lected indicated it was still somewhat impure. The literature[12] reports b_4 60–75°.

2,4-Dinitrophenylosazone. 2,4-Dinitrophenylhydrazine (0.1 g.), 5 ml. of 95% ethanol, 1 ml. of water and 0.5 ml. of concentrated hydrochloric acid were heated on a steam cone until complete solution was obtained and then *ca.* 0.1 g. of VIII was added. The osazone derivative started precipitating in about 5–10 min., but heating was continued until no more precipitate formed. The precipitate filtered, and recrystallized from ethanol–ethyl acetate and ethanol gave bright orange crystals, m.p. 240–241° (dec.). The literature reports m.p. 235–236° (dec.)[13] and m.p. 242°.[8]

Anal. Calcd.: C, 42.86; H, 3.39; N, 23.52. Found: C, 42.94; H, 3.25; N, 22.76.

The same procedure repeated with IV, VI, and IX gave the same derivative as shown by melting point and mixture melting point.

Reaction of ethanol with products of reaction of N-bromosuccinimide with I. *N*-Bromosuccinimide was treated with I (1.05 g.) in 50 ml. of carbon tetrachloride as previously described. The reaction mixture was cooled and 134 mg. of succinimide (13.5 %) filtered off. The filtrate was stripped of solvent and the residue refluxed overnight with 25 ml. of absolute ethanol. Ethanol was removed by distillation and the residue extracted with carbon tetrachloride and water. The carbon tetrachloride layer dried over magnesium sulfate and distilled gave 0.36 g. (17%) of VIII, $b_{1.2}$ 51–54°, n_D^{25} 1.4710. The product was confirmed as VIII by comparison of its infrared spectrum with the product obtained from the reaction of *N*-bromosuccinimide with I in ethanol.

The water extract, distilled free of water and crystallized from 95% ethanol gave 121 mg. (13%) succinimide.

Reaction of ethanol with VI. Five ml. of absolute ethanol and 6.3 mg. of VIb were combined and refluxed overnight. The mixture was distilled free of most of solvent and then poured into an evaporating dish. The air dried residue taken up in 95% ethanol and chilled gave VIb, m.p. 127–129°. Identification was based on the melting point and comparison of the infrared spectrum with that of the starting material.

The same procedure repeated with VIa gave similar recovery of only starting material when the residue was crystallized from aqueous ethanol.

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(12) J. G. M. Brenner and D. G. Jones, Brit. Patent 605,107 (1948).

(13) S. Swadesh and A. P. Dunlop, *J. Org. Chem.*, **14**, 692 (1949).

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE UPJOHN CO.]

Selective Sulfonation of Amino Groups in Amino Alcohols

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The use of the pyridine–sulfur trioxide complex as a sulfonating reagent for amino alcohols has been investigated in an aqueous alkaline medium. Under these conditions, the reagent selectively sulfonates the amino group. The reaction is applicable to insoluble or soluble amino alcohols, and has also been applied to the hydroxyamino acid, serine.

The discovery of sulfonated amino groups in heparin,[1] a naturally occurring sulfated polysaccharide having alternating glucosamine and glucuronic acid units, has stimulated considerable interest in the preparation of synthetic compounds having similar constituents. For the preparation of such sulfated polysaccharides, one of the starting materials has been chitosan, the polyglucosamine which is readily obtained by alkaline hydrolysis of chitin, a naturally occurring polyacetylglucosamine present in crab shells.

In our earlier work with chitosan[2] we reported the sulfation of this polyglucosamine as a heterogeneous phase with liquid sulfur dioxide–sulfur trioxide. In this system the reaction of the amino groups was not complete, and the yields of product were variable because of the heterogeneous reaction medium. Doczi[3] and coworkers reported the preparation of sulfated chitosans having a high degree of sulfonation on the amino group, although the details of their procedure were not disclosed. Wolfrom[4] also obtained an *N*-sulfated-*O*-sulfated chitosan by the use of chlorosulfonic acid and pyridine in a heterogeneous system.

In attempting to obtain improved yields of sulfated chitosans having a high degree of *N*-sulfonation, we have studied a number of sulfonation systems. From these experiments, a method has been obtained for the selective sulfonation of amino groups in the presence of hydroxyl groups which now appears to be a reaction of general application. The procedure makes use of a well-known reagent, the pyridine–sulfur trioxide complex,[5] which has been previously reacted with both amines and

(1) (a) J. E. Jorpes, H. Bostrom, and V. Mutt, *J. Biol. Chem.*, **183**, 607 (1950). (b) K. H. Meyer and D. E. Schwartz, *Helv. Chim. Acta*, **33**, 1651 (1950).

(2) L. L. Coleman, L. P. McCarty, D. T. Warner, R. F. Willy, and J. H. Flokstra, presented before the Division of Medicinal Chemistry of the American Chemical Society, Los Angeles, Calif., March 1953; see Abstracts of Papers, 123rd Meeting, p. 19 L.

(3) J. Doczi, A. Fischman, and J. A. King, *J. Am. Chem. Soc.*, **75**, 1512 (1953).

(4) M. E. Wolfrom, T. M. Shen, and C. G. Summers, *J. Am. Chem. Soc.*, **75**, 1519 (1953).

(5) P. Baumgarten, *Ber.*, **59**, 1976 (1926).

alcohols. However, when it is utilized in an aqueous medium under mild alkaline conditions (*p*H 9–10), pyridine–sulfur trioxide has been found to sulfonate the amino group exclusively in all of the amino alcohols we have tested.

The reaction of pyridine–sulfur trioxide in aqueous alkaline medium is useful with insoluble or soluble amino alcohols. In fact our first application of the process was to the *N*-sulfonation of the insoluble polyglucosamine, chitosan. An aqueous suspension of chitosan at *p*H 9–10 was stirred at room temperature while small portions of pyridine–sulfur trioxide were added together with dilute sodium hydroxide to maintain the alkalinity.[6] Under these mild conditions the insoluble chitosan was readily converted to the sodium salt of the soluble *N*-sulfochitosan; and as the reaction proceeded, the product dissolved in the aqueous medium so that a fresh surface for reaction was continually exposed on the remaining insoluble particles. When approximately 50% of the amino groups had reacted the solid phase had disappeared; and by continuing the addition of the pyridine–sulfur trioxide at *p*H 9–10, a nearly quantitative conversion of chitosan to *N*-sulfochitosan was obtained.

The resulting product contained one sulfamate group for each hexosamine unit, uniformly distributed throughout the polysaccharide chain; and it could be precipitated as a finely divided amorphous powder from a 1% salt solution with 2 volumes of ethanol. The *N*-sulfochitosan has no detectable *in vivo* or *in vitro* anticoagulant activity. However, the amorphous product is readily obtained in a highly reactive particulate form, which can be further sulfated with sulfur dioxide–sulfur trioxide to yield *N*-sulfated, *O*-sulfated chitosans. The latter have considerable anticoagulant activity. An example illustrating the use of the sulfur dioxide–sulfur trioxide procedure with *N*-sulfochitosan is given in the experimental section.

The selective *N*-sulfonation of diethanolamine, 3-aminopropanol, and DL-serine also has been successfully carried out with the aqueous pyridine–sulfur trioxide system. It is interesting to note that amino alcohols may be selectively *O*-sulfated with chlorosulfonic acid in carbon tetrachloride by the procedure of Reeves and Guthrie.[7] Two of the amino alcohols used by these workers were also included in our experiments for comparison purposes. Therefore, it is possible to convert amino alcohols to either *N*-sulfo or *O*-sulfo derivatives by the proper selection of reagents.

(6) Excess alkali should be avoided to prevent extensive conversion of pyridine–sulfur trioxide to the red complex described by P. Baumgarten, *Ber.*, **59**, 1166 (1926). Sodium carbonate may also be used as the alkaline reagent.

(7) W. A. Reeves and J. D. Guthrie, *J. Am. Chem. Soc.*, **75**, 4101 (1953).

EXPERIMENTAL

Preparation of chitosan. Chitin[8] (95 g.) was suspended in about 1.5 l. of 50% sodium hydroxide, and the suspension was refluxed for 24 hr. with stirring. After cooling, the hydrolyzate was poured into 8 l. of water, and the insoluble chitosan was allowed to settle overnight. The supernatant liquid was discarded, and the residual solid was washed four times by decantation with water (5-l. portions) and four times with ethanol (500-ml. portions). The chitosan was then washed once with benzene and dried in a vacuum oven at 50°. The product weighed 62.8 g. (84%).

Anal. Calcd. for $(C_6H_{11}O_4N)_x$: C, 44.7; H, 6.88; N, 8.69; N as $—NH_2$, 8.69; acetyl, 0.0. Found: C, 47.1; H, 7.05; N, 7.97; N, as $—NH_2$, 8.01; acetyl, 1.31 (equivalent to about 6% of the amino groups present).

N-Sulfochitosan. A suspension of 54 g. of chitosan in 1000 ml. of water was dissolved by the addition of 30 ml. of concentrated hydrochloric acid. The chitosan hydrochloride was then converted to a flocculent precipitate of free chitosan by the gradual addition of 48 ml. of 30% sodium hydroxide with stirring. The suspension was stirred thoroughly at room temperature, and pyridine–sulfur trioxide was added to the aqueous medium in small portions. As the pyridine–sulfur trioxide reacted, the *p*H of the reaction medium was maintained in the range 9–10 by the continuous slow addition of a 30% sodium hydroxide solution. In this way a total of about 280 g. of pyridine–sulfur trioxide and about 465 ml. of 30% aqueous sodium hydroxide were added over a period of about 20 hr. During the course of the additions, the chitosan reacted completely and a clear, light brown solution resulted. This solution was concentrated *in vacuo* to about 1.5 l., and the concentrate was dialyzed in cellulose casings against deionized water to remove the sodium sulfate, pyridine, and other lower molecular weight materials. The brown solution in the dialysis bag was concentrated *in vacuo* to 1260 ml. and adjusted to a *p*H of 9–10. Sodium chloride (12.6 g.) was dissolved in the clear solution, and the product was precipitated as a light tan solid by the addition of 2.5 l. of absolute ethanol. The mixture was refrigerated overnight; and the solid was then removed by filtration, and washed with two portions of absolute ethanol and three portions of acetone on the filter. After drying *in vacuo* at 50°, the product weighed 81.1 g. (92% yield).

Anal. Calcd. for $[(C_6H_{10}O_4N)SO_3Na]_x$: C, 27.37; H, 3.82; N, 5.32; N as $—NH_2$, 0.0; S, 12.18. Found: C, 27.78; H, 4.05; N, 5.66, N as $—NH_2$, 0.18; S, 12.35.

Sodium N-bis(2-hydroxyethyl)sulfamate. Diethanolamine (10.5 g.) was dissolved in 125 ml. of water (*p*H 10.8). This solution was stirred at room temperature and pyridine–sulfur trioxide was added in small portions together with dilute sodium hydroxide to maintain the *p*H in the range of 9–10. A total of 17.7 g. of pyridine–sulfur trioxide and 52 ml. of 10% sodium hydroxide were added over a period of 1.5 hr.

The reaction mixture was then concentrated *in vacuo* to 100 ml. to remove excess pyridine. Sodium chloride (1 g.) was dissolved in the clear solution, and 500 ml. of absolute ethanol was added. After refrigerating the mixture overnight, 3.3 g. of solid was removed. This solid contained considerable inorganic material. The filtrate was mixed with 400 ml. of acetone, and crystals began to separate. After refrigerating the solution overnight 9.05 g. of solid was removed by filtration. The silvery leaflets melted at 218–220° on a Fisher block. The yield of pure product was 44%.

Anal. Calcd. for $C_4H_{10}O_5NSNa$: C, 23.19; H, 4.86, N, 6.76; Na, 11.10. Found: C, 23.23; H, 5.04; N, 7.10; Na, 11.10.

DL-*N-Sulfoserine.* DL-Serine (10.2 g.) was suspended in 100 ml. of water and dissolved by the addition of 36 ml. of

(8) The chitin was obtained from Bioproducts, Ltd., Warrenton, Ore., and was ground in a Wiley mill using a 100-mesh sieve before the deacetylation.

10% sodium hydroxide solution to yield a clear solution with *p*H 10.05. This clear solution was then reacted with 18.1 g. of pyridine-sulfur trioxide which was added in portions over about 3.75 hr. During this addition the *p*H was regulated in the range of about 9 to 10 by the continuous addition of 10% sodium hydroxide solution. At the end of this reaction time, the light yellow solution was concentrated *in vacuo* to 225 ml., and 525 ml. of absolute ethanol was added to yield a slightly milky supernatant liquid and an oily layer. The supernatant liquid was decanted and discarded, and the oily layer was thoroughly mixed with acetone and refrigerated overnight to yield 23.3 g. of solid product. Ten grams of this solid product was heated to boiling with 100 ml. of absolute ethanol and the milky supernatant liquid was decanted and discarded. The residual solid was then extracted twice with 150-ml. portions of aqueous ethanol (60 parts ethanol and 40 parts water by volume). These two extracts were combined, and treated with acetone to incipient turbidity at room temperature. After about 1 hr., a small quantity of solid material was removed by filtration, and the clear filtrate was mixed with an equal volume of acetone. Fluffy needle crystals separated from the solution, and after being refrigerated overnight, 5.5 g. (57% yield) of product was removed by filtration. This material was recrystallized from an alcohol-water mixture by the addition of acetone to incipient turbidity at 30°. The product was obtained as white needle crystals by refrigeration, 2.63 g., m.p. 205.8–206.8° (with dec.).

Anal. Calcd. for the disodium salt of DL-*N*-Sulfoserine, $C_3H_5O_6NSNa_2$: C, 15.73; H, 2.20; N, 6.11; N as $—NH_2$, 0.0; S, 13.99; Na, 20.08. Found: C, 15.51; H, 2.89; N, 6.27; N as $—NH_2$, 0.08; S, 13.82; Na, 19.45.

Sodium N-(3-hydroxypropyl)sulfamate. 3-Aminopropanol (7.5 g.) was dissolved in 130 ml. of water to yield a solution of *p*H 11.8. This solution was reacted with 12.6 g. of pyridine–sulfur trioxide added in portions over a period of 2.5 hr., with sufficient 10% NaOH added gradually to maintain a *p*H of about 11.3. At the end of the reaction time, the clear straw-colored solution was concentrated *in vacuo* to 80 ml., and the inorganic salts were precipitated by the addition of 250 ml. of absolute ethanol. After refrigeration, the finely divided inorganic material was removed by filtration. The filtrate yielded no further precipitate with another 50 ml. of alcohol. The reaction product, 4.46 g. (25%), was then precipitated by the addition of 1000 ml. of acetone. The solid was recrystallized once from hot 95% ethanol by the addition of acetone and a second time from absolute ethanol.

Anal. Calcd. for $C_3H_8O_4NSNa$: C, 20.34; H, 4.55; N, 7.91; N as $—NH_2$, 0.0; S, 18.09; Na, 12.98. Found: C, 21.2; H, 4.9; H, 7.89; N as $—NH_2$, 0.06; S, 17.88; Na, 13.5.

O-Sulfation of N-sulfochitosan. The sodium salt of *N*-sulfochitosan was prepared for the reaction with sulfur dioxide–sulfur trioxide by sifting it through a 100-mesh screen and allowing the solid to air-dry several days (10–12% adsorbed moisture). The air-dried material (32.5 g., 0.12 mole, dry basis) was placed in a flask equipped with a stirrer, dropping funnel, and dry ice condenser, and protected from moisture with a slow stream of dry nitrogen passing through a sulfuric acid exit bottle. The flask was cooled in an acetone–Dry Ice bath and dry sulfur dioxide was condensed in the flask to a total volume of about 250 ml. Liquid sulfur trioxide (40 g., 0.5 mole) was distilled into the dropping funnel under anhydrous conditions. The reaction vessel was well insulated with Vermiculite and the suspension of sodium *N*-sulfochitosan in liquid sulfur dioxide was allowed to warm to gentle reflux with continuous stirring. To the resulting stirred suspension in liquid sulfur dioxide, the sulfur trioxide was added slowly over a 20-min. period; and the reaction was then allowed to proceed under reflux with stirring for a total time of about 8 hr. The reaction product was filtered rapidly by suction with a sintered glass funnel, and washed 4 times on the filter by suspending the solid in 200-ml. portions of carbon tetrachloride. The washed solid was added to 1.5 l. of ice-water mixture containing 25 g. of sodium bicarbonate, and the resulting cold solution was adjusted to *p*H 9 with 20% sodium hydroxide solution. Inorganic salts were removed by dialysis in Visking cellulose casing until the solution in the casing gave a negative test for sulfate ion.[9] The sulfate-free solution was concentrated under reduced pressure to 875 ml. About 8.7 g. of sodium chloride was dissolved in the solution and the product was precipitated as a pasty solid by the addition of 2 l. of acetone. After refrigerating overnight, the supernatant liquid was decanted, and the pasty solid was macerated with two portions of absolute ethanol and two portions of acetone. After drying in a vacuum oven at 55°, the granular product weighed 52.4 g. (83% yield). *In vitro* activity = 60 units (Toronto)/mg. The *in vivo* activity was greater than 100% of heparin.[10] The analyses indicated that about 75% of the hydroxyl groups were sulfated.

Anal. Calcd. for $[C_{12}H_{17}O_8N_2(SO_3Na)_5]_x$ or 3 *O*-sulfate and 2 *N*-sulfate groups per anhydrodisaccharide unit: C, 17.31; H, 2.06; N-total, 3.36; N as $—NH_2$, 0.0; S, 19.25; Na, 13.8. Found: C, 17.74; H, 2.42; N-total, 3.19; N as $—NH_2$, 0.38 (Van Slyke) and 0.1 (titration); S, 18.32; Na, 13.9.

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(9) In some instances the addition of barium chloride to the test portion yielded a precipitate of the barium salt of the sulfated chitosan. However, this precipitate readily dissolved in dilute sodium hydroxide, in contrast with barium sulfate.

(10) The procedure of M. H. Kuizenga, J. W. Nelson and G. F. Cartland, *Am. J. Physiol.*, **139**, 612 (1943) was used for *in vitro* assay. *In vivo* assay was made by intravenous injection in rabbits. The chitosan derivative and heparin were compared on an equal weight basis as to duration of clotting time above the twice normal level following administration.