926. *Sulphates of Monosaccharides and their Derivatives. Part I. Preparation.*

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D-Glucose, D-galactose, methyl α- and β-D-glucoside, as well as methyl β-D-galactoside have been directly sulphated with the pyridine–sulphuric anhydride reagent, and chromatographic techniques have been developed for the isolation of the sulphates. Comparison with glucose 3-, glucose 6-, and galactose 6-sulphate, synthesised by routes which define their structures, supports the view that the direct sulphation of glucose and of galactose occurs mainly at position 6.

Carbohydrate sulphates occur widely in Nature,[1,2] and the simple monosaccharide sulphates have received considerable study.[1] The present study concerns the preparation and properties of simple sugar sulphates in the hope that the results will be useful in the structural analysis of polysaccharide sulphates.

Monosaccharides have been sulphated by chlorosulphonic acid,[3] sulphuryl chloride,[4] and pyridine–sulphuric anhydride,[5,6] but purification of the products has been difficult and almost the only criterion of purity has been the analytical data. Although the formation of crystalline alkaloid salts was a step forward in purifying sugar sulphates, several recrystallisations of, for example, the brucine salt failed to yield a completely pure product from the direct sulphation of glucose.[7,8] More recently, the use of paper chromatography has demonstrated the complex nature of the mixtures obtained by direct sulphation of hexoses.[8,9] We describe here some methods which have been developed for the preparation and purification of monosaccharide sulphates.[9]

Table 1. *The sulphation of monosaccharides.*

Sugar (1 mol.)	Reagent * (mols.)	Temp.	Time (hr.)	Yield (g.) †	Sugar (1 mol.)	Reagent * (mols.)	Temp.	Time (hr.)	Yield (g.) †
Mannitol ...	1·5	35°	120	5·6	Galactose	1·5	18°	24	7·1
Glucose ...	3·0	35	24	7·4	,,	3	35	48	7·4
,, ...	3·0	65—70	6	9·3	,,	3	55	9	12·0
					,,	3	65—70	6	11·8

* Pyridine-sulphuric anhydride. † From 5 g. sugar.

In the present study, direct sulphation was effected by the pyridine–sulphuric anhydride reagent. Preliminary experiments (cf. Table 1) showed that temperatures as high as 70° may be used with advantage, especially when excess of the reagent is employed. We followed the reaction by means of paper electrophoresis in neutral buffers. The products from galactose are thereby readily separated into unchanged sugar, monosulphate, disulphate, and trisulphate (when present) in 2 hr. with a potential gradient of 20 v./cm. The neutral buffers used initially were sodium acetate or phosphate but later an acetic acid–pyridine buffer (pH 6·5) was preferred since it could be readily removed from the paper by drying in air and, in some cases, resulted in increased sensitivity to the spray reagents.

The mixture obtained by sulphation of galactose was used to investigate methods of

[1] Percival, *Quart. Rev.*, 1949, **3**, 369.
[2] Mori, *Adv. Carbohydrate Chem.*, 1954, **8**, 315; Trans. Fourth Conf. on Polysaccharides in Biology, The Josiah Macey Jr. Foundation, New York, 1959.
[3] Neuberg and Liebermann, *Biochem. Z.*, 1921, **121**, 326.
[4] Levene and Meyer, *J. Biol. Chem.*, 1922, **53**, 437.
[5] Baumgarten, *Ber.*, 1926, **59**, 1166.
[6] Duff, *J.*, 1949, 1597.
[7] Egami, *J. Chem. Soc. Japan*, 1942, **63**, 763.
[8] Dodgson and Spencer, *Biochem. J.*, 1954, **57**, 310; *Ann. Reports*, 1956, **53**, 320.
[9] Cf. Turvey and Clancy, *Nature*, 1959, **183**, 537.

purification. Ion-exchange chromatography has been used by others [10] for resolution of monosaccharide phosphate mixtures; for the sulphation mixture graded elution from an anion-exchange resin with salt solutions gave only incomplete separation, and recovery of sugar sulphates from the large volumes of salt solutions was laborious. Nevertheless, where the mixture was non-reducing and contained only one sulphated component, *e.g.*, a glycoside and its monosulphate derivative, separation on ion-exchange resins was successful. The sulphate was converted into the acid form and then absorbed on an anion-exchange resin, neutral material being washed through with water. Subsequent elution of the resin with dilute aqueous ammonia, followed by cautious evaporation of the eluate, gave the sugar sulphate as the ammonium salt. These ammonium salts usually had characteristic melting or decomposition points but tended to be hygroscopic and were converted into sodium or barium salts for storage.

The most practical method of separating the products of sulphation was by partition chromatography on columns of cellulose powder (best prepared by packing with dry powder and consolidation by mechanical vibration). As solvent systems, ethanol–acetic acid–water and ethanol–formic acid–water give equally good results. The former system readily separated the galactose sulphation mixture (barium salts) into unchanged galactose and monosulphate, disulphate being subsequently eluted with water. Lloyd [11] has recently used a similar method, in which the sulphation mixture (as the free acids) is separated on columns of cellulose. Previous workers [7,8] have reported that direct sulphation of hexoses gives the 6-sulphate with smaller amounts of isomeric monosulphates. In the products of sulphation of glucose and of galactose we have detected three monosulphates by paper chromatography and agree that one monosulphate predominates. In

TABLE 2. *Physical properties of sugar sulphates.*

Sugar sulphate	$[\alpha]_D$ in H_2O	Relative † R_F value	Sugar sulphate	$[\alpha]_D$ in H_2O	Relative † R_F value
D-*Glucose*			D-*Galactose*		
3-(barium sulphate)	+33·0°	0·29	6-(barium sulphate)	+33·9°	0·20
6-(barium sulphate)	+29·0	0·18	barium sulphate *	+33·1	0·20
barium sulphate *	+28·7	0·18	di(barium sulphate) *	+38·1	0·05

* Prepared by direct sulphation of sugar. † Compared with that of glucose in ethyl acetate–acetic acid–water.

the eluate from cellulose columns, the minor components appeared in the early portions of the monosulphate fraction and hence, by selection of only the peak and later portions of this fraction, the major component was obtained in each case in a chromatographically and ionophoretically pure form (Table 2).

For reference, definitive syntheses of D-galactose 6-sulphate and of D-glucose 3- and 6-sulphate were undertaken. Glucose 3-sulphate and galactose 6-sulphate were prepared by sulphation of the respective di-*O*-isopropylidene derivatives,[12] removal by hydrolysis of the acetone residues, and resolution on cellulose columns. D-Glucose 6-sulphate has been prepared [11] by sulphation of 1,2,3,4-tetra-*O*-acetyl-D-glucose; but we preferred to use 1,2-*O*-isopropylidene-D-glucofuranose 3,5-orthoborate as the intermediate, since it is easily prepared and the blocking groups are readily removed, after sulphation, with little hydrolysis of sulphate groups. Separation on a cellulose column then gave D-glucose 6-sulphate in a chromatographically and ionophoretically pure form. The physical constants of the synthetic sugar sulphates are listed in Table 2.

Many workers have concluded that the main product of direct sulphation of galactose and of glucose is probably, in each case, the 6-sulphate and, indeed, this is to be expected on the basis of the known greater reactivity of the primary hydroxyl group. Comparison of the monosulphates prepared by direct sulphation of glucose and galactose with the

[10] Khym and Cohn, *J. Amer. Chem. Soc.*, 1953, **75**, 1153.
[11] Lloyd, *Nature*, 1959, **183**, 109.
[12] Percival and Soutar, *J.*, 1940, 1475.

respective 6-sulphates, synthesised as described, on the basis of optical rotations (Table 2) supports this view, which was confirmed by comparison of R_F values on paper chromatograms run in the normal solvent systems and in a system containing cetylpyridinium chloride.[13] The disulphates prepared by direct sulphation are probably mixtures, since paper chromatography has indicated that galactose di(barium sulphate) contains at least two components.

Kinetic studies of the hydrolysis of ester sulphate and glycosidic linkages necessitated the preparation of methyl glycoside monosulphates. Methyl β-D-galactopyranoside monosulphate was prepared by direct sulphation of the glycoside [14] and separation of the monosulphate fraction on a cellulose column. The product was free from disulphate but still contained a trace of non-sulphated glycoside, which was removed by the ion-exchange resin method. The galactoside monosulphate was chromatographically homogeneous and

TABLE 3. *Physical properties of sulphates of glycosides and sugar ketals.**

Sulphate	M. p.†	$[\alpha]_D$ in H_2O
Me β-D-galactoside ammonium sulphate	174—175°	−15·6°
Me β-D-galactoside di(barium sulphate)	—	+7·0
Me α-D-glucoside ammonium sulphate	165—166	+78·6
Me β-D-glucoside ammonium sulphate	155	−13·3
Di-*O*-isopropylidene-D-galactose 6-(ammonium sulphate)	130	−24·7
Di-*O*-isopropylidene-D-glucose 3-(ammonium sulphate)	153—155	+5·0

* Prepared by direct sulphation of glycoside or isopropylidene derivative. † Usually with decomp.

was shown to be the 6-sulphate by periodate oxidation (to be described in a later communication). Direct sulphation of methyl α- and β-glucopyranoside [12,14] gave the respective monosulphates with no disulphate, and ion-exchange separations yielded both monosulphates in chromatographically pure forms. The physical constants of the synthetic products are listed in Table 3. Further studies on these sugar sulphates will also be reported later.

Since this paper was first prepared, Lloyd [15] has described other methods for the preparation and purification of sugar sulphates. He uses column electrophoresis in separating the monosulphate fraction from the disulphate and parent sugar present as impurities in a crude monosulphate preparation.

EXPERIMENTAL

General Methods.—All solutions were evaporated under diminished pressure below 40°.

(a) *Analytical.* Total sulphur was determined, after digestion of the sugar sulphate with fuming nitric acid,[16] gravimetrically as barium sulphate or by the titrimetric method of Belcher, *et al.*[16] Barium was determined as barium sulphate or by dissolution of the barium salt in ammoniacal ethylenediaminetetra-acetic acid (EDTA) and titration with magnesium chloride.[17]

(b) *Paper chromatography.* Whatman No. 1 or No. 54 paper was used in the descending technique with the solvent systems, (i) butan-1-ol–acetic acid–water (4 : 1 : 5, by vol., organic phase), (ii) ethyl acetate–acetic acid–water (6 : 3 : 2, by vol.), and (iii) butan-1-ol–ethanol–water (3 : 1 : 1, by vol.) containing cetylpyridinium chloride (3 g./100 ml.). System (ii) gives good resolution of isomeric monosulphates in 3 days (see Table 2). The use of system (iii) for the separation of sugar sulphates has already been described.[13] Carbohydrate zones were detected with silver nitrate [18] (non-reducing and reducing sugar sulphates) or with *p*-anisidine hydrochloride [19] (reducing sugar sulphates). The latter reagent is very sensitive, especially when the papers are viewed in ultraviolet light.

[13] Rees, *Nature*, 1960, **185**, 309.
[14] Duff and Percival, *J.*, 1941, 830.
[15] Lloyd, *Biochem. J.*, 1960, **75**, 478.
[16] Belcher, Bhasin, Shah, and West, *J.*, 1958, 4054.
[17] Belcher, Gibbons, and West, *Chem. and Ind.*, 1954, 127.
[18] Trevelyan, Procter, and Harrison, *Nature*, 1950, **166**, 444.
[19] Hough, Jones, and Wadman, *J.*, 1950, 1702.

(c) *Paper electrophoresis.* Buffers used initially were 0·067M-phosphate (pH 6·8) or 0·2M-acetate (pH 7·0), but in later experiments 0·1M-acetic acid, adjusted to pH 6·5 with pyridine, was preferred. Whatman No. 54 paper was used throughout and potentials of 10—20 v./cm. were applied for 2—4 hr. After the papers had been dried in air, sugar zones were detected as for the chromatograms.

(d) *Column chromatography.* Whatman standard-grade cellulose powder was washed with water, ethanol, and ether before being dried at 35°. The dry powder was poured into the column to give a 10 cm. length and the column was then vibrated on the arm of a vibrating shaker until the powder had settled. More cellulose was then added and the process repeated until the required length was obtained. The column was finally washed with the solvent until coloured impurities were no longer eluted. The sample (as barium salts), dissolved in the minimum of water, was absorbed on the top of the column and elution was continued with the solvent. Ethanol–acetic acid–water (80 : 1 : 19, by vol.) was used as solvent initially; the acetic acid was replaced by formic acid (0·33 vol.) in later separations. Fractions (50 ml. each) were collected and the elution pattern of the column was established by analysing portions (1 ml. each) of each fifth fraction for sugar by the anthrone–sulphuric acid method.[20] Fractions were also examined periodically by paper chromatography and electrophoresis, and then bulked as desired. The solutions were evaporated to dryness or to small volume and the sulphates precipitated by pouring into ethanol (20 vol.).

(e) *Ion-exchange separation of non-reducing sugar sulphates.* A column of the cation-exchange resin "ZeoKarb" 225 (H^+ form) was connected in series with, and above, a column of the anion-exchange resin, "De-Acidite" E or F (free-base form). The mixture to be resolved (as a 1% solution in water) was allowed to percolate slowly down the columns. Elution with distilled water was continued until the eluate was free from sugar (anthrone–sulphuric acid test). The columns were then disconnected and the anion-exchange resin irrigated with 2N-ammonia until all carbohydrate material had been eluted. Excess of ammonia was removed from the eluate by passage of a stream of air and, when neutral, the solution was evaporated to dryness (25°). The ammonium salt of the sugar sulphate was dried *in vacuo* over phosphoric anhydride.

Sulphation of Hexoses and Derivatives.—The sugar or sugar derivative (5 g.), dissolved in dry pyridine (100 ml.), was treated with the pyridine–sulphuric anhydride reagent [5] (3 mol.), and the mixture was stirred under anhydrous conditions on a water bath. When cool, water (100 ml.) was added; the solution, after being stirred for 1 hr., was adjusted to pH 9 with saturated aqueous barium hydroxide, and the precipitated barium sulphate was removed on the centrifuge. The solution was evaporated at 35°, water being added periodically to maintain the volume until all pyridine had been removed. Excess of barium was precipitated with carbon dioxide, and the filtrate evaporated to dryness. If colloidal precipitation retained barium carbonate, the residue was dispersed in a small volume of water, filtered, and poured into ethanol (20 vol.). The precipitated sugar sulphates were washed with ethanol and ether and dried over phosphoric anhydride. Some typical yields are given in Table 1. Examination of the products by paper electrophoresis usually indicated the presence of monosulphate and smaller amounts of disulphate as well as unchanged sugar. If the reaction was prolonged (9—10 hr. at 70°) traces of a trisulphate were also detected.

Ion-exchange Separation of Galactose Sulphates.—The mixture (5 g.) obtained by sulphating galactose at 55° for 9 hr. was dissolved in water (50 ml.), and the solution passed through a column of "ZeoKarb" 215 (H^+ form). The combined eluate and washings (250 ml.) were

Eluant	Vol. (l.)	Eluate	Eluant	Vol. (l.)	Eluate
0·005M-Na_2SO_4	4·25	Monosulphate + trace of galactose	0·1M-Na_2SO_4	1·0	Monosulphate
			2N-NH_3	0·5	Mono- and di-sulphate
0·005M-Na_2SO_4	0·25	Monosulphate	0·4M-Na_2CO_3	0·5	Mono- and di-sulphate
0·01M-Na_2SO_4	2·5	Monosulphate			

percolated through a column (2 × 22 cm.) of "Amberlite" IR-4B (free base form) and the column was washed with water until all non-sulphated material had been eluted. From this eluate, galactose (0·36 g.) was recovered. The column was then eluted as indicated in the Table. Fractions were evaporated to dryness and examined by paper electrophoresis.

[20] Draywood, *Ind. Eng. Chem., Analyt.*, 1946, **18**, 499.

Cellulose-column Separations.—(a) *Galactose sulphates.* The mixture (5 g.) of galactose sulphates was applied to a cellulose column (4 × 42 cm.), and the column eluted with the ethanol–acetic acid–water solvent. Fractions (25 ml. each) were collected and grouped before being evaporated to dryness. Fractions 1—15 gave galactose (0·57 g.). Fractions 16—23 contained a mixture (0·19 g.) of galactose and small amounts of monosulphate; fractions 24—47 contained a mixture (0·21 g.) of monosulphates with traces of galactose. Fractions 48—100 contained the monosulphate (1·06 g.) in a chromatographically and ionophoretically pure form (Table 2) [Found: Ba, 21·4. $(C_6H_{11}O_9S)_2Ba$ requires Ba, 21·0%]. A portion (0·5 g.) of the barium salt was converted into the sodium salt by passage through a column of " ZeoKarb " 225 (sodium form) and evaporation of the eluate. The product (0·37 g.) had $[\alpha]_D^{18}$ +42·9° (in H_2O). Fractions 101—130 gave a mixture (0·09 g.) of monosulphate, and disulphate and fractions 154—174 (eluted with water) gave the disulphate (1·68 g.; Table 2) (Found: Ba, 27·4. $C_6H_{10}O_{12}S_2Ba$ requires Ba, 28·8%). A later examination of this batch by paper chromatography indicated that it probably contained two isomeric disulphates.

(b) *Glucose sulphates.* A similar resolution of the mixture (5 g.) obtained by direct sulphation of glucose at 65—67° for 6 hr. gave the monosulphate as main component (0·74 g.; Table 2) [Found: Ba, 21·4; S, 9·4. $(C_6H_{11}O_9S)_2Ba$ requires Ba, 21·0; S, 9·8%].

Methyl β-D-*Galactopyranoside Ammonium Sulphate.*—Methyl β-D-galactopyranoside (21·5 g.; m. p. 175°, $[\alpha]_D^{20}$ −0·69°) was sulphated at 65—70° for 5 hr. to give a product (45·5 g.), which contained unchanged galactoside and mono- and di-sulphate. The product (5 g.) was partly resolved on a cellulose column (45 × 4 cm.) into main fractions: (i) a mixture (0·35 g.) of galactoside with a trace of the monosulphate; (ii) a mixture (0·34 g.) of galactoside and monosulphate; (iii) monosulphate (2·4 g.) containing traces of galactoside; and (iv) methyl β-D-galactopyranoside di(barium sulphate) (1·05 g.; Table 3) (Found: Ba, 26·6. $C_7H_{12}O_{12}S_2Ba$ requires Ba, 28·0%). Fraction (iii) (1·03 g.) was further separated into unchanged galactoside and methyl β-D-galactopyranoside ammonium sulphate (0·65 g.; Table 3), m. p. 149—150°, decomp. 174—175° (Found: S, 11·1. $C_7H_{17}O_9NS$ requires S, 11·1%). A larger-scale resolution of the sulphation mixture (15 g.) gave the monosulphate (4·54 g.), and the disulphate (3·99 g.).

Methyl α-D-*Glucopyranoside Ammonium Sulphate.*—Methyl α-D-glucopyranoside (63·5 g.; m. p. 135—136°, $[\alpha]_D^{14}$ +158·8°) was sulphated at 65—70° for 5 hr. to give a product (146 g.) containing only the monosulphate and unchanged glucoside. This was resolved by ion-exchange into methyl glucoside (4 g.) and methyl α-D-glucopyranoside ammonium sulphate (96 g.; Table 3), m. p. 165—167° (decomp.) (Found: S, 11·7. $C_7H_{17}NO_9S$ requires S, 11·1%).

Methyl β-D-*Glucopyranoside Ammonium Sulphate.*—Sulphation of methyl β-D-glucopyranoside (8·1 g.; m. p. 105—106°, $[\alpha]_D^{14}$ −34·0°) gave a product (18·3 g.) containing only monosulphate and unchanged glucoside. The product (10 g.) was resolved, as above, into methyl glucoside (0·2 g.) and methyl β-D-glucopyranoside ammonium sulphate (5·0 g.; Table 3), m. p. 155° (decomp.) (Found: S, 11·3. $C_7H_{17}NO_9S$ requires S, 11·1%).

D-*Galactose* 6-(*Barium Sulphate*).—1,2:3,4-Di-*O*-isopropylidene-α-D-galactose [21] (20 g.; $[\alpha]_D^{14}$ −41·9°) was sulphated with 1·5 mol. of reagent at 70° for 4 hr., to give a mixture (24·1 g.) of starting material and the monosulphate. The mixture (5 g.) was separated by ion-exchange into starting material (1·87 g.) and 1,2:3,4-di-*O*-isopropylidene-D-galactose 6-(ammonium sulphate) (2·59 g.; Table 3), m. p. 130° (decomp.) (Found: S, 9·1. $C_{12}H_{23}O_9NS$ requires S, 9·0%). The sulphation mixture (15 g.) was heated at 100° in 1% acetic acid (300 ml.) for 3 hr., giving a mixture (12·2 g.) of galactose and galactose 6-sulphate. This mixture (10 g.) was separated on a cellulose column (50 × 8 cm.) and yielded D-galactose 6-(barium sulphate) (4·3 g.; Table 2) [Found: Ba, 20·3; S, 9·7. $(C_6H_{11}O_9S)_2Ba$ requires Ba, 21·0; S, 9·8%]. A portion (2 g.) was converted into the sodium salt by passage through " ZeoKarb " 225 (sodium form). The galactose 6-(sodium sulphate) had $[\alpha]_D^{14}$ +50·0° (Found: S, 11·2. $C_6H_{11}NaO_9S$ requires S, 11·4%).

D-*Glucose* 3-(*Barium Sulphate*).—1,2:5,6-Di-*O*-isopropylidene-D-glucofuranose [22] (20 g.; m. p. 105—106°, $[\alpha]_D^{14}$ −17·1°) was sulphated, as above, to give a mixture (30·4 g.). From the mixture (2·5 g.) was isolated di-*O*-isopropylideneglucofuranose 3-(ammonium sulphate) (1·52 g.; Table 3) (Found: S, 8·7. $C_{12}H_{23}NO_9S$ requires S, 9·0%). Treatment of the sulphation product (9·5 g.) with 1% acetic acid gave a mixture (6·7 g.), which on a cellulose column gave D-glucose 3-(barium sulphate) (5·5 g.; Table 2) [Found: Ba, 20·9; S, 9·7. $(C_6H_{11}O_9S)_2Ba$ requires

[21] Van Grunenberg, Bredt, and Freudenberg, *J. Amer. Chem. Soc.*, 1938, **60**, 1507.
[22] Bell, *J.*, 1935, 1874.

Ba, 21·0; S, 9·8%]. The sodium salt, prepared as above, had $[\alpha]_D^{20}$ +29·2° (Found: S, 10·7. $C_6H_{11}NaO_9S$ requires S, 11·4%).

D-*Glucose* 6-(*Barium Sulphate*).—1,2-*O*-Isopropylidene-α-D-glucofuranose 3,5-orthoborate, prepared by Vargha's method,[23] had $[\alpha]_D^{20}$ +7·3°. A portion (9·67 g.) was sulphated with 1·5 mol. of reagent at 65—70° for 6 hr., to give a mixture (17·4 g.) containing starting material and monosulphate. The mixture (16·6 g.) was heated at 100° with 1% acetic acid (300 ml.) for 3 hr. and then evaporated to afford an amorphous solid (15·8 g.). Separation of this product (8·8 g.) on a cellulose column (90 × 7 cm.) as above gave D-glucose 6-(barium sulphate) (2·52 g.; Table 2) [Found: Ba, 21·3; S, 9·8. $(C_6H_{11}O_9S)_2Ba$ requires Ba, 20·9; S, 9·8%].

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[23] Vargha, *Ber.*, 1933, **66**, 706.