room temperature. The hydrogenation was stopped after 15 min., at which time the hydrogen uptake was 1.1 moles. The solution was filtered to remove the catalyst and the ethanol was evaporated. The product, 11β,12β-epoxy-3β-hydroxy-5-pregnen-20-one (XXI) was crystallized from hexane, then methanol, m.p. 174–179°, no ultraviolet absorption. Infrared analysis (potassium bromide disk) showed bands at 3400 (hydroxy), 1697 (20-ketone), 875 (epoxy), and 808 cm.$^{-1}$ (Δ^5).

U. S. DEPARTMENT OF AGRICULTURE
PHILADELPHIA, PA.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, SYRACUSE UNIVERSITY]

Monosaccharide Sulfates. I. Glucose 6-Sulfate. Preparations, Characterization of the Crystalline Potassium Salt, and Kinetic Studies[1]

KENNETH B. GUISELEY AND PAUL M. RUOFF

Received July 8, 1960

Speed of preparation and yield and purity of product are improved when glucose is sulfated directly using pyridine–sulfur trioxide in *N,N*-dimethylformamide. Purification of a directly sulfated glucose mixture ultimately leads to the crystalline, nonhygroscopic potassium salt of the 6-sulfate.

Two unequivocal syntheses of glucose 6-sulfate are described involving the removal of the protecting groups from 1) barium 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose 6-sulfate and 2) barium 1,2;3,5-di-*O*-benzylidine-α-D-glucofuranose 6-sulfate.

Kinetic studies on the hydrolysis of the pure potassium salts of glucose 6-sulfate and ethyl sulfate were made, adapting the recently reported colorimetric determination of sulfate ions with barium chloranilate. The energy of activation for acid hydrolysis was determined for each compound. The effect of nitrogenous bases upon the hydrolysis of sulfate from unpurified glucose sulfate in buffered, slightly acid, or basic solution was determined.

Although glucose 6-sulfate has been known in varying degrees of purity for about forty years,[2–8] no one has succeeded in preparing a crystalline metallic salt. Soda and Egami[9] investigated the products of the direct sulfation of glucose and found, in addition to the 6-sulfate, a disulfate, which they concluded was the 1,6-sulfate. More recently, Dodgson and Spencer[10] reported a means of purifying the product of directly sulfated glucose by repeated recrystallization of the brucinium salt. Lloyd[11] has described a definitive synthesis for glucose 6-sulfate.

This paper describes first, some modifications of the direct method of synthesis and two indirect syntheses for glucose 6-sulfate, and, subsequently, characterization of the crystalline potassium salt and kinetic studies on the hydrolysis of the ester sulfate.

The most common direct sulfation procedure employs chlorosulfonic acid in chloroform-pyridine as the sulfating agent,[5] and results in a product which contains about 15% of glucose disulfate,[12] unless the purification technique of Dodgson and Spencer[10] is employed, in which case most of the disulfate is removed.

A more recent method[8] uses pyridine-sulfur trioxide in pyridine but gives a product containing up to 30% disulfate.[13] Furthermore, the use of barium carbonate does not insure complete removal of the pyridinium ion during the neutralization step.

The method finally worked out[14] for the direct sulfation of glucose employed pyridine–sulfur trioxide, with *N,N*-dimethylformamide as a solvent. The method minimizes polysulfation produced to a large extent by the heterogeneity of

---

(1)(a) Abstracted from the dissertation submitted by Kenneth B. Guiseley to the Graduate School of Syracuse University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. (b) Presented at the Cleveland Meeting of the American Chemical Society, April 1960 (Abstracts of that meeting, p. 1D). (c) This investigation was supported by a research grant (RG-4997(C3)) from the National Institutes of Health, Public Health Service.

(2) C. Neuberg and L. Liebermann, *Biochem. Z.*, **121**, 326 (1921).

(3) H. Ohle, *Biochem. Z.*, **131**, 601 (1922).

(4) T. Soda, *Biochem. Z.*, **135**, 621 (1923).

(5) T. Soda, *Bull. Chem. Soc. Japan*, **8**, 37 (1933).

(6) T. Soda and W. Nagai, *J. Chem. Soc. Japan*, **59**, 135 (1938).

(7) E. G. V. Percival and T. H. Soutar, *J. Chem. Soc.*, 1475 (1940).

(8) R. B. Duff, *J. Chem. Soc.*, 1597 (1949).

(9) T. Soda and F. Egami, *J. Chem. Soc. Japan*, **63**, 465 (1942).

(10) K. S. Dodgson and B. Spencer in *Methods of Biochemical Analysis*, Vol. IV, edited by D. Glick, Interscience, New York, 1957, pp. 211 ff.

(11) A. G. Lloyd, *Nature*, **183**, 109 (1959).

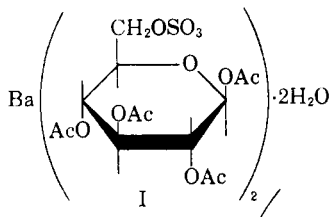
(12) T. Soda and H. Egami, *J. Chem. Soc. Japan*, **61**, 683 (1940).

(13) K. S. Dodgson and B. Spencer, *Biochem. J.*, **57**, 310 (1954).

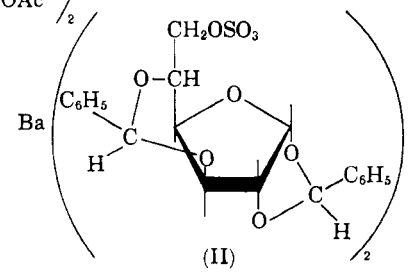
(14) Previously sulfamic acid was tried as a sulfating agent since it was known [M. E. Cupery, *Ind. Eng. Chem.*, **30**, 627 (1938)] to sulfate primary alcohols preferentially unless a suitable base was present [R. L. Burwell, Jr., *J. Am. Chem. Soc.*, **71**, 1769 (1949)]. However, as we later realized, the solvent, *N,N*-dimethylformamide, was basic enough to catalyze the sulfation of secondary alcohols, with the result that the products contained glucose disulfate. In addition, it was difficult to remove ammonium ion during neutralization and barium sulfamate after precipitation. Because of these factors, the method was not pursued further.

Duff's[8] procedure, and eliminates the involved chloride ion removal characteristic of chlorosulfonic acid sulfations. As a result, the method is faster than either of the other two, gives a product which is purer than that made by Duff's method, and, while giving a yield at least comparable to that of the chlorosulfonic method, is easier to perform.[15]

In the quest for pure glucose 6-sulfate, two definitive syntheses were achieved. The first involved sulfation of 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose, and removal of the protective acetyl groups after isolation of the crystalline barium 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose 6-sulfate (dihydrate) (I).



I



(II)

Shortly after this work was completed, Lloyd[11] reported a similar synthesis involving the same intermediate, but using different techniques.

In the second unequivocal synthesis, 1,2;3,5-di-*O*-benzylidene-α-D-glucofuranose (obtained directly from glucose[16]) was sulfated and the resulting barium 1,2;3,5-di-*O*-benzylidene-α-D-glucofuranose 6-sulfate (II) treated with dilute acid to remove the benzylidene residues.

Although both procedures were significant as definitive syntheses, they were not practical as preparative methods since the overall yields from glucose were less than 5%.

When the mixture of products from the direct sulfation of glucose was subjected to paper chromatography (1-butanol - ethanol - water - ammonia, 40:12:20:1[17]), a separation into three components resulted. The R_f values of 0.33, 0.20, and 0.10 corresponded to glucose, glucose 6-sulfate, and glucose disulfate, respectively. The spots for the sulfate salts had the same R_f, regardless of the salt used—barium, potassium, brucinium, and others.

Purification on a macro scale was attempted, using a cellulose column and essentially the same developing solution used with the paper strips, but as it was too time-consuming to prepare large quantities of pure glucose 6-sulfate in this manner, the chromatographic method was not further studied. However, a small quantity of the disulfate was isolated by this technique.

Because the definitive syntheses and the column chromatography method could not be conveniently scaled up, the recrystallization of brucinium glucose 6-sulfate (from direct sulfation) was reexamined as a means of preparing several grams of the pure substance. In our hands, the water-ethanol solvent pair used by Dodgson and Spencer[10] had been ineffectual in removing the disulfate, and was replaced by water-acetone, a solvent mixture used by Soda[4] only a few years after glucose "monosulfate" was first prepared. After only three recrystallizations, 98 mole per cent brucinium glucose 6-sulfate was obtained. From this the potassium salt was prepared in the conventional manner and dried by lyophilization. Subsequently, an aqueous solution of the salt deposited crystals upon spontaneous evaporation of the water. The crystalline potassium glucose 6-sulfate was anhydrous and non-hygroscopic in contrast to the amorphous potassium salt prepared by precipitation in ethanol.[10]

Hydrolytic studies were made on both purified and unpurified directly sulfated glucose. The course of the reaction was followed using the colorimetric sulfate method of Bertolacini and Barney[18] with suitable modifications.

Kinetic studies were made on the hydrolysis of sulfate from the purified potassium glucose 6-sulfate; for comparison, similar studies were made with potassium ethyl sulfate. Pseudo-first order rate constants were determined at three temperatures, and the energy of activation calculated for each compound from an Arrhenius plot. To show the dependence of the rate constants upon the substrate and acid concentrations, each was varied while holding the other and the temperature constant.

The rapid hydrolysis of part of the ester sulfate in unpurified glucose sulfate (direct sulfation) at *p*H 5.1 in the presence of hydrazine,[12] led to a means of analysis for the disulfate.

EXPERIMENTAL[19]

Pyridine-sulfur trioxide was prepared in essentially the same manner described in *Inorganic Syntheses*[20] except that

(15) After this work was completed we noted that sulfur trioxide–*N*,*N*-dimethylformamide complex in an excess of dimethylformamide was used in the homogeneous sulfation of chitosan [M. L. Wolfrom and T. M. Shen Han, *J. Am. Chem. Soc.*, **81**, 1764 (1959)].

(16) H. B. Wood, Jr., H. W. Diehl, and H. G. Fletcher, Jr., *J. Am. Chem. Soc.*, **79**, 3862 (1957).

(17) S. Suzuki, N. Takahashi, and F. Egami, *Biochim. et Biophys. Acta*, **24**, 444 (1957).

(18) R. J. Bertolacini and J. E. Barney II, *Anal. Chem.*, **29**, 281 (1957).

(19)(a) All melting points are uncorrected. (b) Microanalyses were performed at the Spang Microanalytical Laboratory, Ann Arbor, Mich. (c) All evaporations *in vacuo* were carried out with a Rinco Rotating Evaporator, Model 1007 4 IN.

Sulfan B (General Chemical Division, Allied Chemical and Dye Corporation's brand of stabilized sulfur trioxide) was used in place of chlorosulfonic acid in order to prepare the compound free from both chloride and sulfate ions. In a 5-l., three necked flask, equipped with a dropping funnel, thermometer (extending nearly to the bottom), and electric stirrer (loose-fitting glass seal) were placed about 2.25 l. of dry, alcohol-free chloroform and 200 ml. (*ca.* 2.5 moles) of anhydrous pyridine. During the dropwise addition of 80 ml. (1.92 moles) of Sulfan B, the temperature was kept between 0° and 10°. The product was filtered off by suction, washed with 500 ml. of ice-cold, dry, alcohol-free chloroform, and aspirated under a rubber dam. It was dried in a vacuum desiccator over concentrated sulfuric acid by continuous aspiration to yield about 235 g. (80%).

Chlorosulfonic acid was purified by distilling the practical grade material in an all-glass apparatus, using the fraction boiling at 156–158°.

Paper chromatography was carried out on Whatman No. 1 paper, using the solvent mixture of Suzuki *et al.*[17]: 1-butanol, ethanol, water, ammonia: 40:12:20:1. The chromatograms were run in the ascending manner with either glucose or a salt of glucose 6-sulfate spotted alongside to give reference points. After completion of a run, the strips were quickly dried in an oven at 115° and sprayed with a 3% solution of *p*-anisidinium chloride in butanol. Color was developed by heating the sprayed paper at 110–115° for *ca.* 10 minutes.

Direct sulfation procedures. A. *Chlorosulfonic acid in pyridine.* The method of Dodgson and Spencer[10] was followed exactly and gave a 16.5% yield of brucinium glucose 6-sulfate.

B. *Pyridine–sulfur trioxide in pyridine.* Duff's method[8] was followed exactly, giving a gummy product which proved to consist mainly of the pyridinium salt, rather than the expected barium salt of glucose 6-sulfate. Conversion to the barium salt was effected by dissolving the sticky pyridinium salt in water, and adding barium hydroxide solution to *p*H 7.5. Concentration *in vacuo* removed the liberated pyridine and water. This process of neutralization and evaporation was repeated until the sirupy product, when dissolved in water, became neutral. After removal of the barium sulfate by centrifugation, the solution was concentrated to a syrup, and the product precipitated by pouring the syrup slowly into 850 ml. of absolute ethanol. After standing overnight, the barium glucose sulfate was filtered off, washed with ether to facilitate drying, aspirated under a rubber dam, and dried over phosphorus pentoxide *in vacuo;* Yield, 11.4 g. (59.5%). Polysulfation is indicated by high barium analysis.

Anal. Calcd. for $Ba(C_6H_{11}O_9S)_2$: Ba, 20.95. Found: Ba, 25.3.

C. *Pyridine-sulfur trioxide in N,N-dimethylformamide.* By warming to about 50°, 21.6 g. (0.12 mole) of anhydrous D-glucose was dissolved in 300 ml. of anhydrous dimethylformamide in a 1-l., three necked flask equipped with an air condenser and drying tube, ground glass stirrer, and dropping funnel. With stirring, a solution of 19.0 g. (0.12 mole) of pyridine–sulfur trioxide in about 125 ml. of dry dimethylformamide was added to the solution (at room temperature) over a period of 1.5 hr. Stirring was continued for an additional hour, after which, the dimethylformamide was removed *in vacuo* at less than 35°, and the resulting syrup taken up in 125 ml. of water. Barium hydroxide was added to *p*H 10.0, then carbon dioxide to a *p*H of 7.5. Concentration, *in vacuo*, removed water and liberated pyridine. The paste was taken up in 100 ml. of water. If the *p*H of the suspension were under 7.0, more barium hydroxide was added to bring the *p*H to 7.0–7.5, the water was evaporated *in vacuo*, and the process repeated until neutrality was permanent. After warming the suspension to 40°, barium sulfate was centrifuged off, and the solution treated with charcoal. The *p*H was checked and brought to 7.0 with barium hydroxide, if necessary. After concentration of the solution to a medium-thick syrup *in vacuo*, the product was precipitated as follows: methanol was added to the syrup a few milliliters at a time, with swirling, to redissolve the precipitated barium glucose sulfate. When it appeared that solution would not be possible after the addition of another increment, about 500 ml. of methanol was added in one portion. The flask was stoppered and shaken vigorously for about half a minute. The flask was placed on an automatic shaker overnight to "digest" the fine white precipitate of barium glucose sulfate. The resulting granular product was filtered off by suction, washed once with methanol and twice with ether, then dried *in vacuo* over phosphorus pentoxide. A second fraction could usually be obtained from the filtrate after the ether washings had been mixed in. Yield: first fraction, 16.6 g. (40%); second fraction, 5.8 g. (14%).

Anal. Calcd. for $Ba(C_6H_{11}O_9S)_2$: Ba, 20.95. $Ba(C_6H_{11}O_9S)_2 \cdot 2H_2O$: Ba, 19.85. Found: Ba, 1st fraction 19.8; 2nd fraction, 18.7. Despite the apparently good analysis, paper chromatography showed the presence of three compounds: glucose, glucose 6-sulfate, and glucose disulfate.

Pure brucinium α-D-glucose 6-sulfate. Twenty-five grams of crude barium glucose 6-sulfate was dissolved in 175 ml. of water, and the solution passed over an Amberlite IR-120 column (1.5 × 40.0 cm.), hydrogen-ion form, at the rate of 3.0 ml. per min. Ethanolic brucine was added to the combined effluent and rinsings to *p*H 6.0. After removal of the ethanol *in vacuo*, the water solution was washed twice with 75-ml. portions of chloroform, then twice with 50-ml. portions of ether. The solution was concentrated to 150–200 ml., *in vacuo*, and warmed to about 40°. Acetone was added to incipient cloudiness, while the solution was kept close to 40°. Upon slow cooling, and finally refrigeration, rosettes of very fine crystals formed. These were filtered off, washed with 90% acetone, then acetone, and allowed to air-dry. Three recrystallizations of this brucinium salt, performed in the same manner (x g. of brucinium salt dissolved in $3x$ ml. of water, warmed to 40°, and treated with approximately $12x$ ml. of acetone) with one charcoal treatment, yielded 15.2 g. (30.5%, based on crude barium salt) of product which, as shown in the next section, proved to be 98 mole per cent pure brucinium glucose 6-sulfate. $[\alpha]_D^{20}$ −1.57° (5 min.) → −6.53° (equi.) (*c.* 4.835, in water).

Analytical method for the quantitative determination of disulfate in purified brucinium α-D-glucose 6-sulfate. A 0.4144-g. sample of brucinium α-D-glucose 6-sulfate was weighed into a 10-ml. volumetric flask and dissolved in about 3 ml. of warm water. To this was added 4 ml. of 1*M* acetate buffer (*p*H 5.1) and 1.2 ml. of 1*M* hydrazine in aqueous acetic acid (*p*H 5.1). The mixture was diluted to the mark with water, mixed well, and incubated at 37.5° ± 0.5° for 30 hr. A 5-ml. aliquot was then analyzed as follows: The aliquot, contained in a 20-ml. beaker, was neutralized to *p*H 4.0 on the *p*H meter, using 0.64*N* hydrochloric acid (a little over 2 ml. was required). Five milliliters of a 1:1 mixture of 0.05*M* potassium hydrogen phthalate buffer and 95% ethanol was added and the *p*H readjusted to 4.0 with acid. The solution was transferred to a 25-ml. volumetric flask. After rinsing the electrodes into the beaker with about 1 ml. of water and 5 ml. (pipet) of 95% ethanol, the rinsings were transferred to the flask, the beaker rinsed into the flask with 5 ml. (pipet) more of 95% ethanol, and the flask made up to the mark with water. A blank was prepared similarly from a 5-ml. aliquot of a solution containing 40 ml. of 1*M* acetate buffer, *p*H 5.1, and 12 ml. of 1*M* hydrazine in acetic acid (*p*H 5.1) in a total volume of 100 ml. To each 25-ml. volumetric flask was added 0.050 g. of barium chloranilate.[18] The flasks were shaken once a minute for 15 min., the solutions centrifuged to clarity, and the per cent transmittance read on a Fisher Electrophotometer (or other suitable instrument) using the 525B green filter on the D scale. From a standard curve prepared from known concentrations of sulfate, the amount of sulfate liberated was determined, and

(20) W. C. Fernelius, *Inorganic Syntheses*, Vol. II, McGraw-Hill, New York, 1946, p. 173.

from that, the mole per cent of disulfate in the sample. (As hydrazine barely catalyzes the hydrolysis of sulfate from the 6-position, but does cause quantitative hydrolysis of sulfate from the 1-position of the 1,6-disulfate under the conditions employed, the amount of sulfate liberated is proportional to the amount of disulfate present.)

Crystalline potassium β-D-glucose 6-sulfate. To a solution of 15.0 g. of pure brucinium α-D-glucose 6-sulfate in 500 ml. of water, was added 1*N* potassium hydroxide to *p*H 9.5. The mixture was chilled in an ice bath for a few minutes, after which the precipitated brucine was filtered off. The filtrate was washed twice with chloroform, 100 ml., 80 ml., then twice with ether, 100 ml., 80 ml., and concentrated *in vacuo* to about 35 ml. Absolute ethanol was added to incipient cloudiness. If a gelatinous precipitate formed, it was centrifuged off. On standing, potassium β-D-glucose 6-sulfate crystallized out. This was filtered off and washed with 50–60% ethanol, then ethanol. As the compound was not hygroscopic, as contrasted with the amorphous form,[10] it could be air-dried. One recrystallization yielded 4.20 g. (65%, based on brucinium salt), m.p. 170–173° dec. $[\alpha]_D^{20}$ +16.46° (after 5 min.) → +37.35° (equi.) (*c* 2.983, in water).

Anal. Calcd. for $KC_6H_{11}O_9S$: K, 13.11; C, 24.16; H, 3.72; S, 10.75. Found: K, 12.99; C, 24.13; H, 3.68; S, 10.85.

Its density, determined by the flotation method, was 1.816 ± 0.004 g./ml.

X-ray powder diffraction data (nickel-filtered Cu Kα radiation): 12.50 (2), 6.544, 5.746, 5.698, 4.861 (1, strongest), 4.668, 4.651, 4.568, 4.518 (3), 4.110, 3.974 (1) 3.882, 3.703 (4), 3.666, 3.465, 3.329, 3.264, 3.177, 3.117, 3.008, 2.980, 2.958, 2.898, 2.851, 2.818, 2.810, 2.776, 2.724, 2.673 (5), 2.555.

Barium 1,2,3,4-tetra-O-acetyl-β-D-glucopyranose 6-sulfate (I). To a solution of 5.22 g. (0.015 mole) of 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose[21] in 20 ml. of anhydrous pyridine contained in a 50-ml. glass-stoppered flask, was added 2.39 g. (0.015 mole) of pyridine-sulfur trioxide in one portion. The mixture was shaken until solution was complete. After standing at room temperature overnight, the reaction mixture was dissolved in 50 ml. of water, and the solution neutralized (*p*H 7.5) with aqueous barium hydroxide. The resulting suspension was concentrated to dryness *in vacuo*, and the residue taken up in 60 ml. of water. (The *p*H was adjusted to 7.0 with barium hydroxide, if acidic; the neutralization and evaporation steps were repeated until the suspension had a *p*H of 7.0 upon being taken up in water.) Insoluble inorganic salts were removed by centrifugation and the solution treated with charcoal, if necessary. The solution was concentrated *in vacuo* to a thick syrup which was redissolved in 30–40 ml. of methanol. Ether was added to cloudiness and the solution chilled until crystallization was complete. After being washed with methanol-ether, 1:1, and ether, the product was dried *in vacuo* over phosphorus pentoxide. Recrystallization by evaporation of a 98% ethanol solution of the compound (method of Ohle[3]) gave 2.3 g. (30%) of white crystals, m.p. 105–106° dec., $[\alpha]_D^{20}$ + 11.8° (*c*. 2.995, in water).

Anal. Calcd. for $Ba(C_{14}H_{19}O_{18}S)_2 \cdot 2H_2O$: Ba, 13.36; C, 32.71; H, 4.12; S, 6.24. Found: Ba, 13.35; C, 32.32; H, 3.80; S, 6.18.

X-ray powder diffraction data (nickel-filtered Cu Kα radiation): 11.7 (1, strongest), 9.24 (3), 8.20 (5), 6.53 (4), 5.77, 5.01, 4.70, 4.47, 4.31, 4.09, 3.848 (2), 3.614, 3.462, 3.366, 3.272, 3.152, 3.023, 2.936.

Barium glucose 6-sulfate from I. A solution of 1.00 g. of barium 1,2,3,4-tetra-*O*-acetyl-β-D-glucopyranose 6-sulfate (dihydrate) (I) in 10 ml. of absolute methanol, was cooled to 0° and saturated with dry ammonia.[22] After the solution had stood overnight at room temperature, the methanol and ammonia were removed *in vacuo* and a few milliliters of absolute ethanol evaporated from the residue to insure dryness. Following this, the residue was washed three times with absolute ethanol in order to remove acetamide, then taken up in 20 ml. of water. The solution was treated with charcoal, filtered, and concentrated *in vacuo*. Addition of absolute methanol gave 0.35 g. (53%) of product after filtering, washing, and drying.

Anal. Calcd. for $Ba(C_6H_{11}O_9S)_2 \cdot 2H_2O$: Ba, 19.85. Found: Ba, 19.02.

The x-ray diffraction pattern of the brucinium salt made from this barium salt was identical with that of the standard (see below).

1,2;3,5-Di-O-benzylidene-α-D-glucofuranose was prepared according to the method of Wood, Diehl, and Fletcher.[16] Recrystallization of the compound from ethyl acetate gave crystals melting at 160–161° as reported. However, when the reaction was scaled up by a factor of four, the melting point of the product could not be raised over 155–157°, neither by this method of recrystallization, nor the ammoniacal methanol method of Wolfrom and Tanghe.[23]

Barium 1,2;3,5-di-O-benzylidene-α-D-glucofuranose 6-sulfate (II). A solution of 3.11 g. (0.0087 mole) of 1,2;3,5-di-*O*-benzylidene-α-D-glucofuranose in 24 ml. of anhydrous pyridine was shaken in a 50-ml. glass-stoppered flask with 1.67 g. (0.0105 mole, *ca.* 20% excess) of pyridine-sulfur trioxide until solution was complete (10 min.). After an additional 10 min., the solution was poured into 20 ml. of cold water, and barium hydroxide solution was added to *p*H 8.2. Carbon dioxide was bubbled in to *p*H 7.5 and the solvents were removed *in vacuo*. The paste was treated with 50% ethanol to dissolve the product, and the *p*H adjusted to 7.0. Insoluble barium salts were centrifuged off and the resulting solution concentrated to a paste again (*in vacuo*). The neutralization-evaporation procedure was repeated until the *p*H of the solution was 7.0. The solution was dried until it solidified, then dried *in vacuo* over phosphorus pentoxide. The yield was 3.00 g. (68%) of amorphous material melting at 134–139° dec.

Anal. Calcd. for $Ba(C_{20}H_{19}O_9S)_2$: Ba, 13.62: C, 47.65; H, 3.80; S, 6.36. Found: Ba, 13.46; C, 47.95; H, 3.86; S, 6.18.

Barium glucose 6-sulfate from II. The protective benzylidene groups were removed by hydrolysis in dilute acid. In a typical experiment, 2.7 g. (0.00268 mole) of barium dibenzylidene-glucose 6-sulfate was placed in a 100-ml. round bottomed flask with 50 ml. of 1% acetic acid containing 5% ethanol. The mixture was refluxed for a total of 1.25 hr. Solution was complete after 1 hr., indicating that the reaction was nearly over, since the product, but not the starting material, was soluble in so aqueous a medium. After cooling, the solution, now milky with a suspension of benzaldehyde, was washed with three 30-ml. portions of ether, then concentrated to dryness *in vacuo*. The crude product, free from both benzaldehyde and acetic acid, was dissolved in a little water, treated with charcoal, filtered, neutralized to *p*H 7.0 with barium hydroxide solution, and concentrated to a syrup *in vacuo*. Absolute ethanol was added to precipitate the product. After standing overnight, it was filtered off, dried *in vacuo* over phosphorus pentoxide, and weighed: 1.1 g. (59.5%).

The brucinium salt was prepared as above to yield 0.5 g. (23%, based on barium salt) of fine white crystals with m.p. 182° dec. Its X-ray diffraction pattern was taken as a standard for brucinium α-D-glucose 6-sulfate (nickel-filtered Cu Kα radiation): 9.50, 8.54 (5), 7.92, 7.36, 6.90, 6.39 (5), 5.99, 5.66, 5.32 (5), 4.68 (1, strongest), 4.41, 4.14, 4.01, 3.797 (3), 3.636 (2), 3.490 (5) 3.370, 3.242, 3.126 (4), 2.994.

Anal. Calcd. for $C_{29}H_{38}O_{18}N_2S \cdot 2H_2O$: C, 50.43; H, 6.13; N, 4.06; S, 4.64. Found: C, 50.19; H, 6.11; N, 3.75; S, 4.24.

(Removal of the protecting groups from barium 1,2; 3,5-di-*O*-benzylidene-α-D-glucofuranose 6-sulfate by hydro-

(21) E. C. Horning, *Org. Syntheses*, Coll. Vol. III, 432 (1955).

(22) B. Helferich and J. Becker, *Ann.*, 440, 16 (1924).

(23) M. L. Wolfrom and L. J. Tanghe, *J. Am. Chem. Soc.*, 59, 1597 (1937).

TABLE I
KINETIC DATA

Substrate	Temp., ±0.1°	Substrate, (Mole/l.)	HCl, (Mole/l.)	Pseudo-1st Order Rate Constants, Min.$^{-1}$ (least squares)
Potassium glucose 6-sulfate	37.0	0.0600	0.487	$3.37 \pm 0.17 \times 10^{-6}$
	52.0	0.0600	0.503	$2.95 \pm 0.15 \times 10^{-5}$
	70.0	0.0600	0.495	$3.36 \pm 0.16 \times 10^{-4}$
	70.0	0.0287	0.490	$3.62 \pm 0.18 \times 10^{-4}$
	52.0	0.0600	1.24	$8.37 \pm 0.42 \times 10^{-5}$
Potassium ethyl sulfate	37.0	0.0600	0.485	$1.08 \pm 0.05 \times 10^{-6}$
	52.0	0.0600	0.519	$1.10 \pm 0.06 \times 10^{-5}$
	70.0	0.0600	0.505	$1.22 \pm 0.06 \times 10^{-4}$
	70.0	0.030	0.499	$1.26 \pm 0.10 \times 10^{-4}$
	52.0	0.0600	1.23	$2.89 \pm 0.29 \times 10^{-5}$

genolysis in aqueous ethanol over palladium black[24] at 1–2 atm. of hydrogen, failed. However, when the free acid was used, hydrogen uptake was observed, but was believed to be due to hydrogenation of the benzaldehyde produced by hydrolysis.)

Separation of the products of direct sulfation of glucose by column chromatography. Solka-Floc was washed twice with about four to five times its weight of the following solvent mixture: 1-butanol-ethanol-water, 40:12:20. A slurry of the washed cellulose was then poured into a column and packed by passing the solvent mixture through, under 3–4 p.s.i. air pressure until the effluent was colorless, and the cellulose had completely settled.

A trial run was made on a cellulose column 1.29 × 29 cm. A solution containing 0.1 g. of the crude product was prepared as follows: The mixture was dissolved in 1 ml. of water; to this was added 0.6 ml. of ethanol, followed by butanol to a point just short of cloudiness. This solution was placed on the column and eluted with the solvent mixture described above, under gravity. Fractions (3-ml.) were collected on a Rinco Automatic Fraction Collector, and analyzed by paper chromatography:

Fraction No.	Material Present
0–13	—
14–20	Glucose
21–39	Glu-6-SO_4
40–70	Glu-diSO_4

Because the method seemed successful, a larger column (3.0 × 78 cm.) was prepared. A solution of the mixture [5 g. of the crude product in 20 ml. of water, plus 12 ml. of absolute ethanol, then butanol to incipient cloudiness (*ca.* 10 ml.)] was placed on the column and eluted. That the solution contained too much material for separation was demonstrated by a break-through of unresolved starting material (1.9 g., 38%) in the volume 500–1000 ml. The attempted separation was repeated with 1.5 g. of the mixture dissolved in 15 ml. of water + 9 ml. of ethanol + 10 ml. of butanol. Again, there was a break-through of unresolved components, occurring this time in the range 700–820 ml. Various groups of fractions were collected and combined. The solvent was evaporated from the last group of fractions collected (2030–2650 ml.) to yield 0.12 g. of material with an analysis corresponding to 26.1% barium. Ba glu-mono-SO_4 requires 20.95% Ba. Ba glu-di-$SO_4 \cdot 3H_2O$ requires 26.0% Ba.

Kinetic studies on pure potassium glucose 6-sulfate and potassium ethyl sulfate. Hydrolysis of sulfate was followed colorimetrically.[18] The solutions were thermostated to ±0.1°. The exact concentration of acid was determined by titrating aliquots of the solution after a run had been made, and subtracting the contribution of the bisulfate ion produced by the hydrolysis.

Materials. Potassium ethyl sulfate (Eastman Kodak, white label, 10 g.) was purified by dissolving it in water (100 ml.), adding barium hydroxide solution to *p*H 9.0, and centrifuging off the resulting barium sulfate. The clear, sulfate-free solution was passed over an Amberlite IR-120 column, hydrogen-ion form, to remove barium ion. The effluent was neutralized to *p*H 7.2 with potassium hydroxide solution, concentrated to about 25 ml. *in vacuo*, and lyophilized.

Anal. Calcd. for $KC_2H_5O_4S$: K, 23.8. Found: K, 23.1.

Potassium glucose 6-sulfate was prepared by addition of potassium hydroxide solution to pure brucinium glucose 6-sulfate (17 g.) dissolved in water (700 ml.), followed by adequate washings with chloroform and ether. For the kinetic runs, the crystalline material was not yet available. Instead, the solution of potassium glucose 6-sulfate was concentrated to *ca.* 60 ml. *in vacuo*, and lyophilized to give 6.7 g. of pure material.

Anal. Calcd. for $KC_6H_{11}O_9S.H_2O$: K, 12.36. Found: K, 12.43.

Barium chloroanilate was used as supplied by Fisher Scientific Company.

Procedure. The following general method was used in the kinetic determinations having substrate concentration equal to 0.0600*M* and acid concentration 0.5*M*: The desired amount of substrate (0.9490 g. of potassium glucose 6-sulfate; 0.4927 g. of potassium ethyl sulfate) was weighed into a 50-ml. volumetric flask and dissolved in a little water. To the solution was added 25.0 ml. of 1*M* hydrochloric acid and sufficient water to dilute the contents to volume. After thorough mixing, the flask was thermostated. Analyses were made at appropriate time intervals by neutralizing a 5-ml. aliquot contained in a 20-ml. beaker, with 1.2*M* ammonium hydroxide to a *p*H of 2.5–2.8 (*ca.* 2 ml. required), then with 0.12*M* ammonium hydroxide to *p*H 3.0. Five milliliters of 1:1 0.05*M* potassium hydrogen phthalate buffer and 95% ethanol was then added, and the *p*H very carefully adjusted to 4.12–4.15 with the 0.12*M* ammonium hydroxide (accurate *p*H control was very essential). The solution was transferred to a 25-ml. volumetric flask. The electrodes were rinsed off into the beaker with about 1 ml. of water, then 5 ml. of 95% ethanol (pipet). This was transferred to the volumetric flask, and the beaker again rinsed into the flask with 5-ml. more alcohol (pipet). The solution was then diluted to volume with water. A blank was prepared similarly from a 5 ml. aliquot of 0.5*M* hydrochloric acid. (This blank, thus prepared, was also used for diluting purposes; see below.) To each 25-ml. volumetric flask was added 0.050 g. of barium chloranilate. The flasks were shaken once a minute for 15 min., the solution centrifuged to clarity, and the per cent transmittance read on a Fisher Electrophotometer (or other suitable instrument), using the 525B green filter on the D scale. From a standard curve prepared from known concentrations of sulfate, the amount

(24) E. C. Horning, *Org. Syntheses*, Coll. Vol. III, 685 (1955).

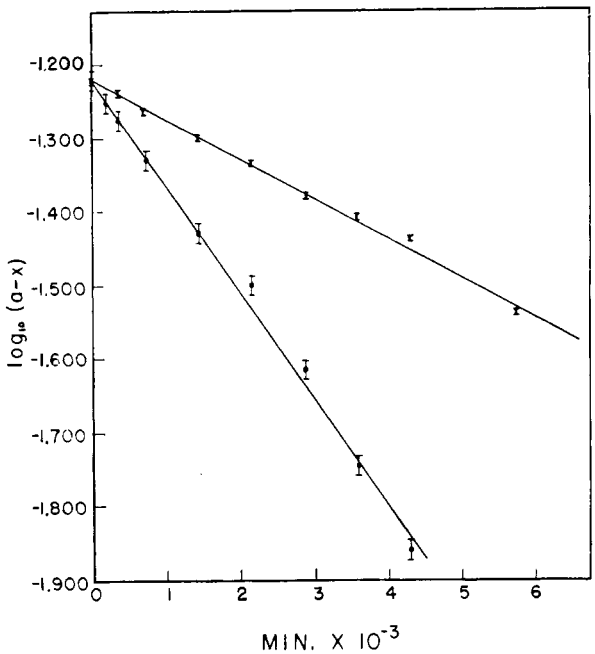


Fig. 1. First order plot (least squares) for hydrolysis of sulfate from 0.0600*M* K glu 6-SO_4 (∓) and 0.0600*M* K $C_2H_5SO_4$ (I) in 0.5*M* HCl at 70.0°

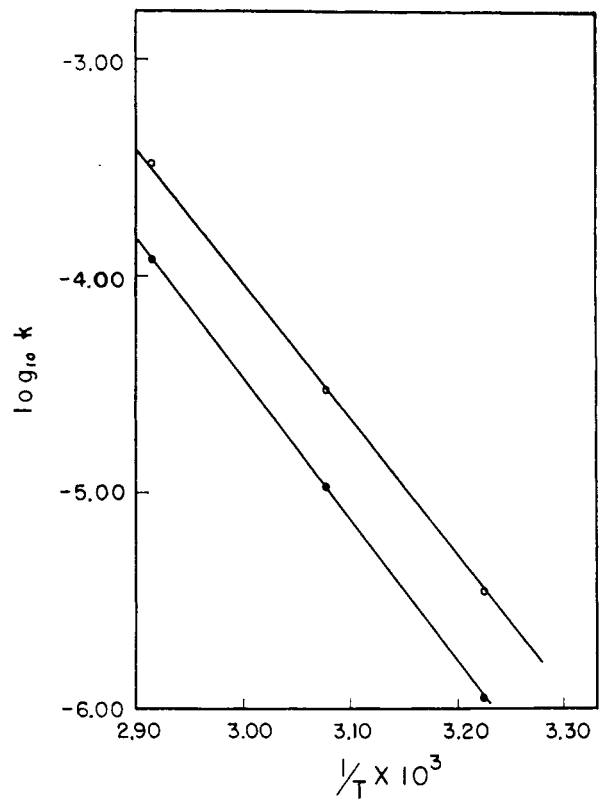


Fig. 2. Arrhenius plot for hydrolysis of sulfate from K glu 6-SO_4 (○) and K $C_2H_5SO_4$ (●) in 0.5*M* HCl

of sulfate liberated was determined. If an aliquot was known to contain an amount of sulfate greater than the upper limit of the standard curve, a dilution was made as follows: Following the neutralization of the sample, and dilution to 25 ml., an aliquot of suitable size was withdrawn and placed in another (dry) 25-ml. volumetric flask. After dilution to volume with prepared blank, this solution was treated with barium chloranilate in the usual manner, an appropriate factor being used when calculating the amount of sulfate present in the original 5-ml. aliquot of reaction mixture.

In the other two sets of kinetic runs, certain modifications were made: For 0.030*M* substrate in 0.5*M* hydrochloric acid, half as much substrate was used; the amount of acid and method of analysis were unchanged. For 0.0600*M* substrate in 1.25*M* hydrochloric acid, the acid added initially, was 2.5*M* (25 ml.), the amount of substrate being the same as before. For analysis of this mixture, 2 ml. was pipetted from the reaction flask and added to 3 ml. of water contained in a 20-ml. beaker. From here on, the method was as described above.

Hydrolytic studies on unpurified glucose sulfate. A. *At pH 5.1.* In a typical experiment, 1.6214 g. of impure glucose sulfate was dissolved in 15 ml. of water, and the solution passed over an ice-water jacketed Amberlite IR-120 column, hydrogen-ion form (1.0 × 13.5 cm.). The free acid was thoroughly removed from the column with water. The effluent was neutralized to *p*H 7.0 with sodium hydroxide solution, then concentrated to a few milliliters *in vacuo*. The solution of sodium glucose sulfate was transferred quantitatively to a 25-ml. volumetric flask and diluted to volume with water. To prepare a solution for hydrolytic study, the following solutions were combined in a 25 ml. volumetric flask, and diluted to volume with water: 8 ml. of the sodium glucose sulfate solution, 10 ml. of 1*M* acetate buffer, *p*H 5.1, 3 ml. of 1*M* catalyst (hydrazine, semicarbazide, imidazole, *dl*-serine, or L-glutamine) adjusted to *p*H 5.1. Final concentrations were: substrate, 0.060*M*, catalyst, 0.120*M*, buffer, 0.40*M*. Blanks were prepared similarly, omitting the glucose sulfate solution; a control was prepared by omission of the catalyst. The flasks were immersed in a constant temperature bath, 37.5 ± 0.1°. At intervals, 5-ml. aliquots were withdrawn and analyzed by the method described in an earlier section (determination of disulfate) for the case of the *p*H 5.1 acetate buffer.

B. *At pH 8.9.* Conditions were the same as in part A, except that the buffer stock solution was 1*M* ammonium chloride–ammonium hydroxide, and the catalyst stock solutions were adjusted to *p*H 8.9 with ammonium hydroxide. The analyses were made as before, except that 0.22*M* hydrochloric acid was used to lower the *p*H of the aliquot to 4.0. Also, suitable blanks and controls were run.

RESULTS AND DISCUSSION

Kinetic studies on pure potassium glucose 6-sulfate and pure potassium ethyl sulfate. The results of the kinetic studies are given in Table I.

Figure 1 shows a typical least squares plot of the data for one of the kinetic runs, while in Fig. 2, the temperature dependence of the sulfate hydrolysis is shown for both potassium glucose 6-sulfate and potassium ethyl sulfate. From the latter curves, the activation energies were calculated and found to be 29 kcal per mole for the glucose 6-sulfate anion, and 30 kcal per mole for the ethyl sulfate anion.

A rough comparison can be made between the rates of hydrolysis of phosphate and sulfate from the 6-position of glucose. Robison[25] found the first order rate constant for the hydrolysis of phosphate from glucose 6-phosphate in 1*M* hydrochloric acid at 100° to be 2.2 × 10^{-4} $min.^{-1}$. By extrapola-

(25) R. Robison, *Biochem. J.*, **26**, 2191 (1932).

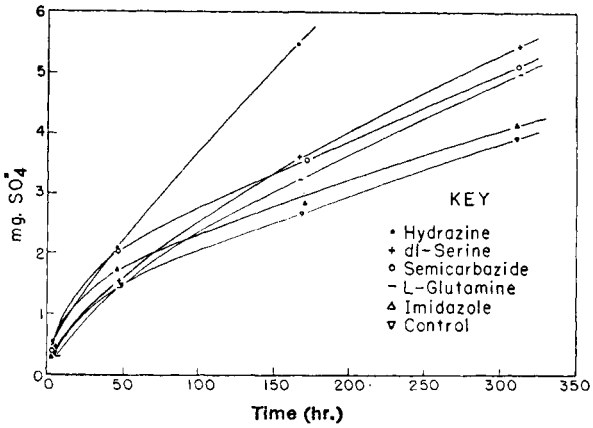


Fig. 3. Hydrolysis of impure Na glu 6-SO_4 (0.06*M*) in the presence of some nitrogenous bases (0.12*M*), in 0.40*M* acetate buffer (*p*H 5.1) at 37.5°

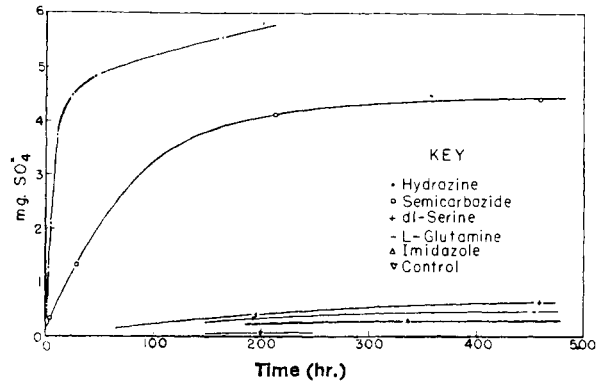


Fig. 4. Hydrolysis of impure Na glu 6-SO_4 (0.06*M*) in the presence of some nitrogenous bases (0.12*M*), in 0.40*M* ammonium buffer (*p*H 8.9) at 37.5°

tion of the Arrhenius plot, the rate constant for the hydrolysis of glucose 6-sulfate in 0.5*M* hydrochloric acid at 100° can be found. As, at the concentrations used, the rate was roughly proportional to the concentration of acid, this value can be doubled to give an approximate value for the rate constant at 100° in 1*M* hydrochloric acid: 1.97×10^{-2} min.$^{-1}$, which is about two orders of magnitude as great as the value for the phosphate. It will be interesting to compare more of these as more data become available.

Hydrolytic studies on unpurified glucose 6-sulfate (ca. 15% disulfate). The results of these preliminary studies are shown in Figs. 3 and 4. As already reported by Soda and Egami,[12,26] hydrazine and semicarbazide were very effective in catalyzing the hydrolysis of sulfate from the disulfate impurity at *p*H 5.1, but much less so in the case of the 6-sulfate (major constituent of the unpurified material). At *p*H 5.1, the other bases examined showed very little activity. At *p*H 8.9, there was a general hydrolysis brought about by the base, although imidazole, L-glutamine, semicarbazide, and *dl*-serine exhibited a small catalytic effect. Hydrazine again showed a strong tendency to accelerate the hydrolytic process.

X-ray crystallographic data on pure potassium β,D-glucose 6-sulfate. From a Weissenberg picture of a single crystal of potassium β-D-glucose 6-sulfate, taken and interpreted by William O. Roberts, the lattice constants of the unit cell (monoclinic) were tentatively assigned the following values: $a_0 = 12.99$ Å, $b_0 = 7.71$ Å, $c_0 = 5.77$ Å, $\beta = 106.6°$. From the density of the compound, 1.816 ± 0.004 g./cc., it followed that there were two molecules per unit cell. However, when d values were obtained from the powder pattern, using a recording diffractometer, *hkl* values could be assigned to only half the d values. There is evidence that grinding causes a partial phase change. More work is currently underway to determine if this is the case.

Polarimetric studies on pure brucinium α-D-glucose and pure potassium β-D-glucose 6-sulfate. Polarimetric readings were obtained at several times during the mutarotation of these two compounds and a plot of $\log_{10}(\alpha_\infty - \alpha)$ *vs. t*, made for each. Extrapolation to zero time gave rotational values of +14.2° for potassium β-D-glucose 6-sulfate and −1.28° for brucinium α-D-glucose 6-sulfate at 20°. After taking into account the rotation of the brucinium ion, the relatively amounts of α- and β-forms in the equilibrium mixture were found to be 81.5% and 18.5%, respectively.

NOTE ADDED IN PROOF: Two significant contributions on monosaccharide sulfates appeared in the December issue of the Journal of the Chemical Society, S. Peat *et al.*, *J. Chem. Soc.*, 4761 (1960) and D. Grant and A. Holt, *J. Chem. Soc.*, 5026 (1960).

(26) H. Egami, *J. Chem. Soc. Japan*, 59, 1034 (1938).

SYRACUSE 10, N. Y.