A NEW SYNTHESIS OF PHOSPHORYL-ENOLPYRUVIC ACID

BY ERICH BAER AND HERMANN O. L. FISCHER*

(From the Banting and Best Department of Medical Research, Banting Institute, University of Toronto, Toronto, Canada)

(Received for publication, March 26, 1949)

While investigating the enzymatic conversion of \( \text{d-glyceric acid-3-phosphoric acid} \) to pyruvic acid and phosphoric acid, an essential step both in glycolysis and fermentation, Meyerhof and Lohmann (1) observed the formation of a hitherto unknown organic phosphoric acid ester. They succeeded in isolating the new intermediary compound by virtue of the ease of crystallization of the silver-barium salt, and established its constitution as that of phosphoryl-enolpyruvic acid (PEPA). The presence of optical activity in the isolated product thus led Meyerhof and Lohmann to propose and favor a formula (B) which differed from the anticipated structure (A). They realized, of course, the possibility that the activity observed could have been due to contamination by an optically active substance.

\[
\begin{align*}
\text{CH}_2\text{=C-COOH} & \quad \text{OPO}_2\text{H}_2 \\
\text{OH} & \\
\text{(A)} & \\
\text{CH}_2\text{=C-C} & \quad \text{OPO}_2\text{H}_2 \\
\text{\textasterisk} & \\
\text{(B)} & 
\end{align*}
\]

The synthesis of compound A, accomplished by Kiessling (2) in Meyerhof's laboratory, by the interaction of pyruvic acid and phosphorus oxychloride in quinoline, produced a substance which proved to be identical with the natural product and thus established its constitution.

Kiessling's synthesis produced the PEPA, however, in a yield of at best 3 to 5 per cent. In view of the current biochemical interest in this substance a more practical method of preparation was desirable. Despite the apparent simplicity of the molecule, the development of an efficient synthesis of PEPA has proved to be a difficult problem. Various approaches over a period of years have been investigated in this laboratory, and the method which was finally developed is illustrated in the accompanying reaction scheme. Although the new procedure produces the compound in a yield several times that obtained by Kiessling's method and in a higher state of purity, it cannot be regarded as completely

* Present address, Virus Laboratory, Forestry Building, University of California, Berkeley 4, California.
satisfactory. However, no better method of preparing this substance is known at the present time.

In its present form the synthesis is as follows: \(\beta\)-Chlorolactic acid (I) is phosphorylated by means of phosphorus oxychloride in the presence of dimethylaniline. The phosphorylation product, a mixture of organic phosphoric acid esters, is not isolated but is treated immediately with an ethanolic solution of potassium hydroxide to remove the elements of hydrochloric acid from the phosphoryl-\(\beta\)-chlorolactic acid (II). The PEPA thus formed is precipitated, together with other organic phosphates, as the ethanol-insoluble barium salt; it is finally isolated by means of its silver salt (III) and its characteristic silver-barium salt (IV) (1).

The silver barium salt, after purification by precipitation from dilute nitric acid, is free from inorganic phosphate and other esters of phosphoric acid, gives the correct analysis for \(C_3H_2O_6P_{BaAg} + 2H_2O\), and contains pyruvic acid, phosphoric acid, silver, and barium in the theoretical ratios of \(1:1:1:1\). Its mercuric acetate-labile phosphorus is identical with the total organic phosphorus, a characteristic of PEPA (1). In alkaline solution the substance consumes 6 moles of iodine, producing 1 mole of iodoform and 1 mole of inorganic phosphate. On acid hydrolysis it forms quantitatively pyruvic acid and phosphoric acid, the rate of hydrolysis being identical with that reported for the natural compound (1).

The crystal form of the silver-barium salt of PEPA varies somewhat with the conditions of crystallization, but the most typical form and arrangement are shown in Fig. 1.

\[
\text{CH}_2\text{Cl} \text{CHCOOH} \xrightarrow{\text{POCl}_3, \text{dimethyl-aniline}} \text{CH}_2\text{Cl} \text{CHCOOH} \xrightarrow{\text{KOH}, 90\% \text{C}_2\text{H}_6\text{OH}} \text{CH}_2\text{COOK} \text{CH}_2\text{COO}\text{Ba} \xrightarrow{\text{AgN}_2\text{O}_3} \text{CH}_2\text{COOAg}^* \text{Ba(NO}_3)_2 \xrightarrow{\text{Nitric acid}} \text{CH}_2\text{COOAg}^* \text{OPO}_3\text{AgK} \xrightarrow{\text{Nitric acid}} \text{CH}_2\text{COOAg}^* \text{OPO}_3\text{Ba} \]

*Placement of the silver and barium in these positions is arbitrary.*

The starting material for the synthesis of PEPA, the \(\beta\)-chlorolactic acid, is not at present available commercially. Its preparation by oxidation of epichlorohydrin with nitric acid has been described by von Richter.
The β-chlorolactic acid thus prepared contains, however, considerable amounts of oxalic acid. In the experimental section of this paper a modified procedure is described for the preparation of pure β-chlorolactic acid by nitric acid oxidation of either epichlorohydrin or glycerol-α-monochlorohydrin.

In view of the greater reactivity of iodo compounds in general, it was thought that the phosphoryl-β-iodolactic acid would, on treatment with alkali, form the PEPA more readily than the corresponding bromo and chloro compounds. Accordingly the investigation was begun with β-iodolactic acid as starting material. The required β-iodolactic acid was prepared from β-chlorolactic acid by halogen exchange as described by Glinsky. The only available abstract of this communication (5) is, however, so lacking in detail that it became necessary to reestablish the conditions of synthesis. The procedure as finally carried out in this laboratory is described in detail below.

Later it was found that the over-all yield of PEPA obtained by direct phosphorylation of β-chlorolactic acid is greater (based on the weight of β-chlorolactic acid) than that obtained by phosphorylation of β-iodolactic acid derived from β-chlorolactic acid. In the event that the β-iodolactic acid becomes more readily available than the corresponding chloro compound, the present procedure for the synthesis of PEPA can be readily adapted to its use as starting material.

Work is in progress in this laboratory on the synthesis of other enol-
phosphates of biological interest by methods similar to that described in this paper.

**EXPERIMENTAL**

*Phosphoryl-enolpyruvic Acid*

*Phosphorylation*—A solution of 9.96 gm. (0.08 mole) of \( \beta \)-chlorolactic acid (m.p. 78–80°) in 140 ml. of dry dimethylaniline\(^1\) was prepared in a 1 liter three-necked flask equipped with an oil-sealed mechanical stirrer, calcium chloride tube, and dropping funnel. The flask was then immersed in an ice bath and the stirrer set in motion. As soon as the temperature of the solution had reached 2°, a solution of 13.5 gm. (0.088 mole) of freshly distilled phosphorus oxychloride in 60 ml. of dry dimethylaniline was added dropwise over a period of 5 to 6 minutes. 15 minutes after the last of the phosphorus oxychloride had been added, the ice bath was removed and the reaction mixture kept at room temperature for a period of 20 minutes. At the end of this time the speed of the stirrer was increased and a solution of 40 gm. of potassium hydroxide in 400 ml. of 90 per cent ethanol at room temperature was added quickly. The temperature of the mixture rose immediately to 45°. After 2 minutes at this temperature, the flask was immersed in an ice bath and the mixture brought to 30°. The vigorously stirred mixture was maintained at this temperature for 1 hour by placing the flask in a water bath at 30–32°. The suspension was then centrifuged and the precipitate washed at the centrifuge twice with 100 ml. portions of 95 per cent ethanol. The mixture of potassium salts was dissolved in 240 ml. of water and the solution freed of inorganic phosphate by adding ammoniacal magnesia mixture\(^2\) until a test showed that all free phosphate was removed. Approximately 150 ml. of the magnesia mixture were sufficient. An excess was avoided. The ammonium magnesium phosphate was removed by centrifugation and the decanted supernatant liquid mixed with a solution of 48 gm. of barium acetate in 120 ml. of water. The water-insoluble precipitate of barium salts was removed by centrifugation and the decanted supernatant liquid (525 ml.) was diluted gradually with an equal volume of 95 per cent ethanol. After standing for 2 hours at room temperature, the ethanol-insoluble precipitate was centrifuged and washed at the centrifuge consecutively with 150 ml. portions of 95 per cent ethanol, 99 per cent ethanol, and ether.

\(^1\) Dried over solid sodium hydroxide.

\(^2\) Prepare as follows: Dissolve 400 gm. of magnesium chloride hexahydrate and 300 gm. of ammonium chloride in 1500 ml. of warm water. Add ammonium hydroxide until the solution is alkaline to litmus. After 1 hour filter the solution and add hydrochloric acid to the filtrate until acid to litmus. Shortly before use 15 ml. of concentrated ammonia per 100 ml. of magnesia mixture are added.
The paste was freed from organic solvent in a stream of air and the resulting fine white powder dried *in vacuo* (0.2 mm.) over phosphorus pentoxide to constant weight.

The mixture of barium salts weighed 13.0 gm. and was free from inorganic phosphate. According to the value for mercuric acetate-labile phosphorus (2.74 per cent) the barium salts contained 1.93 gm. (14.3 per cent) of phosphoryl-enolpyruvic acid. The phosphorus labile to mercuric acetate amounted to only 60.1 per cent of the total organic phosphorus (45.7 per cent). The difference was accounted for by the presence of considerable amounts of other esters of phosphoric acid. The isolation of the pure enol-phosphate was accomplished via the silver and silver-barium salt.

Isolation of Phosphoryl-enolpyruvic Acid As Silver-Barium Salt—The barium salt mixture (13.0 gm.) was dissolved in 250 ml. of 0.2 N nitric acid and the solution was freed from barium ions by adding dropwise 5 N sulfuric acid until, in a centrifuged sample, the presence of a small excess of sulfuric acid could be detected. The barium sulfate was removed by centrifugation and the decanted supernatant liquid mixed with a concentrated aqueous solution of 18.0 gm. of silver nitrate. After the removal of a small precipitate of silver chloride concentrated ammonia was added cautiously drop by drop until the mixture was weakly but distinctly alkaline to litmus, and the voluminous precipitate of silver salts was removed by centrifugation. The well drained silver salts were dissolved in 350 ml. of 0.2 N nitric acid and the solution freed by centrifugation from a small amount of dark brown impurity, added to a solution of 11.0 gm. of barium nitrate in 180 ml. of water. This mixture was quickly poured into 350 ml. of 0.2 N ammonia at room temperature. After 100 ml. of 95 per cent ethanol had been added, the flask was placed in an ice box for a period of at least 3 hours. The colorless silver-barium salt was centrifuged, washed at the centrifuge consecutively with 50 ml. portions of 50 per cent ethanol, 99 per cent ethanol, and ether, and dried *in vacuo* (0.2 mm.) over phosphorus pentoxide for a period of 20 hours.

The white crystalline product weighed 5.3 gm. and gave the following analysis: total P 5.6 per cent; mercuric acetate-labile P 5.2 per cent; organic but not mercuric acetate-labile P 0.2 per cent; inorganic P 0.2 per cent. Theoretical values, total P 6.95 per cent; mercuric acetate-labile P 6.95 per cent.

The silver-barium salt was readily obtained in analytically pure state by reprecipitation from dilute nitric acid.

*In order to prevent discoloration of the silver or silver-barium salts all operations from here on must be carried out in subdued light, and the finished preparations should be stored in darkness.*
Purification of the Silver-Barium Salt—The crude silver-barium salt (5.3 gm.) was dissolved in 110 ml. of 0.2 N nitric acid and the solution was freed, by centrifugation, of a small amount of brown impurities. To the decanted supernatant were added 13 ml. of a 40 per cent barium acetate solution and the mixture was placed immediately in a dark room for a period of 24 hours. When undisturbed during this time, most of the voluminous precipitate changed into a mass of well formed and dense crystals (see Fig. 1), leaving the impurities in a finely divided and easily suspensible state. The mixture was gently agitated, and, after the heavy material had settled, the supernatant liquid carrying the suspended impurities was decanted. This flotation process was repeated twice with 10 ml. portions of 50 per cent ethanol. Finally the crystalline material was transferred, with 20 ml. of 50 per cent ethanol, to a Büchner funnel, washed with 40 ml. of 99 per cent ethanol, and dried in vacuo (10 mm.) at room temperature over phosphorus pentoxide for a period of 18 hours. The colorless silver-barium salt of phosphoryl-enolpyruvic acid weighed 3.6 gm. (10 per cent of the theoretical amount) and was analytically pure.

\[
C_{3}H_{12}O_{2}P_{2}Ba_{2}Ag + 2H_{2}O \text{ (446.3)}
\]

Calculated. C 8.07, H 1.35, P (total organic) 6.95, P (mercuric acetate-labile) 6.95, Ag 24.17, Ba 30.78
Found. C 8.31, H 1.29, P (total organic) 6.96, P (mercuric acetate-labile) 6.96, Ag 24.08, Ba 30.80

Oxidation with Hypoiodite—A solution of PEPA, prepared by triturating 129.4 mg. of silver-barium salt with dilute hydrochloric acid and removing the silver chloride by centrifugation, was mixed with 25.0 ml. of 0.100 N iodine solution and made alkaline to litmus. After standing 30 minutes, the solution was acidified and the remaining iodine was determined by titration with 0.100 N sodium thiosulfate solution. The iodine consumed amounted to 17.0 ml. of 0.100 N solution; i.e., 97.7 per cent of the amount calculated for 6 atoms of iodine.

Determination of Pyruvic Acid—A dilute solution of PEPA, obtained by triturating 150.4 mg. of silver-barium salt with three 5 ml. portions of 2.5 N hydrochloric acid and removal of the silver chloride by centrifugation, was mixed with a warm solution of 250 mg. of 2,4-dinitrophenylhydrazine in 15 ml. of 2.5 N hydrochloric acid, and the mixture was kept in a boiling water bath for a period of 90 minutes. After standing for 2 hours in an ice box at 6° the hydrazone was collected by suction on a crucible with a fritted glass disk, washed with dilute hydrochloric acid and water, and dried in vacuo (0.2 mm.) over phosphorus pentoxide. The weight of the precipitate was 90.0 mg. The crucible was washed
with dilute sodium bicarbonate solution and water until the filtrate was colorless, dried, and weighed as before. The loss in weight, equal to the amount of 2,4-dinitrophenylhydrazone of pyruvic acid, was 89.0 mg. or 97.9 per cent of the theoretical value.

The sodium bicarbonate solution, on acidification, yielded pyruvic acid 2,4-dinitrophenylhydrazone, which after recrystallization from 99 per cent ethanol, melted with decomposition at 218–219°. The melting point of an authentic sample of pyruvic acid 2,4-dinitrophenylhydrazone was 218–219° (decomposition).

**Acid Hydrolysis of Phosphoryl-enolpyruvic Acid**—To prepare a solution of PEPA in hydrochloric acid, 37.3 mg. of the silver-barium salt were tritutated three times with 10 ml. portions of 1 N hydrochloric acid. After each trituration the solid was centrifuged, the supernatant liquid decanted, and the precipitate resuspended in hydrochloric acid. The supernatant solutions were combined and the volume brought to 50 ml. with 1.0 N hydrochloric acid. A series of stoppered Pyrex test-tubes, each containing 2.0 ml. of the PEPA solution (1.67 × 10⁻³ M), was kept in a strongly boiling water bath. At intervals of 5 minutes tubes were removed and their inorganic phosphorus determined colorimetrically. The rate of hydrolysis was found to be identical with that reported for the natural PEPA. Both rates of hydrolysis are plotted in Fig. 2.

**β-Chlorolactic Acid**

To 440 ml. of cold nitric acid (sp. gr. 1.42) in a 5 liter round bottomed flask surrounded by an ice bath, 110.5 gm. (1.0 mole) of glycerol-α-mono-chlorohydrin were added from a separatory funnel over a period of 10 minutes. The flask was then placed on a boiling water bath and its contents heated until reaction set in, as indicated by the appearance of gas bubbles. The water bath was then immediately removed and the reaction allowed to proceed spontaneously. After approximately 10 minutes the vigorous reaction had subsided and the mixture was heated on a boiling water bath for half an hour. The cooled reaction mixture was transferred to a distilling flask and concentrated as far as possible in vacuo (8 to 10 mm. of Hg) at a bath temperature not exceeding 55°. The partially crystallized residue was dissolved in 370 ml. of distilled water, the solution was made neutral to litmus with anhydrous sodium carbonate, and the sodium oxalate was removed by filtration with suction. The remainder of the oxalic acid was removed by adding to the filtrate dropwise a concentrated aqueous solution of calcium chloride until no more precipitate formed, and filtering the mixture. Concentrated

This reaction is accompanied by the evolution of large amounts of nitrogen oxides and therefore must be carried out under a fume hood.
sulfuric acid, equivalent to the amount of anhydrous sodium carbonate used above, was added slowly and with stirring to the ice-cold filtrate and the solution was extracted six to eight times with 230 ml. of ether. The combined ether extracts were evaporated under diminished pressure and the residue heated in vacuo to 60° for 2 hours. The remaining solid was dissolved in 120 ml. of hot n-butyl ether and the solution placed in ice for 3 hours. The colorless crystals were collected on a Büchner funnel, washed on the filter with low boiling petroleum ether, and dried in vacuo. The β-chlorolactic acid melted at 78–80° and weighed 38.5 gm. (32 per cent of the theoretical amount).

By substituting an equivalent amount of epichlorohydrin (92.5 gm.) in the above procedure, β-chlorolactic acid of the same purity (m.p. 78.5–80°) and practically the same yield (37.8 gm., 30.3 per cent of the theoretical amount) was obtained.

**β-Iodolactic Acid**

A solution of 93.4 gm. (0.75 mole) of β-chlorolactic acid and 170 gm. (1.13 mole) of anhydrous sodium iodide* in 550 ml. of dry acetone was

* Dried in the oven at 120°.
E. BAER AND H. O. L. FISCHER

prepared in a 1 liter round bottomed flask with ground glass joint bearing a reflux condenser and a calcium chloride tube. The reaction flask was placed in a water bath of 65° and the solution refluxed for 24 hours. The mixture was then cooled to room temperature, filtered, and the sodium chloride washed on the filter several times with a small amount of dry acetone. The combined filtrates together with 46 gm. (0.31 mole) of dry sodium iodide were refluxed for 24 hours. The cooled solution was filtered, the sodium chloride washed with dry acetone, and the combined filtrates concentrated under reduced pressure to a thick syrup. The partially solid residue was dissolved in 160 ml. of distilled water and the solution decolorized with solid sodium bisulfite. After the addition of 40 ml. of 5 N sulfuric acid the solution was extracted twice with 150 ml. of petroleum ether (b.p. 30-60°) and five times with 130 ml. of peroxide-free ether. The combined ether extracts were dried with anhydrous sodium sulfate, the ether removed under diminished pressure, and the residue was kept for several hours in a vacuum of 10 mm. at a bath temperature of 55°. The dark brown mass was spread on a clay plate to remove oily impurities and then thoroughly dried in vacuo over phosphorus pentoxide. For purification the crude β-iodolactic acid, weighing 131 gm., was dissolved in 260 ml. of dry ethyl acetate and reprecipitated by the addition of 560 ml. of carbon tetrachloride. To increase the yield the mixture was placed in a dry ice-acetone bath at -65° for half an hour. The solid was filtered by suction on a Büchner funnel, washed with cold (-30°) carbon tetrachloride until both crystals and filtrate were colorless, and dried in vacuo. Yield of pure β-iodolactic acid, 106 gm. (65.5 per cent of the calculated amount); m.p. 99-100° (6).

SUMMARY

1. A new and more efficient synthesis of phosphoryl-enolpyruvic acid (PEPA) is described.

2. The synthesis involves three steps: (a) phosphorylation of β-chlorolactic acid with phosphorus oxychloride; (b) removal of the elements of hydrochloric acid from phosphoryl-β-chlorolactic acid by treatment with alkali; (c) isolation of the phosphoryl-enolpyruvic acid as the silver-barium salt.

3. The properties of the phosphoryl-enolpyruvic acid from β-chlorolactic acid were found to be identical with those reported for the natural PEPA (Meyerhof and Lohmann) and the synthetic PEPA obtained by phosphorylation of pyruvic acid (Kiessling).

The petroleum ether extraction removes higher boiling condensation products of acetone.
We wish to express our sincere appreciation to Professor W. Stanley Hartroft, University of Toronto, for his kindness in preparing the photomicrograph.

BIBLIOGRAPHY

A NEW SYNTHESIS OF PHOSPHORYL-ENOLPYRUVIC ACID
Erich Baer and Hermann O. L. Fischer


Access the most updated version of this article at http://www.jbc.org/content/180/1/145.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/180/1/145.citation.full.html#ref-list-1