D-ERYTHROSE METABOLISM IN A STRAIN OF *ALCALIGENES FAECALIS*

HOWARD H. HIATT¹ AND B. L. HORECKER

The National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda, Maryland

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Except for the oxidation of erythritol to L-erythrulose by *Acetobacter* (Bertrand, 1900) little is known of the metabolism of tetroses in microorganisms. Barker and Lipmann (1949) found erythritol to be esterified and oxidized by *Propionibacterium pentosaceum*, although a tetrose intermediate was not identified. The utilization of D-erythrose by mammalian liver slices has been reported by Abraham and Chaikoff (1955). Accumulating evidence for the biological significance of this sugar includes the recent demonstration of D-erythrose-4-phosphate (E-4-P) as an intermediate in the pentose phosphate pathway (for a review see Horecker and Mehler, 1955) and in the biosynthesis of shikimic acid (P. R. Srinivasan and D. B. Sprinson, *personal communication*). Chemical synthesis of E-4-P has been achieved by Ballou *et al.* (1955). The synthetic compound has been reported by Kornberg and Racker (1955) to be identical with the enzymatically formed product.

To explore further the pathways by which erythrose may be metabolized, bacteria which were able to utilize this compound as their sole carbon source were isolated by enrichment culture. From one of these organisms a cell-free preparation capable of metabolizing erythrose was prepared. Preliminary studies suggest that E-4-P is produced in this cell-free system.

MATERIALS AND METHODS

Erythrose preparation. D-Erythrose was synthesized according to the method of Rappoport and Hassid (1951) with some modifications to facilitate relatively large-scale runs. 4,6-Ethylidene glucose was oxidized with periodate to 2,4-ethylidene erythrose. This product was hydrolyzed by heating at 100 C for 1 hr with 2 ml of "dowex-50-H⁺" per g of ethylidene erythrose. During this time water was added as needed to maintain constant volume. The product

was cooled, decolorized with charcoal, and deionized by passage through a mixed-bed resin ("amberlite MB-3"). To remove acetaldehyde formed from the ethylidene derivative the solution was distilled at constant volume under reduced pressure at 25 C, concentrated at low pressure, and finally lyophilized to yield a colorless syrup.

The hydrolysis of ethylidene erythrose at 100 C at constant volume without refluxing and the subsequent distillation at constant volume of the deionized erythrose solution prior to lyophilization were effective in removing the significant quantities of acetaldehyde found to accompany the final product prepared without these steps.

In a typical synthesis 13 g of dry D-erythrose syrup was obtained from 28.2 g of ethylidene glucose. This product was found to have a specific rotation $[\alpha]_{20}^{25} -22.1^\circ$, and a periodate consumption of 2.5 μ moles per 120 μ g of syrup. The reducing power (Nelson, 1944; Somogyi, 1945) was 49 per cent of that given by an equal weight of glucose. By the tetrose assay of Dische (*personal communication*), 5.9 μ g of erythrose gave absorption ($D_{480} - D_{480}$) comparable to that given by 5 μ g of the threose standard. Ascending chromatography on Whatman #1 paper in acetone-water (80:20), followed by spraying with aniline hydrogen phthalate reagent (Partridge, 1949), showed a single spot with R_f of 0.72, in the same position as threose and erythrulose.

Other methods. Studies of bacterial metabolism were carried out in the conventional Warburg apparatus. Respiratory quotients were determined by the direct method of Warburg (Umbreit *et al.*, 1949).

Cell-free extracts (McIlwain, 1948) were prepared by grinding in a mortar 1 g of organisms for 2 min at 0 C with 2.5 g of "alumina A-301" (Aluminum Company of America, 325 mesh) and extracting with 7.0 ml of 0.05 M 2-amino-2-(hydroxymethyl)-1,3-propanediol-HCl buffer (*tris* buffer), pH 7.4, and centrifuging in the Sorvall SS-1 centrifuge.

¹ Present address: Department of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, Mass.

Figure 1. Uptake of O_2 with resting cell suspensions of erythrose-grown *Alcaligenes faecalis* in the presence of several substrates. Each cup contained a resting cell suspension of a 20 hr culture of the organism, 30 mg (wet weight); K phosphate buffer, pH 7.4, 20 μ moles; and 10 per cent KOH, 0.2 ml; in the center well. The total volume was 3.0 ml. At the arrow 6 μ moles of substrate were tipped in from the side arm. Incubation was at 37 C and the gas space contained air.

Erythrose utilization by cell-free extracts was measured by determination of reducing power of the supernatant solution obtained by centrifugation after treatment at 0 C with 5 volumes of absolute alcohol and with 0.02 volume of 20 per cent barium acetate.

ATP³² was synthesized from adenylic acid and P³² by oxidative phosphorylation using mouse liver mitochondria prepared by the method of Kielley and Kielley (1951). Crystalline aldolase was prepared according to Taylor *et al.* (1948). Sedoheptulose diphosphate (SDP) was prepared according to the method of Smyrniotis and Horecker (1956). L-Erythrulose-*o*-nitrophenylhydrazine was prepared by the procedure of Müller *et al.* (1937). Synthetic D-erythrose-4-phosphate was kindly provided by Dr. C. E. Ballou of the University of California. Other substances were obtained from commercial sources. Formaldehyde was determined by the method of MacFadyen (1945), and acetate by the procedure of Rose *et al.* (1954). Glycolaldehyde was assayed according to Dische and Borenfreund (1949). Sedoheptulose was analyzed by the orcinol reaction (Horecker *et al.*, 1953). P³² was counted with a Geiger-Mueller tube.

Bacterial growth. The organisms were isolated from a suspension of Potomac River mud on a medium that included K_2HPO_4 , 0.15 per cent; KH_2PO_4 , 0.05 per cent; $MgSO_4 \cdot 7H_2O$, 0.02 per cent; $(NH_4)_2SO_4$, 0.10 per cent; and D-erythrose, 0.10 per cent. After pure cultures were obtained,

bacterial growth was found to be optimal on a medium which contained K_2HPO_4 , 0.15 per cent; KH_2PO_4 , 0.05 per cent; $MgSO_4 \cdot 7H_2O$, 0.02 per cent; NH_4NO_3 , 0.10 per cent; yeast extract, 0.1 per cent; $FeSO_4$, 0.001 per cent; $CaSO_4$, 0.001 per cent; and D-erythrose, 0.5 per cent; in tap water and distilled water in a 1:1 ratio.

RESULTS

Several organisms were isolated but only two, a strain of *Aerobacter aerogenes*, and one of *Alcaligenes faecalis*, utilized the substrate in substantial quantity. Optimal growth of both organisms occurred at 0.5 per cent concentration of substrate with less growth at higher and at lower concentrations. The optimum temperature was 37 C for either organism. Both *Aerobacter aerogenes* and *Alcaligenes faecalis* are aerobic organisms, although the latter does not require vigorous aeration. With both organisms the repeated transfer in erythrose led to more rapid utilization of this substrate. The yield of *Alcaligenes faecalis* was approximately 1 g wet weight per g of substrate. It was with this organism that most of the studies with resting cells and with cell-free extracts were carried out.

Resting Cell Studies

Respiratory quotient with D-erythrose. A suspension of resting cells of *Alcaligenes faecalis*, to which 4 μ moles of D-erythrose were added, consumed 8.4 μ moles of O_2 in excess of endogenous. During the same period an excess of 8.8 μ moles of CO_2 was produced, giving an R.Q. of 1.05. The final reaction mixture contained a trace of formaldehyde, but no acetate or glycolaldehyde could be detected.

Utilization of substrates. Cells grown on D-erythrose consumed erythritol and L-erythrulose nearly as well as D-erythrose (figure 1). Succinate was rapidly and nearly completely oxidized (table 1, exp. 1), while much less stimulation of O_2 consumption was noted on addition of D-ribose. In other experiments lactate and glycolate were shown to be rapidly oxidized, while tartrate was inert. Fructose was consistently metabolized more rapidly and more completely than was glucose (table 1, exp. 2).

Resting cells grown on glucose adapted to erythrose in 5 to 16 hr under test conditions similar to those given in figure 1. However, adaptation was demonstrable only in cultures

TABLE 1
*O₂ consumption by resting-cell suspensions of erythrose-grown *Alcaligenes faecalis**

Experiment	Substrate	Amount	Initial Rate	Total Extra O ₂ *
		μmoles	μl O ₂ per hr	μmoles
1	None	—	100	—
	Erythrose	3	460	6.2
	Succinate	3	900	8.8
	Ribose	3	170	1.6
2	None	—	50	—
	Erythrose	50	320	—
	Fructose	50	210	—
	Glucose	50	120	—

* In excess of endogenous.

The test conditions were as in figure 1, except that 25 mg (wet weight) of washed cells were used in experiment 2.

carried on erythrose until the final transfer to glucose. After several transfers in glucose the organisms failed to adapt to erythrose during the course of the manometric experiments (up to 24 hr). Cells grown on fructose adapted to erythrose within 1 hr. Cells grown on erythritol readily oxidized erythrose and L-erythrulose (figure 2). Cell growth on fructose and on erythritol exceeded by approximately 20 per cent that observed on erythrose.

Studies with Cell-Free Extracts

Extracts of alumina-ground *Alcaligenes faecalis* were found to be capable of metabolizing erythrose at a rate of 1 μmole per 15 mg wet weight of cells per 23 min. This compares with an erythrose consumption by resting cells of approximately 2.3 μmoles per 15 mg wet weight of cells per 23 min. Such extracts were unstable even in the frozen state, although fresh extracts prepared from cells kept frozen for several weeks were fully active.

Crude extracts of erythrose-grown cells were also found to contain glucose and fructose kinases, hexose phosphate isomerase, glucose-6-phosphate dehydrogenase, and transketolase, but no 6-phosphogluconic dehydrogenase. In contrast to the results obtained with whole cells, extracts of cells grown on erythritol were found to be incapable of utilizing erythrose.

Efforts to purify the enzymes responsible for erythrose utilization were unsuccessful. The

Figure 2. Uptake of O₂ with resting cell suspensions of erythritol-grown *Alcaligenes faecalis*. Test conditions were as in figure 1. At the arrow 6.0 μmoles of substrate were tipped in from the side arm.

erythrose-consuming activity disappeared on freezing or on treatment with protamine, manganoous salts, ammonium sulfate, or calcium phosphate gel. This difficulty prevented separation of substantial quantities of the primary reaction product, which was further metabolized in the reaction mixtures. However, several observations provided presumptive evidence for the formation of E-4-P or a precursor of this substance.

ATP requirement. In the presence of ATP the bacterial extracts catalyzed the conversion of erythrose to a product which was removed by barium-ethanol treatment. This loss of soluble reducing power when ATP was present suggests the formation of a phosphate ester. Even in the absence of barium-ethanol treatment, however, reducing power after incubation with ATP was less than that present at the start of incubation, indicating further metabolism of the phosphate ester by the bacterial extract.

TABLE 2
*ATP requirement for erythrose utilization by cell-free extract of *Alcaligenes faecalis**

Time	Erythrose Remaining	
	Complete	No ATP
min	μmoles	μmoles
0	3.00	3.10
10	1.90	3.50
23	0.50	3.00

The incubation mixture contained D-erythrose, 3.0 μmoles; MgCl₂, 10 μmoles; tris buffer, pH 7.6, 10 μmoles; and *Alcaligenes faecalis* extract, 0.24 ml in a total volume of 0.50 ml. The complete system contained, in addition, 10 μmoles of ATP. After 10 and 23 min at 25 C, 0.1 ml aliquots were removed for determination of barium-ethanol-soluble reducing power.

Figure 3. Incorporation of P^{32} in sedoheptulose diphosphate (SDP) by a cell-free extract of *Alcaligenes faecalis*. The incubation mixture contained: ATP 32 , 1.5 μ moles; specific activity 5×10^5 cpm per μ mole, D-erythrose, 3 μ moles; MgCl $_2$, 50 μ moles; SDP, 50 μ moles; crystalline rabbit muscle aldolase, 1.4 mg; phosphate buffer, 10 μ moles; and *Alcaligenes* extract, 1.5 ml; in a total volume of 4.78 ml, pH 7.4. After 20 min at 25 C, the mixture was put on a dowex-1-formate, (200-400 mesh, 10 per cent cross-linked) column, 0.8 cm 2 by 4.0 cm. The column was washed with water, and gradient elution (Busch *et al.*, 1952) was carried out with 0.5 M Na formate in 0.2 M formic acid in the reservoir and 200 ml of distilled water in the mixing bottle. Fractions of 4.0 ml were collected at a rate of 0.36 ml per min. The radioactivity present in tubes #1 and #10-15 was shown to be associated with nucleotide by absorption at 260 m μ , that in #16-21 with inorganic phosphate, and that in #32-50 with SDP. Thirty-seven μ moles of SDP were recovered from the column in tubes containing 169,000 cpm. The "tailing" shown in the elution of the SDP is attributable to the fact that this procedure was carried out at 3 C. The SDP appears in much more concentrated fashion when the elution is carried out at room temperature.

A phosphate ester chromatographically similar to chemically synthesized erythrose-4-phosphate was detected in the incubation mixtures. Following treatment with charcoal to remove nucleotides, the solutions were spotted on Whatman #1 paper, and chromatographed in ascending fashion with methanol-formic acid-water (80:15:5). With the Bandurski and Axelrod (1951) spray reagent for phosphate esters these chromatograms were found to contain a phosphate ester with an R_f of 0.46, similar to that of synthetic E-4-P. In addition, such incubation mixtures were found to contain a phosphate ester with electrophoretic mobility like that of E-4-P.

In an effort to label the product, the bacterial extract and ATP 32 were incubated with and without erythrose followed by treatment with

charcoal to remove the nucleotides. More radioactivity was consistently found in the charcoal supernatant solution from those incubation mixtures containing erythrose. However, the presence of ATPase in the extracts led to the appearance of substantial amounts of P^{32} in the charcoal-treated mixtures incubated without erythrose, and efforts to isolate the labeled product were unsuccessful.

Trapping experiments with sedoheptulose diphosphate. E-4-P has been shown to condense reversibly with dihydroxyacetone phosphate in the presence of muscle aldolase, forming sedoheptulose 1,7-diphosphate (SDP) (Horecker *et al.*, 1955). Thus, if SDP and aldolase are added to the reaction mixtures, labeled E-4-P produced would be expected to exchange and become incorporated into the diphosphate ester. Accordingly, SDP and aldolase were incubated with ATP 32 and the *Alcaligenes* extract with and without erythrose. SDP was then isolated from the incubation mixture and its radioactivity determined. More than 22 per cent of the counts added as ATP 32 was incorporated in the SDP incubated in the presence of D-erythrose (figure 3). The specific activity of the SDP recovered in the control experiment without erythrose was less than 10 cpm per μ mole, compared with a specific activity of 4600 cpm per μ mole in the run with erythrose.

DISCUSSION

The capacity to metabolize D-erythrose, previously demonstrated in plant (Hough and Jones, 1953) and in animal tissues (Abraham and Chaikoff, 1955; Breusch, 1951), has now been observed in microorganisms. This characteristic has been developed in a strain of *Alcaligenes faecalis* by adaptation. The pathways through which the tetrose is metabolized by the bacterial as well as by the plant and animal systems remain unknown. Initial reduction of erythrose to erythritol followed by phosphorylation is a possibility suggested by the studies with *Propionibacterium pentosaceum* of Barker and Lipmann (1949). However, no evidence for such a reaction was obtained although the presence of endogenous reducing substances could not be excluded. Similarly, isomerization to erythrulose, a product of erythritol oxidation in cultures of *Acetobacter suboxydans* (Bertrand, 1900), may occur prior to phosphorylation. L-Erythrulose

was oxidized more slowly than D-erythrose by resting cell suspensions of erythrose-grown organisms, but the significance of this observation is unknown since D-erythrulose, which was not tested, may be metabolized more readily than the L-isomer.

Studies with cell-free extracts of *Alcaligenes faecalis* indicated that phosphorylation takes place during erythrose metabolism and strongly suggested that E-4-P is formed. This compound did not accumulate in the crude system, but in the experiment with SDP a trapping mechanism was provided for any E-4-P formed. The following reactions might account for the incorporation of P³² into SDP in the bacterial extracts:

The formation of E-4-P from erythrose by the extract could of course result from the activity of an erythrokinae. However, several alternative mechanisms can be formulated which would have the net result of producing the esterification of D-erythrose. One of these involves the enzymes transketolase and fructokinase:

A second possible mechanism involves the enzymes aldolase and a sedoheptulose-1-phosphate kinase:

In each case the over-all reaction would be:

All of the reactions are known except for the conversion of sedoheptulose-1-phosphate to SDP. Studies to investigate the feasibility of these reaction sequences with purified enzymes are now under way.

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SUMMARY

A strain of *Alcaligenes faecalis* and one of *Aerobacter aerogenes* capable of utilizing D-erythrose have been isolated by enrichment culture. The metabolism of erythrose by the former organism has been studied.

A cell-free system capable of decomposing erythrose has been prepared from *Alcaligenes faecalis*. The formation of a phosphate ester product is suggested by a requirement for ATP for the utilization of erythrose. In the presence of erythrose, sedoheptulose diphosphate and ATP³², P³²-labeled sedoheptulose diphosphate is formed. This result is consistent with the formation of D-erythrose-4-phosphate.

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