Previous methods of preparing glucose-6-phosphate for use in biological experimentation were based on the isolation of this ester from a crude mixture of hexose monophosphates, obtained by yeast fermentation. The isolation was achieved by fractional crystallization of the brucine salts (1) or by preferential hydrolysis of the fructose component which leaves a considerable portion of the aldose phosphate intact (2). It has also been prepared by allowing phosphoglucomutase to act on glucose-1-phosphate (3). A chemical synthesis of the Robison ester was reported by Levene and Raymond (4), who treated monoacetone glucose with phosphorus oxychloride in pyridine at low temperatures. The yield of barium salt was only fair and it was necessary to purify the product by several recrystallizations of the brucine salt before it exhibited the proper rotation. These workers also attempted to phosphorylate glucos-1,2,3,4-tetraacetate but obtained an impure product in very poor yield.

The necessity of obtaining quantities of pure glucose-6-phosphate for enzymatic investigations prompted an attempt to devise a practical synthesis of the compound. The steps involved are shown in the accompanying diagram. Advantage was taken of the use of diphenylchlorophosphonate (5) as a phosphorylating agent. This substance reacts with only 1 mole of the substance to be phosphorylated, does not cause the formation of chlorohydrin compounds, as does POCl₃ (6), and the substituted diphenylphosphoric esters formed usually lend themselves to purification by recrystallization more readily than the products obtained when phosphorus oxychloride is employed. The phenyl groups are removed readily by reductive cleavage with hydrogen in the presence of platinum oxide catalyst.

The position of attachment of the phosphate group to the glucose molecule was insured by using as a starting material 1,2,3,4-tetraacetyl-β-D-glucopyranose (7, 8), in which carbon atom 6 has the only free hydroxyl group. The original procedure for the preparation of 1,2,3,4-tetraacetyl-β-D-glucopyranose (7) has been improved by Reynolds and Evans (8), but

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in an earlier study (9), in which this glucose tetraacetate was used in the synthesis of gentiobiose for biological experimentation, some difficulty was experienced in obtaining consistently good yields by their crystallization procedures. It has now been found that dibutyl ether is more reliable than either ethyl ether or petroleum ether for the crystallization of the tetraacetate and yields a product of sufficient purity for phosphorylation without recrystallizing.

After the reductive cleavage of the phenyl groups from the phosphory-
lated glucose tetraacetate, the acetyl groups were removed by catalytic
saponification with potassium methylate in anhydrous methanol. The
potassium salt of glucose-6-phosphate was precipitated from the solution as
the deacetylation proceeded; it was purified by washing several times with
anhydrous methanol and dried in vacuo over P₂O₅. The acetyl groups
could also be removed by acid in aqueous solution, or by the slow addition
of the stoichiometric amount of sodium or potassium hydroxide solution to
an alcoholic solution of the ester, and the product was then isolated as the
barium salt. When the acetyl groups were removed with aqueous alkali,
some discoloration occurred (removable with charcoal) and the final prod-
uct contained appreciable quantities of fructose-6-phosphate, the presence
of which was indicated by optical rotation, the quantitative Seliwanoff
test, and enzymatically catalyzed equilibria. For biological purposes the
potassium salt has the advantage that it is readily soluble in water and may
be used directly without the inconvenience of removing the cation.

The purity of the glucose-6-phosphate was determined not only by
analyses and optical rotation but also by quantitative enzymatic studies.
Synthetic glucose-6-phosphate was converted by phosphohexoisomerase
to the same equilibrium (32 per cent fructose-6-phosphate to 68 per cent
glucose-6-phosphate) as was pure fructose-6-phosphate, prepared according
to Neuberg, Lustig, and Rothenberg (10).

When calculated on the basis of the anion portion of the salts, the optical
rotation of our potassium salt is almost exactly that of the calculated value
([α]d₂⁰ = [α]₀ × 1.18 (1)) for Robison’s purest barium salt obtained from
the several times recrystallized brucine salt of natural aldose mono-
phosphate.

The barium salt of 6-phosphogluconic acid has been prepared from
synthetic glucose-6-phosphate by the procedure of Robison and King (1).

EXPERIMENTAL

1,2,3,4-Tetraacetyl-β-D-glucopyranose (I)—This starting material was
prepared from 6-trityltetraacetyl-β-D-glucose, according to the procedure
of Helferich and Klein (7), except that the crystallization was made from a
concentrated chloroform solution (not sirup) by the slow addition of
dibutyl ether. The first crop of crystalline tetraacetyl-β-D-glucopyranose
corresponded to a yield of 67 per cent and had a melting point of 124–127°.
This material was phosphorylated either directly or after recrystallizing
from chloroform by the addition of dibutyl ether which gave the pure
substance, m.p. 128–129°.

1,2,3,4-Tetraacetyl-6-diphenylphosphono-β-D-glucopyranose (II)—To a
cooled solution of 7.1 gm. of 1,2,3,4-tetraacetyl-β-D-glucopyranose in 20
cc. of anhydrous pyridine, 6.0 gm. of diphenylechlorophosphonate (5) were
SYNTHESIS OF GLUCOSE-6-PHOSPHATE

added dropwise with continuous shaking and cooling in an ice bath. The reaction began at once and within a few minutes a copious crystalline precipitate of pyridine hydrochloride appeared. The mixture was kept in the ice bath for 15 minutes and then placed in a refrigerator at 10° overnight. A few drops of ice water were added to hydrolyze the excess of acid chloride and after one-half hour the product was separated by pouring slowly into 600 cc. of ice water under continuous stirring. When the precipitate became granular, it was filtered off and again stirred up in fresh ice water. The product was filtered off, washed with cold water, and dissolved in 100 cc. of chloroform. The chloroform solution was washed once with dilute HCl and two times with distilled water, was dried with anhydrous sodium sulfate, and evaporated under reduced pressure to a sirup. The product was crystallized by careful addition of petroleum ether (b.p. 60–80°), swirling, and allowing to stand for several hours; the process may be hastened by seeding or scratching. The product was filtered with suction, washed with petroleum ether, and dried. Yield, 10.9 gm. (92 per cent of the theoretical). It melted at 64–66° and was of sufficient purity for subsequent use. The pure substance may be obtained by recrystallizing from isopropyl ether, or from acetone by the addition of water, m.p. 68°. It is soluble in chloroform, acetone, benzene, and ethyl alcohol.

\[ [\alpha]_D^{+2} = +16.5° \ (c = 1.37 \text{ in anhydrous pyridine}). \]

\[
C_{27}H_{39}O_{19}P \ (580.5) \ 
\text{Calculated.} \quad C \ 53.8, \ H \ 5.04, \ P \ 5.34 \\
\text{Found.} \quad " \ 53.8, " \ 5.03, " \ 5.24 \\
" \ 53.8, " \ 5.08, " \ 5.32
\]

1,2,3,4-Tetraacetyl-\(\beta\)-D-glucose-6-phosphoric Acid (III)—A solution of 7.0 gm. of tetraacetyl-6-diphenylphosphono-\(\beta\)-D-glucopyranose (II) in 70 cc. of anhydrous methanol\(^1\) was shaken in an atmosphere of pure dry hydrogen at a pressure slightly greater than 1 atmosphere with 0.7 gm. of platinum oxide (Adams’ catalyst). When the reduction neared completion, the free acid began to crystallize in fine needles. The absorption of hydrogen stopped abruptly when the theoretical quantity (8 moles) had been consumed; this required from 2.5 to 4.5 hours in several runs. After warming to dissolve the product, the catalyst was removed by filtering or centrifuging. An equal volume of petroleum ether was added in portions to the filtrate and crystallization allowed to proceed during slow cooling. The crystals were filtered with suction, washed with petroleum ether, and dried in vacuo at room temperature. Yield, 3.6 gm. (65 per cent of the theoretical). The product melted at 126–128°, and contained the theoretical quantity of organic phosphorus. When recrystallized from anhydrous methanol by slow addition of petroleum ether, the substance melted

\(^1\) Prepared according to Lund and Bjerrum (11).
at 127–128°. A second crop of crystals of the original purity may be obtained by evaporating the mother liquors to dryness under reduced pressure at a bath temperature of 25° and recrystallizing the product from methanol-petroleum ether. The analyses indicated that the substance crystallized with 1 mole of methanol which could not be removed by heating in vacuo without causing further decomposition. It was demonstrated by electrometric titration that the methanol was not esterified with the phosphoric acid residue. The presence and identity of the methanol were established by converting it to methyl iodide, which was trapped in dimethylaniline (12). The trimethylphenylammonium iodide obtained (a) melted at 227°, that from α-methyl glucoside (b) melted at 227°, that from pure methanol (c) melted at 230°, and mixtures of (a) and (c) melted at 227°. Phillips (13) reported the melting point of trimethylphenylammonium iodide to be 231.6°.

\[ \alpha_0^{25} = +17.4° \text{ (c = 1 (of methyl alcololate) in anhydrous pyridine); calculated for solvent-free compound } \alpha_0^{25} = +18.7°. \]

Reduction in Anhydrous Ethanol—The reductive cleavage of 3.8 gm. of the diphenyl compound (II) was carried out in 25 cc. of anhydrous ethanol with 0.4 gm. of platinum oxide. The product, which crystallized from the solvent as the reduction proceeded, was so sparingly soluble in hot ethanol that additions of anhydrous acetone were required to separate it from the catalyst. Slow evaporation of the solvents under reduced pressure caused the product to crystallize. After filtering and drying in vacuo over CaCl₂ and paraffin, the product weighed 2.42 gm. (78 per cent of the theoretical) and melted at 126-127°. It was recrystallized from anhydrous ethanol and dried as above for analysis.

The solvent of crystallization was characterized by distillation (at the melting point of the compound) into a pyridine solution of 3,5-dinitrobenzoyl chloride, isolation, and recrystallization of the ester formed; m.p. 93°, authentic ethyl 3,5-dinitrobenzoate melted at 93°, mixed m.p. 93°.

\[ \alpha_0^{25} = +16.9° \text{ (c = 1 (of ethyl alcololate) in anhydrous pyridine); calculated for solvent-free compound } \alpha_0^{25} = +18.7°. \]

Potassium Glucose-6-phosphate (IV)—To 3.3 gm. of tetraacetylglucose-6-phosphoric acid (III) (from methanol) partially dissolved in 75 cc. of cold
anhydrous methanol, a sufficient quantity of potassium methoxide in anhydrous methanol\(^2\) to neutralize the free acid groups was added dropwise with shaking. Complete solution was attained after the first few drops were added. Cleavage of the acetyl groups was initiated by the addition of a catalytic excess of 1.5 milliequivalents of potassium methoxide. The potassium salt of glucose-6-phosphate began to separate at once. The cleavage was allowed to proceed at refrigeration temperature in a tightly stoppered flask overnight. The product was separated by centrifuging, was washed four times with anhydrous methanol, once with each of the following methanol-ether mixtures: 80:20, 50:50, 20:80, and twice with anhydrous ethyl ether. After drying \textit{in vacuo} at room temperature, the product weighed 1.65 gm. (68.5 per cent of theory). It was essential to use only anhydrous solvents and thoroughly dried equipment in order to obtain good yields.

\[ \left[ \alpha \right]_D^{25} = +21.2^\circ \quad (c = 1.3 \text{ in water}) \]

\( \text{C}_{6}\text{H}_{11}\text{O}_{4}\text{PK}_2 \) (336.3). Calculated. C 21.4, H 3.29, P 9.21

Found. " 21.5, " 3.26, " 9.15

From the combined mother liquor and methanol washings an additional quantity of glucose-6-phosphate was obtained as the barium salt. The slightly turbid alcohol solutions were treated with an excess of BaBr\(_2\) in anhydrous methanol. When the barium salt had settled, it was separated by centrifuging, washed with absolute alcohol, and finally with ether. After purification, as described in the following section, the barium salt weighed 0.6 gm. (21 per cent of original theory); thus the combined yield of potassium and barium salts was 89.5 per cent of theoretical. To obtain all of the product as the barium salt, BaBr\(_2\) solution was added after the deacetylation by potassium methoxide was completed, and the barium salts were again purified, as described in the following section.

\textit{Deacetylation with Acid}—0.5 gm. of tetraacetylglucose-6-phosphoric acid (III) was dissolved in 35 cc. of 0.66 \(N\) HBr and the solution was heated on the steam bath for 3 hours. After cooling, pulverized barium hydroxide was added to neutrality. The solution was filtered and 4 volumes of ethanol were added. When the precipitate had settled, the supernatant liquor was decanted. The precipitate was washed in succession with 90 per cent ethanol, absolute ethanol, 75 per cent ethanol-25 per cent ether, 25 per cent ethanol-75 per cent ether, and finally with dry ether. After drying in air, the barium glucose-6-phosphate was dissolved by extracting successively with 20, 10, and 5 cc. portions of distilled water. To the clear filtrate 4 volumes of ethanol were added and the product was separated

\(^2\) Prepared by the cautious addition of clean potassium metal to anhydrous methanol. Solutions of 1 to 2 \(N\) were used.
and dried as above. The barium salt (0.33 gm.) was free of inorganic phosphate and on the basis of its organic phosphorus content was 93 per cent pure (yield = 72 per cent of theory). Its rotation (purity based on phosphorus analysis) was $\left[\alpha\right]_{D}^{24} = +17.9^\circ$.

**Biological Activity**—The following data indicate that the synthetic glucose-6-phosphate is quantitatively biologically active. A partially purified preparation of phosphohexoisomerase (free of phosphoglucomutase activity) from rat muscle converted 32 per cent of the synthetic ester to fructose-6-phosphate at equilibrium at 25°C. Under the same conditions, pure fructose-6-phosphate, prepared according to Neuberg, Lustig, and Rothenberg (10), was converted to an equilibrium of 68.4 per cent glucose-6-phosphate to 31.6 per cent fructose-6-phosphate. Fructose phosphate was determined in these experiments by a quantitative Seliwanoff test (2).

**SUMMARY**

A new procedure is described for the synthesis of glucose-6-phosphate. 1,2,3,4-Tetraacetyl-β-d-glucopyranose has been phosphorylated with diphenylchlorophosphonate in pyridine. Subsequent removal of the phenyl groups by means of hydrogen and platinum oxide, followed by saponification of the acetyl groups, gave pure glucose-6-phosphate in good yield. The ester was isolated as a crystalline dipotassium salt which is readily soluble in water and can be used directly for enzymatic experimentation. The barium salt may be prepared by an alternative procedure.

The synthetic ester is a convenient source of 6-phosphogluconic acid.

**BIBLIOGRAPHY**

PHOSPHORIC ESTERS OF BIOLOGICAL IMPORTANCE: I. THE SYNTHESIS OF GLUCOSE-6-PHOSPHATE
Henry A. Lardy and Hermann O. L. Fischer


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