220. *Deoxy-sugars. Part XVI. A Study of the Stabilities of Some Phosphoric Acid Derivatives of* D-*Galactose and* 2-*Deoxy-*D-*galactose.*

By A. B. Foster, W. G. Overend, and M. Stacey.

The rates of dephosphorylation of certain phosphoric acid derivatives of D-galactose and 2-deoxy-D-galactose by acid and alkaline reagents and also by an enzyme isolated from *Actinomyces* strain A, have been investigated. Results obtained confirm the view that in 2-deoxyhexoses the methylene (deoxy) group renders substituents in the molecule relatively more labile than in the normal hexose analogue.

In the preceding paper the syntheses were described of galactose-3 and -6 phosphoric acid and of the corresponding 2-deoxy-analogues. In common with most phosphoric acid derivatives of sugars, these compounds were difficult to crystallize and characterize, but they have been purified as their acridine salts. In further work with these compounds it was decided to measure their comparative rates of dephosphorylation.

The action of dilute aqueous mineral acids on the phosphoric acid derivatives of sugars has been employed by numerous workers as a means of correlating the lability of the phosphoric acid residue with its position in the sugar molecule. For example, recent results which Todd and Michelson (*J.*, 1949, 2476) obtained by this method clearly indicate that, in the ribose molecule, a phosphoric acid residue located at position 3 of the sugar is the most labile. Phosphoric esters at positions 2 and 5 in this sugar molecule are definitely less labile. Gulland and Smith (*J.*, 1947, 338) have employed similar methods with uridine-2′ phosphate, and Friedkin (*J. Biol. Chem.*, 1950, **184**, 449) has shown that 50% of the phosphorus of deoxyribose-1 phosphate is released as inorganic phosphate within 10—15 minutes on hydrolysis at pH 4 at 23°.

We have used this method, employing 0·1N-sulphuric acid, to study the lability of the phosphoric acid derivatives of D-galactose and 2-deoxy-D-galactose already described.

Briefly, the technique adopted was to heat aliquots (2 c.c.) of a solution (1 mg. of P/c.c.) of the phosphoric acid derivative in 0·1N-sulphuric acid, in sealed glass tubes, on a boiling water-bath. At suitable intervals the liberated phosphate ion was estimated colorimetrically (Allen, *Biochem. J.*, 1940, **34**, 858). It was found that D-galactose-3 phosphoric acid was more labile than the corresponding 6-phosphoric acid, in agreement with the work described above (Todd and Michelson, *loc. cit.*). For the derivatives of 2-deoxy-D-galactose, it was shown that the phosphoric acid residues located at both $C_{(3)}$ and $C_{(6)}$ in the sugar are much more labile under acid conditions than are the residues in the corresponding derivatives of D-galactose. The hydrolyses are represented graphically in Fig. 1. It is seen that 2-deoxy-D-galactose-3 phosphoric acid had undergone 84% hydrolysis in 5 hours whereas in the same time the corresponding derivative of D-galactose had undergone only 13% hydrolysis. The activation of the phosphoric acid residue in this derivative of 2-deoxy-D-galactose may be attributed to the presence of the methylene (deoxy) group at position 2. The influence of this group on the glycosidic centre in 2-deoxy-hexoses and -pentoses has already been reported

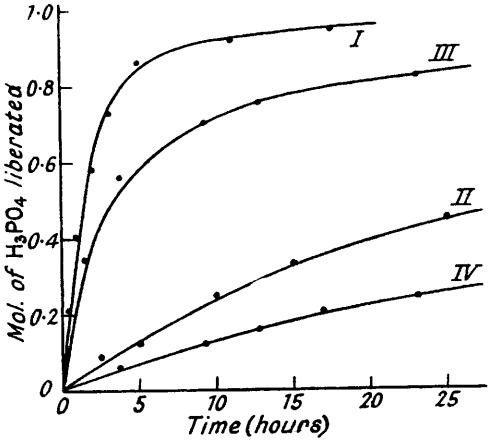
(Stacey *et al.*, *J.*, 1949, 2836, 2846; 1950, 671, 738). It was shown particularly that the glycosidic methyl group in sugars of this type was much more labile than that in the corresponding hexose series. The present work has confirmed this finding (see Fig. 2).

More surprising than the above results was the fact that the lability of the phosphoric acid residue at position 6 in 2-deoxy-D-galactose was greater than that in the corresponding derivative of D-galactose. From Fig. 1 it can be seen that in 5 hours 2-deoxy-D-galactose-6 phosphoric acid had undergone 58% hydrolysis, whereas the corresponding derivative of D-galactose had only undergone 7% hydrolysis. These facts appear to indicate that the methylene (deoxy) group at position 2 in the deoxy-sugar does not merely activate the substituents at the adjacent $C_{(1)}$ and $C_{(3)}$, but activates the molecule as a whole, although the degree of activation of substituents at the adjacent positions 1 and 3 is greater than that of substituents at the more remote position 6.

When the phosphoric acid derivatives of 2-deoxy-D-galactose and of D-galactose were submitted to treatment with 0·1N-sodium hydroxide under conditions analogous to those employed in the acid treatment described above, dephosphorylation was essentially complete in each case in 5 minutes. The rapid rate of dephosphorylation is probably not caused solely

FIG. 1.

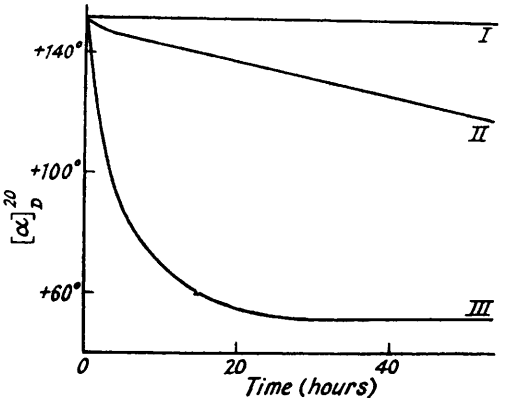
Acid hydrolysis.



I, 2-*Deoxy*-D-*galactose*-3 *phosphoric acid.*
II, D-*Galactose*-3 *phosphoric acid.*
III, 2-*Deoxy*-D-*galactose*-6 *phosphoric acid.*
IV, D-*Galactose*-6 *phosphoric acid.*

FIG. 2.

Hydrolysis of α-methyl-D-galactoside and α-methyl-2-deoxy-D-galactoside with sulphuric acid.



I, α-*Methyl*-D-*galactoside in* 2N-*sulphuric acid.*
II, α-*Methyl*-D-*galactoside in* 12N-*sulphuric acid.*
III, α-*Methyl*-2-*deoxy*-D-*galactoside in* 2N-*sulphuric acid.*

by the lability of the phosphoric acid substitutents towards alkali, since, as is well known, sugar derivatives having a free reducing group are rapidly transformed and destroyed by being heated with alkali, and phosphoric acid would be liberated.

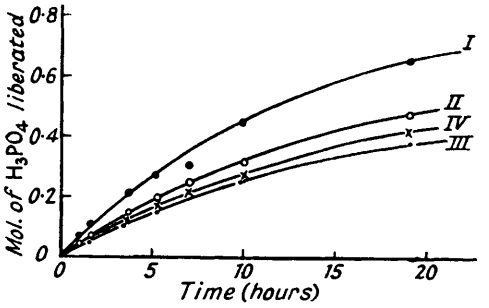
The mechanism of changes undergone during alkaline dephosphorylation needs careful investigation. It should be noted that Percival and Percival (*J.*, 1945, 874) found a slower hydrolysis for methylglucoside phosphates, *i.e.*, where the $C_{(1)}$ is blocked and other hydroxyl groups are free, and we now have shown that alkaline hydrolysis of certain phosphoric esters of D-galactose and 2-deoxy-D-galactose in which $C_{(1)}$ is blocked resulted in a comparatively large decrease in the rate of dephosphorylation. The rates of alkaline dephosphorylation of 4 : 6-benzylidene α-methyl-2-deoxy-D-galactoside-3 phosphoric acid, 3 : 4-*iso*propylidene α-methyl-2-deoxy-D-galactoside-6 phosphoric acid, 4 : 6-ethylidene 1 : 2-*iso*propylidene D-galactose-3 phosphoric acid, and 1 : 2-3 : 4-di*iso*propylidene D-galactose-6 phosphoric acid, are represented graphically in Fig. 3. It is clear from the graphs that on increasing the strength of the alkali to 1·0N, hydrolysis of the most labile phosphoric acid derivative, namely, 4 : 6-benzylidene α-methyl-2-deoxy-D-glactoside-3 phosphoric acid, was 65% complete after 20 hours.

Finally the rates of enzymic dephosphorylation were compared. The enzyme used was isolated from the bacteriolytic system of *Actinomyces* strain A described by Muggleton and Webb (*Biochim. Biophys. Acta*, in the press). It was obtained from a purified culture filtrate from organisms growing on a synthetic medium and was isolated by dialysis and dried from the

frozen state. The enzyme mixture was complex but contained a phosphatase which could effect the dephosphorylation of the sugar phosphates under examination. Fig. 4 shows the liberation of phosphoric acid with time, at pH 7 and 37°, by the action of the enzyme on dilute aqueous solutions of 2-deoxy-D-galactose-3 and -6 phosphoric acid and of the corresponding derivatives of D-galactose. It can be seen that the derivatives of D-galactose were dephosphorylated slowly (20% in 23 hours) and approximately at the same rate. The corresponding derivatives of 2-deoxy-D-galactose were dephosphorylated much more rapidly, for in 23 hours 2-deoxy-D-galactose-3 phosphoric acid had undergone 66% dephosphorylation and the 6-phosphoric acid had been dephosphorylated to the extent of 32%. The rapid decrease

FIG. 3.

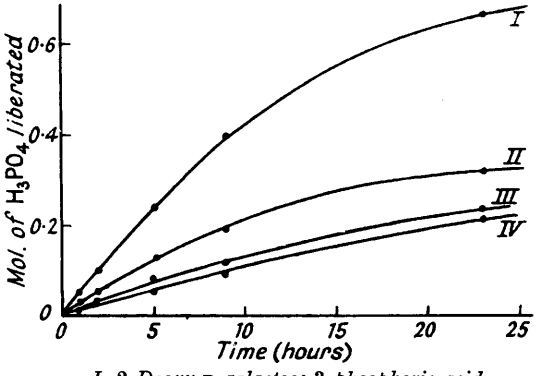
Alkaline hydrolysis of the phosphoric acid derivatives of D-*galactose and* 2-*deoxy*-D-*galactose.*



I, ● 4 : 6-*Benzylidene* α-*methyl*-2-*deoxy*-D-*galactoside*-3 *phosphoric acid.*
II, ○ 3 : 4-iso*Propylidene* α-*methyl*-2-*deoxy*-D-*galactoside*-6 *phosphoric acid.*
III, — · — 1 : 2-3 : 4-*Diisopropylidene* D-*galactose*-6 *phosphoric acid.*
IV, × 4 : 6-*Ethylidene* 1 : 2-iso*propylidene* D-*galactose*-3 *phosphoric acid.*

FIG. 4.

Enzymic dephosphorylation of the phosphoric acid derivatives of D-*galactose and* 2-*deoxy*-D-*galactose.*



I, 2-*Deoxy*-D-*galactose*-3 *phosphoric acid.*
II, 2-*Deoxy*-D-*galactose*-6 *phosphoric acid.*
III, D-*Galactose*-6 *phosphoric acid.*
IV, D-*Galactose*-3 *phosphoric acid.*

in the rates of dephosphorylation after 10 hours may be due to several causes. For example, as the enzymic hydrolysis proceeded, the liberated phosphoric acid displaced the pH of the solution towards the acid region. This effect was not completely counteracted by the veronal buffer used and tended to inhibit the action of the enzyme. This buffer was employed instead of the more usual phosphate buffer, because the enzymic hydrolysis was followed by phosphorus determinations (see Experimental section).

The results described may be interpreted as indicating that the phosphoric acid residue located at position 3 in the 2-deoxy-D-galactose molecule is considerably activated in comparison with that in the corresponding hexose derivative. This may be attributed to the influence of the methylene group at position 2 in the former substance. In agreement with the suggestion made earlier that the molecule of 2-deoxy-D-galactose as a whole is activated by the methylene

group is the fact that the 6-phosphoric acid derivative of the deoxy-sugar is also more rapidly dephosphorylated by the enzyme than is the corresponding D-galactose-6 phosphoric acid.

EXPERIMENTAL.

Hydrolysis of α-Methyl-2-deoxy-D-galactoside with Sulphuric Acid.—α-Methyl-2-deoxy-D-galactoside (0·1 g.) was dissolved in water (5 c.c.) ($[\alpha]_D^{20}$ +152° for this solution), and 12N-sulphuric acid (1·0 c.c.) was added. The ensuing hydrolysis was followed polarimetrically :

Time (hours)	0·03	0·3	1·03	2·25	5·75	17·75	29	40
$[\alpha]_D^{20}$	+150°	+145°	+133°	+114°	+84°	+62°	+52°	+52°

Neutralisation of the solution with barium carbonate followed by removal of the insoluble barium residues and evaporation of the solvent afforded a syrupy product, which readily reduced Fehling's solution. Treatment with a solution of aniline (0·1 g.) in ethanol (2 c.c.) yielded 2-deoxy-D-galactose anilide, m. p. 131—132° alone or on admixture with an authentic specimen.

Hydrolysis of α-Methyl-D-galactoside with Sulphuric Acid.—(*a*) 2N-Sulphuric acid had no effect on the glycoside after 5 days.

(*b*) α-Methyl-D-galactoside (0·1 g.) was dissolved in 12N-sulphuric acid (5 c.c.) and the following changes in optical rotation of the solution were observed polarimetrically :

Time (hours)	0	2	10	50
$[\alpha]_D^{20}$	+150°	+148°	+143°	+120°

Hydrolysis of the Phosphoric Acid Derivatives of D-Galactose and 2-Deoxy-D-galactose.—(a) *By sulphuric acid.* The lead salt of the appropriate phosphoric acid derivative (*ca.* 40 mg.), after being finely powdered and dried *in vacuo* at 100° over phosphoric oxide for 5 hours, was suspended in water (10 c.c.) and decomposed with hydrogen sulphide. The precipitate of lead sulphide was collected and washed by grinding it under water. After a second treatment with hydrogen sulphide the combined filtrate and washings were freed from hydrogen sulphide by aeration. The volume was made up to 25 c.c. and 0·6N-sulphuric acid (6 c.c.) was added. Aliquots (2 c.c.) of the resultant solution were sealed in Pyrex glass tubes and immersed in a bath at 100°. At suitable intervals, the tubes were opened and the contents washed carefully into a standard flask (25 c.c.). After dilution with water, perchloric acid (2 c.c.; 60% solution; *d* 1·54), amidol reagent [2 c.c. of a solution containing amidol (2 g.) and sodium hydrogen sulphite (40 g.) in water (200 c.c.)], 8·3% aqueous ammonium molybdate (1 c.c.), and water (to 25 c.c.) were added successively. The blue colour which developed was measured after 15—30 minutes in a Hilger (Spekker) photoelectric absorptiometer using a red filter (Ilford H.608). The values obtained were standardized by comparison with values obtained using a standard solution of potassium dihydrogen phosphate. This method of determination is essentially that of Allen (*Biochem. J.*, 1940, **34**, 858).

(i) 2-*Deoxy*-D-*galactose*-3 *phosphoric acid.* The lead salt (37 mg.) was treated as above :

Time (hours)	0·5	1·1	2·1	3·0	5·0	11·0	17·5
H_3PO_4, mols. liberated	0·21	0·41	0·58	0·73	0·86	0·92	0·95

(ii) D-*Galactose*-3 *phosphoric acid.* On hydrolysis of the phosphoric acid derivative obtained from its lead salt (36 mg.) or from the dipotassium salt (27 mg.) the following results were obtained :

Time (hours)	2·5	5·0	10·0	15·0	25·0
H_3PO_4, mols. liberated	0·09	0·13	0·25	0·33	0·45

(iii) 2-*Deoxy*-D-*galactose*-6 *phosphoric acid.* This compound (20 mg.) was hydrolysed according to the procedure described, with the results :

Time (hours)	1·5	3·75	9·25	12·75	23·0
H_3PO_4, mols. liberated	0·35	0·56	0·70	0·75	0·82

(iv) D-*Galactose*-6 *phosphoric acid.* Hydrolysis of this compound (20 mg.) gave the results :

Time (hours)	3·75	9·25	12·75	17·0	23·0
H_3PO_4, mols. liberated	0·06	0·12	0·16	0·20	0·24

(b) *By sodium hydroxide.* A solution of the free phosphoric acid derivative was made from the lead salt according to the procedure described. The solution was diluted with 0·6N-aqueous sodium hydroxide (5 c.c.), and aliquots (2 c.c.) were sealed in tubes and the liberated phosphate ion determined as previously described. 2-Deoxy-D-galactose-3 phosphoric acid and D-galactose-3 phosphoric acid were 90% hydrolysed in less than 30 minutes and the corresponding 6-phosphoric acid derivatives were 90% hydrolysed in less than 10 minutes.

(i) 4 : 6-Benzylidene α-methyl-2-deoxy-D-galactoside-3 phosphoric acid (29 mg.) was dissolved in water (25 c.c.) and 6N-sodium hydroxide (5 c.c.) was added. Aliquots (2 c.c.) of this solution were treated as described previously, and the released phosphate ion was determined :

Time (hours)	1·0	1·75	3·75	5·25	7·0	10·0	19·0
H_3PO_4, mols. liberated	0·075	0·11	0·22	0·27	0·30	0·43	0·65

(ii) 4 : 6-Ethylidene 1 : 2-*iso*propylidene D-galactose-3 phosphoric acid (25 mg.) was hydrolysed in a similar manner :

Time (hours)	1·0	1·75	3·75	5·25	7·0	10·0	19·0
H_3PO_4, mols. liberated	0·05	0·07	0·12	0·17	0·22	0·27	0·42

(iii) 3 : 4-*iso*Propylidene α-methyl-2-deoxy-D-galactoside-6 phosphoric acid (23 mg.) was treated according to the same procedure :

Time (hours)	1·0	1·75	3·75	5·25	7·0	10·0	19·0
H_3PO_4, mols. liberated	0·04	0·07	0·15	0·20	0·25	0·27	0·48

(iv) 1 : 2-3 : 4-Di*iso*propylidene-D-galactose-6 phosphoric acid (25 mg.) was hydrolysed as described above :

Time (hours)	1·0	3·75	5·25	7·0	10·0	19·0
H_3PO_4, mols. liberated	0·054	0·11	0·15	0·18	0·25	0·38

Enzymic Dephosphorylation of the Phosphoric Acid Derivatives of D-*Galactose and* 2-*Deoxy*-D-*galactose.*—Samples of the phosphoric acid derivative (*ca.* 20 mg.) were dissolved in water (*ca.* 15 c.c.), and the pH of the solution was adjusted to 6·9—7·1 by titration with 0·1N-sodium hydroxide (end-point determined by pH meter). The solution was diluted to 25 c.c. and treated with veronal buffer (9·4 c.c. of a solution containing 2·02 g. of sodium diethylbarbiturate per 100 c.c.) and 0·1N-hydrochloric acid (8·6 c.c.). The solution (*A*) was warmed at 37°. The enzyme from *Actinomyces* strain A (0·5 g., kindly supplied by Dr. Webb and Dr. Muggleton) was extracted with water (110 c.c.) at room temperature for 30 minutes. Insoluble debris and denatured protein were removed by centrifuging and the straw-coloured supernatant liquid was warmed to 37°. The enzyme solution (25 c.c.) was added to solution *A* and after being shaken the mixture was incubated at 37°. At suitable intervals, samples (5 c.c.) were withdrawn and the inorganic phosphorus was determined by Allen's method (*loc. cit.*).

(a) 2-*Deoxy*-D-*galactose*-3 *phosphoric acid.* The lead salt (37 mg.), converted into the free phosphoric acid by means of hydrogen sulphide as already described, was dissolved in water (25 c.c.) and treated as above :

Time (hours)	0·8	1·9	5·1	9·1	23
H_3PO_4, mols. liberated	0·059	0·097	0·244	0·40	0·66

(b) D-*Galactose*-3 *phosphoric acid.* The dipotasium salt (26 mg.), dissolved in water (25 c.c.), released phosphorus under the influence of the enzyme as shown :

Time (hours)	5·0	9·1	23
H_3PO_4, mols. liberated	0·046	0·094	0·21

(c) 2-*Deoxy*-D-*galactose*-6 *phosphoric acid.* The free phosphoric acid (20 mg.), dissolved in water (25 c.c.), was dephosphorylated as shown below :

Time (hours)	0·9	1·9	5·1	9·1	23
H_3PO_4, mols. liberated	0·038	0·064	0·132	0·185	0·32

(d) D-*Galactose*-6 *phosphoric acid.* The syrupy free phosphoric acid (20 mg.) was dephosphorylated as follows :

Time (hours)	0·9	1·9	5·2	9·2	23
H_3PO_4, mols. liberated	0·022	0·032	0·088	0·103	0·23

The veronal buffer described above was used in preference to the more usual phosphate buffer, because the concentration of phosphate ion would have been too great to permit accurate measurements of the small amounts of phosphate ion liberated. All the hydrolyses described in this paper were carried out in duplicate. Errors did not exceed 3%.

The authors thank the British Empire Cancer Campaign (Birmingham Branch) for financial assistance. One of them (A. B. F.) is indebted to the Brewing Industry Research Foundation for a personal grant.

THE CHEMISTRY DEPARTMENT,
THE UNIVERSITY, BIRMINGHAM, 15.

[*Received, November 29th,* 1950.]