A MUTANT OF ESCHERICHIA COLI DEFICIENT IN PHOSPHOENOLPYRUVATE CARBOXYKINASE ACTIVITY

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The key role of phosphoenolpyruvate (PEP)* in carbohydrate metabolism, particularly gluconeogenesis, is well established (Krebs, 1954; Lardy et al, 1964; Utter, 1963; Utter et al., 1964). PEP arises via the following reactions:

1) $Pyr + ATP + H^+ \underset{K^+,\ Mg^{++}}{\overset{\text{Pyr kinase}}{\rightleftharpoons}} PEP + ADP$ (Lardy and Ziegler, 1945)

2) $Pyr + ATP \xrightarrow[Mg^{++}]{\text{PEP synthase}} PEP + AMP + Pi$ (Cooper and Kornberg, 1965)

3) $OAA + ATP \text{ (or GTP, ITP)} \underset{Mn^{++} \text{ or } Mg^{++}}{\overset{\text{PEPCK}}{\rightleftharpoons}} PEP + ADP + CO_2 \text{ (or GDP, IDP)}$

(Utter and Kurahashi, 1954; Nordlie and Lardy, 1963)

Reaction 1) catalyzed by pyruvate kinase probably plays only a minor role in gluconeogenesis as the equilibrium of the reaction is strongly in favor of pyruvate formation (Krebs, 1954). Cooper and Kornberg (1965) have demonstrated that in Escherichia coli phosphoenolpyruvate synthase catalyzes the formation of phosphoenolpyruvate by reaction 2). Work with rat liver has shown (Lardy et al., 1965; Shargo and Lardy, 1966) that phosphoenolpyruvate carboxykinase (PEPCK),

*Abbreviations used: OAA = oxaloacetic acid; PEP = phosphoenolpyruvate; PEPCK = phosphoenolpyruvate carboxykinase; Pyr = pyruvate.

reaction 3), is sufficiently active to account for the rate of gluconeogenesis observed in that organ. In this communication a mutant of Escherichia coli is described which was isolated on the basis of its inability to grow on succinate as sole source of carbon and which was shown to have abnormally low PEPCK activity. Evidence is presented that PEPCK is essential for growth when tricarboxylic acid cycle intermediates serve as sole source of carbon.

MATERIALS AND METHODS

E. coli K 12 strains AB 257 and AB 257^{suc^-} were employed throughout the present investigation. AB 257 was obtained from Dr. E. A. Adelberg of Yale University. The strain requires methionine for growth. AB 257^{suc^-} was selected from AB 257 on the basis of its inability to utilize succinate as sole source of carbon. AB 257 was submitted to mutagenic treatment with N-methyl-N'-nitro-N-nitrosoguanidine (Adelberg et al., 1965) and a mutant capable of utilizing glycerol but not succinate as sole source of carbon was then selected by the conventional penicillin technique. Bacteria were grown with aeration at 37° on the mineral salts medium "56" (Monod et al., 1951) containing the source of carbon specified and supplemented with l-methionine. Bacterial density was measured turbidimetrically. Bacterial extracts were prepared by treatment of the bacteria, resuspended in Tris buffer, pH 7.6, 0.1 M, in a Raytheon 10 kc magnetostrictive oscillator followed by centrifugation for 30 minutes at 45,000 x g. PEPCK and PEP carboxylase activities were found to be in the supernates. PEPCK was assayed by the isotope exchange method of Utter and Kurahashi (1955). This method is based on the incorporation of $^{14}CO_2$ into a pool of non-radioactive oxaloacetic acid (OAA) in an exchange reaction. The reaction mixture contained the following components in μmoles/ml in a total volume of 1.5 ml: OAA, 40.0; reduced gluthathione 1.6; ATP, 6.0; $MnCl_2$, 5.0; NaF, 10.0; KCl, 10.0; β-mercaptoethanol, 20.0; NaH $^{14}CO_3$ (1 μc), 33.3; Tris (pH 7.6), 100.0; and 1 mg - 2 mg of the bacterial protein. Fig. 1 shows that under the conditions employed, the rate of PEPCK activity was a rectilinear function of both the duration of the assay and the concentration of bacterial protein used. Specific PEPCK ac-

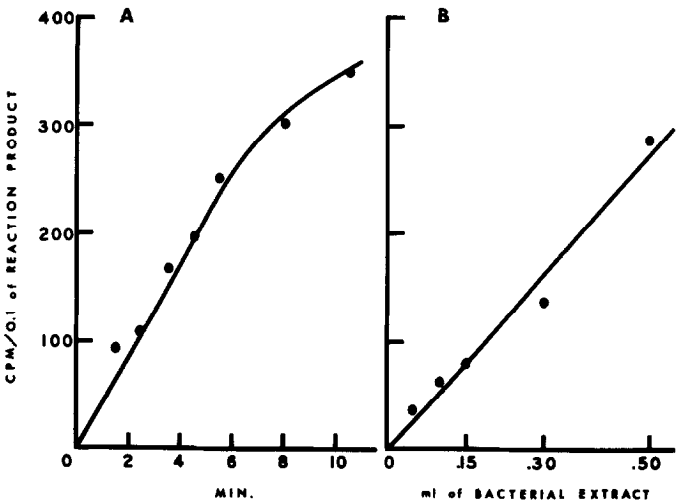

Fig. 1. Activity of PEPCK as a function of reaction time (A) and of bacterial protein concentration (B). AB 257 grown on succinate; 0.5 ml of extract containing 1.65 mg of protein were used and the reaction terminated by the addition of H_2SO_4 to final concentration of 0.6 N at the times indicated (A). Different volumes of the same extract were employed and the reaction terminated after 5 min incubation (B). PEPCK activity is expressed as counts/min/0.1 ml of reaction product. See Utter and Kurahashi (1955) for experimental details.

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tivity is expressed as mμmoles of ^{14}C-labeled OAA formed per mg of bacterial protein per minute at 36° C. That the $^{14}CO_2$ was incorporated into OAA was demonstrated in a preliminary experiment in which ^{14}C-labeled OAA was isolated from the reaction mixture as the 2,4-dinitrophenylhydrazone. The radioactive hydrazone was identified chromatographically as described by El Hawary and Thompson (1953). PEP carboxylase was assayed according to the method of Theodore and Englesberg (1964). Succinic acid-2,3-^{14}C (Calbiochem.) was used to measure the ability of bacteria to take up succinate from the medium. The technique, involving filtration of the bacteria through membrane filters, has been described in an earlier communication (Kessler and Rickenberg, 1963). Protein was determined by the procedure of Lowry et al. (1951).

RESULTS AND DISCUSSION

The growth of strains AB 257 and AB 257^{suc-} on various sources of carbon:

Table I describes the ability of the two strains to use a number of compounds as sole source of carbon. Growth rates of the two strains on sources of carbon which could be utilized by both strains were not significantly different. The utilization of pyruvate was an exception: the mutant grew at only two-thirds of the rate of the wild type strain. Strain AB 257^{suc-} failed to grow on any of the intermediates of the tricarboxylic acid cycle.

Table I

The growth of E. coli strains AB 257 and AB 257^{suc-} on various sources of carbon

	Carbon Source					
Strain	Lactose	Maltose	Glucose	Fructose	Mannose	Galactose
AB 257	+	+	+	+	+	+
AB 257^{suc-}	+	+	+	+	+	+
	Glycerol	Pyruvate	α-Keto-glutarate	Succinate	Fumarate	Malate
AB 257	+	+	+	+	+	+
AB 257^{suc-}	+	+	0	0	0	0

(+) normal growth; (0) no growth.
The concentrations of the compounds employed were as follows: lactose, maltose, 5×10^{-3} M; glucose, fructose, mannose, galactose, 10^{-2} M; glycerol, pyruvate, 2×10^{-2} M; α-ketoglutarate, 1.2×10^{-2} M; succinate, fumarate, malate, 1.5×10^{-2} M.

Uptake of succinate from the medium: The inability of the mutant to grow on succinate was not due to any defect in the transport of succinate. The initial rate of the accumulation of radioactivity derived from the succinate in the medium was similar in both strains irrespective of the source of carbon on which the strains had been grown (Fig. 2). It can also be seen that in the cultures grown on glycerol the accumulation of radioactivity after one minute exposure to the labeled succinate occurred at a higher rate in the wild type than in the mutant strain (Fig. 2A). This difference suggested the utilization of succinate by the wild type at a higher rate than by the mutant.

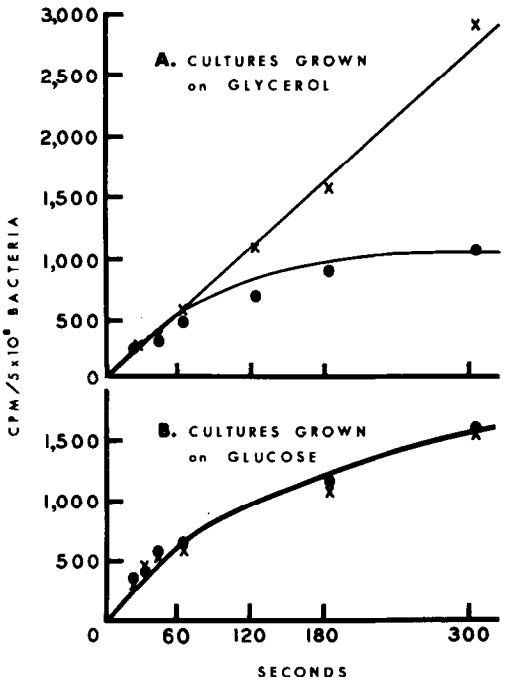

Fig. 2. The uptake of ^{14}C-2,3 succinate by strains AB 257 and AB 257^{suc-}. The succinate was employed at a concentration of 10^{-4} M and had a specific radioactivity of 1.8 x 10^{7} counts/min/micromole. X-X AB 257 ●-● AB 257^{suc-}

PEPCK activity in wild type and mutant strains: Table II shows the PEPCK activity of the wild type and mutant strains grown on a number of different sources of carbon. It can be seen that in the wild type strain the activity of PEPCK was 8 to 14 times higher in cultures grown on succinate than it was in cultures grown on glucose. This suggested that glucose repressed the formation of PEPCK and parallels similar observations by Theodore and Englesberg (1964) in Salmonella and by de Terrontegui et al. (1966) in yeast. The difference between the specific activities of cultures grown on glycerol, fumarate, α-ketoglutarate, malate, and pyruvate and cultures grown on succinate was not sufficiently large to permit one to attribute a specific inducing effect to succinate. Table II also shows that in the mutant grown on either glycerol or glucose the specific PEPCK activity was the same as that of the wild type grown on glucose. The mutant showed the highest PEPCK activity after growth on pyruvate; this activity corresponded to

Table II

The specific activity of PEPCK of E. coli strains AB 257 and AB 257$^{suc^-}$ grown on different sources of carbon

	E. coli strain							
	AB 257				AB 257$^{suc^-}$			
Carbon source	*(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
Glucose	1.7	1.3	1.0	1.7	2.8	2.0	1.6	2.2
Glycerol	10.9	12.9	5.2	9.0	1.8	1.8	1.0	1.8
Succinate	14.8	18.1	14.5	14.3	No growth			
Fumarate	13.2	-	-	-	No growth			
α-Ketoglutarate	10.2	-	-	-	No growth			
Malate	11.2	-	-	-	No growth			
Pyruvate	-	15.9	13.5	12.3	-	4.4	6.6	5.2
**Control	0.4	0.1	0.4	0.5	-	-	-	-

*Numbers refer to different experiments.
**Assayed in the absence of added ATP; bacterial extract derived from AB 257 grown on succinate.

only about one-third of the activity found in the wild type strain grown on pyruvate. The low level of PEPCK activity in the mutant probably accounted for its inability to grow on succinate or other intermediates of the tricarboxylic acid cycle. The fact that PEPCK activity was still detectable in the mutant suggested that the mutation affected the regulation of PEPCK synthesis. Confirmation of this hypothesis requires the purification and characterization of PEPCK from both wild type and mutant strains as well as the identification of regulator and structural genes. The reason why in the mutant pyruvate, but not glycerol, partially derepressed the formation of PEPCK is not clear at present. In this context it should be mentioned that we also isolated a "constitutive" mutant which showed high levels of PEPCK activity even during growth on glucose.

Other workers (Amarasingham, 1959; Theodore and Englesberg, 1964; Ashworth

et al., 1965) have shown that the enzyme PEP carboxylase which catalyzes the carboxylation of PEP to OAA in the following reaction:

$$PEP + CO_2 + H_2O \xrightarrow[Mg^{++},\ Acetyl\ CoA]{PEP\ carboxylase} OAA + Pi$$

is essential for the growth of Enterobacteriaceae on mineral-glucose or mineral-glycerol media, i.e. under conditions where replenishment of the tricarboxylic acid cycle is required. The PEP carboxylase activity of our PEPCK-defective mutant was normal; it was approximately 2-3 times higher in cultures grown on glucose than in cultures grown on glycerol.

The findings reported here demonstrate that in E. coli PEPCK activity is essential for gluconeogenesis when intermediates of the tricarboxylic acid cycle serve as source of carbon. The findings are also in agreement with those of Theodore and Englesberg (1964) in that they show that PEPCK plays no role in the introduction of three-carbon compounds into the tricarboxylic acid cycle.

Note added: Since we submitted this paper, Professor H. L. Kornberg of the University of Leicester has informed us that results quite similar to those presented in this paper have been obtained by Dr. Linda Sage in his laboratory but have not yet been published.

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