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Reduced Nicotinamide Adenine Dinucleotide-Activated Phosphoenolpyruvate Carboxylase in *Pseudomonas* MA: Potential Regulation Between Carbon Assimilation and Energy Production

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Comparison of enzyme activities in crude extracts of methylamine-grown *Pseudomonas* MA (ATCC 23319) to those in succinate-grown cells indicates the involvement of an acetyl coenzyme A-independent phosphoenolpyruvate carboxylase in one-carbon metabolism. The purified phosphoenolpyruvate carboxylase is activated specifically by reduced nicotinamide adenine dinucleotide (K_A = 0.2 mM). The regulatory properties of this enzyme suggests that phosphoenolpyruvate serves as a focal point for both carbon assimilation and energy metabolism.

Phosphoenolpyruvate (PEP) carboxylase (EC 4.1.1.31) catalyzes the formation of oxalacetate from PEP and carbon dioxide. This enzyme, which is widely distributed in plants and microorganisms, has recently been divided into three basic types by Utter and Kolenbrander (19) according to its regulatory properties. Class 1 comprises those forms of the enzyme which are subject to both activation and inhibition. All of the enzymes in this group are activated by acetyl coenzyme A (acetyl-CoA) and inhibited by L-aspartate; however, additional activators and inhibitors have been described. Class 2 includes the group of enzymes that is subject only to inhibition, the most common inhibitors being Krebs cycle intermediates, whereas those enzymes that are subject neither activation nor inhibition are grouped in class 3.

It appears that the PEP carboxylases produced by nonphotosynthetic bacteria grown aerobically are of the type found in class 1. The function of this enzyme is an anaplerotic one, in providing oxalacetate for citrate synthesis (10, 18). The activation of the enzyme by acetyl-CoA and its inhibition by aspartate can thus be rationalized in terms of the metabolic role of the enzyme.

Large et al. (13) first described a class 3-type PEP carboxylase in the nonphotosynthetic bacterium *Pseudomonas* AM-1, aerobically grown on methanol or methylamine as the carbon source. Subsequently, a number of workers (5, 12, 20) have reported class 3-type PEP carboxylases for aerobically grown nonphotosynthetic bacteria with methylamine or methanol as the carbon source.

To investigate in more detail the properties of this apparently anomalous type of PEP carboxylase, we undertook a study of the PEP carboxylase found in methylamine-grown *Pseudomonas* MA (ATCC no. 23319). The results of this study suggest that reduced nicotinamide adenine dinucleotide (NADH) is an activator for this PEP carboxylase. This finding is discussed in terms of regulation between carbon assimilation and energy production.

MATERIALS AND METHODS

The conditions for growth of *Pseudomonas* sp. and the preparation of cell-free extracts have been previously described (1). Acetyl-CoA was prepared by the method of Simon and Shemin (17). All other materials were obtained from commercial sources.

Enzyme assays. Glycerate kinase (EC 2.7.1.31) was assayed by the method of Heptinstall and Quayle (6) as modified by Harder et al. (5).

Enolase (EC 4.2.1.11) and phosphoglycerate mutase (EC 2.7.5.3) were assayed as described by Harder et al. (5) except that with the latter enzyme the amount of 3-phosphoglycerate used was decreased from 5 μmol to 1 μmol.

Serine dehydratase (EC 4.2.1.13) and malic enzyme (EC 1.1.1.39:1.1.1.40) were assayed as previously described (1).

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) was routinely assayed by the method of Canovas and Kornberg (2) except that acetyl-CoA was omitted from the reaction mixture unless otherwise noted.

Reaction mixtures contained 50 mM tris(hydroxymethyl)aminomethane (Tris)-succinate buffer, pH 8.0, 1 mM PEP, 10 mM potassium bicarbonate, 0.11 mM NADH, 5 mM magnesium sulfate, PEP carboxylase, and 4 U of pig heart malic dehydrogenase in a final volume of 1.0 ml. The reaction was initiated by the addition of PEP, and the oxidation of NADH was followed at 340 nm.

Alternatively, three other assay methods were employed to follow the reaction. (i) A schematic diagram of the measurement of oxalacetate formation using citrate synthase is shown in Fig. 1. Reaction mixtures were identical to that described above except NADH and malic dehydrogenase were omitted, and 0.35 mM acetyl-CoA, 0.10 mM dithionitrobenzoate, and 0.4 U of pig heart citrate synthase were added. The reaction was followed by measuring the liberation of thionitrobenzoate at 412 nm.

(ii) For the measurement of oxalacetate formation by reaction with 2,4-dinitrophenylhydrazine, reaction mixtures contained 50 mM Tris-succinate buffer, pH 8.0, 1 mM PEP, 10 mM potassium bicarbonate, 5 mM magnesium sulfate, and PEP carboxylase. At various times a 0.4-ml sample was removed from the reaction mixture and added to 0.1 ml of 0.1% 2,4-dinitrophenylhydrazine in 6 N HCl. After incubation for 10 min at room temperature 0.5 ml of 3.5 N sodium hydroxide was added, and the absorbance at 540 nm was measured.

A standard curve for oxalacetate 2,4-dinitrophenylhydrazone was constructed from freshly prepared solutions of oxalacetate. The concentration of oxalacetate was determined by the malic dehydrogenase reaction, and the concentration of pyruvate was determined by the lactate dehydrogenase reaction. We found the pyruvate concentration to be negligible.

(iii) Measurement of inorganic phosphate release was performed as described by Cottam et al. (3). Reaction mixtures identical to those used in the 2,4-dinitrophenylhydrazine assay were prepared. Samples of 0.7 ml were removed at various times and added to 0.2 ml of 2.5% ammonium molybdate in 1 N NH_2SO_4, and then 0.1 ml of 1% *p*-methylaminophenol sulfate-3% sodium sulfite in water was added. After incubation for 10 min at room temperature the absorbance at 660 nm was determined and compared to a standard curve prepared utilizing potassium phosphate.

Purification of acetyl-CoA-independent PEP carboxylase. (i) Preparation of cell extract. Frozen cells (75 g) of methylamine-grown *Pseudomonas* MA were thawed in 150 ml of 20 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM disodium ethylenediaminetetraacetate and 5 mM 2-mercaptoethanol (buffer A). The cells were suspended with a glass rod and then passed through an Aminco French pressure cell at 9,000 to 16,000 lb/in.^{2} Magnesium sulfate was added to the viscous extract to give a final concentration of 5 mM, and deoxyribonuclease was added to a final concentration of 5 μg/ml. After stirring for 15 min at room temperature, the solution was centrifuged first at 25,000 $\times$ *g* for 20 min and then at 100,000 $\times$ *g* for 1 h; in each case the precipitate was discarded.

$$\text{PEP} + CO_2 \xrightarrow{\text{PEP carboxylase}} \text{oxalacetate} + \text{inorganic phosphate}$$

$$\text{OAA} + \text{acetyl-CoA} \xrightarrow[\text{synthase}]{\text{citrate}} \text{citrate} + \text{CoA}$$

$$\text{CoA} + \text{dithionitrobenzoate} \longrightarrow \text{yellow color (412 nm)}$$

FIG. 1. *Schematic diagram of the measurement of oxalacetate formation using citrate synthase.*

(ii) Acid treatment. To the supernatant solution was added 0.1 N acetic acid until the pH was lowered to 5.2. The solution was centrifuged, and the precipitate was redissolved in a minimal volume of 0.1 M Tris-succinate buffer, pH 7.4. Solid ammonium sulfate was then added to 30% saturation, and the solution was centrifuged. The supernatant was adjusted to 60% ammonium sulfate and centrifuged, and the precipitate was dissolved in a minimal volume of 20 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM disodium ethylenediaminetetraacetate, 5 mM 2-mercaptoethanol, and 50 mM potassium chloride. The redissolved precipitate was dialyzed for 4 to 5 h against this same buffer and then further dialyzed overnight against the same buffer containing 20 mM potassium chloride in place of 50 mM potassium chloride.

(iii) Diethylaminoethyl-cellulose chromatography. The dialyzed solution was applied to a diethylaminoethyl-cellulose column (2.3 by 30 cm) previously equilibrated with buffer A. The column was washed with approximately 2 column volumes of buffer A, and then a linear gradient, consisting of 400 ml of buffer A plus 0.5 M potassium chloride in the reservoir, was used to elute the enzyme. The active fractions were pooled and concentrated by adding solid ammonium sulfate to 80% saturation. The precipitate was collected by centrifugation and redissolved in a minimal volume of buffer A.

(iv) Bio-Gel A 1.5 M chromatography. The redissolved precipitate was applied to a Bio-Gel A 1.5 M column (2.8 by 90 cm) previously equilibrated with buffer A containing 50 mM potassium chloride. The enzyme was eluted with the buffer and concentrated as described above. The enzyme was redissolved in a minimal volume of 10 mM potassium phosphate buffer, pH 6.4, containing 0.1 mM disodium ethylenediaminetetraacetate and 5 mM 2-mercaptoethanol. The enzyme was then dialyzed overnight against the buffer.

(v) Cellulose-phosphate chromatography. The dialyzed enzyme was applied to a cellulose-phosphate column (1.5 by 10 cm) previously equilibrated with the buffer described above. The column was first washed with 20 ml of the above buffer and then 15 ml of this buffer containing 0.1 M potassium chloride. The enzyme was then eluted with a linear gradient consisting of 150 ml of buffer containing 0.1 M potassium chloride in the mixing chamber and 150 ml of buffer containing 0.5 M potassium chloride in the reservoir. The active fractions were pooled, neutralized to pH 7.8 with 1 M Tris-sulfate buffer (the final Tris-sulfate concentration was ca. 50 mM), and precipitated by adding ammonium sulfate to 80% saturation. After centrifugation the precipitate was dissolved in a minimal volume of buffer A and dialyzed against 50 mM Tris-succinate buffer, pH 8.5.

The purified enzyme exhibited only one protein

band when subjected to disc gel electrophoresis using 4% polyacrylamide gels in Tris-glycine buffer, pH 8.5. A summary of the purification procedure is given in Table 1.

RESULTS

Establishment of PEP carboxylase involvement in methylamine metabolism. Studies on the metabolism of methylamine by *Pseudomonas* MA (1, 7–9) have led to the proposal of the metabolic pathway shown in Fig. 2. At the time this pathway was proposed, the identity of the reactions involved in the conversion of serine to malate was unclear. Two possible pathways were evident. The first involves the conversion of serine to pyruvate, followed by reductive carboxylation of pyruvate to yield malate (Fig. 3). The second pathway, originally proposed by Large and Quayle (14), involves the carboxylation of phosphoenolpyruvate, formed via hydroxypyruvate, glycerate, and phosphoglycerate (Fig. 4).

Those enzymes that play a major role in one-carbon metabolism should either be induced or remain at relatively high constitutive levels when the organism is grown on methylamine. Therefore, to determine the pathway for the conversion of serine or malate, the activity of the enzymes shown in Fig. 3 and 4 were measured in cells grown on methylamine and compared to the activity of the same enzyme when succinate was used as the growth source.

As shown in Table 2 the enzymes hydroxypyr-

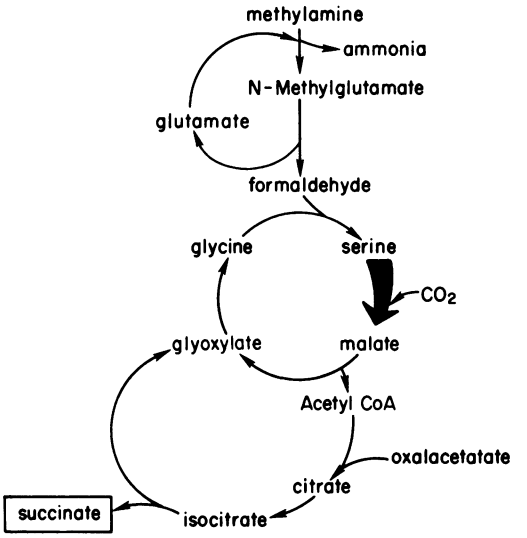

FIG. 2. *Proposed pathway for the metabolism of methylamine by Pseudomonas MA.*

TABLE 1. *Purification of acetyl-CoA-independent carboxylase*

Step	Volume (ml)	Total protein (mg)	Total units (IU)	Sp act (IU/mg)	Recovery (%)
Crude extract	159	1,900	449	0.24	(100)
Acid step	19	214	214	1.00	48
Diethylaminoethyl-cellulose chromatography	8.2	61	182	2.87	41
	4.3	13.6	138	10.1	31
Cellulose-phosphate chromatography	3.7	4.2	67	15.8	15

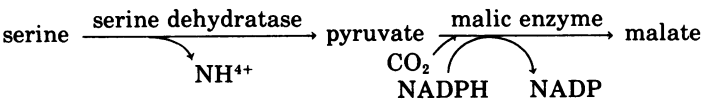

FIG. 3. *Possible pathway for the conversion of serine to malate.*

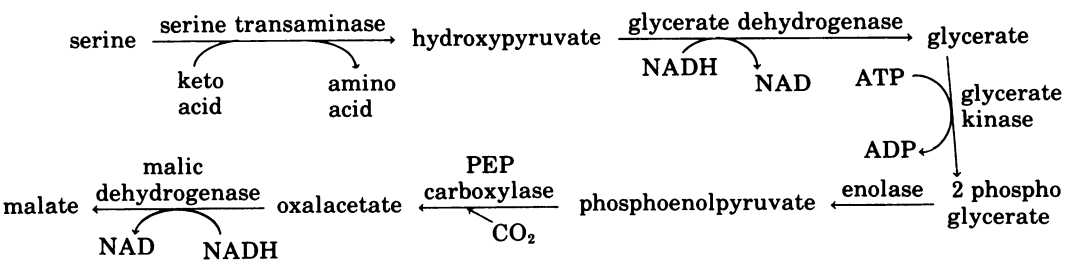

FIG. 4. *Alternate pathway for the conversion of serine to malate (see Fig. 3).*

TABLE 2. *Comparison of various enzyme activities in methylamine-grown cells to succinate-grown cells*[a]

Enzyme	Sp act (μmol/h/mg of protein)		Ratio (methylamine/ succinate)
	Methylamine	Succinate	
Glycerate kinase	2.5	0.15	16.70
Phosphoglyceromutase	34.5	36.5	0.95
Hydroxypyruvate reductase[b]	95.0	<0.1	>950.0
Enolase	13.6	20.0	0.68
PEP carboxylase	5.6	0.1	56.0
Malic enzyme[b]			
NADP linked	1.4	3.2	0.44
NAD linked	2.3	<0.2	>11.50
Serine dehydratase	0.26 (0.8)[c]	0.28 (0.3)	0.93

[a] All assays were conducted as described in Materials and Methods.
[b] Values taken from reference 1.
[c] Values in parenthesis are those previously reported in reference 1.

uvate reductase, glycerate kinase, PEP carboxylase (acetyl-CoA independent), and NAD-linked malic enzyme are all induced in methylamine cells. We previously reported that serine dehydratase levels were elevated upon growth on methylamine (1); however, these more recent studies suggest considerable variation in this activity and do not indicate a consistent elevated level for growth on methylamine. Therefore, we would conclude that the low and variable level of serine dehydratase precludes the functioning of the reactions shown in Fig. 3 as a major metabolic route for malate formation. On the other hand, all of the enzymes required for the reactions shown in Fig. 4 are found in either constitutive or induced levels and thus indicate that the pathway involving PEP carboxylase is the correct one.

The conversion of glycerate to PEP can proceed either by the phosphorylation of glycerate at the 2 position, and the subsequent isomerization to yield PEP, or by phosphorylation of glycerate at the 3 position, requiring the conversion of 3-phosphoglycerate to 2-phosphoglycerate prior to PEP formation. Since crude extracts contained more phosphoglycerate mutase than glycerate kinase activity, a partially purified preparation of glycerate kinase was obtained (Table 3). Using this preparation, glycerate kinase activity equalled the activity for the conversion of glycerate to PEP and was not dependent on added phosphoglycerate mutase or 2,3-diphosphoglycerate (Table 4). The endogenous level of phosphoglycerate mutase could not support this conversion since its activity was less than one-fifth the rate of the over-all reaction (Table 3). Harder et al. (5) have reported a stimulation of phosphoglycerate mutase activity by adenosine triphosphate (ATP); however, no such activation was observed under the assay conditions employed (Table 4).

TABLE 3. *Purification of glycerate kinase*[a]

Step	Total protein	Total act (U)	Sp act (U/mg)
Extract	286	10.3	0.036
40 to 50% $AmSO_4$ fractionation	68.4	7.5	0.110
Sephadex A-25 chromatography	5.1	4.0	0.68

[a] The purification of glycerate kinase was by the procedure of Doughty et al. (4). The ratio of glycerate kinase to phosphoglycerate mutase increased from 0.07 in the extract to 6.0 in the Sephadex A-25 fraction. One unit is defined as the liberation of 1 μmol of ADP per min at 30 C.

PEP carboxylase as a function of growth substrate. Wagner and Quayle (20) showed the induction of an acetyl-CoA-independent PEP carboxylase when *Pseudomonas* AM-1 was grown on methylamine. As shown in Table 5, growth of *Pseudomonas* MA on methylamine also induces an acetyl-CoA-independent PEP carboxylase; however, acetyl-CoA-dependent activity is observed when sucrose, succinate, or lactate is used as a growth source. Although not shown, we also observed an acetyl-CoA-independent PEP carboxylase in methylamine-grown *Pseudomonas* AM-1 and an acetyl-CoA-dependent enzyme when succinate was used as the growth source. It thus seems likely that most, if not all, of the "serine pathway" (14) organisms thus far studied can elicit two types of PEP carboxylases dependent on the growth source utilized.

NADH activation of PEP carboxylase. The acetyl-CoA-independent PEP carboxylase was routinely assayed by coupling this reaction to the malic dehydrogenase reaction. During preliminary experiments designed to measure oxalacetate inhibition of the reaction, the assay

TABLE 4. *2-Phosphoglycerate as a product of the glycerate kinase reaction*[a]

Reaction	Sp act (μmol/min/mg)
(1) Glycerate → PG (glycerate kinase)	0.53
(2) 3PG → 2PG (phosphoglycerate mutase)	0.09 (0.085)
(3) Glycerate → PEP	0.54
Plus 1 mM DPG	0.51
Plus 1 mM DPG + 0.5 U of PG mutase	0.31

[a] All reaction mixtures contained 50 mM Tris-hydrochloride buffer, pH 7.5, 25 mM magnesium sulfate, 100 mM potassium chloride, 2 U of lactate dehydrogenase, and 1 U of pyruvate kinase in a final volume of 1.0 ml. In addition, the specific assays included: glycerate → PG, 0.5 mM ATP and 1 mM calcium glycerate; 3PG → 2PG, 0.2 mM ADP, 5 mM 3PG, 0.1 mM 2,3DPG, and 1 U of enolase (the value in parenthesis included 0.5 mM ATP in the reaction mixture); glycerate → PEP, 0.5 mM ATP, 1.0 mM adenosine diphosphate, 1 mM calcium glycerate, and 1 U of enolase. PG, Phosphoglycerate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; DPG, diphosphoglycerate.

TABLE 5. *PEP carboxylase activity as a function of growth source*[a]

Growth source	PEP carboxylase activity (μmol/h/mg)	
	No acetyl-CoA	Plus acetyl-CoA
Methylamine	5.6	5.8
Sucrose + ammonia	<0.01	5.4
Succinate + ammonia	0.1	4.8
Lactate + ammonia	0.3	3.4

[a] PEP carboxylase activity was determined using the malic dehydrogenase couple assay described in Materials and Methods. When added, the concentration of acetyl-CoA was 0.35 mM.

system was changed to one in which the liberation of inorganic phosphate was measured. Using this assay method virtually no enzyme activity was detected. The addition of NADH, but not malic dehydrogenase, was found to restore enzyme activity. To further verify the activation by NADH, this phenomena was assessed in three different assay systems: inorganic phosphate formation, oxalacetate 2,4-dinitrophenylhydrazone formation, and oxalacetate formation measured with citrate synthase. All three assay systems showed stimulation of the reaction by NADH (Table 6). Similar experiments conducted with methylamine-grown *Pseudomonas* AM-1 indicated no stimulatory effect of NADH on the PEP carboxylase reaction.

To rule out the possibility that an impurity in the NADH preparation was responsible for the observed activation, two different samples of NADH, one referred to as "ultrapure," were tested and found to give identical results. Furthermore, when NADH was oxidized to NAD in the presence of pyruvate and lactate dehydrogenase, the ability to stimulate the PEP carboxylase reaction was abolished (Table 7).

That NADH is not utilized as a substrate during the reaction is shown in Table 8. Although this result shows that NADH is not a substrate, the possibility exists that NADH could react directly with the enzyme and modify it. This possibility seems unlikely since we have found that enzyme incubated in the presence of 0.1 mM NADH and then diluted to 0.02 mM NADH shows the expected activity for the lower NADH concentration. Determination of the K_A for NADH is shown in Fig. 5. A value of ~0.2 mM was obtained.

Other possible effectors of the PEP carboxylase were tested, and activation was only observed with NADH and NADPH, the latter compound being required at a considerably higher concentration (Table 9). Slight inhibition was seen with ATP and adenosine diphosphate; other purine and pyrimidine di- and trinucleotides were ineffective.

DISCUSSION

Significance of NADH activation. The acetyl-CoA-activated aspartate-inhibited PEP carboxylase serves as an anaplerotic enzyme when

TABLE 6. *Comparison of various assay methods for measuring NADH stimulation of PEP carboxylase activity*[a]

Assay method	Sp act (μmol/min/mg)	
	No NADH added	0.11 mM NADH added
Oxalacetate formation—malic dehydrogenase coupled assay		12.16
Oxalacetate formation—2,4-dinitrophenyl hydrazone formation	1.48	12.80
Oxalacetate formation—citrate synthase coupled assay	1.42	12.74
Phosphate formation	1.50	12.70

[a] Reaction mixtures contained 50 mM Tris-succinate buffer, pH 8.0, 1 mM PEP, 10 mM potassium, and the appropriate substrates and enzymes for the coupled assays. The assay procedures are described in Materials and Methods.

TABLE 7. *Demonstration that NADH and not a contaminant of NADH is the activator of PEP carboxylase*[a]

Expt no.	Expt conditions	Sp act (μmol/min/mg)
1	No NADH added	0.9
2	0.06 mM NADH added	5.4
3	0.06 mM NADH converted to NAD added	0.8
4	0.06 mM NADH plus 0.25 mM pyruvate added	5.6
5	0.06 mM NADH boiled in the presence of 2.5 mM pyruvate and then 1 U of lactate dehydrogenase added	5.4

[a] A solution containing 0.60 mM NADH was reacted with excess pyruvate and 1 U of lactate dehydrogenase to convert the NADH to NAD. After complete oxidation of NADH, as ascertained spectrophotometrically, the solution was boiled for 2 min. A sample was removed and added to a standard assay mixture to give a final NADH concentration of 0.06 mM (experiment 3). Controls were run in which pyruvate and NADH were added directly to the assay mixture (experiment 4) and in which lactate dehydrogenase was added to the NADH-pyruvate solution in the boiling water bath (experiment 5). Assays were conducted by measuring inorganic phosphate release as described in Materials and Methods.

TABLE 8. *Stoichiometry of PEP carboxylase reaction*[a]

Time (min)	Concn of reactants (μmol/ml)			
	PEP	OAA	P_i	NADH
0	1.16			0.11
10	1.04	0.13	0.16	0.12
Δ	0.12	0.13	0.16	0.01

[a] Reaction mixtures contained 50 mM Tris-succinate buffer, pH 8.0, 1.16 mM PEP, 10 mM potassium bicarbonate, 0.11 mM NADH, 5 mM $MgSO_4$, and 1.1 mg of PEP carboxylase, specific activity 13.6, in a final volume of 1.0 ml. After incubation for 10 min at 30 C, aliquots were removed for oxalacetate (OAA) and inorganic phosphate (P_i) determinations, while the remainder of the reaction mixture was immersed in a boiling water bath for 1 min. PEP and NADH determinations were performed on the heated solution. PEP concentration was determined using pyruvate kinase-lactate dehydrogenase, and NADH concentration was determined by the absorbance at 340 nm.

an organism utilizes glycolytic intermediates for growth. Thus, when the acetyl-CoA concentration rises, the PEP carboxylase reaction is increased and the oxalacetate concentration rises to condense with acetyl-CoA and form citrate (Fig. 6).

As shown by the present studies, when *Pseudomonas* MA is grown on methylamine, oxalacetate serves as an obligatory intermediate for

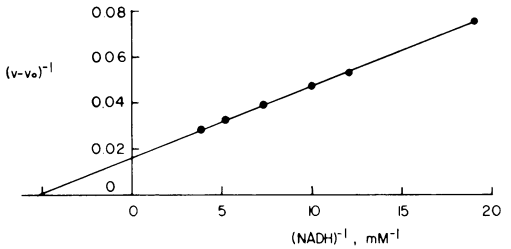

FIG. 5. *Determination of K_A for NADH activation. Reaction mixtures contained 50 mM Tris-succinate buffer, pH 8.0, 10 mM potassium bicarbonate, 5 mM PEP, 5 mM magnesium sulfate, 0.1 mM dithionitrobenzoate, 0.35 mM acetyl-CoA, 0.4 U of pig heart citrate synthase, NADH as indicated and PEP carboxylase in a final volume of 1.0 ml. v-vo is equal to the specific activity of the enzyme at a given NADH concentration minus the specific activity obtained in the absence of NADH. vo = 2.9 μmol/min per mg of protein; V_{max} = V_{max} from graph + vo = 64.6; K_A = 0.196 mM.*

TABLE 9. *Effect of various compounds on the PEP carboxylase reaction*[a]

Effector	Concn (mM)	Sp act (μmol/min/mg)
None		1.68
Fructose 1,6-diphosphate	10.0	2.03
Acetyl-CoA	0.4	1.72
Aspartate	2.5	1.45
Citrate	5.0	1.52
Malate	5.0	1.52
ATP	5.0	0.90
Adenosine diphosphate	5.0	1.10
Guanosine triphosphate	2.5	1.94
Inosine triphosphate	5.0	1.86
Cytosine triphosphate	5.0	1.74
Cytosine diphosphate	5.0	1.82
Uridine triphosphate	5.0	1.88
NADH	0.1	16.30
NAD	1.0	2.00
NADPH	1.0	10.90
NADP	1.0	2.10

[a] Reaction mixtures contained 50 mM Tris-succinate buffer, pH 8.0, 5 mM magnesium sulfate, 1 mM PEP, 8 mM potassium bicarbonate, enzyme, and water in a final volume of 0.50 ml. After incubation at room temperature for 10 min, 0.2 ml was withdrawn and added to 0.1 ml of 2,4-dinitrophenylhydrazine. Oxalacetate 2,4-dinitrophenylhydrazone formation was measured as described in Materials and Methods.

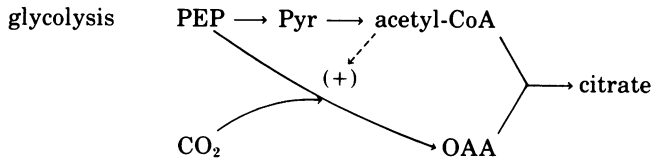

FIG. 6. *Utilization of glycolytic intermediates by an organism for growth.*

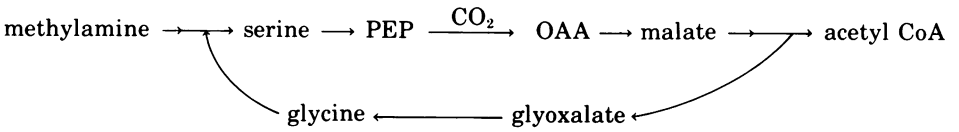

FIG. 7. *Acetyl-CoA formation when Pseudomonas MA is grown on methylamine.*

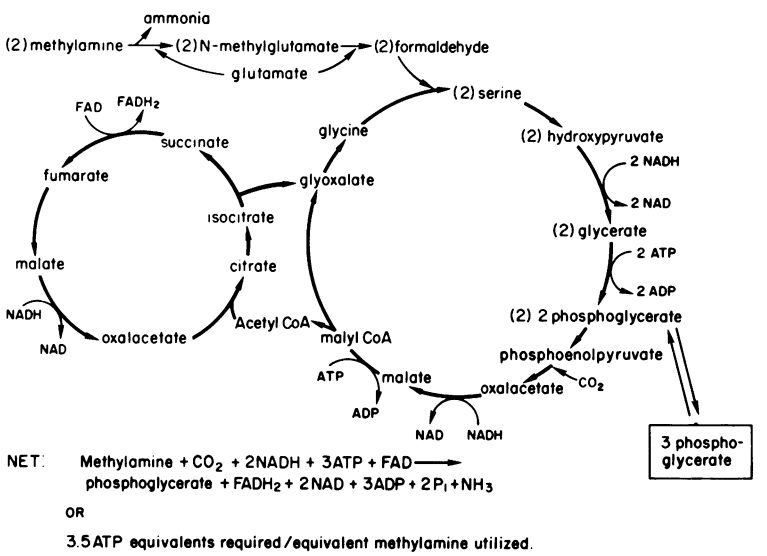

FIG. 8. *Pathway for carbon assimilation as first described by Bellion and Hersh (1) and modified by Harder et al. (5).*

acetyl-CoA formation (Fig. 7). Thus, in this case the activation of PEP carboxylase by acetyl-CoA could serve no beneficial metabolic role.

A comparison of the acetyl-CoA-independent PEP carboxylase from methylamine-grown *Pseudomonas* AM-1 and *Pseudomonas* MA showed that only the *Pseudomonas* MA enzyme is activated by NADH. Although the metabolism of one-carbon compounds in *Pseudomonas* AM-1 is not fully understood (16), it appears that separate pathways for carbon assimilation and energy production are feasible (15). In contrast to the situation in *Pseudomonas* AM-1 and the results of Kung and Wagner (11), we have been unable to detect a separate enzyme system in *Pseudomonas* MA which is capable of producing energy via the direct oxidation of methylamine (9). Therefore, both energy production and carbon assimilation appear to proceed via common intermediates. The pathway for carbon assimilation as first described by Bellion and Hersh (1) and modified by Harder et al. (5) is illustrated in Fig. 8. For the assimilation of 1 molecule of methylamine, 1 NADH molecule and 1.5 ATP molecules are used with the production of 0.5 molecules of $FADH_2$; a net of 3.5 equivalents of ATP are required. The production of one or more ATP molecules during the oxidation of *N*-methylglutamate to glutamate and formaldehyde is possible (9) and would reduce the total ATP requirement to 2.5 or 1.5 equivalents.

Since the organism contains significant levels of the enzymes pyruvate kinase, pyruvate dehydrogenase, malic enzyme and α-ketoglutarate dehydrogenase, two possible energy-yielding pathways diverging from the assimilatory

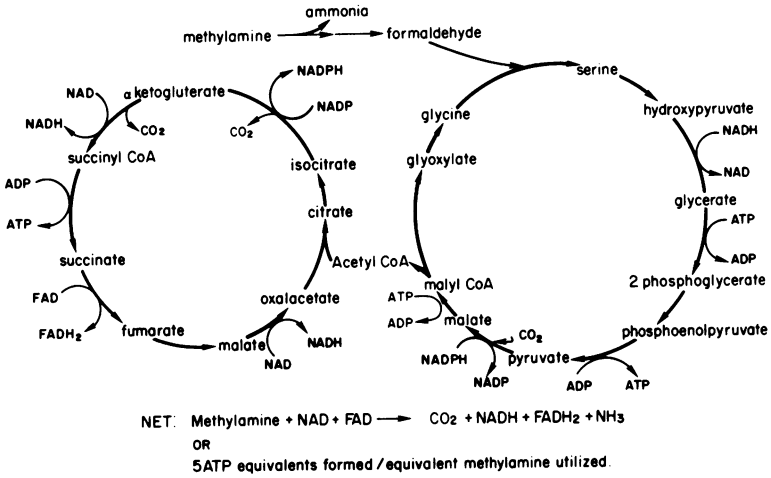

FIG. 9. *Possible energy-yielding pathway in Pseudomonas MA.*

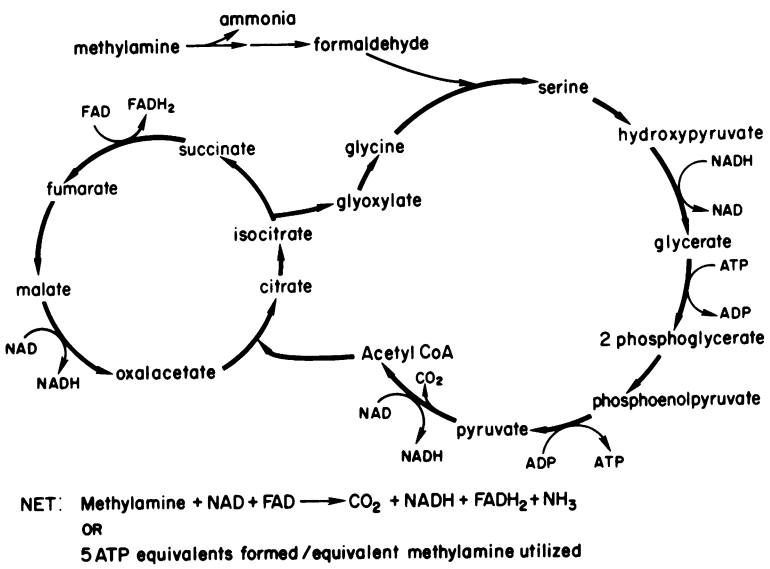

FIG. 10. *Alternate energy-yielding pathway in Pseudomonas MA.*

pathway can be postulated (Fig. 9 and 10).

In either of the schemes the oxidation of methylamine leads to the net formation of one molecule of NADH and one molecule of $FADH_2$, or the equivalent of five molecules of ATP. (If ATP is produced via the *N*-methylglutamate dehydrogenase reaction this number increases to six or seven ATP equivalents.) Thus, it can be seen that the central intermediate in all three pathways is phosphoenolpyruvate. Carboxylation of phosphoenolpyruvate commits the flow of carbon to assimilation, whereas dephosphorylation of phosphoenolpyruvate via pyruvate kinase commits the flow of carbon for energy production. It therefore does not seem unreasonable for the observed NADH activation of PEP carboxylase. When the cell is at a low energy state and the NADH concentration is low, PEP is converted to pyruvate via the action of pyruvate kinase. On the other hand, when the cell has sufficient NADH, PEP carboxylase is activated and the carbon flux leads to carbon assimilation. PEP thus serves as the key intermediate for both energy production and the synthesis of cellular constituents.

The question may be raised as to what controls, if any, are required to prevent the utilization of 2-phosphoglycerate for gluconeogenesis during energy production. Since in the direction of gluconeogenesis NADH is required for

the glyceraldehyde 3-phosphate dehydrogenase reaction, a low NADH level would prevent the utilization of 3-phosphoglycerate and thus permit the flow of carbon into the energy producing pathway.

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