Microbial Growth on C₁ Compounds

4. CARBOXYLATION OF PHOSPHOENOLPYRUVATE IN METHANOL-GROWN *PSEUDOMONAS* AM1*

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Large, Peel & Quayle (1961, 1962) have shown that during growth of *Pseudomonas* AM1 on methanol a substantial fraction (50%) of the cellular carbon passes through a stage which is exchangeable with carbon dioxide. When sodium hydrogen [¹⁴C]carbonate was administered to cells growing on methanol or formate, within the first 10 sec., 40–70% of the incorporated radioactivity appeared in malate and aspartate. This indicates that a carboxylation reaction involving the conversion of a C₃ acid into a C₄ acid is present in the organism. A scheme was proposed (Large *et al.* 1962) in which the conversion of C₃ into C₄ units is a key step in the carbon-assimilation pathway. This paper reports an investigation into the nature of the enzyme(s) involved in the carboxylation reaction, and the enzyme responsible appears to be phosphoenolpyruvate carboxylase [orthophosphate-oxaloacetate carboxylase (phosphorylating), EC 4.1.1.31].

MATERIALS AND METHODS

Special chemicals. NADH, NADPH, lactate dehydrogenase, pyruvate kinase, malate dehydrogenase, phosphoenolpyruvate (silver barium salt), coenzyme A (75% pure) and oxaloacetic acid (80% pure) were obtained from C. F. Boehringer und Soehne, Mannheim, Germany; ADP, GDP and IDP were from Sigma Chemical Co., St Louis, Mo., U.S.A. Other materials were obtained as indicated in the text.

Sodium [¹⁴C]bicarbonate. This was prepared from Ba¹⁴CO₃ (The Radiochemical Centre, Amersham, Bucks.) as described by Large *et al.* (1961).

Acetyl-coenzyme A. This was prepared from acetic anhydride and coenzyme A by the method of Stadtman (1957).

Sodium phosphoenolpyruvate. The silver barium PEP (500 mg.) was dissolved in 0.2N-HNO₃ (the theoretical amount for conversion of the Ag⁺ and Ba²⁺ ions into their nitrates). A slight excess of sodium chloride and sodium sulphate was added, the precipitated AgCl and BaSO₄ were removed by centrifuging and the pH of the supernatant was adjusted to 7 with N-NaOH.

* Part 3: Large, Peel & Quayle (1962).

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Paper chromatography and radioautography. These methods were as described by Large *et al.* (1961). 2,4-Dinitrophenylhydrazones were chromatographed in butan-1-ol-ethanol-aq. NH₃ soln. (sp.gr. 0.88)-water (140:20:1:39, by vol.) (El Hawary & Thompson, 1953). Radioactive spots on chromatograms were assayed directly with a mica end-window Geiger-Müller tube (General Electric Co.; type 2B2). The spots were then identified by co-chromatography.

Growth of the organism. *Pseudomonas* AM1 was grown as described by Peel & Quayle (1961).

Preparation of cell-free extracts. Cells were suspended in 5 ml. of 40 mM-tris hydrochloride, pH 7.5, containing GSH (10 mM), and were subjected for 2–3 min. to the full output at 25 kcy/sec. of a 600 w Mullard ultrasonic oscillator. The resultant debris and unbroken cells were removed by centrifuging at 25000g for 10 min. at 2° in the high-speed angle head of a refrigerated centrifuge (model PR-2, International Equipment Co., Boston, Mass., U.S.A.). Alternatively, the Hughes (1951) press was used for disrupting bacteria (see below).

Purification of the carboxylating enzyme. Step 1: preparation of bacterial extract. Frozen methanol-grown bacteria (21 g., wet wt.) were crushed in a Hughes press at -25°. The crushed cells were extracted with 100 ml. of ice-cold 40 mM-tris hydrochloride, pH 7.5, containing mercaptoethanol (10 mM) and about 1 mg. each of crystalline ribonuclease and deoxyribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks.). The resulting extract was centrifuged at 25000g for 10 min. at 2° and the pellet discarded. All subsequent operations were performed at 2°.

Step 2: treatment with protamine sulphate. To the supernatant (vol. 99 ml., containing 1130 mg. of protein) was added, with stirring, a solution containing 113 mg. of protamine sulphate in 2 mM-sodium acetate buffer, pH 5, and the resulting pink curdy precipitate removed by centrifuging and discarded.

Step 3: ammonium sulphate precipitation and dialysis. To the supernatant (vol. 128 ml.) was added 31.3 g. of ammonium sulphate with stirring, to give 40% saturation. The resulting precipitate was centrifuged down. To the supernatant (136 ml.) was added a further 8.56 g. of ammonium sulphate to bring the percentage saturation to 50. The resulting precipitate was removed by centrifuging. The supernatant (134 ml.) was brought to 60% saturation by the addition of 8.85 g. of ammonium sulphate. The precipitate was centrifuged down, and the supernatant was discarded. The three precipitates were each dissolved in 10 ml. of 50 mM-tris hydrochloride, pH 7.5, and dialysed

overnight against 1.5 l. of 10 mM-tris hydrochloride, pH 7.5. The activity was found to be mainly in the protein fraction that was precipitated between 40 and 50% saturation. This was subjected to ion-exchange chromatography.

Step 4: ion-exchange chromatography. Diethylaminoethylcellulose (DEAE-cellulose, Whatman DE50) (7 g.) was slurried in 5 mM-tris hydrochloride, pH 7.5, and poured into a chromatographic column (2.5 cm. $\times$ 15 cm.). The column was then washed with 1 l. of 5 mM-tris hydrochloride, pH 7.5, and the enzyme extract was applied to the top of the column. The column was then eluted with an increasing gradient of potassium chloride in 5 mM-tris hydrochloride, pH 7.5. This was formed by connecting together the bottoms of two 500 ml. polythene bottles, the first containing 500 ml. of 1M-KCl, the other 500 ml. of 5 mM-tris hydrochloride, pH 7.5. The second bottle was stirred mechanically and the outflow was allowed to drip on to the top of the column. The levels of the solutions in both bottles dropped at the same rate throughout. Fractions (4 ml.) were collected automatically at a flow rate of 40 ml./hr. Under these conditions the carboxylating enzyme was eluted at concentrations of chloride between 0.14 and 0.19M (see Fig. 1). This corresponded to fractions

Fig. 1. Elution of phosphoenolpyruvate carboxylase from the DEAE-cellulose column. The conditions are given in the text. The continuous line A represents the extinction of fractions (vol. 4 ml.) at 280 m μ (1 cm. light-path); ●, carboxylase activity; ▲, gradient of chloride concentration.

52–58, which were combined and the resulting solution (25 ml.) was stored at 2°.

The purification procedure is summarized in Table 1.

Protein estimations. In crude extracts and in steps 1–3 of the purification procedure, protein was determined by the Folin-Ciocalteu method (Lowry, Rosebrough, Farr & Randall, 1951). The protein concentration of the column eluate was determined by measuring the extinction at 260 and 280 m μ (Warburg & Christian, 1941).

Chloride determination. The chloride concentration of the KCl gradient was determined by titration with silver nitrate.

Assay of enzyme activity. (a) Radioactive assay. A portion (0.3 ml.) of enzyme solution was incubated for 30 min. at 30° with 100 μ moles of tris hydrochloride, pH 7.5, 1 μ mole of MgCl₂, 2 μ moles of GSH, 20 μ moles of NaH¹⁴CO₃ (containing 2 μ C of ¹⁴C), 2.5 μ moles of sodium phosphoenolpyruvate, 3 μ moles of NADH and water to 2 ml. After step 3 of the purification it was necessary to add in addition 0.1 ml. of a solution of malate dehydrogenase [containing 375 units by the assay of Ochoa (1955)]. This was prepared by diluting the stock suspension of malate dehydrogenase tenfold with 10 mM-tris hydrochloride, pH 7.5, and dialysing for 2 hr. against 1.5 l. of the same buffer to remove ammonium sulphate. The latter was found to inhibit the carboxylation reaction. The reaction was stopped by the addition of 3 ml. of boiling 95% (v/v) ethanol and 0.1 ml. samples were applied to aluminium planchets (1 in. diameter) and dried in a stream of warm air. The planchets were then irrigated with 0.1 ml. of formic acid (90%, w/v; AnalaR) and dried. Samples so prepared had negligible self-absorption and blank determinations showed that this procedure removes all unchanged [¹⁴C]bicarbonate. The non-volatile radioactive fixation products were then assayed with a gas-flow counter (model D-47, Nuclear Instrument and Chemical Corp., Chicago, U.S.A.) which had an efficiency of 15% under the conditions used. Samples of the tracer solution were diluted 100-fold with 30 mM-NaOH and assayed for radioactivity under the same conditions (omitting the formic acid treatment). The unit of enzyme activity is defined as the amount of enzyme required to fix 1 μ mole of ¹⁴CO₂ into non-volatile products in 30 min.

(b) Spectrophotometric assay. The activity was assayed in a Cary recording spectrophotometer by measuring the rate of oxidation of NADH (decrease in extinction at

Table 1. *Purification of phosphoenolpyruvate carboxylase from Pseudomonas AM 1*

Experimental details are given in the Materials and Methods section.

Step	Fraction	Vol. (ml.)	Activity (units/ml.)	Protein (mg./ml.)	Total units	Total protein (mg.)	Sp. activity (units/mg. of protein)	Purification	Yield (%)
1	Supernatant obtained from crude extract after centrifuging	99	2479	11.4	245 000	1130	217	1.00	100
2	Supernatant after removal of protamine sulphate precipitate by centrifuging	128	1318	5.9	168 700	755	224	1.02	68.9
3	Fraction precipitated by ammonium sulphate (40–50%), after dialysis	11.4	13800	7.9	157 500	90.1	1750	8.06	64.3
4	Selected combined fractions after column chromatography	24.5	3750	0.62	92 000	15.2	6050	27.9	37.6

340 m μ) in the following reaction mixture at 22°. The complete system, contained in 1.5 ml. silica cuvettes (light-path, 1 cm.), consisted of 40 μ moles of tris hydrochloride, pH 8.5, 20 μ moles of NaHCO₃, 0.2 μ mole of NADH, 2 μ moles of MgCl₂, 1 μ mole of sodium phosphoenolpyruvate, 0.03 ml. of the dialysed malate dehydrogenase [113 units by the assay of Ochoa (1955)], 0.05 ml. of enzyme and water to 1 ml. The reaction was started by the addition of phosphoenolpyruvate. One unit was defined as the amount of enzyme required to oxidize 1 μ mole of NADH/min. under these conditions.

Method (a) was used during the enzyme purification procedure, and method (b) for subsequent work with the purified enzyme. Dihydronicotinamide-adenine dinucleotide-oxidase activity precluded the use of method (b) in crude extracts. The oxidase was finally separated from the carboxylating enzyme by the ion-exchange chromatography.

In both assays the measured activity was found to be proportional to the amount of enzyme used.

Estimation of phosphate. Phosphate was determined by the method of Allen (1940).

Estimation of phosphoenolpyruvate. Phosphoenolpyruvate was determined by measuring the decrease in extinction at 340 m μ in the presence of NADH, phosphoenolpyruvate, ADP and lactate dehydrogenase when pyruvate kinase was added (Bucher & Pfeleiderer, 1955). The complete assay system contained, in 3 ml. silica cuvettes: 0.5 μ mole of ADP, 25 μ moles of MgCl₂, 250 μ moles of KCl, 150 μ moles of triethanolamine hydrochloride buffer, pH 7.5, 0.005 ml. of lactate-dehydrogenase suspension, the sample for assay (which contained 0.05–0.15 μ mole of phosphoenolpyruvate) and NADH to give an extinction of 0.55–0.75. The reaction was started by adding 0.005 ml. of pyruvate-kinase suspension, and the decrease in extinction at 340 m μ was measured in a Beckman model DU spectrophotometer. The reaction was complete in 1½ min. under these conditions.

Estimation of pyruvate. This was performed by measuring the decrease in extinction at 340 m μ consequent on the addition of NADH and 0.005 ml. of lactate-dehydrogenase suspension to a sample of the incubation mixture.

RESULTS

Fixation of carbon dioxide in crude extracts

When ultrasonic extracts of freshly harvested methanol-grown *Pseudomonas* AM 1 were incubated with [¹⁴C]bicarbonate in the presence of various substrates, there was a fixation of [¹⁴C]carbon dioxide into non-volatile material (Table 2). The only significant fixation observed under these conditions was in the presence of phosphoenolpyruvate and a boiled cell extract. The major products of this fixation were identified chromatographically as malate and aspartate.

A stimulatory effect on the fixation of [¹⁴C]-carbon dioxide in the presence of phosphoenolpyruvate could also be produced when NADH was substituted for the boiled cell extract. In a reaction mixture which contained 100 μ moles of tris hydrochloride, pH 7.5, 1 μ mole of magnesium chloride, 2 μ moles of reduced glutathione, 20 μ moles of sodium hydrogen [¹⁴C]carbonate (5 μ C of ¹⁴C), ultrasonic extract containing about 2 mg. of protein, 2.5 μ moles of phosphoenolpyruvate, and water to 2 ml., 4.2 μ mole of [¹⁴C]carbon dioxide were fixed into non-volatile ethanol-soluble products after incubation for 30 min. at 30°. The addition of 0.5 ml. of boiled cell extract or 2 μ moles of NADH to the incubation mixture raised the amount of fixation of [¹⁴C]carbon dioxide to 115 and 236 μ mole respectively. The bulk of the radioactivity fixed in the presence of NADH was found to be in malate and fumarate (Table 3). The simplest explanation for these results is that the crude extract can catalyse a carboxylation of phosphoenolpyruvate to oxaloacetate. The oxaloacetate can then be transaminated to aspartate or reduced to malate, or both. The reduction to malate can be

Table 2. *Fixation of [¹⁴C]bicarbonate by crude ultrasonic extracts*

The reaction mixture contained: 50 μ moles of tris hydrochloride, pH 7.5, 1 μ mole of MgCl₂, 2 μ moles of GSH, 16 μ moles of sodium NaH¹⁴CO₃ (20 μ C of ¹⁴C), ultrasonic extract containing 3 mg. of soluble protein, additions as indicated below and water to 1.5 ml. The boiled cell extract was prepared by heating *Pseudomonas* AM 1 (2 g. wet wt.) with 3 ml. of water at 95° for 3 min., the resulting extract was centrifuged and the residue discarded. Incubations were carried out at 30° for 30 min. and terminated by the addition of 3 ml. of boiling ethanol. Samples were assayed for radioactivity after plating on metal planchets, and other samples were analysed by two-dimensional chromatography. These procedures are described in the Materials and Methods section.

Substrate (5 μ moles)	Additions	¹⁴ CO ₂ fixed (μ m-moles)	Percentage of total fixation in				Unidenti- fied
			Malate	Aspartate	Fumarate	Citrate	
Phosphoenolpyruvate	None	66	46	45	6	—	5
Phosphoenolpyruvate	Boiled cell extract (0.5 ml.)	533	53	24	10	5	8
Phosphoenolpyruvate	IDP (2.5 μ moles)	32	37	39	13	—	1
Pyruvate	NADH (2.5 μ moles)	53	79	—	15	—	6
Pyruvate	NADPH (2.5 μ moles)	10	100	—	—	—	—
Pyruvate	ATP (5 μ moles) + acetyl- CoA (0.2 μ mole)	1	—	—	—	—	—
Pyruvate	None	2	—	—	—	—	—
None	None	2	—	—	—	—	—

Table 3. *Products of fixation of [¹⁴C]bicarbonate at different stages of purification*

Assay method (a) was used and the products were chromatographed two-dimensionally, located by radioautography, assayed and identified as described in the Materials and Methods section.

Step of purification	Amount of protein used in incubation (mg.)	¹⁴ CO ₂ fixed in 30 min. (μm-moles)	Percentage of fixed radioactivity in	
			Malate	Fumarate
1	3.42	742	78.7	21.3
3	0.79	1380	79.3	20.7
4*	0.124	653	100	0

* Malate dehydrogenase was added to the reaction mixture at this stage, as described in the Materials and Methods section.

effected by a reduced nicotinamide nucleotide co-enzyme. It seemed probable that the stimulatory effect of the boiled cell extract might be due to its containing reduced nicotinamide nucleotide co-enzymes, since both the addition of the boiled cell extract (Table 2) and NADH (Table 3) resulted in a decrease in the proportion of aspartate and an increase in that of malate and fumarate over that found with the unfortified extract. Fractionation of the enzyme extract was necessary to ascertain whether the reductive carboxylation of the phosphoenolpyruvate was catalysed by one enzyme or, as seemed more likely, by a coupling of two enzymes, e.g.:

The use of a spectrophotometric assay based on measurement of the oxidation of NADH was impracticable during the enzyme purification as the crude extracts rapidly oxidized NADH in the absence of substrate. It was found possible, however, in the presence of a large excess of NADH, to assay the fixation of carbon dioxide by measurement of the NADH- and phosphoenolpyruvate-dependent fixation of [¹⁴C]bicarbonate into non-volatile products as described in the Materials and Methods section. This assay, although tedious, was adopted for the whole of the purification procedure.

Properties of the purified enzyme

Nature of the enzyme-catalysed reaction. The crude enzyme extract catalysed a reductive carboxylation of phosphoenolpyruvate in the presence of NADH. As the enzyme purification proceeded, it was necessary to add malate dehydrogenase to the assay mixture. With the purified enzyme, when malate dehydrogenase was omitted from reaction mixture (b), described in the Materials and Methods section, there was only a slow oxidation of NADH on the addition of phosphoenolpyruvate. When malate dehydrogenase was added to this system,

Fig. 2. Effect of the addition of malate dehydrogenase to the purified phosphoenolpyruvate carboxylase. The first arrow represents the addition of 1 μmole of phosphoenolpyruvate, the second the addition of malate dehydrogenase. The reaction mixture contained carboxylase (31 μg.), 40 μmoles of tris hydrochloride, pH 8.5, 20 μmoles of NaHCO₃, 0.2 μmole of NADH and 2 μmoles of MgCl₂; the total volume was 1 ml. in 1.5 ml. silica cuvettes (light-path 1 cm.). (Taken from a trace by a Cary recording spectrophotometer.)

there was a stimulation in the rate of oxidation of NADH. The extent of this stimulation varied with the pH. At pH 7.0, the stimulation of rate was 1.7-fold, at pH 7.5 2.4-fold, and at pH 8.5 4.8-fold (Fig. 2). A very rapid oxidation of NADH was observed immediately on adding malate dehydrogenase, but the oxidation rate then decreased and settled to a steady lower rate. These results suggest that the purified enzyme is a phosphoenolpyruvate

carboxylase, catalysing reaction (1), and contaminated with a small amount of malate dehydrogenase. The initially very rapid oxidation of NADH, followed by a slower steady rate, is consistent with the rapid initial removal of accumulated oxaloacetate by the added excess of malate dehydrogenase, followed by its removal at the same rate as its formation by the carboxylase, i.e. a measure of the rate of carboxylation. The characterization of the enzyme as a phosphoenolpyruvate carboxylase was confirmed by the following experiments.

Dependence of the reaction on phosphoenolpyruvate and carbon dioxide. The reaction was absolutely dependent on the presence of phosphoenolpyruvate. None of the following compounds could serve as substrate when tested at 1 mM concentration in assay system (b): sodium 3-phosphoglycerate, sodium pyruvate, sodium pyruvate plus ATP (1 mM), L- α -glycerophosphate, lithium hydroxypyruvate, sodium DL-glycerate, DL-serine and potassium DL-lactate.

It was difficult to demonstrate a complete dependence on carbon dioxide. At pH 8.5, with assay system (b), the reaction proceeded at maximal velocity even in the absence of added sodium hydrogen carbonate. However, if the buffer, magnesium chloride and phosphoenolpyruvate solutions were prepared from carbon dioxide-free water, the reaction proceeded at only 60 % of the previous rate. The original rate was completely restored on the addition of sodium bicarbonate. Radioactive carbon from [^{14}C]bicarbonate was incorporated into the reaction products.

Identification of reaction products. When phosphoenolpyruvate was carboxylated in the presence of the enzyme, the liberation of orthophosphate could be detected by inorganic phosphate estimation (Fig. 3). From the initial slope of this curve the specific activity of the enzyme at pH 8.5 and 30° was calculated to be 0.338 μmole of inorganic phosphate liberated/min./mg. of protein. This can be compared with a corresponding spectrophotometric assay of 0.386 μmole of NADH oxidized/min./mg. of protein at pH 8.5 and 22°.

Difficulty was encountered in establishing oxaloacetate as a reaction product owing to its rapid decarboxylation in the reaction mixture. As this decarboxylation was catalysed by the Mg^{2+} ions, the metal-ion concentration was reduced to the minimum necessary for appreciable enzymic reaction (0.5 mM). The reaction was then performed in the following complete reaction mixture: 100 μmoles of tris hydrochloride, pH 8.5, 2.5 μmoles of phosphoenolpyruvate, 0.5 μmole of magnesium chloride, 8 μmoles of sodium hydrogen [^{14}C]carbonate, 62 $\mu\text{g.}$ of enzyme and water to 1 ml. After incubation at 30° for 30 min. 10 μmoles of carrier oxaloacetic acid were added to each tube,

followed by 2 ml. of 0.1 % (w/v) 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid. After standing for 15 min. at 30°, the hydrazones were extracted into 2 ml. of ethyl acetate and samples (0.1 ml.) were chromatographed (see the Materials and Methods section). The radioactivity in the spots of the 2,4-dinitrophenylhydrazones of oxaloacetic acid and pyruvic acid was assayed directly. The dinitrophenylhydrazone of oxaloacetic acid derived from the complete reaction mixture contained 1142 counts/min., whereas that of pyruvic acid (derived from oxaloacetic acid by decarboxylation) was not radioactive. This shows that the phosphoenolpyruvate was being carboxylated at the β -position to give oxaloacetate. No evidence could be found for an enzyme-catalysed exchange of [^{14}C]carbon dioxide with the carboxyl groups of oxaloacetic acid in the absence of phosphoenolpyruvate since no incorporation of radioactivity into the dinitrophenylhydrazone of oxaloacetic acid was observed when 10 μmoles of oxaloacetic acid were substituted for phosphoenolpyruvate in the reaction mixture.

Stoichiometry of the reaction. The changes in concentration of phosphoenolpyruvate, orthophosphate, pyruvate and NADH observed when the carboxylase was coupled with malate dehydrogenase are shown in Table 4. They demonstrate that the disappearance of 1 mole of phosphoenolpyruvate is accompanied by the appearance of 1 mole of orthophosphate and 1 mole of NAD^+ . Thus the coupled reaction can be represented as the sum of reactions (1) and (2).

Metal-ion activation. The reaction was absolutely dependent on the presence of bivalent metal ions, Mg^{2+} ions being the most effective activator; of the other bivalent cations tested, only Mn^{2+} ions showed significant activity (Table 5).

Fig. 3. Formation of inorganic phosphate by phosphoenolpyruvate carboxylase. The system contained 50 μmoles of tris hydrochloride, pH 8.5, 2.5 μmoles of sodium phosphoenolpyruvate, 50 μmoles of NaHCO_3 , 2 μmoles of MgCl_2 and 62 $\mu\text{g.}$ of enzyme; the total volume was 1 ml. The temperature was 30°.

Table 4. *Stoichiometry of the carboxylation of phosphoenolpyruvate*

The complete reaction mixture contained: 40 μ moles of tris hydrochloride, pH 8.5, 20 μ moles of NaHCO_3 , 3 μ moles of NADH, 2 μ moles of MgCl_2 , 1 μ mole of phosphoenolpyruvate, 0.03 ml. of malate dehydrogenase, 31 μ g. of phosphoenolpyruvate carboxylase, water to 1 ml. The mixture was incubated for 90 min. at 30°, and the reaction terminated by heating for 3 min. at 70°. NADH was assayed by the change of extinction at 340 m μ . Other reactants were assayed as described in the Materials and Methods section.

Omissions	Change in concn. of reactant (μ mole/ml.)			
	NADH	Phosphoenolpyruvate	Ortho-phosphate	Pyruvate
None	-0.674	-0.655	+0.646	+0.019
Phosphoenolpyruvate	-0.172	0	0	—
Malate dehydrogenase	-0.487	-0.662	+0.621	+0.072
Boiled enzyme used	-0.122	-0.030	0	—

Table 5. *Effect of metal ions on the rate of the carboxylation reaction*

The reaction mixture contained, in 1.5 ml. silica cuvettes (1 cm. light-path): 1 μ mole of phosphoenolpyruvate, 31 μ g. of purified enzyme, 40 μ moles of tris hydrochloride, pH 8.5, 20 μ moles of NaHCO_3 , 0.2 μ mole of NADH, 0.03 ml. (113 units) of malate dehydrogenase and water to 1 ml. The reaction was started by the addition of 2 μ moles of the metal salt shown below.

Addition	Relative velocity
MgCl_2	100*
MnCl_2	34
CaCl_2	0
SrCl_2	1.75
BaCl_2	1.75
CoCl_2	0
ZnCl_2	0
MgCl_2 + EDTA (2.5 μ moles)	18.6

* This corresponded to 11.8 μ m-moles of NADH oxidized/min.

Inhibition of the reaction. The following compounds at a concentration of 1 mM did not inhibit the carboxylase: sodium iodoacetate, sodium 3-phosphoglycerate, L- α -glycerophosphate, lithium hydroxypyruvate, sodium DL-glycerate, DL-serine, potassium DL-lactate, sodium pyruvate, sodium pyruvate plus 1 mM-ATP, and potassium fluoride (10 mM). The only substances which inhibited the reaction were phosphate and ammonium ions. Both these effects had been noted in crude extracts, and for this reason tris hydrochloride buffers were always used; also, dialysis of the ammonium sulphate precipitate at step 3 of the purification, and dialysis of the malate-dehydrogenase preparation used in the assay of the purified enzyme, were necessary. The effect of the presence of these inhibitors on the rate of the reaction at different phosphoenolpyruvate concentrations was studied. As seen in Fig. 4, the inhibition by 40 mM-phosphate is competitive, whereas the inhibition

Fig. 4. Lineweaver-Burk plot for the carboxylation reaction. The left-hand ordinate refers to assay method (a), the right-hand ordinate to assay method (b). ○, Assay method (a) at pH 7.5 and 30°; ●, assay method (b) at pH 8.5 and 22°; ▲, assay method (b) at pH 8.5 and 22° in the presence of 40 mM-phosphate, pH 8.5; ■, assay method (b) at pH 8.5 and 22° in the presence of 5 mM-ammonium chloride.

by 5 mM-ammonium chloride is non-competitive. The effect with phosphate may be due to competition with the enzyme for Mg^{2+} ions.

Stability and pH optimum. The purified enzyme was stable in 5 mM-tris hydrochloride, pH 7.5, for at least a month at 2°, but was completely inactivated after storage at -15° for 14 days. The activity was completely destroyed in 2 min. at 50°. The enzyme showed a sharp maximum in activity at pH 8.5 (Fig. 5).

Contaminating enzymes. The carboxylase was free of lactate-dehydrogenase and glycerate-dehydrogenase activities as assayed spectrophotometrically, but contained a small amount of malate-dehydrogenase activity. This was measured by omitting added malate dehydrogenase from assay method (b) and replacing phosphoenolpyruvate

Fig. 5. Effect of pH on the specific activity of phosphoenolpyruvate carboxylase.

with oxaloacetate. The specific activity of the contaminating malate dehydrogenase under these conditions was 1.93 μ moles/hr./mg. of protein at pH 7.5 and 2.86 μ moles/hr./mg. of protein at pH 8.5. This exactly accounted for the slight phosphoenolpyruvate-dependent oxidation of NADH observed in the absence of added malate dehydrogenase (Fig. 2). The carboxylase was also free of fumarase. This is evident from Table 3, where the sole product of the reaction with the purified enzyme is malate, whereas at steps 1 and 3 of the purification appreciable amounts of fumarate were also present.

K_m values. The K_m for phosphoenolpyruvate was 0.33 mM at 22° and pH 8.5, with the use of assay method (b) for the determination of reaction rate, and 0.44 mM at 30° and pH 7.5, with assay method (a) (Fig. 4).

The K_m for $MgCl_2$ was found to be 0.196 mM at 22° and pH 8.5, with assay method (b).

In all cases the K_m was determined from a Lineweaver-Burk plot of the reciprocal of reaction velocity against the reciprocal of substrate concentration (Lineweaver & Burk, 1934).

Effect of nucleoside diphosphates. The presence of GDP (1 mM) was without effect on the activity of the carboxylase, and the presence of ADP (1 mM) caused a slight inhibition.

DISCUSSION

The following five enzyme systems are known which catalyse the carboxylation of pyruvate or phosphoenolpyruvate to oxaloacetate or malate:

catalysed by malic enzyme (Ochoa, Mehler & Kornberg, 1948).

where Nu = adenosine, inosine or guanosine, catalysed by phosphoenolpyruvate carboxykinase (Utter & Kurahashi, 1954).

catalysed by phosphoenolpyruvate carboxylase (Bandurski & Greiner, 1953).

catalysed by phosphoenolpyruvate carboxytransphosphorylase (Siu, Wood & Stjernholm, 1961).

catalysed by pyruvate carboxykinase (Utter & Keech, 1960).

An examination of the ability of the cell-free extracts of methanol-grown *Pseudomonas* AM 1 to catalyse carboxylations of the above types indicates that only carboxylation of phosphoenolpyruvate occurs to an appreciable extent (Table 2). Fractionation of the enzyme system responsible shows it to be a phosphoenolpyruvate carboxylase, as in eqn. (5). The absence of malic enzyme, as indicated by the tracer assay, was also indicated by assay involving spectrophotometric measurement of the reduction of nicotinamide nucleotide coenzymes (Ochoa *et al.* 1948). The absence of phosphoenolpyruvate carboxykinase, as suggested by the assay in Table 2, was also indicated by an assay involving incorporation of [^{14}C]carbon dioxide into a pool of oxaloacetate in an exchange reaction (Utter & Kurahashi, 1955). Hence, as has been found in the autotrophic bacterium *Thiobacillus thio-oxidans* (Suzuki & Werkman, 1957, 1958), phosphoenolpyruvate carboxylase appears to play the major role in formation of C_4 dicarboxylic acids, such as oxaloacetate or malate, from C_3 substrates.

Phosphoenolpyruvate carboxylase has been studied extensively in plants. Bandurski (1955) purified the enzyme tenfold from acetone-dried spinach-leaf powder. Tchen & Vennesland (1955) and Walker (1957) studied the enzyme in relatively crude preparations from wheat germ and succulent plants respectively. Other studies in plants have demonstrated similar systems: in succulent plants (Saltman, Kunitake, Spolter & Stitts, 1956), barley roots (Young & Graham, 1958) and spinach chloroplasts (Rosenberg, Capindale & Whatley, 1958). In bacteria, the only detailed report of the occurrence of the enzyme is in the autotroph

T. thio-oxidans (Suzuki & Werkman, 1957, 1958), although the enzyme may well play a role in the incorporation of carbon dioxide into aspartate and oxaloacetate by *Staphylococcus aureus* (Hancock & McManus, 1960) and the minute streptococci (Martin & Niven, 1960). A report that the enzyme occurs in *Propionibacterium shermanii* (Pomerantz, 1958) may need reinvestigation in view of the subsequent demonstration of phosphoenolpyruvate carboxytransphosphorylase (Siu *et al.* 1961) in the same organism.

The present work reveals many similarities in the properties of the enzyme purified from *Pseudomonas* AM 1 with those of the plant enzyme, e.g. K_m values for phosphoenolpyruvate: 0.33 mM for the *Pseudomonas* enzyme, 0.5 mM for the spinach enzyme (Bandurski, 1955), 0.19 mM for the preparation from succulent plants (Walker, 1957). As with the enzyme from spinach, there is a requirement for bivalent cations, of which Mg^{2+} ions are the most active. The other enzyme preparations from plants and *T. thio-oxidans* were equally activated by either Mg^{2+} or Mn^{2+} ions. The enzyme from *Pseudomonas* AM 1 differs from the enzymes obtained from wheat germ and succulent plants in being inhibited, instead of stimulated, by low concentrations of phosphate. The sharp pH optimum of the reaction observed in the present work may be contrasted with the broad optimum over pH 7–9 (Bandurski, 1955) or that at pH 7 (Walker, 1957).

SUMMARY

1. Cell-free extracts of methanol-grown *Pseudomonas* AM 1 have been examined for the presence of enzymes catalysing the carboxylation of pyruvate or phosphoenolpyruvate to C_4 dicarboxylic acids.

2. The only enzyme system found in appreciable activity was a phosphoenolpyruvate carboxylase, similar to that found in plant tissues.

3. The enzyme has been purified 28-fold by protamine sulphate treatment, ammonium sulphate precipitation and diethylaminoethylcellulose column chromatography.

4. The purified phosphoenolpyruvate carboxylase catalyses the formation of oxaloacetate and inorganic phosphate from phosphoenolpyruvate and bicarbonate. The reaction is Mg^{2+} ion-dependent, with a pH optimum of 8.5. It is competitively inhibited by phosphate and non-competitively inhibited by ammonium ions. K_m values of 0.33 mM and 0.196 mM respectively have been found for phosphoenolpyruvate and magnesium chloride at pH 8.5 and 22°.

5. The properties of the enzyme are compared with those of similar enzymes obtained from other sources.

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