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^{13}C-N.M.R. STUDIES OF D-GLUCOSE AND D-GALACTOSE MONOSULPHATES

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ABSTRACT

D-Galactose and D-glucose 2-, 3-, 4-, and 6-sulphates, 2,3,4,6-tetra-*O*-acetyl-β-D-glucose 1-sulphate, and 2-acetamido-2-deoxy-D-glucose 6-sulphate have been definitively synthesised, and the positions of the sulphate groups verified by ^{13}C-n.m.r. spectroscopy. New syntheses are given for D-galactose 3- and 4-sulphates and for D-glucose 4-sulphate. The ^{13}C-n.m.r. spectra showed downfield displacements, with respect to the parent sugar, of the shifts of carbon atoms carrying a sulphate group and smaller upfield shifts for adjacent carbon atoms. The technique of deuterium-induced, differential isotope shift (d.i.s.), ^{13}C-n.m.r. spectroscopy is shown to be valuable for the assignment of chemical shifts.

INTRODUCTION

Authentic monosaccharide sulphates were required for a study of the specificity of mammalian sulphatases; although definitive syntheses of these compounds have been attempted for many years, the lack of a method for the unequivocal determination of the position of the sulphate group has meant that, in some cases, the structure of the product remained doubtful. Infrared absorptions at different positions in the region 810–850 cm^{-1} due to C-O-S bending were claimed[1,2] to be diagnostic for the sulphate groups of primary, secondary axial, or secondary equatorial hydroxyl groups, but it was later found[3] that the position of the absorption also depended upon the nature of any aglycon, or other substituent, and so was not a reliable criterion for the type of sulphate group. For example, the use of i.r. spectroscopy apparently supported the incorrect conclusion that cerebroside sulphate was a substituted galactose 6-sulphate[2]. More recently, p.m.r. spectroscopy has been used[4] with some success to locate the sulphate group in carbohydrate sulphates, but peak assignment in the δ 3.5–5.0 region is difficult because of the coalescence of peaks and the existence of complex multiplets. Recently, a few ^{13}C-n.m.r. studies[5–7] of monosaccharide sulphates have appeared and this has proven to be the best method for determining their structure: a sulphate group shifts substantially downfield, relative to the parent alcohol, the signal from the carbon atom to which it is attached, while the signals from the

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adjacent carbon atoms are shifted upfield. Knowing this, the position of the sulphate group can usually be ascertained, although some of the peak assignments may be tentative.

The advent of the deuterium-induced, differential isotope shift (d.i.s.) method[8] in ^{13}C-n.m.r. spectroscopy has made peak assignment much simpler. This method depends upon the small difference between the resonance frequency of a carbon atom having a hydroxyl substituent and that of the same carbon atom for which the OH has been replaced by OD. A sample of a sugar in H_2O and a deuterium-exchanged sample in D_2O can be examined simultaneously by n.m.r. spectroscopy using a coaxial, dual cell. The magnitude of the shift is dependent upon the position of the hydroxyl group and upon whether or not adjacent hydroxyl groups are present. For the anomeric hydroxyl group, the magnitude of the shift also depends upon whether the adjacent hydroxyl group is in *cis* or *trans* relationship.

DISCUSSION

The existing, definitive routes for the synthesis of D-galactose 2-[9] and 6-sulphates[10], and D-glucose 2-[9], 3-[10], and 6-sulphates[11] have been shown, in this study, to give the required products in pure form. Lloyd's synthesis[12] of 2-acetamido-2-deoxy-D-glucose 6-sulphate *via* 2-acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-β-D-glucopyranose gave, in addition to the required product, significant amounts of unidentified impurities which were only partly removed by chromatography and repeated precipitation by ethanol. In this laboratory, the usual method[9,13] of preparing D-galactose 3-sulphate *via* 4,6-*O*-ethylidene-1,2-*O*-isopropylidene-α-D-galactopyranose gave the 6-sulphate, presumably either by re-arrangement of the protecting groups or by elimination of the ethylidene group during the attempted conversion of 4,6-*O*-ethylidene-D-galactose into its 1,2-*O*-isopropylidene derivative by treatment with acetone and zinc chloride. This elimination has been noted previously[14], and the resulting mixture of 1,2:3,4-di-*O*-isopropylidene-D-galactose and 4,6-*O*-ethylidene-1,2-*O*-isopropylidene-D-galactose was resolved by chromatography. In the present study, the synthesis of D-galactose 3-sulphate was achieved *via* 1,2:5,6-di-*O*-isopropylidene-α-D-galactofuranose obtained from D-galactose in one step. Difficulties were encountered in separating the furanose derivative from the 1,2:3,4-di-*O*-isopropylidene-α-D-galactopyranose also formed in the reaction, and in the recrystallisation of the former, only small amounts of which were obtained, contrary to previous reports[15,16]. Nevertheless, once obtained, this derivative gave high yields of pure D-galactose 3-sulphate.

D-Galactose 4-sulphate has previously been prepared by several methods[17,18], all of which gave poor yields because they required the removal of the aglycon from a sulphated glycoside by catalytic hydrogenation. To overcome this problem, benzyl 2,3,6-tri-*O*-benzoyl-β-D-galactopyranoside[18] was catalytically hydrogenolysed before sulphation; this gave a better yield, but the resulting D-galactose 4-sulphate was shown by ^{13}C-n.m.r. spectroscopy to contain aromatic impurities. A new method

via 1,2,3,6-tetra-*O*-acetyl-D-galactopyranose was therefore devised. Initially, attempts were made to prepare both 1,2,3,6-tetra-*O*-acetyl-D-galactose and 1,2,3,6-tetra-*O*-acetyl-D-glucose from the corresponding 1,2,3,4-tetra-*O*-acetyl compounds by acetyl migration in dilute alkali[19], but sulphation of the unpurified products, followed by de-acetylation, gave mixtures of the 3- and 4-sulphates with, particularly in the galactose sulphates, large proportions of other impurities. Since other workers[20,21] have been successful in obtaining only 1,2,3,6-tetra-*O*-acetyl-D-glucose by this method, it is probable that the reaction time in alkali is critical and should be no longer than one minute[20] if further acetyl migration is to be avoided. Another route to the tetra-acetate was developed by preparing the 4,6-*O*-benzylidene derivative, which was then acetylated to give 1,2,3-tri-*O*-acetyl-4,6-*O*-benzylidene-D-glucose. The benzylidene group was removed by acid hydrolysis and the resulting triacetate was further acetylated with slightly more than one mol. equiv. of acetic anhydride to give mainly the 1,2,3,6-tetra-acetate with some 1,2,3,4,6-penta-acetate. The mixture was sulphated, and the unreacted penta-acetate was removed from the crude product by extraction with chloroform. D-Galactose 1,2,3,6-tetra-acetate was similarly prepared. Pure D-galactose 4-sulphate and D-glucose 4-sulphate were obtained in this way. A similar synthesis had previously been used[22] for D-glucose 4-phosphate; although the product had the expected properties, it could not be adequately characterised at the time.

The synthesis of 2,3,4,6-tetra-*O*-acetyl-β-D-glucose 1-sulphate was that described by Ohle[23]. This ester is quite unstable in aqueous solution[23] and no attempt was made to prepare D-glucose 1-sulphate which would, by analogy with the 1-phosphates, be expected to be even less stable.

The i.r. spectra of the monosaccharide sulphates all showed[24] a characteristic, broad band, due to S=O stretching, at $\sim$1250 cm^{-1} and another, due to C-O-S vibration, in the region of 810–860 cm^{-1}. The latter values are summarised in Table I. The spectra of the 6-sulphates of D-galactose, D-glucose, and 2-acetamido-2-deoxy-D-glucose were, for the most part, in agreement with those reported by Lloyd and Dodgson[2]. Values for the C-O-S vibration previously reported[3,9] for D-galactose 2-sulphate, D-glucose 2-sulphate, and D-galactose 4-sulphate were also close to those found here, except that the spectra of the galactose sulphates had additional shoulders at 817 and 825 cm^{-1}, respectively. The spectrum of galactose 3-sulphate showed two peaks at 862 and 818 cm^{-1}: neither of these agreed with the reported[9] value of 835 cm^{-1}. However, the spectrum published by Jatzkewitz and Nowoczek[25] also showed two peaks in this region. A sample of "galactose 3-sulphate" from their laboratory was shown by ^{13}C-n.m.r. spectroscopy to be an $\sim$60:40 mixture of galactose 3- and 6-sulphates which gave, in this laboratory, an i.r. spectrum identical to that published by the authors[25] and which was intermediate between those of the pure galactose 3- and 6-sulphates. The i.r. spectrum of glucose 3-sulphate resembled that given by Lloyd and Dodgson[2], but the absorption at 814 cm^{-1} (with a weak band at 855 cm^{-1}) did not agree with their reported value of 832 cm^{-1}.

Little information was obtained from the p.m.r. spectra of the monosaccharide

TABLE I

I.R. ABSORPTIONS FOR THE C-O-S VIBRATION, P.M.R. SHIFTS FOR H-1α, AND $J_{1\alpha,2}$ VALUES FOR MONOSACCHARIDE SULPHATES

	C-O-S absorption[a] *(cm^{-1})*	*H-1α shift* *(p.p.m.)*	$J_{1\alpha,2}$[b] *(Hz)*
D-Galactose 2-sulphate	838 (m), 817 (sh)	5.52	3.5
D-Galactose 3-sulphate	862 (m), 818 (m)	5.27	3.5
D-Galactose 4-sulphate	850 (m), 825 (sh)	5.22	3.0
D-Galactose 6-sulphate	818 (m)	5.17	2.5
D-Glucose 2-sulphate	822 (m)	5.41	3.0
D-Glucose 3-sulphate	814 (m)	5.20	3.5
D-Glucose 4-sulphate	817 (m)	5.19	3.0
D-Glucose 6-sulphate	823 (s), 800 (sh)	5.17	2.5
2,3,4,6-Tetra-*O*-acetyl-β-D-glucose 1-sulphate	798 (m), 783–776 (m)	—	—
2-Acetamido-2-deoxy-D-glucose 6-sulphate	816 (m)	5.17	2.0

[a]Key: s, strong; m, medium; sh, shoulder. [b]Values to nearest 0.5 Hz.

sulphates, because of the presence of complex multiplets and of problems of resolution. The doublet due to H-1α was distinctive, because it appeared further downfield than the remaining resonances, and its position and associated coupling constant ($J_{1\alpha,2}$) appear likely to be diagnostic for the different sulphates (see Table I), particularly for the 2-sulphates in which there is a significant downfield shift. Assignment of peaks due to protons other than H-1α would require extensive decoupling and other such procedures.

Earlier reports[5–7] indicated that ^{13}C-n.m.r. spectroscopy was likely to be the best method for determining the position of the sulphate group in monosaccharide sulphates. In carbohydrates, the sulphation of an hydroxyl group has been found[5,6] (and Dr. J. R. Turvey, personal communication) to cause a downfield displacement of between 6 and 10 p.p.m. in the shift of the carbon atom to which it is attached, whereas the shifts of adjacent carbon atoms are displaced upfield by 1 to 2.5 p.p.m. Results for the present study (Table II) are in agreement with this generalisation, although there are two notable exceptions. The sulphate group in 2,3,4,6-tetra-*O*-acetyl-β-D-glucose 1-sulphate caused a downfield displacement of only 2.3 p.p.m.: this might not be unexpected in view of the substantially different environment of the sulphate group in this compound. The other exception was galactose 4-sulphate, where the lower values of the displacements of the adjacent carbon atoms (0.6 to 1.0 p.p.m.) are almost certainly due to the axial configuration of the sulphate.

With the increased amount of data available, it has become apparent that the position of the sulphate group also influences the magnitude of the shift displacement. For example, a sulphate at C-6 tends to give the smallest downfield displacement (6.3 to 6.6 p.p.m.), whereas one at C-3 or C-4 gives the largest (7.1 to 9.4 p.p.m.). A sulphate at C-6 also gives rise to little difference in the displacements of the α and β

TABLE II

^{13}C-N.M.R. SHIFT DIFFERENCES IN WATER BETWEEN POTASSIUM SUGAR SULPHATES AND THE PARENT SUGARS[a]

	Anomer	*Shift differences (p.p.m.)*					
		C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
D-Galactose 2-sulphate	α	+1.83	−7.45	+1.99	−0.19	+0.14	+0.12
	β	+1.61	−8.25	+1.08	−0.08	0.00	+0.13
D-Galactose 3-sulphate	α	+0.02	+1.99	−8.51	+1.68	+0.28	+0.16
	β	+0.26	+1.98	−7.57	+1.74	+0.44	+0.13
D-Galactose 4-sulphate	α	+0.10	−0.10	+0.98	−8.31	+0.64	+0.09
	β	+0.07	−0.05	+1.00	−7.92	+0.85	0.00
D-Galactose 6-sulphate	α	−0.07	+0.12	+0.26	+0.21	+2.16	−6.48
	β	+0.01	+0.15	+0.24	+0.23	+2.42	−6.33
D-Glucose 2-sulphate	α	+1.88	−6.39	+2.14	−0.04	+0.21	+0.07
	β	+1.44	−7.24	+1.31	+0.14	+0.06	+0.16
D-Glucose 3-sulphate	α	+0.10	+1.30	−9.41	+1.35	+0.26	+0.13
	β	+0.28	+1.29	−8.52	+1.34	+0.54	+0.11
D-Glucose 4-sulphate	α	+0.40	+0.23	+1.44	−7.36	+1.74	+0.21
	β	+0.12	+0.20	+1.51	−7.08	+1.60	+0.22
D-Glucose 6-sulphate	α	−0.08	+0.12	+0.13	+0.26	+1.93	−6.51
	β	−0.09	+0.13	+0.19	+0.38	+2.17	−6.36
2,3,4,6-Tetra-*O*-acetyl-β-D-glucose 1-sulphate	β	−2.27	+1.41	+0.22	+0.11	+0.08	+0.33
2-Acetamido-2-deoxy-D-glucose 6-sulphate	α	−0.09	+0.15	+0.06	+0.28	+1.93	−6.55
	β	−0.07	+0.14	+0.20	+0.27	+2.17	−6.33

[a]Positive values are upfield with respect to tetramethylsilane.

TABLE III

^{13}C-N.M.R. CHEMICAL SHIFTS[a] (FIRST LINES), AND OBSERVED AND CALCULATED (IN BRACKETS) D.I.S. SHIFT-VALUES (SECOND LINES) FOR MONOSACCHARIDES AND THEIR SULPHATES

	Chemical and d.i.s. shifts (p.p.m.)					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
α-D-Galactose	93.11	69.18	70.01	70.14	71.30	62.00
	0.14 (0.14)	0.21 (0.20)	0.18 (0.20)	0.16 (0.17)	0.08 (0.06)	0.17 (0.15)
β-D-Galactose	97.28	72.72	73.63	69.58	75.96	61.81
	0.14 (0.14)	0.23 (0.23)	0.19 (0.20)	0.17 (0.17)	0.07 (0.06)	0.16 (0.15)
α-D-Galactose 2-sulphate	91.28	76.63	68.02	70.33	71.16	61.88
	0.12 (0.11)	0.09 (0.06)	0.13 (0.17)	0.16 (0.17)	0.07 (0.06)	0.15 (0.15)
β-D-Galactose 2-sulphate	95.67	80.97	72.55	69.66	75.96	61.68
	0.12 (0.11)	0.11 (0.09)	0.15 (0.17)	0.16 (0.17)	0.07 (0.06)	0.17 (0.15)
α-D-Galactose 3-sulphate	93.09	67.19	78.52	68.46	71.02	61.84
	0.14 (0.14)	0.09[b] (0.17)	0.06 (0.06)	0.12 (0.14)	0.06 (0.06)	0.16 (0.15)
β-D-Galactose 3-sulphate	97.02	70.74	81.20	67.84	75.52	61.68
	0.16 (0.14)	0.19 (0.20)	0.07 (0.06)	0.13 (0.14)	0.06 (0.06)	0.15 (0.15)
α-D-Galactose 4-sulphate	93.01	69.28	69.03	78.45	70.66	61.91
	0.13 (0.14)	0.21 (0.20)	0.17 (0.17)	0.03 (0.03)	0.05 (0.03)	0.15 (0.15)
β-D-Galactose 4-sulphate	97.21	72.77	72.63	77.50	75.11	61.81
	0.15 (0.14)	0.23 (0.23)	0.16 (0.17)	0.04 (0.03)	0.04 (0.03)	0.15 (0.15)
α-D-Galactose 6-sulphate	93.18	69.06	69.75	69.93	69.14	68.48
	0.14 (0.14)	0.20 (0.20)	0.18 (0.20)	0.16 (0.17)	0.04 (0.03)	0.03 (0.00)
β-D-Galactose 6-sulphate	97.27	72.57	73.39	69.35	73.54	68.14
	0.15 (0.14)	0.23 (0.23)	0.20 (0.20)	0.17 (0.17)	0.04 (0.03)	0.03 (0.00)

α-D-Glucose	92.91	72.31	73.60	70.44	72.27	61.46
	0.15 (0.14)	0.20 (0.20)	0.21 (0.20)	0.17 (0.17)	0.04 (0.06)	0.15 (0.15)
β-D-Glucose	96.73	74.97	76.59	70.49	76.76	61.61
	0.14 (0.14)	0.23 (0.23)	0.20 (0.20)	0.15 (0.17)	0.04 (0.06)	0.16 (0.15)
α-D-Glucose 2-sulphate	91.03	78.70	71.46	70.48	72.06	61.39
	0.13 (0.11)	0.11 (0.06)	0.18 (0.17)	0.18 (0.17)	0.09 (0.06)	0.17 (0.15)
β-D-Glucose 2-sulphate	95.29	82.21	75.28	70.35	76.70	61.45
	0.12 (0.11)	0.13 (0.09)	0.18 (0.17)	0.17 (0.17)	0.08 (0.6)	—[c] (0.15)
α-D-Glucose 3-sulphate	92.81	71.01	83.01	69.09	72.01	61.33
	0.15 (0.14)	0.17 (0.17)	0.10 (0.06)	0.13 (0.14)	0.10 (0.06)	0.16 (0.15)
β-D-Glucose 3-sulphate	96.45	73.68	85.11	69.15	76.22	61.50
	0.16 (0.14)	0.20 (0.20)	0.10 (0.06)	0.14 (0.14)	0.09 (0.06)	0.16 (0.15)
α-D-Glucose 4-sulphate	92.51	72.08	72.16	77.80	70.53	61.25
	0.14 (0.14)	0.21 (0.20)	0.16 (0.17)	0.05 (0.03)	0.05 (0.03)	0.15 (0.15)
β-D-Glucose 4-sulphate	96.61	74.77	75.08	77.57	75.16	61.39
	0.14 (0.14)	0.23 (0.23)	0.16 (0.17)	0.05 (0.03)	0.04 (0.03)	0.15 (0.15)
α-D-Glucose 6-sulphate	92.99	72.19	73.47	70.18	70.34	67.97
	0.14 (0.14)	0.20 (0.20)	0.21 (0.20)	0.17 (0.17)	0.05 (0.03)	0.04 (0.00)
β-D-Glucose 6-sulphate	96.82	74.84	76.40	70.11	74.59	67.97
	0.14 (0.14)	0.23 (0.23)	0.21 (0.20)	0.17 (0.17)	0.05 (0.03)	0.04 (0.00)
2-Acetamido-2-deoxy-α-D-glucose	91.67	54.92	71.53	70.93	72.40	61.45
	0.12	0.19	0.18	0.16	0.08	0.16
2-Acetamido-2-deoxy-β-D-glucose	95.76	57.55	74.73	70.69	76.78	61.6
	0.15	0.21	0.20	0.16	0.07	0.15
2-Acetamido-2-deoxy-α-D-glucose 6-sulphate	91.76	54.77	71.47	70.65	70.47	68.00
	0.11	0.19	0.18	0.15	0.05	0.01
2-Acetamido-2-deoxy-β-D-glucose 6-sulphate	95.83	57.41	74.53	70.42	74.61	67.93
	0.15	0.21	0.19	0.16	0.03	0.02

[a]For solutions in H_2O; relative to the chemical shift of 1,4-dioxane (67.4 p.p.m.) and given to the nearest 0.01 p.p.m. [b]Value in doubt because of the proximity of the 1,4-dioxane peak. [c]D_2O shift not known, as peak obscured by C-6α peak.

anomers, whereas a sulphate group at C-2 causes the displacement of the β anomer to be significantly greater than that of the α anomer. The latter effect has also been noted with D-xylose 2-sulphate, but not with L-arabinose 2-sulphate (Dr. J. R. Turvey, personal communication). For a 3-sulphate, the displacements of the β anomer are less than those of the α anomer.

As already mentioned, assignments of chemical shifts in the ^{13}C-n.m.r. spectra were not always straightforward: in particular, it was not possible to give definite assignments to peaks which were in close proximity. The d.i.s. method[8] provided a solution to this problem and all the shifts in question were assigned. The parameters given by Pfeffer *et al.*[8] were used to obtain the calculated d.i.s. values in Table III, which shows that there is generally good agreement between the observed and predicted values. In the glucose series, all of the sulphates gave d.i.s. values slightly higher than calculated both for C-5 (0.01 to 0.04 p.p.m.) and for the carbon atom carrying the sulphate group (0.02 to 0.05 p.p.m.). The latter effect was also noted in galactose 2- and 6-sulphates, where the observed d.i.s. values were 0.02 to 0.03 p.p.m. higher than expected, but not in galactose 3- and 4-sulphates. In the former, this apparent discrepancy could be due to the complication, which has been noted previously[8], that the observed d.i.s. values for C-3 in galactose derivatives tend to be lower (by 0.01 to 0.04 p.p.m., Table III) than predicted. Galactose 4-sulphate is a special case, because it is the only compound in the series in which the sulphate group is axial.

The parameters used to calculate the d.i.s. shifts of glucose, galactose, and their sulphates could not be used with accuracy for 2-acetamido-2-deoxy-D-glucose and its sulphate. However, the observed values (Table III) followed the general trend seen in the simple sugars, and peak assignments were possible by extrapolation from these.

The chemical shifts from the d.i.s. data in water were used to calculate the shift displacements of the monosaccharide sulphates from their parent sugars, which are given in Table II. These values are not significantly different from those calculated from the shifts in D_2O.

EXPERIMENTAL

General methods. — (*a*) *Infrared spectra.* KBr discs were pressed, and the spectra recorded on a Unicam SP1050 spectrophotometer. A peak at $\sim$1650 cm^{-1} occurred in the spectra of all the sugar sulphates and was assumed to be due to water of crystallisation.

(*b*) *N.m.r. spectra.* P.m.r. spectra were recorded on a Varian T-60A spectrometer at 35° in either $CDCl_3$ or D_2O with tetramethylsilane or sodium 3-(trimethylsilyl)propanesulphonate, respectively, as reference.

Decoupled, ^{13}C-n.m.r. spectra were recorded on a Jeol FX90-Q FT spectrometer at 30° and 22.5 MHz: d.i.s. data were obtained by using a coaxial, dual cell, as described by Pfeffer *et al.* (Fig. 1 in ref. 8). The inner compartment contained 100 mg of sample in 1 mL of deionised water, and the outer compartment contained

100 mg of sample, previously exchanged three times with D_2O, in 1 mL of D_2O. One or two drops of 1,4-dioxane were added to both as a standard: this gave two peaks, 0.03 to 0.05 p.p.m. apart, of which the upfield was taken as zero with a shift of 67.40 p.p.m. from tetramethylsilane. Values were taken to the nearest 0.01 p.p.m. All spectra had a width of 1 kHz and a computer memory of at least 16 k (32 k in some cases). Spectra of the coaxial, dual cell with water and a drop of 1,4-dioxane in both compartments gave a difference of 0.12 Hz: the difference was of the same order with water in the inner compartment and D_2O in the outer.

(*c*) *Sulphation*. Pyridine (~ 100 mL) dried over barium oxide was used to dissolve every 5 g of sugar derivative: to this solution was added 1.5 molar equivalents of pyridine-SO_3, and the mixture was kept at the required temperature for the stated time. If necessary, it was then cooled to room temperature, and an equal volume of water was added. After 1 h, the pH of the solution was adjusted to 9 by the addition of saturated, aqueous barium hydroxide, and the precipitate was removed by centrifugation. Pyridine was removed from the solution by evaporation *in vacuo* at ~40° and then by co-evaporation with water (several times, if necessary). Excess Ba^{2+} was removed from the solution by treatment with carbon dioxide and, after concentration as above, the solution was filtered and taken to dryness, to give the crude barium salt of the sugar sulphate.

(*d*) *Acetal hydrolysis*. The sugar acetal (not more than 10 g) was dissolved in the minimum of water and percolated through a column (2.5 × 8 cm) of Dowex 50(H^+) resin which was then washed with water until the pH of the eluate had risen to ~5. The combined eluates (pH ~1) were heated at 80° and the acetal hydrolysis was monitored by p.m.r. spectroscopy. When hydrolysis was complete, the solution was cooled, the pH was adjusted to 9 with barium hydroxide, excess Ba^{2+} was removed with carbon dioxide, the solution was concentrated and, after filtration, taken to dryness.

(*e*) *Deacetylation*. The sugar acetate was dissolved in methanol, cooled in an ice bath, and treated with 0.37M barium methoxide in methanol, as described by Isbell[26]. The solution was kept at 4° for at least 20 h, and the progress of the reaction followed by p.m.r. spectroscopy. When complete, carbon dioxide was passed through the solution for 4–6 h. The methanol was evaporated, a small amount of water was added, barium salts were removed by centrifugation or filtration, and the solution was taken to dryness.

(*f*) *Purification of crude sulphates*. The crude barium sugar sulphate was dissolved in the minimum of water and percolated through a column of Dowex 50(H^+) resin, as described above, to convert it into the free acid. The eluate was made slightly alkaline with ammonium hydroxide solution and taken to dryness, and the crude ammonium sugar sulphate (not more than 10 g) was dissolved in a small volume of mM ammonium hydrogencarbonate. After adjustment of the pH to 8.6 with ammonium hydroxide, the solution was applied to a column (4 × 18 cm) of DEAE-cellulose (DE52, Whatman) equilibrated with mM ammonium hydrogencarbonate (pH 8.6). Free sugars (detected by the anthrone method[27]) were eluted

with 500 mL of mM ammonium hydrogencarbonate (pH 8.6), and then the sugar sulphate was eluted with 1 L of 0.05M ammonium hydrogencarbonate (pH 8.6). The eluate was concentrated to a small volume, water added, and the solution again concentrated; this procedure was repeated several times to remove the ammonium hydrogencarbonate.

In some early experiments, the sugar sulphates were eluted from DEAE-cellulose with a linear gradient from 0.01M to 0.1M ammonium hydrogencarbonate (pH 8.6), but this gave a less satisfactory purification.

The pure ammonium sugar sulphate, which was very hygroscopic in most cases, was then converted into the potassium salt by passage through a column of Dowex 50(K^+) resin. The eluate was evaporated to dryness, and a solution of the residue in a small volume of water was slowly dropped, with vigorous swirling, into ethanol (~10 vol.). Ether (1 or 2 vol.) was added and the mixture left at 4° overnight. The precipitate was then filtered off and dried immediately over phosphorus pentaoxide *in vacuo*. Most of the potassium sugar sulphates were hygroscopic and retained ethanol even after storage for many weeks over phosphorus pentaoxide *in vacuo*, as shown by the appropriate peaks in the p.m.r. and ^{13}C-n.m.r. spectra, and also by the values for C and H (which were invariably high on analysis) and S (which were low).

Conversion of the ammonium salt into the potassium salt *via* the free acid was less satisfactory than the above procedure, because it was accompanied by significant hydrolysis of the sulphate ester linkage.

D-*Galactopyranose 2-sulphate*. — 1,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranose was prepared from D-galactose (32 g) by the method of Helferich and Zirner[28], except that the reaction mixture was left overnight at 4°, instead of 1.5 h at room temperature. The crude product (45 g) was recrystallised from ether containing ~30% (v/v) of chloroform, to give the title product (23.7 g), m.p. 144–146°; lit.[28] m.p. 151°. P.m.r. data ($CDCl_3$): δ 6.28 (d, $J_{1,2}$ 4.0 Hz, H-1), 5.44 (m, H-4), 5.17 (m, H-3), 4.47–3.96 (m, H-2,5,6), 2.17, 2.14, 2.06, and 2.03 (4 s, 4 Ac).

Sulphation of the tetra-acetate (23.5 g) for 3 days at room temperature, with *N*,*N*-dimethylformamide in place of pyridine as solvent, gave 42.4 g of crude product which was deacetylated to give 22.9 g of crude galactose 2-(barium sulphate). The ammonium salt (16.2 g) was divided into three portions for purification as described above and precipitated twice as the potassium salt, to give a total yield of 9.4 g; ν_{max} 3480–3380 (s), 2960–2900 (w), 1265–1233 (s), 1145 (m), 1067 (m), 1026 (s), 919 (w), 882 (w), 838 (m), 817 (sh), 783 (m), 766 (sh), 688 (w), and 618 cm^{-1} (w). P.m.r. data (D_2O): δ 5.52 (d, $J_{1\alpha,2}$ 3.5 Hz, H-1α).

Anal. Calc. for $C_6H_{11}KO_9S \cdot 0.5\ C_2H_5OH$: C, 26.1; H, 4.39; S, 9.96. Found: C, 26.1; H, 4.31; S, 9.98.

D-*Galactopyranose 3-sulphate*. — 1,2:5,6-Di-*O*-isopropylidene-α-D-galactofuranose was prepared from D-galactose by the method of Morgenlie[15]. Although p.m.r. spectroscopy indicated that the crude product contained approximately equal amounts of furanose and pyranose derivatives, the isolation of the former was

difficult and neither the method of Morgenlie[15] nor that of Benzing-Nguyen and Rodén[16] proved satisfactory. Light petroleum (b.p. 40–60°) and cyclohexane were used in the manner described by the latter authors to obtain a partial separation. Addition of light petroleum (b.p. 60–80°) to the viscous, yellow residue from the cyclohexane extraction produced, on slow evaporation, some crystals which were further purified by recrystallisation from ether–light petroleum (b.p. 40–60°) mixtures. A total of only 2.04 g of product was obtained from 37.5 g of D-galactose; m.p. 95–97°; lit.[15] m.p. 97–98°. P.m.r. data ($CDCl_3$): δ 5.87 (d, $J_{1,2}$ 4.0 Hz, H-1), 4.66–3.57 (m, H-2,3,4,5,6), 1.55, 1.45, 1.39, and 1.35 (4 s, 4 Ac).

Sulphation of the foregoing product (1.84 g) for 20 h at room temperature gave 2.28 g of 1,2:5,6-di-*O*-isopropylidene-α-D-galactofuranose 3-(barium sulphate). Hydrolysis of the free acid, as described above, for 30–40 min and subsequent chromatography gave 1.68 g of D-galactose 3-(potassium sulphate); ν_{max} 3555–3440 (s), 2960 (w), 1265–1221 (s), 1150 (m), 1077–1054 (s), 986 (s), 862 (m), 818 (m), and 772 (m). P.m.r. data (D_2O): δ 5.27 (d, $J_{1\alpha,2}$ 3.5 Hz, H-1α).

Anal. Calc. for $C_6H_{11}KO_9S \cdot 0.5\ C_2H_5OH$: C, 26.1; H, 4.39; S, 9.96. Found: C, 26.1; H, 4.62; S, 9.25.

D-*Galactopyranose 4-sulphate.* — D-Galactose (50 g), benzaldehyde (120 mL), and pulverised zinc chloride (40 g) were shaken together for 18 h at room temperature. Ice-cold water (200 mL) and light petroleum (b.p. 60–80°) (100 mL) were added, the layers were separated, and the organic phase was washed several times with water. The combined aqueous layers were washed with light petroleum and then made alkaline by the addition of concentrated, aqueous sodium carbonate. The precipitate was filtered off and washed with water. The filtrates were evaporated almost to dryness and then acetone was added and decanted from the resulting solid; evaporation of the solution gave a gel-like solid which was recrystallised from ethanol to give 4,6-*O*-benzylidene-D-galactopyranose (19.8 g). The latter product (17 g) was treated with dry pyridine (170 mL) and acetic anhydride (25 mL) for 20 h at room temperature. The pyridine was evaporated, complete removal being ensured by the addition of toluene and re-evaporation. The solid residue was recrystallised from ethanol, to give 1,2,3-tri-*O*-acetyl-4,6-*O*-benzylidene-D-galactose (22.3 g; a mixture of anomers).

The triacetate (22.2 g) was hydrolysed as described by Raymond[22], except that light petroleum (b.p. 60–80°) rather than pentane was used to extract the benzaldehyde. The resulting product (15 g) was then treated with 1.3 mol. equiv. of acetic anhydride[22], to give a syrupy mixture (14.7 g) of the required 1,2,3,6-tetra-acetate and the penta-acetate.

The mixture of acetates was sulphated directly for 2 h at 60–70° and worked up as already described except that the aqueous solution was extracted with chloroform to remove the unreacted penta-acetate. This gave 1,2,3,6-tetra-*O*-acetyl-D-galactose 4-(barium sulphate) (11.4 g) which was deacetylated to give crude D-galactose 4-(barium sulphate) (7.8 g); a portion (3.0 g) of this product was purified by chromatography to give D-galactose 4-(potassium sulphate) (2.3 g); ν_{max} 3515–3435

(s), 3010–2870 (w), 1261–1235 (s), 1135 (w,sh), 1090 (m), 1066 (m), 1043 (s), 1005 (w,sh), 921 (m), 850 (m), 825 (w,sh), 726 (w), and 690 (w). P.m.r. data (D_2O): δ 5.22 (d, $J_{1\alpha,2}$ 3.0 Hz, H-1α).

Anal. Calc. for $C_6H_{11}KO_9S \cdot 0.5\ C_2H_5OH$: C, 26.1; H, 4.39; S, 9.96. Found: C, 26.1; H, 4.51; S, 8.83.

D-*Galactopyranose 6-sulphate.* — Sulphation of 1,2:3,4-di-*O*-isopropylidene-α-D-galactose[29] (37 g) at 70° for 4.5 h gave 53 g of crude product. Removal of the protecting groups, as described above, was complete in 30 min and the usual procedure gave 25.8 g of crude D-galactose 6-(ammonium sulphate). Purification of two 8.5-g portions of this product by chromatography gave 3.42 g of D-galactose 6-(potassium sulphate) (3.42 g). The yield was low, because the ammonium salt was precipitated from solution with ethanol, rather than being obtained by evaporation, and this caused considerable losses. The product had ν_{max} 3500–3420 (s), 2970–2935 (w), 1265–1231 (s), 1149 (w), 1095 (sh), 1066 (s), 1025 (s), 996 (s), 818 (m), and 770 (w). P.m.r. data (D_2O): δ 5.17 (d, $J_{1\alpha,2}$ 2.5 Hz, H-1α).

Anal. Calc. for $C_6H_{11}KO_9S \cdot 0.5\ C_2H_5OH$: C, 26.1; H, 4.39; S, 9.96. Found: C, 26.8; H, 4.61; S, 9.92.

D-*Glucopyranose 2-sulphate.* — 1,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranose (34.5 g) was prepared from D-glucose (32 g) in the same way as the corresponding galactose derivative; attempts at recrystallisation were unsuccessful. Direct sulphation of the crude syrup (30.8 g) in *N*,*N*-dimethylformamide, as for the galactose derivative, gave 43.6 g of 1,3,4,6-tetra-*O*-acetyl-D-glucose 2-(barium sulphate), which was deacetylated to give crude D-glucose 2-(barium sulphate) (31.5 g). This contained a large proportion of barium acetate which was incompletely removed by treatment with carbon dioxide for several days. Chromatography of the crude material (8 g), in the usual way, gave pure D-glucose 2-(potassium sulphate) (2.6 g); ν_{max} 3525–3440 (s), 2990–2935 (w), 1264–1243 (s), 1150 (w), 1075 (s), 1035 (s), 990 (s), 822 (m), and 768 (w). P.m.r. data (D_2O): δ 5.41 (d, $J_{1\alpha,2}$ 3.0 Hz, H-1α).

Anal. Calc. for $C_6H_{11}KO_9S \cdot C_2H_5OH$: C, 27.9; H, 4.98; S, 9.31. Found: C, 27.2; H, 4.73; S, 7.24.

D-*Glucopyranose 3-sulphate.* — Sulphation of 1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose was accomplished by the standard method for 5.5 h at 55–65°, and the mixture was left overnight at room temperature before being worked up to give the crude sulphate (22.9 g). Hydrolysis of a portion (22.4 g) for 1.75 h gave crude D-glucose 3-(barium sulphate) (17 g) which was converted into the ammonium salt and purified in two portions to give D-glucose 3-(potassium sulphate) (12.0 g); ν_{max} 3485–3420 (s), 2950–2905 (w), 1262–1230 (s), 1084 (m), 1059–1037 (s), 978 (m), 921–902 (w), 855 (w), 814 (m), and 748 (w). P.m.r. data (D_2O): δ 5.20 (d, $J_{1\alpha,2}$ 3.5 Hz, H-1α).

Anal. Calc. for $C_6H_{11}KO_9S \cdot C_2H_5OH$: C, 27.9; H, 4.98; S, 9.31. Found: C, 27.4; H, 4.90; S, 9.46.

D-*Glucopyranose 4-sulphate.* — 4,6-*O*-Benzylidene-D-glucopyranose[31] (14.3 g) was treated with pyridine (140 mL) and acetic anhydride (23 mL) for 18 h at room

temperature. The mixture was poured, with stirring, into ice-cold water (700 mL) and, after 1 h, the precipitate was filtered off, washed with water, and dried over sulphuric acid, to give 24.1 g of the 1,2,3-triacetate. This material was converted into 1,2,3-tri-*O*-acetyl-D-glucose (14 g) and acetylated, as described for the galactose derivative, to give 1,2,3,6-tetra-*O*-acetyl-D-glucopyranose (14.1 g). Sulphation of this product at 60–70° for 1.5 h gave 1,2,3,6-tetra-*O*-acetyl-D-glucopyranose 4-(barium sulphate) (10.3 g). Deacetylation with barium methoxide for 29 h gave a crude product (7.4 g) which was purified by chromatography to give D-glucose 4-(potassium sulphate) (4 g); ν_{max} 3505–3435 (s), 2960 (w), 2910 (w,sh), 1263–1235 (s), 1150 (w,sh), 1110 (m), 1072 (s), 1032 (s), 981 (s), 917 (w), 817 (m), and 755 (w). P.m.r. data (D_2O): δ 5.19 (d, $J_{1\alpha,2}$ 3.0 Hz, H-1α).

Anal. Calc. for $C_6H_{11}KO_9S \cdot 0.5\ C_2H_5OH$: C, 26.1; H, 4.39; S, 9.96. Found: C, 25.2; H, 4.16; S, 9.57.

D-Glucopyranose 6-sulphate. — Sulphation of 1,2-*O*-isopropylidene-α-D-glucofuranose[30] (5.9 g) was performed for 5.5 h at 65–70° and the mixture was left overnight at room temperature before being worked up as usual. Removal of the protecting groups (45 min) gave crude D-glucose 6-(barium sulphate) which was purified by chromatography to give D-glucose 6-(potassium sulphate) (4.0 g); ν_{max} 3435–3403 (s), 3360–3292 (s,sh), 2966 (w), 2905 (w), 1263 (s), 1226–1214 (m), 1132 (m), 1105 (s), 1081 (s), 1058 (s), 1027 (s), 1011 (s), 980 (s), 937 (w), 897 (w), 823 (s), 800 (m,sh), and 646 (w). P.m.r. data (D_2O): δ 5.17 (d, $J_{1\alpha,2}$ 2.5 Hz, H-1α).

Anal. Calc. for $C_6H_{11}KO_9S \cdot 0.5\ C_2H_5OH$: C, 26.1; H, 4.39; S, 9.96. Found: C, 25.2; H, 4.28; S, 10.3.

2,3,4,6-Tetra-O-acetyl-β-D-glucopyranose 1-sulphate. — 2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranose[32] (16 g) was sulphated with chlorosulphonic acid, as described by Ohle[23], to give 2,3,4,6-tetra-*O*-acetyl-β-D-glucose 1-(pyridinium sulphate) (14 g), m.p. 112–114° without decomposition; lit.[23] m.p. 127° with decomposition. The potassium salt (1.4 g) was obtained by adding a slight excess of potassium acetate in ethanol to an ethanolic solution of the pyridinium salt (2 g) and, after standing some hours at 4°, was filtered off, thoroughly washed with ethanol, and dried *in vacuo* over sulphuric acid. It had ν_{max} 3640–3450 (w), 3040–2910 (vw), 1753 (s), 1441 (w), 1382 (m), 1373 (m), 1308 (m), 1277–1215 (s), 1093 (m), 1066–1018 (s), 988 (w), 905 (w), 881 (vw), 798 (m), 783–776 (m), and 619 (w). ^{13}C-N.m.r. data (water, reference 1,4-dioxane): 174.40, 173.69, 173.39, 173.31 (C=O), 97.25 (C-1β), 73.44 (C-5β), 72.63 (C-2β), 71.73 (C-3β), 68.75 (C-4β), 62.52 (C-6β), 21.00, and 20.92 (Me).

Anal. Calc. for $C_{14}H_{19}KO_{13}S$: C, 36.0; H, 4.11; S, 6.87. Found: C, 35.3; H, 4.02; S, 7.19.

2-Acetamido-2-deoxy-D-glucopyranose 6-sulphate. — 2-Acetamido-1,3,4-tri-*O*-acetyl-2-deoxy-β-D-glucopyranose[33] (5.2 g) was sulphated for 40 h at room temperature followed by deacetylation for 21 h to give crude 2-acetamido-2-deoxy-D-glucose 6-(barium sulphate) (5 g). The potassium salt (1.35 g) was prepared by the usual method, but, even after two fractionations on DEAE-cellulose, the ^{13}C-n.m.r. spectrum indicated the presence of considerable amounts of acetate-containing

impurities. The deacetylation and chromatographic purification was therefore repeated, to give material (0.82 g) which n.m.r. studies showed to be still impure. The product had ν_{max} 3533–3400 (s), 2962 (w), 1577–1569 (m), 1465–1437 (w), 1382 (w), 1262–1228 (s), 1110 (m,sh), 1063 (s), 1007 (s), 985 (m,sh), 940 (w), and 816 (m). P.m.r. data (D_2O): δ 5.17 (d, $J_{1\alpha,2}$ 2.0 Hz, H-1α).

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