Erythritol Metabolism by *Propionibacterium pentosaceum*. The Role of L-Erythrulose 1-Phosphate*

Edward J. Wawszkiewicz†

ABSTRACT: L-Erythrulose 1-phosphate, the compound previously postulated to be the first intermediate following L-erythritol 1-phosphate in the pathway of erythritol metabolism in *Propionibacterium pentosaceum*, has been found in dried-cell preparations metabolizing erythritol in the presence of fluoride. Added to cell-free extracts, this compound can be either reduced to L-erythritol 1-phosphate (with reduced nicotinamide–adenine dinucleotide as H donor) or cleaved between the 3 and 4 positions, depending on the experimental conditions.

Conversely, L-erythritol 1-phosphate can act as substrate for nicotinamide–adenine dinucleotide reduction in these extracts and dihydroxyacetone phosphate and formaldehyde can condense to form erythrulose phosphate.

Previous reports (Wawszkiewicz and Barker, 1965; Wawszkiewicz, 1966) have indicated that erythritol metabolism in *Propionibacterium pentosaceum* proceeds *via* a series of phosphorylated intermediates, of which L-erythritol 1-phosphate is the primary member. Until recently, however, only indirect evidence has been available as to the nature of the intermediate immediately following L-erythritol 1-phosphate in the metabolic sequence. The compound thought most likely to fill this role, L-erythrulose 1-phosphate, has been synthesized by Gillett and Ballou (1963) and has been shown by these investigators to undergo slow reduction to L-threitol 1-phosphate in a reaction catalyzed by rabbit muscle glycerol 3-phosphate:NAD[1] oxidoreductase (EC 1.1.1.8). We have now reinvestigated the complement of products arising in dried-cell preparations of *P. pentosaceum* metabolizing erythritol in the presence of fluoride to see if erythrulose 1-phosphate can be found among the sugar phosphates which accumulate. Using fractionated cell-free extracts of erythritol-adapted cells we have studied L-erythritol 1-phosphate oxidation and L-erythrulose 1-phosphate reduction. We have also investigated the breakdown and synthesis of erythrulose 1-phosphate labeled with ^{14}C in the 4 position.

Materials and Methods

Preparation of Enzymically Active Materials. P. pentosaceum E 2.1 was grown on the medium of Shetter (1956) modified by the use of tap water in place of distilled water in its preparation. Cells were dried according to the method of Barker and Lipmann (1949). Cell extracts were made from cells which had been frozen at −70° and stored at −10°. Suspensions (25 ml) containing equal parts by weight of packed wet cells and 0.2 M Tris-HCl buffer at pH 7.4 were shaken with 30-g quantities of 0.18-mm glass beads in a Merkenschlager *et al.* (1957) mechanical cell homogenizer (a product of B. Braun, Germany) for 45 sec at 4000 rpm. The resulting mixture of beads, extract, and cell debris was centrifuged for 1 hr at 24,000*g* and the supernatant extract (which usually contained approximately 20 mg of protein/ml) was then decanted. Enough solid ammonium sulfate (Mann Research Laboratories, Enzyme Grade) was added to it with constant stirring at 0° to yield a 40% saturated solution. After a 15-min equilibration period the resulting suspension was centrifuged for 1 hr at 24,000*g*. The supernatant liquid was discarded and the protein precipitate was either dissolved in a minimum quantity of 0.2 M Tris–HCl (pH 7.4) buffer to yield a solution containing approximately 35 mg of protein/ml or stored at 0°. Precipitates so stored retained their L-erythritol 1-phosphate:NAD oxidoreductase activity for several months. Concentrated cell extract used in some experiments was a saturated solution of protein precipitated from crude extracts by the addition of ammonium sulfate to 80% saturation. ATP:erythritol phosphotransferase (EC 2.7.1.27) was prepared from diluted raw cell extracts by the method of Holten and Fromm (1961). Erythrulose 1-phosphate:formaldehyde lyase (EC 4.1.2.2) was obtained as a rat liver extract (Charalampous and Mueller, 1953). Potato orthophosphoric monoester phosphohydrolase (EC

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† Present address: Institute for Biomedical Research, Chicago, Ill. 60610.

[1] Abbreviations used: NAD, nicotinamide–adenine dinucleotide; NADP, nicotinamide–adenine dinucleotide phosphate; ATP, adenosine triphosphate.

2.1.3.2) was purchased from Sigma Chemical Co., St. Louis.

Preparation of Substrates. Randomly labeled erythritol-^{14}C (2.26 mc/mmole) was obtained from Nuclear Research Chemicals, Inc., Orlando, Fla., and was purified by descending paper chromatography with *n*-butyl alcohol–ethyl alcohol–water (52.5:32:15.5, v/v). L-Erythrulose was prepared by the method of Charalampous and Mueller (1953). D-Erythrose and D-erythrose 4-phosphate were obtained from Calbiochem, Los Angeles. L-Erythritol 1-phosphate was prepared by the method of Holten and Fromm (1961). L-Erythrulose 1-phosphate was a generous gift from Professor C. E. Ballou. C_4-labeled erythrulose-^{14}C 1-phosphate was prepared by the method of Charalampous and Mueller (1953) from formaldehyde-^{14}C (0.5 mc/mmole, Nuclear Research Chemicals, Inc.) and unlabeled dihydroxyacetone phosphate (Sigma Chemical Co.). Enzymatically reduced NAD and NADP were also purchased from Sigma.

Separation and Identification of Reaction Mixture Components. Dried-cell reaction mixtures were analyzed by paper chromatography in two dimensions as previously described (Wawszkiewicz, 1961; Wawszkiewicz and Barker, 1966). Free sugars or sugar alcohols were separated by electrophoresis on 8-in. wide strips of Whatman no. 1 paper in a Savant high-voltage plate electrophoresis apparatus. The buffer used was saturated boric acid adjusted to pH 6.0 with sodium hydroxide. Electrophoresis was carried out for 4 hr at 1800–2000 V, 20–25 ma. Under these conditions caffeine (used as an electroosmosis marker) and threitol have identical mobilities, both migrating 18.5 cm toward the cathode, erythritol moves slightly toward the cathode and erythrulose moves 3–4 cm toward the anode. Radioactive materials on chromatograms were detected by standard radioautographic methods on Kodak No-Screen Medical X-ray film or by scanning the papers directly in a P10 gas (argon–methane) automatic chromatogram scanner (Kisieleski and Smetana, 1958). Both free sugars and sugar phosphates on chromatograms were located by the periodate–benzidine method of Gordon *et al.* (1956).

Determinations. Protein was estimated by the procedure of Lowry *et al.* (1951). Reduction or oxidation of nicotinamide nucleotide coenzymes was measured spectrophotometrically at 340 mμ in a Beckman DU spectrophotometer fitted with a Gilford multiple-sample absorbance recorder.

Results

L-Erythritol 1-Phosphate Oxidation and L-Erythrulose 1-Phosphate Reduction. In previous studies (E. J. Wawszkiewicz and H. A. Barker, submitted for publication), we demonstrated with dried-cell extracts of *P. pentosaceum* that L-erythritol 1-phosphate can act as an H donor for 2,6-dichlorobenzenone indophenol reduction at physiological pH levels. Until recently, however, we have found it impossible to show the coupling of its oxidation with reduction of one of the nicotinamide nucleotide coenzymes. We have now observed the latter, a slow ($\Delta OD_{340\,m\mu}$/min per mg of protein = 0.025 in a 1-cm light-path cuvet), enzyme-dependent reduction of NAD in the presence of 40–70% saturation ammonium sulfate fractions of fresh cell extracts and of chromatographically pure L-erythritol 1-phosphate, at pH 9.2. Previous evidence had indicated that of the several possible products of L-erythritol 1-phosphate oxidation, L-erythrulose 1-phosphate is the one most likely to be formed in our system. Our cell extract fractions which at high pH values slowly oxidize L-erythritol 1-phosphate actively reduce L-erythrulose 1-phosphate at pH 6.8 with reduced NAD as the H donor. These extract fractions will also reduce L-erythrulose but, as seen in Table I, the rate of the latter reduction is approximately one-eighth that of the reduction of the phosphorylated derivative. Whether or not both reductions are catalyzed by the same enzyme remains to be determined. D-Erythrose and D-erythrose 4-phosphate seem to have no effect on the system.

TABLE I: Oxidation of Reduced NAD in the Presence of Various Substrates.[a]

Substrate	$\Delta OD_{340\,m\mu}$/min
L-Erythrulose 1-phosphate	0.243
L-Erythrulose	0.027
D-Erythrose 4-phosphate	0.000
D-Erythrose	0.000

[a] A solution of a 40–70% saturation ammonium sulfate cell extract precipitate was prepared as described under Materials and Methods and diluted with enough water to yield a mixture containing approximately 2 mg of protein/ml. Quantities (0.01 ml) were added at zero time to 0.65-ml silica cuvets of 1-cm light path containing 100 μmoles of potassium phosphate buffer at pH 6.8, 0.1 mg of reduced NAD, and either 0.4 μmole of L-erythrulose 1-phosphate or 2 μmoles of L-erythrulose or 0.4 μmole of D-erythrose 4-phosphate or 0.8 μmole of D-erythrose dissolved in enough water to yield a final volume of 0.49 ml. Optical density changes at 340 mμ were measured against a water blank. The values recorded above have been corrected for that oxidation of reduced NAD which takes place in the presence of enzyme but in the absence of added substrate ($\Delta OD_{340\,m\mu}$/min of 0.005).

The Product of L-Erythrulose 1-Phosphate Reduction. The product of L-erythrulose 1-phosphate reduction has the chromatographic properties of L-erythritol 1-phosphate in both the phosphate ester solvents which we have used (see Materials and Methods) in analyzing reaction mixtures. However, since L-erythritol

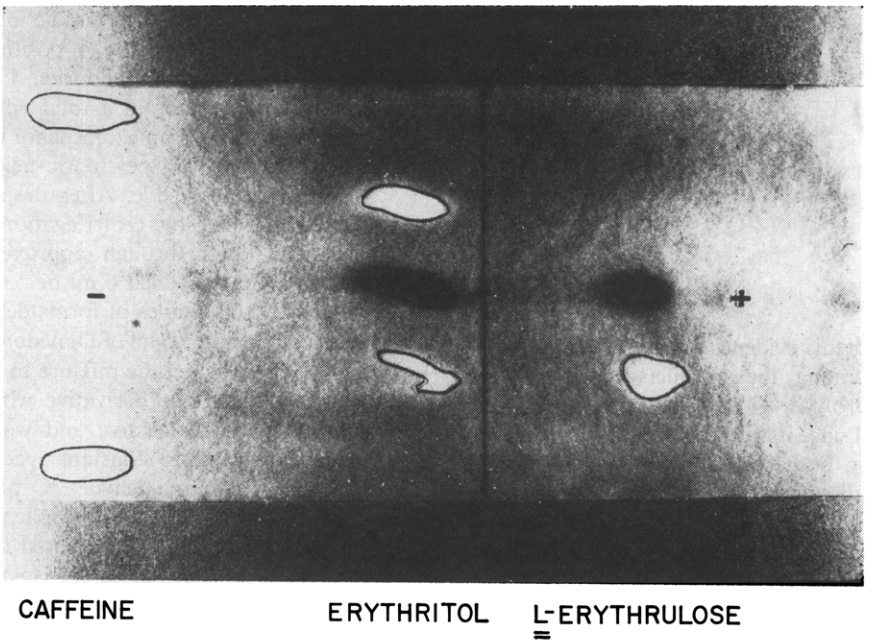

FIGURE 1: High-voltage electrophoretogram and (superimposed) its radioautogram showing the hydrolyzed product of ^{14}C-labeled L-erythrulose 1-phosphate reduction. The conditions of the experiment are given in the text.

1-phosphate, L-threitol 1-phosphate, and indeed even L-erythrulose 1-phosphate have identical mobilities in these solvents (nor can they be separated from each other electrophoretically), it has been necessary to remove the phosphate group from the reduction product in order to ascertain its structure. To increase the sensitivity of the analytical procedure we used erythrulose-^{14}C 1-phosphate labeled in the 4 position as starting material. We incubated 0.4 μmole of potassium phosphate buffer at pH 7.4, 1 mg of reduced NAD, and a solution of 40–70% saturation ammonium sulfate precipitate capable of oxidizing 0.3 μmole of reduced NAD/min with L-erythrulose 1-phosphate as the H acceptor, in a total volume of 1.3 ml, at 30° for 10 min, then adjusted the pH of the solution to 5.0 with dilute acetic acid and added 0.2 ml of a solution of acid phosphatase capable of hydrolyzing 0.06 μmole of monophosphate ester/min at 37°. We incubated the mixture at that temperature for 30 min, then added 3 ml of 11.5% trichloroacetic acid solution, centrifuged down the precipitate, extracted the supernatant liquid five times with 7-ml quantities of ether, passed the aqueous layer through approximately 1 ml of Dowex 50 (H^+), then through an equal volume of Dowex 2 (HCO_3^-), and finally brought it to dryness over phosphorus pentoxide *in vacuo*. We then subjected the residue to coionophoresis with unlabeled erythritol and L-erythrulose in a boric acid buffer as described under methods. A radioautogram of the paper strip revealed two spots exactly congruent with the erythritol and erythrulose spots located with periodate–benzidine (see Figure 1). Although there was also a trace streak of radioactive material running from the erythritol spot toward the cathode no discrete spot occurred in the region into which threitol would have been expected to migrate (*i.e.*, parallel to the caffeine area). In a control experiment in which cell extract was omitted from the reaction mixture only one radioactive spot was detected on the electrophoretogram, exactly congruent with L-erythrulose. We therefore conclude that under the conditions of the experiment erythrulose 1-phosphate is reduced primarily to erythritol 1-phosphate and that the reduction is catalyzed by an enzyme other than glycerol phosphate dehydrogenase (also present in our extracts), known slowly to catalyze the reduction of L-erythrulose 1-phosphate to the L-threitol derivative. We choose tentatively to call the enzyme L-erythritol 1-phosphate:NAD oxidoreductase but, of course, we cannot say what the limits of its specificity are until it has been purified. The enzyme is active with NADP as well as with NAD but the rate of its activity with the former is only one-half that observed with NAD.

The Presence of L-*Erythrulose 1-Phosphate in Fluoride-Poisoned Cell Suspensions Metabolizing Erythritol.* In a system containing this enyzme and other enzymes involved in the metabolism of erythritol *via* its L-1-phosphate derivative one might expect to find, with fluoride added to stop the reactions at the glyceric acid phosphate level and to inhibit phosphatases, traces of L-erythrulose 1-phosphate accumulating as an intermediate during erythritol metabolism. Evidence suggesting that an erythrulose phosphate may accumulate in reaction mixtures of this kind has already been presented (Wawszkiewicz and Barker, 1965; E. J. Wawszkiewicz and H. A. Barker, submitted for publi-

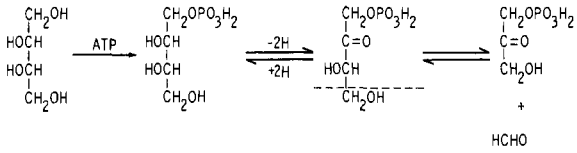

FIGURE 2: Initial reactions in erythritol metabolism by *P. pentosaceum*.

cation). However, this evidence is based on the finding of erythrulose among the products resulting from phosphatase hydrolysis of all the reaction mixture components. And since it is possible that the erythrulose found could have been present as such prior to the hydrolysis, more direct evidence on this point is needed. When synthetic L-erythrulose 1-phosphate became available and its chromatographic properties had been determined a rational approach to its isolation presented itself. We incubated a reaction mixture consisting of 100 mg of dried cells, 4.42 μmoles of randomly labeled erythritol-^{14}C (10 μc), 10 μmoles of sodium pyruvate, 40 μmoles of sodium fluoride, and 40 μmoles of potassium phosphate buffer at pH 6.7, all in a volume of 1 ml of water, for 10 min at 23°, then worked it up for analysis and chromatographed it in two dimensions as previously described (Wawszkiewicz, 1961; E. J. Wawszkiewicz and H. A. Barker, submitted for publication). Since L-erythrulose 1-phosphate has the same mobility in both chromatography solvents as does L-erythritol 1-phosphate, the region of the chromatogram containing the latter should also hold whatever L-erythrulose 1-phosphate may accumulate. Accordingly we eluted the L-erythritol 1-phosphate region from the chromatogram, freed the radioactive materials in it from picric acid by chromatography in solvent I, incubated them with acid phosphatase, and subjected the products to electrophoresis in boric acid buffer as in the previous experiment. Scanning the electrophoretogram with a gas-flow counter revealed a small but distinct peak of radioactivity in the erythrulose region and the congruence of this radioactivity with the erythrulose spot was confirmed by radioautography. Since no other sugar or sugar alcohol which we have tested migrates to the erythrulose region under the conditions of the electrophoresis, and free erythrulose could not have been present in the L-erythritol 1-phosphate region of the chromatogram, we conclude that the original reaction mixture contained an erythrulose phosphate. Because our enzyme system is known to exhibit L-erythritol 1-phosphate oxidoreductase activity we can assume with some confidence that this erythrulose phosphate is the L sugar phosphorylated in the 1 position.

Cleavage of L-*Erythrulose 1-Phosphate.* L-Erythrulose 1-phosphate is known to undergo enzymic cleavage to formaldehyde and dihydroxyacetone phosphate in a variety of systems (Charalampous and Mueller, 1953; Mueller *et al.*, 1955; Gillett and Ballou, 1963). To determine whether this cleavage can occur in *P. pentosaceum* we incubated 0.16 μmole of erythrulose-^{14}C 1-phosphate labeled in the 4 position (40,000 cpm) with 100 μmoles of sodium fluoride, 1 ml of cell extract concentrate, and water in a total volume of 2 ml for 45 min at 30° under an atmosphere of nitrogen, then added 0.5 ml of 60% perchloric acid and 0.5 ml of 5 N potassium hydroxide. After the resulting mixture had been clarified by centrifugation we passed the supernatant liquid through approximately 5 ml of Dowex 2 (HCO_3^-), added 6 ml of 2 M sodium acetate to the eluate, 100 μmoles of formaldehyde dissolved in 0.1 ml of water, and 1.5 ml of Dimedon reagent (Reeves, 1941). We then heated the mixture to boiling and after cooling it isolated the derivative which had formed. It proved to be radioactive and we recrystallized it from alcohol–water to constant specific activity. The total amount of formaldehyde-^{14}C which could be so isolated was 3720 cpm. In a duplicate experiment in which cell extract had been omitted from the reaction mixture only 26 cpm could be isolated as Dimedon derivative. We conclude, therefore, that our cell extract does contain an enzyme or enzymes capable of cleaving the erythrulose 1-phosphate at the 4 position.

Synthesis of L-*Erythrulose 1-Phosphate.* The equilibrium of the reaction L-erythrulose 1-phosphate ⇌ formaldehyde + dihydroxyacetone phosphate, whether catalyzed by rabbit muscle fructose 1,6-diphosphate D-glyceraldehyde 3-phosphate lyase (EC 4.1.2.13; Gillett and Ballou, 1963) or by erythrulose 1-phosphate formaldehyde lyase (EC 4.1.2.2; Charalampous, 1954) favors synthesis of the tetrulose phosphate. Incubation of radioactive formaldehyde and unlabeled dihydroxyacetone phosphate with a cell extract containing either enzyme should therefore lead to the formation of labeled erythrulose 1-phosphate. We incubated 21 ml of cell extract concentrate with 0.18 ml of 0.01 M sodium iodoacetate for 10 min at 30° under nitrogen, then added to it solutions containing, respectively, 50 μmoles of dihydroxyacetone phosphate, 0.9 μmole of sodium iodoacetate, 7.8 μmoles of formaldehyde-^{14}C (3.9 μc), and water to bring the mixture to a final volume of 3 ml, and continued the incubation for 45 min. We then added an equal volume of 11.5% trichloroacetic acid, centrifuged down the precipitate, extracted the supernatant liquid seven times with 5-ml quantities of ether, and analyzed its contents on a column of Dowex 1 (monochloroacetate$^-$) according of the method of Charalampous and Mueller (1953). We treated the radioactive material in the peak suspected of containing tetrulose phosphate with acid phosphatase and subjected the products to electrophoresis in boric acid buffer as in previous experiments. Scanner tracings of the electrophoretogram revealed peaks at the erythrulose and erythritol regions, also in the region of uncomplexed materials such as glycerol. We conclude that while erythrulose phosphate is indeed formed from formaldehyde-^{14}C and dihydroxyacetone phosphate in our system, once formed it is rapidly reduced to erythritol phosphate and also metabolized to other products not separable from erythrulose phosphate on the chromatography column.

Discussion

The results of the present experiments support the role (see Figure 2) previously postulated (Wawszkiewicz and Barker, 1965) for L-erythrulose 1-phosphate in the metabolism of erythritol by *P. pentosaceum*. Although both the oxidation of L-erythritol 1-phosphate to L-erythrulose 1-phosphate and the subsequent cleavage of the latter to formaldehyde and dihydroxyacetone phosphate are reactions whose equilibria lie definitely to the left, it is easy to imagine that less reversible reactions further on in the metabolic sequence might enable them to serve a catabolic function. Erythrulose 1-phosphate itself has been previously detected in tissues of higher plants (Mueller *et al.*, 1955) and of animals (Charalampous and Mueller, 1953). The results reported here, however, constitute the first definite indication that this compound can play the role of key intermediate in a pathway of carbohydrate metabolism.

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