Organophosphorus Compounds. (II)*

A Novel Synthesis of Glucose-6-phosphate

Takeshi HASHIZUME**, Kenji FUJIMOTO***, Hideo UNUMA****
Kouichi TAKINAMI***** and Keiichi MORIMOTO******

(Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University)

Received January 6, 1960

The phosphorylation of 1, 2, 3, 4-tetraacetyl β-D-glucopyranose (V) in anhydrous dioxane was carried out using tetra-(*p*-nitrophenyl) pyrophosphate (III) as phosphorylating agent, obtaining bis-(*p*-nitrophenyl) 1, 2, 3 ,4-tetraacetyl β-D-glucose-6-phosphate (VI) in crystalline form. Mild acidic hydrolysis of the compound (VI) gave glucose-6-phosphate (VII) in good yield.

In 1914, Harden and Young found that the hexose-monophosphate is formed in the process of yeast juice fermentation. Later in 1931, Robison and King[1] were able to isolate glucose-6-phosphate in pure form. A number of chemical and enzymatic synthetic methods have so far been reported because of the biochemical significance and the investigations of the chemical structure. These chemical synthetic methods except enzymatic methods are summerized as follows :

(1) The phosphorylation of 1, 2-isopropylidene-glucose or 1, 2, 3, 4-tetraacetylglucose by (a) phosphorus oxychloride in pyridine[2], (b) bis-(benzyl) chlorophosphate[3], (c) bis-(phenyl) chlorophosphate[4][5].

(2) The phosphorylation of 5, 6-anhydro-1, 2-0-isopropylidene-D-glucofuranose in water with dipotassium or sodium hydrogen phosphate[6], and this method is recommended to introduce isotopic phosphorus, described above.

(3) Direct phosphorylation of free glucose by (a) tetraphosphoric acid in water[7], (b) meta-phosphoric acid in acetonitrile[8][9][10][11][12].

Among these procedures, the method (1) (c) has been used widely because of the purity and the yield of the preparation.

Recently, however, tetra-(*p*-nitrophenyl) pyrophosphate was successfully used for the synthesis of guanosine-5′-phosphate by Khorana and his co-wor-

---

* The part I of this article was published in the *Memoirs of the College of Agriculture, Kyoto University*, No. 81, p. 1-40 (March 1959), and a part of this article was presented at the Union Meeting of Agr. Chem. Soc. Japan, Kansai and Western Japan Section, May 1957, Kagoshima.

** 橋爪 健.

*** 藤本賢治 Present address: Sekisui Chemical Sponge Company, Ltd., Osaka.

**** 鵜沼英夫 Present address: Takeda Pharmaceutical Company, Ltd., Osaka.

***** 滝波弘一 From April 1960: Aji-no-moto Company, Ltd., Central Chemical Laboratory, Kawasaki.

****** 森本圭一 From April 1960: Kirin Beer Company, Ltd; The Research Laboratory, Yokohama.

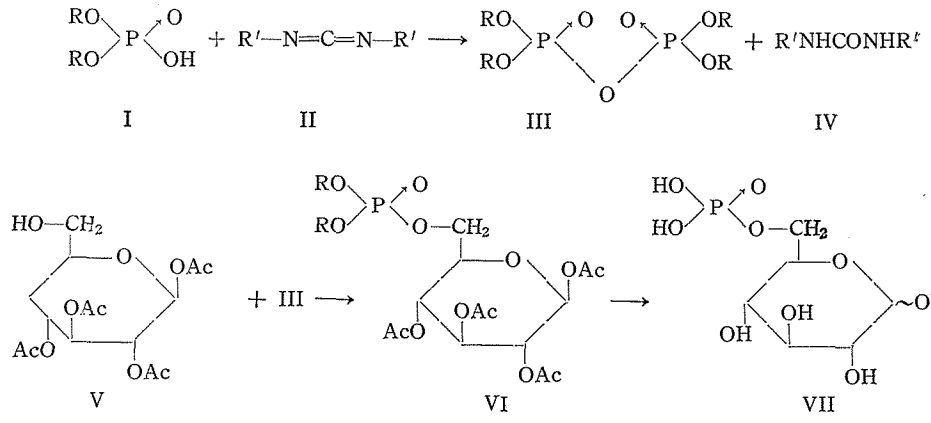
kers[13].

Then, the authors applied this reagent for the synthesis of glucose-6-phosphate. Preliminary phosphorylation of anhydrous ethanol, cyclohexylamine and isomeric menthols[14] were attempted in anhydrous *p*-dioxane obtaining ethyl bis-(*p*-nitrophenyl) phosphate, cyclohexylamino bis-(*p*-nitrophenyl) phosphate and bis-(*p*-nitrophenyl) menthyl phosphates in good yield. Subsequently, the phosphorylation of 1, 2, 3, 4-tetraacetyl β-D-glucose (V) was carried out in anhydrous *p*-dioxane at room temperature and proceeded without the formation of secondary reaction products. Bis-(*p*-nitrophenyl) 1, 2, 3, 4-tetraacetyl β-D-glucose-6-phosphate (VI) was isolated in good yield from the phosphorylated product. The *p*-nitrophenyl groups and the acetyl groups could be eliminated simultaneously on acid hydrolysis with 0.1 *N* hydrochloric acid producing glucose-6-phosphate (VII) in the yield of about 83%. The quantity of *p*-nitrophenol liberated was spectrophotometrically determined at 440 millimicrons. It was observed that the liberation of *p*-nitrophenol occured instantly and quantitatively. Although the mechanism will be discussed in the following paper, it should be noted that both of the *p*-nitrophenyl groups of the compound (VI) were liberated simultaneously in this acid hydrolysis.

In the case of the synthesis of guanosin-5′-phosphate, Khorana *et al* used the combination of hydrolysis by alkali and snake venom phosphodiesterase in order to remove both of the *p*-nitrophenyl groups. In case of the synthesis of glucose-6-phospate, however, acidic hydrolysis provides a simple and practical way for the removal of protective *p*-nitrophenyl groups.

The yield of crystalline barium glucose-6-phosphate was comparatively higher than those of the other methods. As the criterion of the purity of the preparation, the analysis of phosphorus[15][16] and determination of rotatory power were employed. Both the values were satisfactory as shown in the part of Experimental. Besides, the compound migrated chromatographically as a single component using several solvent systems shown in Experimental.

Table 1.



R : *p*-nitrophenyl group R′ : *p*-tolyl group

Table 2. Rf-Values of D-Glucose Derivatives (Ascending).

	i-PrOH-NH_3-H_2O (70 : 5 : 25 v/v)	80% EtOH cont'g 0.64% boric acid	EtOAc-Pyr-H_2O (2 : 1 : 1 v/v, top layer)
3, 5, 6-Trimethyl-D-glucose	0.77		
Glucose-6-phosphate	0.10	0.13	
Glucose-4, 6-cyclic phosphate	0.35	0.36	0.35
1, 6-Anhydro-D-glucose	0.64	0.67	0.66
3, 6-Anhydro-D-glucose	0.65		0.70
Glucose-6-dimethylphosphate	0.64	0.69	0.79
D-Glucose	0.44	0.51	0.44

Table 3. Rf-values of D-glucose monophosphates (Descending).

	n-BuOH-Pyr-H_2O (1 : 1 : 1 v/v)	80% EtOH cont'g 0.64% Boric acid
D-Glucose	0.51(5.4/10.5)	0.58(5.2/9.0)
D-Glucose-6-phosphate	0.24(2.6/10.5)	0.31(2.8/9.0)
D-Glucose-4, 6-cyclic phosphate	0.35(3.7/10.5)	0.43(3.9/9.1)

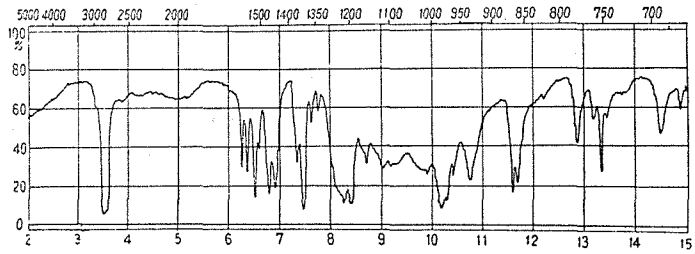


Fig. 1. IR-Spectrum of Bis-(*p*-nitrophenyl) phosphate (nujol mull).

EXPERIMENTAL

N, N-Bis-(*p*-tolyl) thiourea. A solution of 214 grams (2 moles) of *p*-toluidine and 91 grams (1.2 mole) of carbon disulfide in one liter of 95% ethanol was refluxed for six hours (During the reaction, copious amount of the crystals of N, N'-bis-(*p*-tolyl) thiourea separated generating hydrogen sulfide gas.). The reaction mixture was cooled overnight, the crystals were filtered, washed with a small quantity of ethanol, and dried. Recrystallized from ethanol, yield: 196 grams (66%), m.p. 180-181° (Reported 180-181°[17]). Further crops were obtained from the mother liquor. For the practical use no recrystallization seems to be required.

Sodium *p*-nitrophenolate. One hundred grams of *p*-nitrophenol was dissolved in 500 ml. of ether and aqueous sodium hydroxide solution (twenty nine grams of sodium hydroxide in fifty ml. of water) was slowly added. The yellow colored crysals separated were filtered, washed with ether and ethanol, and dried over anhydrous phosphoric acid *in vacuo* at 100°. Yield : 140 grams

(98.7%).

Tris-(*p*-nitrophenyl) phosphate. The compound was synthesized in slightly changed manner in the molar ratio that Khorana described. Fifty six grams (0.348 mole) of anhydrous sodium *p*-nitrophenolate was placed in 100 ml. of anhydrous ether containing 15.6 grams of freshly distilled phosphorus oxychloride was added with shaking at room temperature. The reaction mixture was refluxed for two hours. The products were filtered and washed thoroughly with water till the washings were colorless. The colorless crystals were dried over anhydrous phosphoric acid *in vacuo*. Recrystallized from freshly distilled glacial acetic acid, yield: 41 grams (87.3%), m.p. 156–158°.

Anal. Calcd for $C_{18}H_{12}N_3O_{10}P$: C, 46.85; H, 2.62; N, 9.12; P, 6.73;
Found : C, 46.71; H, 2.67; N, 8.87; P, 6.61;

Bis-(*p*-nitrophenyl) phosphate. Towenty grams of tris-(*p*-nitrophenyl) phosphate was dissolved in 200 ml. of warm *p*-dioxane and cooled to room temperature under running water. To the solution were added 25 ml. of 4 *N* lithium hydroxide and 75 ml. of distilled water, and shaken for two hours. A hundred ml. of water was added and the pH was adjusted *ca.* 7 by the addition of Amberlite IR-120 (H^+-form). The resin was removed by the filtration and the filtrate was eveporated to viscous residue using rotatory film evaporator. The residue was dissolved in 150 ml. of water and the pH was adjusted to 4 by the further addition of the resin, and then filtered. Combined filtrate and washings were extracted with ether till the ether extracts were colorless with alkali. The aqueous layer was warmed to remove ether and slowly acidified with about 40 ml. of concentrated hydrochloric acid, obtaining the crystals melted at 167–172°. Yield: 13.0 grams. Recrystallized twice from anhydrous ethylacetate, m.p. 176–177°.

Anal. Calcd for $C_{12}H_9N_2O_8P$: C, 42.40; H, 2.67; N, 8.28; P, 9.10;
Found : C, 42.51; H, 2.80; N, 8.10; P, 8.89;

The IR-spectrum (nujol mull) of this compound is shown in Figure 1.

6-Trityl 1, 2, 3, 4-tetraacetyl β-D-glucose. The compound was synthesized in the method described before[18] and the preparation of the m.p. 166–167° was used.

1, 2, 3, 4-Tetraacetyl β-D-glucose. The compound was synthesized in the method described before[18], and the preparation of the m.p. 126.5–127° was used.

Ethyl bis-(*p*-nitrophenyl) phosphate : 3.40 grams (0.01 mole) of bis-(*p*-nitrophenyl) phosphate was dissolved in 25 ml. of warm anhydrous *p*-dioxane. The solution was cooled under running water, and 1.11 gram (0.005 mole) of freshly prepared bis-(*p*-tolyl) carbodiimide* was added. Bis-(*p*-tolyl) urea separated at once, and was removed by filtration. To the filtrate was 0.33 ml. (0.005 mole) of anhydrous ethanol added. The reaction mixture was mechanically shaken for forty hours at room temperature. Further precipitate occur-

* Fresh preparation gave better yield.

ed during shaking was filtered off. The filtrate was evaporated to crystalline residue which was extracted with a small amount of chloroform thrice, washed with saturated sodium bicarbonate and water, dried with magnesium sulfate. Chloroform was removed under reduced pressure at 40° resulting nice crystalline residue. Recrystallized from ethanol, m.p. 137-138.2°. No depression was observed on mixing with the authentic specimen of ethyl bis-(*p*-nitrophenyl) phosphate. Yield: 1.5 gram (81.5%).

Anal. Calcd for $C_{14}H_{13}N_2O_8P$: C, 45.65; H, 3.54; N, 7.60; P, 8.42;
Found : C, 45.71; H, 3.59; N, 7.55; P, 8.55;

Bis-(*p*-nitrophenyl) 1,2,3,4-tetraacetyl-β-D-glucopyranose-6-phosphate: 13.60 grams (0.04 mole) of bis-(*p*-nitrophenyl) phosphate was dissolved in 100 ml. of warm anhydrous *p*-dioxane. The solution was cooled rapidly to room temperature under running water. 4.44 grams (0.02 mole) of bis-(*p*-tolyl) carbodiimide was added and shaken after a few minutes. 6.67 grams (0.0192 mole) of 1, 2, 3, 4-tetraacetyl β-D-glucopyranose was added and shaken mechanically for twenty hours at room temperature. Diluted with a small amount of chloroform and precipitated bis-(*p*-tolyl) carbourea was separated by filtration. The filtrate was washed with 5% sodium bicarbonate, (since further precipitation of bis-(*p*-tolyl) carbourea occured, this was separated by filtration) water, and then dried with anhydrous magnesium sulfate. Chloroform was removed by evaporation under reduced pressure at forty degree. Semicrystalline residue was dissolved in methanol, water was added till turbid, seeded, scratched and then stayed in the cold, obtaining colorless* crystals. m.p. 138-141°. Yield: 23.8 grams (89%). Recrystallized from methanol, m.p. 144-145°.

Anal. Calcd for $C_{26}H_{27}O_{17}N_2P$: C, 46.6; H, 4.03; N, 4.18; P, 4.62;
Found : C, 46.81; H, 4.31; N, 4.15; P, 4.71;

$[\alpha]_D^{24} = +20.9$ (at 2% anhydrous pyridine solution)

Barium D-glucose-6-phosphate. 6.7 grams (10 millimoles) of bis-(*p*-nitrophenyl) 1, 2, 3, 4-tetraacetyl-β-D-glucopyranose-6-phosphate was dissolved in 70 ml. of anhydrous *p*-dioxane and 70 ml. of 0.2 *N* hydrochloric acid was added and heated in the boiling water bath for three hours. The hydrolysate was evaporated using the rotatory film evaporator *in vacuo* to viscous syrup in which crystalline *p*-nitrophenol was observed. A small amount of water (*ca.* 30 ml.) was added and extracted thrice with ether. Aqueous layer was neutralized with solid pulverized barium hydroxide to the pH 7, and insoluble solid materials were filtered off. The filtrate was seeded with the authentic specimen of barium glucose-6-phosphate, scratched and then kept in the cold. The crystalline precipitate of barium glucose-6-phosphate was filtered, washed in turn with 95% ethanol, ether, and dried over anhydrous phosphoric acid *in vacuo* at 100°**. Yield: 3.3 grams (83.3%, as $C_6H_{11}O_9PBa$).

Anal. Calcd for $C_6H_{11}O_9PBa$: P, 5.96; Found P, 6.08.

$[\alpha]_D^{15} = +17.5$ at 1% aqueous solution)

* Impure compound shows slightly pale yellow color.

** Barium glucose-6-phosphate exists as the heptahydrate in normal condition.

Paper chromatography of glucose-6-phosphate Paper chromatography was used extensively for analyzing the reaction products and for identification purposes. The solvent system used were :

1. isopropyl alcohol-ammonia-water (70 : 5 : 25 v/v)
2. 80% aqueous ethanol containing 0.64% boric acid
3. *n*-butyl alcohol-pyridine-water (1 : 1 : 1 v/v)
4. ethyl acetate-pyridine-water (2 : 1 : 1 v/v, top layer)

The chromatograms were run in the ascending and descending manners at room temperature and for the detection of the spots were the following coloring reagents employed.

1. 1% aniline oxalate.
2. Periodate benzidine reagent.
3. Wade Morgan reagent.
4. Hanes Isherwood reagent.

The Rf-values observed are shown in Tables 2 and 3.

ACKNOWLEDGMENT

The authors wish to express their sincers thanks to Professor T. Mitsui (Department of Agricultural Chemistry, Kyoto University) and Mr. Kurihara (Division of Chemistry, Institute for Infections Deseases, Tokyo University) for microanalysis, and to Assistant Professor Z. Kumazawa (Department of Agricultural Chemistry, Kyoto University) for the infrared absorption spectra.

REFERENCES

(1) Rebison, R. and King, E. J., *Biochem. J.*, **25**, 323 (1931).
(2) Levene, P. A. and Raymond, A. L., *J. Biol. Chem.*, **92**, 757 (1931).
(3) Atherton, F. R., Howard, H. T. and Todd, A. R., *J. Chem. Soc.*, 1106 (1948).
(4) Lardy, H. A. and Fischer, H. O. L., *J. Biol. Chem.*, **164**, 513 (1946).
(5) "Biochemical Preparations," Vol. II, p. 39 (1952).
(6) Lampson, G. P. and Lardy, H. A., *J. Biol. Chem.*, **181**, 693 (1949).
(7) Seagmiller, J. E. and Horecker, B. L., *ibid.*, **192**, 175 (1951).
(8) Viscontini, M. and Oliver, C., *Helv. Chim. Acta*, **36**, 466 (1953).
(9) Karrer, P. and Viscontini, M., *ibid.*, **29**, 711 (1946).
(10) Viscontini, M., Ebnöther, C. and Karrer, P., *ibid.*, **34**, 1834 (1951).
(11) Viscontini, M., Ebnöther, C. and Karrer, P., *ibid.*, **34**, 2199 (1951).
(12) Viscontini, M., Ebnöther, C. and Karrer, P., *ibid.*, **35**, 457 (1952).
(13) Chambers, R. W., Moffatt, J. G. and Khorana, H. G., *J. Amer. Chem. Soc.*, **77**, 3416 (1955).
(14) Hashizume, T. and Tagaki, W., The 153rd Meeting of Agr. Chem. Soc. Japan, Kansai Section (Oct. 1958, Kyoto).
(15) Fleury, P. and Leclerc, M., *Bull. Soc. Chim. Biol.*, 201 (1943).
(16) Hashizume, T. and Takinami, K., Unpublished.
(17) Braun, J. and Beschke, E., *Ber.*, **39**, 4373 (1906).
(18) Hashizume, T., *Memoirs of the College of Agr., Kyoto University*, No. 81 (Chem. Series No. 31) (March 1959) p. 16.