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Itaconate, an Isocitrate Lyase-Directed Inhibitor in *Pseudomonas indigofera*

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Enzymes catalyzing steps from ethanol to acetyl-coenzyme A, from malate to pyruvate, and from pyruvate to glucose 6-phosphate were identified in ethanol-grown *Pseudomonas indigofera*. Enzymes catalyzing the catabolism of glucose to pyruvate via the Entner-Doudoroff pathway were identified in glucose-grown cells. Phosphofructokinase could not be detected in *Pseudomonas indigofera*. Itaconate, a potent inhibitor of isocitrate lyase, abolished growth of *P. indigofera* on ethanol at concentrations that had little effect upon growth on glucose. The data obtained through enzyme analyses and studies of itaconate inhibition with both extracts and toluene-treated cells suggest that itaconate selectively inhibits and reduces the specific activity of isocitrate lyase.

The glyoxylate cycle is an anaplerotic (17) metabolic sequence that appears to function in a variety of microorganisms (18, 19, 22), certain germinating fatty seedlings (for a brief review, see reference 2), some nematodes (1, 5, 20, 37-39), and the adult worm *Monolithiformis dubius* (21). In seedlings (2) and the parasitic nematode *Ascaris lumbricoides* (1), the cycle functions during development in the conversion of lipids to saccharides.

At least two enzymes, isocitrate lyase (EC 4.1.3.1) and malate synthase (EC 4.1.3.2), function uniquely in the glyoxylate cycle. In some bacteria, isocitrate lyase is derepressed by growth on C_2 compounds, whereas malate synthase is constitutive (19). In other bacteria such as *Pseudomonas indigofera*, the control is more relaxed in that relatively high levels of isocitrate lyase occur under growth conditions ostensibly not requiring function of the glyoxylate cycle (12, 13, 26-28).

Itaconate is a potent inhibitor (K_i = 0.9 μM at a pH of 6.8 and 30°C) of isocitrate lyase from *P. indigofera* (45). It seemed possible that this compound could be used to specifically inhibit isocitrate lyase and, therefore, the glyoxylate cycle in growing cells of *P. indigofera*. We now describe evidence that itaconate is indeed an isocitrate lyase-directed inhibitor.

(Some of this work was taken in part from a thesis submitted by S. P. to Washington State University, Pullman, Wash., in partial fulfillment of the requirements for the M.S. degree, 1976.)

MATERIALS AND METHODS

Materials. DL-Isocitrate (trisodium) was purchased from California Corp. for Biochemical Research; yeast extract and agar were from Difco Laboratories, and itaconic acid was a product of the Matheson Co. Phosphoenolpyruvate (PEP), oxaloacetic acid, sodium glyoxylate, fructose 1,6-diphosphate (FDP), fructose 6-phosphate, glyceraldehyde 3-phosphate diethylacetal, 3-phosphoglycerate, 6-phosphogluconate, cysteine, bovine serum albumin, glutathione, dithiothreitol, coenzyme A (CoA), acetyl-CoA, L-malate, oxidized and reduced nicotinamide adenine dinucleotide (NAD^+ and NADH), oxidized and reduced nicotinamide adenine dinucleotide phosphate ($NADP^+$ and NADPH), adenosine 5'-diphosphate, adenosine 5'-triphosphate, guanosine 5'-triphosphate, inosine 5'-triphosphate, inosine 5'-diphosphate, phenylmethylsulfonyl fluoride, morpholinosulfonate, lactic dehydrogenase, pyruvate kinase, α-glycerophosphate dehydrogenase, glucose 6-phosphate dehydrogenase, phosphoglucoisomerase, and FDP aldolase were purchased from Sigma Chemical Co. Sodium pyruvate and glycine were the products of Nutritional Biochemicals Corp. Sodium butyrate and ethyl alcohol were products of J. T. Baker Chemical Co. and U.S. Industrial Chemicals Co., respectively.

Growth experiments. The organism used in these studies, *P. indigofera* M1, was maintained as described previously (22, 24). To inoculate a seed culture, cells from a stock slant were suspended in 0.9% sterile NaCl and added to a medium that contained 0.54% K_2HPO_4, 0.3% KNO_3, 0.1% $MgSO_4 \cdot 7H_2O$, 0.01 volume of a trace minerals solution (46), and the specified final percentage of carbon source. The phosphate solution, which had been adjusted to a pH of 7.2 with HCl, was sterilized separately and added

after cooling to the other sterile mineral constituents. In the case of the seed culture containing sodium butyrate, the medium was supplemented with 0.05% Difco yeast extract. Seed cultures were grown with shaking under air at 30°C for 24 h and transferred to 10 volumes (150 ml) of the same medium, except that the yeast extract supplement was eliminated from the butyrate-containing medium and, in some cases, itaconate was added at the specified final concentration. After inoculation, the main cultures were shaken under air at 150 rpm and 30°C. Growth was monitored in flat-bottomed, 500-ml Erlenmeyer flasks, each of which had been fitted with a tubular side arm, by periodic turbidimetric measurements with a Klett-Summerson filter photometer. The relationship between Klett values and dry weight (milligrams per milliliter) was established experimentally.

Glucose was autoclaved separately from the other medium constituents. Itaconate was prepared by neutralization of itaconic acid with NaOH and was sterilized by filtration. Ethyl alcohol was added without sterilization.

Cell-free preparations. Main cultures that had been grown for 12 to 14 h on 17 mM glucose or for 16 to 18 h on 52 mM ethanol in the absence of itaconate were harvested in the late log phase and washed once with TME buffer [pH 7.7, 25°C; 0.1 tris(hydroxymethyl)aminomethane (Tris), 5 mM $MgCl_2$, and 1 mM ethylenediaminetetraacetate]. Cells that had been grown on 52 mM ethanol and varying concentrations of itaconate were harvested at the end of exponential growth (1.0 to 1.1 mg [dry weight] per ml) and washed as described above. Wet-packed cells (1 to 2 g) were suspended in 3 ml of TME buffer containing 1 mM phenylmethylsulfonyl fluoride and broken at 2°C by one or two passages through a French press at 16,000 lb/in^2. The suspension was then centrifuged at 18,000 × *g* for 15 min, and the resultant supernatant solution was again centrifuged at 130,000 × *g* (average force field) for 1 h. The final supernatant solution, $S_{130,000}$, was generally used for enzyme assays. In some cases, however, to remove small molecules the supernatant was added to a Sephadex G-25 column (1.7 by 17 cm) that had been equilibrated with TME buffer and was eluted with TME buffer (4°C). The void volume was used for enzyme assays and will be referred to as $S^{SPD}_{130,000}$. In some cases, as specified, the extracts were dialyzed against 100 volumes of TME buffer containing 1 mM phenylmethylsulfonyl fluoride.

Preparation of toluenized cells. Main cultures (300 ml) were grown on 52 mM ethanol in 1-liter Erlenmeyer flasks as described in the preceding section, harvested in the late log phase of growth, and washed once with a buffer (pH 7.7) containing 0.1 morpholinosulfonate, 5 mM $MgCl_2$, and 1 mM ethylenediaminetetraacetate. A 10-ml cell suspension in the same buffer containing 0.5 g of wet-packed cells was adjusted to 37°C; 0.05 volume of toluene was added, and the suspension was then maintained at 37°C for 10 min. After chilling, cells were harvested by centrifugation, washed twice with TME buffer, and resuspended in 5 ml of same buffer, and portions were then assayed for enzymes as described.

Enzyme assays. All assays were conducted at 30°C. Activities were determined over a range of protein (or toluenized cell) concentrations, and specific activities are expressed as micromoles of substrate disappearing (or product appearing) per minute per milligram of protein in a range of linearity between protein concentration and rate. Protein was determined in both the final supernatant fraction and toluenized cells by the microbiuret method (10). Rates for NADH-utilizing reactions were corrected for the presence of NADH dehydrogenase (EC 1.6.9.93) in the high-speed supernatant fraction, $S_{130,000}$, or in toluenized cells. When conducted, toluenized cell assays were carried out under conditions similar to those used to measure the enzyme of interest in the final high-speed supernatant solution. In some cases the blank was supplemented with toluene-treated cells.

Alcohol dehydrogenase (EC 1.1.1.1) was measured spectrophotometrically in the direction of NAD^+ reduction at pH 8.8 in 0.019 M sodium pyrophosphate containing 7.5 mM NAD^+ and 10 mM ethyl alcohol.

Aldehyde dehydrogenase (EC 1.2.1.3) was measured spectrophotometrically in the direction of NAD^+ reduction by acetaldehyde by the method of Dawes and Foster (8), except that CoA was omitted and 1 mM NAD^+ was used.

Acetyl-CoA synthase (EC 6.2.1.1) was measured colorimetrically by trapping the acetyl-CoA formed with hydroxylamine (15).

Isocitrate lyase (EC 4.1.3.1) was measured colorimetrically (35) by a method based upon oxidation of the phenylhydrazone of glyoxylate (23), and malate synthase (EC 4.1.3.2) was determined spectrophotometrically by measuring glyoxylate-dependent acetyl-CoA disappearance (25).

Assays for PEP carboxykinase (EC 4.1.1.32) were made with $S_{130,000}$, $S^{SPD}_{130,000}$, and toluenized cells, both in the direction of oxalacetate decarboxylation and PEP carboxylation using, respectively, inosine 5'-triphosphate, guanosine 5'-triphosphate, and adenosine 5'-triphosphate or the diphosphates (4, 11). For spectrophotometric assays in the decarboxylation direction, commercial pyruvate kinase and lactate dehydrogenase (both from Sigma Chemical Co.) were added.

$NADP^+$-malic enzyme (EC 1.1.1.40) was measured spectrophotometrically in the direction of malate-dependent NADPH production in the presence of 1 mM $MnCl_2$ (14).

PEP synthase was determined by measuring adenosine 5'-triphosphate-dependent pyruvate disappearance (7). The stoichiometry between pyruvate disappearance and PEP appearance was established after termination of the reaction with 4% $HClO_4$, reneutralization with KOH, and subsequent spectrophotometric assays measuring, first, pyruvate and, next, PEP by using commercial pyruvate kinase and/or lactate dehydrogenase. The presence of pyruvate orthophosphate dikinase (EC 2.7.9.1)

was probed by the same method, except that $S^{sp}_{130,000}$ was utilized and dependence upon 1.0 mM orthophosphate was examined.

Fructose diphosphatase (EC 3.1.3.11) was determined spectrophotometrically by measuring FDP-dependent NADPH formation in the presence of excess phosphoglucoisomerase and glucose 6-phosphate dehydrogenase (36).

The enzymes of glucose metabolism were assayed spectrophotometrically by the method described in the references cited: hexokinase, EC 2.7.1.2 (15), glucose 6-phosphate dehydrogenase, EC 1.1.1.49 (41), phosphohexose isomerase, EC 5.3.1.9 (32), and phosphofructokinase, EC 2.7.1.1 (43), except that, in the latter assay, dithiothreitol, β-mercaptoethanol, or glutathione was substituted for ethanethiol, and no exogeneous triose phosphate isomerase was added, due to its presence in $S_{130,000}$. FDP aldolase (EC 4.1.2.13) was measured by the method of Richards and Rutter (34); the triose phosphates were measured by the oxidation of NADH in the presence of α-glycerophosphate dehydrogenase (EC 1.1.1.8). High levels of triose phosphate isomerase (EC 5.3.1.1) were found (31) to be present in $S_{130,000}$. Thus, the FDP-dependent oxidation of 2 μmol of NADH reflected the cleavage of 1 μmol of FDP. NAD^+- and $NADP^+$-dependent glyceraldehyde 3-phosphate dehydrogenases (EC 1.2.1.12 and 1.2.1.13, respectively) were determined as described by O'Brien (31). DL-Glyceraldehyde 3-phosphate was obtained by treating the diethylacetal of the barium salt with Dowex 50 (H^+ form) as described by the supplier. The inorganic phosphate content of the glyceraldehyde 3-phosphate was 0.065 mol/mol. 3-Phosphoglycerate kinase (EC 2.7.2.3) was determined spectrophotometrically (32), except that no exogenous glyceraldehyde 3-phosphate dehydrogenase was added. Phosphoglycerate mutase (EC 2.7.5.3) and enolase (EC 4.2.1.11) were measured together (32) by using 0.25 M Tris-chloride buffer, pH 7.6 (25°C). Pyruvate kinase (EC 2.7.1.40) was measured spectrophotometrically at 340 nm. The reaction mixture contained 50 mM Tris-chloride (pH 7.5, 25°C), 0.125 mM NADH, 0.25 mM $MgCl_2$, 15 mM adenosine 5'-diphosphate, 1.5 mM PEP, and 30 U of lactate dehydrogenase (EC 1.1.1.27). Endogenous lactate dehydrogenase was assayed at 340 nm in an incubation mixture containing 50 mM Tris-chloride (pH 7.5, 25°C), 0.125 mM NADH, 0.25 mM $MgCl_2$, and 0.125 mM pyruvate. PEP carboxylase (EC 4.1.1.31) was measured by the method of Canovas and Kornberg (3). Pyruvate carboxylase (EC 6.4.1.1) was measured as described by Seubert and Weicher (42), except that exogenous malate dehydrogenase was omitted because of the high endogenous levels. The first two unique enzymes of the Entner-Doudoroff pathway, 6-phosphogluconate hydrolyase (EC 4.2.1.12) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14), were assayed together (41); 6-phosphogluconate dehydrogenase was assayed as described in the same paper (41).

RESULTS

Effect of itaconate upon growth. Itaconate at 10 mM almost completely arrested growth on 52 mM ethanol as measured during a 24-h period, whereas it had almost no effect upon growth on 17 mM glucose (Fig. 1). However, cells eventually assimilated ethanol in the presence of either 5 or 10 mM itaconate, although 10 mM itaconate reduced the growth rate (Fig. 1). The effects upon growth on these substrates or on butyrate in the presence of other concentrations of itaconate are summarized in Table 1. Once again it is evident that itaconate selectively inhibited the growth rate observed with ethanol. Moreover, it also arrested growth on butyrate in comparison with that on glucose.

There was no growth on itaconate alone at concentrations in the range of 1 to 60 mM, nor was there any evidence of diauxic growth in experiments where both the growth substrate and itaconate were furnished. That itaconate was not metabolized by *P. indigofera* was further indicated by the fact that no disappearance could be spectrophotometrically detected when toluene-treated cells (see below) were incubated with this compound at 0.05 mM (pH 7.7); the same concentration gave almost complete inhibition of isocitrate lyase in toluene-treated cells (J. E. Rogers and B. A. McFadden, unpublished data).

Assays of cell-free preparations and toluene-treated cells. The specific activities of various enzymes known to function uniquely in two-carbon assimilation in numerous organisms are shown in Table 2. Of interest is the fact that PEP carboxykinase could not be detected in *P. indigofera*. However, $NADP^+$-malic enzyme was present; in addition, an enzyme that catalyzed the adenosine 5'-triphos-

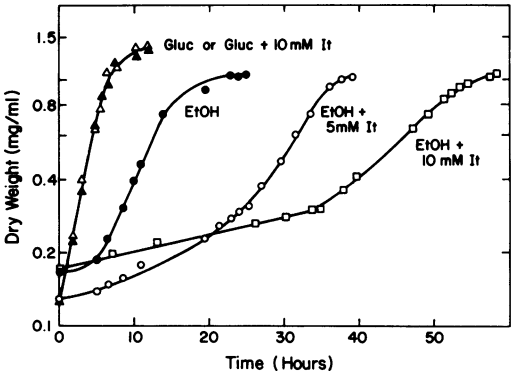

FIG. 1. *Semilogarithmic plot of growth of P. indigofera upon 17 mM glucose (Gluc)* (▲), *17 mM Gluc plus 10 mM itaconate (It)* (△), *52 mM ethanol (EtOH)* (●), *52 mM EtOH plus 5 mM It* (○), *or 52 mM EtOH plus 10 mM It* (□). *The zero-time cell concentration for the culture in 17 mM glucose* (▲) *was 0.065 mg (dry weight) per ml.*

TABLE 1. *Effect of itaconate upon growth of P. indigofera at pH 7.0 and 30°C in a minerals salts medium containing only the carbon sources specified*

Reading no.[a]	Klett value[b]														
	Glucose (17 mM) plus itaconate (mM):					Ethanol (52 mM) plus itaconate (mM):					Sodium butyrate (27 mM) plus itaconate (mM):				
	0	20	30	40	60	0	20	30	40	60	0	20	30	40	60
1	13	26	26	28	10	29	30	30	33	20	42	53	54	56	58
2	44	40	42	44	26	40	37	32	35	22	70	76	69	67	62
3	70	70	63	60	39	58	43	37	39	25	90	83	77	73	63
4	129	94	98	87	62	73	41	39	40	25	115	95	87	80	67
5	168	130	115	103	73	85	43	38	38	27	133	100	90	82	62
6	210	162	143	129	93	127	45	42	41	27	190	120	106	88	68
7	237	197	170	152	111	162	50	45	45	29	230	131	112	94	68
8	244	258	246	220	162	218	54	54	57	29	290	146	123	103	68
9	256	254	258	244	220	200	50	41	43	22	300		190	128	77

[a] Reading numbers 1 to 9 for glucose correspond to: 0, 1.5, 3.0, 4.5, 5.5, 6.5, 7.5, 10.17, and 12.0 h, respectively. For ethanol they correspond to: 3.5, 4.5, 5.67, 6.67, 7.33, 8.75, 10.5, 24.67, and 47.5 h, respectively. For sodium butyrate they correspond to: 0, 9.25, 11.5, 14.5, 16.0, 19.0, 21.0, 23.5, and 32.0 h, respectively.

[b] One unit in the Klett filter photometer is equivalent to 5 μg of cells (dry weight) per ml.

TABLE 2. *Specific activities of enzymes of two-carbon assimilation in $S_{130,000}$ from ethanol- and glucose-grown cells and in toluene-treated (Tl-t) cells*

Enzyme	Sp act after:		
	Ethanol growth		Glucose growth ($S_{130,000}$)
	Tl-t	$S_{130,000}$	
NAD^+-alcohol dehydrogenase	0.013	0.025	0.0033
NAD^+-aldehyde dehydrogenase	0.011	0.016	0.0006
Acetyl-CoA synthase	0.042	0.086	0.027
Isocitrate lyase	1.39	1.17	0.12
Malate synthase	0.20	0.55	0.055
$NADP^+$-malic enzyme	0.044	0.036	0.034
NAD^+-malic enzyme		ND[a]	
PEP-synthesizing system[b]	0.19	0.086	0.102
Fructose diphosphatase	0.042	0.018	0.179
PEP carboxykinase	ND	ND[c]	

[a] Not detectable.

[b] Adenosine 5'-triphosphate-dependent conversion of 1 mol of pyruvate to 1 mol of PEP was observed with Tl-t, $S_{130,000}$, and $S^{SPD}_{130,000}$ fractions. With the latter, there was no dependence upon added 1 mM orthophosphate.

[c] Also not detectable in $S^{SPD}_{130,000}$.

phate-dependent conversion of 1 mol of pyruvate to 1 mol of PEP was detected. This latter conversion, which was independent of added phosphate using gel-filtered extract (Table 2), may therefore have reflected catalysis by PEP synthase and not pyruvate phosphate dikinase. Nevertheless, the possibility that the latter enzyme was responsible for the observed conversion could not be rigorously excluded because of the possibility that orthophosphate was present in the reagents or was generated during the assay. Accordingly, this conversion is attributed to a PEP-synthesizing system.

In Table 3 it is established that itaconate concentrations in the range of 0.014 to 1 mM and, in most cases, as high as 5 mM yielded little or no inhibition of any of the enzymes except isocitrate lyase assayed in either $S_{130,000}$ or toluene-treated cells. Itaconate was a linear uncompetitive inhibitor with respect to isocitrate cleavage in toluene-treated cells (Fig. 2). The K_{ESI} was 0.020 mM and the K_m for D$_s$-isocitrate was 0.086 mM. With respect to the latter, it has been established that D$_s$-isocitrate is the substrate for isocitrate lyase from *P. indigofera* and that the enantiomer is non-inhibitory (25).

In Table 4, the specific activity of each enzyme detected and represented in Table 2 is normalized after growth in the presence of several concentrations of itaconate with respect to that (taken as 100) observed after growth in the absence of itaconate. For these comparisons, cells harvested from the late log phase were utilized. As evident, only the specific activity of isocitrate lyase was reduced by growth in the presence of 2 to 15 mM itaconate; at the latter concentration, the specific activity of isocitrate lyase was reduced 33-fold. The reduced specific activity of isocitrate lyase could not be attributed to carryover of endogenous itaconate into the assay mixture because dialysis of the extract derived from cells grown in the presence of 5 or 10 mM itaconate did not increase the specific activity. Of interest is the fact that the presence of itaconate during growth on ethanol

led to somewhat higher levels of NAD+-alcohol dehydrogenase, acetyl-CoA synthase, and NADP+-malic enzyme and at concentrations of 2 and 5 mM led to higher levels of NAD+-aldehyde dehydrogenase (Table 4). At 10 and 15 mM itaconate, levels of the latter enzyme were, however, about that for growth on ethanol alone. Of special interest was the markedly higher level of fructose diphosphatase observed in cells grown on ethanol in the presence of 1 and 2 mM itaconate (Table 4). An explanation for these itaconate-induced trends must await further experimentation.

The data in Table 5 suggest that the route of glucose catabolism in *P. indigofera* involves the Entner-Doudoroff pathway (9). Of interest is the fact that FDP aldolase detected after growth of cells on ethanol or glucose is inhibited by 90 and 97%, respectively, in the presence of 0.2 mM ethylenediaminetetraacetate (data not shown). Thus, this activity appears to reflect a class II aldolase (40). In contrast, Stribling and Perham (44) detected a mixture of class I and II aldolases after growth of *E. coli* on pyruvate or lactate but could detect only the ethylenediaminetetraacetate-sensitive class II aldolase after growth on glucose. Of further interest is our failure to detect phosphofructokinase in *P. indigofera* grown on either ethanol or glucose (Table 5). Because this enzyme could be readily detected (specific activity, 0.080) in *Escherichia coli* grown on the 17 mM glucose-minerals medium used in the present research, phosphofructokinase may indeed be absent in *P. indigofera*, in which case the presence of phosphohexose isomerase in glucose-grown cells is of unknown significance. The level of the latter enzyme was not reduced by the inclusion of 15 mM itaconate during growth on ethanol (Table 5).

TABLE 3. *Effect of itaconate upon enzymes of two-carbon assimilation in cell-free preparations of toluene-treated P. indigofera derived from cells grown on 52 mM ethanol*

Enzyme	% inhibition[a] by the specified concn (mM) of itaconate with:	
	$S_{130,000}$	Toluene-treated cells
NAD+-alcohol dehydrogenase	0 at 0.5–5	0 at 0.5–5
NAD+-aldehyde dehydrogenase	10 at 2–5	0 at 1–5
Acetyl-CoA synthase	0 at 0.5–5	0 at 1–5
Isocitrate lyase	38–96 at 0.014–0.23[b]	K_i = 0.02 (see Fig. 2)
Malate synthase	0 at 0.5–1	0 at 0.5–1
NADP+-malic enzyme	7 at 2	10 at 1–5
PEP-synthesizing system	0 at 0.5–5	8 at 1–5
Fructose diphosphatase	0 at 0.5–5	10 at 1–5

[a] Where a single percent inhibition is specified, it is the average observed in the itaconate concentration range defined. The concentration of itaconate was 0.5, 1.0, 2.0, or 5.0 mM.

[b] Percent inhibitions were 38, 61, 80, 86, 86, and 96 at 0.014, 0.029, 0.057, 0.11, 0.17, and 0.23 mM itaconate, respectively.

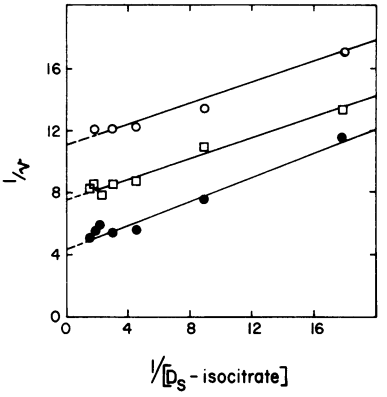

FIG. 2. *Double-reciprocal plots for the cleavage reaction catalyzed by isocitrate lyase in toluene-treated cells of P. indigofera at pH 7.7 in the absence of itaconate (●) or in the presence of 0.014 mM (□) or 0.028 mM itaconate (○). The slopes are identical at the 95% confidence level.*

TABLE 4. *Relative specific activities of enzymes of two-carbon assimilation in $S_{130,000}$ of P. indigofera grown on 52 mM ethanol with various concentrations of itaconate*

Enzyme	Relative sp act after growth in the presence of itaconate at (mM):					
	0	1.0	2.0	5.0	10.0	15.0
NAD+-alcohol dehydrogenase	(100)[a]	112	68	128	160	128
NAD+-aldehyde dehydrogenase	(100)	94	138	294	94	81
Acetyl-CoA synthase	(100)	101	125	128	157	151
Isocitrate lyase	(100)		60	47	26	3
Malate synthase	(100)	90	108	89	84	99
NADP+-malic enzyme	(100)	89	108	111	127	178
PEP-synthesizing system	(100)	107			149	108
Fructose diphosphatase	(100)	400	300		180	128

[a] For actual specific activities, see Table 2.

TABLE 5. *Enzymes related to glucose metabolism in $S_{130,000}$ after growth on 17 mM glucose or 52 mM ethanol in the presence or absence of itaconate*

Enzyme	Sp act ($\times 10^3$) after growth in the presence of:			
	Glucose	Ethanol	Ethanol + 10 mM itaconate	Ethanol + 15 mM itaconate
Hexokinase	230			
Glucose 6-phosphate dehydrogenase				
NADP+	284	8.6		
NAD+	70	1.2		
Phosphohexose isomerase	47	3[a]		5[b]
Phosphofructokinase	ND[c]	ND		
FDP aldolase[d]	72	10		
Triose phosphate isomerase	2,020	3,110		
3-Phosphoglycerate kinase	18	5		
Phosphoglycerate mutase and enolase	6	9		
Gyceraldehyde 3-phosphate dehydrogenase				
NAD+	278	1.4		
NADP+	33	17	14	19
Pyruvate kinase	162	63		
Lactic dehydrogenase	7	4		
Pyruvate carboxylase[e]	ND	ND		
PEP carboxylase	900	133		
6-Phosphogluconate hydrolyase and 2-oxy-3-deoxy-6-phosphogluconate aldolase	7.2	ND		

[a] Dialyzed for 4 h against 100 volumes of TME containing 1 mM phenylmethylsulfonyl fluoride at 4°C. The buffer was changed once after 2 h.

[b] Dialyzed as described in footnote *a*; the specific activity was determined at one protein concentration only.

[c] Not detectable.

[d] Cells were broken in in 0.1 M Tris-chloride buffer containing 5 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride.

[e] Cells were broken in in TME containing 1 mM phenylmethylsulfonyl fluoride and 10 mM glutathione.

The findings that an NAD+-glyceraldehyde 3-phosphate dehydrogenase was apparently induced by growth on glucose whereas the NADP+-enzyme was found after growth of *P. indigofera* on either glucose or ethanol (Table 5) are consistent with observations made with *P. citronellolis* (31). The average specific activity of the NADP+-enzyme in extracts from cells grown in the presence of 10 and 15 mM itaconate did not differ from that for cells grown on ethanol alone (Table 5).

The following enzymes assayed in the $S_{130,000}$ fraction from ethanol-grown cells were not inhibited by 1 to 5 mM itaconate: phosphohexose isomerase, 3-phosphoglycerate kinase, phosphoglycerate mutase plus enolase, NADP+-glyceraldehyde 3-phosphate dehydrogenase, PEP carboxylase, and triose phosphate isomerase.

DISCUSSION

The present work suggests that ethanol is converted to acetyl-CoA in *P. indigofera* through steps catalyzed by NAD+-alcohol and NAD+-aldehyde dehydrogenases and by acetyl-CoA synthase. Earlier work established that enzymes of the tricarboxylic acid cycle (28), as well as isocitrate lyase and malate synthase (26), are also present in ethanol-grown cells. The present communication establishes that malic enzyme and a PEP-synthesizing system provide a link between the tricarboxylic acid cycle and gluconeogenesis in *P. indigofera*. It is significant that PEP carboxykinase could not be detected in this pseudomonad. The present research also establishes that fructose diphosphatase is detectable after growth on ethanol, as would be expected, but that, curiously, this enzyme is elevated in cells grown on glucose (Table 2). It seems unlikely that fructose diphosphatase functions in glucose catabolism.

In Fig. 3, the pathways of ethanol and glucose metabolism are shown. Because phosphofructokinase could not be detected in glucose- or ethanol-grown cells but could be readily detected in glucose-grown *E. coli*, we conclude that it is absent from *P. indigofera*. Indeed, this latter organism has the potential to catabolize glucose via the Entner-Doudoroff pathway as evidenced by the fact that the presence of 6-phosphogluconate hydrolyase and 2-keto-3-

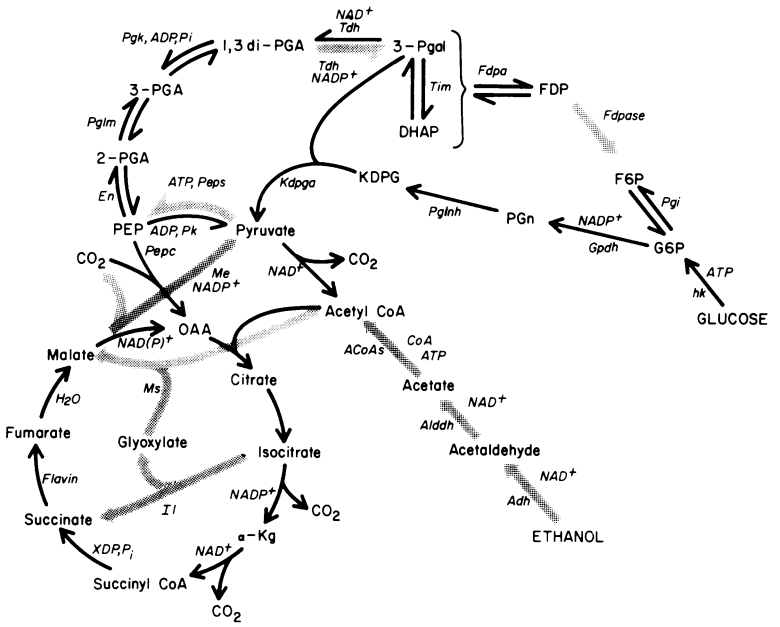

FIG. 3. *Metabolism of ethanol and glucose by P. indigofera. Shaded arrows identify enzymatic steps that are required for metabolism of ethanol but not for that of glucose. Abbreviations: Adh, alcohol dehydrogenase; Alddh, aldehyde dehydrogenase; ACoAs, acetyl-CoA synthase; Ms, malate synthase; Il, isocitrate lyase; Me, malic enzyme, Pepc, phosphoenolpyruvate carboxylase; Peps, phosphoenolpyruvate-synthesizing system; Pk, pyruvate kinase, En, enolase; Pglm, phosphoglyceromutase; Pgk, 3-phosphoglycerate kinase; Tdh, triosephosphate dehydrogenase; Tim, triose phosphate isomerase; Fdpa, fructose diphosphate aldolase; Fdpase, fructose diphosphatase; Pgi, phosphoglucoisomerase (phosphohexose isomerase); Hk, hexokinase; Gpdh, glucose 6-phosphate dehydrogenase; Pglnh, phosphogluconate hydrolyase; and Kdpga, 2-keto-3-deoxy-6-phosphogluconate aldolase.*

deoxy-6-phosphogluconate aldolase is induced by growth on glucose.

The research described, then, provides a firm experimental foundation for the delineation of enzymatic differences between ethanol and glucose metabolism in *P. indigofera*. In essence, the enzymatic steps that appear to function in ethanol utilization but are not required for glucose metabolism (shaded in Fig. 3) are catalyzed by: alcohol dehydrogenase, aldehyde dehydrogenase, acetyl-CoA synthase, isocitrate lyase, malate synthase, malic enzyme, the PEP-synthesizing system, and fructose diphosphatase. Presumably, the selective inhibition by itaconate of growth on ethanol in comparison with that on glucose reflects the sensitivity of one or more of these steps to itaconate, a substance that is not utilized for growth. Our data suggest that the sensitive step is catalyzed by isocitrate lyase and that the level of isocitrate lyase is reduced as well. That this is the correct interpretation is strengthened by the selective inhibition by itaconate of growth on butyrate. The assimilation of butyrate also requires function of the glyoxylate (19, 26) and tricarboxylic acid cycles and gluconeogenesis.

The use of toluene treatment to increase the permeability of *P. indigofera* has confirmed the interpretations based on assays of cell-free preparations. Of particular interest has been the finding that itaconate is highly selective in the inhibition of isocitrate lyase in toluene-treated cells. In this connection, the findings that the K_m for D$_s$-isocitrate and the K_i for itaconate are 0.086 and 0.02 mM, respectively, at pH 7.7 (30°C) in toluene-treated cells are in excellent accord with the analogous values of 0.11 (35) and 0.013 (33) mM obtained with homogeneous isocitrate lyase from *P. indigofera*. The observed uncompetitive inhibition by itaconate in toluene-treated cells is also in accord with the mechanism of inhibition observed at one concentration of itaconate with the pure enzyme from *P. indigofera* (33). Finally, recovery experiments done with itaconate incubated with toluene-treated cells have suggested that this pseudomonad does not metabolize itaconate—a finding in accord with the failure of growing cells to utilize itaconate. In two other species of *Pseudomonas*, however, itaconate is

utilized via itaconyl-CoA and citramalyl-CoA. The latter compound is then cleaved to acetyl-CoA plus pyruvate (6, 29, 30).

In summary, the present evidence establishes that itaconate is taken up by cells of *P. indigofera* and that it selectively reduces the level of isocitrate lyase and inhibits the glyoxylate cycle at the step catalyzed by isocitrate lyase. The extracellular concentration required for inhibition of assimilation via the glyoxylate cycle is, however, about three orders of magnitude higher than that required for effective in vitro inhibition of isocitrate lyase. This probably reflects the lack of a transport system for itaconate in *P. indigofera*, although a lowered intracellular sensitivity of isocitrate lyase to itaconate cannot be excluded. We continue to test itaconate as an isocitrate-lyase-directed inhibitor in other organisms that require the function of the glyoxylate cycle for growth.

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