Carbon Assimilation by *Pseudomonas oxalaticus* (OX 1)

2. FORMATE AND CARBON DIOXIDE UTILIZATION BY CELL-FREE EXTRACTS OF THE ORGANISM GROWN ON FORMATE*

By J. R. QUAYLE AND D. B. KEECH

*Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry,
University of Oxford*

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Isotopic experiments performed on cultures of *Pseudomonas oxalaticus* (OX 1) growing on formate as sole carbon and energy source (Quayle & Keech, 1958, 1959) indicated the existence in the organism of a carbon dioxide-fixation cycle similar to that found in photosynthetic tissue (Bassham *et al.* 1954) and autotrophic bacteria (Santer & Vishniac, 1955; Trudinger, 1955).

An investigation of the ability of cell-free extracts of *P. oxalaticus* (OX 1) to incorporate formate carbon and carbon dioxide into 3-phosphoglyceric acid and certain C_4 acids is reported in this paper.

METHODS AND MATERIALS

Maintenance and growth of the organism. The maintenance and growth of stock cultures of *Pseudomonas oxalaticus* (OX 1) have been described (Quayle & Keech, 1959). Large quantities of the organism were grown in 40 l. of medium inoculated with 800 ml. of a bacterial suspension in the logarithmic phase of growth. The growth vessel used was a modified stainless-steel container of a commercial washing machine (Hunt, Rogers & Hughes, 1959). Growth was maintained at 30° under conditions of vigorous aeration, formic acid (90%, w/v; A.R.) being added to keep the pH at 7.5–8.5. After 42 hr. the bacteria (31 g. wet wt.) were harvested in a Sharples centrifuge and stored at –10°.

Preparation of cell-free extracts. The harvested cells (0.5–2.0 g. wet wt.) were suspended in 2 vol. of mm-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7.8, containing about 200 μ g. each of crystalline ribonuclease and deoxyribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks). Extracts were then prepared by crushing the frozen suspension in a Hughes press (Hughes, 1951), at –20°. The crushed material was homogenized with a glass homogenizer and the resulting extract was used for some experiments. More frequently, the supernatant fraction obtained from the homogenate by centrifuging at 15 000 g for 20 min. in a refrigerated angle-head centrifuge was used. This fraction always contained 12–20 mg. of protein/ml.

Experiments with radioactive fixation. The standard-assay mixture for measuring isotope incorporation into 3-phosphoglyceric acid (PGA) from [14 C]bicarbonate or [14 C]-formate contained: 5 μ moles of tris buffer, pH 7.8, 2.5 μ -

moles of $MgCl_2$, 0.5 μ mole of ribulose 1:5-diphosphate, 2.5 μ moles of reduced glutathione (GSH), 0.5–2.5 μ moles of tracer, 0.05 ml. of bacterial extract and water to a final volume of 0.35 ml. The reaction was allowed to continue for 15 min. at 30°.

Fixation under anaerobic conditions was studied in a type of micro-Thunberg tube (Fig. 1). The tubes were blown from 6 mm. glass tubing and the appropriate solutions introduced into the bulbs with a hypodermic syringe. The tubes were evacuated to a pressure of 14 mm. Hg with a water pump and refilled with O_2 -free N_2 , the procedure being repeated six times in succession. The tubes were finally evacuated and sealed with a coal gas- O_2 hand blow-pipe. The solutions were mixed by tipping and incubation was at 30°. The reaction was terminated by plunging the tubes into liquid air, and by opening the tubes while still frozen, pipetting in 1 ml. of ethanol and transferring the tubes to a boiling-water bath for 15 sec. The contents were then taken out of the tubes and treated similarly to the products of the aerobic experiments.

Measurement of radioactivity and analysis of labelled compounds. Direct measurement of the total radioactivity incorporated by bacterial extracts into non-volatile compounds was made by stopping the enzymic reaction with 3 vol. of hot ethanol. Samples (up to 0.25 ml.) of the reaction mixture were then plated in a stream of warm air on to glass planchets mounted on a rotating turntable. The plates were irrigated with 0.2 ml. of formic acid (90%, w/v; A.R.), and dried and counted with an end-window, lead-shielded G.M. tube (General Electric Co. Ltd.)

Fig. 1. Micro-Thunberg tube. After filling, the tubes were sealed off at the position indicated by the arrow.

* Part 1. Quayle & Keech (1959).

Samples of the ethanolic extracts of the reaction mixture were centrifuged and the residues washed with 0.2 ml. of hot aqueous 20% (v/v) ethanol. The combined supernatant fractions were evaporated to dryness at 60° under N_2 and redissolved in aqueous 20% (w/v) ethanol ready for application to the origin position of paper chromatograms. The resulting chromatographic analysis, radioautography and identification of labelled compounds followed the procedures previously described (Quayle & Keech, 1959). An independent measurement was made of the relation between the count of a radioactive sample on a glass planchet and on paper after chromatography. Under the conditions used, the planchet count was 1.5 times as high as the count obtained *in situ* after chromatography.

The formation of radioactive oxaloacetate in a reaction mixture was demonstrated by the addition of 5 μ moles of carrier oxaloacetate immediately before adding 0.5 ml. of 2:4-dinitrophenylhydrazine (0.1% in 2*N*-HCl). The mixture was incubated at 30° for 15 min. and the resulting hydrazone extracted into 2 ml. of ethyl acetate; it was extracted from the ethyl acetate with 1 ml. of 2*M*- Na_2CO_3 , acidified with HCl and re-extracted with 2 ml. of ethyl acetate. A sample of this solution was chromatographed in the solvent system of El Hawary & Thompson (1953).

Preparation of tracer solutions and manometric methods. The procedures used have been described previously (Quayle & Keech, 1959).

Chemicals. A sample of ribulose 1:5-diphosphate, used in the initial experiments, was a gift from Dr J. A. Bassham (Radiation Laboratory, University of California, Berkeley 4). The larger quantities subsequently used were prepared in this Laboratory from ribose 5-phosphate and adenosine triphosphate (ATP) (both chemicals from Schwarz Laboratories Inc., New York, U.S.A.), purified phosphoriboisomerase and phosphoribulokinase prepared from spinach according to the method of Horecker, Hurwitz & Weissbach (1956) being used. Glycic acid was a gift from Dr P. W. Kent. Pyridine nucleotides were purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. Tetrahydrofolic acid was a gift from Dr R. Kisluk. Sedoheptulose was a gift from Dr A. A. Benson (Department of Agricultural

and Biological Chemistry, Pennsylvania State University, University Park, Pennsylvania).

Protein determinations. Protein was estimated by the method of Folin & Ciocalteu (1927).

RESULTS

Fixation of [^{14}C]bicarbonate by the cell-free supernatant fraction

Ribulose 1:5-diphosphate-dependent carboxylation. [^{14}C]Bicarbonate fixation into phosphorylated compounds by the cell-free supernatant fraction of extracts of *P. oxalaticus* was found to be dependent upon the addition of ribulose 1:5-diphosphate to the reaction mixture (Table 1). This activity was not removed from the extract by centrifuging for 30 min. at 100 000 *g* (Table 7). The requirement for ribulose 1:5-diphosphate as a substrate for the carboxylation reaction could be met by the addition of ribose 5-phosphate plus ATP to the reaction mixture (Table 2).

The observed fixation of [^{14}C]bicarbonate into ribulose 1:5-diphosphate was enhanced by the presence of $MgCl_2$ and GSH. Substitution of phosphate buffer for tris buffer reduced the amount of fixation to less than one-half of the previous activity. The carboxylating enzyme showed considerable sensitivity to the pH of the medium (Table 3), maximum incorporation being observed at pH 8.0. Increase in ribulose 1:5-diphosphate concentration beyond 1.4 mM, under the experimental conditions used, proved inhibitory, although at this concentration fixation was proportional to both the protein and [^{14}C]bicarbonate concentrations (Table 4). The rate of the ribulose 1:5-diphosphate-dependent carboxylation was linear over 15 min. (Fig. 2).

Table 1. *Effect of various factors on the fixation of [^{14}C]bicarbonate in the presence of ribulose 1:5-diphosphate*

The complete system contained 0.42 μ mole of sodium bicarbonate (10 μ C of ^{14}C), 0.05 ml. of *P. oxalaticus* extract (supernatant fraction), compounds added as indicated and water to 0.35 ml. Incubation was at room temperature for 15 min. and the reaction was stopped with 3 vol. of hot ethanol. Samples (0.1 ml.) were plated on glass planchets, and treated with formic acid, dried and the radioactivity was counted. Different bacterial extracts were used for Expts. 1 and 2.

Expt.	Additions	Total radio-activity fixed (counts/min.)
1	Tris buffer (pH 7.8; 5 μ moles) + ribulose 1:5-diphosphate (0.5 μ mole) + $MgCl_2$ (2.5 μ moles) + GSH (2.5 μ moles)	5 770
	Tris buffer (pH 7.8; 5 μ moles) + ribulose 1:5-diphosphate (0.5 μ mole) + $MgCl_2$ (2.5 μ moles)	4 090
	Tris buffer (pH 7.8; 5 μ moles) + $MgCl_2$ (2.5 μ moles) + GSH (2.5 μ moles)	20
2	Tris buffer (pH 7.8; 5 μ moles) + ribulose 1:5-diphosphate (0.5 μ mole) + $MgCl_2$ (2.5 μ moles) + GSH (2.5 μ moles)	12 300
	Tris buffer (pH 7.8; 5 μ moles) + ribulose 1:5-diphosphate (0.5 μ mole) + GSH (2.5 μ moles)	9 330
	Phosphate buffer (pH 7.8; 5 μ moles) + ribulose 1:5-diphosphate (0.5 μ mole) + $MgCl_2$ (2.5 μ moles) + GSH (2.5 μ moles)	4 380

Table 2. Fixation of [^{14}C]bicarbonate in the presence of ribose 5-phosphate and adenosine triphosphate

The complete system contained 0.05 ml. of bacterial extract (supernatant), 5 μmoles of tris buffer (pH 7.8), 2.5 μmoles of MgCl_2 , 2 μmoles of GSH, 1.25 μmoles of $\text{NaH}^{14}\text{CO}_3$ (containing 10 μC of ^{14}C), additions as indicated and water to 0.35 ml. Incubation was at 30° for 15 min. and the reaction was stopped with 3 vol. of hot ethanol. Resulting precipitates were washed with 0.2 ml. of ethanol (20%, v/v); half quantities of the resulting combined supernatants were chromatographed and the radioactivity incorporated into the products was counted.

Additions	Total radioactivity fixed (counts/min.)
None	20
ATP (4 μmoles)	590
Ribose 5-phosphate (2 μmoles) + ATP (4 μmoles)	3280

Table 3. Effect of pH on the fixation of [^{14}C]bicarbonate in the presence of ribulose 1:5-diphosphate

The complete system contained 0.42 μmole of sodium bicarbonate (10 μC of ^{14}C), 0.05 ml. of *P. oxalaticus* extract (supernatant fraction; 25.9 mg. of protein/ml.), 5 μmoles of tris buffer, 0.5 μmole of ribulose 1:5-diphosphate, 2.5 μmoles of MgCl_2 , 2.5 μmoles of GSH and water to 0.35 ml. Incubation was at room temperature for 15 min. and the reaction was stopped with 3 vol. of hot ethanol. The radioactivity in 0.1 ml. samples was assayed as described in Table 1.

pH	Total radioactivity fixed (counts/min.)
7.6	4360
7.8	7320
8.0	8070
8.2	6360
8.4	5920

Chromatographic analysis, followed by radioautography, of the products of the incubation showed that 80–90% of the radioactivity was present as PGA (Table 5). Alanine, aspartic acid, serine, malic acid and glyceric acid contained most of the remaining radioactivity.

No stimulation of ribulose 1:5-diphosphate-carboxylating activity in resin-treated extract (Dowex-1, chloride form; 200–400 mesh), by addition of diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) in either

Fig. 2. Incorporation of [^{14}C]bicarbonate into PGA in the presence of ribulose 1:5-diphosphate. The incubation mixture was the same as that in Table 7, 0.45 μmole of $\text{NaH}^{14}\text{CO}_3$ (10 μC of ^{14}C) being used as tracer. Incubation and assay for radioactivity were as described in Table 1.

Table 4. Dependence of fixation of [^{14}C]bicarbonate on ribulose 1:5-diphosphate, bicarbonate and enzyme concentrations

The complete systems contained 5 μmoles of tris buffer (pH 7.8), 2.5 μmoles of MgCl_2 , 2.5 μmoles of GSH, additions as indicated and water to 0.35 ml. The supernatant fraction of the *P. oxalaticus* extract contained 17.9 mg. of protein/ml. Incubation was at room temperature for 15 min. and treatment was as described in Table 1.

Expt.	Additions		Total radioactivity fixed (counts/min.)
	Added to each tube	Variable addition	
1	$\text{NaH}^{14}\text{CO}_3$ (0.4 μmole , 10 μC) + supernatant (0.05 ml.)	Ribulose 1:5-diphosphate { (0.2 μmole) (0.5 μmole) (1.0 μmole)	2440 3130 2170
2	Supernatant (0.05 ml.) + ribulose 1:5-diphosphate (0.5 μmole)	$\text{NaH}^{14}\text{CO}_3$ { 0.2 μmole , 5 μC 0.4 μmole , 10 μC 0.8 μmole , 20 μC	1460 3130 6241
3	Ribulose 1:5-diphosphate (0.5 μmole) + $\text{NaH}^{14}\text{CO}_3$ (0.4 μmole , 10 μC)	Supernatant { 0.025 ml. 0.050 ml. 0.100 ml.	1307 3130 5200

oxidized or reduced forms, ATP, tetrahydrofolic acid, biotin, pyridoxal phosphate, coenzyme A or cyanocobalamin, singly or together, was observed.

The formation of PGA from [^{14}C]bicarbonate and ribulose 1:5-diphosphate in the extract shows the presence in this organism of an enzyme similar to the carboxydismutase found in photosynthetic tissue (Weissbach, Smyrniotis & Horecker, 1954; Quayle, Fuller, Benson & Calvin, 1954) and autotrophic bacteria (Santer & Vishniac, 1955; Trudinger, 1955). The increased incorporation of radioactivity observed in the presence of GSH and magnesium chloride, pH optimum and the inhibition observed in the presence of inorganic phosphate or excess of ribulose 1:5-diphosphate are all

properties possessed by purified spinach carboxydismutase (Weissbach, Horecker & Hurwitz, 1956; Jakoby, Brummond & Ochoa, 1956).

Fixation of [^{14}C]bicarbonate in the presence of pyruvate and phosphopyruvate. The existence of a 'malic enzyme' (Ochoa, Mehler & Kornberg, 1948) catalysing the reductive carboxylation of pyruvate

was suggested by the fixation of [^{14}C]bicarbonate into malic acid in the presence of pyruvate and either reduced DPN (DPNH) or reduced TPN (TPNH) (Table 6, Expt. 1); the latter coenzyme was more effective than the former. Malic acid contained over 90% of the radioactivity fixed.

In the presence of phosphopyruvate incorporation of [^{14}C]bicarbonate into malate, citrate, aspartate and oxaloacetate (Table 6, Expts. 2 and 3) was observed. The appearance of malate and oxaloacetate as reaction products indicated the presence of a phosphopyruvate carboxylase (Bandurski & Greiner, 1953) catalysing the irreversible carboxylation of phosphopyruvate

or a nucleoside diphosphate-dependent oxaloacetate carboxylase or both (Utter & Kurahashi, 1954): phosphopyruvate + CO_2 + inosine diphosphate $\rightleftharpoons$ oxaloacetate + inosine triphosphate. The stimulation of fixation into oxaloacetate after the addition of inosine diphosphate (Table 6, Expt. 3) is evidence for the existence of the last-named enzyme.

Table 5. *Products of fixation of [^{14}C]bicarbonate in the presence of ribulose 1:5-diphosphate*

The incubation mixture was similar to that described in Table 3; 5 μmoles of tris buffer (pH 7.8) was used. The reaction products were analysed chromatographically as in Table 2.

Reaction product	Radioactivity incorporated (counts/min.)	Percentage of total non-volatile radioactivity incorporated into mixture
PGA	17 500	80
Aspartate	130	<1
Serine	880	4
Malate	820	4
Glycerate	353	2
Alanine	1 428	6.5
Unknown compounds	670	3

Table 6. *Fixation of [^{14}C]bicarbonate in the presence of pyruvate and phosphopyruvate*

Micromanometer cups, flushed with $\text{N}_2 + \text{CO}_2$ (95:5), were used. The main compartments contained 20 μmoles of tris buffer (pH 7.4), 3 μmoles of MgCl_2 , 5 μmoles of GSH, 11 μmoles of $\text{NaH}^{14}\text{CO}_3$ (20 μc) and additions as indicated; the side arms contained 0.05 ml. of bacterial extract (homogenate) and water to bring total volume in the flasks to 0.7 ml. Flasks were tipped after equilibration for 2 min. and incubated at 30° for 45 min. In Expts. 1 and 2 the reaction was stopped by adding 2 ml. of hot ethanol; the resulting precipitates were washed with ethanol (20%, v/v), and the contents of the combined supernatants were chromatographed and the products assayed for radioactivity as described in Table 2. In Expt. 3, 5 μmoles of oxaloacetate was added before stopping the reaction with acid 2:4-dinitrophenylhydrazine solution. Resulting hydrazones were chromatographed and assayed for radioactivity as described above.

Expt.	Additions	Radioactivity (counts/min.)			
		Malate	Citrate	Aspartate	Oxaloacetate 2:4-dinitrophenylhydrazone
1	Pyruvate (4 μmoles)	0	0	0	—
	Pyruvate (4 μmoles) + DPNH (10 μmoles)	72	0	0	—
	Pyruvate (4 μmoles) + TPNH (10 μmoles)	1340	0	0	—
2	None	0	0	0	—
	Phosphopyruvate (5 μmoles)	5700	1000	2200	—
	Phosphopyruvate (5 μmoles) + inosine diphosphate (3.5 μmoles)	4050	720	2390	—
3	None	—	—	—	0
	Phosphopyruvate (5 μmoles)	—	—	—	1430
	Phosphopyruvate (5 μmoles) + inosine diphosphate (3.5 μmoles)	—	—	—	3190

The fact that citrate and aspartate appeared in Expt. 2 and not in Expt. 1 indicates that, under the experimental conditions, i.e. with DPNH or TPNH added, no appreciable amounts of oxaloacetate are formed.

Fixation of [^{14}C]formate in the presence of ribulose 1:5-diphosphate

Fixation of formate carbon into phosphorylated compounds by the cell-free supernatant fraction, as with [^{14}C]bicarbonate fixation, was found to be dependent on the addition of ribulose 1:5-diphosphate (Table 7). Analysis of the reaction products by chromatography and radioautography revealed a pattern of labelled compounds similar to that obtained with [^{14}C]bicarbonate as tracer; 80–90 % of the radioactivity appeared in PGA. No stimulation of this activity was observed in resin-treated extract (Dowex 1, chloride form; 200–400 mesh) by addition of the same cofactors as were tested in the carboxylation reaction.

It was important to know whether this incorporation of formate carbon depended upon either a prior transformation of the formate to carbon dioxide (followed by a carboxylation of ribulose 1:5-diphosphate, as demonstrated in the previous

experiments) or the formation of a C_1 unit, different from carbon dioxide, which was involved in PGA production from ribulose 1:5-diphosphate. The following experiments were performed to distinguish between these possibilities.

Removal of [^{14}C]formate-fixing activity by centrifuging. The ability to incorporate formate carbon was completely removed by centrifuging the extract at 100 000 g for 30 min. (Table 7). Recombination of the particulate fraction (14 000–100 000 g) with the supernatant fraction restored the capacity of the system to incorporate [^{14}C]formate to 65 % of the original value in the whole extract. The particulate fraction possessed the ability to incorporate carbon both from [^{14}C]formate and [^{14}C]bicarbonate in the presence of ribulose 1:5-diphosphate (Table 8). However, the incorporation from both substrates was reduced by over 75 % after washing it twice in small volumes of tris buffer. Recombination of the washed particles with the supernatant fraction restored the [^{14}C]formate-fixing activity.

Oxidation of formate by the particulate fraction. The cell-free extract rapidly oxidized formate, and in a Warburg manometer 98 % of the theoretical oxygen uptake was observed (Table 9). After

Table 7. *Separation by centrifuging of [^{14}C]formate- and [^{14}C]bicarbonate-fixing activities*

The reaction mixture contained 5 μmoles of tris buffer (pH 7.8), 2.5 μmoles of MgCl_2 , 2.5 μmoles of GSH, 0.05 ml. of extract, 0.2 μmole of ribulose 1:5-diphosphate, tracer as indicated and water to 0.35 ml. Incubation was at 30° for 15 min. Radioactivity incorporated was assayed as described in Table 1.

Omissions	Centrifugal interval ($10^{-3} \times$ relative centrifugal force) (g)	Fraction	Total radioactivity (counts/min.) incorporated/mg. of protein	
			$\text{NaH}^{14}\text{CO}_3$ (1.2 μmoles , 5 μC)	$\text{H}^{14}\text{CO}_3\text{Na}$ (1.2 μmoles , 5 μC)
None	—	Whole extract	4000	6400
Ribulose 1:5-diphosphate	—	Whole extract	330	300
None	0–14	Supernatant	5270	2140
None	14–100	Supernatant	5650	380
Ribulose 1:5-diphosphate	14–100	Supernatant	—	310
None	14–100	Supernatant	}	4200
	14–100	+ Particulate		

Table 8. *Effect of washing on the [^{14}C]formate- and [^{14}C]bicarbonate-fixing activities of the particulate fraction*

Particulate fraction obtained from 0.8 g. (wet wt.) of bacteria was disrupted in a Hughes press and homogenized in 2 ml. of mM-tris buffer (pH 7.8). The centrifugal fraction 1600–10 000 g for 15 min. was resuspended in 0.7 ml. of tris buffer to give fraction R_1 ; this procedure was repeated twice in succession to give R_2 and R_3 respectively. Fractions were tested for enzymic activity as described in Table 7.

Omissions	Fraction	Total radioactivity (counts/min.) incorporated in assay mixture	
		$\text{NaH}^{14}\text{CO}_3$ (1.2 μmoles , 5 μC)	$\text{H}^{14}\text{CO}_3\text{Na}$ (1.2 μmoles , 5 μC)
None	R_1	2600	1280
None	R_2	540	1010
None	R_3	430	175
Ribulose 1:5-diphosphate	R_3	55	110

centrifuging, all the oxidizing activity was found in the particulate fraction, whereas the supernatant fraction was devoid of such activity.

These experiments demonstrate the existence in the extract of a system capable of ribulose 1:5-diphosphate-dependent incorporation of formate: an oxidation of the formate to carbon dioxide catalysed by the particulate fraction, followed by carboxylation of ribulose 1:5-diphosphate by the supernatant fraction. The experiments do not, however, show if this pathway was responsible for all the observed [^{14}C]formate fixation, or whether, by the concerted action of supernatant and particulate fractions, some C_1 unit other than carbon dioxide was involved.

It has been shown that hypophosphite, an inhibitor of formic dehydrogenase in some organisms (Takamiya, 1953), stops the oxidation of formate by washed suspensions of *P. oxalaticus* at a concentration of mM (Quayle & Keech, 1959). Carboxydismutase is not inhibited by this concentration of hypophosphite and hence the study of the effect of hypophosphite on formate incorporation into PGA in the cell-free extract seemed an obvious approach to the problem. It was found, however, that formate oxidation, as well as formate incorporation

into PGA, by the cell-free extract was completely unaffected by the presence of mM-hypophosphite. The non-inhibition by hypophosphite of formate oxidation in the extracts was an unexpected finding, since with other organisms the compound functions as an inhibitor of formate oxidation both in whole cells and cell-free extracts (Campbell, 1954, 1955; H. L. Kornberg & A. M. Gotto, personal communication), and requires further investigation. The alternative approach of studying [^{14}C]formate incorporation under anaerobic conditions was then tried.

Anaerobic fixation of [^{14}C]formate. The experiments were performed in micro-Thunberg tubes filled with nitrogen at a pressure of about 15 mm. Hg. Under these conditions the incorporation into PGA was reduced to one-third of the value obtained under aerobic conditions (Table 10). The addition of methylene blue to the system increased the anaerobic incorporation of radioactive carbon into PGA to 72% of the aerobic incorporation. Radioactive malate was formed under all conditions.

DISCUSSION

Evidence for the operation of a carbon dioxide-fixation cycle in *P. oxalaticus* (OX 1) grown on formate, similar to that found in photosynthetic and other autotrophic organisms, has been presented in the preceding paper. Confirmation of this is provided in the present work by demonstration of the presence of the two enzymes peculiar to such a cycle, namely phosphoribulokinase and carboxydismutase, in the cell-free bacterial extracts. Phosphoriboisomerase is also present. This enzyme is not peculiar only to the Calvin cycle of carbon dioxide fixation, but is also involved in reactions of the pentose phosphate cycle.

The participation of a C_1 unit other than carbon dioxide in the observed formation of PGA from ribulose 1:5-diphosphate and formate in the

Table 9. *Oxidation of formate by the cell-free extract*

Particulate fraction was obtained from the bacterial extract by centrifuging (0–30 000 *g* for 15 min.). Manometer cups contained: main compartment, whole extract (12 mg. dry wt.), or particulate fraction (11 mg. dry wt.), in 71 mM-sodium phosphate (pH 7.0); side arm, substrate; centre well, 400 μmoles of KOH. Total volume of contents was 3 ml. Incubation was at 30° for 1 hr.

Fraction	Formate (μmoles)	O_2 uptake ($\mu\text{l.}$)	Theoretical O_2 uptake ($\mu\text{l.}$)
Whole extract	None	20	—
Whole extract	20	218	224
Particulate	20	213	224
Supernatant	20	13	224

Table 10. *Anaerobic fixation of [^{14}C]formate by the extract*

The supernatant fraction obtained after centrifuging at 1600 *g* for 10 min. was used in the following reaction mixture: 5 μmoles of tris buffer (pH 7.8), 2.5 μmoles of MgCl_2 , 2.5 μmoles of GSH, 0.05 ml. of extract, 2.5 μmoles of $\text{H}^{14}\text{CO}_2\text{Na}$ (10 μC) and water to 0.35 ml. Anaerobic tests were carried out in sealed micro-Thunberg tubes filled with N_2 under reduced pressure. Incubation was at 30° for 15 min. Reaction was stopped with 1 ml. of hot ethanol and the products were chromatographed and the radioactivity was counted as described in Table 2.

Additions	Experimental conditions	Radioactivity (counts/min.) incorporated	
		PGA	Malate
None	Aerobic	0	0
None	Anaerobic	0	0
Ribulose 1:5-diphosphate (0.5 μmole)	Aerobic	1570	511
Ribulose 1:5-diphosphate (0.5 μmole)	Anaerobic	570	108
Ribulose 1:5-diphosphate (0.5 μmole) + methylene blue (0.3 μmole)	Anaerobic	1250	190

extracts is discounted on the basis of the following facts:

(a) The formate-fixing activity of the extract disappears on centrifugal removal of a particulate fraction which is capable of oxidizing formate to carbon dioxide.

(b) PGA formation from formate and ribulose 1:5-diphosphate is greatly reduced under anaerobic conditions, when formate cannot be oxidized to carbon dioxide.

The fact that PGA production was not completely arrested anaerobically is considered to be an experimental limitation rather than evidence for some incorporation of formate itself. The presence of a formic dehydrogenase in the crude extract could lead to anaerobic dismutation of formate to carbon dioxide and formation of a reduced coenzyme. Re-oxidation of this coenzyme by an electron acceptor could then lead to a continuous oxidation of formate. The formation of radioactive malate under anaerobic conditions is evidence for one such system operating in the extracts. The synthesis of malate by reductive carboxylation of pyruvate, or carboxylation of phosphopyruvate followed by reduction of the resulting oxaloacetate, would accomplish the re-oxidation of a reduced pyridine nucleotide; such enzymes were present in the extract.

The presence of these carboxylating enzymes in the cell could also explain the observation (Quayle & Keech, 1959) that radioactive carbon from [^{14}C]bicarbonate or [^{14}C]formate was rapidly incorporated into malate by growing cells.

SUMMARY

1. Reactions of [^{14}C]formate and [^{14}C]bicarbonate in cell-free extracts (prepared with a Hughes press) of *Pseudomonas oxalaticus* (OX1) grown on formate have been studied.

2. The extracts catalyse the incorporation of [^{14}C]bicarbonate into 3-phosphoglycerate in the presence of either ribulose 1:5-diphosphate or ribose 5-phosphate plus adenosine triphosphate. These findings show the existence of phosphoriboisomerase, phosphoribulokinase and carboxydismutase in the extracts.

3. 3-Phospho[^{14}C]glyceric acid is also formed in the extracts from [^{14}C]formate and ribulose 1:5-diphosphate. This enzymic activity is removed by centrifuging the extract and restored by recombining the particulate and supernatant fractions.

4. Incubation under anaerobic conditions reduced the phosphoglycerate formation from [^{14}C]formate to one-third of that observed on aerobic incubation. Addition of methylene blue restored the anaerobic incorporation to 72% of that observed under aerobic conditions.

5. The particulate fraction of the extract oxidizes formate to carbon dioxide.

6. These results show that the ribulose 1:5-diphosphate-dependent incorporation of [^{14}C]formate in the extract is preceded by oxidation of the formate to carbon dioxide. The small amount of formate incorporation still observed under anaerobic conditions is considered to be due to experimental limitation and not to some incorporation of formate itself.

7. The extracts incorporate [^{14}C]bicarbonate into malate in the presence of pyruvate and reduced di- or tri-phosphopyridine nucleotide. The latter coenzyme was the more effective. [^{14}C]Bicarbonate incorporation into malate and oxaloacetate in the presence of phosphopyruvate is catalysed by the extracts.

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