OXIDATIVE METABOLISM AND THE GLYOXYLATE CYCLE IN *PSEUDOMONAS INDIGOFERA*

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ABSTRACT

MCFADDEN, BRUCE A. (Washington State University, Pullman, Wash.) AND WILLIAM V. HOWES. Oxidative metabolism and the glyoxylate cycle in *Pseudomonas indigofera*. J. Bacteriol. **84**:72-76. 1962.—Oxidative patterns of *Pseudomonas indigofera* have been investigated. Intact cells oxidize acetate, ethanol, fumarate, glyoxylate, α -ketoglutarate, malate, oxaloacetate, pyruvate, and succinate to greater than 35% of completion. Isocitrate is oxidized to 21% of completion. Citrate is not oxidized by whole cells but is oxidized by cell-free preparations, as are fumarate, isocitrate, malate, and succinate. These patterns are suggestive of the operation of the tricarboxylic acid cycle. Investigations of levels of isocitrate lyase and malate synthase as functions of growth substrate have been conducted. Assays for these enzymes in "soluble" preparations were performed under ostensibly optimal conditions for catalysis. Growth substrates used at 0.3% were: (i) ethanol, (ii) glucose, (iii) succinic acid, and (iv) yeast extract. Specific activities of isocitrate lyase were: for (i) 3.80, (ii) 0.61, (iii) 1.47, and (iv) 1.33; activities of malate synthase were: for (i) 0.18, (ii) 0.032, (iii) 0.021, and (iv) 0.029. Additionally, the isocitrate lyase level from butyrate-grown cells was similar to that for ethanol-grown cells; the specific activity of malate synthase was about 60% as high. Specific activities of these enzymes were reproducible when conditions of sonic disruption were standardized. Longer durations of disruption decreased both activities.

The naming, isolation, and partial description of two strains of *Pseudomonas indigofera* were described by Elazari-Volcani (1939). Virtually nothing is known of the metabolism of this species. The reisolation of one strain and an investigation of its nutrition have recently delineated several metabolic traits (McFadden and

Howes, 1961a). For example, rapid aerobic growth on ethanol indicated catalysis by isocitrate lyase (D_s -isocitrate glyoxylate-lyase, EC 4.1.3.1) and malate synthase (L -malate glyoxylate-lyase, EC 4.1.3.2) to provide precursors for cellular syntheses, as was hypothesized by Kornberg and Krebs (1957). [The systematic names and numbers for these enzymes are essentially those suggested in *Report of the Commission on Enzymes of the International Union of Biochemistry*, 1961, p. 125, Pergamon Press, New York. Although the Commission recommended the systematic name L_s -isocitrate glyoxylate-lyase for isocitrate lyase, the configuration of the α -carbon of natural isocitrate and therefore of the substrate has recently been established as D_s (Kaneko and Katsura, 1960).] It was therefore of interest to establish that these enzymes were present in ethanol-grown cells and to investigate the effects of growth substrates on levels of these catalysts. The present publication details these investigations and presents evidence suggestive of the operation of the tricarboxylic acid (TCA) cycle in *P. indigofera*.

MATERIALS AND METHODS

Growth and manometric studies. The strain of *P. indigofera* used had been isolated by enrichment from soil (McFadden and Howes, 1961a). For manometric studies it was cultured in 1% yeast extract (Difco), harvested, and washed once by centrifugation in 0.1 M potassium phosphate (pH 7). Cells were then suspended in this buffer with added $MgCl_2$ (2×10^{-4} M). Weights (expressed as dry weights) of bacteria per unit volume were determined by standard turbidimetric procedures.

Disruption of washed cells suspended in 0.1 M potassium phosphate (pH 7) was accomplished in 20 min, using a Raytheon 10-kc sonic oscillator at full power with tap water as coolant. Cell debris and undisrupted cells were removed

by centrifugation at $5,000 \times g$ for 20 min at 4 C.

Conventional Warburg respirometers were used at 30 C for manometric studies.

Cultures used for studies of isocitrate lyase and malate synthase were inoculated and grown exactly as previously described for heterotrophic cultures of *Hydrogenomonas facilis* (McFadden and Howes, 1962). The basal inorganic medium employed was that described by McFadden and Howes (1961a), and was adjusted to a pH of 7 when necessary to compensate for added substrate.

Materials. The following were suppliers of chemicals. J. T. Baker: glucose, potassium acetate; California Corporation for Biochemical Research: D,L-isocitric lactone, α -ketoglutaric acid, D,L-malic acid, oxaloacetic acid; Eastman: fumaric acid (practical); Matheson, Coleman and Bell: sodium butyrate; Merck: succinic acid; Nutritional Biochemicals: sodium pyruvate; Pabst: coenzyme A (CoA); Pfizer: potassium citrate (USP); Sigma: sodium glyoxylate monohydrate; U.S. Industrial Chemicals: ethanol.

D,L-Isocitric lactone was converted to the salt by treatment with KOH (3.5 moles per mole of lactone) for 10 min at 100 C.

Preparation and assay of enzymatic fractions. "Soluble" preparations containing isocitrate lyase and malate synthase were prepared as described for *H. facilis* (McFadden and Howes, 1962). Techniques employed for assay and stoichiometric studies of malate synthase are described there, as are the definitions of specific activity and enzyme unit for both enzymes. Assays for isocitrate lyase were conducted under conditions found optimal by McFadden and Howes (1961b). Under these conditions and with 0.10 to 1.55 units of enzyme, the initial rate was a function of enzyme concentration only and was constant for a given level of enzyme.

RESULTS

Manometric studies. Manometric investigations with intact cells revealed that all intermediates of the TCA cycle, with the exception of citrate, were oxidized (Table 1). Actually, isocitrate was not consistently oxidized, suggesting poor cellular penetration of the six-carbon intermediates. Variable results may have reflected only differing physiological states. Cells desiccated in vacuo over CaCl_2 still did not oxidize citrate but did

TABLE 1. *Oxidative studies*

Compound	Theoretical O ₂ uptake	Observed O ₂ uptake	Endogenous O ₂ uptake	Per cent of theoretical O ₂ uptake ^a
	<i>μliters</i>	<i>μliters</i>	<i>μliters</i>	
<i>With intact cells^b</i>				
Acetate	180	219	127	51
Citrate	202	310	328	0
Ethanol	268	190	55	50
Fumarate	216	157	62	44
Glyoxylate	90	192	159	37
D _s -Isocitrate (as D,L)	202	370	328	21
α -Ketoglutarate	180	134	62	40
D,L-Malate	268	508 ^c	328	67
Oxaloacetate	112	334	286	43
Pyruvate	224	100	40	27
Succinate	236	177	62	49
<i>With cell-free preparations^d</i>				
Citrate	404	670	604	16
Fumarate	108	648	604	41
D _s -Isocitrate (as D,L)	404	696 ^c	604	23
α -Ketoglutarate	90	619	604	17
D,L-Malate	536	650	604	9
Oxaloacetate	224	617	604	6
Succinate	118	646	604	36

^a To obtain these figures, observed O₂ consumption was corrected for endogenous O₂ consumption by direct subtraction.

^b Cell densities of 4.0 to 5.6 mg per ml were employed. All oxidations were terminal within 40 to 110 min except where noted.

^c Oxidation was somewhat less than terminal.

^d Cell-free preparations were made as described in the text; 2.0-ml portions of cell-free preparation containing 56 mg of protein were used. All oxidations were terminal at 130 min, except where noted.

oxidize isocitrate. Hence, an examination of cell-free preparations for oxidative capacity was undertaken. The results are presented in Table 1. Acetate, ethanol, glucose, glyoxylate, and pyruvate were not oxidized by these preparations. Furthermore, the significance of oxidation of α -ketoglutarate and oxaloacetate is dubious because of low O₂ consumption relative to endogenous consumption. As can be noted, endogenous O₂ consumption was extensive. Efforts to reduce this by starvation prior to disruption were unsuccessful. While aging of these preparations at 4 C diminished the endogenous oxidative

rate, it also sharply reduced rates of oxidation with all substrates tested. Specific oxidative rates of various substrates (i.e., rates per mg of protein) were very low with fresh preparations. No extensive efforts have yet been made to find alternate methods of cell disruption which might preserve oxidative capacity to a larger degree.

Conditions of assay for malate synthase. Optimally conditions for subsequent assays were established by studies of cofactor requirements, reaction kinetics, and pH optimum. Initial studies of a given reaction parameter were conducted under conditions found optimal for the enzyme in "soluble" preparations from *H. facilis* (McFadden and Howes, 1962). Cells grown on 0.3% ethanol (v/v) served as the source of enzyme. The pH optimum was 8.5. The same maximal rate occurred at pH 8.5 for 0.038 to 0.15 M tris(hydroxymethyl)aminomethane (tris). Magnesium chloride gave maximal stimulation between 2.5×10^{-3} and 7.5×10^{-3} M. The reaction was zero order with respect to 1.33×10^{-4} to 6.7×10^{-4} M glyoxylate. The reaction was zero order with respect to 1.3×10^{-4} to 1.1×10^{-3} M acetyl-CoA. The reaction was first order with respect to 0.20 to 0.53 units of malate synthase. No requirement for a thiol, i.e., reduced glutathione, was demonstrable.

As a result of these investigations, a reaction mixture of 1.5 ml of the following composition was used for all subsequent assays: 0.4 μ mole acetyl-CoA, 0.8 μ mole glyoxylate, 7.7 μ moles MgCl_2 , 122 μ moles tris (pH 8.5), and 0.20 to 0.53 units of enzyme.

The stoichiometry observed (Table 2) was that expected for the malate synthase reaction. Efforts to demonstrate reversibility of the reaction have been unsuccessful. This coincides with results of several other studies (Wong and Ajl, 1957; Dixon, Kornberg, and Lund, 1960; Marcus and Velasco, 1960; Yamamoto and Beevers, 1961).

Effects of growth substrates on isocitrate lyase and malate synthase. The effects of substrates on specific activities of isocitrate lyase and malate synthase are presented in Table 3. In experiment 1, growth on ethanol resulted in the highest activities for both enzymes. While it seemed reasonable that growth on substrates other than ethanol actually reduced levels of both enzymes, there remained the possibility that inhibitor was present in preparations where low activities were observed. However, incubation of "soluble"

TABLE 2. *Stoichiometry of the malate synthase reaction^a*

Compound	Change due to reaction
	μ moles
Glyoxylate	-0.20 ^b
Acetyl-CoA	-0.20
Malate	0.20

^a Cells were cultured on 0.3% (v/v) ethanol.

^b This value is corrected, reflecting a slight aerobic disappearance of glyoxylate independent of acetyl-CoA.

TABLE 3. *Effects of growth substrates on isocitrate lyase and malate synthase*

Substrate	Specific activity	
	Isocitrate lyase	Malate synthase $\times 10^2$
<i>Experiment 1^a</i>		
0.3% (v/v) Ethanol	3.80	18.0
0.3% Glucose ^b	0.61	3.2
0.3% Succinic acid	1.47	2.1
0.3% Yeast extract	1.33	2.9
<i>Experiment 2^c</i>		
0.3% (v/v) Ethanol	2.73	41
0.3% Sodium butyrate	2.93	25

^a Cells were cultured to the late logarithmic phase (20 hr), with cell densities being 1.0 to 1.1 mg/ml except for the succinate culture which contained 0.5 mg/ml. Enzyme assays were performed 24 hr after sonic disruption. The "soluble" preparations were stored at 4 C.

^b Filtrates from a 250-ml culture were slightly ninhydrin positive. Paper chromatography of these filtrates revealed no detectable aspartate or glutamate.

^c Cultures were grown to the same average cell density, 0.98 mg/ml. Each value of specific activity is an average of two values obtained from parallel experiments.

preparation from glucose-grown cells (0.30 mg protein) with that from ethanol-grown cells (0.12 mg protein) under standard assay conditions resulted in additivity of rates for both enzymatic reactions. Furthermore, rates of both reactions catalyzed by the preparation from ethanol-grown cells were unaltered by the addition of a boiled preparation from glucose-grown cells.

Although butyrate was originally included in

experiment 1 (Table 3), growth at 20 hr was poor. Consequently, experiment 2 (Table 3) was conducted. Values should not be compared with those for experiment 1, since malate synthase activity is unstable to storage at 4 C; up to 50% is lost as a result of storage for 24 hr.

As can be seen, growth on butyrate diminishes the specific activity of malate synthase about 40% while that of isocitrate lyase is essentially unchanged. Although the depression of 40% appeared to be significant, the reproducibility and effects of sonic disruption as related to activities of malate synthase and isocitrate lyase were investigated. Sonic treatment of cells grown on 0.3% (v/v) ethanol for 5 min (see McFadden and Howes, 1962, for conditions and for preparation of enzymes) resulted in duplicate specific activities for isocitrate lyase of 3.26 and 3.20 under standard assay conditions. However, treatment for 15 min resulted in duplicate values of 2.33 and 2.13. Corresponding values for malate synthase were 0.42 and 0.39 (5-min disruption) and 0.24 and 0.24 (15-min disruption). Thus, agreement of duplicates is good, and a longer treatment period results in extensive loss of both activities. It is conceivable that shorter sonic treatments at lower temperatures, or milder means of cell rupture, would result in higher specific activities for both enzymes. Cognizance of this could be of importance to future efforts to purify these enzymes, especially if efficiencies of cell breakage were not greatly impaired.

DISCUSSION

Sonic disruption greatly damages oxidative capacity of *P. indigofera*, and, furthermore, the residual activity in cell-free preparations is labile to storage. However, oxidative patterns with cell-free preparations and intact cells are consistent with function of the TCA cycle. It seems likely that the discrepancy between theoretical and observed O₂ consumption by non-proliferating cells reflects both assimilation and the difficulty in correcting for endogenous O₂ consumption, as has been shown for *Nocardia corallina* (Midwinter and Batt, 1960) and *Bacillus cereus* (Clifton and Sobek, 1961).

Kornberg and Krebs (1957) first recognized the metabolic consequences of microbial growth on two-carbon compounds. For such growth, reactions were required which would result in the continual synthesis of TCA intermediates needed for synthetic purposes. That these re-

actions are catalyzed, in most cases, by isocitrate lyase and malate synthase has been well documented (Kornberg and Elsdén, 1961). These authors also summarized data showing that, in general, levels of isocitrate lyase are more mutable than malate synthase, being enhanced more by growth on two-carbon compounds. Thus, the elevated levels of isocitrate lyase observed in *P. indigofera* grown on ethanol and butyrate (a presumed precursor of C₂-units) are expected. Calley, Dagley, and Hodgson (1958) found that growth of a vibrio on octanoate resulted in a specific activity of this enzyme comparable with that obtained for extract from cells grown on acetate. Indeed, extracts converted octanoate essentially quantitatively to acetate. Our data suggest that both butyrate and ethanol are metabolized via acetyl-CoA in *P. indigofera*. In fact, observations that the specific activity of malate synthase from butyrate-grown cells was 60% that from ethanol-grown cells, while isocitrate lyase levels were similar, are consistent with the slower growth observed on butyrate. It appears that somewhat diminished malate synthase catalysis may partially limit butyrate growth relative to growth on ethanol. In this connection, it is noteworthy that growth on acetate is markedly inferior to that on butyrate. Perhaps acetate activation is limiting.

It is generally assumed that carboxylation of pyruvate or phosphoenolpyruvate (or both) replenishes TCA intermediates during microbial growth on glucose. If, however, these reactions are slow while growth rates are high, catalysis by isocitrate lyase and malate synthase may contribute to cellular syntheses, especially if there is a defect in the TCA cycle. Shiio, Otsuka, and Takahashi (1961) observed such a defect in a strain of *Brevibacterium flavum* which accumulated glutamate when grown on glucose. Under these growth conditions, both isocitrate lyase and malate synthase were present at substantial levels. Such an explanation does not seem to apply to *P. indigofera*, where specific activities for these enzymes from yeast extract-grown cells are comparable to those from cells grown on glucose. Furthermore, glucose-grown cells do not accumulate glutamate or aspartate. It is clear that in several organisms, in addition to *P. indigofera*, extensive catalysis by isocitrate lyase and malate synthase may occur during growth on substrates other than C₂-compounds (see, for example, McFadden and Howes, 1962).

Although an exhaustive survey was not conducted, apparently in *P. indigofera* isocitrate lyase is somewhat less mutable by growth substrate than malate synthase, in contrast to general observations (Kornberg and Elsdén, 1961). Of interest is the finding that the specific activity of isocitrate lyase from succinate-grown cells is 40% that from cells cultured on ethanol. This is in contrast to reports (Kornberg, Gotto, and Lund, 1958; Barnett and Kornberg, 1960; Calley et al., 1958) that growth on succinate strongly suppresses the specific activity of isocitrate lyase relative to that for growth on acetate. Furthermore, provision of succinate to cultures grown on acetate resulted in apparent cessation of isocitrate lyase synthesis during further cell division (Kornberg et al., 1958; Kornberg, Collins, and Bigley, 1960). In view of these striking discrepancies, more research is needed before a general concept can be developed concerning the control of isocitrate lyase and malate synthase by repression or feed-back inhibition.

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