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The Sulfation of Chitin in Chlorosulfonic Acid and Dichloroethane

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RECEIVED MARCH 29, 1954

Chitin has been sulfated in a mixture of chlorosulfonic acid and dichloroethane. Some properties of the sulfated product have been noted.

Chitin has for many years been regarded as an attractive starting material for the synthesis of heparin-like anticoagulants by sulfation[1] because the acetylglucosamine unit of chitin provided at least some resemblance to the glucosamine moiety of heparin.[2] The recently reported experiments on the sulfation of chitosan[3] provide a sulfated anticoagulant which should resemble heparin more closely because sulfated chitosan contains sulfamic groups similar to those present in heparin.

It has been shown[4] by workers in this field that the average molecular weight of sulfated polysaccharides must be lowered to avoid the toxicity encountered in early work. Piper[5] concluded that sulfated chitin was unsuitable for use *in vivo* because of certain evidences of toxicity.

In our re-examination of the sulfation of chitin we hoped that it might be possible by lowering the average molecular weight of the product to avoid the toxicity observed by Piper. This hope was realized, but other signs of toxicity were still seen even at the lower molecular weight.[6]

We first examined the conventional pyridine–chlorosulfonic acid sulfation, varying the time and temperature of reaction, in order to obtain products of varying molecular weight. The main disadvantages of this process are, first, a lack of reproducibility and, second, a very poor color of the finished product due to interaction of pyridine with the polysaccharide at the elevated temperature.

We then turned to sulfation procedures which did not require pyridine. Claesson[7] sulfated cellulose and other polysaccarides in undiluted chlorosulfonic acid. Hadidian[8] sulfated hyaluronic acid in the same sulfating agent. The procedure to be described differs from those used by Claesson and Hadidian in the use of an inert diluent which increased the ease of control of the sulfation. Meyer, Piroue and Odier[9] used sulfur dioxide as solvent for chlorosulfonic acid in the sulfation of chondroitin sulfuric acid. Pulver[10] used formamide as solvent for chlorosulfonic acid in the same sulfation. In these latter examples, the aim was to avoid degradation of chain length of the polysaccharide. In contrast, the procedure described below is of value because the degradation of chain length necessary in this case goes on simultaneously with sulfation at or below room temperature.

(1) The short name "sodium chitin sulfate" will be used to describe the sodium salt of the product obtained on esterification of poly(1-4)N acetylglucosamine by chlorosulfonic acid.

(2) (a) S. Bergstrom, *Naturwissenschaften*, **23**, 706 (1935); (b) P. Karrer, H. Koenig and E. Usteri, *Helv. Chim. Acta*, **26**, 1296 (1943); (c) T. Astrup, I. Galsmar and M. Volkert, *Acta Physiol. Scand.*, **8**, 215 (1944).

(3) (a) J. Doczi, A. Fischman and J. A. King, THIS JOURNAL, **75**, 1512 (1953); (b) M. L. Wolfrom, T. M. Shen and C. G. Summers, *ibid.*, **75**, 1519 (1953); (c) L. L. Coleman, L. P. McCarty, D. T. Warner, R. F. Willy and J. H. Flokstra, Abstracts, Am. Chem. Soc., 123rd Meeting, page 19L.

(4) (a) K. N. von Kaulla and E. Husemann, *Experientia*, **2**, 222 (1946); (b) T. Astrup and J. Piper, *Acta Physiol. Scand.*, **9**, 351 (1945); (c) K. W. Walton, *Br. J. Pharmacology and Chemother.*, **7**, 370 (1952).

(5) J. Piper, *Acta Pharmacol. Toxicol.*, **2**, 317 (1946).

(6) L. W. Roth, I. M. Shepperd and R. K. Richards, *Proc. Soc. Exp. Biol. Med.*, **86**, 315 (1954).

(7) P. Claesson, *J. prakt. Chem.*, [2] **19**, 1 (1879).

(8) Z. Hadidian, U. S. Patent 2,599,172.

(9) K. H. Meyer, R. P. Piroue and M. E. Odier, *Helv. Chim. Acta*, **35**, 574 (1952).

Experimental

To 100 ml. of redistilled dichloroethane (b.p. 82–84°) and 20 ml. of redistilled chlorosulfonic acid maintained under dry nitrogen at 10° was added 8 g. of chitin,[11] previously dried in high vacuum over concentrated sulfuric acid. The bath temperature was raised to 25° and sulfation was continued for two hours with stirring.

The stirrer was then stopped, and the solvent and unused chlorosulfonic acid were removed by nitrogen pressure through a siphon. The residue was stirred with 100 ml. of fresh dichloroethane for 5 minutes. This dichloroethane was removed and a second 100-ml. portion of fresh dichloroethane added. The bath was cooled to 0° and 70 ml. of dry redistilled triethylamine was added dropwise at such a rate that the inside temperature did not rise above 20°; 40 ml. of water was then added.

To the water-dichloroethane-triethylamine emulsion was added 320 ml. of isopropyl alcohol. The product was allowed to settle, or was centrifuged, the supernatant was decanted, and the sirup was triturated with 150 ml. of isopropyl alcohol. The sirup was dissolved in 240 ml. of 75% aqueous ethyl alcohol, and 20 ml. of 5 *N* sodium hydroxide was added. The sirupy sodium salt of chitin sulfate was allowed to settle for one hour, the supernatant was decanted carefully and the product was taken up in water and dialyzed in a cellophane membrane against distilled water until free of inorganic sulfate. The aqueous solution was brought to neutral *p*H with a small amount of 1 *N* sodium hydroxide. The solution was then concentrated *in vacuo* to 100 ml. and the product was precipitated by addition of 400 ml. of redistilled denatured ethanol[12] and 0.6 ml. of saturated aqueous sodium chloride. The finely divided amorphous precipitate was collected on a sintered glass funnel and washed with 200 ml. of redistilled absolute denatured ethanol[13] and 50 ml. of diethyl ether.

For the physical data, see Table I, product 1. Since the product cannot be precisely defined, a range of observed values is given rather than single values. Measurements of viscosity of aqueous solutions of the product were carried out in Ostwald-Fenske viscosimeters in a constant temperature bath at 25°.

TABLE I

DATA ON SAMPLES OF SODIUM CHITIN SULFATE

Product	1	2	3	4
Yield, g.	11–12	10.5–11.5	9.5–11.0	8–9
Sulfur, %	13.2–14.5	14.5–15.0		13.9–14.5
Nitrogen, %	2.5–3.2			
In vitro potency				
I.U./mg.	26–34	26–30	22–26	24–27
η_{sp}/c, *c* 1, water, 25°	0.30–0.38	0.26–0.30	0.25–0.30	0.35–0.40
η_{sp}/c, *c* 1, 25° 0.9% NaCl,	0.14–0.16			
$[\alpha]^{25}D$, *c* 5.0 water	−25°			

(10) R. Pulver, U. S. Patent 2,612,499.

(11) Bioproducts Oregon, Ltd., Warrenton, Oregon.

(12) 5% methanol–5% water–90% ethanol by volume.

(13) Approximately 5% methanol to 95% ethanol by volume.

Stability of Sodium Chitin Sulfate.—In contrast to some sulfated polysaccharides, the sodium salt of chitin sulfate had only a moderate stability. The solid product stored in ordinary screw top containers showed a slow drop in anticoagulant potency. In unbuffered aqueous solutions stored at room temperature the *p*H fell because of the release of sulfate groups, and the fall of potency and viscosity was accelerated by the resulting acidity.

The stability of aqueous solutions could be improved by the following treatment. To the dialyzed solution, concentrated to 100 ml. as described above, was added sufficient 1 *N* sodium hydroxide to raise the *p*H of the solution to 11.0-11.5. The product was then precipitated from solution with the amounts of alcohol and saturated salt used previously. It was necessary to redissolve and reprecipitate the product to reduce the excess alkalinity still present in the solid. The finely divided precipitate was washed with alcohol and ether and was dried in high vacuum over concentrated sulfuric acid. The potency as well as the relative viscosity of a solution of the stabilized product was unchanged from the values recorded for unstabilized material. When a 5% aqueous solution of the product was autoclaved for 20 minutes at 15 lb. gage pressure, the *p*H of the solution fell from 8.5 to 6.5 with only a 5% drop in anticoagulant activity. In contrast to this, the *p*H of a solution of unstabilized material, adjusted approximately to the same starting *p*H, fell from 8.6 to 1.8 with complete loss of anticoagulant activity.

Solutions of similar good stability were also obtained by omitting dialysis. The sodium salt was precipitated from the 75% alcohol solution of the triethylamine salt of chitin sulfate as already described. When the sirupy product was taken up in 100 ml. of water, sufficient residual sodium hydroxide remained to give a solution of the desired *p*H range, 11.0-11.5. The product was precipitated by addition of 400 ml. of denatured ethanol,[12] and 0.6 ml. of saturated NaCl solution, and was filtered off, redissolved and reprecipitated. For data on this type of product see product 2 of Table I.

Peroxide Treatment of Chitin Sulfate.—Chitin sulfate prepared as described above gave yellow aqueous solutions at a concentration of 5%. This color could be improved by a peroxide treatment carried out in the following manner. To the 240 ml. of the solution of the triethylamine salt in 75% ethanol was added 4 ml. of 30% hydrogen peroxide. After 30 minutes, 5 g. of Darco G-60[14] decolorizing carbon was added, and the solution was stirred for ten minutes, and was filtered through a tightly packed bed of Solkafloc SW-40A[15] on a sintered glass funnel. The sodium salt was precipitated as a sirup from the filtrate by addition of 5 *N* sodium hydroxide in the manner already described, and was either subjected to dialysis followed by alcohol precipitation (product 3, Table I) or was simply twice precipitated from aqueous solution (product 4). Stabilization of dialyzed samples was carried out in the manner already described. Peroxide treated samples gave 5% aqueous solutions which appeared colorless to the eye.

Absorption Spectrum.—The absorption spectrum exhibited no peaks and the absorption declined rapidly from very high values at 220 mμ to low readings at 600 mμ. A wave length of 340 mμ was chosen arbitrarily for measurements of absorbance. Readings were made in a Beckman model CU spectrophotometer. Typical values of the absorbance of 1% aqueous solutions at 340 mμ in a 1-cm. cell are as follows: peroxide treated, dialyzed, 0.03; non-peroxide treated, dialyzed, 0.29.

Nitrogen Assay.—The nitrogen assay of dialyzed samples varied from 2.5 to 3.2% and the molecule still contained some triethylamine. When the sulfated product was dialyzed against a large excess of sodium hydroxide (0.1 *N*) or sodium acetate, and then against distilled water, the nitrogen content could be reduced to 2.3%. The theoretical nitrogen value for a molecule with sulfur of 14.5% is 3.6%. Therefore, one-third of the nitrogen has been cleaved by this method of sulfation. The nature of the cleavage product is not known. Paper chromatography of hydrolysates failed to show the presence of either glucose or desoxyglucose.

Molecular Weights.—Light scattering measurements were carried out on dialyzed sodium salts by Mr. G. H. Barlow of the Department of Physical Chemistry. Only samples not treated with peroxide were used. Measurements were made on solutions between 1 and 1.5% concentration in 0.9% sodium chloride. Separate portions of the salts were dried in high vacuum over concentrated sulfuric acid and molecular weights were corrected for moisture content.

Some osmometric measurements of molecular weights in sodium chloride also were done by Mr. E. C. Olson of the Department of Physical Chemistry, with results from 4 to 8% below the light scattering values.

TABLE II

MOLECULAR WEIGHTS AND VISCOSITIES OF CHITIN SULFATE

Sample	η^{sp}/c, c 1% in water	η_{sp}/c, c 1% in NaCl, 0.9%	Average molecular weight
1	0.85	0.25	17,100[16]
2	.30	.14	12,100
3	.35	.14	14,800
4	.35	.15	14,700

Acknowledgment.—Our thanks are due to Mr. E. F. Shelberg and staff for microanalyses, to Mr. G. H. Barlow for molecular weight determinations, and to Dr. L. W. Roth and Miss I. M. Shepperd for Pharmacological tests, and to Messrs. R. T. Olsen and R. A. Rieker for potency assays.

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(14) Atlas Powder Co., New York 17, N. Y.

(15) Brown Co., Berlin, New Hampshire.

(16) Reaction conditions, 2 hours at 0° in dichloroethane and chlorosulfonic acid.