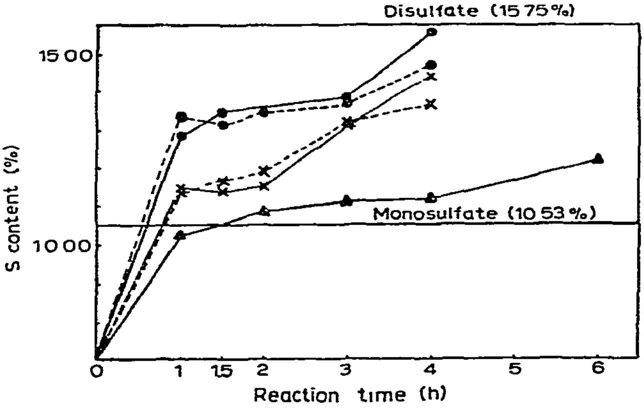

Fig. 2. Effect of temperature and reaction time on sulfation of chitin with concentrated sulfuric acid. ———, Nondialyzable fraction; and - - - - - -, dialyzable fraction; ●, reaction at 5°; ×, at −5°, and ▲, at −20°.

stages. The reaction temperature and the length of time, especially the former, influenced the depolymerization of chitin. After 4 h at 5°, 94% of the sulfated chitin was recovered in the dialyzable fraction which had an *Mn* value of 6,200 (D.P. 16), whereas all the products obtained from the reaction at −20° for 1–6 h are non-dialyzable (Fig. 3). These results also suggest that the depolymerization of chitin is the greatest at the time of dissolution.

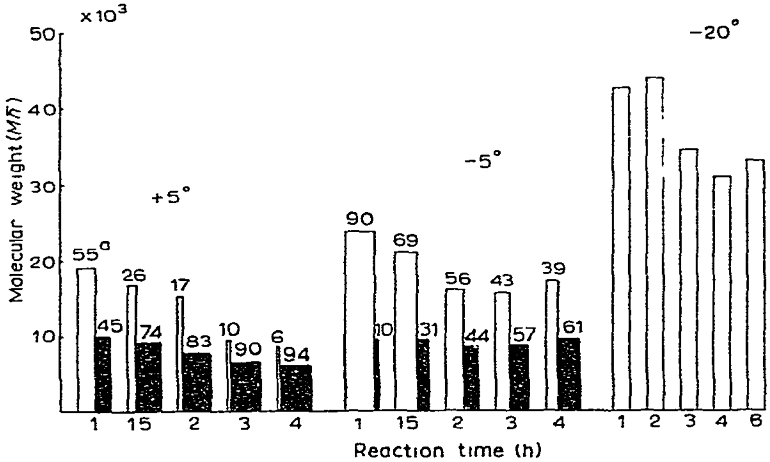

Fig. 3. Effect of reaction temperature and reaction time on the depolymerization of chitin with concentrated sulfuric acid. The figures on top of bars represent the yield of nondialyzable fraction (empty bars) and dialyzable fraction (full bars).

It may be concluded that the reaction consists of two processes, an intensive depolymerization and sulfation at the time of dissolution of the solid polysaccharide into concentrated sulfuric acid, followed by a gradual depolymerization and sulfation.

The second process is easier to control by changing the temperature and length of the reaction time than is the first process.

EXPERIMENTAL

Materials. — Chitin (N, calc. 6.89%; found 6.34%), was prepared from crab shells according to the method of Hackmann[11]; the $\bar{Mw}$ of the chitin was assumed to be at least $>1.2{\sim}5\times10^5$ from the $\bar{Mw}$ of chitosan. Chitosan ($\bar{Mw}$ $1.2{\sim}5\times10^5$ by light-scattering method) was prepared from the purified chitin by the procedure of Wolfrom[12], (*N*-Ac 1.71%, corresponding to 92% deacetylation). Microcrystalline cellulose "Avicel" (D.P. *ca.* 200) was obtained from American Viscose Division of FMC Co., Marcus Hook, Pennsylvania U.S.A., sodium chondroitin 6-sulfate (Lot No. N3012, S 6.65%, $\bar{Mw}$ 65.000 by light-scattering method) from Seikagaku Kogyo Co. Ltd., Chuō-ku, Tokyo, Japan, and Dextran (Lot No. 8687, $\bar{Mw}$ 39.500 by light-scattering method) from Pharmacia, Uppsala, Sweden. Sodium alginate and amylopectin, obtained from the Daiichi Pure Chemicals Co. Ltd., Chuō-ku, Tokyo, Japan, were dialyzed and reprecipitated with ethanol prior to use. Concentrated sulfuric acid used throughout this investigation was a reagent grade containing 96% of sulfuric acid by weight. Organic solvents were dried and redistilled before use. Type 36/32 of Visking tube was used for dialysis.

Paper electrophoresis and thin-layer chromatography. — Paper electrophoresis of sulfated polysaccharides was carried out on Toyo Roshi No. 51 paper with a buffer solution (pH 5.8) consisting of pyridine (5 ml), acetic acid (1 ml), butyl alcohol (5 ml), and water (250 ml), at a potential of 23 V/cm, for 15–30 min. The spots were detected with a u.v. light as dark spots on a light background, and then stained by 1% (w/v) ethanolic Toluidine Blue.

Determinations of sulfur content and molecular weight. — The sulfate content was determined by the Dodgson turbidimetric method[13]. The number-average molecular weight ($\bar{Mn}$) was calculated from the value obtained by the hypoiodite method[1]. Weight-average molecular weight ($\bar{Mw}$) was determined by the light-scattering method using a Shimadzu Light-scattering Photometer. The determination was performed on five concentrations of a sample in 0.1M sodium chloride and at ten angles (30–135°) using the wave-length of 436.1 nm.

Treatment of polysaccharides (except chitosan) with concentrated sulfuric acid. — The finely powdered polysaccharide was dried *in vacuo* over phosphorus pentaoxide for 2 h at 80–85°*. The dried polysaccharide (2 g) was added gradually to concentrated sulfuric acid (20 ml) at −5° to form a homogeneous solution which was kept for 2 h at the same temperature under vigorous stirring. Dry ether (200 ml) was added to the mixture being kept at −5°. The precipitate was filtered off or centrifuged, washed several times with cold ether, and left to dry. The sulfated products were dissolved in ice-water (100 ml), and the solution was neutralized with cold sodium hydroxide

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*Amylopectin was dried at room temperature.

solution, and dialyzed five times against distilled water (800 ml). After being tested for the absence of inorganic sulfate, the nondialyzable fraction was concentrated to 10–20 ml at a temperature below 40° and centrifuged. The supernatant was added to ethanol (10 vol.) and the nondialyzable product was filtered off and washed successively with ethanol and ether. The powdery product was dried over phosphorus pentaoxide for 2 h at 80°, and its molecular weight ($\bar{M}_w$ and $\bar{M}_n$) and sulfur content were determined.

The pooled, dialyzable fractions were concentrated to *ca.* 100 ml. A 10% solution of barium acetate was added to remove inorganic sulfate and, after centrifugation, the supernatant solution was applied to a column of Dowex 50 (W-×2, 50–100 mesh, Na^+, 1.5×15 cm) to remove the barium ions. The effluent and washings were combined, evaporated at a temperature below 40° to 10–20 ml, and then centrifuged. The dialyzable product was precipitated and analyzed as described for the nondialyzable product.

Treatment of chitin with concentrated sulfuric acid. — To examine a time course of the reaction at various temperatures, chitin was treated with sulfuric acid as follows. Finely powdered chitin (10 g) was added gradually to 100 ml of concentrated sulfuric acid kept at a constant temperature, under vigorous stirring [At −20°, a mixture of concentrated sulfuric acid (85 ml) and tetrahydrofuran (15 ml) was used]. A homogeneous solution was obtained within 30 min after the addition of chitin powder. An aliquot (*ca.* 20 ml) of the reaction mixture was taken at five intervals. The temperature being kept constant, cold ether (10 vol.) was immediately added to each aliquot. The reaction product was filtered off or centrifuged, purified by dialysis, isolated, and analyzed as just described.

Treatment of chitosan with concentrated sulfuric acid. — Finely powdered chitosan (2 g, dried over phosphorus pentaoxide for 3 h at 75°) was added to concentrated sulfuric acid (40 ml) with vigorous stirring during 15 min at 0°. The mixture became gelatinous and, after 2 h at 0°, ether (400 ml) was added with stirring at −20°. The precipitate was collected on a sintered-glass filter, washed several times with cold ether, and dissolved in ice–water (100 ml). Cold sodium hydroxide solution was immediately added to the solution to pH 7.6. After being dialyzed against tap water for 2 days and tested for the absence of inorganic sulfate, the nondialyzable fraction was concentrated to *ca.* 10 ml at a temperature below 40° and centrifuged. Ethanol (100 ml) was added and the precipitate was filtered off, washed successively with ethanol and ether, and dried over phosphorus pentoxide for 2 h at 80°.

Determination of N-*sulfate groups and of inorganic sulfate*[10,13,14]. — A solution of sulfated chitosan ($\bar{M}_w$ 31.000, S 17.29%, 94.40 mg) in 40 mM hydrochloric acid (10 ml) was heated in a tightly stoppered tube on a boiling water bath. An aliquot (2 ml) was removed at intervals of 1, 2, 3, 4, and 5 h, cooled in ice–water, and centrifuged. To the supernatant (1 ml) was added 1 ml of a solution containing 10.70 g of cetylpyridinium chloride in 500 ml of 0.3M hydrochloric acid. The mixture was kept for 20 min at 37°, and then centrifuged at 1.5×10^4 r.p.m. for 20 min. The supernatant (0.2 ml) was mixed with a solution (4.8 ml) prepared by dissolving gelatin (0.5 g) in distilled water (100 ml) at 60°, keeping the solution overnight, and mixing it with powdered barium chloride (0.5 g) and concentrated hydrochloric acid (1.25 ml). After 15 min at room temperature, the turbidity of the solution was measured at 500 nm. A calibration curve was prepared with potassium sulfate solutions (40–200 μg/0.2 ml).

Determination of D-*glucose in sulfated cellulose.* — A sample ($M\bar{w}$ 6.200, S 13.62%, corresponding to 1.2 moles of sulfate per mole of glucose) of sulfated cellulose was analyzed by the anthrone method[14] (D-Glucose found: 0.971, 0.969 mole).

A solution of the same sample (10 mg) in M hydrochloric acid (1 ml) was heated in a sealed tube for 5 h at 105–110°. The hydrolyzate was treated with barium acetate to remove inorganic sulfate, and the acidic solution was evaporated *in vacuo* to remove the volatile acids. The residual solution was analyzed by thin-layer chromatography on cellulose powder "Avicel-SF" in pyridine–ethyl acetate–acetic acid–water (5:5:1:3, v/v), R_F 0.72; butyl alcohol–acetic acid–water (3:1:1, v/v), R_F 0.23; and butyl alcohol–pyridine–water (6:4:3, v/v), R_G 1.00. Observed for D-glucose: R_F 0.72, 0.26, and R_G 1.00. The spots were detected with aniline hydrogen phthalate and with alkaline silver nitrate.

Determination of N-*acetyl content and molar ratio of hexosamine to uronic acid units.* — The *N*-acetyl content of sulfated chitin was analyzed by the method of Kuhn and Roth[16]. The molar ratios of hexosamine to uronic acid units in chondroitin 6-sulfate and its sulfated products were analyzed by the carbazole[17] and Elson-Morgan tests[18], respectively.

Sephadex-gel filtration of sulfated chondroitin 6-sulfate. — A sample ($M\bar{w}$ 9.900, S 12.06%) of sulfated chondroitin 6-sulfate was subjected to gel filtration on Sephadex G-100. A mixture of the sample (12.69 mg), Blue dextran (4 mg), and D-glucose 6-sulfate (2 mg) in 2 ml of 0.1M sodium chloride was applied to a column (2.5 × 90 cm) of Sephadex G-100, and eluted with 0.1M sodium chloride at a flow rate of 39 ml/h. Fractions of 4.5 ml each were collected, and the uronic acid content was determined by the Dische carbazole method[17]. The concentrations of Blue dextran and D-glucose 6-sulfate were determined by the absorbancy at 660 nm and the 3,6-dinitrophthalic acid method[19], respectively.

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