330. *Sulphates of Monosaccharides and Derivatives. Part II.*[1] *Periodate Oxidation.*

By J. R. Turvey, M. J. Clancy, and T. P. Williams.

The oxidation with periodate of glucose 3- and 6-sulphate and of galactose 6-sulphate has been studied under various conditions of pH; the results are compared with the oxidations of the analogous phosphates. The monosulphate prepared by direct sulphation of methyl β-D-galactopyranoside has been shown by periodate oxidation to be the 6-sulphate.

Sugar sulphates are difficult to characterise since few of these salts have definite melting points and they do not readily form crystalline derivatives. Paper chromatography can be used to differentiate between certain isomeric sulphates [1,2] but other methods for their characterisation seemed desirable.

The use of periodate oxidation in structural investigations of carbohydrates is well established but there are few reports on its reaction with carbohydrate sulphates. Barry, Dillon, and their co-workers [3] used this reagent in structural investigations of a number of sulphated polysaccharides from algæ and assumed that the sulphate groups are stable to the reagent. Holt [4] briefly reported that the sulphate groups in some sugar sulphates are liberated on periodate oxidation but are stable in other cases. The reactions of the analogous sugar phosphates with periodate have, however, received considerable attention. Reducing sugar phosphates are usually considered as being oxidised in ring form with production of intermediate formyl esters [5,6] but the consumption of periodate and the end products of the reaction depend on the conditions of the reaction and on the position of the phosphate group. Ribose 5-phosphate and glucose 6-phosphate consume 3 and

[1] Part I, *J.*, 1960, 4761.

[2] Dodgson and Spencer, *Biochem. J.*, 1954, **57**, 310; Lloyd, *Nature*, 1959, **183**, 109; Rees, *Nature*, 1960, **185**, 309.

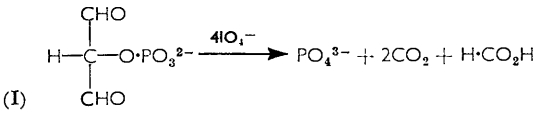
[3] Barry and Dillon, *Proc. Roy. Irish Acad.*, 1945, **50***B*, 349; Dillon and O'Colla, *ibid.*, 1951, **54***B*, 51; Barry and McCormick, *J.*, 1957, 2777.

[4] Holt, Inst. Seaweed Res., Ann. Reports for 1958, p. 15.

[5] Morrison, Rouser, and Stotz, *J. Amer. Chem. Soc.*, 1955, **77**, 5156.

[6] Loring, Moss, Levy, and Hain, *Arch. Biochem. Biophys.*, 1956, **65**, 578; but see Distler, Merrick, and Roseman, *J. Biol. Chem.*, 1958, **230**, 497.

4 mol. of periodate, respectively, and give the relatively stable glycollaldehyde phosphate [7-10] although, at pH 8, this ester is slowly hydrolysed to inorganic phosphate and glycollaldehyde, which is then further oxidised.[7] Glucose 2-, 3- and 4-phosphate and ribose 2- and 3-phosphate are all oxidised to the same intermediate tartrondialdehyde phosphate (I). This then undergoes overoxidation to yield inorganic phosphate, carbon dioxide and formic acid, with the consumption of 4 mol. of periodate: [8]

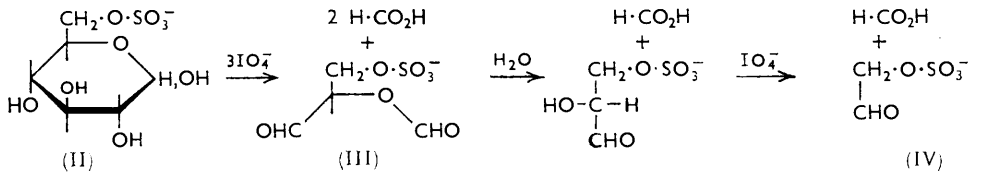


(I)

We now report a comparative study of the periodate oxidation of synthetic sugar sulphates [1] and of sugar phosphates.

To avoid the precipitation of insoluble barium salts, the sugar sulphates were used as either sodium or potassium salts. Oxidations were carried out with an excess of sodium metaperiodate either in unbuffered (initial pH 4·2—4·8) or in buffered solution. In the former case, both the consumption of periodate and the liberation of formic acid were measured but, in the latter cases, only the consumption of periodate was determined. Under alkaline conditions, a lower concentration of the periodate was used in order to prevent precipitation of disodium paraperiodate [11] and, with the same object, potassium salts of the sugar sulphate and buffers were preferable.

The oxidation of galactose 6-sulphate and of glucose 6-sulphate in an unbuffered solution (pH 4·2 falling to 3) followed the course shown in Fig. 1. With each sulphate there was an initial rapid (1 hr.) consumption of over 3 mol. of periodate with the liberation of 2 mol. of formic acid, the latter rising to 3 mol. after a few hours, and finally both the periodate consumption and formic acid liberation became constant at about 3·5 mol. An oxidation sequence such as that depicted for glucose 6-sulphate (II) would require the rapid consumption of 3 mol. of oxidant and the liberation of 2 mol. of acid with the formation of 2-*O*-formylglyceraldehyde 3-sulphate (III). Hydrolysis of the formyl ester, which is relatively slow at pH 4,[12] would be followed by further oxidation to glycollaldehyde sulphate (IV), in which 1 mol. of periodate is consumed and 2 mol. of formic acid are liberated. Neither the periodate consumed nor the formic acid liberated reached the theoretical values of 4 mol., but similar incomplete oxidation has been reported for ribose 5-phosphate in unbuffered solution, which consumes only 2·5 instead of 3 mol. of oxidant.[7] Explanations, based on the stability of the intermediate formyl ester,[7] do not, however, agree with the fact that, in the case of the hexose 6-sulphates the formic acid liberated was equivalent to the oxidant ultimately consumed. Loring *et al.*[9] have shown that, at neutral pH, ribose 5-phosphate consumes the expected 3 mol. of periodate. In phosphate buffer at pH 6·6, the 6-sulphates of glucose and galactose consumed 3·87 and 3·82 mol.



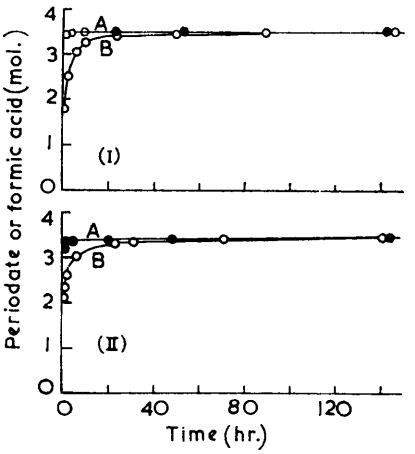
(II) (III) (IV)

of oxidant respectively. In an alkaline buffer (borate, pH 8·2) approximately the same figures were obtained although uptake of oxidant was faster, presumably owing to faster

[7] Marinetti and Rouser, *J. Amer. Chem. Soc.*, 1955, **77**, 5345.
[8] Courtois and Ramet, *Bull. Soc. Chim. biol.*, 1945, **27**, 610.
[9] Loring, Levy, Moss, and Ploeser, *J. Amer. Chem. Soc.*, 1956, **78**, 3724.
[10] Khym, Doherty, and Cohn, *J. Amer. Chem. Soc.*, 1954, **76**, 5523.
[11] Jeanloz and Forchielli, *J. Biol. Chem.*, 1951, **188**, 361.
[12] Neumüller and Vasseur, *Arkiv Kemi*, 1953, **5**, 235.

hydrolysis of the formyl esters in alkaline media. Bicarbonate buffer (pH 7·8), which is reported as favouring complete oxidation,[13] did not further increase the consumption of periodate. In no case did the consumption of periodate exceed 4 mol., thus clearly demonstrating that the glycollaldehyde sulphate is more stable to hydrolysis than glycollaldehyde phosphate, which is stable at neutral or slightly acid pH (refs. 8—10, 14) but is slowly hydrolysed at pH 8 (ref. 7). The stability of the ester link of glycollaldehyde sulphate was also indicated by the fact that no inorganic sulphate could be detected in the products of oxidation of either of the 6-sulphates, even after 216 hr. at pH 8·2.

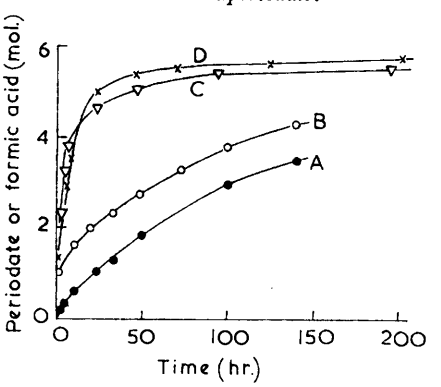
FIG. 1. *Oxidation of sugar sulphates with sodium metaperiodate.*



(I) *Galactose 6-sulphate*; (II) *Glucose 6-sulphate.*

A, *Periodate consumed*; B, *Formic acid liberated.*

FIG. 2. *Oxidation of glucose 3-sulphate with sodium metaperiodate.*

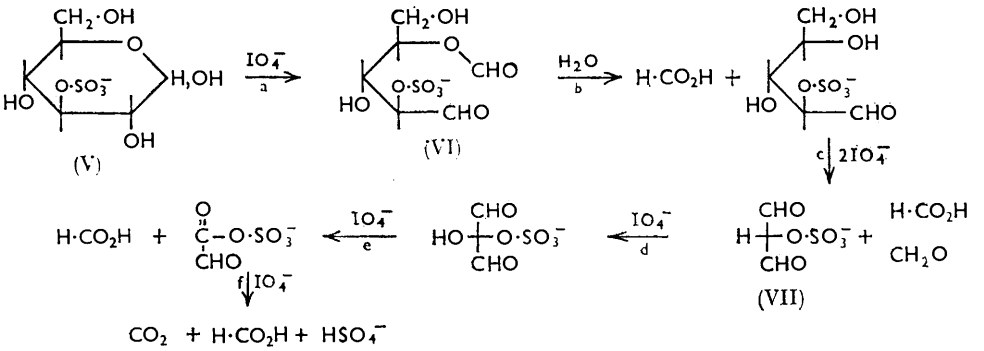


A, *Formic acid liberated in unbuffered solution*; B, *Periodate consumed in unbuffered solution*; C, *Periodate consumed in borate buffer* (pH 8·2); D, *Periodate consumed in bicarbonate buffer* (pH 7·8).

The oxidation of glucose 3-sulphate followed the course shown in Fig. 2. In unbuffered solution, 1 mol. of periodate was rapidly consumed with the liberation of very little formic acid and thereafter the oxidation proceeded slowly until, at 140 hr., over 4 mol. of oxidant had been consumed and over 3 mol. of formic acid liberated. The discontinuity of the curve after the consumption of 1 mol. of oxidant was not as sharp as was expected. The sequence of reactions a ⟶ f is suggested for the oxidation of the 3-sulphate (V). This would require rapid consumption of 1 mol. of oxidant (reaction a) and then a slow further consumption as the 4-*O*-formylarabinose 2-sulphate (VI) was hydrolysed. In unbuffered solution (pH 4·8 falling to 3) this hydrolysis would be slow. In the oxidation of 3-*O*-methylglucose, Hough *et al.*[15] observed a sharp inflection in the curve after the consumption of 1 mol. of oxidant, corresponding to the formation of the relatively stable 4-*O*-formyl-2-*O*-methylarabinose. As seen in Fig. 3 for glucose 3-sulphate, we did not observe this sharp discontinuity even when the solution was buffered at pH 3·6, *i.e.*, in the region of greatest stability of formyl esters (pH 3—5).[12] Two explanations are possible: either the sulphuric hemiester group promotes the hydrolysis of the formyl ester [4] or the molecule is not being oxidised entirely in the pyranose form, a proportion being oxidised in the open-chain form. In alkaline buffer (borate, pH 8·2) there was no discontinuity in the

[13] O'Dea and Gibbons, *Biochem. J.*, 1953, **55**, 580; Jeanloz, *Helv. Chim. Acta*, 1944, **27**, 1509.
[14] Euler, Karrer, and Becker, *Helv. Chim. Acta*, 1936, **19**, 1060; Kiessling and Meyerhof, *Biochem. Z.*, 1938, **296**, 410.
[15] Hough, Taylor, Thomas, and Woods, *J.*, 1958, 1212.

region of 1 mol. of oxidant consumed, the curve smoothly approaching a limiting value of 6 mol. of periodate. The use of bicarbonate buffer at pH 7·8, to favour complete oxidation,[13] gave essentially the same results as the borate buffer. If the oxidation follows the sequence depicted in reactions a ⟶ f, then at the " half-way stage " [the



tartrondialdehyde sulphate (VII) stage] the amount of oxidant consumed and formic acid liberated would be 3 and 2 mol., respectively, and this 3 : 2 ratio is observed in the unbuffered solution (*ca.* 55 hr.). The final stages of the oxidation (reactions d ⟶ f) represent a normal overoxidation although the course may not be exactly as depicted. These reactions should result in an uptake of 3 mol. of periodate and the liberation of inorganic sulphate and are in contrast to the analogous oxidation of tartrondialdehyde phosphate, during which 4 mol. of periodate are consumed.[8] In the borate buffer after 100 hr., over

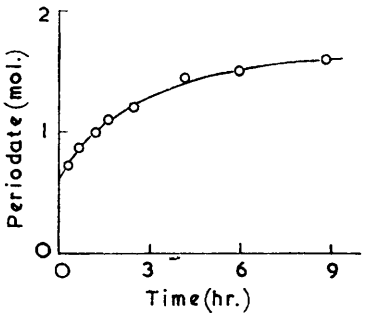
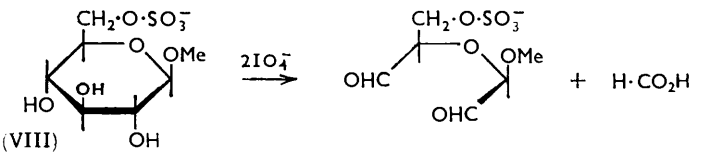


FIG. 3. *Oxidation of glucose 3-sulphate with sodium metaperiodate in acetate buffer* (pH 3·6).

80% of the sulphate had been liberated when 5·4 mol. of periodate had been consumed, and in bicarbonate buffer after 49 hr. the corresponding figures were 70% sulphate liberated and 5·35 mol. of periodate consumed. In no case was there any evidence that the total consumption of periodate would exceed 6 mol. and, indeed, the final stages of the oxidation were so slow that it was not possible to follow the consumption of periodate even to 6 mol.



Direct sulphation of methyl β-D-galactopyranoside and separation of the products has given a monosulphate as the main product.[1] By analogy with the sulphation of monosaccharides, this product should be methyl β-D-galactopyranoside 6-sulphate (VIII) and

it was of interest to see if this compound would be oxidised by periodate in the expected manner. On oxidation this glycoside sulphate consumed 2 mol. of periodate with the liberation of 1 mol. of formic acid in 30 min. and thereafter underwent little further oxidation, in agreement with the predicted values. This result confirms the assignment of the sulphate group to the 6-position in this glycoside sulphate, since only 1 mol. of oxidant would be consumed if the sulphate group were on $C_{(2)}$ or $C_{(4)}$ and none if it were on $C_{(3)}$.

Experimental

General Methods.—The sugar sulphates were dried *in vacuo* over phosphoric anhydride at 60° before use. Oxidations were carried out at room temperature and in darkness,[16] with *ca.* 10 mol. of sodium metaperiodate.

(a) *Determination of periodate reduced.* For sulphates of reducing sugars, Linstedt's buffer method [17] was used, whereby an aliquot part of the reaction mixture is added to an excess of phosphate buffer (pH 7), followed by potassium iodide, and the liberated iodine is titrated with sodium thiosulphate. For the glycoside sulphate, Malaprade's acid method [18] was used.

(b) *Determination of formic acid.* This was estimated iodometrically, after destruction of the excess of periodate with ethylene glycol.[18]

Oxidation of Reducing-sugar Sulphates.—(a) *Unbuffered solution.* To the barium salt of the sugar sulphate (250 mg.) in water (10 ml.) was added 8% aqueous sodium sulphate (2 ml.), and the mixture was shaken. After centrifugation, the supernatant solution (10 ml.) was pipetted into the reaction flask, together with 0·3M-sodium metaperiodate (20 ml.), 0·01N-acetic acid (8 ml.), and water to 50 ml. At suitable time intervals, portions (1 ml. each) were removed, added to a mixture of 0·5M-phosphate buffer (pH 7·0; 5 ml.) and 10% aqueous potassium iodide (1 ml.), and the iodine was titrated with 0·05N-sodium thiosulphate. Other portions (2 ml. each) were added to ethylene glycol (0·5 ml.) and, after 5 min., the formic acid was determined iodometrically. A solution containing the reagents but no sugar sulphate was used as a control in each case. The results are given in Figs. 1 and 2.

(b) *Glucose 3-sulphate in acetate buffer.* The reaction mixture contained the sodium salt (5 ml.; 100 mg.) of the sugar sulphate (prepared as above), 0·2M-acetate buffer (pH 3·6; 5 ml.), 0·3M-sodium metaperiodate (10 ml.), and water to 25 ml. A similar mixture containing no sugar sulphate was used as control. The consumption of periodate was determined as above (Fig. 3).

(c) *In phosphate buffer.* The barium salt of the sugar sulphate (40 mg.) in water (5 ml.) was added to 3% potassium sulphate (1 ml.) and, after mixing and centrifugation, the supernatant solution (5 ml.) was placed in the reaction flask. The other reactants were 0·2M-potassium phosphate buffer (pH 6·6; 10 ml.), 0·15M-sodium metaperiodate (5 ml.), and water to a final volume of 50 ml. At intervals, portions (3 ml. each) were withdrawn for estimation of periodate by the buffer method as above. A control solution, containing all the reagents except the sugar sulphate, was similarly treated. The results are given in Table 1.

(d) *In borate buffer.* The reaction mixture contained the sugar solution (5 ml.) as above, 0·2M-borate buffer (pH 8·2; 25 ml.), 3% aqueous potassium sulphate (1 ml.), 0·15M-sodium metaperiodate (5 ml.), and water to 100 ml. A similar solution, containing the reagents but no sugar sulphate, was used as control. The consumption of periodate was determined as for the phosphate buffer (Table 2 and Fig. 2).

For the determination of sulphate liberated during the reaction, portions (5 ml. each) of the reaction mixture were removed at intervals and to each was added 10% aqueous potassium iodide (1 ml.), 0·45M-stannous chloride in 2N-hydrochloric acid (1 ml.) and water to 10 ml. When all the liberated iodine had been reduced, portions (1 ml. each) were removed for the determination of sulphate by the method of Jones and Letham.[19] The amount of sulphate present in the control was deducted from that found for the reaction mixture. Previous tests had shown that stannous chloride was an effective reagent for removing iodine liberated by

[16] Head and Hughes, *J.*, 1952, 2046.
[17] Lindstedt, *Arkiv Kemi, Min. Geol.*, 1945, **20***A*, No. 13.
[18] Malaprade, *Bull. Soc. chim. France*, 1926, **39**, 325; 1928, **43**, 683.
[19] Jones and Letham, *Chem. and Ind.*, 1954, 662.

periodate and iodate, both of which interfere with the method for determining sulphate. In the cases of glucose 6-sulphate and of galactose 6-sulphate, no liberation of sulphate could be detected, but for glucose 3-sulphate the results are given in Table 2.

TABLE 1. *Consumption of periodate by sugar sulphates at* pH 6·6.

	Galactose 6-sulphate.								
Time (hr.)	0·10	0·20	0·50	1·33	4·17	6·25	24	48	72
Consumption (mol.)	2·90	3·42	3·55	3·62	3·62	3·66	3·76	3·81	3·87
	Glucose 6-sulphate.								
Time (hr.)	0·10	0·25	0·50	0·9	2·5	5·5	7·0	24	72	125
Consumption (mol.)	2·5	3·21	3·37	3·44	3·44	3·48	3·51	3·57	3·70	3·82

TABLE 2. *Oxidation of sugar sulphates in alkaline solutions.*

	Galactose 6-sulphate. (*Consumption of periodate in mol.*)								
Time (hr.)	0·06	0·47	1·6	3·0	18·1	22·0	48	91·5	216
Borate buffer	—	3·48	3·46	3·5	—	3·61	3·64	—	3·88
Bicarbonate buffer...	1·39	3·70	3·85	3·84	3·85	—	3·91	3·93	—
	Glucose 6-sulphate. (*Consumption of periodate in mol.*)								
Time (hr.)	0·20	0·50	1·0	3·0	24	72	164	240	
Borate buffer	3·20	3·27	3·31	3·40	3·41	3·53	—	3·91	
Bicarbonate buffer...	2·40	3·19	3·51	3·57	—	3·70	3·72	—	
	Glucose 3-sulphate. (*Liberation of sulphate as % of total.*)								
Time (hr.)	0·08	0·20	6·0	22·3	48·6	100			
Borate buffer	0	—	38	—	—	88			
Bicarbonate buffer...	—	1·5	19	53	70	—			

TABLE 3. *Oxidation of methyl* β-D-*galactopyranoside sulphate by sodium metaperiodate.*

Time (hr.)	0·15	0·25	0·50	1·0	1·5	14·5
Periodate consumed (mol.) ...	1·78	—	2·08	2·08	2·08	2·09
Formic acid liberated (mol.)	—	0·73	0·84	0·97	0·93	1·29

(e) *In bicarbonate buffer.* The conditions were as for the oxidations in borate buffer except that the borate buffer was replaced by N-potassium hydrogen carbonate (5 ml.), giving pH 7·8 in the reaction mixture. The results are given in Fig. 2 and Table 2.

Oxidation of Methyl Galactoside Sulphate.—The ammonium salt (60 mg.) was oxidised in 0·049M-sodium metaperiodate (25 ml.). At intervals, portions (0·5 ml. each) were added to 2·5% aqueous potassium iodide (2 ml.) and 3N-sulphuric acid (2 ml.) for the determination of the consumption of periodate by the acid method. Portions (2 ml. each) were also removed at intervals for the determination of formic acid. The results are given in Table 3.

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CHEMISTRY DEPARTMENT, UNIVERSITY COLLEGE OF NORTH WALES,
BANGOR, CAERNARVONSHIRE. [*Received, September 19th,* 1960.]