TABLE I

THE SURFACE ACTIVITY OF VARIOUS LACTIC ACID GLYCERIDES AT AN OIL/WATER INTERFACE AT 37°

Additive (1% by weight dissolved in cottonseed oil)	Interfacial tension, dynes cm.$^{-1}$
None	23.6
1-Palmitoyl-2,3-dilactyllactin	13.7
1-Palmitoyl-2,3-dilactin	11.3
1-Monoölein	10.3
1-Mono-O-palmitoyllactin	8.9
1-O-Palmitoyllactyl-2,3-dilactin	7.3

1-Mono-O-palmitoyllactin, metastable form: m.p. 34.2; stable form (from ethyl acetate and melt): m.p. 70°; L.S., 45.8; S.S., 5.20m, 4.50m+, 4.19vs, 3.99w, 3.86w, 3.67m−, 3.46m−.

1-Palmitoyl-2,3-dilactin, stable α-form: m.p. 31.5°; L.S., 31.5; S.S., 4.20s.

1-O-Palmitoyllactyl-2,3-dilactin, stable α-form: m.p. 26.1°; L.S., 42.7; S.S., 4.13s+.

1-Palmitoyl-2,3-dilactyllactin, stable α-form: m.p. 12.1°; L.S., 42.0; S.S., 4.14s.

Tri-O-palmitoyllactin, α: m.p. 30.5°; L.S., 28.9; S.S., 4.15s; stable form (from hexane): m.p. approximately 50°; L.S., 30.7; S.S., 5.91m, 4.71m, 4.51m, 4.28s−, 4.05s.

1-O-Palmitoyllactyl-2,3-dipalmitin, α: m.p. 41.0°; L.S., 50.0; S.S., 4.15s+; super-α (from hexane or ethyl acetate or from α after one week at 38°): m.p. 63.0°; L.S., 42.0; S.S., 5.26w, 4.42vw+, 4.07vs, 3.68vw (a third form of a less pure specimen, from hexane: m.p. 58.2°; L.S., 44.9; S.S., 4.40w, 4.26s+, 4.02s−, 3.85s, 3.47vw).

Surface Activity.—One of the interesting features of the lactic acid glycerides is their surface activity. The compounds 1-palmitoyl-2,3-dilactin and 1-O-palmitoyllactyl-2,3-dilactin, although triglycerides, both contain two unesterified hydroxyl groups in the molecule and have surface activities similar to those of monoglycerides. The interfacial tensions of several lactic acid glycerides dissolved in cottonseed oil at an oil–water interface are listed in Table I. The interfacial tension was determined by a drop-weight method[8] at a temperature of 37°. Distilled water was used and the oil was a refined, bleached, and deodorized cottonseed oil.

Discussion

The chief feature of interest in the phase behavior of these compounds is the stability in an α-phase of the three glycerides 1-palmitoyl-2,3-dilactin, 1-O-palmitoyllactyl-2,3-dilactin and 1-palmitoyl-2,3-dilactyllactin. This behavior is reminiscent of the high, but not complete, alpha stability of 1-stearoyldiacetin, etc.[9] The alpha stability of the dimorphic tri-O-palmitoyllactin is also quite high. The 1-O-palmitoyllactyl-2,3-dipalmitin shows unusual behavior with a normal α-form and a form from melt called super-alpha because of its single strong, short spacing and its resemblance to forms of that name observed for 2-butyroyldipalmitin and 2-butyroyldistearin.[10] (A third form was obtained from hexane with a specimen of lower purity.)

The acid compound, O-palmitoyllactic acid, appeared to be monomorphic.

The monoglyceride, 1-mono-O-palmitoyllactin, was dimorphic but with a lower melting form so fleeting as to defy X-ray characterization.

The influence of the lactic moiety on surface activity is not easily assessed. As might be expected, the possession of two hydroxyl groups by triglyceride compounds gives them a surface activity comparable to that of monoölein. It is of interest that for the first time synthetic triglycerides have been observed which show both surface activity and stability in the α-phase.

(8) W. D. Harkins and F. E. Brown, THIS JOURNAL, **41**, 499 (1919).
(9) F. L. Jackson and E. S. Lutton, *ibid.*, **74**, 4827 (1952).
(10) F. L. Jackson, R. L. Wille and E. S. Lutton, *ibid.*, **73**, 4280 (1951).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE OHIO STATE UNIVERSITY]

The Sulfonation of Chitosan[1,2]

BY M. L. WOLFROM AND (MRS.) T. M. SHEN HAN

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Sulfation of chitosan with pyridine–chlorosulfonic acid yielded an amorphous sodium salt which had an anticoagulant activity of 56 I. U./mg. It had a molecular weight (by light scattering) of 456,000 or a D. P. of 1280. It was approximately twice as toxic as heparin. A homogeneous sulfation method was established, using the sulfur trioxide–N,N-dimethylformamide complex in an excess of N,N-dimethylformamide. Sulfated chitosan thus obtained had an anticoagulant activity of 50 I. U./mg. and a D. P. (by light scattering) of 530. Its acute LD_{50} (mouse, intravenous) was about equal to that of heparin.

In a preliminary communication[2] from this Laboratory, we reported the preparation of a sulfated chitosan with a high degree of anticoagulant activity by treatment of chitosan with chlorosulfonic acid in pyridine. Essentially simultaneously with the publication of our results, Doczi and co-workers[3] reported the preparation of a sulfated chitosan, although no experimental details were given or have appeared to date. Coleman and associates[4] reported the preparation of sulfated chitosan of low anticoagulant activity by treatment of chitosan with sulfur dioxide and sulfur trioxide. Ricketts[5] also treated dried chitosan with chlorosulfonic acid in pyridine and isolated a product which exhibited very little anticoagulant activity.

We report herein the details of our sulfation[2] of essentially (90%) N-deacetylated chitosan with chlorosulfonic acid and pyridine. The success of

(1) Supported by the Bristol Laboratories, Inc., Syracuse, N. Y., under contract with The Ohio State University Research Foundation, Project 432(1951–1954).

(2) Reported in part in THIS JOURNAL, **75**, 1519 (1953), and in U. S. Patent 2,832,766 (1958).

(3) J. Doczi, A. Fischman and J. A. King, *ibid.*, **75**, 1512 (1953).

(4) L. L. Coleman, L. P. McCarty, D. T. Warner, R. F. Willy and J. H. Flokstra, *Abstracts Papers Am. Chem. Soc.*, **128**, 19L (1953); British Patent 746,870 (1956); *C. A.*, **51**, 1258 (1957).

(5) C. R. Ricketts, *Research*, **6**, 17S (1953).

this reaction depends upon the surface condition of the chitosan. We found that a suitable surface activation apparently produced some purification and yielded a colloidal suspension readily susceptible to sulfation. In addition to the surface condition of the chitosan, the purity of the pyridine employed was considered critical. This type of sulfation yielded a product isolated as an amorphous, water-soluble sodium salt. It was levorotatory and contained two N-sulfate and two O-sulfate groups per anhydrodisaccharide unit. It showed an anticoagulant activity of 56 International Units (I. U.) per mg. and a degree of polymerization of 1280 as determined by light scattering. Its acute LD_{50} (mouse, intravenous) was found to be twice that of heparin, which itself has a low acute toxicity. The presence of acid-sensitive N-sulfate groups in the sulfated chitosan was demonstrated by observing the rate of evolution of nitrogen in the Van Slyke deamination apparatus (Fig. 1). A similar behavior has been reported with heparin.[6] Thus, both contain the same type of sulfoamino linkages.[7,8] The sulfated chitosan reacts much more rapidly (Fig. 1) under these conditions than does heparin, in accordance with the general results of Foster, Martlew and Stacey,[9] who have shown that such an effect in the unsulfated amines can be ascribable to the presence of an α-D-hexosamine linkage in the heparin (N-desulfated) and to a β-D-linkage in the chitosan. In our experiments, the unsulfated amines would be intermediates in the reaction with sulfuric and nitrous acids under the Van Slyke assay conditions.

The high anticoagulant activity exhibited by our preparation of sulfated chitosan is further evidence in support of the findings of this Laboratory[10] in regard to the positive contribution of the sulfoamino linkage to the anticoagulant activity of sulfated polysaccharides of the heparin type. The work of Doczi,[3] Hiyama[11] and their associates on their preparations of sulfated chitosan and other polysaccharides also confirms this point.

We now report further a homogeneous sulfation method for chitosan, using a sulfur trioxide–N,N-dimethylformamide complex in an excess of N,N-dimethylformamide. This complex is a colorless, crystalline substance that is soluble in N,N-dimethylformamide. It has been employed for the sulfation of vat dyes[12] and for the formation of mixed anhydrides with the salts of amino acids.[13] It has been shown[14] that N,N-dimethylformamide reacts with benzoyl bromide to form a strong elec-

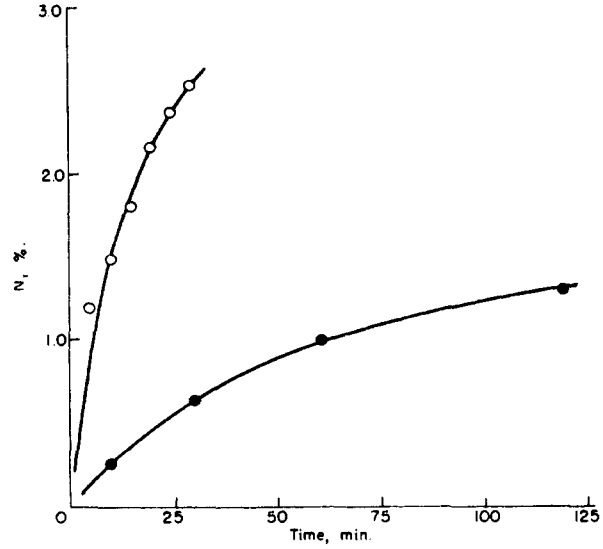
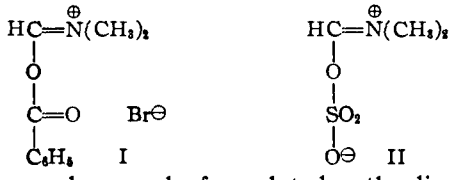
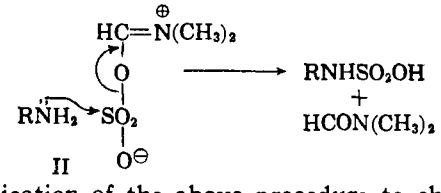


Fig. 1.—Variation of amino nitrogen (Van Slyke) with time; O, sulfated chitosan, prepared from pyridine and chlorosulfonic acid; ●, crystalline barium acid heparinate, data of ref. 6.

trolyte which is a benzoylating agent. It has been suggested[15] that the electrolyte is a salt (I) resulting from the O-acylation of N,N-dimethylformamide by benzoyl bromide. By analogy, the sulfur tri-



oxide complex may be formulated as the dipolar ion II. The reaction of II with a nucleophilic group, such as a primary amine or a secondary or primary alcohol, should involve attack at the sulfur atom to effect sulfation on nitrogen or oxygen with the concomitant release of N,N-dimethylformamide.



Application of the above procedure to chitosan yielded an amorphous product, isolated as a levorotatory sodium salt, containing one sulfoamino group and one sulfate acid ester group per monomer unit. The light scattering method established a degree of polymerization of 530 for this product, which is a lower value than that obtained on the product sulfated by the pyridine–chlorosulfonic acid method. Its anticoagulant activity was 50 I.U./mg. and its acute LD_{50} was about that of heparin. This lower toxicity may have been effected by the smaller molecular size of the polysaccharide, in accordance with what is known concerning dextran toxicity effects.

The new sulfation method established here has several advantages. The solution of II in N,N-dimethylformamide is stable toward storage and can

(6) M. L. Wolfrom, D. I. Weisblat, J. V. Karabinos, W. H. McNeely and J. McLean, THIS JOURNAL, **65**, 2077 (1943).

(7) J. E. Jorpes, H. Bostrõm and V. Mutt, *J. Biol. Chem.*, **183**, 607 (1950).

(8) M. L. Wolfrom and W. H. McNeely, THIS JOURNAL, **67**, 748 (1945).

(9) A. B. Foster, E. F. Martlew and M. Stacey, *Chemistry & Industry (London)*, 825 (1953).

(10) M. L. Wolfrom, R. Montgomery, J. V. Karabinos and P. Rathgeb, THIS JOURNAL, **72**, 5796 (1950).

(11) N. Hiyama, M. Maki and Y. Miyazawa, *Hirosaki Med. J.*, **7**, 284 (1956); *C. A.*, **51**, 1398 (1957).

(12) S. Coffey, G. W. Driver, D. A. W. Fairweather, F. Irving and Imperial Chemical Industries, Ltd., British Patents 610,117 (1948) and 642,206 (1950); *C. A.*, **43**, 3205 (1948), and **45**, 3412 (1951).

(13) G. W. Kenner and R. J. Stedman, *J. Chem. Soc.*, 2069 (1952).

(14) H. K. Hall, Jr., THIS JOURNAL, **78**, 2717 (1956).

(15) D. Davidson, *Trans. N. Y. Acad. Sci.*, Ser. II, **20**, 320 (1958).

be handled with ease and accuracy. Sulfations can be effected at low temperatures and the powerful solvent action of N,N-dimethylformamide should allow many of these to be carried out in a homogeneous fashion.

Acknowledgment.—The counsel of Dr. T. Y. Shen in portions of this work is gratefully acknowledged.

Experimental

Activation of Chitosan.—Chitosan was utilized that had been prepared from shrimp shell chitin by the method of Rigby.[16] It was found to be 90% N-deacetylated. An amount of 10 g. of flaked chitosan was suspended with stirring in 1 liter of 2% aqueous acetic acid until most of the solid dissolved. The insoluble residue was removed by centrifugation and the clear solution was neutralized with 2.5 *N* sodium hydroxide. The white precipitate formed was collected and washed successively with distilled water (4 times), ethanol, absolute ethanol, ether and freshly distilled, pure, dry pyridine and finally suspended in 80 ml. of dry pyridine.

Sulfation of Chitosan with Chlorosulfonic Acid and Dry Pyridine.—An amount of 60 ml. of freshly distilled, pure pyridine was placed in a three-necked flask previously cooled in an ice-bath. To the cooled pyridine was added slowly, through a dropping funnel, 10 ml. of chlorosulfonic acid over a period of 30–40 min. To this mixture, 40 ml. of the above described suspension of chitosan in pyridine (containing 3.5 g. of chitin) was added and the whole was heated on a boiling water-bath for 1 hr. After cooling to room temperature, the reaction mixture was poured into 200 ml. of water, to give a clear brown solution, and 75 ml. of 2.5 *N* sodium hydroxide was added. The sodium salt of crude sulfated chitosan was then precipitated with 500 ml. of ethanol. The precipitate was redissolved in 200 ml. of water and subjected to dialysis in a seamless tubing[17] for three days against distilled water. After the solution was concentrated under reduced pressure to 100 ml., 10 ml. of saturated sodium chloride solution was added to the concentrate and the product was precipitated as its sodium salt with 150 ml. of ethanol; yield 3.2 g., $[\alpha]^{25}D$ −23° (*c* 1.5, water). No inorganic sulfate was detectable.

Anal. Calcd. for $[C_{12}H_{18}O_6(NCOCH_3)_2(OSO_3Na)_2]_{0.11}$ + $[C_{12}H_{18}O_6(NSO_3Na)_2(OSO_3Na)_2]_{0.89}$: C, 20.65; H, 2.64; N, 3.92; Na (H_2SO_4 ash), 12.00; S, 16.70; N-acetyl (as CH_3CO), 1.63. Found: C, 20.54; H, 2.95; N, 3.41; Na(H_2SO_4 ash), 11.31; S, 16.25; N-CH_3CO,[18] 1.63; -NH_2 (by ninhydrin), absent; mol. wt. (by light scattering[19]), 456,000.

Sulfation of Chitosan with Sulfur Trioxide–N,N-Dimethylformamide Complex.—Commercial N,N-dimethylformamide was redistilled through a heated Vigreux column (4″) and the fraction of b.p. 152° was collected. Care was taken to protect the distillation vessel from moisture.

Sulfur trioxide was generated by heating 30% oleum over phosphoric anhydride in a Claisen flask, and was conducted into a receiver containing N,N-dimethylformamide so that the sulfur trioxide was absorbed immediately. When the solution became saturated with the complex, a white deposit began to appear and the distillation was stopped. Sufficient N,N-dimethylformamide was added to the solution to dissolve the excess complex and the final concentration (2.5 *N*) was obtained by titrating 2 ml. of the solution in water with 0.1 *N* sodium hydroxide. The solution was kept in a glass-stoppered (silicon grease) bottle, preferably at 15°. It generally developed a yellow color on standing.

An amount of 2 g. of the chitosan was activated as described above except that the pyridine was replaced by N,N-dimethylformamide. The activated material was placed in a three-necked flask fitted with a drying tube, mercury-sealed mechanical stirrer and a dropping funnel. To this was added, at room temperature, 30 ml. of sulfur trioxide–N,N-dimethylformamide. The chitosan dissolved and the solution was stirred for 12 hr. The crude product was isolated as the sodium salt by the addition of solid sodium bicarbonate to the reaction mixture, followed by the filtration of the insoluble inorganic salts and addition of ethanol to the filtrate to complete the precipitation of the sulfated chitosan. The precipitate was redissolved in 500 ml. of water and was subjected to dialysis for 3 days. The purified product was obtained by precipitation with ethanol in the presence of a trace of saturated sodium chloride; $[\alpha]^{27}D$ −17° (*c* 1.67, water).

Anal. Calcd. for $[C_{12}H_{18}O_6(NCOCH_3)_2(OSO_3Na)_2]_{0.11}$ + $[C_{12}H_{18}O_6(NSO_3Na)_2(OSO_3Na)_2]_{0.89}$: S, 16.70; N-acetyl (as CH_3CO), 1.63. Found: S, 16.33; N-$COCH_3$, 1.63; -NH_2 (by ninhydrin), absent; mol. wt. (by light scattering[19]), 186,000.

Bioassays.—The *in vitro* anticoagulant activity was determined by the method of Kuizenga and associates.[20] Citrated sheep plasma was used. The sulfated chitosan, prepared from pyridine and chlorosulfonic acid, had an activity of 56 International Units (I. U.) per mg. This compound showed an acute LD_{50} (mouse, intravenous) of 380 mg./kg. The sulfated chitosan prepared from sulfur trioxide–N,N-dimethylformamide had an anticoagulant activity of 50 I. U./mg. and showed a marked decrease in toxicity with an LD_{50} (mouse, intravenous)[21] of 775 mg./kg. while heparin exhibits the comparable LD_{50} of 750 mg./kg.

(16) G. W. Rigby, U. S. Patent 2,040,879 (1936); *C. A.*, **30**, 4598 (1936).

(17) Visking Co., Chicago, Ill., wall thickness 0.0023 in.

(18) A. Chaney and M. L. Wolfrom, *Anal. Chem.*, **28**, 1614 (1956).

(19) Acknowledgment is made to Professor Quentin Van Winkle of this department for advice and assistance in this measurement.

(20) M. H. Kuizenga, J. W. Nelson and G. F. Cartland, *Am. J. Physiol.*, **139**, 612 (1943).

(21) Performed by Dr. H. L. Dickison of Bristol Laboratories, Inc., Syracuse, N. Y.

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COMMUNICATIONS TO THE EDITOR

SYNTHESIS OF HEXABORANE

Sir:

New conversions of pentaborane-11 by basic reagents[1] give nearly 30% yields of hexaborane, which thus may become as available as other polyboranes. Effective for this conversion are the non-volatile liquid from the B_5H_{11}-$[(CH_3)_2N]_2BH$ reaction,[1] trimethylamine, dimethyl ether, and "diglyme" (β,β′-dimethoxydiethyl ether), the last working well with flowing B_5H_{11} vapor. Both ethers liberate much diborane and tetraborane, which are convertible to pentaborane-11 for recycling. Hydrogen formation is negligible.

The Non-Volatile Catalyst.—The reaction of B_5H_{11} with $[(CH_3)_2N]_2BH$ (1.75:0.748 mmoles; 17 hr. at −78°, warming to 0° during 9 hr.) gave 11% B_2H_6, 19% B_4H_{10}, 37% B_5H_9, 8.4% B_6H_{10}[2] and an oil which was used four times to convert 0.322–0.840 mmole samples of B_5H_{11}. Yield-ranges were: 22–24% B_2H_6, 0.2–3.9% $(CH_3)_2NB_2H_5$,

(1) J. L. Boone and A. B. Burg, THIS JOURNAL, **80**, 1519 (1958).

(2) All yields are based on boron in the consumed B_5H_{11}.