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Erythritol Catabolism by *Brucella abortus*

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Cell extracts of *Brucella abortus* (British 19) catabolized erythritol through a series of phosphorylated intermediates to dihydroxyacetonephosphate and CO_2. Cell extracts required adenosine 5'-triphosphate (ATP), nicotinamide adenine dinucleotide (NAD), Mg^{2+}, inorganic orthophosphate, and reduced glutathione for activity. The first reaction in the pathway was the phosphorylation of meso-erythritol with an ATP-dependent kinase which formed D-erythritol 1-phosphate (D-*erythro*-tetritol 1-phosphate). D-Erythritol 1-phosphate was oxidized by an NAD-dependent dehydrogenase to D-erythrulose 1-phosphate (D-*glycero*-2-tetrulose 1-phosphate). *B. abortus* (US-19) was found to lack the succeeding enzyme in the pathway and was used to prepare substrate amounts of D-erythrulose 1-phosphate. D-Erythritol 1-phosphate dehydrogenase (D-*erythro*-tetritol 1-phosphate: NAD 2-oxidoreductase) is probably membrane bound. D-Erythrulose 1-phosphate was oxidized by an NAD-dependent dehydrogenase to 3-keto-L-erythrose 4-phosphate (L-*glycero*-3-tetrosulose 4-phosphate) which was further oxidized at C-1 by a membrane-bound dehydrogenase coupled to the electron transport system. Either oxygen or nitrate had to be present as a terminal electron acceptor for the oxidation of 3-keto-L-erythrose 4-phosphate to 3-keto-L-erythronate 4-phosphate (L-*glycero*-3-tetrulosonic acid 4-phosphate). The β-keto acid was decarboxylated by a soluble decarboxylase to dihydroxyacetonephosphate and CO_2. Dihydroxyacetonephosphate was converted to pyruvic acid by the final enzymes of glycolysis. The apparent dependence on the electron transport system for erythritol catabolism appears to be unique in *Brucella* and may play an important role in coupling metabolism to active transport and generation of ATP.

Smith and co-workers (20, 33, 37) first described the unique role of erythritol in the pathogenesis and physiology of the genus *Brucella*. Extensive growth of *Brucella* occurs in fetal tissues and fluids of pregnant cows, sheep, goats, and sows, leading to endotoxin shock and abortion. In contrast to domestic animals, the bacteria cause a chronic disease in man in which cells of the reticuloendothelial system are parasitized. It is significant that erythritol is present only in fetal fluids and tissues of animals which suffer acute infectious abortions; however, considerable controversy has been raised concerning possible relationship(s) between erythritol utilization and virulence (23, 24). Infections with *B. melitensis* and *B. suis* have been enhanced by co-injection of erythritol (20). Erythritol may play some selective role in tissue localization, since most maternal pathogens do not localize in fetal tissues. It is possible that, through the centuries of association with domestic animals, a unique enzyme system for erythritol catabolism has evolved which is important in the physiology of *Brucella*.

Anderson and Smith (1) reported that *B. abortus* preferentially utilized erythritol in a complex medium containing high concentrations of D-glucose and amino acids. The 4-carbon polyol served as a general carbon source for *B. abortus* as shown by the distribution of radioactivity after exhaustion of [^{14}C]erythritol from growth media: bacteria, 23%, medium, 37%, and carbon dioxide, 40%. Cell extracts prepared by various methods and supplemented with nicotinamide adenine dinucleotide (NAD), NAD phosphate (NADP), adenosine 5'-triphosphate (ATP), and Mg^{2+} did not metabolize erythritol.

The ability to catabolize erythritol is almost universal in the genus *Brucella*. McCullough and Beal (22) studied the utilization of carbohydrates by 12 strains of *Brucella* and found that erythritol was the only sugar which supported growth of all strains. More recently, several hundred strains of *Brucella* were examined for ability to catabolize erythritol, and only the culture of *B. abortus* used for vaccine production in the United States was negative (18, 23, 24).

Since animal tissues do not appear to catab-

olize erythritol, it should be possible to improve chemotherapy with analogues of erythritol or intermediates of erythritol breakdown without toxicity to the host. The growth of *B. abortus* within bovine phagocytes was inhibited by 2-fluoro-D,L-erythritol, under conditions where extracellular streptomycin had no bactericidal effect (32). Once the pathway of erythritol catabolism is known, new analogues can be synthesized.

Examples of erythritol utilization are limited in bacteria and fungi. The pathway of erythritol catabolism has been characterized in *Propionibacterium pentosaceum* (36) and erythritol was shown to be oxidized to L-erythrulose by cell-free extracts of *Enterobacter aerogenes* (17) and the wood-rotting fungus *Schizophyllum commune* (8). Slotnik and Dougherty (31) reported that all strains of *Serratia marcescens* utilize erythritol as a sole carbon source.

In this report we have described the pathway of erythritol catabolism in *B. abortus* (British 19) which proceeds via a series of membrane-bound dehydrogenases and requires a functional electron transport system. The pathway may be important in membrane energization (13) and may partially explain the biochemical basis of tissue localization exhibited by these bacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. abortus*, British strain 19 and U.S. strain 19, were obtained from B. L. Deyoe, National Animal Research Laboratories, Ames, Iowa. Cells were grown on a rotary shaker in 250-ml Erlenmeyer flasks containing tryptose, yeast extract, vitamins, salts, and glucose or erythritol as previously described (27). The cells were harvested in late log phase at an absorbancy at 620 nm (A_{620}, B & L spectrophotometer) of 6 to 7. The cells were centrifuged at 6,000 $\times$ *g* in a Sorvall RC-2B centrifuge for 20 min, washed once with 0.25 volume of 0.05 M *N*-2-hydroxyethyl-piperazine-*N*-2'-ethanesulfonic acid (HEPES)-NH_4OH buffer (pH 7.4), and resuspended to an A_{620} of 40 in the same buffer by swirling with sterile glass beads. Bacterial suspensions to be used for cell extract preparation were stable at 3 to 5 C for up to 10 days.

Cell extract preparation. Cells were broken with a Bronwill MSK cell homogenizer by the method of Robertson and McCullough (28) with minor modifications. A suspension of *B. abortus* (19 ml; A_{620} of 40) and 1 ml of dithiothreitol (DTT) (2×10^{-3} M) were added to a precooled (−5 C), 40-ml, glass-stoppered bottle containing 10 g of 0.17-mm glass beads (B. Braun Melsungen Aparatebau). After 4 min of homogenization, the beads were allowed to settle, and the supernatant fluid was removed and centrifuged at 7,700 $\times$ *g* for 20 min to remove unbroken cells and debris. The supernatant fraction of the centrifugation (3.5 mg/ml, pH 7.2) is hereafter referred to as cell extract (CE).

Preparation of Brucella membranes. To prepare *Brucella* membranes, cells were disrupted as described in CE preparation except that, after the beads settled, the supernatant was decanted and 1 mg each of ribonuclease (EC 3.1.4.22) and deoxyribonuclease (EC 3.1.4.5) was added with stirring for 20 min. The suspension was centrifuged at 3,020 $\times$ *g* for 10 min to remove unbroken cells and debris. The opaque supernatant was centrifuged at 25,000 rpm in an SW41 rotor for 1 h at 4 C (Beckman model L2-65B). The high-speed supernatant was decanted and the membranes were resuspended in 5 to 10 ml of 0.1 M HEPES containing 10^{-4} M DTT with the aid of a variable-speed homogenizer (Tri-R Instrument Co.).

Measurement of respiration. Membrane-bound dehydrogenases were assayed with a Clark oxygen electrode (Yellow Springs Instrument Co.). The reaction reservoir contained: substrate, 3 to 6 μmol; membranes, 1 to 3.2 mg of protein; and 0.1 M HEPES, 100 μmol; in a total volume of 3 ml. Reactions were conducted at 37 C and were started by addition of substrate after 5 min of temperature equilibration. The maximum dissolved oxygen at 37 C was calculated to be 0.22 μmol (0.45 μg-atom) per ml of reaction mixture.

The activity of the overall pathway was determined by oxygen consumption using standard manometric techniques. The main compartment of each 15-ml flask contained (in micromoles): reduced glutathione, 9; $MgSO_4$, 12; $MnSO_4$, 6; $(NH_4)_2HPO_4$, 25; ATP, 5; NAD, 10; as well as 1 ml of CE and distilled water to 2.75 ml. The side arm contained 0.25 ml of [U-^{14}C]-erythritol (25 μmol, 0.05 μCi). The center well contained 0.2 ml of a solution (1:2, vol/vol) of ethanolamine in ethylene glycol monomethyl ether, which was used to trap $^{14}CO_2$. At termination of an experiment, the contents of the main compartment were withdrawn and added to an equal volume of cold 0.6 M perchloric acid. The suspension was centrifuged at 10,000 $\times$ *g* for 10 min in a Sorvall RC-2B and the supernatant was removed and adjusted to pH 6.8 with 1 M potassium hydroxide. The solution was left overnight at 5 C to be assayed later for intermediates of erythritol breakdown or pyruvic acid. The contents of the center well were removed and added to 10 ml of XDC scintillation fluid (9) for determination of radioactivity in a Packard Tri-Carb liquid scintillation spectrometer (model 3375B, Packard Instrument Co., Inc.), with an efficiency of 75% for ^{14}C.

Trapping of intermediates with hydrazine. The reaction mixture contained 35 mg of *B. abortus* (British 19) CE protein and (in millimoles): reduced glutathione, 0.09; NAD, 0.10; ATP, 0.5; $(NH_4)_2HPO_4$, 0.25; hydrazine sulfate, 1.0; [^{14}C]-erythritol (2.5 μCi), 0.5; and $MgCl_2$, 1.0. The final volume was 25 ml with incubation at 37 C for 5 h. The reaction was stopped by addition of 5 ml of 1.8 M $HClO_4$ and the denatured protein was removed by centrifugation at 10,000 $\times$ *g* for 10 min. The supernatant was adjusted to pH 6.8 with 2 N KOH and stored overnight at 4 C. The supernatant was decanted, 5 ml of 1 M $Ba(C_2H_3O_2)_2$ was added with stirring, the pH was adjusted to 7.0 with 1 N NaOH,

and the suspension was centrifuged at 10,000 × *g* for 10 min. The supernatant was adjusted to pH 2.0 with 6 N HBr, followed by addition of 1 g of Norit. The Norit was removed by filtration and the filtrate was adjusted to pH 6.7 with 1 N NaOH. Four volumes of ethanol were added to precipitate the barium salt of the hydrazone(s), and the solution was stored overnight at 4 C. The product was collected by centrifugation at 10,000 × *g* for 10 min, washed with 85% ethanol, and dried in vacuo. The yield of hydrazone(s) was 30% as determined by recovery of radioactivity. The barium and hydrazine were removed by dissolving the salt, adjusting the pH to 3.0, and passing the solution through a small Dowex 50W-X8(H+) column.

Preparation of D-erythritol 1-phosphate (D-erythro-tetritol 1-phosphate). CE (10 ml) was treated with 100 μg each of ribonuclease and deoxyribonuclease with stirring for 20 min, filtered through a membrane filter (0.45 μm, Millipore Corp.), and chromatographed on a column of Sephadex G-200. The fractions with erythritol kinase activity were pooled and concentrated with an Amicon Diaflo apparatus and fractionated (40 to 70% cut) with ammonium sulfate. The reaction mixture contained the 40 to 70% $(NH_4)_2SO_4$ fraction (4.4 IU, 5.6 mg of protein) and (in millimoles): HEPES-NH_4OH (pH 7.4), 0.1; [U-^{14}C]erythritol (2 μCi), 0.25; ATP, 0.25; $MgCl_2$, 0.5; and DTT, 0.0003. The final volume of 3 ml was incubated at 37 C with 115 oscillations per min for 105 min and terminated by addition of $HClO_4$ to 0.3 M. The denatured protein was removed by centrifugation at 10,000 × *g* for 10 min. The supernatant was decanted and adjusted to pH 6.8 with KOH and stored at 5 C for 2 h. The resulting supernatant was decanted and the barium salt of the sugar phosphate was prepared by the method of Anderson and Wood (2). The yield was 12% based on ^{14}C recovery. D-Erythritol 1-phosphate was synthesized as described by MacDonald et al. (21).

Preparation of L-erythritol 1-phosphate (L-erythro-tetritol 1-phosphate). The reaction mixture contained (in millimoles): ATP, 1; erythritol, 2; $MgCl_2$, 2; triethanolamine (TEA)-NH_4OH buffer (pH 8.0), 2; NaF, 0.6; and 1 ml of erythritol kinase (1.55 mg of protein, 10.86 IU, purified from *P. pentosaceum*) (15), in a total volume of 30 ml. The mixture was incubated with stirring at 37 C for 4.5 h, at which time 60% of the ATP was consumed. Acetic acid (3.1 ml, 2.2 M) was added, and the suspension was filtered. The pH of the filtrate was adjusted to 6.7 with NaOH, and 1 ml of 2 M $Ba(C_2H_3O_2)_2$ was added with stirring. After centrifugation of the mixture at 10,000 × *g* for 10 min, the supernatant fluid was decanted and adjusted to pH 2.0 with HBr. The supernatant fluid was treated with acid-washed charcoal until there was minimal absorption at 260 nm. The pH of the solution was then adjusted to 8.5 with NaOH and the barium salt was precipitated with 4 volumes of ethanol. After overnight storage at 4 C, the precipitate was collected by centrifugation, washed with 90% ethanol, air-dried, treated with Dowex 50 (H^+) plus distilled water, and filtered. The solution was adjusted to pH 8.5 with cyclohexylamine and evaporated to dryness. The product was recrystallized from ethanol and dried in a desiccator. The yield was 25% based on ^{14}C recovery.

Preparation of 3-keto-L-erythrose 1-phosphate (L-glycero-3-tetrosulose 4-phosphate). The reaction contained 30 ml of CE prepared by centrifugation at 100,000 × *g* and (in millimoles): HEPES-NH_4OH (pH 7.4), 1.5; niacinamide, 0.025; DTT, 0.006; $MgCl_2$, 0.3; NAD, 0.1; ATP, 1; sodium pyruvate, 2; [U-^{14}C]erythritol (4 μCi), 1.02; KOH, 2.2; 10 μliters of type III beef heart lactate dehydrogenase (EC 1.1.1.27, 3.2 IU, 100 μg of protein), and distilled water to 45 ml. The mixture was incubated at 37 C with shaking for 3 h, and the reaction was terminated by addition of 5 ml of perchloric acid (3 M) and centrifuged at 10,000 × *g* for 15 min. The supernatant was adjusted to pH 6.8 with KOH and stored at 3 to 5 C for 2 h. The effluent was decanted, and 5 ml of 1 M $Ba(C_2H_3O_2)_2$ was added with stirring. The pH was adjusted to 7.0 with NaOH and the precipitate was removed by centrifugation at 10,000 × *g* for 15 min. The supernatant was adjusted to pH 2.0 with HBr (6 M) followed by addition of 2.5 g of acid-washed charcoal with stirring. The mixture was filtered and the filtrate was adjusted to pH 6.7 with hydrazine hydrate. The sugar phosphate was precipitated with 4 volumes of ethanol, and the solution was stored overnight at 5 C. The precipitate was collected by centrifugation, washed with 85% ethanol, and dried in a vacuum desiccator. The yields ranged from 50 to 55% based on ^{14}C recovery.

Inhibition of triosephosphate isomerase. The phosphate isomerase activity in cell extracts of *B. abortus* was inhibited using the active site reagent, glycidol 1-phosphate (29). Four milliliters of CE was incubated with 4.4 μmol of glycidol 1-phosphate and 0.22 μmol of phosphoenolpyruvate (PEP) at room temperature, the PEP being added to protect enolase (21). The excess glycidol 1-phosphate was removed by dialysis for 2.4 h against 20 volumes of 0.1 M HEPES containing 100 μg of niacinamide per ml and 10^{-4} M DTT. Inactivated preparations were incubated with erythritol or phosphorylated intermediates using the incubation mixture described earlier.

DCIP enzyme assays. The assay mixture contained 10 μmol of HEPES, 0.03 μmol of dichlorophenol indophenol (DCIP), 0.2 μmol of NAD, 10 μliters of diaphorase (type III, pig heart, 2 IU), 10 μliters of CE (30 to 50 μg of protein), and distilled water to 0.3 ml. Some assays included addition of 2 μmol of $MgCl_2$. Once the blank rate was negligible, the reaction was initiated with 0.5 μmol of substrate. The light path was 1 cm and the reaction was monitored at A_{600}.

NAD-linked dehydrogenase assays. The reaction mixture in 0.3 ml contained 140 μg of CE protein (supernatant from 100,000 × *g* centrifugation) and (in micromoles): HEPES-NH_4OH (pH 7.4), 10; reduced glutathione, 0.9; NAD, 0.2; $MgCl_2$, 2.0; and substrate, 0.5. The absorbance was monitored at 340 nm, and the reaction was started by addition of CE.

Radioactive kinase assay. The radioactive kinase assay of Newsholme et al. (25) was used with the following modifications. The reactions were incubated at 30 C and contained 10 μliters of CE (30 to 50 μg of CE protein) and (in micromoles): HEPES-NH_4OH (pH 7.4), 0.4; MgCl, 0.2; ATP, 0.1; and [U-^{14}C]erythritol (0.1 μCi), 0.1. At timed intervals, the reactions were stopped by addition of

15 μliters of hot ethanol, and the contents were centrifuged. The supernatant (20 μliters) was spotted on the center of a Whatman diethylaminoethyl (DEAE) filter disk (DE 21, 2.4 cm, Reeve Angel), air-dried for 0.5 h, and washed with 200 ml of distilled, deionized water.

Paper chromatography. Descending paper chromatography was performed on Whatman 3MM paper in a chromatocab (Warner-Chilcott Laboratories Instruments Div.). After 6 to 8 h of development, the papers were removed, air-dried, and then sprayed for polyols with periodate-*p*-anisidine spray of Bragg and Hough (7) or for organic phosphate compounds (4).

Preparation and spectra of MBTH derivatives. Methylbenzothiazolone hydrazone hydrochloride (MBTH) derivatives of intermediates containing a carbonyl group were prepared by the procedure of Paz et al. (26). The spectra were obtained in 1-ml cuvettes at room temperature using a Cary model 14 spectrophotometer. The samples were incubated intermittently in a 40 C water bath until the spectra of the derivatives had stabilized.

Analytical methods. Periodate oxidations of carbohydrates were performed as described by Jackson (16). The reaction mixture (5.5 ml) contained (in micromoles): acetic acid, 40; $NaIO_4$, 5; and substrate, 0.2 to 1.0. The reaction was incubated up to 60 min in the dark and stopped with 0.2 ml of 0.2 M Na_2AsO_2. Formaldehyde was determined by the chromatropic acid procedure (35) and formate was determined by the thiobarbituric acid assay (5). Protein was determined with the micro-biuret procedure (3). β-Keto acids were detected using the *p*-nitroaniline diazo reagent (19).

Periodate disappearance was measured by the method of Dixon and Lipkin (12). Pyruvate, dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate were assayed enzymatically (10). Erythritol was measured by a coupled spectrophotometric enzyme assay at 340 nm which contained (in micromoles): TEA·HCl-NH_4OH buffer (pH 7.4), 100; $MgCl_2$, 2; ATP, 1; PEP, 0.5; NADH, 0.1; pyruvate kinase (EC 2.7.1.40, 3 IU); lactate dehydrogenase (16 IU); 5 μliters of erythritol kinase (*P. pentosaceum*, 10 μg of protein, EC 2.7.1.27, 0.5 IU); and up to 75 μliters of solution containing 10 to 40 nmol of erythritol in a final volume of 0.3 ml.

Characterization of isolated intermediates. D- and L-erythritol 1-phosphate, synthesized chemically and enzymatically, were characterized by paper chromatography against reference standards, periodate degradation (16), and hydrolysis with alkaline phosphatase with determination of either erythritol or inorganic phosphate. The MBTH derivatives (26) of D-erythrulose 1-phosphate and 3-keto-L-erythrose 4-phosphate were prepared in addition to the above parameters.

The hydrazone isolated by paper chromatography, D-erythrulose 1-phosphate (D-*glycero*-2-tetrulose 1-phosphate) prepared with CE of *B. abortus* (US-19) (J. F. Sperry and D. C. Robertson, submitted for publication), and synthetic D-erythrulose 1-phosphate were tested as substrate for glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) and fructose 1,6-diphosphate aldolase (EC 4.1.2.13) as described by Chu and Ballou (11). The reaction mixture contained 10.0 μmol of TEA·HCl (pH 7.6), 0.11 μmol of reduced NAD (NADH), 1.0 μmol of D-erythrulose 1-phosphate, and 0.03 IU of glycerol 3-phosphate dehydrogenase in a total volume of 0.3 ml. The glycerol 3-phosphate dehydrogenase level was 10-fold that which gave A_{340} of 0.06/min (3×10^{-3} IU) with DHAP as substrate. The same reaction mixture, containing glycerol 3-phosphate dehydrogenase, was used for incubations with fructose 1,6-diphosphate aldolase (0.03 IU).

The concentration of L-erythritol 1-phosphate, D-erythritol 1-phosphate, D-erythrulose 1-phosphate, and 3-keto-L-erythrose 4-phosphate was determined from the specific activity of the [^{14}C]erythritol added to each reaction mixture which was established using the enzymatic assay for erythritol and counting a sample in XDC scintillation fluid (9). D-Erythritol 1-phosphate and L-erythritol 1-phosphate were digested with alkaline phosphatase and the erythritol was determined enzymatically: a 1:1 ratio of ^{14}C to erythritol was routinely observed. D-Erythrulose 1-phosphate was determined with glycerol 3-phosphate dehydrogenase (10), and the amount reduced with NADH agreed with the calculated specific activity.

Materials. Tryptose, yeast extract, and potato infusion agar were obtained from Difco Laboratories. Trypticase soy agar was purchased from BBL. All standard biochemicals, unless otherwise indicated, were obtained from Sigma Chemical Co. *p*-Nitroaniline was purchased from Eastman Kodak Co. MBTH was purchased from Aldrich Chemical Company. Naphthalene (purified) and ammonium sulfate were purchased from Mallinckrodt Chemical Works. [U-^{14}C]erythritol (3.6 mCi/mmol) was obtained from Amersham/Searle. All other chemicals were of reagent grade and were purchased from commercial sources.

RESULTS

CE preparation and cofactor requirements. CE of *B. abortus* which metabolized erythritol to pyruvate and CO_2 were prepared using the MSK cell homogenizer (Table 1). The carbon recovery was 95% and probably accounts for all the major end products, since pyruvate was not degraded by these extracts (Table 1). The minimal erythritol utilization shown was due to each reaction mixture containing 1.7 mg of CE protein. Increasing the CE protein threefold increased erythritol breakdown and the accumulation of products by as much as 14-fold (data not shown). The basis of the concentration effect is unknown. The activity of the overall pathway could be measured either by oxygen consumption or by the accumulation of pyruvic acid. Optimal enzymatic activity was obtained when the bacteria were disrupted in HEPES buffer as compared to 0.05 M phosphate buffer (pH 7.4) or 0.05 M tris(hydroxymethyl)amino-

methane-hydrochloride buffer (pH 7.4) (data not shown).

Dialyzed extracts were used to establish that ATP and NAD were required for both oxygen uptake and pyruvic acid accumulation (Table 2). The minimal activity in the absence of exogenous NAD probably reflects membrane-bound cofactor.

Phosphorylation of erythritol and product identification. The phosphorylation of erythritol by an ATP-dependent kinase (ATP: erythritol 1-phosphotransferase) was shown using a radiochemical kinase assay (Fig. 1). High levels of NADH oxidase activity did not permit the use of coupled spectrophotometric kinase assay. The phosphorylation was linear for 10 min, and no radioactivity was abosrbed to the DEAE filters when CE of *B. abortus* was incubated with [^{14}C]erythritol in the absence of ATP. In later experiments, erythritol kinase was coupled to the subsequent dehydrogenase in the pathway using DCIP as an artificial electron acceptor (Table 3). There was no dye reduction until ATP was added to the reaction mixture, which further indicates that phosphorylation precedes dehydrogenation.

The product of erythritol kinase in CE of *B. abortus* (British 19) could be either D- or L-erythritol 1-phosphate. L-Erythritol 1-phosphate prepared with partially purified erythritol kinase from *P. pentosaceum* was not converted to pyruvate and CO_2 with oxygen comsumption, in contrast to the reaction mixture which contained synthetic D-erythritol 1-phosphate (Table 4). The erythritol kinase in CE of *B. abortus* was purified by Sephadex G-200 and $(NH_4)_2SO_4$

TABLE 1. *End products of erythritol catabolism by CE of B. abortus (British 19)*

Expt[a]	Erythritol consumed[b] (μmol)	$^{14}CO_2$ (μmol)	Pyruvate (μmol)
1	4.31	4.44	3.93
2	3.66	4.29	4.07
Avg	3.99	4.37	4.00

[a] Experiments were run in 15-ml Warburg flasks at 37 C for 3 h. Each reaction mixture contained 1.7 mg of CE protein and (in micromoles): HEPES-NH_4OH (pH 7.4), 50; niacinamide, 0.2; reduced glutathione, 9.0; $MgSO_4$, 12.0; $MnSO_4$, 6; $(NH_4)_2HPO_4$, 25; ATP, 5.0; NAD, 10; and [^{14}C]erythritol (0.2 μCi), 25. The center well contained 0.2 ml of ethanolamine in ethylene glycol monomethyl ether (1:2, vol/vol) to trap $^{14}CO_2$. Reactions were terminated with 0.6 M $HClO_4$ and processed for product analysis.

[b] Calculated as micromoles added minus residual micromoles of erythritol.

TABLE 2. *Requirements for erythritol catabolism by cell extracts*

Reaction mixture[a]	O_2 uptake (μmol)	Pyruvate (μmol)
Complete	16.3	4.31
-ATP	0.5	0
-NAD	4.7	1.20

[a] The CE was dialyzed for 2.5 h against 20 volumes of 0.05 M HEPES-NH_4OH (pH 7.4) containing 100 μg of niacinamide per ml and 10^{-4} M DTT. Conditions and reaction mixture were as described in Table 1.

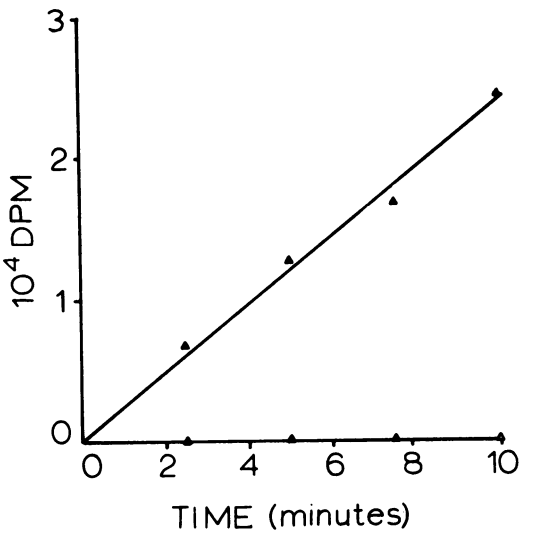

FIG. 1. *Radiochemical assay for erythritol kinase, using [^{14}C]erythritol plus ATP (▲) and [^{14}C]erythritol minus ATP (△). Each assay contained 50 μg of CE protein.*

TABLE 3. *Enzyme activities of the erythritol catabolic pathway[a]*

Substrate	Activities[b]	
	British 19 CE	U.S. 19 CE
Erythritol	0	0
Erythritol + ATP	0.010	0.013
D-Erythritol-1-PO_4	0.040	0.032
D-Erythrulose-1-PO_4[c]	0.023	0

[a] The assays were run at 600 nm and 23 C in micro cuvettes using a Gilford spectrophotometer and contained 35 μg of CE protein, 2 IU of pig heart diaphorase, and (in micromoles): HEPES-NH_4OH (pH 7.4), 10; DCIP, 0.03; and NAD, 0.2. The absorbance was followed until the blank was negligible, and the reaction was started by addition of 0.5 μmol of substrate.

[b] Micromoles of product formed per minute per milligram of protein.

[c] $MgCl_2$ (2 μmol) was added.

fractionation. The partially purified preparation converted [^{14}C]erythritol in the presence of ATP to a sugar phosphate which migrated with an R_f of synthetic D-erythritol 1-phosphate. The radioactive peak coincided with that detected with the phosphate spray. Periodate consumption, although somewhat low, was similar for the product of *B. abortus* (British 19) erythritol kinase and the synthetic product (Table 5). The reason for the occasional low consumption of periodate is unknown; however, the stoichiometry of products in later experiments was close to the expected value of 1.0. These data indicated the incorporation of a phosphate group in one of the primary hydroxyl groups of erythritol (21) and that the product of the erythritol kinase in CE of *B. abortus* was D-erythritol 1-phosphate.

Identification of D-erythrulose 1-phosphate as oxidation product of D-erythritol 1-phosphate dehydrogenase. The oxygen uptake observed with CE of *B. abortus* and D-erythritol 1-phosphate (D-*erythro*-tetritol 1-phosphate) (Table 4) suggested that a dehydrogenase (D-*erythro*-tetritol 1-phosphate:NAD$^+$ 2-oxidoreductase) was the second enzyme in the pathway and was detected using CE, D-erythritol 1-phosphate, and the artificial electron acceptor, DCIP (Table 3). The enzyme was inactive with NADP; thus the reaction appeared to be specific for NAD.

TABLE 4. *Catabolism of erythritol catabolic intermediates by CE of B. abortus (British 19)*

Substrate[a]	O_2 uptake (μmol)	$^{14}CO_2$ (μmol)	Pyruvate (μmol)
Erythritol	21.4	10.9	11.0
D-Erythritol-1-PO_4	9.2	ND	3.1
L-Erythritol-1-PO_4	2.6	0	0
D-Erythrulose-1-PO_4	8.7	5.1	5.4
3-Keto-L-erythrose-4-PO_4	4.8	4.0	4.0

[a] The experimental conditions were as described in Table 1, except that each reaction contained 3.5 mg of CE protein. The amounts of ^{14}C substrates were as follows: erythritol, 25 μmol (4,440 dpm/μmol); D-erythrulose 1-phosphate, 10 μmol (10,950 dpm/μmol); and 3-keto-L-erythrose 4-phosphate, 10 μmol (9,840 dpm/μmol). ND, Not determined.

The possible products of D-erythritol 1-phosphate oxidation included D-erythrulose 1-phosphate, L-erythrulose 4-phosphate, and L-erythrose 4-phosphate. Since either a keto or aldehyde group was possible, hydrazine was employed as a trapping agent. Addition of hydrazine to a reaction mixture containing *B. abortus* (British 19) CE and other cofactors required for activity of the pathway resulted in the accumulation of at least 2 hydrazones. After removal of the barium and hydrazine, the preparation was examined by paper chromatography using methanol, ammonia, and water (6:1:3, vol/vol/vol) as the solvent. Two radioactive spots, both polyol and phosphate positive, with R_f values of 0.55 and 0.63, were detected. Both sugar phosphates were eluted with distilled water, and the sugar phosphate with an R_f of 0.55 contained 70% of the radioactivity applied to the paper. Substrate amounts of the major product were isolated by streaking on Whatman 3MM paper and chromatographic elution of the band with distilled water. The isolated intermediate was metabolized by CE of *B. abortus* (British 19), reduced by α-glycerolphosphate dehydrogenase (α-GDH) and not cleaved by fructose 1,6-diphosphate (FDP) aldolase. The

TABLE 5. *Characterization of phosphorylated intermediates of the erythritol pathway in B. abortus (British 19)*

Characteristic	D-Erythritol 1-phosphate	D-Erythrulose 1-phosphate	3-Keto-L-erythrose 4-phosphate
R_f (MeOH-NH_4OH-H_2O) 6:1:3, vol/vol/vol	0.73(0.73)[a]	0.55(0.56)[a]	0.67
P_i/erythritol	0.88	–	–
Periodate degradation			
Consumption (μmol of IO_4/μmol of substrate)	1.41(1.45)[a,b]	1.9	1.41
Products (μmol/μmol of substrate)			
Formaldehyde	0.98[b]	0.95	0.00
Formic acid	0.97	0.97	1.41
MBTH derivative λ_{max}	–	318	304
Substrate for:			
Glycerol 3-phosphate dehydrogenase	ND[c]	+	ND
Fructose 1,6-diphosphate aldolase	ND	–	ND

[a] Numbers in parentheses were obtained with synthetic compounds provided by C. E. Ballou.
[b] Periodate consumption and products were from different experiments.
[c] ND, Not determined.

reduction by α-GDH indicated a carbonyl group alpha to the phosphate ester group; hence, the product could be either D- or L-erythrulose 1-phosphate. The D-erythritol 1-phosphate oxidation product was not split by FDP aldolase, known to act between trans hydroxyl groups (11); thus, the hydrazone was tentatively identified as that of D-erythrulose 1-phosphate (D-*glycero*-2-tetrulose 1-phosphate). Finally, D-erythrulose 1-phosphate prepared by chemical synthesis was converted by CE of *B. abortus* (British 19) to pyruvic acid and CO_2 with oxygen consumption (Table 4).

The oxygen uptake with CE and D-erythrulose 1-phosphate suggested yet another dehydrogenase in the pathway. It was not possible to separate D-erythrulose 1-phosphate from the contaminating hydrazone by column chromatography using Dowex 1X-8 (either formate or bicarbonate form), and only limited amounts (40 to 50 μmol) could be isolated by preparative paper chromatography. Fortunately, D-erythrulose 1-phosphate dehydrogenase (D-*glycero*-2-tetrulose 1-phosphate:NAD^+ 4-oxidoreductase) was found to be absent in CE of *B. abortus* used for vaccine production in the United States (Table 3) (J. F. Sperry and D. C. Robertson, manuscript in preparation), which presented a rapid and convenient method of preparation. The sugar phosphate product formed by *B. abortus* (US-19) CE showed an R_f of 0.55 using methanol-ammonia-water (6:1:3, vol/vol/vol), similar to chemically synthesized D-erythrulose 1-phosphate, and served as substrate for α-GDH (Table 5). The phosphate ester was not cleaved by FDP aldolase and the periodate consumption was that expected of D-erythrulose 1-phosphate.

Oxidation of D-erythrulose 1-phosphate. D-Erythrulose 1-phosphate dehydrogenase was demonstrated in CE of *B. abortus* (British 19) by coupling the enzymatic activity to DCIP (Table 3). High levels of NADH oxidase activity prevented following the reduction of NAD at 340 nm. Diaphorase activity was 100-fold that of NADH oxidase; thus, dye reduction was a valid indication of dehydrogenase activity. The enzyme was NAD dependent, and no activity was observed unless magnesium was added to the reaction mixture.

Since CO_2 was one of the products of the pathway, it was obvious that an oxidative cleavage of a terminal carbon was one of the latter steps. The oxygen uptake with D-erythrulose 1-phosphate, and the consumption of 1.5 mol of oxygen per mol of pyruvic acid formed, suggested oxidation at C4 to a carboxyl group, with a possible aldehyde intermediate. Although formaldehyde dehydrogenase activity was present in CE, no formate dehydrogenase could be detected (unpublished data). Neither unlabeled formic acid nor unlabeled formaldehyde diluted the specific activity of $^{14}CO_2$ released during catabolism of [^{14}C]erythritol by CE of *B. abortus* (British 19). Hence, it was concluded that CO_2 was a primary product of the decarboxylation step and not formic acid or formaldehyde which was oxidized to CO_2.

Electron transport system and erythritol catabolism. Early experiments suggested that molecular oxygen was essential to the operation of the pathway; thus anaerobic conditions were employed to trap intermediates between D-erythrulose 1-phosphate and the decarboxylation step. Under anaerobic conditions, little or no CO_2 was released or pyruvate formed with erythritol, D-erythritol 1-phosphate, or D-erythrulose 1-phosphate in the presence of excess NAD and ATP. An NAD-generating system consisting of sodium pyruvate and lactate dehydrogenase did not increase the levels of intermediates (Table 6).

Artificial electron acceptors were used to ascertain whether molecular oxygen was directly involved. Addition of 10 mM nitrate stimulated the anaerobic breakdown of erythritol to levels which approached aerobic control levels (Table 6). Sodium nitrite and hydroxylamine had no effect on the anaerobic inhibition of erythritol catabolism, which suggested an important role for a one-step dissimilatory nitrate reductase in *B. abortus*. These data strongly implied that electron transport was essential to erythritol utilization by *B. abortus*.

Inhibitors of the electron transport system were used to probe the interactions between erythritol catabolism, molecular oxygen, and

TABLE 6. *Effects of oxygen and electron acceptors on erythritol catabolism*

System[a]	Addition (10 mM)	O_2 uptake (μmol)	$^{14}CO_2$ (μmol)	Pyruvate (μmol)
Aerobic		16.66	5.38	3.62
	KNO_3	9.72	7.11	6.92
Anaerobic			0.27	0.00
Anaerobic + LDH[b]	Sodium pyruvate		0.37	0.00
Anaerobic	KNO_3		4.75	2.89
Anaerobic	$NaNO_2$		0.29	0.00
Anaerobic	NH_2OH		0.23	0.00

[a] The reaction mixture (3 ml) contained 3.5 mg of CE protein: other conditions were the same as in Table 1, except when flused with nitrogen.

[b] Contained 5 μliters of LDH (16 IU) and 25 μmol of sodium pyruvate, as an NAD-generating system.

the nitrate reductase system. As indicated in Table 7, amytal, dicumarol, and HOQNO caused significant inhibition of erythritol catabolism as measured by release of $^{14}CO_2$ (similar data were obtained by following the accumulation of pyruvic acid). It should be noted that KNO_3 was present in all anaerobic experiments as a terminal electron acceptor. These data plus experiments on the electron transport system of *B. abortus* (R. F. Rest and D. C. Robertson, submitted for publication) show that particulate dehydrogenases of the erythritol pathway, flavoproteins, ubiquinone, and cytochromes *b* and *c* can be coupled to nitrate via the one-step dissimilatory nitrate reductase.

Soluble and membrane-bound enzymes of erythritol catabolism. It was now apparent that some of the enzymes involved in erythritol catabolism were membrane bound. Centrifugation at 100,000 × *g* resolved the CE into supernatant and membrane fractions. Erythritol kinase was in the soluble fraction; however, D-erythritol 1-phosphate and D-erythrulose 1-phosphate dehydrogenases were only partially solubilized during disruption (Table 8). Both dehydrogenases may be membrane-bound enzymes within the cell.

Identification of D-erythrulose 1-phosphate dehydrogenase product. The product of D-erythrulose 1-phosphate dehydrogenase accumulated when the high-speed supernatant was incubated with [^{14}C]erythritol, ATP, and an NAD-generating system. The sugar phosphate was isolated as a barium salt. A single radioactive sugar phosphate spot, R_f 0.67 (similar to minor product trapped with hydrazine), was detected after removal of the barium with Dowex 50 W-X8 (H^+) and paper chromatography in the alkaline solvent system. The λ max of the MBTH derivative resembled that of an aldehyde and was quite distinct from D-erythrulose 1-phosphate (Table 5) which resembled pyruvic acid. It should be noted that the periodate degradation studies (Table 5) were performed under similar conditions, and that the rate of periodate consumption by 3-keto-L-erythrose 4-phosphate was markedly slower than for either D-erythritol 1-phosphate or D-erythrulose 1 phosphate. Even though the yield of formic acid was less than the expected value of 2.0 μmol/μmol of substrate, there was good agreement between the amount formed and the periodate consumed. Also, no formaldehyde was detected which indicated that the terminal carbon had been oxidized to an aldehyde. The λ max of the MBTH derivative and periodate oxidation products of the intermediate were consistent with the oxidation of the C4 carbon from a primary alcohol to an aldehyde, with the product being 3-keto-L-erythrose 1-phosphate (L-*glycero*-3-tetrosulose 4-phosphate).

TABLE 7. *Effect of electron transport system inhibitors on erythritol catabolism by CE of B. abortus (British 19)*

Inhibitor	Concn (M)	O_2[a]	$^{14}CO_2$ (μmol)	Inhibition (%)
None		+	2.88	
None		−	3.47	
Amytal	10^{-3}	+	0.35	80.8
Amytal	10^{-3}	−	0.13	96.2
Dicumarol	10^{-4}	+	0.61	78.8
Dicumarol	10^{-4}	−	1.07	69.2
HOQNO	5×10^{-6}	+	3.08	−6.9
HOQNO	5×10^{-6}	−	0.69	80.1

[a] +, aerobic; −, flushed with nitrogen.

[b] Experimental conditions were as described in Table 1, except that each reaction mixture contained 3.5 mg of CE protein and 30 μmol of KNO_3.

TABLE 8. *Distribution of enzyme activities in supernatant and membrane fractions of B. abortus (British 19)*

Enzyme	Units/mg of protein	
	Supernatant[a]	Membrane[b]
Erythritol kinase	0.209[c]	ND
D-Erythritol-1-PO_4 dehydrogenase	0.039	0.120
D-Erythritol-1-PO_4 dehydrogenase	0.060	0.007
3-Keto-L-erythrose-4-PO_4 dehydrogenase	0	0.038

[a] Determined by NAD reduction in a 0.3-ml reaction mixture which contained 140 μg of CE protein and (in micromoles): HEPES-NH_4OH (pH 7.4), 10; reduced glutathione, 0.9; NAD, 0.2; $MgCl_2$, 2.0; and substrate, 0.5.

[b] Determined with the oxygen electrode in a 3.0-ml reaction mixture which contained 2.6 mg of membrane protein and (in micromoles): HEPES-NH_4OH (pH 7.4), 150.0; $MgCl_2$, 20.0; and substrate, 3.0. Assays were run at 37 C.

[c] ATP (1.0 μmol) was added to the reaction mixture.

Oxidation of 3-keto-L-erythrose 4-phosphate by membranes. Incubation of 3-keto-L-erythrose 1-phosphate with the membrane fraction of 100,000 × *g* centrifugation resulted in the formation of 3-keto-L-erythronate 4-phosphate (L-*glycero*-3-tetrulosonic acid 4-phosphate) (Fig. 2) and was not detected with membranes alone. This oxidative activity is present only in the membrane fraction (Table 8) and

could be assayed using the oxygen electrode without additional cofactors. The complete electron transport system was reduced when the membranes were incubated with the sugar phosphate. The β-keto acid has not been isolated for chemical characterization.

Decarboxylation and formation of DHAP. The decarboxylation step was enzyme mediated and present in the soluble fraction of the 100,000 × *g* centrifugation (Table 9). Small amounts of $^{14}CO_2$ were detected with either the soluble or membrane fractions; however, when the two were combined, the yield of $^{14}CO_2$ was increased ninefold, which supports an oxidation and subsequent decarboxylation.

Isolation of the decarboxylase (L-*glycero*-3-tetrulosonic acid 4-phosphate 1-carboxy-lyase) product was complicated by the extremely high turnover number (10 IU/ml) of triose phosphate isomerase in CE of *B. abortus*. Isotopic dilution experiments failed to distinguish between DHAP and D-glyceraldehyde 3-phosphate. The active site reagent, glycidol 1-phosphate, was used to inhibit triose phosphate isomerase activity, and the treated CE were then incubated with various sugar phosphates. The similar inhibition observed between D-erythrulose 1-phosphate and DHAP (Table 10) strongly suggests that DHAP is the product of the erythritol pathway. DHAP can be converted to D-glyceraldehyde 3-phosphate and metabolized to pyruvic acid by the enzymes of the latter enzymes of the glycolytic (Embden-Meyerhof) pathway (28). Due to very high triose phosphate isomerase in CE and only 99% inhibition by glycidol-phosphate, DHAP did not accumulate in reaction mixtures.

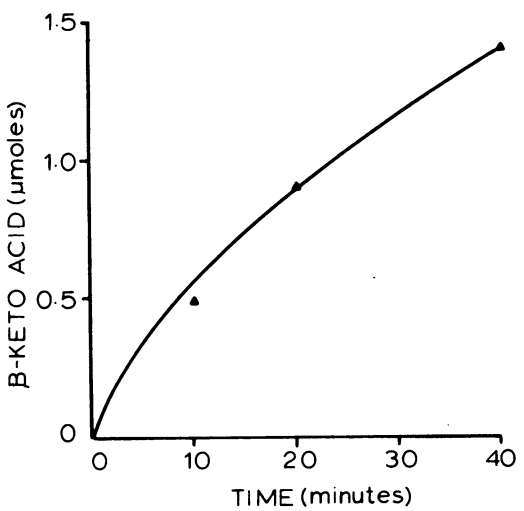

FIG. 2. *Production of β-keto acid, as measured by diazo reagent assay. The reaction mixture was incubated at 37 C and contained 20 mg of B. abortus (British 19) membrane protein, 100 μmol of HEPES-NH_4OH (pH 7.4), and 10 μmol of 3-keto-L-erythrose 4-phosphate in a total volume of 2.5 ml.*

TABLE 9. *Catabolism of 3-keto-L-erythrose-4-PO_4 by supernatant and membrane fractions*

Supernatant[a]	Membrane[a]	O_2 uptake (μmol)	$^{14}CO_2$ (μmol)
+	−	2.3	0.76
−	+	4.0	0
+	+	18.5	6.28

[a] Each reaction mixture contained 8 mg of supernatant protein and 2.5 mg of membrane protein as indicated; other experimental conditions were as indicated in Table 1, except that 10 μmol of 3-keto-L-erythrose-4-PO_4 (9,840 dpm/μmol) was substituted for erythritol.

TABLE 10. *Effects of glycidol-PO_4 on erythritol catabolism in cell extract*

Substrate[a]	Glycidol-PO_4[b]	Pyruvate (μmol)	Inhibition (%)
D-Erythrulose-1-PO_4	−	5.32	
D-Erythrulose-1-PO_4	+	1.33	75.0
D-Glyceraldehyde-3-PO_4	−	8.16	
D-Glyceraldehyde-3-PO_4	+	7.41	9.2
Dihydroxyacetone-PO_4	−	7.91	
Dihydroxyacetone-PO_4	+	1.48	81.3

[a] Substrate was 10 μmol.

[b] The reaction mixture contained 3.5 mg of control or glycidol-PO_4-treated CE and the other conditions were the same as Table 1.

DISCUSSION

The proposed pathway of erythritol catabolism in *B. abortus* (British 19) (Fig. 3) is unique in that a functional electron transport system is apparently required for conversion of 3-keto-L-erythrose 1-phosphate (L-*glycero*-3-tetrosulose 4-phosphate) to 3-keto-L-erythronate 4-phosphate (L-*glycero*-3-tetrulosonic acid 4-phosphate). Further, D-erythritol 1-phosphate: NAD^+ 2-oxidoreductase) was shown to be membrane bound and partially solubilized during disruption. It is also possible that the enzyme exists in multiple forms. D-Erythritol 1-phosphate (D-*erythro*-tetritol 1-phosphate) (Rest and Robertson, submitted for publication) has

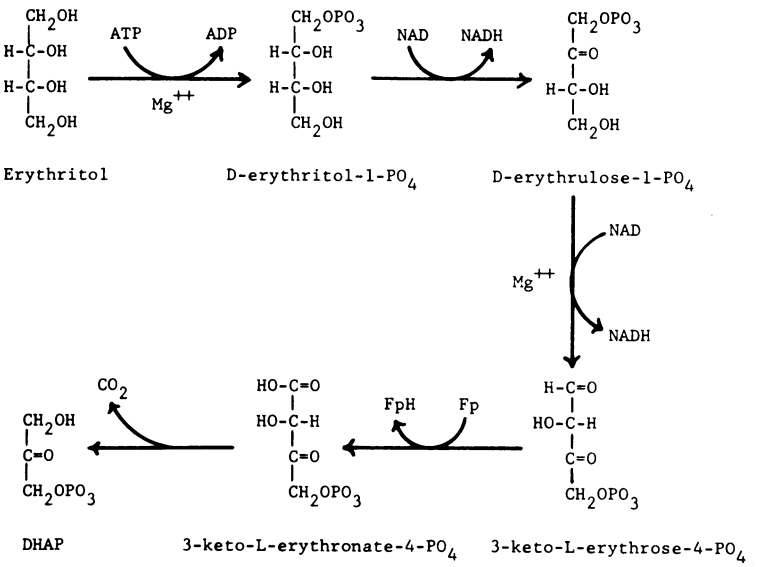

FIG. 3. *Proposed pathway for erythritol catabolism in B. abortus.*

been shown to act as an electron donor for the *Brucella* electron transport system. Hence it appears that energy metabolism and erythritol dissimilation are tightly coupled in *Brucella*.

The erythritol kinase (ATP:erythritol 1-phosphotransferase) in *B. abortus* forms D-erythritol 1-phosphate, whereas the kinase in *P. pentosaceum* (30) converts erythritol to L-erythritol 1-phosphate. L-Erythritol 1-phosphate prepared with a partially purified enzyme preparation from *P. pentosaceum* was not catabolized by CE of *B. abortus* and, in fact, exhibited moderate inhibition as assayed by the conversion of erythritol to pyruvic acid and CO_2.

Erythritol kinase was partially purified from CE of *B. abortus* by Sephadex G-200 chromatography and $(NH_4)_2SO_4$ fractionation for synthesis of D-erythritol 1-phosphate. The isolated sugar phosphate migrated as a single spot during paper chromatography using both radioactivity and phosphate spray as markers. The periodate consumption indicated that phosphorylation was not at C2 or C3 and the product served as substrate for α-GDH.

The next intermediate, D-erythrulose 1-phosphate (D-*glycero*-2-tetrulose 1-phosphate), could be trapped by addition of hydrazine to reaction mixtures; however, it was not possible to obtain substrate amounts by this method. CE of *B. abortus* (U.S. 19) were found to lack D-erythrulose 1-phosphate dehydrogenase and were used to synthesize the intermediate in 55% yield. The D configuration was indicated since the isolated product was not cleaved by FDP aldolase and was reduced by α-GDH. Only one spot was detected by paper chromatography (radioactivity and phosphate spray) with an R_f similar to synthetic D-erythrulose 1-phosphate. Periodate degradation products were consistent with D-erythrulose 1-phosphate being the next intermediate in the pathway.

The oxidation of D-erythrulose 1-phosphate by an NAD-dependent dehydrogenase (D-*glycero*-2-tetrulose 1-phosphate:NAD^+ 1-oxidoreductase) could be demonstrated by coupling to DCIP; however, the product was not isolated until it became evident that 3-keto-L-erythrose 4-phosphate dehydrogenase was membrane bound and tightly coupled, or was an integral part of the electron transport system. The CE was centrifuged at 100,000 $\times$ *g* to remove membranes, and the soluble fraction was used as a source of enzymes to convert D-erythrulose 1-phosphate to 3-keto-L-erythrulose 4-phosphate. Alternatively, since D-erythritol 1-phosphate dehydrogenase is partially solubilized, erythritol was converted to the same aldehyde intermediate by addition of ATP and NAD with lactate dehydrogenase and pyruvic acid added to oxidize NADH formed by the two dehydrogenases. The MBTH derivative of the product of D-erythrulose 1-phosphate dehydrogenase was indicative of an aldhyde, and periodate degradation indicated that primary hydroxyl at C4 was oxidized to an aldehyde.

Initially, it appeared that an oxidase mediated the next step in the pathway. The oxidation of D-erythrulose 1-phosphate could be demonstrated anaerobically, yet no release of $^{14}CO_2$ occurred unless molecular oxygen was present.

Artificial electron acceptors were employed to determine whether oxygen was directly or indirectly involved in the pathway. Zobel and Meyer (38) demonstrated that, of 425 strains of *Brucella*, all reduced nitrates to nitrites, indicating the presence of nitrate reductase. When nitrate was added as an acceptor, the yields of pyruvic acid and CO_2 were almost those obtained in the presence of O_2. A nitrate reductase has been shown to be coupled to the electron transport system of *B. abortus* via flavoproteins, cytochrome *b*, and cytochrome *c* (Robertson and Rest, submitted for publication). When the membrane fraction was added to the 100,000 $\times$ *g* supernatant, oxygen was consumed with formation of a β-keto acid from 3-keto-L-erythrose 1-phosphate. 3-Keto-L-erythrose 4-phosphate served as an electron donor with reduction of the complete electron transport system as was observed with D-erythritol 1-phosphate (Robertson and Rest, submitted for publication). 3-Keto-L-erythrose 4-phosphate has not been isolated due to the unfavorable equilibrium of the membrane-bound 3-keto-L-erythrose 4-phosphate dehydrogenase (L-*glycero*-3-tetrosulose 4-phosphate:ferricytochrome 1-oxidoreductase), which is also the rate-limiting enzyme in the pathway. The rate of the overall pathway seems to depend on the decarboxylase pulling the oxidation to the β-keto acid which can then be converted to DHAP.

The decarboxylation was shown to be an enzyme-mediated reaction with the decarboxylase (L-*glycero*-3-tetrulosonic acid 4-phosphate 1-carboxy-lyase) in the soluble fraction of the 100,000 $\times$ *g* centrifugation. It was not possible to completely inhibit or separate triose phosphate isomerase from the decarboxylase activity, and hence the triose phosphate product could not be isolated. Isotopic dilution experiments pointed to D-glyceraldehyde 3-phosphate of DHAP as the product; however, extremely high triose phosphate isomerase activity complicated these experiments. The active site reagents for triose phosphate isomerase chloroacetol 1-phosphate (14) and glycidol 1-phosphate (32), were used to identify the triose phosphate product of the decarboxylase. Chloroacetol 1-phosphate was not extensively used since it inhibited the conversion of D-erythrulose 1-phosphate to a triose phosphate and the formation of pyruvate from D-glyceraldehyde 3-phosphate. In contrast to these results, glycidol 1-phosphate inhibited triose phosphate isomerase activity by 99% and inhibited conversion of D-glyceraldehyde 3-phosphate to pyruvate by only 9%. The CE treated with glycidol 1-phosphate was incubated with D-erythrulose 1-phosphate, D-glyceraldehyde 3-phosphate, and DHAP. The comparative yields of pyruvate observed with D-erythrulose 1-phosphate and DHAP suggested that DHAP was the product of the decarboxylase.

Smith et al. (32) were unable to obtain cell extracts of *B. abortus* (544) prepared in phosphate buffer which catabolized erythritol. High ATPase activity has been observed with extracts prepared in this manner (unpublished data). Either HEPES or TEA buffer are acceptable for preparation of active CE.

Smith et al. (33) proposed that the ability to catabolize erythritol was a virulence factor for *Brucella*. Although erythritol utilization is important in determining tissue localization in pregnant cows, goats, sheep, and hogs, there is no erythritol within macrophages or fetal tissues of animals in which the disease is of a chronic long-term nature. Further, *B. canis* and *B. ovis* are pathogenic for dogs and sheep, respectively, but neither species can utilize erythritol as a carbon source. Nevertheless, since brucellosis is a disease transmitted from animals to man, the information in this report is useful in designing new approaches to chemotherapy in order to control the reservoir of infection. It is noteworthy that domestic animals can tolerate *Brucella* infections much better than can man.

Brucella lack fructose 6-phosphate kinase and FDP aldolase (28); thus, the hexose monophosphate pathway is the only primary pathway of glucose dissimilation. As much as 80% of glucose is lost as CO_2 (27) which reflects the extensive operation of the hexose monophosphate pathway and the tricarboxylic acid cycle. The energy yield of the hexose monophosphate pathway is minimal and D-glyceraldehyde 3-phosphate which acts as a carrier in the pathway has to be routed through the latter enzymes of glycolysis to pyruvate for oxidation by the tricarboxylic acid cycle. Any series of reactions which might generate ATP, either NADH oxidation and oxidative phosphorylation or substrate level phosphorylation, may contribute to what seems to be an "energy-poor" cell. When points of regulation of the erythritol pathway and more details of membrane energization are known, the role(s) of erythritol and the biochemical basis of tissue localization exhibited by *Brucella* may be better understood.

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