*Chemical Studies on Charoninsulfuric Acid**

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Introduction

T. Soda and his co-workers have found that several marine molluscs are the best materials for the preparation of sulfatases. For example, the visceral hump of *Charonia lampas* (*Tritonium nodiferm*) contains very active phenosulfatase, glucosulfatase and chondrosulfatase. So they considered that it must contain natural substrates for sulfatases. In fact, a polysaccharide sulfuric ester was obtained from the mucus of *Charonia lampas* by T. Soda and F. Egami[1] in 1938 and named

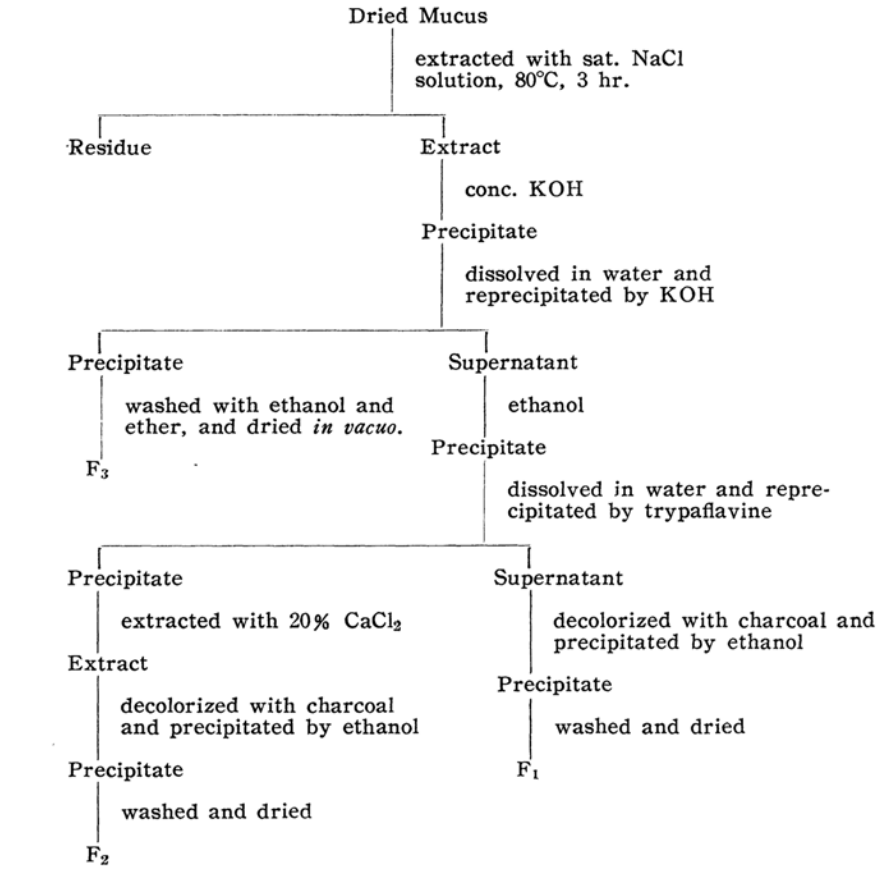
* Most part of this communication was already presented before the 27th Congress of the Biochemical Society of Japan (Kyoto, April 5, 1955) by S. Suzuki and before the 8th General Assembly of Physiolosists, Biochemists and Pharmacologists in USSR (Kiev, May 24, 1955) by F. Egami.

1) T. Soda and F. Egami, *Bull. Chem. Soc. Japan*, **13**, 652 (1938); *J. Chem. Soc. Japan*, **59**, 1417 (1938).

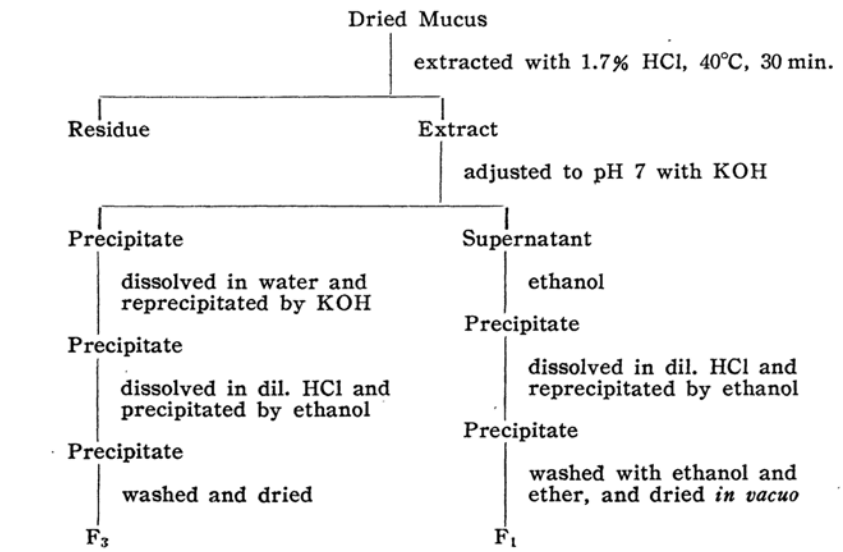
TABLE I

METHODS OF PREPARATION

Method 1.



Method 2.



charoninsulfuric acid. The chief characters of the preparation obtained by them were as follows:

Sodium charoninsulfate is free from N, and contains 15% of ethereal sulfate S and 36% of ash.

Lison's metachromatic reaction is intensely positive.

By sulfatase preparation of *Charonia lampas*, it liberates sulfuric acid and gives reducing sugars, one of which is regarded as D-glucose from the specific optical rotation and its fermentability by baker's yeast.

It has a strong inhibitory action on blood coagulation.

From these results, they considered charoninsulfuric acid to be a desamino-mucoitin polysulfuric acid.

In 1948, the further investigation on the chemical nature of charoninsulfuric acid was undertaken by T. Soda and H. Terayama[2]. The most important contribution by them was the discovery that the purified preparation did not contain uronic acid and the polysaccharide fraction gave only D-glucose after acid hydrolysis. Moreover they desulfated charoninsulfuric acid by heating it in methanolic hydrogen chloride and obtained a white powder, which gave an X-ray diffraction pattern identical to that of cellulose. From this and other qualitative results, they concluded that charoninsulfuric acid is a cellulose sulfate or sulfuric ester of a glucan quite similar to cellulose.

In 1954 we repeated this investigation in order to ascertain the conclusion of Soda by chemical and enzymatic methods. It is the purpose of the present paper to describe our experiments and results.

Experimental*

I. Preparation and General Properties

Material.—The mucus of *Charonia lampas* was defatted and dried by hot alcohol and acetone: N, 5~10%; ethereal sulfate S, 6~7%. The mucus may be regarded as a mucoprotein.

Extraction.—Charoninsulfuric acid can be extracted from the dried mucus by concentrated mineral salt solution or diluted mineral acid.

Methods of Separation.—As will be shown later, charoninsulfuric acid is not a definite chemical individual, but a mixture of glucan sulfates with different sulfur contents and different solubilities, and hence this had to be taken into consideration when preparing samples. Our methods of preparation were as follows:

1) Salting out by KCl: Charoninsulfuric acid with high sulfur content can be salted out by KCl as the potassium salt.
2) Precipitation by Trypaflavine: Charoninsulfuric acids, except those of very low sulfur content (ca 2%), are precipitated with trypaflavine, and then from this precipitate charoninsulfuric acid with a relatively low sulfur content may be extracted by concentrated $CaCl_2$ solution.
3) Precipitation by KOH: Charoninsulfuric acid with high sulfur content can be precipitated by KOH at the concentration of ca 0.5 N.
4) Precipitation by Benzidine: The benzidine salt of charoninsulfuric acid with high sulfur content is also difficultly (less difficultly than trypaflavine salt) soluble. Charoninsulfuric acid may be extracted from the precipitate by NaOH solution.
5) Precipitation by Ethanol: Charoninsulfuric acid is precipitated with an equal volume of ethanol from aqueous solutions.

Combining these methods of fractionation, various charoninsulfuric acids with different sulfur contents were obtained. Typical methods of preparation are shown in Table I. The properties of charoninsulfuric acids with different sulfur contents are summarized in Table II.

TABLE II

PROPERTIES OF CHARONINSULFURIC ACIDS

Charonin-sulfuric acids	S content %	Salting out by KCl	Precipi-tation by KOH	Precipi-tation by trypaflavine	I_2-KI reaction
F_1	1~ 2	−	−	−	red
F_2	2~ 5	+	−	+	−
F_3	10~18	+	+	+	−

The possibility that while charoninsulfuric acid itself is a substance with definite sulfur content, the preparations thus obtained are mixtures of charoninsulfuric acid and glycogen (or similarly nonsulfated glucans) need not be considered because of the following facts:

1) Charoninsulfuric acid with low sulfur content (2~5%) is precipitated by trypaflavine, and from the precipitate charoninsulfuric acid is extracted by $CaCl_2$ solution. Calcium charoninsulfate is obtained by alcohol precipitation. Even by repetition of this process, we could not separate any nonsulfated glucans and obtained the same charoninsulfate with a low sulfur content.
2) By Grassman's continuous electrophoresis on paper[3], we could confirm the fact that glycogen moves towards the cathode (probably owing to the electroendosmose) and charoninsulfuric acids towards the anode. The latter, when mixed with the former, can be separated easily by the apparatus.

Thus we have been led to the conclusion that there are various charoninsulfuric acids with different sulfur contents. Sodium charoninsulfate with the highest sulfur content obtained so far contained 20.3% ethereal sulfate sulfur.

II. Investigation on the Chemical Nature of the Glycan.

Desulfation.—Charoninsulfuric acid with a high sulfur content (more than 5%) can be desulfated by heating it in methanol-HCl (1~1.5%) for several hours. The desulfated product was named "charonin". It is dissolved in Schweit-

2) T. Soda and H. Terayama, *J. Chem. Soc. Japan*, **69**, 65 (1948).

* A part of the experiments was executed in the Marine Biological Laboratory of Nagoya University.

3) W. Grassman and K. Hanning, *Z. Physiol. Chem.*, **292**, 32 (1953).

zer's reagent. When the solution was neutralized with acetic acid, a part of the charonin was precipitated. From the supernatant a further amount of precipitate was produced by adding methanol. The former was washed with diluted HCl, methanol and ether, and the white powder thus obtained was provisionally named Fraction A. The latter was purified by repeated reprecipitation and named Fraction B. So far the analysis for various charoninsulfuric acids with different sulfur contents showed that the less the sulfur content was the less the proportion of Fraction A to Fraction B (*vide infra*); fraction F_1 with the lowest sulfur content may be regarded as Fraction B itself. The properties of charonin, Fraction A, and Fraction B are summerized in Table III. It is remarkable that Fraction B gives a red color with iodine solution. Because Fraction A does not inhibit the color reaction of Fraction B, it seems that charonin is probably not a simple mixture, but a compound of Fraction A and Fraction B, which are combined with a rather weak bond and decomposed by the action of Schweitzer's reagent. The weak reducing power of Fraction A and B may be attributed to the hydrolysis by Schweitzer's reagent.

TABLE III

PROPERTIES OF CHARONIN, FRACTIONS A AND B

Substances	I_2–KI reaction	I_2–KI–H_2SO_4 reaction	$ZnCl_2$–I_2 reaction	Reducing power*
Charonin	negative	yellow	yellow	0.00
Fraction A	negative	blue	blue	0.02
Fraction B	red	red	red	0.03
Cellulose	negative	blue	blue	
Amylose	blue	blue	blue	
Starch	purple	purple	purple	

* The value represents mg. glucose which corresponds to the reducing power per mg. of the substance (Somogyi's method (4)).

Constitution of Fraction A.—The desulfated preparation used for X-ray investigation by Soda and Terayama corresponds to this fraction.

It was confirmed by paper chromatography that sulfuric acid hydrolysis of this fraction resulted in the exclusive production of D-glucose:

Solvents, *n*-BuOH: AcOH: H_2O (4 : 1 : 2), *n*-BuOH: Ether: H_2O: Ammonia (40 : 10 : 49 : 1), *n*-BuOH: Pyridine: H_2O (6 : 4 : 3)

Aniline hydrogen phthalate was used to locate the sugar.

Octaacetylcellobiose (m.p. 220°C) was obtained by acetolysis of Fraction A. No melting point depression occured when mixed with octaacetylcellobiose prepared from filter paper. Moreover as shown in Table IV, it was hydrolysed by Irpex-cellulase prepared by the method of Nisizawa[5], but not at all by crystalline α-amylase (Taka-amylase) prepared by the method of Akabori[6] and sweet potato β-amylase prepared by the method of Balls[7]. In the reaction mixture after hydrolysis by Irpex-cellulase, glucose and cellobiose were detected by paper chromatography. Cellobiosazone (m.p. 210°C) was obtained after fermenting out the glucose by yeast (Rasse XII). It should be noted here that this Fraction A gives, similar to cellulose, a blue color reaction with I_2-KI-H_2SO_4 reagent.

TABLE IV

HYDROLYSIS BY IRPEX-CELLULASE

Substrate	Substrate concentration. %	Incubation time, hrs.	Increase of reducing power* per mg. of substrate
Charonin	0.50	72	0.19
Fraction A	0.50	72	0.33
Fraction B	0.50	72	0.04
Cellobiose	0.12	137	0.00
Soluble starch	0.25	66	0.00
Laminarin	0.30	66	0.00
Hydrocellulose	0.50	24	0.18
Carboxymethyl-cellulose	0.25	24	0.45

Conditions: 0.1 M acetate buffer, pH 4.0, temp. 30°C.

* The reducing power was calculated as mg. cellobiose (Somogyi's method).

Notwithstanding these findings confirming the existence of cellulose structure in Fraction A, several properties of the fraction are not consistent with a cellulose structure. For example, as shown in Table V, the periodate consumption was lower than expected from a cellulose structure.

TABLE V

PERIODATE CONSUMPTION BY FRACTIONS A AND B

Substance	Reaction time, hrs.	Periodate consumption, mol. per glucose unit
Fraction A	120	0.65
Fraction B	68	0.93
Cellulose	93	1.08

4) M. Somogyi, *J. Biol. Chem.*, **160**, 61 (1945).

5) K. Nisizawa and T. Kobayasi, *J. Agr. Chem. Soc. Japan*, **27**, 239 (1953): K. Nisizawa, T. Kobayasi and N. Ichikawa, *Symposia on Enzyme Chem.*, **10**, 7 (1954).

6) S. Akabori, B. Hagihara and T. Ikenaka, *Proc. Japan Acad.*, **27**, 350 (1951).

7) A.K. Balls, R.R. Thompson and M.K. Walden, *J. Biol. Chem.*, **173**, 9 (1948).

So the constitution of Fraction A cannot yet be established completely and it must be further studied. However we may be able to conclude that the main part of Fraction A has a cellulose structure, i.e. β-1, 4′-glucan constitution.

Constitution of Fraction B.—Fraction B, which is soluble in water and gives a red iodine reaction, was also found to be composed entirely of glucose (by reducing power measurement and by paper chromatography after sulfuric acid hydrolysis). As previously shown in Table IV, this fraction was also, although far less easily than Fraction A, hydrolysed by Irpex-cellulase to give glucose and cellobiose. The most remarkable characteristic of this fraction is the fact that it was hydrolysed by α-and β-amylases (Table VI). In the reaction mixture after hydrolysis by both amylases, maltose and relatively small amounts of glucose were detected by paper chromatography. Because this fraction consumed periodate as expected from an 1, 4′-glucoside glucan structure (Table V), it seems to contain amylose structure. From these results, Fraction B may be regarded as containing both α-1, 4′- and β-1, 4′-glucoside bonds.

TABLE VI

ACTIONS OF α-AND β-AMYLASES

Substrate	Increase of reducing power* per mg. substrate	
	α-Amylase	β-Amylase
Charonin	0.02	0.00
Fraction A	0.00	0.00
Fraction B	0.36	0.09
Glycogen	0.32	0.09

Conditions: Substrate conc. 0.13%, M/30 acetate buffer, pH 5.6 (α−), 4.7 (β−), incubation time 18 hrs., temp. 37°C; reaction mixtures contained 0.055% NaCl in the case of α-amylase.

* The reducing power was calculated as mg. maltose (Somogyi's method).

III. Discussion on the Constitution of Charoninsulfuric Acid

From the foregoing results, it is quite certain that charoninsulfuric acid is a mixture of glucan polysulfates with different sulfur contents. The nature of the glucan seems to be very complex. A part of it has a cellulose structure and the other part an amylose structure; however the nature of the linkage combining the two parts are as yet unknown. At any rate, it is decomposed by Schweitzer's reagent after desulfation into two parts, i.e. Fraction A, insoluble in water, composed mainly of cellulose structure, and Fraction B, soluble in water, composed mainly of amylose structure. So far as we have analysed various charoninsulfuric acids, we have found that a parallelism exists between the sulfur content and the cellulose structure (Table VII). It seems that biological sulfation may be preceded or accompanied by biological transformation of amylose to cellulose.

TABLE VII

THE YIELDS OF FRACTIONS A AND B FROM VARIOUS CHARONINSULFURIC ACIDS WITH DIFFERENT SULFUR CONTENTS

Material	S Content %	Yield* of Fraction A	Yield* of Fraction B
F_3	10.0	214	43
	9.2	200	50
F_2	4.0	12	270
F_1	1.1	0	390

* The values represent mg. each fractions obtained from 1 g. of charoninsulfulic acids.

The question might arise, whether a Walden inversion may take place during the desulfation process of charoninsulfuric acids. However, because the preparation F_1 with only one to two % of sulfur gave only glucose after direct hydrolysis by sulfuric acid, it is quite reasonable to assume that glucose must exist as such in charoninsulfuric acid.

Summary

We have obtained charoninsulfuric acid free from glycogen (or similar glucans) and confirmed that it is a mixture of glucan polysulfates with different sulfur contents. The desulfated product (charonin) was decomposed by Schweitzer's reagent into two parts, Fractions A and B. From Fraction A, which is insoluble in water and gives a blue color reaction with I_2-KI-H_2SO_4, glucose (after hydrolysis by sulfuric acid), cellobiose (after hydrolysis by Irpex-cellulase) and octaacetylcellobiose (after acetolysis) were obtained. From Fraction B, which is soluble in water and gives a red iodine reaction, glucose, cellobiose and maltose (after hydrolysis by amylases) were obtained. This fraction consumed periodate as expected from 1, 4′-glucoside glucan. From these results, this glucan (charonin) may be regarded as having both cellulose and amylose structure. Moreover it was found by analysis of various charoninsulfuric acids that a close relation existed between the sulfur content of charoninsulfuric acid and the constitution of glucan, i.e. the less the sulfur content was, the less was the cellulose structure.

We wish to express our thanks to Prof. T. Soda for his kind encouragement and interest. We gratefully acknowledge the gift of amylases by Prof. S. Akabori and amylose by Prof. Z. Nikuni. The expense of this study was defrayed in part by a grant from Seikagaku-Kenkyujo Ltd., to which our thanks are due.

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