APPENDIX

Preparation of D-Glycerate 3-O-Sulphate, DL-Glycerate 3[³⁵S]-O-Sulphate and D-Glucose 6-O-Sulphate

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The studies described in the main paper (Fitzgerald & Dodgson, 1971) necessitated the preparation of a number of sulphate esters the synthesis of which had either not been reported before or which were difficult to obtain in pure form on a large scale. The ³⁵S-labelled and unlabelled 3-O-sulphate esters of glyceric acid belong to the first category and the 6-O-sulphate ester of D-glucose to the latter. The problem relating to the synthesis of the glycerate esters is to ensure that the ester sulphate group is present at position C-3 rather than C-2, whereas the problem with the glucose ester is to remove contaminating isomers (cf. Meezan, Olavesen & Davidson, 1964) and the large quantities of salt that accompany column fractions (cf. Lloyd, Large, Davies, Olavesen & Dodgson, 1968).

METHODS

Definitive synthesis of dipotassium D-glycerate 3-O-sulphate. This was achieved by treating potassium D-serine O-sulphate with nitrous acid, a reaction that forms the basis of the Van Slyke gasometric method for determining amino acid nitrogen (Van Slyke, 1912) and that ensures that the ester sulphate group will be located at C-3 in the sulphated glycerate product:

Potassium D-serine O-sulphate (1.5g prepared by the method of Tudball, 1962) was dissolved in 10ml of water and acidified to pH 1.5 with 2M-HCl. The solution was stirred and cooled to 0°C in an ice bath. NaNO₂ (0.7g) dissolved in 7ml of water was added slowly (approx. 15 min) and stirring was continued for a further 30 min at 0°C and for 1 h at room temperature. The mixture was passed through a pad (9 cm × 2 cm) of Dowex 50 ion-exchange resin (H⁺ form; 20–50 mesh), supported on a Buchner funnel, to remove cations and the acid solution was adjusted to pH 7.5 with a saturated solution of Ba(OH)₂·8H₂O. Precipitated BaSO₄ was removed by centrifuging and the supernatant was concentrated *in vacuo* at 40°C to about 3ml. Ethanol was added dropwise to the cooled solution (0°C) until an orange oil separated. The supernatant was decanted and further ethanol added to it until a white solid separated. The solid was filtered off, dissolved in 5 ml of water and again passed through a pad of

Dowex 50 (H⁺ form) to remove Ba²⁺ ions. The acid solution was adjusted to pH 7.5 with 5% (w/v) KOH and concentrated *in vacuo* at 40°C to small volume. Ethanol was added dropwise and the supernatant was decanted from any further orange oil (or solid) that separated. Addition of ethanol to the supernatant was continued until a white solid was precipitated, and this was separated at the pump, washed with ethanol and then with ether and recrystallized from ethanol–water (1:1, v/v). The yield was 220mg. The product moved as a single component on paper electrophoresis and on paper chromatography with solvent 1 (see Fitzgerald & Dodgson, 1971). The i.r. spectrum was in accord with a structure of D-glycerate 3-O-sulphate, and glyceric acid and inorganic SO₄²⁻ ions were liberated after hydrolysis of the compound with M-HCl for 15 min at 100°C (Found: K, 33.9; SO₄²⁻, 41.8. Calc. for C₃H₄K₂O₇S: K, 33.9; SO₄²⁻, 41.7%).

The corresponding L-isomer can be made in an identical manner starting with L-serine O-sulphate, although consistently lower yields have been obtained in this case.

Preparation of dipotassium DL-glycerate 3[³⁵S]-O-sulphate by direct sulphation. Calcium DL-glycerate (Sigma Chemical Co., St Louis, Mo., U.S.A.) was ground to a fine powder and dried over CaCl₂ *in vacuo* at room temperature. The dried powder (1.82g) was suspended in 20ml of dry dimethylformamide and the whole cooled to –10°C before adding (with stirring) 0.01 mol of chlorosulphonic acid containing 35 μl of chloro[³⁵S]sulphonic acid (specific radioactivity 6.35 mCi/mmol). The acid was added dropwise over a period of 20 min, when the temperature was allowed to rise to 0°C and stirring was continued for a further 10 min. After the mixture had been kept at room temperature for 2.5 h an equal volume of ice-cold water was added with vigorous stirring, when the clear solution became turbid. The pH was adjusted to 7.0 with 0.1M-KOH and the whole was concentrated to dryness *in vacuo* at 30°C. The dried material was suspended in 20ml of water and the suspension was filtered. The filtrate was treated with acetone (3 vol.) and precipitated material was removed by filtration. The filtrate was concentrated to low bulk *in vacuo* at 30°C and passed through a column (2.5 cm × 25 cm) of Dowex 50 (X8; Ba²⁺ form; 100–200 mesh). The cloudy eluate was filtered through a Millipore filter (0.45 μm) and the clear filtrate passed through a second Dowex 50 column (H⁺ form). The acid eluate was neutralized with 0.5M-KOH and concentrated *in vacuo* at 30°C to less than 1 ml. Portions of the concentrate were subjected to thick-layer chromatography on Silica Gel G with solvent 2 (Fitzgerald & Dodgson, 1971). Radioautographs revealed the presence of a major component (*R_F* 0.35) and a minor component (*R_F* 0.11). Areas

of the plates corresponding to the major component were scraped off and suspended in aq. 50% (w/v) acetone (0.5 g of dry gel/ml). The suspension was filtered through Whatman no. 1 filter paper and material retained by the paper was dried in air and re-extracted twice with acetone as before. The combined filtrates from the extracts were concentrated *in vacuo* at 30°C to the smallest possible volume and the ester was precipitated by the dropwise addition of 3 vol. of ethanol. Precipitated material was separated and dried *in vacuo* over CaCl_2 . The yield was 300 mg and the specific radioactivity was $0.48 \mu\text{Ci/mg}$. The material was homogeneous on paper electrophoresis and chromatography, mobilities being identical with those exhibited by authentic D-glycerate 3-O-sulphate. The i.r. spectrum of the material was identical with that of the authentic compound. The labelled ester was stable for periods up to 6 months when stored either as a dry solid at 2°C or as a frozen aqueous solution at -20°C.

Preparation of D-glucose 6-O-sulphate on a large scale. A crude preparation of the barium salt of D-glucose 6-O-sulphate was obtained on a large scale (52.7 g) by the pyridine- SO_3 method of Lloyd (1960), except that the reaction mixture was shaken at 30°C for 5 h.

The crude preparation was fractionated (in 11 g quantities, each dissolved in 40 ml of water) on a Dowex 1 (X8; formate form; 200–400 mesh) column (4.5 cm $\times$ 45.0 cm). To remove unchanged glucose the column was washed with water until the effluent no longer reacted with anthrone reagent (Siefter, Dayton, Novic & Muntwyler, 1950). The column was then eluted with a linear gradient of formic acid (0–50%, v/v), and the effluent (4 litres), which contained small quantities of D-glucose 3-O-sulphate, was discarded. Further elution was with 0.3 M-ammonium formate in 50% (v/v) formic acid until the effluent was anthrone-negative (2 litres). Any disulphated glucose remains on the column under these conditions. The anthrone-positive fractions were combined and concentrated *in vacuo* at 25°C to 100 ml. Ammonium formate in the concentrate was converted into formic acid by passage through two columns (each 4.0 cm $\times$ 35.0 cm) of Dowex 50 (X8; H^+ form; 100–200 mesh). The acid eluate was concentrated as before and the concentrate was extracted with successive portions (4 vol.) of ether until samples were negative for formate by the chromotrophic acid test (Vogel, 1953). The extracted concentrate was adjusted to pH 7.4 with 0.5 M-KOH and concentrated *in vacuo* at 35°C to low bulk. Potassium D-glucose 6-O-sulphate was precipitated by the addition of 5 vol. of ethanol (96%, v/v) and collected by centrifuging. The material was washed with ethanol followed by acetone and dried *in vacuo* at room temperature over CaCl_2 . The yield (from 11 g of crude material) was 4–5 g. Samples from two

independent preparations were analysed [Found (preparation 1): C, 23.6; H, 3.8; K, 13.8, S, 9.6; (preparation 2): C, 23.2; H, 3.9; K, 14.3; S, 9.4; $\text{C}_6\text{H}_{11}\text{KO}_3\text{S}$ requires C, 24.2; H, 3.7; K, 13.1; S, 10.7%].

Paper chromatography on Whatman no. 1 paper with butan-1-ol-acetic acid-water (4:1:2, by vol.) showed the product to be free from glucose (AgNO_3 reagent of Trevelyan, Procter & Harrison, 1950) and from inorganic SO_4^{2-} ions [BaCl_2 -rhodizonate method as described by Lloyd (1960)]. Absence of glucose was confirmed by the fact that glucose oxidase was without action on the final product (cf. Lloyd *et al.* 1968).

Chromatography on thin layers formed from slurries of Silica Gel G (30 g) in 0.1 M-boric acid (50 ml) was employed to show the absence of contaminating D-glucose 3-O-sulphate. 2-Methoxyethanol-pyridine-acetic acid-water (8:4:1:1, by vol.) was the developing solvent and other details of the procedure have been described by Wusteman, Dodgson, Lloyd, Rose & Tudball (1964). Chromatography under these conditions separated authentic samples of D-glucose 3-O-sulphate (R_F 0.48) and D-glucose 6-O-sulphate (R_F 0.70), kindly supplied by Mr D. I. Bain and prepared by the methods of Dodgson & Spencer (1954) and Lloyd *et al.* (1968) respectively.

The i.r. and n.m.r. spectra of the product were identical with those of authentic D-glucose 6-O-sulphate.

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