reduced diphosphopyridine nucleotide of 2.7×10^{-6} *M*. The calculated concentration of hydroxypyruvic acid in the assay cuvette is 2.95×10^{-6} *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]

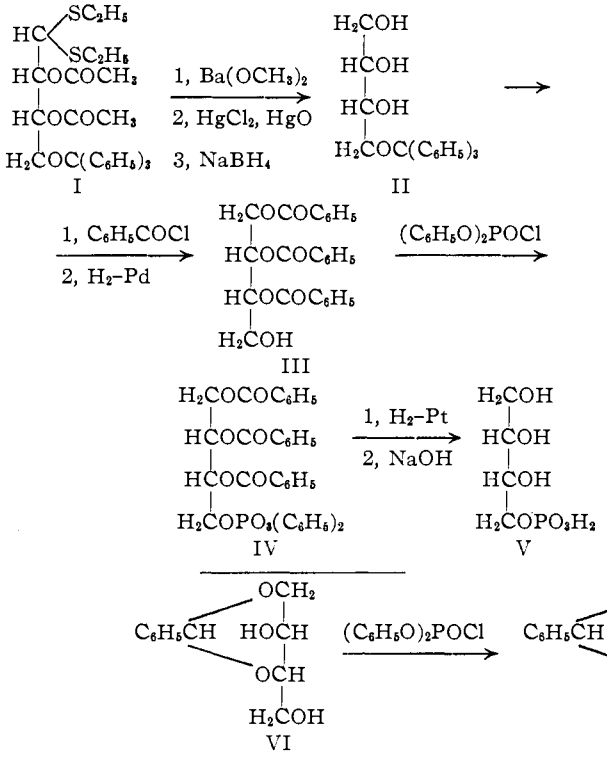
The Enantiomorphic Erythritol 4-Phosphates

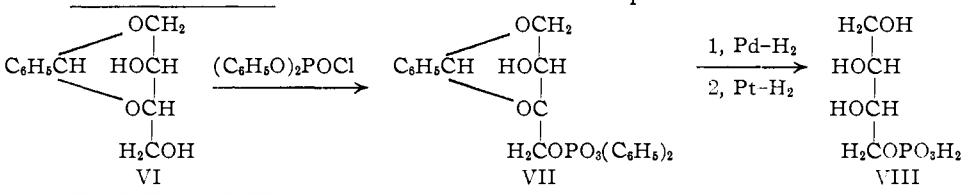
By D. L. MacDonald, Hermann O. L. Fischer and Clinton E. Ballou

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).

phosphate group has been shown by periodate oxidation to be in a primary position. Since this compound has a rotation very similar to that of our synthetic D-erythritol 4-phosphate, it may be concluded that the two are identical.

It is apparent that the preparation of the 4-carbon sugar phosphates by synthesis will be of great value in the work of elucidating the mechanisms by which these substances are metabolized. Aside from assisting in the proof of structure and the assignment of configuration, the syntheses will make these compounds readily available in reasonable amounts for further studies.

Experimental

4-*O*-Trityl-D-erythritol Tribenzoate.—4-*O*-Trityl-2,3-di-*O*-acetyl-D-erythrose diethyl mercaptal[2] (5.00 g.) was deacetylated with barium methylate in methanol. The solution was concentrated *in vacuo*, taken up in 125 ml. of acetone and placed in a three-necked flask equipped with a reflux condenser and a mercury-sealed stirrer. To the solution there was added 7.5 g. of mercuric oxide, 2.5 ml. of water, and with vigorous stirring, 7.5 g. of mercuric chloride dissolved in 25 ml. of acetone. The mixture was refluxed gently for one hour with stirring and then filtered into about 2 g. of mercuric oxide. The solvent was removed *in vacuo*, the residue was extracted several times with chloroform (total 125 ml.) and filtered and the chloroform solution was washed several times with an equal volume of ice-water. After drying (sodium sulfate) the solvent was removed at reduced pressure, and the residue taken up in 100 ml. of ethanol. A solution of sodium borohydride (0.6 g.) in water (5 ml.) was added; the grey precipitate, which formed at once, was removed by centrifugation after one hour, and the supernatant was concentrated *in vacuo*. The sirupy residue was dried by azeotropic distillation following the addition of benzene. The dried sirup was dissolved in anhydrous pyridine (50 ml.), and benzoyl chloride (12 ml.) was added while cooling the mixture in ice-water. After standing 18 hours, the excess benzoyl chloride was decomposed with a little ice, and the pyridine removed at reduced pressure. The residue was dissolved in methylene chloride and washed with 1 *N* sulfuric acid, 1 *N* potassium carbonate and with water, and dried (sodium sulfate). The solvent was removed at reduced pressure and the residue taken up in 40 ml. of *n*-propanol, from which there crystallized 4.39 g. (72%) of material, m.p. 137–140°. Recrystallization from *n*-propanol gave 4.17 g. of colorless material, m.p. 141–142°, $[\alpha]^{25}D$ +12.2° (*c* 2.5, dry dioxane).

Anal. Calcd. for $C_{44}H_{36}O_7$ (676.7): C, 78.07; H, 5.36. Found: C, 77.92; H, 5.42.

4-*O*-Trityl-D-erythritol.—The above tribenzoate (1.5 g.) was dissolved in 10 ml. of hot dry dioxane, 15 ml. of dry methanol was added and to the warm solution was added 0.3 ml. of 0.5 *N* barium methylate. After 90 minutes, the solution was concentrated *in vacuo*, the residue was taken up in methylene chloride and washed twice with water. The solution was dried (sodium sulfate) and the solvent removed at reduced pressure; the resulting viscous sirup crystallized spontaneously after one week. Two recrystallizations from benzene by addition of petroleum ether (b.p. 60–70°) and seeding, gave 0.51 g. (63%) of material, m.p. 86–88°, $[\alpha]^{23}D$ −1.2° (*c* 3, dry dioxane).

Anal. Calcd. for $C_{23}H_{24}O_4$ (364.4): C, 75.78; H, 6.64 Found: C, 75.83; H, 6.61.

The 4-*O*-trityl-D-erythritol can also be obtained directly from 4-*O*-trityl-2,3-diacetyl-D-erythrose diethyl mercaptal in a yield of 54% by following the demercaptalation and reduction steps, but omitting the benzoylation step described above for the preparation of the tribenzoate.

1,2,3-Tri-*O*-benzoyl-D-erythritol.—Two grams of finely powdered 4-*O*-trityl-D-erythritol tribenzoate was shaken with hydrogen in 100 ml. of absolute ethanol with 2 g. of reduced and washed palladium catalyst (5% palladium chloride-on-carbon).[5] After 20 hours, when the starting material had all dissolved, the catalyst was centrifuged off, and the supernatant was concentrated *in vacuo* to dryness. The crystalline residue was extracted twice with 50-ml. portions of warm petroleum ether (b.p. 30–60°) to remove triphenylmethane, and the crystalline tribenzoate was collected by filtration. It weighed 1 g. (78%). Two recrystallizations from absolute ethanol give the pure compound with m.p. 135–136°, $[\alpha]^{26}D$ −56.2° (*c* 5, chloroform).

Anal. Calcd. for $C_{25}H_{22}O_7$ (434): C, 69.1; H, 5.07. Found: C, 69.1; H, 4.97.

Phosphorylation.—One gram of the crude tri-*O*-benzoyl-D-erythritol was dissolved in 5 ml. of pyridine at 5°, and 1 g. of diphenyl phosphorochloridate was added dropwise. After 1 hour at 5°, the reaction mixture had solidified. After an additional 16 hours at 5°, 50 ml. of water was added with mixing, and the crystalline solid was filtered off. It was washed free of pyridine, then dried in air. The product weighed 1.5 g. (90%). A sample was recrystallized twice from absolute ethanol and dried in a high vacuum at 57°. It melted at 128–132° and showed $[\alpha]^{25}D$ −1.1° (*c* 2.5, chloroform).

Anal. Calcd. for $C_{37}H_{31}O_{10}P$ (666): C, 66.7; H, 4.65; P, 4.65. Found: C, 66.9; H, 5.09; P, 4.50.

D-Erythritol 4-Phosphate.—The crude product from above (1.2 g.) was shaken in 100 ml. of absolute ethanol with 0.5 g. of platinum oxide catalyst and hydrogen gas to remove the phenyl groups. The hydrogen uptake ceased after 4.5 hours at 960 ml. (theory 690). The catalyst was centrifuged off, and 15 ml. of 1 *N* potassium hydroxide was added to the supernatant alcoholic solution. After 15 hours to complete saponification, the alcohol was removed by concentration *in vacuo*. The residue, in 50 ml. of water, was filtered through Filter-cel to remove a little carbon, and the filtrate was treated batchwise with 20 ml. of Dowex 50 (H^+ form, 2 meq./ml.) to remove the cations. After filtration to remove the resin, the solution was brought to about *p*H 9 with cyclohexylamine, and then was concentrated *in vacuo* to a white solid product. This dicyclohexylammonium D-erythritol 4-phosphate crystallized from hot absolute ethanol as fine needles. After drying in a high vacuum over phosphorus pentoxide at room temperature for three hours, the substance melted at 183–186° (softened above 177°). It showed $[\alpha]^{25}D$ −2.3° (*c* 3, water); $[\alpha]^{25}D$ +2.6° (*c* 1.5, free acid in water).

Anal. Calcd. for $C_{16}H_{37}O_7PN_2$ (400): C, 48.0; H, 9.25; N, 7.0; P, 7.75. Found: C, 48.3; H, 8.90; N, 6.85; P, 7.46.

A 40.3-mg. sample of the above salt (0.100 mmole) consumed 0.19 mmole of periodate. The consumption of 2 moles of periodate per mole of the erythritol phosphate confirms the assignment of the phosphate group to a terminal position.

2,4-*O*-Benzylidene-D-erythritol (1,3-*O*-Benzylidene-L-erythritol).—Ten grams of 4,6-*O*-benzylidene-D-glucose (m.p. 167–170°)[3] was oxidized to 2,4-*O*-benzylidene-D-erythrose as has been described.[6] The ethyl acetate extract was concentrated at reduced pressure and then ethanol was added and removed *in vacuo*. The residue was dissolved in 200 ml. of ethanol and a solution of 2 g. of sodium borohydride in 20 ml. of water was added. After one hour at room temperature, the solution was concentrated *in vacuo*, the residue was dissolved in 250 ml. of ethyl acetate and the solution was washed twice with 50-ml. portions of 10% aqueous sodium sulfate. The dried (sodium sulfate) solution was concentrated to dryness at reduced pressure, to give 7.0 g. (89%) of colorless crystals, which were recrystallized from 35 parts of chloroform to give 5.56 g. of material with m.p. 135–136°, $[\alpha]^{23}D$ −43.0° (*c* 2, methanol). Addition of three volumes of petroleum ether to the mother liquors gave a further 0.70 g. of material of the same m.p. making the purified yield 80%.

Anal. Calcd. for $C_{11}H_{14}O_4$ (210.2): C, 62.85; H, 6.71. Found: C, 62.67; H, 6.81.

L-Erythritol 4-Phosphate.—To an ice-cold solution of 2.82 g. of diphenyl phosphorochloridate in 10 ml. of anhydrous pyridine, 2.00 g. of 1,3-*O*-benzylidene-L-erythritol was added, and the solution was kept for 36 hours at 5°. The excess acid chloride was decomposed by the addition of ice, and the solution was concentrated *in vacuo*. The residue was taken up in methylene chloride and washed with cold

(5) C. E. Ballou and H. O. L. Fischer, THIS JOURNAL, **76**, 3188 (1954).

(6) J. C. Sowden, *ibid.*, **72**, 808 (1950).

1 *N* sulfuric acid, 1 *N* potassium carbonate and water and dried (sodium sulfate). The solvent was removed at reduced pressure, and the residue, dissolved in methanol, was filtered from traces of insoluble matter and concentrated *in vacuo* to give 3.39 g. (80%) of a viscous sirup.

This sirup was taken up in 100 ml. of dry methanol and reduced with hydrogen and 2 g. of reduced and washed 5% palladium chloride on charcoal.[5] The hydrogenation was complete in 4.5 hours with the uptake of 470 ml. of hydrogen. The catalyst was removed by centrifugation and the solution was returned to the hydrogenation vessel and reduced using 0.5 g. of platinum oxide catalyst. The hydrogenation proceeded rapidly, with the uptake of 1960 ml. of hydrogen in one hour. The catalyst was centrifuged off, the phosphate ester converted to its salt by the addition of 3.5 ml. of cyclohexylamine, and the solution was concentrated to dryness *in vacuo*. The crystalline residue was dissolved in a minimum of hot 95% ethanol and allowed to crystallize at −10°. The yield was 1.80 g. (47%), m.p. 186–190° with some decomposition, $[\alpha]^{23}D$ +2.3° (*c* 5, water). A further 0.25 g. (6%) of material of the same melting point was obtained by addition of ether to the mother liquors, followed by recrystallization of the precipitated material from ethanol.

Anal. Calcd. for $C_{16}H_{37}O_7N_2P$ (400): C, 48.0; H, 9.25; N, 7.00; P, 7.75. Found: C, 47.81; H, 9.41; N, 7.19; P, 7.88.

On treatment with sodium periodate, the material consumed 1.95 moles of periodate per mole of compound, indicating that the phosphate group was on a terminal, not a secondary, position.

Acknowledgment.—The work was supported in part by grants from the Nutrition Foundation, the Eli Lilly Research Grants Committee, and the U. S. Public Health Service (Research Grant A-884).

BERKELEY 4, CALIF.

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

Formation of D-Erythritol 4-Phosphate by Propionibacterium Pentosaceum[1]

BY JANETTE K. SHETTER

RECEIVED MARCH 13, 1956

Extracts of *Propionibacterium pentosaceum* catalyze the phosphorylation of erythritol by adenosine triphosphate. The product of the reaction has been isolated and characterized as D-erythritol 4-phosphate.

Dried cells of *Propionibacterium pentosaceum* have been shown to catalyze a phosphate transfer between adenosine triphosphate (ATP) and erythritol.[2] In the presence of pyruvate and fluoride, erythritol disappears with the uptake of orthophosphate and the formation of an approximately equivalent amount of a difficultly hydrolyzable phosphate ester which was thought to be phosphoerythronic acid. However, the phosphate esters formed from erythritol by this organism have not been characterized adequately. In view of the recent work[3–8] indicating that erythrose phosphate plays an important role in the metabolism of plants and animals, it seemed desirable to study the metabolism of erythritol by *P. pentosaceum* in more detail. This paper describes the isolation and characterization of the phosphate ester formed by reaction between erythritol and ATP.

Experimental

Bacterial Preparation.—*Propionibacterium pentosaceum*, strain E.21 was grown in 12-liter flasks containing 10 liters of medium of the following composition in grams per liter of distilled water: sodium lactate 15, Difco yeast extract 7.5, Difco tryptone 4.0, ammonium sulfate 1.5, dipotassium hydrogen phosphate trihydrate 3.76, sodium dihydrogen phosphate monohydrate 1.13, and magnesium sulfate heptahydrate 0.05. The medium was inoculated with 5% (v./v.) of an active culture in the same medium and incubated at 30° for about 50 hours, by which time most of the lactate was decomposed. To adapt the cells to erythritol, 0.5 g. of erythritol was then added per liter as a sterile solution, and the incubation was continued for an additional 12 to 18 hours when most of the erythritol was decomposed. The bacteria were harvested in a Sharples centrifuge, washed three times and resuspended in 20 ml. of 0.05 *M* triethanolamine buffer *p*H 7.0 per liter of growth medium. To disrupt the cells, 50 ml. of the suspension and 2 g. of fine carborundum were treated for 20 minutes at 0 to 5° in a 10 KC Raytheon Sonic oscillator. The resulting suspension was centrifuged for 15 minutes at 15,000 × *g* and the clear supernatant extract was stored at −10°. Such a preparation can be kept at least three weeks without loss of activity.

Assay.—The activity of the extracts was roughly assayed by determining the difference in rate of decomposition of ATP, measured as acid-labile phosphate, between an erythritol containing reaction mixture and a control without substrate. The reaction mixture contained 0.005 *M* sodium iodoacetate, 0.005 *M* magnesium chloride, 0.043 *M* sodium fluoride, 0.1 *M* triethanolamine *p*H 7.0, 0.005 *M* ATP, 0.05 *M* erythritol (when present), and bacterial extract equivalent to 12 mg. of dry cells per ml. The mixture was incubated for 1 hour at 30° and then both inorganic phosphate and acid-labile phosphate were determined. Good extracts have an activity corresponding to the phosphorylation of about 0.3 μmole of erythritol per mg. dry cells per hour under the test conditions.

Materials.—ATP^{32}, labeled in the two terminal positions with P^{32}, was prepared from rat liver mitochondria by the method of Kielley and Kielley,[9] starting with 45 μmoles of orthophosphate having a specific activity of approximately 67 μcuries per μmole. P^{32}-Labeled L-α-glycerophosphate was prepared by the method of Kornberg and Pricer.[10] The dicyclohexylammonium salt of synthetic D-erythritol 4-phosphate was donated by Dr. C. E. Ballou.[11]

Analyses.—Orthophosphate was determined by the Fiske-SubbaRow method[12] and total phosphate by the same

(1) This work was supported in part by a Research Grant (E-563) from the U. S. Public Health Service, Department of Health, Education and Welfare.

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