Anal. Calcd. for $C_{12}H_{28}NO_4P$: P, 11.0. Found: P, 11.1.

Diethyl *t*-Butyl Phosphate.—Sodium *t*-butoxide was prepared by refluxing 125 ml. (1.32 moles) of *t*-butyl alcohol containing 3.45 g. (0.15 mole) of finely cut sodium until reaction was complete. To this mixture was added gradually with stirring and cooling 25.9 g. (0.15 mole) of diethyl phosphorochloridate at 25–30°. After stirring for 2 hours at room temperature the excess *t*-butyl alcohol was removed *in vacuo*, 25 ml. of water added to dissolve the sodium chloride, and the resulting solution extracted with ether. Removal of the ether and distillation gave 9.8 g. (31%) of diethyl *t*-butyl phosphate, b.p. 63–66° (1 mm.), $n^{35}D$ 1.4042 and d^{35}_4 1.0105.

Anal. Calcd. for $C_8H_{19}O_4P$: P, 14.8. Found: P, 15.2.

To 21 g. (0.1 mole) of diethyl *t*-butyl phosphate was gradually added 16 g. (0.1 mole) of bromine at 10–15°. Distillation gave a high boiling product and 18 g. (83%) of 1,2-dibromo-2-methylpropane, b.p. 140–154°, and $n^{35}D$ 1.4992 (literature[5] b.p. 145–148°, $n^{25}D$ 1.5050).

(5) N. A. Milas and C. N. Winnick, THIS JOURNAL, **71**, 748 (1949).

Anal. Calcd. for $C_4H_8Br_2$: Br, 74.1. Found: Br, 74.3.

The high-boiling product, 10.2 g., b.p. 136–148° (1 mm.), may be a mixture of diethyl hydrogen phosphate and diethyl phosphorobromidate. Analysis indicated 17.9% phosphorus, 10.8% bromine and 4.9 milliequivalents of acid per gram.

Acknowledgment.—We are indebted to the Westvaco Chlor-Alkali and Niagara Chemical Divisions of Food Machinery and Chemical Corporation for permission to publish this work. We also wish to express our thanks to Dr. P. F. Derr and Mr. D. K. Chapman for assistance with the spectrographic work, to Messrs. G. S. Haines and C. R. Walker for analytical data, to Messrs. J. Bekenstein, R. B. Greenlee and C. R. Smoot for assistance in carrying out the experimental work, and to Dr. G. M. Kosolapoff for advice during the course of this work.

SOUTH CHARLESTON 3, W. VA.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

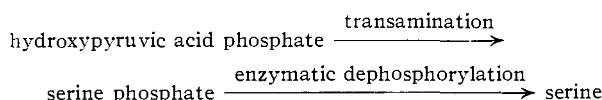
The Synthesis and Properties of Hydroxypyruvic Acid Phosphate

BY CLINTON E. BALLOU AND ROBERT HESSE

RECEIVED FEBRUARY 27, 1956

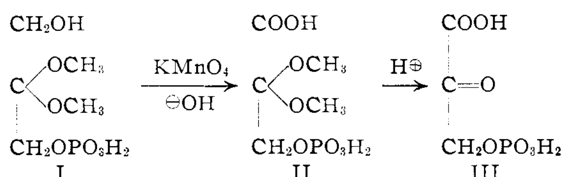
Hydroxypyruvic acid phosphate, a possible intermediate in the biosynthesis of serine phosphate, has now been obtained by synthesis and some of its properties have been determined. This synthetic material has been shown by other workers to be a good precursor of serine in a partially purified enzyme system prepared from rat liver.

Hydroxypyruvic acid phosphate has been postulated as a possible precursor of serine phosphate and of serine *via* the pathway[1]



However, since the hydroxypyruvic acid phosphate has not been available either by isolation or synthesis, it has been impossible to test its biological activity in such a role.

We wish to report the synthesis of hydroxypyruvic acid phosphate by a definitive method, using as starting material the cyclohexylammonium salt of dihydroxyacetone phosphate dimethyl or diethyl ketal.[2] The reaction scheme is outlined below.



The cyclohexylammonium salt of dihydroxyacetone phosphate dimethyl ketal (I) was converted to the potassium salt, which was oxidized to II with potassium permanganate. The salt of II was converted to the free acid by passage through a column of Dowex 50 (H^+). The acid eluate was neutralized with cyclohexylamine to give the salt, which was isolated in crystalline form from water by the addition of acetone.

The titration curves for I and II show the presence of a carboxyl group in the oxidation product, II (one mole per mole of primary or secondary phosphate), and are confirmation of the assigned structure.

The ketal structure of II is more stable at the *p*H of its own free acid than is that of I, being only slowly hydrolyzed to the ketone III, at 40°. However, incubation of the acidic solution at 40° for 4 days does hydrolyze the ketal, with the liberation of very little inorganic phosphate. The ultraviolet absorption spectra of the ketal and of the hydrolyzed compound show a shift in absorption toward the longer wave lengths following the hydrolysis of the ketal structure, which may serve as the basis of a useful spectrophotometric assay. The preparation of III by way of the diethyl ketal is in some respects preferable, since hydrolysis of the diethyl ketal proceeds about three times more readily than the dimethyl ketal.

III has the following properties. The phosphate group was liberated by acid hydrolysis at a rate comparable to that of dihydroxyacetone phosphate (half-time of hydrolysis about 20 minutes in 1 *N* hydrochloric acid at 90°).[2] In contrast, liberation of the phosphate group in alkali was more difficult, requiring 15 hours at room temperature in 1 *N* alkali for the elimination of 50% of the phosphate. Conditions for the quantitative elimination of phosphate in alkali were not found. Dephosphorylation

(1) H. J. Sallach, in H. B. McElroy and B. Glass, "A Symposium on Amino Acid Metabolism," Johns Hopkins Press, Baltimore, Md., 1955, p. 782.

(2) C. E. Ballou and H. O. L. Fischer, THIS JOURNAL, **78**, 1659 (1956).

of III by an acid phosphatase yielded hydroxypyruvic acid (92% of the theoretical amount), characterized by its reduction by lactic dehydrogenase in the presence of reduced diphosphopyridine nucleotide.[3] Hydroxypyruvic acid phosphate itself was not reduced by lactic dehydrogenase at a rate that could be conclusively attributed to reduction of the phosphorylated compound.[4] The hydroxypyruvic acid phosphate prepared in this Laboratory has been shown by Ichihara and Greenberg[5] to give a good yield of serine by transamination with glutamic acid in a crude enzyme preparation from rat liver. Whether transamination precedes or follows dephosphorylation is not known.[6]

Experimental

Hydroxypyruvic Acid Phosphate Dimethyl Ketal (II).—To a solution of 1.0 g. of I in 25 ml. of 1 *N* potassium hydroxide, was added 50 ml. of water. The solution was concentrated to a sirup on the water pump at 35° to remove the cyclohexylamine. The sirup, dissolved in 25 ml. of water, was then cooled in ice-water, and 0.75 g. of potassium permanganate was added. The solution was mixed and allowed to return to room temperature as the permanganate dissolved. The reaction vessel was then stoppered and left for 36 hours at room temperature. At that time the excess oxidant was decomposed by the cautious, dropwise addition of a 30% solution of hydrogen peroxide (about 100 drops). The mixture was filtered through a pad of celite to remove the manganese dioxide, the clear filtrate was passed through a column of Dowex 50 (acid form, about 30 ml.) and the acid eluate was collected. The column was washed with water until the eluate was no longer acidic (*p*H 5–6). Cyclohexylamine was immediately added to the eluate to raise the *p*H to 8, and the solution was then concentrated to dryness on the water pump, bath temperature 40–45°. The dry residue was extracted with 25 ml. of warm (40–45°) absolute ethanol, and about 60 ml. of ether was added to completely precipitate any inorganic phosphate. The solution was filtered by suction, and the filtrate was concentrated to dryness on the water pump. The dry, crystalline residue was taken up in 1 ml. of water, a few more drops of cyclohexylamine were added, and acetone was added until just before turbidity appeared. The solution was allowed to stand until crystallization commenced, at which point it was placed in the refrigerator (5°) and left there overnight. The crystals were collected by filtration and washed with acetone, the yield of air-dried material being 1.10 g. (87%) with m.p. 183–185° (some browning at 180°).

For analysis, the product was dried for several hours at room temperature in a high vacuum over phosphorus pentoxide.

Anal. Calcd. for $C_{23}H_{50}O_8N_3P \cdot H_2O$: C, 50.7; H, 9.6; N, 7.7; P, 5.7; OCH_3, 11.4; H_2O, 3.3. Found: C, 50.9; H, 9.7; N, 7.4; P, 5.8; OCH_3, 12.2; H_2O, 3.5.

The diethyl ketal of II was prepared in an identical manner from dihydroxyacetone phosphate diethyl ketal. Solubility differences in the product required elimination of the warm absolute ethanol extraction to remove inorganic phosphate, and the product was crystallized directly from a little water by the addition of acetone. After drying at 60° over phosphorus pentoxide in a high vacuum, the product melted at 170–173° dec. with slight browning at 165°.

Anal. Calcd. for $C_{25}H_{54}O_8PN_3 \cdot H_2O$: C, 52.4; H, 9.8; N, 7.3; P, 5.4; OEt, 15.7. Found: C, 52.5; H, 8.2; N, 7.1; P, 5.4; OEt, 15.3.

Titration of I and II.—To a solution of 21.6 mg. (50 µmoles) of the cyclohexylammonium salt of I, and 74.6 mg. of potassium chloride in 5 ml. of water was added 100 µ equivalents of 1 *N* hydrochloric acid. The solution was quickly titrated on an automatic recording *p*H meter. This titration must be performed rapidly after the addition of acid, for the ketal structure of I is easily hydrolyzed in acid solution. The titration required 98 µ equivalents of sodium hydroxide (theoretical for two acid groups is 100 µ equivalents), and showed apparent *pK*'s of 2.3 and 5.9.

A titration performed under the same conditions on II (27.3 mg., 50 µmoles) required 150 µ equivalents of alkali, theoretical for 3 acid groups, and showed apparent *pK*'s of 2.2, 3.0 and 6.4.

Hydroxypyruvic Acid Phosphate. From the Dimethyl Ketal.—A solution of 100 mg. of II in 10 ml. of water was swirled for a minute with about 2 ml. of Dowex 50 (H^+) to remove the cyclohexylamine. After filtration, the solution was kept at 40° for 4 days. At the start of the hydrolysis, a descending chromatogram (butanol:acetic acid:water, 35:10:25; Whatman #1 acid washed paper) showed only the ketal with R_f 0.52. As hydrolysis proceeded, a new organic phosphate compound with R_f 0.32 appeared along with a trace of inorganic phosphate. About 50% of the ketal was hydrolyzed in 24 hours, and hydrolysis was practically complete in 4 days, when the chromatogram no longer showed the faster component. About 7% of the organic phosphate appeared as inorganic phosphate.

This solution may be neutralized and used as such as the source of hydroxypyruvic acid phosphate.

A barium salt was prepared by adding 70 mg. of barium acetate to the above acidic solution, followed by ethanol to precipitate the salt. It was an amorphous material that gave a rather poor analysis.

Anal. Calcd. for $C_3H_5O_7PBa_{1.5} \cdot 2H_2O$: Ba, 48.2; P, 7.3. Found: Ba, 47.3; P, 7.5.

From the Diethyl Ketal.—A solution of 26.3 mg. of cyclohexylammonium hydroxypyruvic acid phosphate diethyl ketal in 2.0 ml. of water was swirled with 1 ml. of Dowex 50 (H^+) and filtered. The filtrate was kept at 40° for 36 hours, when the ketal was completely hydrolyzed. This solution was 0.023 molar in hydroxypyruvic acid phosphate. Its absorption curve shows a maximum at 230 mµ, the molecular extinction coefficient being 240. No attempt was made to isolate a solid derivative of this substance. About 7% of the phosphate had been split off by the mildly acidic treatment to give inorganic phosphate and presumably hydroxypyruvic acid (see next section).

Reaction of Hydroxypyruvic Acid Phosphate and its Dephosphorylated Product with Lactic Dehydrogenase.—An assay system containing 2.84 ml. of 0.015 *M* *p*H 7.5 glycylglycine buffer, 0.15 ml. of reduced diphosphopyridine nucleotide (1.75×10^{-3} *M*) and 0.125 µmole of lithium pyruvate had an optical density at 340 mµ of 0.425. After addition of 0.01 ml. of lactic dehydrogenase the optical density changed at the rate of about 0.125 unit per minute to a value of 0.185. This corresponded to 95% of the amount of lithium pyruvate added.

A similar assay containing 2.3 µmoles of hydroxypyruvic acid phosphate instead of the lithium pyruvate, and ten times as much enzyme had an optical density change of 0.332 over a 3-hour period. This change, which corresponds to 7% of the added substrate, could be due to hydroxypyruvic acid formed by acid dephosphorylation.

A solution of 0.1 ml. of 0.023 *M* hydroxypyruvic acid phosphate in 2.0 ml. of *p*H 5 acetate buffer containing 0.01 *M* magnesium chloride was treated with 0.5 ml. of an acid phosphatase preparation (ammonium sulfate fractionated "Polydase"). After 2 hours, all of the organically bound phosphate had been released. An assay for hydroxypyruvic acid with lactic dehydrogenase of an 0.10-ml. aliquot of this solution (0.088 µmole), gave an optical density change of 0.167 unit, corresponding to a concentration change of

(3) A. Meister, *J. Biol. Chem.*, **197**, 309 (1952).

(4) In interpreting very slow enzyme-catalyzed reactions in which high concentrations of substrate and enzyme are required to give a measurable rate, some authors too readily attribute to the observed change in optical density a specific reaction. For example, the reported oxidation of D-erythrose 4-phosphate catalyzed by glyceraldehyde phosphate dehydrogenase (H. L. Kornberg and E. Racker, *Biochem. J.*, **61**, iii, (1955)) proceeds so slowly that the reaction could be due to an enzyme contaminant or a trace of D-erythrose, D-erythrose 2-phosphate, or D-erythrose 3-phosphate that might well be present.

(5) A. Ichihara and D. M. Greenberg, *Proc. Nat. Acad. Sci. (U. S.)*, **41**, 605 (1955).

(6) NOTE ADDED IN PROOF.—In more recent studies these workers have demonstrated the route: D-glyceric acid → D-glyceric acid 3-phosphate → hydroxypyruvic acid phosphate → serine phosphate → serine, using fractionated protein preparations from sheep liver.

reduced diphosphopyridine nucleotide of 2.7×10^{-5} *M*. The calculated concentration of hydroxypyruvic acid in the assay cuvette is 2.95×10^{-5} *M*. Thus, the yield of lactic dehydrogenase reducible substance, presumed to be hydroxypyruvic acid, was 92%.

Acknowledgment.—The support of Grant A884 from the United States Public Health Service is gratefully acknowledged.

Berkeley, California

[Contribution from the Department of Biochemistry, University of California, Berkeley]

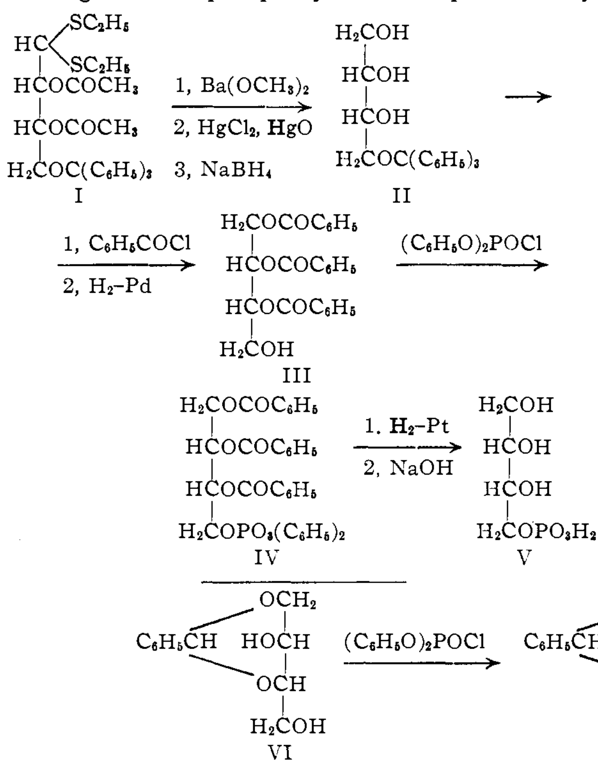
The Enantiomorphic Erythritol 4-Phosphates

By D. L. MacDonald, Hermann O. L. Fischer and Clinton E. Ballou

Received March 13, 1956

The enantiomorphic forms of erythritol 4-phosphate have been synthesized from D-glucose by reactions which permit the assignment of stereochemical configuration to each isomer. The two phosphates were isolated readily as their crystalline cyclohexylammonium salts.

Evidence for the metabolism of erythritol by *Propionibacterium pentosaceum via* a pathway involving its direct phosphorylation was presented by



Barker and Lipmann in 1949.[1] The primary product was suggested to be an erythritol phosphate.

We have recently become interested in the four carbon sugars, and have described a synthesis of D-erythrose 4-phosphate by which this important biological intermediate was obtained pure for the first time.[2] By a happy coincidence, we have been able to correlate our further efforts at the synthesis of erythritol phosphates with those of Janette Shetter,[4] in the Department of Plant Biochemistry, who was attempting to isolate and characterize the presumed erythritol phosphate resulting from the enzymatic phosphorylation of erythritol.

The synthesis of D-erythritol 4-phosphate was successfully carried out according to the following scheme (I–V). The starting material I was prepared as described in our paper on D-erythrose 4-phosphate.[2]

The final product was obtained as the crystalline cyclohexylammonium salt. It consumed two moles of periodate per mole, as required if the phosphate occupied a terminal position. The D-configuration is fixed by the mode of synthesis from a D-erythrose derivative obtained originally from D-glucose. The D-erythritol phosphate cyclohexylammonium salt showed a specific rotation (D-line of sodium) of $-2.3°$ in water, while the free acid gave $+2.6°$ in water.

The enantiomorphic L-erythritol 4-phosphate was also prepared from D-glucose. The hexose was converted into the known 4,6-*O*-benzylidene-D-glucose[3] which was oxidized with periodate, and the resulting D-erythrose derivative reduced to 2,4 *O*-benzylidene-D-erythritol. This compound can also be considered as a derivative of L-erythritol, namely, 1,3-*O*-benzylidene-L-erythritol. The further steps in the synthesis (VI–VIII) leading to L-erythritol 4-phosphate are illustrated by the formulas below. The L-erythritol 4-phosphate was isolated, like its enantiomorph as the crystalline cyclohexylammonium salt. The periodate consumption was two moles per mole of compound, clearly indicating that the phosphate group had been introduced in the primary hydroxyl group and not on the one unblocked secondary hydroxyl. The specific rotation (D-line of sodium) of the L-erythritol 4-phosphate cyclohexylammonium salt was $+2.3°$ in water.

Shetter[4] has succeeded in isolating an erythritol phosphate prepared by the phosphorylation of erythritol with adenosine triphosphate in the presence of extracts of *P. pentosaceum*. This ester was isolated as the cyclohexylammonium salt, and the

(1) H. A. Barker and F. Lipmann, *J. Biol. Chem.*, **179**, 247 (1949).

(2) C. E. Ballou, H. O. L. Fischer and D. L. MacDonald, This Journal, **77**, 2658, 5967 (1955).

(3) L. Zervas, *Ber.*, **64**, 2289 (1931).

(4) J. K. Shetter, This Journal, **78**, 3722 (1956).