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.

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

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

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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

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method after digesting with a sulfuric acid–nitric acid mixture. Acid-labile phosphate was defined as the orthophosphate formed during 7 minutes hydrolysis in 1 *N* hydrochloric acid at 100°.

Formation of Erythritol Phosphate.—To prepare sufficient erythritol phosphate for isolation, 500 ml. of a reaction mixture was used containing 200 ml. of *P. pentosaceum* extract and the following components: sodium iodoacetate 0.005 *M*, magnesium chloride 0.008 *M*, sodium fluoride 0.045 *M*, erythritol 0.025 *M*, potassium bicarbonate saturated with carbon dioxide 0.1 *M*, and sodium ATP^{32} 0.008 *M* containing 0.25 μcurie P^{32} per μmole. This reaction mixture was incubated 2 hours at 30°. A control reaction mixture without erythritol was incubated under the same conditions.

Isolation Procedure.—The reaction was stopped by boiling the solution for 3 minutes. After centrifuging off the coagulated protein, cations were removed by stirring the solution with 100 ml. of Dowex-50-H$^+$. After filtration the nucleotides were removed by adsorption on 20 ml. of acid washed charcoal, Nuchar C-190. The charcoal eluate and washings were lyophilized, dissolved in water, neutralized with ammonium hydroxide to *p*H 8.5 and made up to 100 ml. At this stage the erythritol reaction mixture contained 81% of the initial P^{32} whereas the control reaction mixture contained 46%.

The charcoal eluates were adsorbed on 9.6 cm.^{2} × 27 cm. Dowex-1-formate, 8% crossed linked, 200–400 mesh columns and were eluted with a solution containing 0.1 *M* ammonium formate and 0.01 *M* sodium tetraborate. Most of the P^{32} appeared in two broad overlapping zones with peaks at 580 and 880 ml. of total eluate. Paper chromatography with *n*-butanol–acetic acid as a developing solvent showed that all fractions in these zones contained both orthophosphate and a phosphate ester of lower R_f. The 580 and 880 ml. peaks, containing a total of 4.3 mmoles of phosphate, apparently represent orthophosphate and erythritol phosphate, respectively. The elution pattern of the control experiment on Dowex-1-formate showed a single P^{32} peak corresponding to 0.64 mmole of orthophosphate.

All fractions from the Dowex-1 column containing the phosphate ester were combined, cations were removed with Dowex-50, the solution was evaporated to dryness, and formic and boric acids were removed as their volatile methyl esters by distillation with anhydrous methanol.[13] The residue was dissolved in water, neutralized to *p*H 8.5, and the orthophosphate was precipitated with an excess of barium bromide. The barium salt of the phosphate ester was precipitated by addition of 6 volumes of ethanol. The precipitate was dissolved in water, barium ions removed with Dowex-50-H$^+$, and the free acid was neutralized with a 1 *M* solution of cyclohexylamine.[11,14] On evaporation, 203 mg. of a crystalline dicyclohexylamine salt was obtained which was recrystallized from 50 ml. of absolute ethanol to yield 116 mg. of an almost colorless product that was dried *in vacuo* at 25° over phosphorus pentoxide.

Identification of Erythritol Phosphate.—Elementary analysis of the dicyclohexylamine salt gave the following values.

Anal. Calcd. for $C_{16}H_{37}O_7N_2P$ (400.45): C, 48.0; H, 9.3; N, 7.00; P, 7.75. Found: C, 47.6; H, 9.45; N, 7.0; P, 7.5.

The compound was shown to be a phosphate ester of erythritol by hydrolysis with human seminal phosphatase and identification of the resulting erythritol by paper chromatography. The phosphatase preparation was made by precipitating seminal plasma proteins with 0.8 saturated ammonium sulfate, which effectively removed the fructose present, and redissolving in water to the original volume. The hydrolysis was done with 0.1 ml. of this preparation, 10 μmoles of sodium citrate buffer *p*H 5.4, and 6.2 μmoles of erythritol phosphate in a total volume of 0.5 ml. Appropriate controls with no substrate and with glycerophosphate in place of erythritol phosphate were incubated simultaneously under conditions (36°, 2 hours) shown to cause complete hydrolysis of glycerophosphate. Paper chromatography of 0.01 ml. of the reaction mixtures along with known samples of glycerol, erythritol, adonitol and mannitol in butanol–pyridine–water 4:1:1,[15] and phenol–water, followed by spraying with a periodate reagent (ref. 16, test 1) gave evidence for the presence of erythritol in the isolated compound. A negative reaction with *p*-anisidine reagent[17] on a duplicate chromatogram indicated that the periodate-positive compound was not a reducing sugar.

The position of the phosphate group on the erythritol ester could be determined by periodate oxidation since erythritol 3-phosphate should react with one equivalent of periodate without formation of formate, whereas erythritol 4-phosphate should react with two equivalents of periodate and produce one mole of formate. Only two erythritol phosphates are possible, since the 1- and 4-positions, and the 2- and 3-positions are equivalent. Accordingly, 3.1 μmoles of the isolated compound, calculated from its P content, was incubated with 10 μmoles of potassium periodate in 1 ml. for 45 minutes at 37° and back titrated with thiosulfate; 6.2 μmoles of periodate was consumed indicating that the phosphate is at the 4-position. In a similar experiment with 10 μmoles of erythritol phosphate, 9.5 μmoles of formic acid was found by titration following vacuum distillation of the acid by the method of Grant.[18]

The isolated compound and synthetic D-erythritol 4-phosphate[11] showed identical chromatographic behavior on Whatman No. 1 paper, as indicated by an orthophosphate test[19] following treatment with ultraviolet light,[20] in the following solvents: *t*-butyl alcohol–water containing 4 g. of picric acid per 100 ml. 80:20[19]; ethyl acetate–acetic acid–water 3:3:1[21]; acetone–50% monochloroacetic acid 7:3[22]; and *n*-butanol–acetic acid–water 52:13:35.[23]

The specific rotation at 589 mμ of the isolated erythritol phosphate was −2.6° in water. This is sufficiently close to the value of −2.3° for synthetic D-erythritol 4-phosphate[11] to establish the identity of the two compounds.

Discussion

Although the results demonstrate the conversion of erythritol to D-erythritol 4-phosphate, the role of this compound in the metabolism of erythritol by *P. pentosaceum* is not established. Earlier evidence[2] for the formation of 4-phosphoerythronic acid, a possible product of erythritol phosphate oxidation, is inconclusive. Recent experiments show that dried cell preparations sometimes oxidize erythritol more rapidly than erythritol phosphate. Since the preparation contains an active phosphatase, the possibility that erythritol phosphate is hydrolyzed before oxidation cannot yet be excluded.

The attempt to separate orthophosphate and erythritol phosphate on a formate column by the addition of borate[24] was unsuccessful, indicating that borate does not form a strong complex with this ester. This is in agreement with the work of Böeseken[25] who found that straight chain polyalcohols with two or three adjacent *cis* hydroxyl groups form relatively weak borate complexes.

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