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On the Synthesis of the Phosphoric Acid Esters,¹ II. Synthesis of Some Glucose Monophosphoric Acid Esters and their Behavior toward Yeast.

By

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While the authors were engaged in experimentation upon the present problem, R. Robison's article² on the hexose monophosphoric acid ester appeared. According to his statement, a hexose monophosphoric acid ester was produced with hexose diphosphoric acid ester in the course of alcoholic fermentation of hexose by yeast juice or zymin in presence of a phosphate, and it was also fermented by yeast juice or zymin, the rate of fermentation being equal approximately with that of a mixture of phosphate and glucose.

C. Neuberg and his collaborators have synthesised hexose monophosphoric acid esters by the interaction of phosphorus oxychloride and some hexoses³ or sucrose⁴ in presence of calcium carbonate, and also by the partial hydrolysis⁵ of hexose diphosphoric acid ester. They claimed the calcium salts of galactose and fructose phosphoric esters were fermented well by living yeast and the salt of the ester obtained by

¹ Previous article, S. Komatsu and S. Kumamoto: On Phosphoric Acid Hexahydrophenol ester, *These Memoirs*, **6**, 45 (1922).

² *Biochem. J.*, **16**, 809 (1922).

³ *Ber. D. Chem. Ges.*, **43**, 2060 (1910); *Biochem. Zs.*, **26**, 514 (1910).

⁴ *Biochem. Zs.*, **56**, 5 (1911).

⁵ *Ibid.*, **88**, 432 (1918).

the second method was fermented slowly by the same yeast, but that of glucose phosphoric ester was not fermented entirely.

These phosphoric acid esters, differing from one another in their chemical properties and especially in their behavior toward living yeast, seem to be isomeric substances; the isomerism should be attributed to the position in which the phosphoric acid radical is attached to the sugar molecule and also the molecular structure of the sugars. The questions, however, of the isomerism and of the fate and rôle of these esters in the course of alcoholic fermentation have left unsettled.

The authors, therefore, have undertaken the present work to settle the above questions by studying the behavior of the synthesised hexose monophosphoric acid esters of known constitution and to contribute to the progress of fermentation theory.

1. Glucose-1-Phosphoric Acid Ester, (α or β).

This substance was obtained from tetracetyl glucose-1-phosphoric acid ester by partial hydrolysis with baryta water, which was prepared by the action of phosphorus oxychloride on pentacetyl glucose in the presence of barium hydroxide.

8 gm. pentacetyl glucose (M.P. 131°), prepared by the method of Königs and Knorr¹, were suspended in 200 c.c. of water and cooled to -5°, and then 50 gm. of barium hydroxide were added. To the mixture, 5 gm. phosphorus oxychloride dissolved in 50 c.c. dry ether, were added from a dropping funnel under vigorous stirring. The stirring was continued for 3 hours after all of the phosphorus oxychloride solution had been added, and the reaction product was made up to 800 c.c. with water, and 32 gm. of barium hydroxide were added. The mixture was shaken for 24 hours at room temperature, filtered and the residue was washed several times with conc. baryta water until no more chlorine ion was present in the washing. The insoluble residue was then extracted with 400 c.c. of water, and the

¹ Ber. D. Chem. Ges., **34**, 957 (1901).

extraction was repeated five times. The extracts combined together, carbon dioxide was passed, and the carbonate was filtered. The filtrate was concentrated to a small volume under diminished pressure, and there appeared barium carbonate which was filtered off. The filtrate was poured into a large volume of absolute alcohol, where the barium salt of the ester was precipitated. The salt thus obtained (amounting to 2 gm.), was purified by precipitation with absolute alcohol from its aqueous solution.

The purified salt, dried at 78° in vacuo on phosphorus pentoxide, was analysed: it gave C=17.70; H=2.97; P=7.64; Ba=34.70; Ash=56.53; (C₆H₁₁O₆PO₃Ba requires C=18.21; H=2.81; P=7.84; Ba=34.73; Ash=56.74 as Ba₂P₂O₇). The specific rotatory power in the aqueous solution was:

$$[\alpha]_{\text{D}}^{25} = + \frac{0.68 \times 100}{4.551 \times 1} = +14.9^{\circ}$$

It was a white powder and reduced Fehling's solution only when it was subjected to prolonged boiling with the solution or to hydrolysis with dilute acid. It yielded an osazone when the salt was heated on a water bath with phenyl hydrazine in presence of acetic acid, which melted at 203–204°. The barium salt was precipitated by alkali from its aqueous solution, but not by lead acetate. The ester, on hydrolysing with acid, yielded a substance of strong dextrotatory power.

Considering the method of formation of the ester and its properties above mentioned, it will be decided that the phosphoric acid radical of the ester is attached to the terminal aldehydic carbon atom of the glucose molecule.

2. Glucose-3-Phosphoric Acid Ester.

This substance was prepared from 1-2-5-6-diacetone glucose-3-phosphoric acid by partial hydrolysis, which was obtained by the action of phosphorus oxychloride on diacetone glucose¹ (M.P. 109–110°, $[\alpha]_{\text{D}}^{25}$

¹ K. Freudenberg and O. Ivers: Ber. D. Chem. Ges., 55, 929 (1922).

$= -18.7^\circ$) in almost the same manner as P. A. Levene and G. M. Meyer¹.

5 gm. of the thoroughly dried diacetone glucose were reacted with 2.7 gm. phosphorus oxychloride in presence of dry pyridine, at a low temperature such as -35° by cooling with a mixture of solid carbon dioxide and ether. The reaction product was alkalinized with barium hydroxide, and pyridine was removed by distillation under reduced pressure. To remove chlorine ion, it was kneaded with silver sulphate in a mortar, and the excess of silver was removed by sulphuretted hydrogen. An excess of barium hydroxyde was added, carbon dioxide passed into the solution and filtered. The filtrate was concentrated under diminished pressure to a small volume, and then poured into a large volume of absolute alcohol. Leaving the solution standing over night, a translucent gelatinous precipitate was formed which was filtered and dried in a desiccator on calcium chloride. The filtrate was concentrated under reduced pressure and then allowed to evaporate to dryness in a desiccator. Both substances were almost insoluble in alcohol and acetone, and were one and the same differing only in content of diacetone glucose as an admixture, and the latter substance, therefore, was removed by extraction with ether. The yield of the pure substance free from diacetone glucose was about 6 gm. which was dried in vacuo on phosphorus pentoxide at 100° and was analysed: it gave C=29.98; H=4.40; P=6.64; Ba= 27.24; Ash=46.60; ($C_{12}H_{19}O_6PO_3Ba$ requires C=30.29; H=4.00; P=6.53; Ba=28.89; Ash=47.18 as $Ba_2P_2O_7$);

The specific rotatory power in water solution:

$$[\alpha]_D^{18^\circ} = + \frac{0.63 \times 100}{9.134 \times 1} = +6.80^\circ$$

which was quite different from that ($[\alpha]_D^{20^\circ} = -2.48^\circ$) of the barium salt of 1-2-5-6-diacetone glucose, phosphoric acid mentioned by Drs. P. A. Levene and G.M. Meyer². The analytical data agree with that for the

¹ J. Biol. Chem., **43**, 236 (1921).

² Loc. cit.; J. Biol. Chem., **53**, 43 (1922).

barium salt of diacetone glucose phosphoric acid. It did not reduce Fehling's solution unless it was previously hydrolysed with acid. It is strange that the specific rotatory power and the solubilities in alcohol and acetone coincide well with those of Levene and Meyer's barium salt of 1-2-monoacetone glucose-3-phosphoric acid.

To remove the acetone radical from the ester, 2 gm. of the barium salt of 1-2-5-6-diacetone glucose-3-phosphoric acid dissolved in 100 cc. of $\frac{N}{2.5}$ sulphuric acid and kept at 40° for 24 hours. The reaction product was neutralised with barium carbonate and made alkaline slightly by barium hydroxide and filtered, and the filtrate was concentrated to one half of the original volume under diminished pressure and filtered again. The filtrate concentrated under reduced pressure to a small volume, poured into absolute alcohol, making the concentration of alcohol to 75 per cent. Whereas the barium salt was precipitated, filtered, washed with 80 per cent alcohol, and dried on calcium chloride in a vacuum desiccator. The yield was 1.1 gm.

The sample dried at 78° in vacuo on phosphorus pentoxide, was analyzed: it gave C=18.22; H=3.27; P=7.25, 7.38; Ba=33.45, 31.17; Ash=56.19; ($C_6H_{11}O_6PO_3Ba$ requires C=18.21; H=2.81; P=7.84; Ba=34.73; Ash=56.74 as $Ba_2P_2O_7$);. The specific rotatory power in water solution, after 20 minutes:

$$[\alpha]_D = + \frac{0.95 \times 100}{3.241 \times 1} = +29.2^\circ$$

which diminished slightly after 24 hours. The aqueous solution reduces Fehling's solution easily.

The ester was designated glucose-3-phosphoric acid ester, since we admitted no transposition of the phosphoric acid group had occurred during the above treatment.

3. Glucose-6-Phosphoric Acid Ester.

It was prepared analogously with the preparation of glucose-6-

sulphuric acid described by H. Ohle¹ and T. Soda².

16 gm. of the dried powdered glucose were suspended in 80 c.c. of dry pyridine. The mixture, under cooling to -5° and vigorous stirring, was reacted with 12 gm. phosphorus oxychloride in 30 c.c. of chloroform, keeping down the reaction temperature so that it should not rise above 0° . The reaction product was allowed to remain at -5° for 3 hours after all of the phosphorus oxychloride had been added and then ice water was added. It was then brought to room temperature and stirred over night. The solution was subjected to distillation under diminished pressure to remove pyridine, and diluted with water and alkalinized distinctly with baryta water, and then filtered. The excess of barium was removed by means of carbon dioxide, and the filtrate from barium carbonate was concentrated under reduced pressure to a syrup. To remove hydrochloric acid from the syrup, it was diluted with water and treated with silver sulphate, and then with sulphuretted hydrogen. When the latter had been removed by a current of air, the solution was filtered and a small excess of barium hydroxide was added and carbon dioxide was passed. The solution free from barium carbonate, was concentrated under diminished pressure and poured into absolute alcohol, the concentration of alcohol was kept in the neighbourhood of 75 per cent. The barium salt precipitated was separated from the solution by centrifuging, washed with 75 per cent alcohol, and again centrifuged. The barium salt, thus obtained, weighed 3.5 gm.

For the purification, it was dissolved in a small volume of water, alkalinized with baryta water, filtered and precipitated with absolute alcohol and then washed with 80 per cent alcohol. The precipitate was dissolved again in water and filtered and then neutralized with carbon dioxide. The precipitate dried to constant weight under reduced pressure on phosphorus pentoxide at 78° , and analyzed: it gave C=18.61; H=3.28; P=8.42; Ba=33.81; ($C_6H_{11}O_6PO_3$ Ba requires C=

¹ *Biochem. Zs.*, **131**, 601 (1922).

² *Ibid.*, **135**, 621 (1923).

18.21 ; H=2.81 ; P=7.84 ; Ba=34.73 as Ba₂P₂O₇ ;). The specific rotatory power in water solution :

$$[\alpha]_D = + \frac{0.66 \times 100}{3.460 \times 1} = +19.1^\circ ; \text{whence } [\alpha]_D = +29.0^\circ \text{ for the free ester.}$$

The rotatory power diminished a little after 24 hours. The salt is a white powder and reduces Fehling's solution readily.

The allocation of phosphoric acid radical in the glucose molecule was assigned to be the 6th carbon atom from analogy with the sulphuric acid ester¹ and also from the chemical properties of the ester.

4. Neuberg's Glucose Phosphoric Acid Ester².

The calcium salt of this ester was prepared by the action of phosphorus oxychloride on glucose in the presence of calcium carbonate. The salt dried to constant weight in vacuo on sulphuric acid, was analyzed : it gave C=21.82 ; H=4.23 ; P=9.69 ; (C₆H₁₁O₉P Ca+2H₂O requires C=21.55 ; H=4.49 ; P=9.28 ;). The specific rotatory power in aqueous solution :

$$[\alpha]_D^{15^\circ} = + \frac{0.68 \times 100}{2.316 \times 1} = +29.3^\circ (\text{after 20 minutes})$$

$$[\alpha]_D^{15^\circ} = + \frac{0.58 \times 100}{2.316 \times 1} = +25.0^\circ (\text{constant}) \text{ and that calculated as free esters } [\alpha]_D = 30.5^\circ$$

Accordingly the Neuberg's ester seems to be the same substance as the 6-phosphoric acid ester.

5. Behavior of the Esters toward Yeast and Zymin.

In the experiment, the aqueous solution of the sodium salts of the esters was used which prepared by neutralizing the free esters with sodium hydroxide using phenolphthalein as indicator. The free esters were formed by the interaction of barium or calcium salts of the esters and calculated quantities of sulphuric acid and centrifuging off

¹ Loc. cit.

² Loc. cit.

the sulphate. The velocity of fermentation was determined by measuring the volume of carbon dioxide generated, using Einhorn's fermentation saccharometer. The yeast employed, was a fresh pressed bottom yeast from the Suita brewery (Dai Nippon Bakushu Kabushiki-kaisha, Suita, near Osaka).

Exp. I.

(a) 10 c.c. 2 per cent (calc. as free ester) solution of the 1-ester+1 gm. pressed yeast.

(b), (c) and (d) 3-, 6- and Neuberg's esters respectively with pressed yeast.

(e) 10 c.c. 2 per cent glucose solution+1 gm. pressed yeast. These were incubated at 26° and the quantity of CO₂ measured in c.c.

Time (in hr.)	(a)	(b)	(c)	(d)	(e)
2	0.3	0	0.3	0	6.0
5	"	"	"	0.1	—
6	"	"	"	"	—

None of the esters, as seen in the experimental results, was fermented by the yeast.

Exp. II.

The action of Schroder's zymin on the esters was studied, and the zymin was prepared according to the directions of A. Harden.¹

(a) 10 c.c. 2 per cent (calc. as free ester) solution of the 1-ester+1 gm. zymin+0.1 c.c. toluene.

In (b), (c) and (d) the 1-ester in (a) was replaced by 3-, 6- and Neuberg's esters respectively.

(e) 10 c.c. of the 1.38 per cent glucose solution (equivalent to the esters) +1 gm. zymin+0.1 c.c. toluene+0.05 gm. of Na₂HPO₄·12H₂O :

These were incubated at 25° and the quantity of CO₂ was measured in c.c.

Time (in hr.)	(a)	(b)	(c)	(d)	(e)
1.0	0.4	0.3	0.1	0	2.0
1.5	0.8	1.4	0.6	0.1	3.0
2.0	1.5	2.4	1.2	0.2	3.5
2.5	2.4	3.3	1.8	0.25	4.2
3.0	3.5	4.1	2.2	0.4	4.8

All of the esters were fermented by zymin, and especially 1- and 3-esters

¹ A. Harden: *Alcoholic Fermentation* 1923, p28.

worked more rapidly.

Exp. III.

(a) 15 c.c. 5 per cent glucose solution+3 c.c. 2 per cent solution of the 1-ester (calc. as free ester)+1 gm. zymin+0.1 c.c. toluene. In (b), (c) and (d) the 1-ester in (a) was replaced by 3-, 6- and Neuberg's esters respectively.

(e) 15 c.c. 5 per cent glucose solution+3 c.c. Na_2HPO_4 solution (0.274 gm. Na_2HPO_4 12 H_2O in 10 c.c., equivalent to the esters)+1 gm. zymin+0.1 c.c. toluene.

(f) 15 c.c. 5 per cent glucose solution+3 c.c. H_2O +1 gm. zymin+0.1 c.c. toluene.

These were incubated at 24° and the quantity of CO_2 was measured in c.c.

Time (in hr.)	(a)	(b)	(c)	(d)	(e)	(f)
1.0	0.2	0.2	0.2	0.2	0.2	0.3
1.5	0.5	0.5	0.3	0.4	0.6	0.4
2.0	0.9	0.7	0.5	0.6	1.3	0.5
2.5	1.25	1.1	0.75	0.8	1.95	0.7
3.0	1.65	1.35	1.1	1.05	2.7	0.8
3.5	2.1	1.8	1.5	1.4	3.3	1.0

Among the esters, 1- and 3-esters exert some positive effects on the zymin action.

Exp. IV

(a) 15 c.c. 5 per cent glucose solution+4 c.c. of the 2 per cent solution of the 1-ester (calc. as free ester)+ a residue obtained from 2 gm. zymin by washing with water 3 times+0.1 c.c. toluene.

(b) and (c) the 1-ester in (a) was replaced by the 3- and 6-esters respectively.

(d) 15 c.c. 5 per cent glucose solution+4 c.c. H_2O + the washed zymin +0.1 c.c. toluene.

These were incubated at 24° and gas was measured in c.c.

Time	(a)	(b)	(c)	(d)
11.5 hrs.	0	0	0	0
4 days	0	1.5	0	0

Exp. V.

In (a), (b), (c) and (d) the mixtures of the corresponding cases in Exp.

IV had added 1 c.c. 10 per cent $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ solution respectively.

Time	(a)	(b)	(c)	(d)
12 hrs.	0	0	0	0
4 days	2.4	7.0	3.0	2.0

(In (d), after one day, some water extract of zymin was added.)

Iodoform reaction in the fermented liquids was positive.

The mixture of glucose and the 3-ester was fermented by the washed zymin, while that of glucose and other esters were fermented very slowly only when they were accompanied by sodium phosphate.

From these results the following conclusions are drawn :

1. None of the esters was fermented by living yeast while the 1- and 3- phosphoric acid esters, on the one hand, were fermented rapidly by zymin, the rate being approximately equal to that attained with a mixture of phosphate and glucose, and other hand, they accelerate the fermentative action of zymin on glucose. It was, therefore, concluded that the 1- and 3-esters will maintain some important relation to the alcoholic fermentation of sugars in presence of phosphate.
2. Moreover, the fact that the 3-ester 4 days after the experiment had started, began to accelerate the action of zymin, freed from the co-enzyme, on glucose with or without phosphate, which suggested that some resemblance in behavior existed between the ester and co-enzyme.
3. Now, it is highly probable that the hexose monophosphoric acid ester, taking the investigation of Robison into consideration, will play an important rôle in the alcoholic fermentation of sugars as the di-phosphoric acid ester does, which was fully revealed by the extensive investigation of A. Harden and W. J. Young¹. Admitting that Robison's statement was correct, the idea was naturally deduced that some of our esters will actually be formed in the course of the alcoholic fermentation of sugars in presence of phosphate by yeast juice.
4. Among them all, the 3-phosphoric acid ester from its behavior

¹ Proc Chem. Soc., **21**, 184 (1905); Proc. Roy. Soc. B, **80**, 299 (1908); *Ibid.*, **81**, 336 (1909); *Ibid.*, **82**, 321 (1910); *Biochem. Zs.*, **173**, 178 (1911); Proc. Roy. Soc. B, **81**, 525 (1909).

towards zymine or washed zymine, was regarded as the very ester which the authors had expected would be formed in the fermentative liquor. Final decision, however, was postponed until further work should be done, since none of our esters coincide, in specific rotatory power, with Robison's ester.

Nov. 1923. Laboratory of Organic- and Bio-Chemistry.