Erythritol Metabolism by Propionibacterium pentosaceum

THE OVER-ALL REACTION SEQUENCE*

Randomly labeled erythritol-14C has been synthesized and incubated with fluoride-poisoned suspensions of dried cells of Propionibacterium pentosaceum. Therefrom labeled glycerol-3-P, glyceraldehyde-3-P, erythritol-1-P, ribose-5-P, xylulose-5-P, glucose-6-P, fructose-6-P, mannose-6-P, fructose-1,6-di-P, and sedoheptulose-7-P were isolated. Erythritol-14C-1-P accumulated more rapidly than did the other phosphates. Even under conditions in which its dephosphorylation is inhibited it was metabolized to the same products as was erythritol-14C. Also, in short term experiments the addition of unlabeled l-erythritol-1-P to cell preparations actively metabolizing erythritol-14C reduced the amount of label normally found as glyceraldehyde-3-P. This supports the view that in P. pentosaceum erythritol metabolism proceeds via a series of phosphorylated intermediates, of which l-erythritol-1-P is the primary member. L-Erythritol-1-P can serve as hydrogen donor for the reduction of 2,6-dichloroindophenol in cell-free extracts. Although the product of the oxidation was not isolated, indirect evidence suggests that it is l-erythrose-1-P and that this compound is the next intermediate in the erythritol fermentation. Labeling dilution experiments indicated that D-erythrose-4-P is not a direct intermediate in the sequence. The formation of labeled formaldehyde in the reaction mixtures lends support to the view that l-erythrose-1-P, formed from l-erythritol-1-P, undergoes cleavage between the C2 and C3 positions to yield formaldehyde and dihydroxyacetone-P. This cleavage, combined with reactions known to occur in the propionic acid bacteria, provides a source of the labeled C3, C5, C6, and C7 sugar phosphates accumulating in the cell suspensions and an explanation for the substantial quantities of formic acid produced in the fermentation of erythritol.

Propionibacterium pentosaceum is one of the few microorganisms capable of anaerobic growth with the 4-carbon polyalcohol, erythritol, as the sole source of carbon (1). The major products of its fermentation of erythritol are propionic, succinic, acetic, and formic acids (2). Suspensions of P. pentosaceum cells dried in thin layers in vacuum over phosphorus pentoxide at 4° metabolize erythritol in the presence of sodium pyruvate and sodium fluoride, with a concomitant uptake of inorganic phosphate from the medium and the accumulation of difficulty hydrolyzable phosphate esters. In the experiments of Barker and Lipmann (3), cell suspensions metabolizing erythritol under these conditions appeared to produce acid not accounted for as glyceric acid-3-P. Since no appreciable carbon dioxide is released during the fermentation of erythritol by P. pentosaceum, Barker and Lipmann postulated that their cell suspensions were converting erythritol to a 4-phosphate, oxidizing the latter to erythrose-4-P, and then oxidizing that, via a diphosphate intermediate, to 4-phosphophorycetic acid. Shetter (4) succeeded in isolating the erythritol phosphate which accumulates in the reaction mixtures and identifying it as d erythritol-1-P (l erythritol-1-P), and Holten and Fromm (5) partially purified the kinase which mediates its formation. We have now determined the nature of the major products accumulating in fluoride-poisoned, pyruvate-enriched cell suspensions incubated with randomly labeled erythritol-14C, have studied the effects of some unlabeled suspected intermediates on the system, and have investigated certain aspects of its enzymic complement.

EXPERIMENTAL PROCEDURE

Preparation of Enzymically Active Materials—Propionibacterium pentosaceum, E2.1, obtained from the collection of Pro-
fessor C. B. van Niel, was grown at 30°, usually in 20-liter quanti-
ties, in the medium described by Shetter (4). The inoculum
was a culture, 10% the volume of the final one, growing actively
in the Shetter medium modified by the addition of 2.5 g of so-
dium thiglycolate per liter. After the final culture had been
incubated for 50 hours, 0.5 g of erythritol was added per liter
and the incubation was allowed to continue for another 12 to 18 hours.
The erythritol-adapted cells so obtained were harvested in a
Sharples centrifuge, washed three times with distilled water, dried
according to the method of Barker and Lipmann (3) over phos-
phorus pentoxide at 4° in a vacuum, and stored over calcium
chloride at -10°. No appreciable loss in enzymatic activity was
observed in such preparations during storage periods of up to 3
weeks. Cell-free extracts were prepared by subjecting suspensions
containing 1.5 g of such dried cells and 1 g of carborundum
in 15 ml of 0.5 m triethanolamine-HCl buffer at pH 7.5 to the
action of a 10-kc Raytheon sonic oscillator for 20 min at 0-5°
and centrifuging the resulting material for 40 min at 13,500 ×
g. Pooled human seminal plasma, stored at -10° for approxi-
mately 1 year, was used as source of orthophosphoric monoester
hydrolyase (acid phosphatase, EC 2.1.3.2).

Determination—Orthophosphate was determined by the
method of Fiske and SubbaRow (6). Protein was estimated by
the method of Lowry et al. (7), with crystalline bovine serum
albumin as a standard. Radioactivity values were calculated
from counts of samples dried in nickel-plated, cup-shaped
planchets or as barium carbonate precipitates on filter paper
disks mounted on stainless steel rings. To ensure an even dis-
tribution of the material in the pluchets small amounts of 95%
ethyl alcohol were added to the samples before they were dried.
The planchets and ring-mounted filter paper discs were counted with
a Tracerlab SC-16 windowless gas flow counter after having been
sprayed with Krylon to prevent contamination of the in-
strument by loose radioactive particles. Counts were registered
on a Berkeley 2051 decimal scaler. The values were corrected
for background and, in the case of barium carbonate samples, for
self absorption. Radioactive spots on chromatograms were
counted directly with a Sharp monitor ratemeter LMR-168. A
Beckman DU spectrophotometer was used in experiments in-
volving the measurement of optical density changes.

Separation and Detection of Products of Erythritol Metabolism—
Air-dried Whatman No. 1 filter paper, which had been saturated
with a 1% oxalic acid solution by descending flow and rinsed
with distilled water until the washings were almost neutral, was
used in all chromatographic and ionophoretic procedures involving the
separation of phosphate esters. Since the residual acidity of
acid-washed paper interfered with the detection of free sugars,
unwashed Whatman No. 1 paper was used for the separation of
the latter compounds. Sugar phosphates were separated chromato-
graphically by a previously described two-dimensional method (8).
For counting and degradative purposes radioactive phosphate esters as well as free labeled sugars so separated were cluted from the chromatograms and freed of picric acid by a
second run in Solvent I. Solvents used to develop chromatograms of free sugars included a mixture of n-butyl alcohol, ascic acid, and water in the proportions by volume of 52:13:35, a mixture of n-butyl alcohol, ethyl alcohol, and water in the proportions, by volume, of 52.5:32:15.5, and phenol-water.
The latter contained 39.5 ml of water per each 100 g of phenol
purified by the method of Draper and Pollard (9). The location of unlabeled phosphate esters on chromatograms was determined
by the method of Bandurski and Axelrod (10). Unlabeled
sugars were detected by spraying the chromatograms with 5%
silver nitrate solution to which concentrated ammonium hy-
droxide had been added until the precipitate which first formed
just redissolved, drying the papers, and then heating them for
several minutes at 100°. This technique could be used only
with chromatograms which had finally been developed with a
neutral solvent. Radioactive materials on chromatograms were
detected by standard radioautographic methods with the use of
Kodak "no screen" x-ray film. Paper electrophoresis was
washed with a 1% oxalic acid solution by descending flow and rinsed with
alkaline human seminal plasma, stored at -10° for approxi-
mately 1 year, was used as source of orthophosphoric monoester
hydrolyase (acid phosphatase, EC 2.1.3.2).

Identification of Products of Erythritol Metabolism—While
specific details are given with the evidence for the identity of various reaction mixture components, the techniques of co-
ionophoresis and co-chromatography found general application.
Unknown, radioactive compounds were mixed with known,
unlabeled markers before being subjected to ionophoresis or
paper chromatography. Congruence of radioactivity and
location of marker compound was considered an evidence of
identity. Suspected phosphate esters were enzymically hydro-
lyzed by the action of acid phosphatase before being co chro-
matographed with free sugars or sugar acids. In general, 5 μl of
a solution of the unknown compound, containing about 1000
cpm of 14C (approximately 0.005 to 0.02 μmole of ester) were
mixed with 5 μl of 1 m ammonium acetate, pH 5, and 10 μl of
human seminal plasma. The mixture was incubated in a
capillary tube for 2 hours at 37°, then mixed with unlabeled marker. In order to detect sedoheptulosan formation from 14C-labeled compounds suspected of being phosphate esters of sedo-
heptulose, the unknown material was incubated with phosphatase
as described above, 1 μmole of unlabeled sedoheptulose was
added, and the entire mixture was subjected to the action of 1 N
HCl in a sealed capillary tube at 100° for 45 to 60 min and then
analyzed by paper chromatography.

Preparation of Randomly Labeled Erythritol-14C—Chroma-
tographically pure, randomly labeled d-glucose-14C prepared ac-
cording to the method of Putman, Hassid, Krotkov, and Barker
(12) was oxidized to d-ß-formyl-ß-erythritol-14C by the method
of Perlin and Brice (13). This was hydrolyzed to n-erythrose-
14C and the latter was reduced to i-erythritol-14C with potas-
sium borohydride. Unlabeled glucose (45.8 mg dissolved in
0.092 ml of water) was added to 4.2 mg of randomly labeled glu-
cose containing 1 μC of 14C. The resulting solution was mixed
with 5 ml of glacial acetic acid. Then 12.8 ml of a solution of
500 μg of lead tetraacetate in 25 ml of glacial acetic acid were
added to it in five equal portions at 1-min intervals with constant
stirring. The reaction was carried out at room temperature,
and 15 min after the initial addition of lead tetraacetate it was
stopped by the introduction of 0.83 ml of a solution of 1.9 g of
oxalic acid in 25 ml of glacial acetic acid. After 1 hour the sus-
pension was clarified by centrifugation and the supernatant
liquid was decanted. The precipitate was extracted with 10 ml
of ethyl acetate, and the extract and original supernatant solution
were combined and brought to dryness under reduced pressure.
The residue was then taken up in 2 ml of 0.05 N HCl and incu-
bated at 50° for 2 hours. The solution was then adjusted to pH
3 by the addition of 0.1 N KOH, and to it were added cautiously,
in small portions, 100 mg of potassium borohydride. The copious
lead precipitate which appeared eventually formed a pellet at the
bottom of the reaction vessel. After 1 hour the liquid above it
was passed through 10 ml of Dowex 50-H+. The eluate and washings were brought to dryness under reduced pressure and the residue, taken up in a little water, was transferred to a polyethylene planchet. After the contents of the planchet had been dried over P₂O₅ in a vacuum, the boric acid in the residue was removed by the addition of three 1-ml portions of absolute methyl alcohol, each of which was allowed to evaporate spontaneously. The final product, a yellowish syrup-like material, was taken up in a little water and subjected to repeated paper chromatography, with phenol-water as the first solvent and n-butyl alcohol-acetic acid-water as the second. The yield of chromatographically pure erythritol thus obtained was 35% of that predicted by theory. In one experiment, randomly labeled erythritol-¹⁴C (2.26 mc per mmole), obtained from Nuclear Research Chemicals, Orlando, Florida, and purified by paper chromatography with n-butyl alcohol-ethyl alcohol-water, was used.

Preparation of Randomly Labeled Fuctose-¹⁴C 6-Phosphate—Randomly labeled f-fructose-¹⁴C (principal side product in the preparation of n-glucose-¹⁴C) was incubated with crystalline yeast ATP-d-hexose 6-phosphotransferase (EC 2.7.1.1; a gift of Dr. A. Ramel) and commercially available ATP. The product was purified by successive paper chromatography in phosphate Solvents I and II and a final development with Solvent I.

Preparation of Suspected Intermediates—L-Erythritol was prepared by the method of Charalampous and Mueller (14). L-Erythritol-1-P was prepared either by chemical synthesis (15) (a gift of Professor C.E. Ballou) or biologically (4) (a gift of Dr. J.K. Shetter). L-Erythritol-¹⁴C-1-P was isolated chromatographically from fluoride-poisoned suspensions of dried cells of P. pentosaceum metabolizing randomly labeled erythritol-¹⁴C. n-Erythrose-4-P, n-erythrulose-1-P, L-erythrulose-1-P, and d-erythrulose-4-P were obtained as the cyclohexylamine salts of their dimethyl- or diethyldiethylacetals or -ketals, prepared by synthetic methods which have previously been described (16-18), and were gifts of Professor C.E. Ballou. To bring each substance into solution preparatory to converting it to the free sugar phosphate, 20 μmoles of each compound were mixed with 0.25 ml of wet Dowex 30-H⁺ in pieces of glass tubing 5 to 6 mm in diameter constricted at one end and plugged at that end with glass wool. Water was passed through these microcolumns until each eluate amounted to 0.8 ml. The eluates were then incubated at 40°C to hydrolyze the free acetal or ketals (n-erythrose-4-P dimethylacetal, 16 hours; n-erythrulose-1-P dimethylketone, n-erythrulose-4-P dimethylketone, and L-erythrulose-1-P dimethylketone, 6 hours). The compounds in solution were stored as the free acids, at -10°C, and were used within 2 weeks of hydrolysis.

Preparation of Reduced Nicotinamide Nucleotide Coenzymes—Enzymically reduced NAD and NADP were purchased from Sigma. In some experiments NADP (from Sigma) was reduced chemically by the method of Kaplan, Colowick, and Neufeld (19).

RESULTS

Major Products Accumulating during Erythritol-¹⁴C Metabolism by Fluoride-poisoned, Dried Cells Supplemented with Pyruvate—Randomly labeled erythritol-¹⁴C was incubated with suspensions of dried cells as described in the legend to Fig. 1, and the reaction products were analyzed chromatographically. While inevitable losses of radioactivity occurred during the various manipulations (because of nonspecific surface adsorption phenomena, etc.), recoveries of 80 to 90% of the total radioactivity in the initial alcoholic extracts were usually obtained. Fig. 1 is a photograph of a radioautogram taken from a typical chromatogram. The evidence for the identity of the various numbered spots is based upon the following.

1. Glycerol: co-chromatography with unlabeled glycerol in phenol-water and in n-butyl alcohol-ethyl alcohol-water.

2. Glycerol-3-P: co-chromatography with unlabeled glycerol-3-P in sugar phosphate Solvents I and II; co-chromatography in phenol-water with unlabeled glycerol of material released by treatment with seminal phosphatase.

3. Glyceric acid-3-P: co-ionophoresis with unlabeled glyceric acid-3-P in 0.1 M ammonium acetate buffer at pH 5.4; co-chromatography with unlabeled glyceric acid of material treated with seminal phosphatase, in phenol-water and in n-butyl alcohol-ethyl alcohol-water.

4. Erythritol: co-chromatography with unlabeled erythritol in phenol-water and in n-butyl alcohol-ethyl alcohol-water.

5. Erythritol-1-P: co-chromatography with unlabeled l-erythritol-1-P in both sugar phosphate solvents; co-ionophoresis with unlabeled l-erythritol-1-P in 0.1 M ammonium formate buffer at pH 5.4; co-chromatography with unlabeled erythritol in phenol-water and in n-butyl alcohol-ethyl alcohol-water of material released by treatment with seminal phosphatase.

6. Xylulose-5-P: not positively identified but presumed to be xylulose-5-P on the basis of chromatographic mobilities in sugar phosphate Solvents I and II and Rₜ value of product, resulting from seminal phosphatase treatment, subjected to ionophoresis in 0.05 M sodium borate buffer.

7. Fructose-6-P: co-chromatography with unlabeled fructose-6-P in both sugar phosphate solvents; co-ionophoresis with fructose-6-P in 0.1 M ammonium acetate buffer at pH 5.4; co-chromatography with unlabeled fructose of material released by seminal phosphatase treatment in phenol-water and in n-butyl alcohol-ethyl alcohol-water.

8. Fructose-1,6-di-P: chromatographic mobilities in sugar phosphate Solvents I and II; co-chromatography of product of seminal phosphatase hydrolysis with unlabeled fructose, with phenol-water and n-butyl alcohol-ethyl alcohol-water as solvents.

9. Mannose-6-P and sedoheptulose-7-P: co-chromatography with unlabeled sedoheptulose-7-P in both sugar phosphate solvents, co-ionophoresis with sedoheptulose-7-P in 0.1 M ammonium acetate buffer at pH 5.4; co-chromatography with sedoheptulose of material resulting from seminal phosphatase treatment in phenol-water and in n-butyl alcohol-ethyl alcohol-water.

10. Ribose-5-P: not positively identified but presumed to be ribose-5-P on the basis of its chromatographic mobilities in sugar phosphate Solvents I and II.

11. Region of free sugars: chromatography in phenol-water and n-butyl alcohol-ethyl alcohol-water. Not all the individual spots were identified, but the mixture included fructose, glucose, mannose, and sedoheptulose.

12. Glucose-6-P: co-chromatography in sugar phosphate solvents with unlabeled glucose-6-P; co-ionophoresis with unlabeled glucose-6-P in 0.1 M ammonium acetate buffer at pH 5.4; co-chromatography of product resulting from seminal phosphatase hydrolysis with unlabeled glucose in phenol-water and in n-butyl alcohol-ethyl alcohol-water.
FIG. 1 (upper). Radioautogram of chromatogram of products resulting from erythritol-\(^{14}\)C metabolism in the presence of fluoride. Reaction mixtures (1 ml), containing 40 \(\mu\)moles of potassium phosphate buffer, pH 6.7, 40 \(\mu\)moles of sodium fluoride, 10 \(\mu\)moles of sodium pyruvate, 1 to 10 \(\mu\)moles of randomly labeled erythritol-\(^{14}\)C, 100 mg of dried cells, and water to volume, were incubated at room temperature (23°) for 8 min. The cells were ground in the phosphate-fluoride solution and introduced into 2-ml reaction vessels (polyethylene planchets) in which the requisite amounts of pyruvate and erythritol-\(^{14}\)C stock solutions had previously been dried. Inactivation of the mixtures was accomplished by transferring them to 5-ml quantities of boiling 96\% ethyl alcohol, boiling the suspensions for 1 min, immediately cooling them to 0° in an ice bath, and removing the precipitated protein by centrifugation. The supernatant alcoholic extracts were brought to dryness in 50-ml glass beakers in a current of cold air. The residues, taken up in 2 to 5 ml of water, were allowed to react with 0.2 ml of 0.2 M magnesium mixture (0.2 M magnesium acetate in 0.2 M ammonium acetate solution), and the resulting suspensions, cooled in ice, were brought to pH 8.3 with 1 N NaOH (phenolphthalein was used as internal indicator) and then clarified by centrifugation. The supernatant solutions obtained were passed through approximately 0.5-ml volumes of Dowex 50-H\(^+\), and the eluates, collected in silicone-treated polyethylene planchets, were brought to dryness in a current of cold air without being neutralized (neutralization having been found to enhance the decomposition of the phosphate esters during drying). The resulting residues were taken up in a few drops of water, spotted on paper, and chromatographed in two dimensions (method for phosphate esters) as described in the text. The evidence for the identity of the numbered compounds is also given in the text. They are 1, glycerol; 2, glycerol-3-P; 3, glyceraldehyde-3-P; 4, erythritol; 5, erythritol-1-P; 6, xylulose-5-P; 7, fructose-6-P; 8, fructose-1,6-di-P; 9, mannose-6-P and sedoheptulose-7-P; 10, ribose-5-P; 11, region of free sugars; and 12, glucose-6-P.

FIG. 2 (lower). Portion of a radioautogram of a chromatogram of products arising during erythritol-\(^{14}\)C metabolism in the presence of fluoride treated with seminal phosphatase. The conditions of the experiment are given in the text.

To take advantage of certain chromatographic separations possible with free sugars but not possible with their phosphorylated derivatives, a desalted extract residue from a reaction mixture was prepared as described in the legend to Fig. 1 and subjected to the action of seminal phosphatase in 0.33 M ammonium acetate buffer at pH 5, then chromatographed in two dimensions, with the use of phenol-water in the first direction and \(n\)-butyl alcohol-ethyl alcohol-water in the second. Fig. 2 is a photograph of a portion of a radioautogram of the chromatogram. The spots labeled in the photograph were identified by co-chromatographing the radioactive materials with the corresponding unlabeled compounds in the same solvents. As can be seen, mannose and sedoheptulose are not separated from each other; the presence of both compounds in the area indicated was confirmed by subjecting the material eluted from this area to ionophoresis in 0.05 M sodium borate. The presence of erythrose in the area indicated was confirmed by reducing the material eluted from the region with potassium borohydride in the manner outlined in the description of the preparation of randomly labeled erythritol-\(^{14}\)C and subjecting the reduced material to ionophoresis in saturated boric acid buffer, adjusted to pH 6.0, for 4 hours at 2000 volts (20). Under these conditions erythritol is well separated from threitol. Upon chemical reduction, erythrose would be expected to yield both erythritol and threitol, while erythrose (from which erythrose is not separated by the chromatographic procedure) would yield only erythritol. Both radioactive erythritol and radioactive threitol were produced during the reduction.

Formaldehyde is a product of one of the reactions postulated to explain the accumulation of the various phosphate esters, and it was therefore of interest to attempt its isolation from reaction mixtures. A 1-ml reaction mixture containing approximately
9.5 × 10⁶ cpm of randomly labeled erythritol-¹⁴C, prepared as described in the legend to Fig. 1, was incubated for 6 min. Water (1 ml) was then added, and the new mixture was placed in a bath of boiling water for 4 min, then cooled. An additional 0.5 ml of water was introduced, and the mixture was centrifuged. The supernatant solution was decanted, and 1 ml of it was desalted in the ordinary manner. Ionophoresis of the desalted material in 0.1 M ammonium formate buffer at pH 3.7 yielded 7.25 × 10⁴ cpm of glyceric acid-3-P. To another 1 ml of initial water extract, 3 ml of 1 M sodium acetate, 0.5 ml of a standard solution of formaldehyde (containing 196.4 μmoles of formaldehyde per ml), and 1.5 ml of Dimedon reagent (50 mg of 5,5-dimethyl- dihydroresorcinol per ml of 95% ethyl alcohol (21)) were added. The mixture was placed in a bath of boiling water for 10 min, then allowed to stand at room temperature for 1 hour. The copious precipitate which formed was separated by centrifugation, washed twice with a total volume of 9 ml of water, then crystallized five times by being dissolved in a minimum amount of absolute ethyl alcohol (from 6 to 10 ml, depending on the crystallization) followed by the addition of an equal amount of water. Small portions of the precipitates were spread on metal planchets, dried, weighed, and counted. Within experimental error, the specific activity of the samples reached a constant value of 19 cpm per mg. Assuming that the quantity of formaldehyde actually present in the reaction mixture before the addition of carrier was nil and that 291 mg of Dimedon derivative should have been formed from the carrier, the total amount of formaldehyde-¹⁴C that could be isolated was 5.53 × 10³ cpm.

Kinetics of Phosphate Ester Accumulation—It was thought that an insight into the sequence of reactions involved in erythritol metabolism by P. penibaceum might be obtained by studying the kinetics of the accumulation of the various phosphate esters from erythritol itself and from L-erythritol-1-P. In some early experiments with erythritol-¹⁴C, fructose-6-P appeared to accumulate more rapidly than did the other esters with the exception of erythritol-1-P. The kinetics of its metabolism was therefore also of interest. Reaction mixtures were prepared as described in the legend to Fig. 1, and labeled substrates were added at zero time. At various intervals, 0.2-ml samples were removed and introduced into 5-ml volumes of boiling 80% ethyl alcohol; the resulting extracts were then desalted in the usual manner and analyzed chromatographically in two dimensions. Radioactive areas on the papers were counted directly, and the results were plotted as in Figs. 3 and 4. As can be seen in Fig. 3, all the sugar phosphate esters arising during erythritol metabolism, except fructose-1,6-di-P, accumulate from the very beginning of the incubation period, and the ester which accumulates most rapidly is erythritol-1-P. We have found that the same C₅, C₆, and C₈ phosphate esters as those indicated in Fig. 3 accumulate when L-erythritol-¹⁴C is metabolized in the presence of fluoride and again that, within experimental error, the accumulation of these esters is linear with time. Fig. 4 shows the transformation of fructose-6-P to glucose-6- and mannose-6-P. Sedoheptulose-7-P and glyceric acid-3-P also accumulate in these cell suspensions.

Orthophosphoric Hydrolase Activities in Dried Cell Preparations—Since the products of L-erythritol-1-P metabolism by fluoride-poisoned, dried cells are the same as those of erythritol metabolism, it was important to determine whether erythritol-1-P might be dephosphorylated by our cell suspensions and whether this could occur during the metabolism experiments. L-Erythritol-1-P was itself available only in limited quantities and therefore was replaced as substrate by glycerol-P in our initial studies. The results, shown in Fig. 5, indicate that cell suspensions do contain considerable phosphatase activity and that...
FIG. 5. Orthophosphoric hydrolase activity in dried cell preparations. Dried cells were ground in 0.5 M ethylenediaminetetraacetic acid buffer at pH 6.5 and samples of the suspension thus obtained were variously diluted with more buffer; 0.2 ml of each of the dilutions was mixed with 0.2 ml of 0.1 M sodium glycerophosphate (52% of) at zero time and incubated for 40 min at 29°. Controls, containing water rather than the sodium glycerophosphate solution, were incubated under the same conditions. Aliquots (0.5 ml) from each of the reaction mixtures were withdrawn at the times indicated and diluted to 10 ml with 3% trichloracetic acid. The supernatant solutions were analyzed for orthophosphate. The amount of orthophosphate released from the glycerophosphate was calculated by subtracting the orthophosphate in the control reaction mixtures from the orthophosphate present in the glycerophosphate reaction mixtures.

This activity is dependent on cell concentration. The cell suspensions actually appear to contain at least two phosphatases active on glycerol-P (see Fig. 6); one of these has maximum activity at pH 5.0, the other at pH 8.5. However, as can be seen in Table I, L-erythritol-1-P can be dephosphorylated by the cell suspensions, sodium fluoride, when present in concentrations similar to those obtaining in the metabolism experiments, effectively inhibits this hydrolysis.

Oxidation-Reduction Reactions Catalyzed by Dried Cell Extracts —If, as will be proposed, L-erythritol-1-P is the primary intermediate in the metabolism of erythritol by *P. pentosaceum*, the next step in the metabolic reaction sequence might be expected to be its transformation to some oxidized product. The oxidation might well be linked to the reduction of one or both of the nicotinamide nucleotide coenzymes and, conversely, the reduction of the oxidized product would then be expected to be coupled to the oxidation of the reduced coenzymes. With dried cell extracts we have not been able to demonstrate either NADH or NADPH in our dried cell extracts, but glyceraldehyde-3-P, dihydroxyacetone-P, ribose-5-P, and glucose-6-P are all active hydrogen donors. D-Erythrose-4-P, d-erythulose-1-P, and D-erythulose-4-P, possible products of erythritol P oxidation, have no effect on the rate of oxidation of reduced NAD or

seen in Table II, erythritol itself will not reduce NADH or NADPH in our dried cell extracts, but glyceraldehyde-3-P, dihydroxyacetone-P, ribose-5-P, and glucose-6-P are all active hydrogen donors. D-Erythrose-4-P, d-erythulose-1-P, and d-erythulose-4-P, possible products of erythritol P oxidation, have no effect on the rate of oxidation of reduced NAD or

### Table I

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<thead>
<tr>
<th>Incubation time (min)</th>
<th>Net orthophosphate released</th>
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Fig. 6. The effect of pH on orthophosphoric hydrolase activity. Reaction mixtures, containing 20 mg of dried cells and 20 µmoles of sodium glycerophosphate in 0.4 ml of 0.35 M buffers of various pH values, were incubated for 10 min at 30°. The following buffers were used over the indicated ranges: sodium tartrate, pH 3.0 to 4.0; sodium acetate, pH 4.5 to 5.0; sodium phosphate, pH 5.5 to 6.0; ethylenediamine-HCl, pH 6.5 to 7.5; and Tris-HCl, pH 8.0 to 9.0. At the end of the incubation period 0.1-ml samples were withdrawn and made up to 10 ml with 3% trichloracetic acid, and the supernatant extracts were analyzed for total orthophosphate. Controls containing no glycerophosphate were incubated under the same conditions and analyzed for endogenous orthophosphate release. The net release of orthophosphate from glycerophosphate at different pH values is plotted.
produced by our dried cell extracts. However, L-erythritol-1-P, another possible oxidation product (unfortunately not available for test at the time the experiments reported here were completed), serves as a very active hydrogen acceptor from reduced NAD in fractionated fresh cell extracts (23).

**Dilution of Label in Glyceric Acid 3-Phosphate by Unlabeled Substrates—Unlabeled intermediates directly involved in the reaction sequence leading from erythritol to glyceric acid-3-P should act as traps for label when added to reaction mixtures producing glyceric acid-14C-3-P from erythritol-14C. Their presence in such reaction mixtures should decrease the amount of 14C converted to glyceric acid-14C-3-P per unit time. Table III shows the results of an experiment in which unlabeled, suspected intermediates were added to dried cell preparations metabolizing erythritol-14C and the amount of glyceric acid-14C-3-P produced in 10 min was measured. A discernible reduction of glyceric acid-14C-3-P production occurred when unlabeled L-erythritol-1-P, L-erythritol, and L-erythrose-1-P were added. In view of the inaccuracy of the assay procedure (±25%), we believe that the changes in glyceric acid-14C-2-P production apparently induced by the addition of L-erythrose-1-P and L-erythrose-4-P have questionable significance. However, the results do clearly indicate that the addition of L-erythrose-4-P is without effect.

**Discussion**

The pathway of erythritol metabolism in _P. pentosaceum_, as we conceive it on the basis of the results of these experiments, is outlined in Fig. 8. Sodium fluoride, added to our cell suspensions as an inhibitor of 2-phospho-D-glycerate hydrolyase (EC 4.2.1.11), acts also as a phosphatase inhibitor, and in its presence any sugar phosphate ester intermediates prior to phosphoenolpyruvic acid in the pathway of erythritol metabolism would be expected to accumulate. As is clear from the results, the major products of erythritol-14C metabolism by _P. pentosaceum_ dried cell suspensions poisoned by fluoride and supplemented with pyruvate (supplied as energy source) are indeed several sugar phosphates, of which L-erythritol-1-P is the one which accumulates most rapidly. This confirms and extends the findings of Shetter (4), who had previously isolated and identified this compound from dried cell suspensions. Shetter

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### Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>NAD* reduction</th>
<th>NADP* reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythritol</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>DL-Glycerol-5'-P (50% a)</td>
<td>0.030</td>
<td>0.000</td>
</tr>
<tr>
<td>DL-Glyceraldehyde-3-P</td>
<td>0.143</td>
<td></td>
</tr>
<tr>
<td>Dihydroxyacetone-P</td>
<td>0.168</td>
<td></td>
</tr>
<tr>
<td>UDP-Nucleotide-5'-P</td>
<td>0.208</td>
<td>0.338</td>
</tr>
<tr>
<td>UDP-Glucose-4'-P</td>
<td>0.232</td>
<td>0.398</td>
</tr>
</tbody>
</table>

* For this determination the cell extract was prepared without mercaptoethanol and the pH of the assay mixture was adjusted to 10 with 1 M KOH.
also showed that the addition of l-erythritol-1-P to fluoride-
poisoned cell preparations could enhance their oxygen uptake, which suggested that this compound could in some way be
oxidized by the cells. However, since free erythritol was even
more active than erythritol-P in stimulating oxygen uptake by
cell suspensions, the possibility remained that erythritol metabo-
lism in this organism actually proceeds via a pathway not in-
volving phosphorylated intermediates. This possibility has
now been rendered highly unlikely by our observation that no
appreciable accumulation of materials other than phosphorylated
compounds occurs when fluoride-poisoned, dried cells metabolize
erythritol-14C. Although free radioactive sugars are detectable
on our reaction mixture chromatograms, they occur only in the
amounts expected as the result of that sugar phosphate hydrolysis
which unavoidably takes place during the analytical manipula-
tions.

If erythritol itself were the species oxidized by the cells, the
primary product formed from it would be expected to be either
erthyrose or erythrulose. One or both of these compounds might
then, under the proper conditions, be expected to act as hydrogen
acceptor in a reversal of the oxidation reaction. α-Erythrose
was not reduced by any of our preparations, nor has it nor any
of its phosphate derivatives been identified on our chromatograms.
And although, in experiments not reported here, β-
erthrose has been found to stimulate reduced NAD oxidation in
crude, fresh cell extracts, the rate of reduced NAD oxidation
induced by its presence is only one-eighth that induced by the
presence of its 1-phosphate derivative. Also, while its addition
to reaction mixtures metabolizing erythritol-14C did reduce the
rate of glyceric acid-14C-3-P production (see Table III), this
result is difficult to interpret because the unlabeled erythritol
produced from the erythrulose during the experiment would have
the same effect.

We have shown that l-erythritol-1-P can be hydrolyzed by
phosphatases present in the dried cell suspensions. But we
have also shown that this hydrolysis does not occur to any ap-
preciable extent under the conditions and during the times of
our metabolism experiments. The fact that in these experi-
ments l-erythritol-1-P is metabolized to the same products as is
erythritol itself lends strong support, therefore, to the view that
this compound is a primary intermediate in erythritol metabo-
lism. We have shown that this compound can act as hydrogen
donor for 2,6-dichloroindophenol reduction at physiological
pH values. This suggests that it can be oxidized by the cells
directly. We have been unsuccessful in isolating the oxidized
product from systems containing the reduced dye. But that
product would be expected to be either an erythrose or eryth-
rulose phosphate. β-Erythrose-4-P, a postulated intermediate
in the scheme of Barker and Lipmann (3) and an intermediate of
general significance in carbohydrate metabolism, has been shown
to not influence the amount of labeled glyceric acid-3-P arising
from erythritol-14C metabolism. It therefore cannot be the
oxidized product in question. Although both β-erythritol-1-P
and β-erythrose-4-P do appear to exert some effect on glyceric
acid-14C-3-P production, that effect is slight and, in fact, in the
case of the 4-phosphate is actually opposite to what an unlabeled
intermediate would be expected to induce. So both of these
esters, too, can safely be ruled out as possible oxidation products.

This leaves for consideration l-erythrose-1-P, a compound the
addition of which to reaction mixtures metabolizing erythritol-
14C reduces substantially the amount of glyceric acid-14C-3-P
produced from erythritol-14C per unit time and the production
of which from erythritol had been surmised from the finding of
erythrulose on chromatograms of reaction mixture components
subjected to treatment with acid phosphatase.

We have not found any evidence for the occurrence of the
erthronic acid mono- and diphosphates which had been postu-
lated as intermediates by Barker and Lipmann (3), and we now
believe that l-erythrulose-1-P is the only 4-carbon phosphate
ester, other than l-erythritol-1-P, which is formed during eryth-
ritol metabolism by our cell suspensions. Furthermore, since we
have been unable to demonstrate the presence of a kinase for l-
erthryulose in our preparations, we consider it highly likely that
whatever l-erythrulose-1-P occurs in them arises either by the
oxidation of l-erythritol-1-P or by the reversal of the cleavage
reaction which we envisage as determining its subsequent fate.
The latter reaction, involving a split of the erythrose-1-P molecule
between carbon atoms 3 and 4, has been studied by Chara-
lampus (24) with an enzyme, erythrose 1-phosphate formal-
dehyde lyase (EC 4.1.2.2), from rat liver and by Mueller, Quinn,
and Rueckert (25) in a Swiss chard homogenate. Although its
equilibrium actually favors synthesis, the reaction could never-
theless be used for producing dihydroxyacetone-P and formal-
dehyde from erythritol-1-P. Our isolation of formaldehyde-
14C from a cell preparation metabolizing erythritol-14C lends
considerable plausibility to the suggestion that this is indeed the
mechanism by which l-erythrulose-1-P is catabolized by our
system.

A cleavage of this kind provides, in addition, a ready explana-
tion for a phenomenon not heretofore understood. Little
formic acid is produced from most substrates by the propionic
acid bacteria. Glycerol, for example, when fermented by P.
pentosaceum yields no formate at all, while 100 mmoles of man-
nitol and of arabinojubone yield only 0.29 and 0.21 mmoles of formate,
respectively. In contrast, formate does occur in significant
quantities when erythritol is fermented and is actually the second
most abundant end product (20.1 mmoles from 100 mmoles of
substrate) of the fermentation of erythritol by Propionibacterium
arabinojubone (2). Since formate can be produced from formal-
dehyde by formate hydratase (EC 1.2.1.1) (26) and other aldehyde dehydrogenases, the formaldehyde
produced from erythritol via erythrulose-P might very well be a
precursor to the formic acid accumulating during the erythritol
fermentation. Although each molecule of erythritol metabolized
via erythrulose-P would be expected to yield 1 molecule of formal
dehydode, and hence 1 molecule of formate, the amount of formic acid which is isolated from erythritol fermentations is much less than would be predicted on the basis of this formulation and, as a matter of fact, in our trapping experiments the amount of formaldehyde-\(^{14}\)C isolated corresponds to only 20\% of the amount of glyceric acid-\(^{14}\)C-3-P (presumably arising from dihydroxyacetone-P) produced. This finding should cause no surprise, however: formaldehyde is known to be utilized in various C\(_1\) metabolism reactions and is probably very quickly removed from the pool of intermediates as soon as it is formed. At least two mechanisms by which formaldehyde could enter the metabolic scheme here proposed, other than by the reversal of the \(\text{L-erythrose-1-P} \rightarrow \text{pentose phosphate}\) cleavage, come immediately to mind: (a) a condensation with \(\text{L-erythrose-1-P}\) to yield a pentose phosphate (a reaction of this kind may occur in rat liver (27)) and (b) a condensation with pentose-P to yield a C\(_6\)-P, as happens in certain pseudomonads (28).

The dihydroxyacetone-P which would be formed by a C\(_2\), C\(_4\) cleavage of erythrose-\(\text{P}\) would be expected to enter the well known reactions of the glycolytic pathway to yield glyceraldehyde-\(\text{P}\), the glyceraldehyde diphosphates, and glyceraldehyde-3-P. This last would yield phosphaenolpyruvate or pyruvate and the latter, by entering the reactions of the Swick and Wood cycle (29), succinate and propionate, the two main end products of the fermentation. Acetate, another end product, would also be expected to arise from pyruvate, via acetyl-CoA (30). In a side reaction catalyzed by \(\text{L-glycerol 3-phosphate:NAD oxidoreductase}\) (EC 1.1.1.8), known to be present in our system, dihydroxyacetone-P would be expected to be reduced to glycerol-P, a product accumulating in substantial amounts in our reaction mixtures. That dihydroxyacetone-P could be converted to glyceraldehyde-P in our preparations is suggested by the fact that it will reduce NAD\(^+\) in a system containing \(\text{n-glyceraldehyde-3-phosphate}\) oxidoreductase (EC 1.2.1.12). We have, of course, isolated glyceraldehyde-\(^{14}\)C-3-P from reaction mixtures metabolizing erythritol-\(^{14}\)C, also from them fructose-\(^{14}\)C-1, 6-di-P, the product resulting from the condensation of dihydroxyacetone-\(^{14}\)C-P with glyceraldehyde-\(^{14}\)C-P. Fructose-1, 6-di-P can yield fructose-6-P and, as is clear from Fig. 4, the latter has been shown to be in equilibrium with glucose-6-P and mannose-6-P in our system.

The formation of the pentose \(\delta\)-phosphates which we observe might be expected to occur, since both \(\text{d-glucose 6-phosphate}\) NADP oxidoreductase (EC 1.1.1.49) and \(\text{6-phospho-d-glucanate:NADP oxidoreductase}\) (EC 1.1.1.44) activities have been inferred to be present in sonic extracts of \(\text{P. pentosaceum}\) (31) and the presence of pentose-P isomersases in a system such as ours might be predicted. Of course, in the living cell the carbon dioxide which is released in the formation of ribulose-5-P from gluconic acid-6-P must quickly be taken up by other metabolic reactions since no net carbon dioxide evolution is observed during the erythritol fermentation.

Sedoheptulose-7-P would be expected to be produced from the pentose phosphates by the action of sedoheptulose 7-phosphate: \(\text{d-glyceraldehyde 3-phosphate glycolaldehyde dehydratase}\) (EC 2.2.1.1). On the other hand, because the addition of unlabeled \(\text{d-erythrose-4-P}\) seems to have no effect on the labeling of the erythritol-\(^{14}\)C metabolism products accumulating in our preparations, these must lack sedoheptulose 7-phosphate: \(\text{d-glyceraldehyde 3-phosphate dihydroxyacetone transferase}\) (EC 2.2.1.2).

The metabolic scheme discussed above accounts for all the major products of erythritol metabolism accumulating in our dried cell preparations poisoned by fluoride and is consistent with our various experimental observations. It also accounts for the major end products of the erythritol fermentation by growing cells and, in addition, offers an explanation for the production of formic acid from erythritol by the propionic acid bacteria. While our experiments do not rule out such alternate pathways of erythritol metabolism as that, for example, involving a C\(_2\), C\(_2\) split of erythrose-P, that particular cleavage has not yet been shown to occur in our system and we believe the pathway proposed here, involving the C\(_2\), C\(_4\) cleavage, the simplest summary of the available information possible at this time.

Acknowledgments—The authors wish to express particular thanks to Professor Clinton E. Ballou for his generous gifts of the synthetic sugar phosphate esters used in this study and to Dr. Edison W. Putman for his valuable assistance during the preparation of randomly labeled glucose-\(^{14}\)C.

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