Carbon Assimilation by *Pseudomonas oxalaticus* (OXI)

4. METABOLISM OF OXALATE IN CELL-FREE EXTRACTS OF THE ORGANISM GROWN ON OXALATE*

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The oxidation level of oxalate is such that incorporation of its carbon into cell constituents must involve a net reduction. If it is the sole carbon source for microbial growth then it must also undergo condensation reactions resulting in the synthesis of, for example, C₃ and C₄ units necessary for formation of carbohydrate and amino acid carbon skeletons (for a review of this aspect, see Kornberg, 1959). Previous work, mainly with whole cells of *Pseudomonas oxalaticus* (Quayle & Keech, 1959a, 1960) indicated that a reductive step precedes any condensation reactions, and that this initial reduction reaction is that of oxalyl-coenzyme A to glyoxylate. It was also suggested that successive steps involve condensation of two molecules of the glyoxylate to tartronic acid semi-aldehyde, which in turn is reduced to glyceric acid by a reaction sequence similar to that proposed for the growth of *Pseudomonas* species on glycollate (Kornberg & Gotto, 1959).

The formation and metabolism of oxalyl-coenzyme A, including its reduction to glyoxylate by reduced triphosphopyridine nucleotide in cell-free extracts of *P. oxalaticus*, are reported in this paper. Part of this work has been presented to the Biochemical Society (Quayle, Keech & Taylor, 1960).

METHODS AND MATERIALS

Maintenance and growth of the organism. The growth conditions have been described by Quayle & Keech (1960).

Isotopic materials. Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks.

Preparation of cell-free extracts. Harvested cells (1.25–5 g.) were suspended in 4 vol. of suitable buffer, as indicated in the text, and subjected to ultrasonic vibrations for 2–3 min. with a 25 kcyc./sec. Mullard ultrasonic generator type E 7590 B (Mullard Ltd., London); to minimize heating, the transducer assembly was immersed in a rapidly flowing stream of tap water. The resulting suspension was centrifuged at 25 000 g for 20 min. in a refrigerated angle-head centrifuge; this crude supernatant fraction which contained 10–30 mg. of protein/ml. was used as such in

many experiments. For some purposes the crude extract was either dialysed for 3 hr. against 2 l. of 10 mM-buffer at 3° before use, or the protein was precipitated at 0° and pH 7.0 by the addition of saturated (NH₄)₂SO₄ solution to a final strength of 80% of saturation. The resulting precipitated protein was collected by centrifuging at 0°, dissolved in 5–10 ml. of 10 mM-buffer and dialysed for 3 hr. against 2 l. of the same buffer at 3°.

In one experiment (Table 8) a bacterial extract was prepared by crushing a frozen suspension of the bacteria, containing 1 g. wet wt. suspended in 2 ml. of 2-amino-2-hydroxymethylpropane-1.3-diol (tris) buffer (0.01 M, pH 7.3) in a Hughes (1951) press at –20°. The crushed material was homogenized and about 200 µg. each of crystalline ribonuclease and deoxyribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks.) were added.

Manometric methods. The decarboxylation and oxidation of oxalate was measured in conventional Warburg manometric apparatus.

Radioactive assay of ¹⁴CO₂, [¹⁴C]oxalate and [¹⁴C]formate. ¹⁴CO₂ was converted into Ba¹⁴CO₃ by precipitation with BaCl₂ and was then plated on paper disks (2.4 cm. diam.) as described by Sakami (1955a). The samples were assayed for radioactivity in a gas-flow counter (Model D-47, Nuclear Instrument and Chemical Corp., Chicago, U.S.A.), and corrected for self-absorption. Study of the enzymic decarboxylation of oxalic acid to formic acid involved the use of [¹⁴C]oxalic acid of known specific activity in terms of Ba¹⁴CO₃ derived from it by oxidation. The specific activity of the [¹⁴C]oxalate was determined by oxidation to ¹⁴CO₂ with KMnO₄ (0.4 N, in 2 N-H₂SO₄) at 60–80° and the evolved ¹⁴CO₂ was trapped in CO₂-free NaOH solution as described by Sakami (1955b). The resulting Na¹⁴CO₃ was converted into Ba¹⁴CO₃ and assayed as above. The amount of [¹⁴C]-formate evolved from [¹⁴C]oxalic acid by the cell-free extracts was estimated by adding 1.96 m-moles of unlabelled formic acid to the acidified reaction mixture at the end of the reaction. The formic acid was steam-distilled out of the mixture and was then decarboxylated by Mg²⁺ ions in acetate buffer as described by Sakami (1955c); the yields of CO₂ obtained were 70–80% of theoretical. The specific activity of the evolved ¹⁴CO₂ was measured as Ba¹⁴CO₃, and from this value the yield of [¹⁴C]formate from the [¹⁴C]oxalate in the original reaction mixture was calculated.

Chromatographic and radioautographic analysis. The general techniques used for chromatography and radioautography of radioactive compounds have been described by Quayle & Keech (1959b). Formate was chromatographed in ethanol-aq. NH₃ soln. (sp.gr. 0.880)-water

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Table 1. *Decarboxylation of oxalate by the crude bacterial extract*

The main compartment of each Warburg vessel contained 50 μ moles of sodium potassium phosphate buffer (pH 6.9) in Expt. 1, or 50 μ moles of tris buffer (pH 6.9) in Expt. 2, and ATP and oxalate as indicated; the two side arms contained respectively 0.5 ml. of cell-free extract, and 0.25 ml. of 10N-H₂SO₄ in Expt. 1 or 0.2 ml. of aq. 50% trichloroacetic acid (w/v) in Expt. 2. Total vol. of contents, 2 ml. in Expt. 1 or 2.2 ml. in Expt. 2. The flasks were flushed with N₂ and incubated at 30° for 30 min. in Expt. 1, or 15 min. in Expt. 2, before the acid was tipped in from the second side arms. In Expt. 2 the flask contents were centrifuged, the solid residues extracted with 1 ml. of water, and the combined supernatants were made up to 4 ml. and neutralized with 0.25 ml. of m-Na₂CO₃. Samples (0.5 ml.) were analysed by ion-exchange chromatography on columns of Dowex-1 (Cl⁻ form). The extracts were prepared from 1 g. of cells subjected to ultrasonic vibrations for 4 min. in 3 ml. of 0.1M-phosphate buffer (pH 6.9) in Expt. 1, or 5 ml. of 0.01M-tris buffer (pH 6.9) in Expt. 2, followed by centrifuging.

Expt. no.	Amount of oxalate in incubation mixture (μ moles)	Amount of ATP in incubation mixture (μ moles)	CO ₂ evolved (μ moles)	Amount of adenine nucleotides present after incubation (μ moles)		
				AMP	ADP	ATP
1	20	0	0.8	—	—	—
	20	1	3.2	—	—	—
	20	5	10.6	—	—	—
	20	10	16.4	—	—	—
	20	20	19.4	—	—	—
Analysis of original ATP solution used	—	—	—	0	0	22.0
2	20	22	13.1	2.5	5.2	14.3
	0	22	0.31	2.5	5.2	15.0

(80:4:16, by vol.; Long, Quayle & Stedman, 1951). Succinyl-, formyl- and oxalyl-coenzyme A were chromatographed in phenol-water (1:1, v/v; Fink & Fink, 1949; Jakoby, Ohmura & Hayaishi, 1956) and located by spraying with 5% (w/v) FeCl₃.6H₂O in ethanol (0.1N in HCl) (Stadtman & Barker, 1950).

Preparation of succinyl-coenzyme A. The method of Simon & Shemin (1953) was used with slight modification. Coenzyme A (CoA, 15 mg., Pabst Laboratories, Milwaukee, Wis., U.S.A.) was dissolved in 2 ml. of water at 0°, and 0.25 ml. of aqueous 0.1M-succinic anhydride was added; the pH was adjusted to 7 with 0.1N-NaOH, and the solution kept at 0° for 20 min. At the end of this time a sample gave a negative nitroprusside test for free thiol groups; this became positive after a sample was first treated with methanolic KOH, indicating that the formation of the succinyl-CoA was complete (Stadtman, 1957). The succinyl-CoA content of the solution, determined by the hydroxamic acid method of Lipmann & Tuttle (1945), showed yields of acyl-CoA of 30–50% of the theoretical yield expected from the weight of CoA taken. It was used immediately after preparation, owing to its instability.

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

Analysis of adenine nucleotides. Mixtures of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) were separated by standard procedures of ion-exchange chromatography. Approx. 3 μ moles of nucleotide, contained in a 0.5 ml. solution, was adsorbed on a column (4 cm. long $\times$ 1.5 cm. diameter) of Dowex-1 (Cl⁻ form, 200–400 mesh). The AMP, ADP and ATP were successively eluted from the column by washing with 10-ml. portions of 0.01N-HCl, 0.01N-HCl-0.05M-NaCl and 0.01N-HCl-0.2M-NaCl respectively. Their quantities were assayed spectrophotometrically at 260 m μ .

Chemicals. A sample of pure sodium glyoxylate was a gift from Dr I. Zelitch.

Table 2. *Stoichiometry of the decarboxylation of oxalate*

The complete system in the main compartments of the Warburg vessels contained 50 μ moles of tris-HCl buffer (pH 7.0), 20 μ moles of ATP, 20 μ moles of [¹⁴C]oxalate (0.125 μ Ci of ¹⁴C) of known specific activity in terms of derived Ba¹⁴CO₃; the two side arms contained respectively 0.5 ml. of cell-free extract (or boiled extract) and 100 μ moles of H₂SO₄ (0.2 ml.). Total volume of contents was 2.2 ml. The flasks were flushed with N₂ and incubated at 30° for 60 min. before the acid was tipped in. The extract was prepared from 1.25 g. of cells subjected to ultrasonic vibrations in 5 ml. of 10 mM-tris-HCl buffer (pH 7.0) for 3 min. and centrifuged. The contents of the flasks were analysed for [¹⁴C]formate by isotope dilution. In this measurement, 1.96 m-moles of formic acid was added to the flask contents; the formic acid was then isolated, purified, and decarboxylated to ¹⁴CO₂. The ¹⁴CO₂ was converted into Ba¹⁴CO₃, the specific activity of which was measured.

Omissions	Reaction products	
	CO ₂ (μ moles)	Formate (μ moles)
None	15.2	14.1
ATP	5.1	3.5
Boiled extract used in place of enzyme extract	0	0

RESULTS

Decarboxylation of oxalate

The crude supernatant fraction from cell-free extracts of *P. oxalaticus* prepared by ultrasonic disintegration catalysed a rapid decarboxylation of oxalate in the presence of ATP (Table 1). ATP

was required in catalytic quantities and up to 1 mole of carbon dioxide was evolved from 1 mole of oxalate. Other experiments have shown that no more than 1 mole of carbon dioxide could be evolved from 1 mole of oxalate even in the presence of more than 1 mole of ATP. The requirement for ATP could be met with inosine triphosphate, uridine triphosphate or cytidine triphosphate.

Products and stoichiometry of the decarboxylation. With [^{14}C]oxalate as substrate in the above reaction mixture, the major product was identified chromatographically as [^{14}C]formate. The stoichiometry of the reaction was determined by using [^{14}C]oxalate of known specific activity and estimating the resulting [^{14}C]formate by the method of isotope dilution (Table 2). The evolution of 15.2 μmoles of CO_2 was also accompanied by the formation of 14.1 μmoles of formate; these yields were decreased to one-third when ATP was left out of the reaction mixture. That the reaction is an enzymic one is shown by the complete loss of activity after boiling the enzyme extract for 5 min.

Cofactor requirements. Jakoby *et al.* (1956) described a partially purified enzyme preparation, obtained from an organism grown in the presence of oxalate, which catalysed an anaerobic decarboxylation of oxalate. The system required substrate quantities of ATP and catalytic quantities of acetate, CoA, thiamine pyrophosphate (TPP) and magnesium ions. These authors suggested that the decarboxylation proceeded by the following reactions:

The function of the ATP, acetate and CoA in the overall reaction was to synthesize acetyl-CoA by the action of acetyl-CoA synthetase (acetic thio-kinase). The enzyme preparation used was prepared from bacterial extracts, which had been subjected to ultrasonic vibrations, by precipitation with ammonium sulphate between 25 and 50% saturation.

An enzyme fraction was prepared in a similar fashion from oxalate-grown *P. oxalaticus* and was tested for decarboxylation activity manometrically. However, the carbon dioxide output was too small to be measured in this way, hence [^{14}C]oxalate was used as substrate and the evolved $^{14}\text{CO}_2$ was collected in the centre wells of the Warburg vessels and assayed as barium [^{14}C]carbonate.

The decarboxylation was dependent on the presence of acetate, ATP and CoA (Table 3); omission of any of these cofactors caused a six- to eight-fold diminution of activity. This closely

Table 3. *Cofactors for the decarboxylation of oxalate*

The complete system in the main compartments of the Warburg vessels contained 100 μmoles of sodium potassium phosphate buffer (pH 6.9), 100 μmoles of NaF, 10 μmoles of MgCl_2 , 10 μmoles of GSH, 20 μmoles of sodium acetate, 10 μmoles of ATP, 0.25 μmole of CoA, 0.1 μmole of TPP, 2.1 μmoles of [^{14}C]oxalate (1.25 μC of ^{14}C) of known specific activity in terms of derived $\text{Ba}^{14}\text{CO}_3$; the two side arms contained respectively 0.25 ml. of enzyme extract, and 2.5 m-moles of H_2SO_4 (0.25 ml.); the centre wells contained 0.2 ml. of CO_2 -free 2N-KOH. The total volume was 2.75 ml. The flasks were flushed with N_2 and incubated at 30° for 30 min. before the acid was tipped in. They were then shaken for a further 30 min. before the contents of the centre wells were analysed by adding 115 μmoles of Na_2CO_3 to each and measuring the specific activity of the [^{14}C]carbonate as $\text{Ba}^{14}\text{CO}_3$. The enzyme fraction was prepared by precipitating the protein from a sonic extract of 4 g. wet wt. of bacteria with $(\text{NH}_4)_2\text{SO}_4$ between 25 and 50% saturation. The precipitated protein was dissolved in 1 ml. of 50 mM-sodium potassium phosphate buffer (pH 6.9) and dialysed for 4 hr. against 4 l. of the same buffer containing 20 m-moles of GSH.

Omissions	CO_2 evolved (μmoles)
None	0.031
Sodium acetate	0.0059
ATP	0.0039
CoA	0.0062
TPP	0.031

followed the findings of Jakoby *et al.* (1956). Considerable variation of the overall reaction rate attained with the complete system was encountered from one enzyme preparation to another; in no case, however, did the rate exceed 2 μmoles of carbon dioxide evolved/30 min. The same results were found when the protein fraction precipitating between 0 and 80% of ammonium sulphate saturation was used. These rates, which are of the same order as those reported by Jakoby *et al.* (1956), are nevertheless a small fraction of the decarboxylation rates which crude extracts were capable of catalysing in the presence of ATP, which suggested that some essential cofactor had been lost on purification. This was confirmed by the finding that oxalate decarboxylation by the 0–80% ammonium sulphate fraction was greatly stimulated by the addition of either boiled extract or yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.). Since the yeast extract contained succinate, the effect of addition of this compound to the oxalate-decarboxylation system was tested.

Succinate requirement. Oxalate was rapidly decarboxylated by the 0–80% ammonium sulphate fraction, in the presence of ATP, CoA, TPP, magnesium chloride, reduced glutathione (GSH) and catalytic quantities of succinate (Fig. 1). In

the absence of succinate, CoA or oxalate no decarboxylation was detected manometrically. Varied requirements for the other cofactors were found. The results indicated that oxalate was decarboxylated by the scheme of reactions (1)–(3) except that acetyl-CoA was replaced in (1) by succinyl-CoA. If this were so, the function of the ATP, CoA and succinate would be to generate succinyl-CoA, and therefore the presence of the latter compound should replace the former three cofactors in the decarboxylation. This was found to be the case (Table 4): the presence of 5.7 μ moles of succinyl-CoA caused the decarboxylation of

9.5 μ moles of oxalate. The non-stoichiometric relationship between succinyl-CoA and oxalate decarboxylation indicated that oxalyl-CoA must be regenerated after the decarboxylation step by transfer of the CoA grouping from formyl-CoA, either directly to oxalate, or indirectly via succinate.

Formation of succinyl-coenzyme A from succinate. The preceding results indicate that a succinyl-CoA synthetase is present in *P. oxalaticus* which synthesizes succinyl-CoA from succinate, ATP and CoA. The presence of such an enzyme was demonstrated by the method described by Jones & Lipmann (1955) for acetyl-CoA synthetase; succinate was used in place of acetate, and the buffer used was tris buffer (pH 7.6). In this assay, the succinyl-CoA is detected as its derived hydroxamic acid with a ferric chloride reagent. With either the crude, cell-free extract or the 0–80% ammonium sulphate fraction, rates of succinylhydroxamate formation of 0.8 μ mole/hr./mg. of protein were observed. Negligible hydroxamate formation was observed if acetate, oxalate or formate was substituted for succinate in the assay system. Succinylhydroxamate formed in the complete incubation system was identified chromatographically.

Formation of oxalylhydroxamate and formylhydroxamate. The presence of oxalyl-CoA and formyl-CoA in the incubation mixture during the decarboxylation of [14 C]oxalate was demonstrated by adding hydroxylamine to the mixture and chromatographing the products. After incubation of 4.6 μ moles of succinyl-CoA with 9 μ moles of [14 C]oxalate, radioactive formylhydroxamate and oxalylhydroxamate, and non-radioactive succinylhydroxamate, were detected on the chromatogram (Table 5). The amounts of formylhydroxamate and oxalylhydroxamate isolated were very small: under the conditions of radioassay their radioactivity

Fig. 1. Effect of succinate and other cofactors on the anaerobic decarboxylation of oxalate. The complete system in the main compartment of the Warburg vessels contained 50 μ moles of tris buffer (pH 7.2), 20 μ moles of GSH, 10 μ moles of ATP, 0.5 μ mole of CoA, 0.5 μ mole of TPP, 10 μ moles of MgCl_2 , 1 μ mole of sodium succinate and 20 μ moles of potassium oxalate; the two side arms contained respectively 0.35 ml. of enzyme extract, and 0.25 ml. of aq. trichloroacetic acid (50%, w/v). The total volume was 2.25 ml. The flasks were flushed with N_2 and incubated at 30°. The reaction was stopped and bound CO_2 liberated by tipping in the acid after 70 min. incubation. The enzyme extract was a 0–80% $(\text{NH}_4)_2\text{SO}_4$ fraction. There was no measurable CO_2 output on omitting oxalate, succinate or CoA singly from the incubation mixture. O, Complete system; ●, MgCl_2 omitted; x, GSH omitted; Δ, TPP omitted; ▲, ATP omitted.

Table 4. Replacement of the requirement for succinate, adenosine triphosphate and coenzyme A by succinyl-coenzyme A in the decarboxylation of oxalate

The main compartment of each Warburg vessel contained 50 μ moles of tris buffer (pH 7.2), 20 μ moles of GSH, 0.5 μ mole of TPP, 10 μ moles of MgCl_2 , 20 μ moles of potassium oxalate and additions as indicated; the two side arms contained respectively 0.5 ml. of enzyme extract [0–80% $(\text{NH}_4)_2\text{SO}_4$ fraction], and 0.25 ml. of aq. trichloroacetic acid (50%, w/v). The total volume was 3.6 ml. The flasks were flushed with N_2 , and incubated at 30° for 40 min. before the acid was tipped in.

Additions	CO_2 evolved (μ moles)
None	0
ATP (10 μ moles) + CoA (0.5 μ mole) + succinate (1 μ mole)	17
Succinyl-CoA (5.7 μ moles)	9.5

Table 5. Formation of oxalylhydroxamate and formylhydroxamate during the anaerobic decarboxylation of oxalate

The main compartment of each Warburg vessel contained 50 μ moles of tris buffer (pH 7.2), 20 μ moles of GSH, 0.5 μ mole of TPP, 10 μ moles of $MgCl_2$, 9 μ moles of [^{14}C]oxalate (0.83 μ C of ^{14}C) and succinyl-CoA as indicated; the two side arms contained respectively 0.35 ml. of enzyme extract [0–80% $(NH_4)_2SO_4$ fraction], and 1 m-mole of neutral hydroxylamine (0.5 ml.). The total vol. was 3.65 ml. The flasks were flushed with N_2 and incubated at 30°. After incubation for 8 min. the hydroxylamine was tipped in, the flask shaken for 10 min., and the contents were then tipped into 30 ml. of ethanol and centrifuged. The supernatant was evaporated to dryness under reduced pressure of N_2 , the residue was extracted with 3 ml. of ethanol, concentrated and chromatographed in phenol–water (1:1, w/v). The paper was dried and sprayed with $FeCl_3$ reagent.

Addition	CO_2 evolved (μ moles)	Hydroxamates detected	R_F	Radioactivity detected in hydroxamates (counts/min.)
Succinyl-CoA (4.6 μ moles)	3	Succinyl	0.46	0
		Oxalyl	0.08	32
		Formyl	0.54	270
None	0.7	None	—	—

Fig. 2. Oxidation of oxalate and formate by washed suspensions of *P. oxalaticus*. The main compartments of Warburg vessels contained: 50 μ moles of potassium phosphate buffer (pH 7.0), bacteria (2 mg. dry wt.) washed with 0.9% KCl; the centre wells contained 0.2 ml. of 3N-KOH; the side arms contained 20 μ moles of sodium formate or sodium oxalate. The final volume was 2.2 ml. The flasks were incubated at 30°. O, Formate; ●, oxalate; ▲, endogenous.

corresponded to the presence of 0.17 and 0.01 μ mole respectively. Authentic formylhydroxamate and oxalylhydroxamate were prepared chemically from the monoethyl esters of the parent acids by the method of Hestrin (1949), and succinylhydroxamate was prepared from succinic anhydride as described by Lipmann & Tuttle (1945).

Oxidation of oxalate and formate

Whole cells of *P. oxalaticus* oxidized both oxalate and formate (Fig. 2), the latter substrate after a variable lag of approx. 10 min. With oxalate, there was an initial evolution of carbon dioxide, which was more rapid than its absorption by the alkali in the centre well of the Warburg vessel. This finding suggests that the oxidation of oxalate in whole cells is preceded by decarboxylation to formate.

The organism possesses a diphosphopyridine nucleotide (DPN)-linked formic dehydrogenase; cell-free extracts catalysed the reduction of 5.3 μ moles of DPN/hr./mg. of bacterial protein when assayed according to the procedure of Nason & Little (1955). No reduction of triphosphopyridine nucleotide (TPN) was observed under these conditions.

No evidence was found for the existence of a direct DPN- or TPN-linked oxalic acid dehydrogenase in the cell-free extracts of the organism.

Interconversion of oxalate and glyoxylate

It has been suggested (Quayle & Keech, 1959a) that the primary step in the conversion of oxalate into cell constituents in *P. oxalaticus* is a reduction

of oxalate to glyoxylate, possibly by a CoA- and pyridine nucleotide-dependent reaction:

This reaction would be analogous to the reduction of acetyl-CoA to acetaldehyde observed in *Clostridium kluyveri* by Burton & Stadtman (1953):

Extracts of *P. oxalaticus* catalysed the reverse reaction, i.e. the reduction of TPN in the presence of glyoxylate and CoA (Fig. 3). The reaction was specific for TPN, no detectable reduction of DPN in place of TPN being observed. The rate of TPN reduction markedly increased with increasing pH, the maximum rate being observed at pH 8.5 (Fig. 4). The reaction was inhibited when tris buffer was used in place of pyrophosphate buffer at the same molarity and pH: both the initial rate and the final amount of TPN reduction were approximately halved. The partially purified enzyme (50–80% ammonium sulphate fraction) was not very stable stored at 0° in 10 mM-sodium pyrophosphate buffer (pH 7.0). Such preparations lost up to 80% of their activity at 0° in 24 hr.; they were, however, stable when stored at –15°. In crude, cell-free extracts the enzyme was even less stable, losing 70% of its activity in 6 hr. at 0°.

Fig. 3. Reduction of TPN in the presence of glyoxylate and CoA by a partially purified enzyme preparation from *P. oxalaticus*. The enzyme preparation was obtained from a cell-free extract (32 mg. of protein/ml. in 10 mM-tris buffer) by precipitation at pH 7.0 with $(\text{NH}_4)_2\text{SO}_4$ between 50 and 80% saturation; the resulting protein fraction was dissolved in 5.3 ml. of 10 mM-tris buffer (pH 7.0) and contained 12.4 mg. of protein/ml. The reaction was followed by change of extinction at 340 mμ in a Cary recording spectrophotometer. The reaction system, contained in 3 ml. quartz cells, consisted of: 81 μmoles of sodium pyrophosphate buffer (pH 8.5), 4 μmoles of cysteine, 0.5 μmole of CoA, 0.5 μmole of TPN (omitted from blank cell), 10 μmoles of sodium glyoxylate, 0.1 ml. of enzyme extract and water to 3.0 ml. The reaction was started by addition of the glyoxylate. Further additions of 0.5 μmole of TPN and CoA were made as indicated.

Reaction products and stoichiometry. The only reaction product isolated from the action of the

TPN-linked glyoxylic dehydrogenase was oxalic acid. This was separated from residual glyoxylate by precipitation of the oxalate as its calcium salt.

Such a procedure was used, owing to the unsatisfactory direct chromatographic and electrophoretic separations of these two acids. The precipitation method, however, precluded the use of pyrophosphate as buffer in the incubation mixture, owing to its precipitation as a gel on the addition of calcium ions. Tris buffer did not interfere in this manner and was thus used in place of pyrophosphate, although the reaction gave lower yields of product as a result. With $[^{14}\text{C}]$ glyoxylate as substrate, the incubation was carried out in quartz cells and the reduction of TPN followed spectrophotometrically. The reaction was stopped by heating the reaction mixtures to boiling and the amount of $[^{14}\text{C}]$ oxalate isolated from them was estimated by radioactive assay (Table 6). The identity of the product was

Fig. 4. pH optimum of glyoxylic dehydrogenase. The reaction was followed by measurement of TPN reduction in a Cary recording spectrophotometer at 340 mμ. The reaction system, contained in 3 ml. quartz cells, consisted of: 81 μmoles of sodium pyrophosphate buffer, 4 μmoles of cysteine, 0.5 μmole of CoA, 0.5 μmole of TPN (omitted from blank cell), 10 μmoles of sodium glyoxylate, 0.05 ml. of enzyme extract [0–80% $(\text{NH}_4)_2\text{SO}_4$ fraction, diluted 1:10] and water to 3.0 ml. The reaction was started by addition of the glyoxylate.

Table 6. Oxidation of glyoxylate to oxalate in the presence of coenzyme A and triphosphopyridine nucleotide

The complete system contained: 75 μ moles of tris buffer (pH 8.5), 4 μ moles of cysteine, 1 μ mole of CoA, 1 μ mole of TPN, 0.22 μ mole of sodium [^{14}C]glyoxylate (4.4×10^4 counts/min. of ^{14}C), 0.2 ml. of partially-purified enzyme extract [50–80% $(\text{NH}_4)_2\text{SO}_4$ fraction containing 12.4 mg. of protein/ml.], omissions as indicated and water to 3 ml. After incubation at room temperature for 6 min., the reaction was terminated by boiling for 5 min. after 2 μ moles of carrier potassium oxalate had been added to each tube. Protein was centrifuged down and 0.05 ml. of 0.1M- CaCl_2 added to each supernatant. The calcium oxalate was removed by centrifuging, washed with 0.5 ml. of water, and converted into oxalic acid by treatment with 0.1 mg. of Dowex-1 (H^+ form) and 0.1 ml. of 0.2M-ethylenediaminetetra-acetic acid in a final vol. of 0.6 ml. After centrifuging, the resin was extracted with two 0.5 ml. portions of water which were combined with the supernatant solution. Samples (0.1 ml.) of the resulting solutions were plated on glass planchets and assayed for radioactivity.

Omissions	^{14}C Oxalate formed (counts/min. in total sample)	Net amount of ^{14}C oxalate produced* (μ mole)	Net amount of TPN reduced* (μ mole)
None	12 900	0.054	0.048
TPN	2 060	—	—
CoA	[360]†	—	—
Boiled extract used in place of enzyme extract	[525]†	—	—

* Corrected for the value obtained with TPN omitted.

† Radioactivity not affected by oxalic acid decarboxylase treatment, probably [^{14}C]glyoxylate cross-contamination.

established by showing its decarboxylation to [^{14}C]formic acid on incubation with purified oxalic acid decarboxylase obtained from *Collybia velutipes* according to Shimazono & Hayaishi (1957) (Table 7).

The formation of 0.054 μ mole of oxalic acid was accompanied by the reduction of 0.048 μ mole of TPN (Table 6). Separate experiments in which excess of TPN and CoA were used in the presence of limiting glyoxylate, with pyrophosphate as buffer, showed that under these conditions the TPN reduction proceeds to within 90–95% of the amount of glyoxylate added. No products of the reaction, volatile or non-volatile, other than [^{14}C]oxalate have been detected chromatographically. The results show that the reaction is represented by:

Role of coenzyme A. It seems likely that reaction (6), by analogy with the oxidation of acetaldehyde (Burton & Stadtman, 1953), would proceed via oxalyl-CoA:

The product isolated from the oxidation of glyoxylate in the previous experiment was oxalate and all attempts to detect as oxalylhydroxamate any oxalyl-CoA formed in the reaction have been unsuccessful. This may be due to its enzymic or chemical hydrolysis as in (9) since only catalytic quantities of CoA are required for the oxidation. This effect is shown in Fig. 5 where 0.015 μ mole of CoA (according to the stated purity of the CoA) resulted in the reduction of 0.069 μ mole of TPN in 19 min.; the reaction was still proceeding at the end of this time.

Reversibility of the reaction. In the absence of substrate quantities of oxalyl-CoA, a direct demonstration of the reversibility of the reaction is

not possible. However, oxidation of TPNH, dependent on the presence of ATP, CoA, succinate and oxalate, was catalysed by a 0–80% ammonium sulphate fraction of the cell-free extract (Fig. 6). No TPNH oxidation was observed if oxalate or any of the cofactors was omitted, suggesting that the oxalyl-CoA known to be generated in such an enzyme system from oxalate, ATP, CoA and succinate was being reduced by TPNH. Only trace amounts of glyoxylate could be detected as its 2,4-dinitrophenylhydrazones in the reaction mixture. This is to be expected in view of the presence in the enzyme extract of a highly active enzyme, similar to that discovered in *Escherichia coli* by Krakow & Barkulis (1956), catalysing the condensation

Table 7. Identification of oxalate as the product of oxidation of glyoxylate

A portion (1 ml.) of each of the supernatant solutions described in Table 6 was added to 0.1 ml. of 0.5M-potassium citrate buffer (pH 4.5). Samples (0.25 ml.), adjusted to pH 7.0, were withdrawn for chromatography in ammoniacal aq. ethanol. To the remainder was added 0.1 ml. of oxalic acid decarboxylase and after 1½ hr. incubation 0.1 ml. samples were withdrawn for chromatography. The [¹⁴C]formate was located by radioautography, and co-chromatographed with authentic formate. The radioactive areas on the paper chromatograms were assayed directly for radioactivity.

Omissions (see Table 6)	Before incubation with oxalic acid decarboxylase		After incubation with oxalic acid decarboxylase	
	Radioactivity (counts/min.)		Radioactivity (counts/min.)	
	Origin position of chromatogram	Formate area of chromatogram (<i>R_F</i> 0.54)	Origin position of chromatogram	Formate area of chromatogram (<i>R_F</i> 0.54)
None	535	0	0	263
TPN	96	0	0	69
Boiled extract used	10	0	10	0

Fig. 5. Effect of a limiting amount of CoA on the oxidation of glyoxylate by TPN. The reaction was followed by change of extinction at 340 mμ in a Cary recording spectrophotometer. The reaction system, contained in 1 ml. quartz cells, consisted of: 24 μmoles of sodium pyrophosphate buffer (pH 8.5), 2 μmoles of cysteine, 0.015 μmole of CoA, 1 μmole of TPN, 0.1 ml. of partially purified enzyme [50–60% (NH₄)₂SO₄ fraction containing 2 mg. of protein/ml.], 1 μmole of sodium glyoxylate and water to 1 ml. The reaction was started by addition of enzyme. CoA was omitted from the blank cell.

Fig. 6. Reduction of oxalyl-CoA by TPNH. The reaction was followed by change of extinction at 340 mμ in a Cary recording spectrophotometer. The complete reaction system, contained in 3 ml. quartz cells, consisted of: 75 μmoles of sodium pyrophosphate buffer (pH 7.5), 2 μmoles of cysteine, 10 μmoles of ATP, 0.5 μmole of CoA, 1.0 μmole of succinate, 10 μmoles of potassium oxalate, 0.5 μmole of TPNH (omitted from blank cell), 0.1 ml. of enzyme extract [0–80% (NH₄)₂SO₄ fraction] and water to 3.0 ml. Time of addition of the oxalate is indicated by the arrow.

of two molecules of glyoxylate (Quayle & Keech, 1959a, 1960):

Formation of C₄ acids

The activities of malate synthetase, isocitratase and condensing enzyme in extracts of *P. oxalaticus* were measured by the procedures of Dixon & Kornberg (1959). The activities of the first two enzymes were very low, i.e. 0.48 μmole of acetyl-CoA utilized/hr./mg. of protein for malate synthetase and 0.10 μmole of glyoxylate formed/hr./mg.

of protein for isocitratase. These values compare with 12.4 μmoles of acetyl-CoA utilized/hr./mg. of protein for condensing enzyme.

The low activity of malate synthetase suggests that C₄ acids in oxalate-grown *P. oxalaticus* are made primarily by some route not involving this enzyme. One probable mechanism is carbon dioxide fixation by pyruvate or phosphopyruvate, and enzymes catalysing such reactions were found in the formate-grown organism (Quayle & Keech, 1959c). When extracts of oxalate-grown *P. oxalaticus* were incubated with [¹⁴C]bicarbonate, rapid incorporation of isotope into malate, aspartate and tricarboxylic acid cycle intermediates was

Table 8. Fixation of [^{14}C]bicarbonate in the presence of pyruvate and phosphopyruvate

The reaction mixture contained 20 μmoles of tris buffer (pH 7.3), 60 μmoles of $\text{NaH}^{14}\text{CO}_3$ (20 μc), additions as indicated, 0.05 ml. of bacterial extract (prepared in a Hughes press) and water to a final vol. of 0.60 ml. The reaction was started by addition of extract and the mixture incubated at 30° for 15 min. The reaction was stopped by adding 2 ml. of hot ethanol; the resulting precipitates were centrifuged down and discarded. The supernatants were concentrated, the contents were chromatographed two-dimensionally, and the radioactive compounds, located by radioautography, were assayed for radioactivity *in situ*.

Additions	Radioactivity (counts/min.)					
	Malate	Glutamate	Serine	Citrate	Aspartate	Fumarate
None	30	0	0	0	33	0
Pyruvate (5 μmoles)	79	20	0	20	0	0
Pyruvate (5 μmoles) + DPNH (10 μmoles)	33	0	0	20	0	0
Pyruvate (5 μmoles) + TPNH (10 μmoles)	394	0	0	25	0	0
PEP (3 μmoles)	3420	460	470	255	1790	970
PEP (3 μmoles) + IDP (0.28 μmole)	3140	0	0	183	6400	725

found when the incubation was carried out in the presence of phosphoenolpyruvate (PEP) (Table 8). This indicated either the presence of a PEP carboxylase (Bandurski & Greiner, 1953) catalysing the irreversible carboxylation of PEP:

or a nucleoside diphosphate-dependent oxaloacetate carboxylase (Utter & Kurahashi, 1954):

(IDP, inosine diphosphate; ITP, inosine triphosphate) on both enzymes. The stimulation of total fixation on addition of inosine diphosphate is evidence for the existence of the last-named enzyme. The presence 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 TPNH. DPNH was not active in this system.

DISCUSSION

The work of Jakoby *et al.* (1956) on the decarboxylation of oxalate by a bacterial enzyme system provided the first evidence for the existence of oxalyl-CoA. The work reported in this paper shows that extracts of *P. oxalaticus* also catalyse the formation of this compound. In addition, oxalate is decarboxylated by a similar series of reactions. It is possible that a re-examination of the system of Jakoby *et al.* (1956) would show the two respective reaction sequences to be identical. The overall decarboxylation rates recorded by Jakoby and co-workers are very low compared with those obtained from *P. oxalaticus*. Since the former authors have not recorded figures for the

actual amounts of enzyme extract used to catalyse the decarboxylation, it is not possible to see if the relatively low decarboxylation rates are merely due to the use of protein concentrations lower than have been used in the present study. Comparison of the stoichiometric requirements of the two reaction systems suggests, however, that the co-factors used by Jakoby *et al.* may have effected only a partial reconstitution of full enzymic activity. These workers showed a requirement for substrate quantities of ATP and catalytic quantities of acetate, CoA, TPP and magnesium ions; the ATP, acetate and CoA requirements could be met with substrate quantities of acetyl-CoA or acetyl phosphate. This compares for *P. oxalaticus* with the requirements for catalytic amounts of ATP, succinate, CoA, TPP and magnesium ions; catalytic quantities of succinyl-CoA replace the succinate, ATP and CoA requirement. This suggests the operation of a cyclic mechanism for the decarboxylation of oxalate to formate, in which the CoA grouping from formyl-CoA is transferred back to oxalate by transferase enzyme(s) either directly, or indirectly via succinate (see Fig. 7). A similar type of cycle has been discovered in *Pseudomonas* species for the decarboxylation of malonate to acetate by Hayaishi (1954) and Wolfe & Rittenberg (1954). As will be discussed further, the oxidation of formate probably represents the main energy-yielding reaction of the organism while growing on oxalate, and thus the expenditure of substrate quantities of ATP in converting oxalate into formate could lead to the loss of 30 % of the possible energy yield in the complete oxidation of oxalate to carbon dioxide. It seems more reasonable to expect the alternative cyclic mechanism to operate, and hence, substitution of succinate for acetate in the system of Jakoby *et al.* (1956) might well show the decarboxylation to proceed in an identical fashion with that catalysed by extracts of *P. oxalaticus*.

Oxalylhydroxamate could only be isolated in trace amounts during the decarboxylation of oxalate by extracts of *P. oxalaticus*, indicating a very rapid turnover of small quantities of oxalyl-CoA or instability of the compound or a combination of both factors. Evidence from the reversal of the oxalyl-CoA reductase indicates the instability of the compound at pH 8.5, although it is not yet known if its decomposition is spontaneous or enzymic.

The detection of formylhydroxamate, also in small amounts, is evidence for the existence of formyl-CoA, the formation of which has previously been suspected in extracts of *C. kluyveri* (Stadtman, 1953; Lieberman, 1954) and an oxalate-grown organism (Jakoby *et al.* 1956).

The reduction of oxalyl-CoA by TPNH to glyoxylate in *P. oxalaticus* is taken to be the first reductive step in the incorporation of oxalate into cell constituents. The fact that no such enzyme activity could be found in formate-grown *P. oxalaticus* indicates that this enzyme is under adaptive control and is necessary for the metabolism of oxalate in this organism. The reaction is another example of the reduction of a carboxylic acid group to an aldehyde group via, presumably, an intermediate CoA thioacetal. This has been shown to occur in *C. kluyveri* (Burton & Stadtman, 1953) and in *E. coli* (Dawes & Foster, 1956) where acetyl-CoA is reduced to acetaldehyde with DPNH. The reduction of palmityl-CoA with TPNH in the synthesis of sphingosine by rat-brain tissue (Brady & Koval, 1958), and β -hydroxy- β -methylglutaryl-CoA to mevalonic acid with TPNH by yeast (Ferguson, Dunn & Rudney, 1959), are further examples of this type of reaction.

No evidence has been found for the presence of a direct pyridine nucleotide-linked oxalic dehydrogenase, although a DPN-linked formic dehydrogenase is present. The burst of carbon dioxide before oxygen uptake observed during oxidation of oxalate by washed suspensions of *P. oxalaticus* suggests that in fact oxidation of oxalate proceeds via initial decarboxylation to formate. The formate, on being oxidized by the formic dehydrogenase, could then serve as primary source of reducing power necessary for synthesis of ATP and reduction of oxalate. In order to transfer the reducing power from DPNH to TPN, a transhydrogenase must be present. The fact that [^{14}C]oxalate is

reduced to [^{14}C]glycerate by crude cell-free extracts in the presence of acetyl-CoA and DPNH (Quayle & Keech, 1959*a*, 1960), despite the fact that the oxalyl-CoA reductase is now shown to be strictly TPN-specific, is evidence for the presence of such

Fig. 7. Conversion of oxalate into glyoxylate.

a transhydrogenase. Evidence for the reverse reaction, reduction of DPN by TPNH, was found during assay of the extract for reduced pyridine nucleotide-cytochrome *c* reductase activity. With the assay system described by Brodie (1955), it was found that DPNH reduced cytochrome *c* four times as fast as TPNH did in crude sonic extracts of *P. oxalaticus*. The reduction by TPNH was doubled by the addition of equimolar quantities of DPN, suggesting a reduction of the DPN by the TPNH.

The assignment of formate as the main source of reducing power for the organism allots a role for the oxalate-decarboxylation cycle (see Fig. 7) in terms of generation of formate. Oxalyl-CoA is then situated at the branching point of two reaction sequences: (a) decarboxylation to formate, which is the source of DPNH; (b) reduction by TPNH to glyoxylate, which initiates the synthesis of cell constituents.

A major factor controlling these two alternative pathways is probably the relative levels of the reduced and oxidized pyridine nucleotides. This would enable a sensitive balance between synthesis and breakdown to be maintained.

The oxidation of oxalate to carbon dioxide would involve cycles II and III, the sum of which is

The coupling of the oxidation of oxalate to the synthesis of glyoxylate would involve cycles I-IV, the sum of which is

The need for ATP is presumably met by phosphorylation of ADP coupled to the oxidation of more oxalate.

Further stages in the conversion of glyoxylate into cell constituents may take place by a scheme

similar to that proposed by Kornberg & Gotto (1959) for the metabolism of glycollate by a glycollate-grown organism in which two molecules of glyoxylate are condensed to one molecule of a C_3 compound and one molecule of carbon dioxide; the C_3 compound (probably tartronic acid semialdehyde) is then reduced and phosphorylated to 3-phosphoglyceric acid. Evidence for the occurrence of these reactions in *P. oxalaticus* has previously been presented (Quayle & Keech, 1960). Since only very low activities of malate synthetase have been detected in oxalate-grown *P. oxalaticus*, whereas both a TPN-linked 'malic enzyme' and an inosine diphosphate-stimulated PEP carboxylase are present, it is likely that C_4 acids are made by carbon dioxide fixation with pyruvate or PEP rather than through condensation of acetyl-CoA and glyoxylate by malate synthetase.

Work is currently in progress on the further purification of oxalyl-CoA reductase.

SUMMARY

1. Reactions of oxalate, glyoxylate, formate and bicarbonate in cell-free extracts of *Pseudomonas oxalaticus* (OXI) grown on oxalate have been studied.

2. The extracts catalyse a decarboxylation of oxalate to formate which is dependent on catalytic quantities of succinate, adenosine triphosphate, coenzyme A and thiamine pyrophosphate. Magnesium ions and reduced glutathione accelerate the reaction. Succinate, adenosine triphosphate and coenzyme A can be replaced by catalytic quantities of succinyl-coenzyme A. The extracts catalyse the formation of succinyl-coenzyme A from succinate but no similar direct activation of oxalate, formate or acetate has been detected.

3. During the decarboxylation of oxalate under the above experimental conditions both oxalyl-hydroxamic acid and formylhydroxamic acid have been isolated on addition of hydroxylamine, indicating the formation of coenzyme A esters of oxalic acid and formic acid.

4. It is suggested that oxalyl-coenzyme A is synthesized by transferase action between succinyl-coenzyme A and oxalate. Oxalyl-coenzyme A is decarboxylated to formyl-coenzyme A, the coenzyme A grouping of which can be transferred back to oxalate either directly, or indirectly via succinate.

5. Whole cells of *P. oxalaticus* oxidize both formate and oxalate. Oxidation of oxalate is preceded by a rapid evolution of carbon dioxide suggesting that the oxidation proceeds via formate. Cell-free extracts possess a diphosphopyridine nucleotide-linked formic dehydrogenase but no pyridine nucleotide-linked oxalic dehydrogenase was found.

6. Extracts catalyse a triphosphopyridine nucleotide- and coenzyme A-dependent oxidation of glyoxylate to oxalate. The initial reaction product is considered to be oxalyl-coenzyme A which is hydrolysed under the experimental conditions to oxalate. Indirect evidence indicates the reaction to be reversible, and it is suggested that this is the primary reduction reaction in the conversion of oxalate into cell constituents.

7. The extracts incorporate [^{14}C]bicarbonate into malate and citrate in the presence of pyruvate and reduced triphosphopyridine nucleotide. [^{14}C]-Bicarbonate is incorporated mainly into malate and aspartate in the presence of phosphopyruvate; this fixation is stimulated by inosine diphosphate.

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The Kinetics of Deoxygenation of Human Haemoglobin

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Until 1949, kinetic data for the reactions of haemoglobin with oxygen and carbon monoxide, obtained almost entirely by Hartridge & Roughton (1923, 1925), Roughton (1934) and Millikan (1933) in experiments with sheep haemoglobin, were considered to conform to simple first-order and second-order rate equations, and to be consistent, therefore, with the assumption that the four haems in the molecule behave independently. The best equilibrium data, on the other hand, could only be interpreted by the intermediate-compound theory of Adair (1925) with the assumption of haem interaction (Forbes & Roughton, 1931; Courtice & Douglas, 1947). This situation was reviewed by Roughton (1949), who reported that re-inspection of earlier kinetic data, and new experiments on the deoxygenation of sheep haemoglobin in the presence of sodium dithionite (Legge & Roughton, 1950), gave evidence of haem interaction, notably a significant upward trend in the value of the overall first-order velocity coefficient in the early stages of the deoxygenation reaction. This was confirmed with human haemoglobin under various conditions (cf. Dalziel, 1953, 1954), as will be described here. The further evaluation of the inter-

mediate-compound hypothesis has since been pursued with remarkable success by Roughton, Gibson and their collaborators. For sheep haemoglobin the four equilibrium constants for the oxygen reaction (Roughton, Otis & Lyster, 1955) and the four combination velocity constants for the carbon monoxide reaction (Gibson & Roughton, 1957a) have been determined to within narrow limits. Little further work has been reported for the deoxygenation reaction, however.

For the investigation of the kinetic effects of haem interaction, the oxygen dissociation appeared to be the reaction of choice, since previous work had indicated that the reverse reaction could be eliminated by an excess of sodium dithionite, and that the analysis of consecutive reactions would be simpler than for the reverse, combination reaction. This was the main purpose of the work reported here; a secondary objective was to compare the kinetic properties of haemoglobin from several normal individuals and from persons suffering from various types of anaemia. The work, which was carried out during 1948–53, forms part of a Ph.D. thesis approved by the University of London, and brief accounts have also been published in the literature (Dalziel, 1954, 1955, 1958). It was found that secondary reactions between dithionite-

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