659. *Glucose-2 Phosphate: Its Preparation and Characterisation by Hydrolysis Studies.*

By (Mrs.) Kathleen R. Farrar.

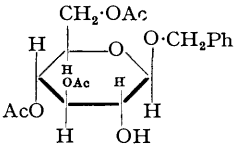
D-Glucose-2 phosphate and β-hexahydrobenzyl-D-glucoside-2 phosphate have been prepared in the form of potassium salts and their rates of acid and alkaline hydrolysis studied. The general problem of carbohydrate phosphoric-ester hydrolysis is discussed.

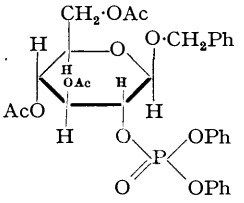
D-Glucose-2 phosphate has not previously been made by an unequivocal method, though Courtois and Ramet (*Bull. Soc. Chim. biol.*, 1945, **27**, 614) prepared 2-phosphogluconic acid, characterised by periodate oxidation, from the mixture of phosphorylated hexoses known as Hatano's ester (*Biochem. Z.*, 1925, **159**, 175), and later, Fleury, Courtois, and Desjobert (*Bull. Soc. chim.*, 1948, **15**, 694) described experiments using glucose-2 phosphate prepared from Hatano's ester, but no details of the fractionation are given and no rotations are recorded. Levene and Meyer (*J. Biol. Chem.*, 1921, **48**, 233) prepared an impure ester, probably mainly a 2-phosphate, by phosphorylation of 3 : 5 : 6-trimethyl α- and β-methylglucosides.

In the present investigations, phosphorylation of 3 : 4 : 6-triacetyl β-benzyl-D-glucoside (I) (Reynolds, *J.*, 1933, 323) with diphenyl chlorophosphonate (Brigl and Müller, *Ber.*, 1939, **72**, 2121) yielded the *diphenyl 2-phosphoric* ester (II). On catalytic hydrogenation (Adams's catalyst) some reducing material was formed, but the only crystalline product isolated was a non-reducing substance which analysed correctly for 3 : 4 : 6-*triacetyl* β-*hexahydrobenzyl*-D-*glucoside*-2 *phosphoric acid* (III). It was converted by potassium methoxide into *dipotassium* β-*hexahydrobenzyl*-D-*glucoside*-2 *phosphate* (IV). This behaviour of the benzyl group on catalytic hydrogenation is in accordance with the results of Richtmyer (*J. Amer. Chem. Soc.*, 1934, **56**, 1633). Attempts were made to remove the benzyl group by using a palladium-black catalyst, followed by Adams's catalyst for subsequent removal of the phenyl groups, but the product after deacetylation was always deficient in phosphorus.

The reaction of diphenyl chlorophosphonate with the recently available 1 : 3 : 4 : 6-tetra-acetyl β-D-glucose (V) (Hardegger and Pasenal, *Helv. Chim. Acta*, 1948, **31**, 281) gave, in good yield, *diphenyl* 1 : 3 : 4 : 6-*tetra-acetyl* β-D-*glucose*-2 *phosphate* (VI), which on hydrogenation with Adams's catalyst in methanol was converted into 1 : 3 : 4 : 6-*tetra-acetyl* β-D-*glucose*-2 *phosphate* (VII). Unlike Fischer and Lardy's 1 : 2 : 3 : 4-tetra-acetyl β-D-glucose-6 phosphate (*J. Biol. Chem.*, 1946, **164**, 513), this compound was very soluble in ethanol. Deacetylation with potassium methoxide in methanol gave, in almost theoretical yield, *dipotassium glucose*-2

phosphate (VIII) as a white powder, $[\alpha]_D^{17}$ +15° in water (c = 2) and $[\alpha]_D^{17}$ +24·4°, rising to +35° after 4 hours, in N/10-sulphuric acid.

(I.) 



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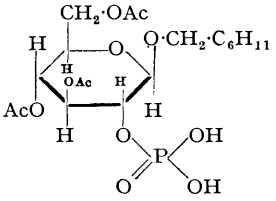
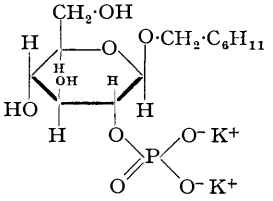
(III.) 



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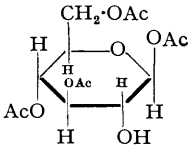
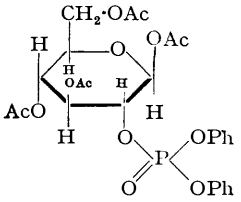
The unimolecular velocity constant for the hydrolysis of glucose-2 phosphate with N/10-sulphuric acid remained constant with time, indicating the purity of the material (cf.

(V.) 



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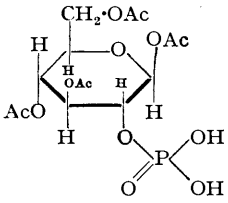
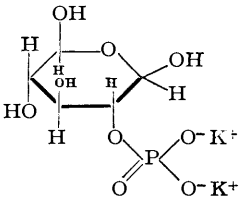
(VII.) 



(VIII.)

Fleury and Courtois, *Bull. Soc. chim.*, 1941, **8**, 75; 1942, **9**, 570). Table I shows the half hydrolysis periods of glucose-2 phosphate and other phosphoric esters under acid or alkaline conditions.

TABLE I.

Substance.	Conditions of hydrolysis.	Half hydrolysis period.	Ref.
Glucose-1 phosphate	0·25N-HCl at 37°	230 mins.	Cori, Colowick, and Cori, *J. Biol. Chem.*, 1937, **121**, 465.
Glucose-2 phosphate	0·1N-H_2SO_4 at 100°	137 mins.	This paper.
	0·1N-NaOH at 100°	97 mins.	This paper.
1 : 2-5 : 6-Di*iso*propylidene glucose-3 phosphate	0·1N-H_2SO_4 at 100°	525 mins.	Levene and Yamagawa, *J. Biol. Chem.*, 1920, **43**, 323.
1 : 2-*iso*Propylidene glucose-3 phosphate	N-$Ba(OH)_2$ at 100°	3 days	Percival and Percival, *J.*, 1945, 874.
Glucose-6 phosphate	0·1N-H_2SO_4 at 100°	38·5 hours	Robison, *Biochem. J.*, 1932, **26**, 291.
1 : 2-*iso*Propylidene glucose-6 phosphate	N-$Ba(OH)_2$ at 100°	*ca.* 3 days	Percival and Percival, *loc. cit.*
Mannose-6 phosphate	0·1N-H_2SO_4 at 100°	38·5 hours	Robison, *loc. cit.*
Fructose-1 phosphate	0·1N-HCl at 100°	33 mins.	Tanko and Robison, *Biochem. J.*, 1935, **29**, 961.
Fructose-6 phosphate	0·1N-HCl at 100°	275 mins.	Fleury, Courtois, and Desjobert, *loc. cit.*
2-Phosphogluconic acid	N-H_2SO_4 at 100°	16 hours	Courtois and Ramet, *loc. cit.*
	N-NaOH at 100°	9 hours	Courtois, *Ann. Ferment.*, 1943, **8**, 105.

When β-hexahydrobenzylglucoside-2 phosphate was hydrolysed by N/10-sulphuric acid at 100°, it was found that k, calculated for a unimolecular reaction, increased from $2{\cdot}9 \times 10^{-5}$ after 30 minutes to $6{\cdot}1 \times 10^{-5}$ after 540 minutes. It may be inferred from this that β-hexahydrobenzylglucoside-2 phosphate is much more slowly hydrolysed than the glucose-2 phosphate ($k = 8{\cdot}4 \times 10^{-5}$) to which it will give rise on hydrolysis of the glycosidic grouping. This is in accordance with the results set out in the table, from which it can be seen that carbohydrate phosphoric esters with a free glycosidic hydroxyl group adjacent to the phosphoric ester grouping are much more readily hydrolysed than esters without such a group.

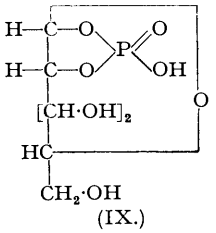
From the table it can be seen that glucose-2 phosphate is clearly distinguished by its hydrolysis rate from the other carbohydrate phosphoric esters.

It is not possible to say definitely whether the glucose-2 phosphate described in this paper is identical with the product from Hatano's ester, prepared by Fleury, Courtois, and Desjobert, and found by them to be 31·5% hydrolysed after 1 hour at 100° with 0·08N-hydrochloric acid (calculated from a graph), which seems to indicate that it may have contained a small proportion of a more labile ester, since the glucose-2 phosphate now described was only 27% hydrolysed after 1 hour's heating at 100° with N/10-sulphuric acid. However, the results indicate rates of acid hydrolysis of the same order.

Glucose-2 phosphate is far less acid-labile than Levene and Meyer's ester (*loc. cit.*), 3 : 5 : 6-trimethyl methylglucoside-2 phosphoric acid, which is probably at least 80% hydrolysed after 1 hour at 100° with N/10-sulphuric acid, though the exact amount of hydrolysis is difficult to estimate, since the substance was contaminated with approximately 20% of a more stable ester. This difference in stability is probably connected with the different ring forms present in the two compounds and has some implications in connection with the enhanced lability of phosphoric ester groupings adjacent to glycosidic hydroxyl groups.

It was shown by Fleury, Courtois, and Desjobert (*loc. cit.*) that the stability of such ester groupings is dependent on acid concentration, although this is not so in the case of glucose and mannose-6 phosphates (Robison, *Biochem. J.*, 1932, **26**, 291). Bailly (*Bull. Soc. chim.*, 1942, **9**, 314, 340, 421, 450) has shown that the α→β-glycerophosphate transformation is dependent on acid concentration, and it seems possible that the acid lability of glucose-2 phosphate and similar esters may be due to a transposition of the phosphate group to the labile 1-position, possibly with intermediate formation of a cyclic intermediate of the type postulated by Bailly, *e.g.*, (IX).

Both in direct transposition and in cyclic-intermediate formation, the product would be of a glycosidic type and would be most readily formed when the ring is in the furanose condition. Levene and Meyer's ester, on acid hydrolysis, will rapidly lose its glycosidic methyl group, but will remain in the furanose form, because of the methyl groups at position 3, 5, and 6. Acid hydrolysis, if occurring through 1-phosphate formation, might therefore be expected to be more rapid than in the case of unsubstituted glucose-2 phosphate.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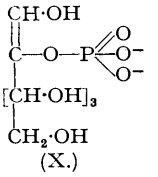
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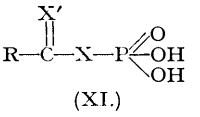
(XI.)

The lability to alkaline hydrolysis of glucose-2 phosphate shows some resemblance to the diose and triose phosphates investigated by Fleury and Courtois (*locc. cit.*). There is no tendency to form a proportion of a more stable ester, as in the case of diose phosphate.

The marked difference in stability to alkali shown by the stable esters in which carbon atom 1 is engaged in glycoside formation, or is oxidised to give an aldonic acid, on the one hand, and the readily hydrolysed glucose-2 and fructose-1 phosphates on the other, may be attributed to the possibility of the formation of ene-diols by the labile esters (Kusin, *Ber.*, 1936, **69**, 1041), *e.g.*, glucose-2 phosphate could form (X). It has been pointed out by Kalckar (*Chem. Reviews*, 1941, **28**, 71) that the labile phosphoric esters, carboxyl, enolic, guanidino- and pyro-phosphates all have the configuration (XI). The ene-diol (X) is a special case of (XI) in which X′ = CH·OH, X = O, and R = $CH_2(OH)\cdot[CH\cdot OH]_3$.

Since β-hexahydrobenzyl-D-glucoside-2 phosphoric acid and 2-phosphogluconic acid cannot

form an intermediate of type (XI), they would not be expected to show any marked lability to alkali.

Experimental.

Reaction of Diphenyl Chlorophosphonate with 3 : 4 : 6-*Triacetyl* β-*Benzyl-*D-*glucoside.*—Diphenyl chlorophosphonate (7·0 g.) and dry pyridine (12 c.c.; thrice distilled from phosphoric oxide) were mixed in a stoppered flask and cooled to 0°. 3 : 4 : 6-Triacetyl β-benzyl-D-glucoside (7·9 g.; dried at 100° for 2 hours *in vacuo*) was added, and the flask kept at room temperature for a week. The flask was then cooled to 0°, and kept cool while ice-cold water (1 c.c.) was added dropwise. After 10 minutes, the product was transferred to a separating funnel by addition of ether (250 c.c.) and water (30 c.c.). After separation of the water, the ethereal layer was washed successively with 0·5N-sulphuric acid (2 × 50 c.c.), water (50 c.c.), saturated sodium hydrogen carbonate solution (50 c.c.), and finally water (50 c.c.). During the washing, it was necessary to add a little ether at intervals to keep the product in solution. The ethereal solution was then dried ($MgSO_4$) and evaporated to 50 c.c., light petroleum (50 c.c.) added slowly, and the solution allowed to crystallise, giving white needles (12 g.) of the 2-*diphenylphosphoric* ester of 3 : 4 : 6-*triacetyl* β-*benzyl-*D-*glucoside* (II); m. p. (after recrystallising from benzene–light petroleum) 88·5°, $[\alpha]_D^{18}$ +6·4° ± 1° (c, 1·872 in chloroform; $l = 1$) (Found: C, 58·8; H, 4·9; P, 49. $C_{31}H_{33}O_{12}P$ requires C, 59·4; H, 5·25; P, 4·9%).

Catalytic Hydrogenation of the Foregoing Ester.—The ester (II) (1 g.) was dissolved in methanol (50 c.c.), and platinum oxide (0·1 g.) added. The mixture was shaken with hydrogen at room temperature and pressure. Maximum uptake (11·5 mols.) was reached after 6 hours. The catalyst was filtered off, and the solution evaporated under reduced pressure. Water (5 c.c.) was added, and the suspension of crystals filtered off and washed with a little water. 3 : 4 : 6-*Triacetyl* β-*hexahydrobenzyl-*D-*glucoside-2 phosphoric acid* (III) was obtained as small needles (0·55 g.), m. p. 202—203° after recrystallising from water; $[\alpha]_D^{18}$ −3° ± 1° in methanol (*c*, 1·37; $l = 1$) (Found: C, 47·2; H, 6·3; P, 6·3. $C_{19}H_{31}O_{12}P$ requires C, 47·3; H, 6·4; P, 6·4%).

Deacetylation of 3 : 4 : 6-*Triacetyl* β-*Hexahydrobenzyl-*D-*glucoside-2 Phosphoric Acid.*—The acid (III) (0·43 g.) was dissolved in dry methanol (5 c.c.) and 0·1N-methanolic potassium methoxide added to pH 10 (B.D.H. Universal indicator paper). After being kept overnight in the refrigerator, the solution was evaporated to 5 c.c., under reduced pressure, and allowed to crystallise. Rosettes of thick needles of *dipotassium* β-*hexahydrobenzyl-*D-*glucoside-2 phosphate* (IV) separated; these were washed with dry methanol and dried *in vacuo* (yield, 0·29 g.); $[\alpha]_D^{18}$ −46·5° ± 1° (*c*, 1·33 in water; $l = 1$) (Found: C, 36·2; H, 5·3; P, 6·9. $C_{13}H_{23}O_9PK_2$ requires C, 36·1; H, 5·3; P, 7·1%).

Reaction of 1 : 3 : 4 : 6-*Tetra-acetyl* β-D-*Glucose with Diphenyl Chlorophosphonate.*—The tetra-acetyl compound (V) (5·05 g.) was dissolved in pyridine (10 c.c.; dried as before), and diphenyl chlorophosphonate (5 g.) added. After 3 days at room temperature, the semisolid mass was cooled in ice, and stirred with water (2 c.c.). The product was dissolved by addition of benzene (80 c.c.) and water (20 c.c.), and the aqueous layer separated. The benzene layer was washed with successive (25 c.c.) portions of N-sulphuric acid (twice), saturated sodium hydrogen carbonate solution (twice), and water. During the washing, it was necessary to add more benzene (25 c.c.) to prevent crystallisation of the product. The benzene solution was dried (Na_2SO_4) and evaporated to a small volume under reduced pressure. Methanol (100 c.c.) was then added, and the product allowed to crystallise. *Diphenyl* 1 : 3 : 4 : 6-*tetra-acetyl* β-D-*glucose-2 phosphate* (VI) was obtained in long white needles (6·4 g.); on evaporation of the mother-liquors, a further 1·6 g. was obtained; m. p. 126—127° after recrystallisation from methanol; $[\alpha]_D^{18}$ +26·3° ± 1° (*c*, 1·899 in chloroform; $l = 1$) (Found: C, 53·3; H, 5·1; P, 5·3. $C_{26}H_{29}O_{13}P$ requires C, 53·8; H, 5·0; P, 5·3%).

Hydrogenation of foregoing (VI). The ester (4 g.) and platinum oxide (0·2 g.) were suspended in methanol (50 c.c.) and shaken with hydrogen at room temperature and pressure. 8 Mols. of hydrogen were taken up during 2 hours. The catalyst was filtered off, and the solution evaporated to dryness. The residue was dissolved in ethyl acetate (20 c.c.) and dried ($MgSO_4$), and the solution filtered; light petroleum (15 c.c.; b. p. 40—60°) was added gradually with warming. Crystallisation soon began, and appeared to proceed best if the flask was warmed on the water-bath from time to time, with occasional scratching. 1 : 3 : 4 : 6-*Tetra-acetyl* β-D-*glucose-2 phosphoric acid* (VII) was obtained; yield, 2·3 g.; m. p. 157—158° after recrystallisation from ethyl acetate by slow addition of light petroleum (b. p. 40—60°), and 160° after two recrystallisations; $[\alpha]_D^{18}$ +15·8° ± 1° (*c*, 2·03 in water; $l = 1$) (Found: C, 38·9; H, 5·0; P, 7·3. $C_{14}H_{21}O_{13}P$ requires C, 39·2; H, 4·9; P, 7·2%).

By use of absolute ethanol instead of methanol in the above experiment better yields were obtained. In one experiment 1·3 g. of (VII) were obtained from 2 g. of the diphenyl ester. It is essential in either case to evaporate off all the alcohol before adding ethyl acetate, as crystallisation is retarded by the presence of ethanol and methanol, and especially by the latter. The product is pure enough for deacetylation without recrystallisation.

Deacetylation of 1 : 3 : 4 : 6-*Tetra-acetyl* β-D-*Glucose-2 Phosphoric Acid.*—The acid (VII) (2·4 g.) was dissolved in methanol (20 c.c.), and the solution filtered, the filter being washed with methanol (5 c.c.) and the filtrate and washings combined. To this solution was added a filtered 0·2N-solution of potassium methoxide in methanol. About 20 c.c. of this solution were required for neutralisation, and 0·1 c.c. excess was then added. Crystallisation began within a few seconds of adding the excess. *Dipotassium glucose-2 phosphate* (VIII) which crystallised was filtered off after 3 hours in the refrigerator; yield, 1·75 g.; $[\alpha]_D^{17}$ +15° ±1° (*c*, 2 in water; $l = 1$), $[\alpha]_D^{17}$ +24·4° ±1° (initial), and $[\alpha]_D^{17}$ +35° ±1° (final) in 0·1N-sulphuric acid (*c*, 1·97; $l = 1$) (Found: C, 21·5; H, 3·2; P, 9·2. $C_6H_{11}O_9PK_2$ requires C, 21·4; H, 3·3; P, 9·2%). The compound reduced Fehling's solution, though decidedly more slowly than does glucose itself.

Hydrolysis of Dipotassium Glucose-2 Phosphate by 0·1N-*Sulphuric Acid.*—(1) A solution containing 0·197 g. of (VIII) in 10 c.c. of 0·1N-sulphuric acid was heated in a sealed tube at 100° for 12 hours. After 12 hours, the solution had rotation +0·59° (Calc. for theoretical liberation of glucose: +0·58°).

(2) A solution of the phosphate (Solution A) containing 0·0885 mg. of phosphorus per c.c. was used. Solution A (2 c.c.) and 0·2N-sulphuric acid (2 c.c.) were sealed in Pyrex tubes, and heated in a boiling water-bath for the stated times. Determinations of free and total phosphorus were made, the Bell–Doisy–Briggs method (*J. Biol. Chem.*, 1920, **44**, 55; 1922, **53**, 13) and a Spekker photometer being used. The results are in Table II, where $k = (2{\cdot}3/t)\log_{10}a/(a - x)$, t being in seconds.

TABLE II.

Time of hydrolysis, hrs.	0·5	1·0	1·5	2	3	4	5	6	7	8
Free phosphorus, %	14·1	26·9	37·4	48·3	61·0	69·0	78·0	84·8	87·8	90·0
	14·6	27·0	38·0	49·3	60·8	69·4	78·1	84·8	88·0	90·0
10^5k	8·5	8·6	8·7	8·8	8·2	8·0	8·4	8·5	8·4	8·1

Mean value of $k = 8{\cdot}4 \times 10^{-5}$. Half-hydrolysis period = 137 mins.

Hydrolysis of Glucose-2 Phosphate by 0·1N-Sodium Hydroxide.—Solution A (2 c.c.) and 0·2N-sodium hydroxide (2 c.c.) were sealed in Pyrex tubes, and heated in a boiling water-bath for the stated times. Determinations of free and total phosphorus were made by means of a Spekker photometer and a modification of the method of Briggs *et al.* (*loc. cit.*) to be more fully described later. Briefly, the modification made use of tartaric acid to overcome the effect of silicate dissolved from the glass by the sodium hydroxide.

Time of hydrolysis, hrs.	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0
Free phosphorus, %	17·8	29·6	41·8	56·4	63·0	66·0	76·5	84·5
10^5k	11	10	10	10	10	10	11	12

Mean value of $k = 11 \times 10^{-5}$. Half-hydrolysis period = 97 mins.

Hydrolysis of β-Hexahydrobenzyl-D-glucoside-2 Phosphate (Potassium Salt).—(i) *By acid.* A solution of the glucoside-2 phosphate (potassium salt) containing 0·0755 mg. of phosphorus per c.c. (solution B) was made. For the hydrolysis, solution B (2 c.c.) and 0·2N-sulphuric acid (2 c.c.) were sealed in Pyrex tubes and heated in a boiling water-bath for the stated times. Phosphorus was determined as previously described.

Time of hydrolysis, hrs.	0·5	1·0	1·5	2·0	3·0	4·0	5·0	7·0	8·0
Free phosphorus, %	5·1	12·0	19·1	26·8	41·5	53·4	63·8	78·5	82·7
10^5k	2·9	3·6	4·0	4·3	5·0	5·3	5·6	6·1	6·1

(ii) *By alkali.* Solution B (2 c.c.) and 0·2N-sodium hydroxide (2 c.c.) were sealed in Pyrex tubes and heated in a boiling water-bath When the solution was examined for free phosphorus by the method described under the alkaline hydrolysis of glucose-2 phosphate, no free phosphorus could be detected within the limits of experimental error, even after 7 hours' hydrolysis.

Note on Preparation of 1-Chloro 3 : 4 : 6-Triacetyl 2-Trichloroacetyl D-Glucose.—This, the starting material for the above syntheses, was made by Brigl's method (*Z. physiol. Chem.*, 1921, **116**, 1) from penta-acetyl β-glucose and phosphorus pentachloride. His yields could only be reproduced by using phosphorus pentachloride made from the trichloride and chlorine (*Inorg. Synth.*, Vol. I): commercial samples of pentachloride gave reduced yields or none, probably owing to their ferric chloride content.

Microanalyses are by Mr. E. Morton. The author thanks Professor E. L. Hirst, F.R.S., for encouragement and advice, and Mr. R. Chalmers for assistance.

THE UNIVERSITY, MANCHESTER, 13. [*Received, August 6th*, 1949.]