419. *Sulphates of Monsaccharides and Derivatives. Part V.*[1] *Products of Sulphation of Galactose and Glucose.*

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Sulphation of galactose and glucose with an excess of pyridine–sulphur trioxide gives complex mixtures containing mono-, di-, tri-, and tetra-sulphates. The main components of such mixtures have been separated and tentatively identified.

It is generally accepted that the chief product obtained by sulphating a hexose under mild conditions is the hexose 6-sulphate. It has been shown, however, that the glucose 6-sulphate, prepared by this method, contained other sulphates as by-products,[2,3] although repeated recrystallisation of the brucine salt effected some purification. Dodgson and Spencer [3,4] have some evidence that one impurity is an isomeric glucose monosulphate (detected by paper chromatography) and that the use of chlorosulphonic acid as sulphating agent gives a purer glucose 6-sulphate than the pyridine–sulphur trioxide reagent.[5] Under more drastic conditions, *e.g.*, at higher temperatures, disulphates are also present in the products [6,7] and Lloyd [8] has described a method for separating a pure hexose 6-sulphate from such mixtures of mono- and di-sulphates. After sulphating glucose with pyridine–sulphur trioxide at 65°, Rees detected five components in the products.[9] We now report a study of the products obtained by sulphating galactose and glucose under specified conditions.

The experimental conditions for the sulphations were chosen to give complex mixtures in that 3 mol. of sulphating reagent were used at 65° for 5 hr. Under these conditions, mono-, di-, and tri-sulphates were readily detected in the products by paper chromatography and electrophoresis. The mixtures obtained in this way were chromatographed on cellulose powder [7] and, where appropriate, the fractions so obtained were separated into pure components by preparative paper chromatography or electrophoresis.

Galactose.—In addition to unchanged galactose, fractions from the cellulose column contained, severally, a mixture of monosulphates, and a di-, a tri-, and a tetra-sulphate. Each of the last three fractions appeared to contain only one component, as judged by chromatographic and electrophoretic examination, but the monosulphate fraction contained two components, which were separated by preparative paper chromatography. The major component was shown to be galactose 6-sulphate, by comparison with an authentic specimen, and the minor component was identified as galactose 3-sulphate on the following evidence. It differed in R_F value from authentic galactose 6- and 4-sulphate but had an M_G value close to that of galactose 6-sulphate, suggesting that it was the 3- rather than the 2-sulphate (see Table). On oxidation with periodate, 1 mol. of oxidant was consumed rapidly and this was followed by further slow oxidation similar to that shown by authentic glucose 3-sulphate,[10] again suggesting the 3- rather than the 2-sulphate.

Since galactose 6-sulphate was the preponderant monosulphate, it is reasonable to suggest that the disulphate would have one of its sulphate groups on position 6. Dodgson and Lloyd [11] have shown by infrared spectroscopy that a galactose disulphate, obtained by sulphation of galactose, has one sulphate at position 6 and that the other sulphate group

[1] Part IV, *J.*, 1962, 2119.
[2] Egami, *J. Chem. Soc. Japan*, 1938, **59**, 1034; 1940, **61**, 593; 1942, **63**, 763.
[3] Dodgson and Spencer, *Biochem. J.*, 1954, **57**, 310.
[4] Dodgson and Spencer, *Ann. Reports*, 1956, **53**, 318.
[5] Duff, *J.*, 1949, 1597.
[6] Lloyd, *Biochem. J.*, 1960, **75**, 478.
[7] Peat, Turvey, Clancy, and Williams, *J.*, 1960, 4761.
[8] Lloyd, *Biochem. J.*, 1962, **83**, 455.
[9] Rees, *Nature*, 1960, **185**, 309.
[10] Turvey, Clancy, and Williams, *J.*, 1961, 1692.
[11] Dodgson and Lloyd, *Biochem. J.*, 1961, **78**, 319.

is not at position 4. In agreement with this, the galactose disulphate isolated was tentatively identified as the 3,6-isomer on the basis of its oxidation by periodate. It consumed 1 mol. of oxidant rapidly and thereafter was slowly oxidised further until a total of more than 2 mol. were consumed. Of the possible disulphates, the 2,6-, 3,4-, and 3,6-isomers would all be expected to consume 1 mol. of oxidant rapidly if oxidised in the ring form,[10] but only the 3,6-isomer would undergo further oxidation with a total consumption in excess of 2 mol. The trisulphate component was tentatively identified as galactose 2,3,6-trisulphate (rather than the alternative 3,4,6-trisulphate) because it consumed very little periodate, whereas the corresponding alcohol, obtained by borohydride reduction, consumed the expected 1 mol. The tetrasulphate is probably galactose 2,3,4,6-tetrasulphate since in no case was there any evidence for sulphation at position 1.

Glucose.—As with galactose, glucose mono-, di-, tri-, and tetra-sulphate were separated on the cellulose column. The monosulphate fraction contained three components, the major component being glucose 6-sulphate. The two components isolated in small amounts were identified as glucose 3-sulphate (by comparison with an authentic specimen) and glucose 4-sulphate (identified by periodate oxidation and a low M_G value similar to that shown by galactose 4-sulphate [1]).

Paper electrophoresis indicated that two disulphates, *A* and *B*, were present but the trisulphate, *C*, appeared to be a single isomer. The 2,3,4,6-tetrasulphate was also isolated and analysed. Properties of these sulphates are listed in the Table. The percentage yields recorded in the Table are based on the weights of isolated material but must be considered as only approximate since quantitative recovery of all the components was not achieved. In addition, it is possible that isomeric sulphates other than those noted occur in the mixtures but their presence was not detected.

Products of sulphation of D-galactose and D-glucose.

Component	Yield * (%)	R_{Gluc} †	M_S ‡	M_G §
D-*Galactose* (sodium salts)				
3-sulphate	1	0·29	1·0	1·33
6-sulphate	20	0·26	1·0	1·30
3,6-disulphate	31		1·7	1·68
2,3,6-trisulphate	23		2·09	1·76
2,3,4,6-tetrasulphate	19		2·22	1·93
D-*Glucose* (barium salts)				
3-sulphate	0·9	0·42	1·0	1·28
4-sulphate	1·5	0·36	1·0	1·02
6-sulphate	27	0·27	1·0	1·27
disulphate *A*	10	0·15	1·71	1·53
disulphate *B*		0·15	1·71	1·36
trisulphate *C*	13	0·07	2·09	1·65
2,3,4,6-tetrasulphate	16	0·02	2·22	1·70

* Yields are expressed as weight percentages of the total dry product of sulphation. † R_F value relative to that of D-glucose in ethyl acetate–acetic acid–water (6 : 3 : 2, by vol.). ‡ M_S is defined as the electrophoretic mobility in 0·1M-acetic acid–pyridine buffer (pH 6·0) compared with that of glucose 6-sulphate. § In borate buffer (see Experimental section).

The main conclusion to be drawn from these results is that the conditions employed here lead to extensive polysulphation. It seems that it is as easy to introduce another sulphate group into an already sulphated sugar as it is to introduce a sulphate group initially into the parent monosaccharide. The use of a smaller excess of sulphating agent diminishes the proportion of polysulphated product,[7,11] but it is unlikely that a monosulphate completely free from di- and higher sulphates can be obtained by varying the conditions of sulphation. Further, when sulphation was allowed to proceed for short periods only, the monosulphate fraction always contained more than one component (unreported experiment).

The composition of the monosulphate fractions, obtained under the conditions used in the main experiments, shows that the predominant product (90—95%) formed is the 6-sulphate, but that it is accompanied by small amounts of 3-sulphate in each case and by some 4-sulphate in the case of glucose. That the 6-sulphate is predominant is expected from the known relative reactivities of the hydroxyl groups in the free sugars, but the absence of 2-sulphates is unexpected since position 2 appears to be the most reactive of the secondary hydroxyl groups in such reactions as toluene-*p*-sulphonylation and benzoylation [12] and, indeed, sulphation of methyl 4,6-*O*-benzylidene-α-D-glucopyranoside gives the 2- rather than the 3-sulphate.[13] The absence of galactose 4-sulphate reflects the difficulty previously encountered in sulphating this position in galactose.[1] The disulphates found are to be expected from a knowledge of the monosulphate components, *i.e.*, the 3,6-isomer in the case of galactose but two isomers in the case of glucose. If we assume that the 6-position is occupied in both glucose disulphates, the expected isomers would be the 3,6- (*A*) and 4,6-disulphate (*B*), the former having the larger M_G value. Only one trisulphate was detected in each mixture. In the glucose series this may be the 3,4,6-isomer but, in the galactose series, it was the 2,3,6-trisulphate, again illustrating the lower reactivity of the 4-position in galactose.

Experimental

General Methods.—The sulphation of monosaccharides at 65° with pyridine–sulphur trioxide (3 mol.) has been described previously.[7] Sugar sulphates were generally isolated as barium salts initially but were converted into sodium salts by passage through the ion-exchanger ZeoKarb-225 (sodium form) for periodate oxidation. The methods of paper chromatography and electrophoresis in neutral buffer have also been described,[7] but in the detection of polysulphates on the paper an " Azure-A " stain [14] was found to be valuable, in addition to the other reagents. For measurement of M_G values and for preparative paper electrophoresis, 0·1M-borate buffer (pH 10) was used with Whatman No. 3MM filter paper and a potential gradient of 40 v per cm., the apparatus being fitted with cooling plates. Sugar sulphates recovered from such papers were freed from borate by chromatography on small columns of charcoal.[15] For measurement of R_{Gluc} values (R_F relative to that of glucose) Whatman No. 3MM paper was used in the solvent system ethyl acetate–acetic acid–water (6 : 3 : 2, by vol.), but reproducible values were difficult to obtain. Values given in the Table were obtained by using freshly prepared solvent in small tanks, where good equilibration was obtained in a relatively short time. Under these conditions, the rates of migration of sugar sulphates were lower than when larger tanks or old solvent were employed.

Sulphation of D-*Galactose.*—The mixture obtained by sulphating galactose at 65° for 5 hr.[7] was shown by paper chromatography to contain at least five components, two of which had the mobilities of galactose and galactose 6-sulphate, respectively. The mixture (1·2 g.) was chromatographed on a cellulose column (70 × 4 cm.), the column being eluted successively with (*a*) 85% aqueous ethanol (3 l.) containing 0·5% (v/v) of formic acid, (*b*) 75% ethanol (10 l.) also containing formic acid, (*c*) 70% ethanol (5 l.) containing formic acid, and finally with water. Fractions (50 ml. each) were collected, analysed with the phenol–sulphuric acid reagent [16] and pooled as appropriate. The eluting solvent (*a*) gave galactose (70 mg.) in fractions 10—60; solvent (*b*) gave two peaks, the first of which was divided into two groups, *D* (fractions 80—134) and *E* (fractions 135—150), and the second into *F* (fractions 151—200) and *G* (fractions 201—217); solvent (*c*) gave a diffuse peak from which *H* (fractions 286—365) and *I* (fractions 366—440) were obtained. The water eluted group *J* (fractions 441—490). Each group of fractions was evaporated to dryness. Examination of the products by paper chromatography and electrophoresis indicated the following compositions: *D* (82 mg.), mainly galactose 6-sulphate but a second monosulphate also present (see below); *E* (114 mg.), pure D-galactose 6-sulphate {the sodium salt had $[\alpha]_D^{16}$ +51·1° (in water) (Found: S, 11·5. Calc.

[12] Sugihara, *Adv. Carbohydrate Chem.*, 1953, **8**, 1.
[13] Guiseley and Ruoff, *J. Amer. Chem. Soc.*, 1962, **27**, 1479.
[14] Spolter and Marx, *Biochim. Biophys. Acta*, 1960, **38**, 123.
[15] Hughes and Whelan, *Chem. and Ind.*, 1958, 884.
[16] Dubois, Gilles, Hamilton, Rebers, and Smith, *Analyt. Chem.*, 1956, **28**, 350.

for $C_6H_{11}NaO_9S$: S, 11·4%)}; *F* (153 mg.), mainly a single disulphate with traces of galactose 6-sulphate; *G* (108 mg.), a single disulphate {the sodium salt had $[\alpha]_D^{16}$ +51·7° (in water) and was subsequently identified as D-*galactose* 3,6-*di*(*sodium sulphate*) (Found: S, 16·1. $C_6H_{10}Na_2O_{12}S_2$ requires S, 16·7%)}; *H* (156 mg.), a single galactose trisulphate, the sodium salt of which was subsequently identified as D-galactose 2,3,6-*tri*(*sodium sulphate*), $[\alpha]_D^{16}$ +35·8° (in water) (Found: S, 20·2. $C_6H_9Na_3O_{15}S_3$ requires S, 19·7%). A portion (20 mg.) of this trisulphate was reduced to the corresponding alcohol with sodium borohydride (20 mg.) in water (4 ml.). After 16 hr., the solution was neutralised with 3N-sulphuric acid and the volume adjusted to 5 ml. Portions of this solution were used for periodate oxidation (see below). Fraction *I* (80 mg.) contained a mixture of tri- and tetra-sulphate; *J* (107 mg.) was a single tetrasulphate, the *sodium salt* of which had $[\alpha]_D^{16}$ +0·9° (in water) (Found: S, 22·4. $C_6H_8Na_4O_{18}S_4$ requires S, 21·8%).

Separation of Galactose Monosulphates.—Mixture *D* (82 mg.) was separated by preparative paper chromatography into *U*, galactose 6-sulphate (65 mg.), and *V*, galactose 3-sulphate (9 mg.). Each was converted into the sodium salt and oxidised with periodate (see below) (Found: S in *U*, 11·5; in *V*, 11·7. Calc. for $C_6H_{11}NaO_9S$: S, 11·4%).

Sulphation of Glucose.—The mixture obtained by sulphating glucose, under the same conditions as for galactose, contained at least 5 components detectable by paper chromatography. A portion (10 g.) was chromatographed on a cellulose column (94 × 7 cm.), which was eluted successively with (*a*) 80% aqueous ethanol (8 l.) containing 0·2% (v/v) of formic acid, which gave glucose and monosulphates, (*b*) 60% aqueous ethanol (5·75 l.) containing 0·3% of formic acid, which eluted di- and tri-sulphates, and (*c*) water (3·2 l.), which eluted tetrasulphate. Fractions (50 ml. each) were collected, examined as before, and grouped into the following: *L* (fractions 1—40; 3·2 g.), glucose with a trace of monosulphate; *M* (fractions 41—70; 1·22 g.), a mixture of three monosulphates (see below) with a trace of glucose; *N* (fractions 71—90; 0·81 g.), mainly glucose 6-sulphate, with traces of other monosulphates; *O* (fractions 91—120; 0·74 g.), glucose 6-(barium sulphate), $[\alpha]_D^{16}$ +28·7° (in water) [Found: Ba, 21·4; S, 9·4. Calc. for $(C_6H_{11}O_9S)_2Ba$: Ba, 21·0; S, 9·8%]; *P* (fractions 121—200; 0·45 g.), two disulphates *A* and *B* (see Table) and galactose 6-sulphate; *Q* (fractions 201—216; 60 mg.), mainly *A* and *B* with traces of a single trisulphate, *C* (see Table); *R* (fractions 217—300; 1·79 g.), a mixture of disulphates, trisulphate, *C*, and a tetrasulphate; and *S* (fractions 301—380; 1·08 g.), D-glucose 2,3,4,6-tetra(barium sulphate), $[\alpha]_D^{16}$ +38·3° (in water) (Found: Ba, 35·1; S, 16·0. $C_6H_8Ba_2O_{18}S_4$ requires Ba, 35·6; S, 16·6%).

Separation of Glucose Monosulphates.—The mixture *M* contained three monosulphates, two of which had the R_F values of glucose 6- and glucose 3-sulphate, severally. Preparative paper chromatography of the mixture (125 mg.) gave a pure sample of the 6-sulphate (82 mg.) (*X*), but the others were incompletely separated. They were eluted together from the paper and separated by paper electrophoresis in borate buffer. The faster-migrating component was identified as glucose 3-sulphate (9 mg.) (*Y*), and the slower was provisionally identified (by M_G value and periodate oxidation) as glucose 4-sulphate (16 mg.) (*Z*). Each monosulphate was converted into the sodium salt and analysed (Found: S in *X*, 11·6; in *Y* 11·5; in *Z* 11·6. Calc. for $C_6H_{11}NaO_9S$: S, 11·4%). Each isomer was also subjected to periodate oxidation (see below).

Periodate Oxidation of Products.—The sugar sulphate (1·8—2·0 mg.) was oxidised with 0·01M-sodium metaperiodate (3 ml.) in the dark at 35° and the consumption of oxidant measured by the method of Aspinall and Ferrier.[17] The results are as follows:

Oxidant consumed in mol. (time in hr. given in parentheses):

U, Galactose 6-(sodium sulphate). 1·41 (0·1), 3·38 (0·5), 3·48 (1·0), 3·72 (4·5).

V, Galactose 3-(sodium sulphate). 0·58 (0·1), 1·04 (0·3), 1·56 (0·6), 1·73 (1·9), 2·30 (5·0), (cf. glucose 3-sulphate [10]).

G, Galactose 3,6-di(sodium sulphate). 0·69 (0·5), 0·70 (1·0), 1·00 (2·0), 1·15 (3·0), 1·31 (5·5), 2·92 (20·0).

H, Galactose 2,3,6-tri(sodium sulphate) 0·07 (1·0), 0·17 (2·0), 0·25 (5·0). After reduction with borohydride, 0·75 (0·25), 0·92 (3·0), 0·99 (7·5).

X, Glucose 6-(sodium sulphate). 2·39 (0·1), 3·15 (0·3), 3·59 (0·75), 3·64 (1·0), 3·71 (1·5), 3·78 (2·0).

[17] Aspinall and Ferrier, *Chem. and Ind.*, 1957, 1216.

Y, Glucose 3-(sodium sulphate). 0·44 (0·1), 1·01 (0·4), 1·44 (0·9), 1·57 (1·5), 1·72 (3·0).
Z, Glucose 4-(sodium sulphate). 0·45 (0·1), 1·11 (0·3), 1·56 (0·6), 2·03 (1·0), 2·76 (2·0), 3·00 (3·0), 3·24 (4·1), (cf. galactose 4-sulphate [1]).

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