but to regard the glycollic oxidase of the sedimentable fractions of tobacco leaves as a contaminant from the supernatant fraction. If the enzyme is associated with any microscopic structure of the tobacco cells, the linkage is labile and is completely disrupted during the disintegration of the leaves. Such a linkage would be quite unlike that holding fumarase on to the mitochondria.

SUMMARY

1. Preparations from tobacco leaves, which sedimented between 1000 and 10 000 g, oxidized glycollate and lactate. The oxidations did not appear to involve cytochromes and were probably brought about by glycollic oxidase.

2. The glycollic oxidase of these preparations was only a small (7.5%) proportion of the total in the leaf extract. The bulk of the activity was in the supernatant fraction and was not sedimented by centrifuging at 55 000 g for 2 hr.

3. In contrast, the sedimentable preparations contained nearly all (85–92%) of the fumarase in the extracts. Ageing at 30°, followed by freezing and thawing, liberated three-quarters of this activity in a form not sedimented at 50 000 g.

4. The fumarase was inhibited by *p*-chloro-mercuribenzoate and iodobenzoate and partially reactivated by cysteine. It was competitively inhibited by phosphate.

5. The fumarase activity was in excess of that

required for its expected role in the oxidation of fumarate by the preparations.

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Carbon Assimilation by *Pseudomonas oxalaticus* (OX1)

3. OXALATE UTILIZATION DURING GROWTH ON OXALATE*

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It has been shown (Quayle & Keech, 1959*a*) that one of the key enzymes of the ribulose 1:5-diphosphate cycle of carbon dioxide fixation (Bassham *et al.* 1954), carboxydismutase, is not synthesized when *Pseudomonas oxalaticus* (OX1) is grown on oxalate. This indicates that such a cycle cannot be of major importance in the conversion of oxalate carbon atoms into cellular material in oxalate-

grown cells, in contrast with formate-grown cells of the same organism (Quayle & Keech, 1959*b, c*). The present work, by giving information on the activities of phosphoribulokinase and phosphoriboisomerase during adaptation of oxalate-grown cells to growth on formate, confirms this conclusion. The sequence of compounds which become labelled when the organism is growing on [¹⁴C]-oxalate has also been determined, and a route for incorporation of oxalate into cellular constituents, based on these results and on reactions observed in

* Part 2: Quayle & Keech (1959*c*).

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crude cell-free extracts, is proposed. A preliminary account of part of this work has been published (Quayle & Keech, 1959d).

METHODS AND MATERIALS

Maintenance and growth of the organism. The maintenance and growth of cultures of *Pseudomonas oxalaticus* (OX1) have been described (Quayle & Keech, 1959b), sodium oxalate being used in place of sodium formate in the liquid media. Large quantities of the organism were grown in 10 l. of medium inoculated with 400 ml. of a bacterial suspension in the logarithmic phase of growth. The growth vessel used was a glass bottle vigorously aerated by blowing in air through a pumice plug fixed to the end of a submerged inlet tube. Growth was maintained at 30°, oxalic acid (A.R.) being added to keep the pH at 7.5–8.5. After 48 hr. the bacteria (15 g. wet wt.) were harvested in a Sharples centrifuge and stored at –10°.

Manometric methods and preparation of tracer solutions. The procedures used have been described previously (Quayle & Keech, 1959b). Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks.

Bacterial incorporation of [¹⁴C]oxalate, [¹⁴C]formate and [¹⁴C]bicarbonate. Bacteria which had been grown in conical (Fernbach) culture flasks were centrifuged at room temperature and resuspended in a small volume of their supernatant medium. A sample (1 ml.) of this suspension, containing from 13 to 24 mg. dry wt. of bacteria, was added either to fresh growth medium (8 ml.) containing 100 mM-sodium oxalate or to 10 mM-potassium phosphate, pH 7.0 (8 ml.), containing 10 mM-sodium oxalate (in the [¹⁴C]oxalate experiment) in a cylindrical vessel (10 cm. × 2.5 cm. diam.) fitted with a sintered-glass bottom. Vigorous aeration in the suspension was maintained by blowing a stream of air through the sintered glass. Incubation was continued for 5 min. (or 1 hr. in the [¹⁴C]bicarbonate experiment) to ensure that growth had recommenced.

In the [¹⁴C]bicarbonate experiment fresh oxalic acid was continually added to keep the pH at 7.5–8.5. At the end of the preincubation period, 200 μ C of ¹⁴C-tracer solution was added to bring the final volume of the suspension to 10 ml. Samples (1 ml.) were withdrawn with a hypodermic syringe at suitable time intervals over 10 min. and transferred immediately to 3 ml. of boiling aqueous 95% (v/v) ethanol.

In the [¹⁴C]formate experiment a parallel manometric measurement of the extent of formate oxidation was made. A sample (2 ml.) of a similarly prepared bacterial suspension was placed in the main compartment of a double-side-arm manometer cup, which contained 400 μ moles of CO₂-free sodium hydroxide in the centre well. The two side arms contained respectively 50 μ C of [¹⁴C]formate in 0.4 ml. of water and 3.3 m-moles of sulphuric acid. The sulphuric acid was tipped in to terminate the reaction and the manometer was then shaken for 1 hr. to ensure that all the liberated ¹⁴CO₂ was trapped in the centre well, whose contents were then transferred with washing to a stoppered tube and made to a total volume of 2 ml. with water. The radioactivity in this solution was assayed as barium [¹⁴C]carbonate.

Treatment of aqueous ethanolic suspensions of cells. The hot ethanolic suspensions described previously were centrifuged and the resulting precipitates extracted with 1 ml.

of hot aqueous 20% (v/v) ethanol. The combined supernatant fractions were evaporated to dryness at 60° under N₂. Those resulting from the [¹⁴C]formate and [¹⁴C]bicarbonate experiments were redissolved in 0.25 ml. of aqueous 20% (v/v) ethanol ready for application to the origin position of paper chromatograms. Those fractions resulting from the [¹⁴C]oxalate experiments were freed from residual [¹⁴C]oxalate by incubation in the following manner with purified oxalate decarboxylase, prepared from *Collybia velutipes* by the method of Shimazono & Hayaishi (1957). The fractions were redissolved in 0.5 ml. of water and treated with a small quantity of Dowex-50 (H⁺ form) to remove cations which inhibited the activity of the decarboxylase. The resin was removed by centrifuging, washed with 0.2 ml. of water and the combined supernatant fraction adjusted to pH 4.5 with 0.1 M-acetate buffer, pH 4.5. A portion (0.1 ml.) of an aqueous solution of purified oxalate decarboxylase was added and the fractions were incubated at room temperature for 5 hr.; 1 μ mole of oxalic acid and a further 0.1 ml. of decarboxylase were then added and incubated for a further 3 hr. Samples (0.5 ml.) of the resulting solutions were applied to the origin position of paper chromatograms.

Chromatographic analysis and radioautography. The procedures used have been described previously (Quayle & Keech, 1959b).

Identification of labelled compounds. The radioactive compounds were eluted from the chromatogram with water and chromatographed in appropriate solvent systems together with authentic samples of the suspected compound. Exact matching of the resulting radioactive areas and the coloured area developed with an appropriate spray reagent was necessary for identification. Carboxylic acids were rechromatographed either in the original solvent systems or in ethanol-aq. NH₃ soln. (sp.gr. 0.880)-water (80:4:16, by vol.; Long, Quayle & Stedman, 1951) for 8 hr. Amino acids were rechromatographed in water-saturated phenol in the presence of NH₃ vapour (Consden, Gordon & Martin, 1944) for 16 hr. or in butanol-acetone-water-diethylamine (10:10:5:2, by vol.; Hardy, Holland & Nayler, 1955) for 8 hr. The last-named solvent was found particularly useful for separating serine, glycine and glutamate. Dephosphorylation of phosphorylated compounds was carried out by incubation of the eluted compounds with a phosphatase fraction, obtained from Polidase-S (Schwarz Laboratories Inc., New York, U.S.A.), by precipitation in 85–100% saturated aqueous ammonium sulphate. The resulting compounds were chromatographed two-dimensionally for 12 hr. in water-saturated phenol and butanol-propionic acid-water (47:22:31, by vol.; Benson *et al.* 1950). The sprays used for locating various compounds on chromatograms were those described by Quayle & Keech (1959b).

Preparation of bacterial extracts for enzyme assay. Samples of bacteria were obtained from culture solutions by centrifuging. The resulting bacterial samples, containing 30–50 mg. dry wt. of bacteria, were each suspended in 1 ml. 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 disrupting the bacteria with a Mullard-MSE ultrasonic cell disintegrator (19 kcyc. at 75 w) for 7 min. at 0°, followed by centrifuging at 25 000 g for 20 min. in a refrigerated centrifuge.

Carboxydismutase assay. A sample (0.05 ml.) of extract, containing about 1 mg. of total protein, was incubated with 5 μ moles of tris buffer, pH 7.8, 2.5 μ moles of magnesium chloride, 2.5 μ moles of reduced glutathione (GSH), 0.2 μ mole of ribulose 1:5-diphosphate, 0.45 μ mole of sodium bicarbonate (10 μ C of 14 C) and water to 0.35 ml. for 15 min. at 30°. The reaction was stopped with 1 ml. of hot ethanol and samples (0.1 ml.) were plated in a stream of warm air on glass planchets mounted on a rotating turn table. The plates were irrigated with 0.2 ml. of formic acid (90%, w/v; A.R.), dried and the radioactivity on them was assayed with an end-window, lead-shielded Geiger-Müller tube (General Electric Co. Ltd.). Carboxydismutase activity is expressed as total radioactivity incorporated (counts/min.)/ μ g. of bacterial protein. Chromatographic analysis and radioautography of the soluble radioactive components showed 3-phosphoglyceric acid (PGA) to contain over 85% of the total radioactivity.

Phosphoriboisomerase assay. The method of Hurwitz, Weissbach, Horecker & Smyrniotis (1956) was used, in which the ribulose 5-phosphate formed from ribose 5-phosphate is estimated by the cysteine-carbazole colorimetric method. One unit of enzyme activity is defined as the amount of enzyme which catalyses the formation of 0.6 μ mole of ribulose 5-phosphate in 10 min. under the experimental conditions.

Phosphoribulokinase assay. The enzyme was assayed by measuring [14 C]3-phosphoglyceric acid formation from ribose 5-phosphate, adenosine triphosphate (ATP) and sodium [14 C]bicarbonate as a result of the following reactions:

Purified carboxydismutase, catalysing reaction (3), was prepared from spinach by the method of Weissbach, Horecker & Hurwitz (1956) and a portion was added to each sample in excess. Phosphoriboisomerase, catalysing reaction (1), was found to be present in all the samples. Under these conditions, phosphoribulokinase, catalysing reaction (2), was the limiting step. The complete assay mixture contained 5 μ moles of tris buffer (pH 7.8), 2.5 μ moles of magnesium chloride, 2.5 μ moles of GSH, 2 μ moles of ribose 5-phosphate, 5 μ moles of ATP, 0.45 μ mole of sodium bicarbonate (10 μ C of 14 C), 0.2 ml. of carboxydismutase solution and 0.05 ml. of cell-free extract to be assayed. The mixture was incubated for 15 min. at 30° and the reaction was stopped with 1 ml. of hot ethanol and samples (0.1 ml.) were assayed on glass planchets for radioactivity as previously described.

Special materials. Acetyl-coenzyme A was a gift of Dr G. H. Dixon. A culture of *Collybia velutipes* was a gift of Mr Kemp, Department of Botany, University of Oxford.

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

RESULTS

Oxidation of formate and oxalate. Washed suspensions of *P. oxalaticus* oxidized both formate and oxalate with Q_{O_2} values ranging from -40 to -150

(μ l. of O_2 consumed/mg. dry wt. of bacteria/hr.), depending on the condition of the culture harvested.

Incorporation of [14 C]formate and [14 C]bicarbonate. The operation of an autotrophic type of metabolism similar to that found in the formate-grown organism, during growth on oxalate should lead to a major incorporation of carbon dioxide by the cells. If in such a case the breakdown of oxalate proceeded via formate, then a major incorporation of formate carbon might also be expected. In order to test these possibilities the incorporation of either [14 C]formate or [14 C]bicarbonate into cells growing on oxalate was measured. In both cases negligibly small incorporation of isotope into the ethanol-soluble fraction of the cells was observed. With [14 C]bicarbonate the only compound that appeared labelled after incubation for 10 min. was malic acid. With [14 C]formate, a parallel determination was carried out in a Warburg manometer cup of the extent of [14 C]formate oxidation that had occurred during the incubation. It was found that the radioactivity of the [14 C]carbonate in the centre well accounted for 31% of the radioactivity added as [14 C]formate at the beginning of the experiment, showing that formate was being metabolized by the cells and not being excluded from them by permeability barriers. These results contrast with the incorporation of [14 C]oxalate

(see later), in which over 30 labelled compounds were obtained after incubation for 2 min., and show that neither formate nor carbon dioxide lies on the main pathway of carbon incorporation from oxalate, unless exogenous formate and carbon dioxide do not mix with endogenous formate or carbon dioxide formed from oxalate. Such a situation might exist because of compartment barriers within the cell or the utilization of the metabolites in an activated form.

The results also show that different metabolic pathways must operate during growth on either formate or oxalate, the autotrophic type of metabolism found with the former growth substrate being replaced in the latter substrate by some process not involving a major incorporation of formate carbon or carbon dioxide.

This change in metabolism with the change of growth substrate was checked by following the changes in activities of carboxydismutase, phosphoribulokinase and phosphoriboisomerase during adaptation of the cells to growth on either oxalate or formate. These three enzymes are necessary for the operation of the ribulose 1:5-diphosphate cycle of carbon dioxide fixation.

Enzymic changes during adaptation of the oxalate-grown organism to growth on formate. *P. oxalaticus* was grown in 2.2 l. of oxalate growth medium at 30° to a density of 0.15 mg. dry wt. of bacteria/ml. and centrifuged. The centrifuged cells were resuspended to a final vol. of 15 ml. in aqueous 0.9% potassium chloride; 2 ml. was retained for enzyme

Fig. 1. Variation with time of enzyme activities during adaptation of oxalate-grown *P. oxalaticus* to growth on formate. ○, Growth of organism; ●, carboxydismutase activity; expressed as counts/min./μg. of protein; △, phosphoribulokinase activity, expressed as counts/min./μg. of protein; ▲, $10^{-1} \times$ pentose phosphate-isomerase activity, expressed as units/mg. of protein.

Fig. 2. Influence of chloramphenicol on the appearance of carboxydismutase activity in oxalate-grown cells suspended in formate growth medium. ●, Control suspension; ○, chloramphenicol added to final concn. of 50 μg./ml. at the time indicated by the arrow.

assay and the remaining 13 ml. resuspended in 5.5 l. of formate growth medium contained in a 10 l. glass jar. Aeration was maintained through a plug of pumice sealed at the end of a submerged glass inlet tube, the temperature being maintained at 30°. Growth was measured nephelometrically and the pH was maintained at 7.0–8.5 by addition of formic acid (90%, w/v; A.R.); samples (750 ml.) were withdrawn at suitable time intervals and the bacteria collected from them by centrifuging. The bacterial samples were assayed for carboxydismutase, phosphoribulokinase and pentose phosphate-isomerase activities. The oxalate-grown cells had negligibly low activities of carboxydismutase and phosphoribulokinase (Fig. 1); these activities rose sharply in the lag period of no growth (12 hr.) and were then maintained during growth on formate. The rise in carboxydismutase activity preceded that of the kinase. A high pentose phosphate-isomerase activity was found in both the oxalate-grown cells and formate adapted cells.

The effect of the presence in the growth medium of chloramphenicol on the appearance of carboxydismutase activity during the lag period was tested. Oxalate-grown *P. oxalaticus* was suspended in 5.6 l. of formate growth medium at a cell density of 0.15 mg. dry wt./ml. and the resulting suspension was divided between two 10 l. growth vessels, which were aerated at 30°. After 2.25 hr. chloramphenicol was added to one flask to a final concentration of 50 μg./ml. Samples (350 ml.) of the two cultures were harvested at time intervals and tested for carboxydismutase activity. No rise in enzyme activity was observed in the flask to which chloramphenicol had been added (Fig. 2), suggesting that the appearance of carboxydismutase activity represents an actual synthesis of new enzyme protein rather than an unmasking of enzyme activity.

Enzymic changes during adaptation of the formate-grown organism to growth on oxalate. *P. oxalaticus* was grown in 1.6 l. of formate growth medium at 30° to a density of 0.17 mg. dry wt. of bacteria/ml. and centrifuged. The centrifuged cells were resuspended to a final vol. of 15 ml. in aqueous 0.9% potassium chloride; 2 ml. was retained for enzyme assay and the remaining 13 ml. resuspended in 2 l. of oxalate growth medium contained in a 2.5 l. bottle. Growth was maintained as described previously under the reverse adaptation experiment, oxalic acid (A.R.) being added to maintain the pH at 7.0–8.5. When the bacterial culture had doubled in cell density, 1 l. was harvested and 1 l. of fresh oxalate growth medium was returned to the culture vessel. This was repeated through seven bacterial generations. During the first generation of growth the carboxydismutase activity was maintained almost unchanged; thereafter decrease in

activity closely followed the theoretical diluting-out to be expected if the enzyme was no longer being synthesized (Fig. 3). After seven generations of growth in a constant concentration of oxalate, formate was added to a concentration one-tenth that of the oxalate. A rapid increase in carboxydismutase activity was observed without a detectable change in growth rate.

Attempted induction of carboxydismutase synthesis under different growth conditions. The following experiment was undertaken to determine whether the stimulation of carboxydismutase synthesis after the addition of formate, as described in the previous experiment, could be repeated under different growth conditions. Starting from inocula

taken from oxalate-agar slopes, separate cultures of organisms were grown in oxalate, lactate and succinate liquid growth media (acetate and ribose were also tried, but the organism failed to grow on these substrates). When the cultures had reached a suitable cell density, sodium formate was added to the growth media to a final concentration of 10 mM. Samples of the bacteria were harvested by centrifuging during the 2 hr. period subsequent to the formate addition and tested for carboxydismutase activity in the manner previously described. In no case was any appreciable carboxydismutase activity observed.

It has previously been observed by Fuller (1956) and Fuller & Gibbs (1959) that a fivefold increase of carboxydismutase activity could be induced in xylose- or arabinose-grown *Escherichia coli* (Crookes strain) by passing air + CO₂ (95:5, v/v) through the

Fig. 3. Change of carboxydismutase activity in formate-grown *P. oxalaticus* plotted against the number of generations grown in oxalate medium after adaptation. 10 mM-Formate was added after the seventh generation was harvested.

Fig. 4. Variation with time of the percentage distribution of ¹⁴C from [¹⁴C]oxalate incorporated into the constituents of the ethanol-soluble fraction of *Pseudomonas oxalaticus* growing on oxalate. Conditions were as described in Table 1. ○, Phosphoglycerate; ●, glycine; △, phosphates of glucose, fructose and sedoheptulose; ▲, alanine; ×, glutamate.

Table 1. Distribution of radioactive carbon amongst the non-volatile labelled components of the ethanol-soluble fractions of growing suspensions of *Pseudomonas oxalaticus* incubated with sodium [¹⁴C]oxalate

Sodium [¹⁴C]oxalate (10 μmoles; 200 μC of ¹⁴C) was added at zero time to 10 ml. of a suspension of bacteria (20 mg. dry wt.) vigorously aerated in 10 mM-sodium oxalate, 10 mM-potassium phosphate, pH 7.0. Samples (1 ml.) were withdrawn by hypodermic syringe at known times and transferred into 3 ml. of hot ethanol. The precipitates were each extracted with 1 ml. of hot aqueous 20% (v/v) ethanol and the extracts were analysed by two-dimensional chromatography. The radioactive areas were located by radioautography and directly counted with a mica end-window Geiger-Müller tube.

Time (sec.)	Radioactivity (counts/min.)								
	Phospho-glyceric acid	Glucose phosphates	Fructose phosphates	Sedoheptulose phosphates	Glycine	Glutamate	Alanine	Other compounds	Total
3	136	0	0	0	340	0	0	38	514
10	224	36	41	20	206	0	0	67	594
16	243	75	51	30	237	0	172	118	926
22	162	168	64	30	265	0	135	103	927
46	250	300	138	50	268	130	328	237	1701

bacterial suspension, which suggested that carbon dioxide partial pressure may affect carboxydismutase synthesis. However, no significant change in carboxydismutase activity in *P. oxalaticus* growing on oxalate was found when the bacterial suspension was aerated with air + CO₂ (95:5, v/v) instead of air alone, even when the cells had adapted from growth on formate.

A comparison between the effect of addition of formate to the growth medium and aeration with air + CO₂ (95:5, v/v) upon the carboxydismutase activity in pentose-grown *E. coli* was made to see whether formate had the same effect as increased CO₂ partial pressure as noted by Fuller & Gibbs. However, no appreciable carboxydismutase activity was observed under either of the two growth conditions with ribose-grown *E. coli* (NCIB no. 8571).

Incorporation of [¹⁴C]oxalate into cells growing on oxalate. A growing suspension of *P. oxalaticus* was incubated with [¹⁴C]oxalate and the course of incorporation of the ¹⁴C into the cells was followed by chromatographic and radioautographic techniques. After incubation for 2 min. over 30 labelled compounds were found in the aqueous ethanol-soluble fractions of the cells. The distribution of radioactivity amongst the labelled compounds formed during the first 46 sec. of incubation is shown in Table 1. The curves obtained by plotting the percentage of the total radioactivity contributed by each constituent of the sample against time of incubation are shown in Fig. 4. Radioactive malate was also formed but it has been included under the heading 'Other compounds' in Table 1, owing to the presence of traces of a radioactive impurity in the [¹⁴C]oxalate. This impurity chromatographed together with authentic malate in all solvents tried and masked the time at which [¹⁴C]malate was formed in the cells. The only labelled compounds formed in 3 sec. and isolated under these conditions were glycine and PGA. During the incubation period of 3–10 sec., the percentage of radioactivity contained in glycine diminished while that in PGA increased. This shows that isotope from [¹⁴C]oxalate enters glycine more rapidly than PGA. Other metabolites such as sugar phosphates, alanine and glutamate become labelled later. The following work with cell-free extracts indicates the possible nature of these initial trans-formations.

Formation of glycerate in cell-free extracts

Formation of glycerate from glyoxylate. Crude cell-free extracts prepared from the organism catalyse a rapid anaerobic decarboxylation of glyoxylate in which 1 mole of CO₂ is formed from 2 moles of glyoxylate, a reaction similar to that discovered in *E. coli* by Krakow & Barkulis (1956). These workers suggested a C₃ compound such as

hydroxypyruvate or tartronic acid semialdehyde as being the reaction product:

When [1-¹⁴C]glyoxylate was incubated anaerobically with an extract prepared from the cells by disruption in a Hughes's (1951) press, the formation of radioactive glycerate was found (Table 2). The yield of glycerate was increased by the addition of reduced diphosphopyridine nucleotide (DPNH).

These reactions are similar to those found in glycollate-grown *Pseudomonas ovalis* Chester by

Table 2. Formation of [¹⁴C]glycerate from [1-¹⁴C]glyoxylate

The complete system contained, in a Thunberg tube: 100 μmoles of potassium phosphate-sodium phosphate, pH 6.9; 20 μmoles of MgCl₂; 10 μmoles of GSH; 2 μmoles of DPNH; 1.2 μmoles of sodium [1-¹⁴C]glyoxylate (0.25 μC); 0.25 ml. of bacterial extract and water to 2.4 ml. [The extract was prepared by disrupting 1 g. of bacteria in 3 ml. of phosphate buffer (0.03 M; pH 6.9) in a Hughes's (1951) press cooled to -25°; the resulting extract was centrifuged at 25 000 g for 20 min. and the residue discarded.] The evacuated Thunberg tubes were incubated at 30° for 15 min. and the reaction was stopped by freezing the tubes in liquid air, opening them and adding 8 ml. of absolute ethanol. After the tubes had thawed, the precipitated protein was centrifuged, washed with 0.2 ml. of aqueous ethanol (20%, v/v) and the combined supernatants were evaporated at 30° under reduced pressure of N₂. The products were analysed by chromatography and radioautography.

Contents of tubes	Radioactivity (counts/min.) in glycerate
Complete system	5650
DPNH omitted	2380
Boiled extract used	0

Table 3. Formation of [¹⁴C]glycerate from [¹⁴C]oxalate

Experimental conditions were the same as those described in Table 2. The complete system contained, in a Thunberg tube: 100 μmoles of potassium phosphate-sodium phosphate, pH 6.9; 20 μmoles of MgCl₂; 10 μmoles of GSH; 2 μmoles of DPNH; 0.25 μmole of acetyl-CoA; 2 μmoles of sodium [¹⁴C]oxalate (1.25 μC); 0.25 ml. of bacterial extract; water to 2.4 ml.

Contents of tubes	Radioactivity (counts/min.) in glycerate
Complete system	710
DPNH omitted	60
Acetyl-CoA omitted	159
DPNH and acetyl-CoA omitted	60
Boiled extract used	0

Kornberg & Gotto (1959), and postulated in a pseudomonad grown on glycine (Callely & Dagley, 1959), and demonstrate a reductive conversion of glyoxylate into glycerate.

Formation of glycerate from oxalate. Formation of [^{14}C]glycerate from [^{14}C]oxalate was also observed in these extracts in the presence of acetyl-coenzyme A (acetyl-CoA) and DPNH (Table 3). No other product was found on the chromatograms and addition of these cofactors separately resulted in little synthesis of glycerate. These results indicate that the following reactions may occur in the crude extract under the test conditions:

Reaction (2), involving an acetyl-CoA transphorase, would lead to oxalyl-CoA, evidence for the existence of which has previously been presented for a bacterium growing in the presence of oxalate (Jakoby, Ohmura & Hayaishi, 1956). The reduction of an acyl-CoA grouping to an aldehyde grouping, as suggested in (3), has already been demonstrated (Burton & Stadtman, 1953) in extracts of *Clostridium kluyveri* with acetyl-CoA:

The over-all effect of reactions (2)–(4) would be to condense oxalate reductively to glycerate and CO_2 :

DISCUSSION

The changes in enzyme activity observed when *P. oxalaticus* adapts from growth on formate to growth on oxalate, and vice versa, show that the ribulose 1:5-diphosphate cycle of carbon dioxide fixation is not used to any major extent during growth on oxalate. The two enzymes that are unique to the cycle, namely phosphoribulokinase and carboxydismutase, are synthesized during growth on formate but are not synthesized (except in two special instances) during growth on oxalate. This shows that a different pathway of carbon assimilation is operating during growth on these two substrates. The conclusion is borne out by the finding that [^{14}C]formate and [^{14}C]bicarbonate are incorporated only to a minor extent during growth on oxalate.

The possibility, nevertheless, remained that during the adaptation of the formate-grown organism to growth on oxalate the synthesis of enzyme(s) catalysing a decarboxylation of oxalate to formate and carbon dioxide, or a complete oxidation to carbon dioxide, might enable the

formate-grown cell to use its existing enzymic apparatus temporarily to incorporate carbon dioxide by the ribulose 1:5-diphosphate cycle in an oxalate growth medium, before control mechanisms stopped the synthesis of key enzymes of this cycle in order to deflect the oxalate carbon into a more favourable assimilation pathway. The synthesis of carboxydismutase observed during the first generation of growth in oxalate by cells previously grown in formate suggested that this might be occurring. However, cells harvested at this particular stage fixed only small quantities of [^{14}C]bicarbonate, compared with the formate-grown organisms

(J. R. Quayle & P. J. Large, unpublished observation), showing that despite the availability both of carbon dioxide and the enzymes necessary to incorporate it by the ribulose 1:5-diphosphate cycle, the operation of the cycle is inhibited.

The factors regulating carboxydismutase synthesis in cells growing on oxalate, subsequent to growth on formate, are not at present understood and need further investigation. The enzyme is synthesized by the cells during the first generation of growth on oxalate subsequent to their adaptation from formate. It seems unlikely that formate can be present in the growth medium after the lag period of 10 hr. that is entailed in the adaptation

from growth on formate to growth on oxalate. Both formate- and oxalate-grown cells possess a formic dehydrogenase which would presumably enable them to oxidize the residual formate to carbon dioxide. In succeeding generations the enzyme is not longer synthesized but is 'diluted out'. These cells after seven generations of growth in the oxalate medium appear to possess some property different from cells which have been growing for a long time on oxalate. This is manifested by a rapid synthesis of carboxydismutase in response to addition of formate to the oxalate growth medium in the cells de-adapted from growth on formate, but not in the oxalate-grown cells. It is not known through how many bacterial generations of growth on oxalate, subsequent to growth on formate, this effect persists. Similar effects may have been observed recently by McCarthy (1959) during a study of the adaptation of *Aerobacter aerogenes* to growth on glucose or succinate. During the adaptation of this organism from growth on glucose to growth on succinate, the succinic-dehydrogenase activity rose approximately 20-fold after a lag period of 14 hr. Transfer of succinate-

grown cells to a glucose medium caused the succinic-dehydrogenase activity to fall to its original level, but not so rapidly as simple dilution of the cell contents with the normal material of glucose-grown cells would require, suggesting that the succinic-dehydrogenase system continued to be formed in excess of normal requirements for some time. When the cells were transferred to succinate medium after three generations of growth in the glucose medium they showed rapid synthesis of succinic dehydrogenase with no lag period, in contrast with cells grown a long time on glucose.

Apart from the special case discussed above, formate appears to act as an inducer of carboxydismutase synthesis only when cells are resuspended in a formate growth medium. If formate is added to a growth medium containing a carbon substrate other than formate, in which the organism is already growing, no detectable carboxydismutase synthesis takes place. No evidence was found for increased partial pressure of carbon dioxide raising the activity of carboxydismutase in *P. oxalaticus* or ribose-grown *E. coli*. The latter finding contrasts with the report of Fuller (1956) and Fuller & Gibbs (1959) of such an effect in xylose- or arabinose-grown *E. coli*.

The results of kinetic analysis of the entry of [^{14}C]oxalate into *P. oxalaticus* growing on oxalate suggest that carbon atoms are incorporated by a pathway which is characterized by the chain of metabolites shown in Scheme 1 (volatile intermediates would not have been detected):

Scheme 1

Studies with cell-free extracts indicate that the transformation of oxalate into PGA may occur by reduction of an 'activated' oxalate to glyoxylate, condensation and decarboxylation of 2 molecules of glyoxylate to 1 molecule of a C_3 compound which is then reduced and phosphorylated to PGA. This series of reactions would then place the glycine in Scheme 1 as arising from glyoxylate; the latter compound, whose presence would not be detected satisfactorily in the chromatographic and radioautographic analyses, actually being on the main pathway and not glycine.

Such a sequence of reactions has many features in common with that proposed for the growth of *Pseudomonas* species on glycolate (Kornberg & Gotto, 1959) and glycine (Callely & Dagley, 1959) respectively. The essential difference lies in the

reduction of oxalate to glyoxylate, the latter compound being the first common intermediary metabolite of these three growth substrates.

The mechanism of the suggested oxalate activation is currently under investigation. The work with a cell-free extract implicates oxalyl-CoA by a transphorase reaction with acetyl-CoA. However, this cannot be the system used for oxalate activation by the whole cell as little acetate activation in the presence of ATP and CoA can be observed in cell-free extracts. Similarly, no direct oxalate activation in the presence of these cofactors has been observed. Recent work indicates that the oxalate is activated instead through a transphorase reaction with succinyl-CoA.

SUMMARY

1. The incorporation of carbon atoms from [^{14}C]oxalate, [^{14}C]formate or [^{14}C]bicarbonate into the aqueous ethanol-soluble fraction of *Pseudomonas oxalaticus* (OX 1) grown on oxalate has been studied.

2. Only isotope from [^{14}C]oxalate appeared in appreciable quantities in the aqueous ethanol-soluble fraction of the cells during 10 min. incubation periods. This shows that carbon dioxide-fixation processes, similar to those found to occur during growth of the organism on formate, do not play a major part during growth on oxalate.

3. This conclusion is supported by the findings that carboxydismutase and phosphoribulokinase are synthesized during growth on formate but not (except under special conditions) during growth on oxalate.

4. Carboxydismutase synthesis has been observed in cells growing on oxalate only if they have previously been growing on formate. Such cells continue to synthesize carboxydismutase during the first generation of growth on oxalate subsequent to their adaptation from growth on formate. Addition of formate to the oxalate growth medium leads to resumed enzyme synthesis. No enzyme is formed if the cells have not been grown previously on formate.

5. The radioactivity incorporated at the earliest times (3 sec.) from [^{14}C]oxalate was present mainly in glycine (65%) and 3-phosphoglyceric acid (25%). After incubation for 45 sec., radioactivity also appeared in alanine, glutamate, malate and phosphates of glucose, fructose and sedoheptulose.

6. The data show that the isotope enters glycine much more rapidly than it enters phosphoglycerate.

7. Cell-free extracts catalyse the formation of glyceric acid and carbon dioxide by a diphosphopyridine nucleotide-linked reductive condensation

of glyoxylate, or oxalate, in the presence of acetyl-coenzyme A.

8. It is suggested that during growth on oxalate, the oxalate, possibly in the form of oxalyl-coenzyme A, is reduced to glyoxylate, which is taken to be the precursor of glycine and phosphoglycerate. The phosphoglycerate formed then enters established routes of biosynthesis.

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The Interconversion of Glutamic Acid and Aspartic Acid in Respiring Tissues

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In previous papers (Krebs, 1950; Ternier, Eggleston & Krebs, 1950) it was briefly reported that glutamic acid, when added in excess to respiring animal-tissue preparations, is rapidly converted into aspartic acid, and that aspartic acid, when added in excess, is converted into glutamic acid. Qualitatively these interconversions are not unexpected: both amino acids are oxidized in animal tissues through the tricarboxylic acid cycle and

therefore form α -oxoglutarate and oxaloacetate as intermediates. As animal tissues all contain aspartic-glutamic transaminase, any excess of the added amino acids may be expected to transaminate with the appropriate α -oxo acid arising as an intermediate. Starting with an excess of glutamate, a trace quantity of oxaloacetate present in the tissue, or formed from glutamate, would thus be expected to react as follows: