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Reaction between Carbohydrates and Sulfuric Acid. (2).[1] Depolymerization and Sulfation of Chondroitin Sulfate by Sulfuric Acid

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When chondroitin sulfate was dissolved in concentrated sulfuric acid, sulfation and depolymerization took place simultaneously during dissolution, and subsequent depolymerization developed gradually with reaction time which had no effect on sulfation. The degree of depolymerization depended on reaction temperature which had a slight effect on sulfation. The degree of sulfation was decisively controlled by temperature at the time of ether addition to the reaction mixture for the separation of products.

By controlling various reaction conditions, *i.e.*, reaction time, reaction temperature, and temperature at the time of ether addition, sulfated chondroitin with various combinations of molecular weight and sulfur content was obtained.

The distribution of molecular size in sulfated chondroitin was investigated by gel-filtration, which suggested a high polydispersity in molecular weight. Chemical analyses of sulfated chondroitin revealed that neither degradation of the uronic acid residue nor deacetylation of the N-acetylgalactosamine residue occurred. The data of periodate oxidation and infrared spectra suggested that the three substitutable hydroxyl groups present in the unit disaccharide of chondroitin sulfate were randomly sulfated.

The first report of this series[1] showed that depolymerization and sulfation of polysaccharides occurred concurrently in cold concentrated sulfuric acid and that degradation of the constituent monosaccharides in the polysaccharides was not observed. It was also confirmed that the degree of depolymerization and sulfation of chitin in this reaction depended on both reaction time and temperature. It is important to establish a method for the preparation of sulfated polysaccharides of different molecular weights and sulfur content in order to elucidate the correlation between the structure of the compounds and their heparin-like activity.[3,4] The present paper describes the effect of reaction conditions on molecular weight and sulfur content of sulfated chondroitin, prepared from chondroitin 6-sulfate with concentrated sulfuric acid, and the chemical properties of various sulfated products.

The properties of sulfated chondroitins obtained under different reaction conditions are summarized in Table I. Because depolymerization as well as sulfation occurs in this reaction, it is important to determine the molecular weight of the reaction products. As shown in Table I, $M\bar{w}$ and $M\bar{n}$ values of sulfated chondroitin samples, IIIa and IVa-1, are nearly identical and the fact suggests that the degree of depolymerization can be discussed from the $M\bar{n}$ values.

In order to see the effect of reaction time on the products, sulfation and depolymerization of chondroitin 6-sulfate with concentrated sulfuric acid at −5° and −20° were examined. Both the yield and $M\bar{n}$ of nondialyzable fractions (Ia-1—3) obtained by reaction at −5° decreased gradually with reaction time, which had no effect on their sulfur content. This phenomenon is inconsistent with the results obtained with chitin, in which sulfation progressed gradually with reaction time.[1] When the reaction took place at −20° (IIa-1—6), no marked change was observed in molecular weight or sulfur content, and very little dialyzable fraction was obtained, even after 7 hr.

1) Part I: K. Nagasawa, Y. Tohira, Y. Inoue and N. Tanoura, *Carbohyd. Res.*, **18**, 95 (1971).
2) Location: *Shirokane 5-9-1, Minato-ku, Tokyo, 108, Japan.*
3) K.H. Meyer, R.P. Piroue and M.E. Odier, *Helv. Chim. Acta*, **35**, 574 (1952).
4) K. Murata, *Naturwissenschaften*, **49**, 39 (1962).

Table I. Reaction Conditions and Analytical Data of Sulfated Chondroitin Prepared from Chondroitin 6-Sulfate

Reaction conditions				Products							
				Nondialyzable fraction				Dialyzable fraction			
Temp. (°C)	Time (hr)	Temp. of Ether addn. (°C)	Method[a]	Sample No.	Yield[b] (%)	S content (%)	Mn̄	Sample No.	Yield[b] (%)	S content (%)	Mn̄
−5	1.5	−30	B	Ia-1	85	13.78	17,900	Ib-1	15	14.39	4,200
−5	2.5	−30	B	Ia-2	65	13.73	15,700	Ib-2	35	13.98	4,000
−5	4	−30	B	Ia-3	52	14.21	12,200	Ib-3	48	14.11	3,500
−20[c]	2	−20	A	IIa-1	100	12.79	23,600	IIb-1	0		
−20[c]	3	−20	A	IIa-2	99	12.70	25,500	IIb-2	1		
−20[c]	4	−20	A	IIa-3	96	12.88	19,900	IIb-3	4		
−20[c]	5	−20	A	IIa-4	96	12.57	23,300	IIb-4	4		
−20[c]	6	−20	A	IIa-5	93	12.85	19,400	IIb-5	7		
−20[c]	7	−20	A	IIa-6	99	12.49	19,500	IIb-6	1		
−5	2	−5	A	IIIa	73 (1.46)	12.06	11,200 (9,900)[e]	IIIb	27 (0.54)	11.59	3,800
−30[d]	2	−30	B	IVa-1	100 (1.97)	11.83	30,100 (27,900)[e]	IVb-1	0		
−15[d]	2	−30	B	IVa-2	99 (1.90)	12.97	21,100	IVb-2	1 (0.02)		
−5	2	−30	B	IVa-3	76 (1.45)	13.99	17,800	IVb-3	24 (0.46)	13.75	3,600
+5	2	−30	B	IVa-4	34 (0.65)	14.09	10,400	IVb-4	66 (1.26)	13.77	3,500
−5[d]	2	−30	B	Va	90 (1.66)	13.55	17,000	Vb	10 (0.18)	12.86	4,000
−5	2	−30	A	VIa-1	85	13.78	17,000	VIb-1	15	13.59	4,600
−5	2	−10	A	VIa-2	82	12.74	14,500	VIb-2	18	12.99	4,400
−5	2	+10	A	VIa-3	68	10.68	11,500	VIb-3	32	9.90	3,700
−5	2	−10	B	VIIa-1	68	13.51	16,400	VIIb-1	32	12.42	3,800
−5	2	+10	B	VIIa-2	68	13.01	15,700	VIIb-2	32	13.01	4,600

a) method A: Ether was added dropwise to the reaction mixture.
method B: The reaction mixture was poured into ether.
b) The figures represent percentage of the yield of nondialyzable fraction or dialyzable fraction to the total yield. Figures in parentheses are the weight (g) obtained from 2 g of the starting material.
c) Concentrated sulfuric acid containing 15% (v/v) of tetrahydrofuran was used.
d) Concentrated sulfuric acid containing 20% (v/v) of ether was used.
e) Figures in parentheses represent the Mw̄ values determined by the light-scattering method.

In order to clarify the effect of reaction temperature on the products, the reaction was carried out at −30°, −15°, −5°, and 5° for 2 hr and each reaction mixture was poured into ether at −30° (IVa-1—4 and IVb-1—4). It was thereby found that depolymerization and sulfation increased with the reaction temperature, except that the approximate sulfur content to each other resulted at −5° and 5°. To examine the effect of the presence of ether in sulfuric acid at −30° and −15°, the reaction was also performed at −5° with sulfuric acid containing 20% (v/v) of ether (Va and Vb), and the sulfur content was a little lower than that in the absence of ether.

As described in the preceding paper,[1] dropwise addition of ether to the reaction mixture at the end of the reaction produced gradual separation of sulfated polysaccharides from it. The data on the products prepared by ether addition at different temperatures (−30°, −10°, and 10°) indicate that the sulfur content of the products (VIa-1—3 and VIb-1—3) was lower when ether was added at a higher temperature than those at a lower temperature. On the other hand, when the order of addition was reversed and the reaction mixture was poured into cold ether, a rapid precipitation of the product occurred and no great change in sulfur content of the products was observed (IVa-3, IVb-3, VIIa-1—2, and VIIb-1—2). Therefore,

it is assumed that in the former case desulfation occurs in sulfuric acid diluted with ether, and its extent depends on the temperature at the time of ether addition. Moreover, the temperature seems to affect the degree of depolymerization, especially in the case of samples VIa-1—3 and VIb-1—3. These facts indicate that the temperature at the time of ether addition is also an important factor, in addition to the reaction time and temperature described in the preceding paper.[1)]

Chondroitin 4-sulfate was treated with concentrated sulfuric acid at −5° for 1—4 hr, and the product was separated from the reaction mixture by adding ether at −30°. No marked difference was observed between chondroitin 4- and 6-sulfate in the depolymerization and sulfation.

Under the reaction conditions described above, sulfated chondroitin having three sulfate groups per unit disaccharide were prepared. However, it was not possible to obtain a fully sulfated product (calculated S content, 15.84%) by this reaction.

From the results of these experiments, the following may be concluded.

1) Sulfation: When solid chondroitin sulfate dissolves in concentrated sulfuric acid, a sulfation reaction occurs but does not increase with reaction time. It is slightly dependent on reaction temperature.

2) Depolymerization: When solid chondroitin sulfate dissolves in concentrated sulfuric acid, depolymerization occurs, particularly at a high temperature, and is followed by gradual depolymerization with reaction time.

3) Desulfation: When ether is added to the reaction mixture to separate the products, desulfation reaction occurs, particularly at a high temperature.

A series of sulfated chondroitin differing widely in molecular weight ($\bar{M}n$ 2100—32600) and sulfur content (S 9.31—14.39%) were prepared by selecting the reaction conditions. Estimation of their biological activities will be reported in a subsequent paper.

The sulfated chondroitin migrated uniformly as a relatively homogeneous single spot on paper electrophoresis.

Gel-filtration of the nondialyzable fractions, obtained by reaction under different conditions, was carried out on Sephadex G-100 column. The elution diagrams showed a high degree of polydispersity in molecular size and dependency of depolymerization on the reaction temperature. When the molecular weight was taken into consideration, the product (IVa-1) obtained by the reaction at −30° was eluted abnormally from the Sephadex column (----in Fig. 1a). This phenomenon is probably due to the molecular asymmetry originating from the skeleton of chondroitin 6-sulfate. On the other hand, elution of the products (IVa-3

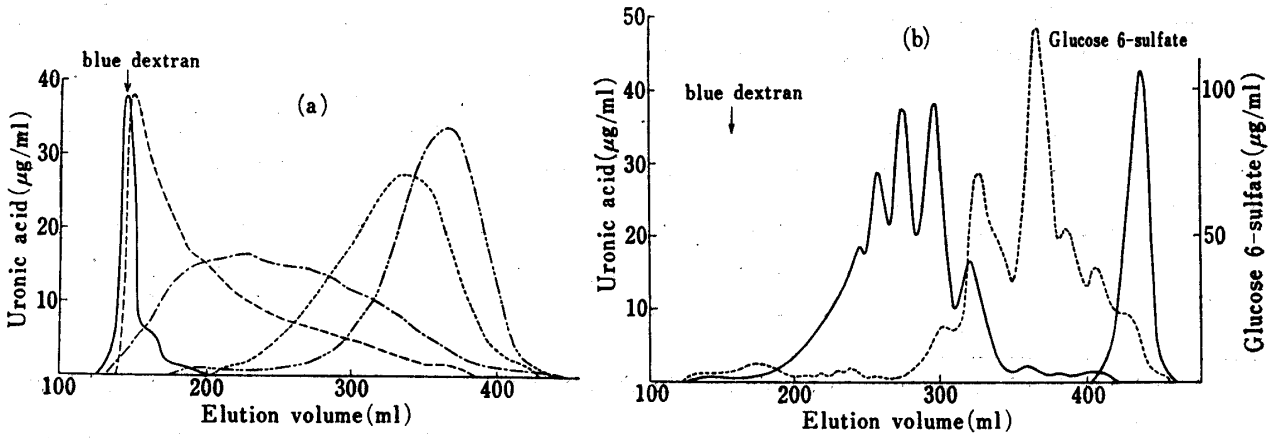

Fig. 1. Elution Diagrams of Sulfated Chondroitin

(a) Each sample was chromatographed individually on Sephadex G-100 column (2.5×90 cm) and eluted with 0.1M NaCl solution.
———: chondroitin 6-sulfate, – – – –: IVa-1, – – – –: IVa-2, – – – –: IVa-3, ———: IVa-4
(b) Each sample was chromatographed on Sephadex G-50 column (2.5×90 cm) and eluted with 0.1M NaCl solution.
———: IVa-4, – – – –: IVb-4

and IVa-4) obtained under a more drastic condition resulted in larger elution volume than would be expected from their molecular weight (------- and ---- in Fig. 1a).

Gel-filtration of the dialyzable and nondialyzable fractions obtained by the reaction at 5° for 2 hr on Sephadex G-50 column showed heterogeneity of the molecular size more distinctly and the one with the smallest molecular size showed the same elution volume as that of D-glucose 6-sulfate (Fig. 1b).

A molar ratio of hexosamine to uronic acid was 1 to 1.02[5] in a sample ($\bar{M}n$ 16700, S 10.80%) with sulfur content corresponding approximately to that of chondroitin disulfate (calculated S content: 10.62%). A molar ratio of a calculated value of N-acetyl group per unit disaccharide in chondroitin disulfate to the experimental value of that in a smaple ($\bar{M}n$ 2900, S 10.67%), prepared under a most drastic condition,[6] was 1 to 0.95.[7] From these results, it was confirmed that neither decomposition of the glucuronic acid moiety nor deacetylation of the N-acetylgalactosamine moiety in the original structure of chondroitin 6-sulfate occurred during this reaction.

Very similar infrared (IR) spectra were obtained from the sulfated products of chondroitin 4- and 6-sulfate which were almost equal in their sulfur content. Both sulfated products have absorptions at 928, 852, and 725 cm^{-1}, characteristic of chondroitin 4-sulfate, and at 1000, 820, ahd 775 cm^{-1}, characteristic of chondroitin 6-sulfate. It is also recognized that the intensity of absorption at 1250 cm^{-1} due to O-sulfate bond increases in the order of mono-, di-, and tri-sulfate. However, no definite information can be obtained from these IR spectra on the fine structure of sulfated chondroitin.

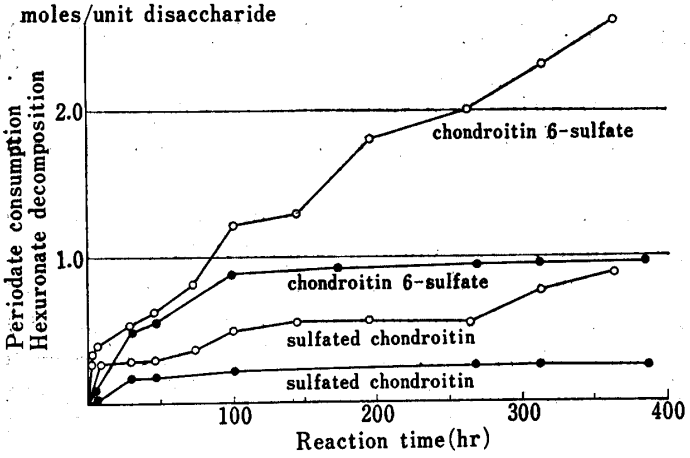

Fig. 2. Periodate Oxidation of Chondroitin 6-sulfate and Sulfated Chondroitin at 25°

○: consumption of periodate ●: decomposition of hexuronate

In order to obtain information on the substitution of hydroxyl groups in chondroitin 6-sulfate with sulfate groups, a sample ($\bar{M}n$ 16700, S 10.80%) with sulfur content corresponding to that of chondroitin disulfate was reacted with periodate at 25°, and the consumption of periodate and the quantity of residual uronic acid were measured. As shown in Fig. 2, one mole of uronic acid per unit disaccharide in chondroitin 6-sulfate was degraded, while only 0.25 mole of uronic acid was degraded in the case of sulfatedchondroitin. Provided there is no difference in the Dische carbazole reaction between chondroitin 6-sulfate and its sulfated product, these results may indicate that at least 25% of total uronic acid in the sample is not substituted with sulfate group.

In order to obtain additional information on the structure of the sulfated products of chondroitin 6-sulfate, an enzymic digestion using purified chondroitinase[8] was applied to sulfated chondroitin prepared from chondroitin 6-sulfate, and the digestion products were

5) A molar ratio of hexosamine to uronic acid in chondroitin 6-sulfate used as a starting material was 1 to 1.09.

6) This sample was prepared by the reaction at 5° for 4 hr and precipitated by the addition of ether at 5°.

7) A molar ratio of the calculated value of N-acetyl group per unit disaccharide to experimental value of that in chondroitin 6-sulfate used as a starting material was 1 to 0.97.

8) T. Yamagata, H. Saito, O. Habuchi and S. Suzuki, *J. Biol. Chem.*, **243**, 1523 (1968).

analyzed by ultraviolet (UV) spectrophotometry and paper chromatography. The results obtained, which suggested random sulfation and depolymerization of chondroitin 6-sulfate, will be published later.

Experimental

Material——Sodium chondroitin 6-sulfate (Lot No. N 3012: $M\bar{w}$ 65900, $M\bar{n}$ 61900,[9] S 6.65%) and sodium chondroitin 4-sulfate (Lot No. WH-48: $M\bar{n}$ 64500,[9] S 6.51%) were supplied by the Seikagaku Kogyo Co., Ltd., Tokyo, Japan. Concentrated sulfuric acid used throughout this investigation was special reagent grade containing 96% of sulfuric acid by weight (sp. gr. 1.84). The organic solvents and reagents were all special reagent grade and used without further purification. Type 36/32 of Visking tube (Visking Co., Ltd., U.S.A.) was used as a Cellophane tube for dialysis of the sulfated products. Type 20/32 of Visking tube was used only in the dialysis of periodate-oxidized products.

Paper Electrophoresis——Paper electrophoresis 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), butanol (5 ml), and water (250 ml), and subjected to a potential gradient of 23 V/cm for 15—30 min. Spots of samples were detected by UV ray as dark spots on a light background, and then stained with 1% ethanolic Toluidine Blue.

Determination of Sulfur Content and Molecular Weight——A sample was hydrolyzed and the liberated inorganic sulfate was determined by turbidimetry.[11] The number average molecular weight ($M\bar{n}$) was calculated from the value obtained by the hypoiodite method.[3] The weight average molecular weight ($M\bar{w}$) was determined by the light-scattering method using a Shimadzu Light-Scattering Photometer. The determination was made 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.

Gel-filtration——A mixture of a sample (13—15 mg), blue dextran (0.4 mg), and D-glucose 6-sulfate (2 mg) dissolved in 2.0 ml of 0.1M sodium chloride solution was applied on a column (2.5 × 90 cm) of Sephadex G-100 or G-50 and eluted with the same solvent. In the case of chondroitin 6-sulfate, samples IVa-1 and IVa-2 shown in Fig. 1a, gel-filtration of Blue dextran was separately carried out. Effluent was collected in 4.5-ml fractions at a flow rate of 20—40 ml/hr. Sulfated chondroitin was assayed for uronic acid by the Dische carbazole method.[12] Blue dextran and D-glucose 6-sulfate were analyzed by the determination of optical density at 635 nm and by the 3,6-dinitrophthalic acid method,[13] respectively.

Determination of N-Acetyl Content and Molar Ratio of Hexosamine to Uronic Acid——The sulfated chondroitin prepared from chondroitin 6-sulfate was analyzed for *N*-acetyl content by the method of Kuhn and Roth,[14] and for molar ratio of hexosamine to uronic acid by the carbazole[12] and Elson-Morgan tests,[15] respectively.

Periodate Oxidation of Sulfated Chondroitin——The procedure applied to heparin[16] was used for oxidation of chondroitin 6-sulfate and its sulfated product ($M\bar{n}$ 16700, S 10.80%). To a solution of 1.00883 g (2.004 mmoles) of chondroitin 6-sulfate or 1.31170 g (2.001 mmoles) of its sulfated product dissolved in 100 ml of water, 100 ml of 0.1M sodium periodate solution was added and the reaction mixture was kept at 25° in the dark. The degree of periodate consumption was determined with 1-ml aliquot of the reaction mixture by the method of Fleury and Lange.[17]

At the same time, another aliquot (20 ml) was used to estimate residual uronic acid. To each aliquot, 0.5 ml of ethylene glycol was added, the mixture was left to stand at room temperature for 30 min, and then reacted with 100 mg of sodium borohydride at room temperature overnight. Excess sodium borohydride was decomposed by the addition of acetic acid (2 ml) and the pH was adjusted to 4.0±0.5 with dilute hydrochloric acid. The treated mixture was dialyzed against tap water for 2 days, nondialyzable fraction was accurately diluted to 100 ml with distilled water, and its uronic acid content was determined by the method described previously.[12]

General Procedure for the Reaction between Chondroitin Sulfate and Concentrated Sulfuric Acid——Finely powdered sodium chondroitin 6-sulfate was dried over phosphorus pentoxide *in vacuo* at 80—85° for 2 hr.

9) The indicated $M\bar{n}$ values determined by the Nelson-Somogyi method[10] were reported by the Research Laboratory of Seikagaku Kogyo Co., Ltd. The $M\bar{n}$ values obtained by the method used in this experiment were 132000 for chondroitin 6-sulfate and 115000 for chondroitin 4-sulfate.
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16) G.J. Durant, H.R. Hendrickson, and Rex Montgomery, *Arch. Biochem. Biophys.*, **99**, 418 (1962).
17) P.F. Fleury and J. Lange, *J. Pharm. Chim.*, [8] **17**, 107, 196 (1933).

Two grams of chondroitin sulfate was added in small portions during 30 min to 20 ml of concentrated sulfuric acid with vigorous stirring at the temperatures indicated in Table I until in homogeneous state. When the reaction was carried out at below −15°, concentrated sulfuric acid containing 20% (v/v) of ether or 15% (v/v) of tetrahydrofuran was used instead of concentrated sulfuric acid to avoid solidification of the reaction medium. The reaction mixture was stirred for a certain period and then treated by one of the following methods:

A) Ether (300 ml) was added dropwise to the reaction mixture.

B) The reaction mixture was poured into ether (300 ml).

The precipitate formed was collected on a sintered glass filter, immediately dissolved in ice-water, and neutralized with 15% sodium hydroxide solution. The neutralized solution was dialyzed against five 800-ml portions of distilled water. The nondialyzable fraction was concentrated to a small volume (*ca.* 5 ml), filtered to remove some impurities, and added to 10—12 volumes of ethanol saturated with sodium acetate. The precipitate formed was collected by centrifugation, washed successively with ethanol and ether, and dried over phosphorus pentoxide *in vacuo* at 80—85° for 2 hr. The pooled dialyzable fractions were combined, concentrated to an appropriate volume (*ca.* 20—50 ml), and treated with 20% barium acetate solution to remove inorganic sulfate. After removal of barium sulfate, the supernatant was applied on a column (1.5 × 15 cm) of Dowex-50 (W-X2, 50—100 mesh, Na^+). The effluent and washings were combined, evaporated to a volume of 5—10 ml, and centrifuged to remove suspended material. The supernatant was added to 10—20 volumes of ethanol saturated with sodium acetate, the precipitate formed was collected by centrifugation, washed successively with ethanol and ether, and dried over phosphorus pentoxide *in vacuo* at 80—85° for 2 hr.

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