Biochem. J. (1968) **107**, 705
Printed in Great Britain

Choice between Autotrophy and Heterotrophy in *Pseudomonas oxalaticus*

GROWTH IN MIXED SUBSTRATES

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(*Received* 17 *January* 1968)

1. The type of metabolism adopted by *Pseudomonas oxalaticus* during growth on a variety of carbon sources was studied. 2. The only substrate upon which autotrophic growth was observed is formate. 3. In mixtures of formate and those substrates upon which the organism can grow faster than on formate, e.g. succinate, lactate or citrate, heterotrophic metabolism results. 4. In mixtures of formate and those substrates upon which the organism can grow at a similar rate to that on formate, e.g. glycollate or glyoxylate, the predominant mode of metabolism adopted is heterotrophic utilization of the C_2 substrate coupled with oxidation of formate as ancillary energy source. 5. *P. oxalaticus* grows on oxalate 30% slower than on formate. In mixtures of formate and oxalate, the predominant mode of metabolism adopted is autotrophic utilization of formate coupled with oxidation of oxalate as ancillary energy source. 6. In mixtures of formate and those substrates upon which the organism grows at a much lower rate than on formate, e.g. glycerol and malonate, the predominant mode of metabolism adopted is autotrophic utilization of formate. 7. It is concluded that synthesis of the enzymes involved in autotrophic metabolism is controlled by a combination of induction and metabolite repression.

The only carbon growth substrate on which it is known that *Pseudomonas oxalaticus* grows autotrophically is formate; the two key enzymes of autotrophic metabolism, ribulose diphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] and phosphoribulokinase (ATP–D-ribulose-5-phosphate 1-phosphotransferase, EC 2.7.1.19), are present in extracts of the organism grown on formate but are not detectable in extracts of cells grown on oxalate, lactate or succinate as carbon source (Quayle & Keech, 1960). This paper describes an extension of these studies over a wider range of single growth substrates and also examines the type of metabolism that is adopted by the organism when it is growing on a mixture of formate and a second substrate.

METHODS AND MATERIALS

Maintenance and growth of organism. Cultures of *P. oxalaticus* (OX1) were maintained as described by Blackmore, Quayle & Walker (1968). The medium used for liquid growth was a modification of that used by Jayasuriya (1955). The final concentrations in the medium (mg./100ml.) were: $(NH_4)_2SO_4$, 50; $MgSO_4,7H_2O$, 20; $CaCl_2,6H_2O$, 1·3; $FeSO_4,7H_2O$, 0·5; $MnSO_4,4H_2O$, 0·225; $Na_2MoO_4,2H_2O$, 0·25; K_2HPO_4, 290; NaH_2PO_4, 260. Carbon sources were used at a concentration of 0·05M. The organism was grown in 600ml. of medium contained in 2l. Erlenmeyer flasks, which were shaken on a rotary shaker at 30°. Growth was followed by measurement of extinction at 540mμ in a spectrophotometer (Unicam model SP.600). Growth rates were obtained by plotting $\log_2$(extinction) [or cell density (μg./ml.)] against time (Monod, 1949).

Manometric methods. Uptake of O_2 was measured in conventional Warburg manometers at 30° with air as the gas phase. A washed suspension of cells, containing 4–6mg. dry wt. of bacteria/ml., was added to 150μmoles of 50mM-sodium phosphate buffer, pH7·0, in the main compartment of the Warburg flask; the final volume in the flask was 3ml. The centre well contained 0·1ml. of 4M-KOH. A solution of substrate (usually 0·4ml. of 0·1M) was placed in the side arm. Equilibration was carried out for 10min., and gas uptake was followed for 40min. after the addition of substrate.

Preparation of cell-free extracts. Samples of bacteria, obtained from 600ml. of growth medium, were suspended in 3ml. of 50mM- sodium phosphate buffer, pH7·0. Extracts were then prepared by disrupting the bacteria with an ultrasonic cell disintegrator (MSE, 100w) for 2min. at 0°, followed by centrifuging at 18000*g* for 10min. in a refrigerated centrifuge. The extracts contained 1·5–6mg. of protein/ml.

Assays of enzymes. Formate dehydrogenase (formate–NAD oxidoreductase, EC 1.2.1.2). The method of Johnson, Jones-Mortimer & Quayle (1964) was used. This assay is based on spectrophotometric measurement of the rate of reduction of NAD^+. The reaction was followed at 340mμ at

30° in a recording spectrophotometer (Beckman model DB). One unit of activity is defined as that amount of enzyme that catalyses the reduction of 1 μmole of NAD^+ in 1 min. under the assay conditions.

Oxalyl-CoA reductase [glyoxylate–NADP oxidoreductase (acylating CoA), EC 1.2.1.17]. The method of Quayle & Taylor (1961) was used. This is based on the spectrophotometric measurement of the rate of reduction of $NADP^+$ in the presence of glyoxylate and CoA at 25°. One unit of enzyme activity is defined as that amount of enzyme that catalyses the reduction of 1 μmole of $NADP^+$ in 1 min. under the assay conditions.

Oxalyl-CoA decarboxylase (oxalyl-CoA carboxy-lyase, EC 4.1.1.8). The method of Quayle (1963*b*) was used. This involves the measurement of the rate of evolution of CO_2 in micromanometer cups under N_2 at 30° in the presence of oxalyl-CoA and thiamine pyrophosphate. One unit of activity is defined as that amount of enzyme that catalyses the evolution of 1 μmole of CO_2 in 1 min. under the assay conditions.

Ribulose diphosphate carboxylase (EC 4.1.1.39). The method of Weissbach, Horecker & Hurwitz (1956) was used. A sample of cell-free extract usually containing 0·1–0·5 mg. of total protein was incubated for 15 min. at 30° with 50 μmoles of tris hydrochloride buffer, pH 7·8, 12·5 μmoles of $MgCl_2$, 6 μmoles of GSH, 40 μmoles of $NaHCO_3$, 0·18 μmole of ribulose diphosphate and water to 1 ml. The reaction was stopped by the addition of 0·02 ml. of 1 N-HCl, followed by heating to 100° for 2 min. Denatured protein was removed by centrifuging and a sample of the supernatant (0·2 ml.) was assayed for 3-phosphoglyceric acid by conversion into 1,3-diphosphoglyceric acid with purified phosphoglycerate kinase (ATP–3-phospho-D-glycerate 1-phosphotransferase, EC 2.7.2.3) and ATP, and reduction to glyceraldehyde 3-phosphate with NADH and glyceraldehyde 3-phosphate dehydrogenase [D-glyceraldehyde 3-phosphate–NAD oxidoreductase (phosphorylating), EC 1.2.1.12]. The oxidation of NADH was followed spectrophotometrically at 340 mμ. One unit of activity is defined as that amount of enzyme that catalyses the carboxylation of 1 μmole of ribulose diphosphate/min. under the assay conditions.

Glyoxylate carboligase. This enzyme was assayed manometrically by measuring the rate of anaerobic evolution of CO_2 from glyoxylate. Double-armed Warburg flasks contained 50 μmoles of sodium phosphate buffer, pH 7·0, bacterial extract and water, to a final volume of 2·0 ml. The flasks were flushed with O_2-free N_2. After equilibration at 30°, 0·2 ml. of sodium glyoxylate (0·2 M) was added from the first side arm. The reaction was terminated after 20 min. by the addition of 0·2 ml. of 2 N-H_2SO_4 from the second side arm. One unit of activity is defined as that amount of enzyme that catalyses the evolution of 1 μmole of CO_2/min. under the assay conditions.

Definition of specific activity. All specific activities are expressed as units of enzyme activity/mg. of protein.

Chemical determinations. Oxalate was estimated in culture media by titration against standard $KMnO_4$ (0·1 N). Samples (3 × 3 ml.) were each heated to 60° with 1 ml. of 2 N-H_2SO_4 and titrated against the $KMnO_4$ soln.

Glycollate was estimated by the method of Dagley & Rogers (1953). This involves heating with conc. H_2SO_4, when the glycollate decomposes to CO_2, H_2 and formaldehyde. The last-named is then estimated by combination with chromotropic acid.

Formate was estimated by the use of purified formate dehydrogenase obtained from oxalate-grown *P. oxalaticus* as described by Johnson *et al.* (1964). Purified enzyme (0·1–0·2 unit) was used to assay up to 0·08 μmole of formate.

Glyoxylate was assayed by using purified lactate dehydrogenase (L-lactate–NAD oxidoreductase, EC 1.1.1.27) (Meister, 1950, 1952). Glyoxylate is reduced to glycollate in the presence of NADH. The total decrease in extinction at 340 mμ was equivalent to the amount of glyoxylate present in the sample.

Protein was determined by the Folin–Ciocalteu method, as described by Lowry, Rosebrough, Farr & Randall (1951).

Special chemicals. Oxalyl-CoA was prepared by the method of Quayle (1963*a*), which involves the thiol–ester interchange between CoA and thiocresyl hydrogen oxalate, followed by ether extraction.

Ribulose 1,5-diphosphate was prepared enzymically from ribose 5-phosphate by the method described by Horecker, Hurwitz & Weissbach (1958). The barium salt of ribulose 1,5-diphosphate was converted into the sodium salt by the addition of 0·2 N-H_2SO_4 until precipitation of $BaSO_4$ was complete, followed by neutralization with 0·01 N-NaOH. Purified enzymes, nicotinamide nucleotides and ATP were obtained from C. F. Boehringer und Soehne, G.m.b.H. Mannheim, Germany.

RESULTS

Growth on formate, oxalate and mixtures of the two substrates

Comparison of metabolism of formate or oxalate as single substrates. The data in Table 1 show that *P. oxalaticus* grows on formate faster than it does on oxalate. The formate-grown organism has a much higher Q_{O_2} with respect to formate than has the oxalate-grown organism with respect either to formate or to oxalate. The specific activity of formate dehydrogenase in an oxalate-grown cell is four times that in a formate-grown cell. It should thus be noted that, despite the much greater

Table 1. *Comparison of metabolism of formate and oxalate by* P. oxalaticus

Growth substrate	Mode of metabolism	Mean generation time (hr.)	Q_{O_2} (μl. of O_2/hr./mg. dry wt. of cells)		Formate dehydrogenase (units/mg. of protein)
			Formate	Oxalate	
Oxalate	Heterotrophic	4·5	49	108	1·4
Formate	Autotrophic	3·25	240	—	0·37

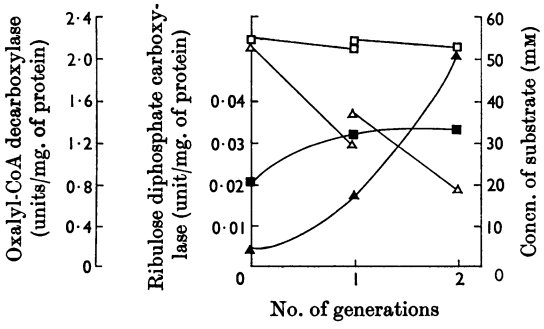

Fig. 1. Metabolism of formate and oxalate by formate-grown *P. oxalaticus*. Formate-grown *P. oxalaticus* was suspended in an equimolar mixture of formate and oxalate and grown for one generation at 30°, as described in the text. The culture was diluted with an equal volume of fresh growth medium and grown for a second generation. Enzyme activities of the organism and the concentration of formate and oxalate in the medium were measured as described in the Methods and Materials section. ■, Specific activity of ribulose diphosphate carboxylase; ▲, specific activity of oxalyl-CoA decarboxylase; □, concn. of oxalate; △, concn. of formate.

economy in terms of energy required for heterotrophic assimilation of oxalate compared with autotrophic assimilation of formate carbon atoms (Blackmore *et al.* 1968), the cell is able to grow on formate considerably faster than it can on oxalate.

Metabolism of a mixture of formate and oxalate by a formate-grown cell. When *P. oxalaticus* is harvested during the period of exponential growth on formate and then resuspended in a growth medium containing an equimolar mixture of formate and oxalate, growth recommences without detectable lag. It seems very likely that at this stage the organism commences to grow autotrophically on formate, as it already possesses the enzymes necessary to do this whereas it does not contain appreciable quantities of the enzymes necessary to metabolize oxalate. Since the organism can grow faster on formate than on oxalate, it is to be expected that on continued growth in a mixture of formate and oxalate the predominant type of metabolism will continue to be autotrophic utilization of formate. The following experiment was designed to test this and to ascertain the utilization, if any, of oxalate under these conditions.

A culture of formate-grown *P. oxalaticus* (600ml., 0·12mg. dry wt./ml.) was harvested by centrifuging and the bacteria were resuspended to a final volume of 9ml. in growth medium containing 50mM-sodium formate and 50mM-sodium oxalate as energy source. A portion (3ml.) was withheld for assay of initial enzyme activities and the remainder was added to 600ml. of the same medium contained in a 2l. conical flask. The cell density of this suspension was 0·06mg. dry wt./ml. The suspension was shaken for 4hr. at 30° on a rotary shaker until the cells had grown through one generation to a density of 0·13mg. dry wt./ml. At this point 90ml. of the suspension was withdrawn and the cells in it were harvested by centrifuging and retained for assay of enzyme activities. A further 300ml. of the suspension was withdrawn and added to 300ml. of fresh medium, bringing the cell density back to 0·065mg. dry wt./ml. The cells were then grown through a second generation and harvested for assay of enzyme activities. During the experiment, the concentrations of oxalate and formate in samples of the growth medium were determined. The results are shown in Fig. 1.

It can be seen that the formate concentration in the growth medium dropped at approx. 20 μmoles/ml./generation compared with 2 μmoles/ml./generation for oxalate. Thus formate utilization is clearly the major type of metabolism adopted over two generations of growth. This may be correlated with the continued synthesis of one of the key enzymes of autotrophic metabolism, ribulose diphosphate carboxylase, during the experiment. The utilization of oxalate may be correlated with synthesis of oxalyl-CoA decarboxylase, a key enzyme of oxalate catabolism. No synthesis of oxalyl-CoA reductase could be detected, indicating that oxalate was not being used as a source of carbon atoms for cell constituents.

Metabolism of a mixture of formate and oxalate by an oxalate-grown cell. The converse experiment to the preceding one was carried out, in which the adaptation of oxalate-grown *P. oxalaticus* to growth in equimolar formate–oxalate medium was followed.

For this experiment, a culture of oxalate-grown *P. oxalaticus* (600ml., 0·22mg. dry wt./ml.) was harvested and the bacteria were resuspended to a final volume of 10ml. in growth medium containing 50mM-sodium formate and 50mM-sodium oxalate as energy source. A portion (6ml.) was withheld for assays of initial enzyme activity and the remainder was added to 600ml. of the same medium contained in a 2l. flask. The cell density of this suspension was 0·08mg. dry wt./ml. The suspension was shaken for 4½hr. at 30° on a rotary shaker, after which time the cell density had increased to 0·16mg. dry wt./ml. At this point, the cells in 90ml. were harvested by centrifuging and retained for assay of enzyme activities. A further 300ml. of the suspension was withdrawn and added to 300ml. of fresh medium, bringing the cell density back to 0·07mg. dry wt./ml. The cells were then grown through a second generation and harvested for assay of enzyme activities. During the experiment the concentrations of formate and oxalate in the growth medium were followed as in the previous

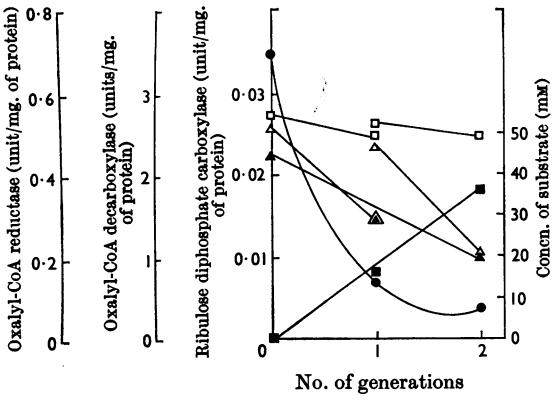

Fig. 2. Metabolism of formate and oxalate by oxalate-grown *P. oxalaticus*. Oxalate-grown *P. oxalaticus* was suspended in an equimolar mixture of formate and oxalate and grown for one generation at 30°, as described in the text. The culture was diluted with an equal volume of fresh growth medium and grown for a second generation. Enzyme activities of the organism and the concentration of formate and oxalate in the growth medium were measured as described in the Methods and Materials section. ●, Specific activity of oxalyl-CoA reductase; ▲, specific activity of oxalyl-CoA decarboxylase; ■, specific activity of ribulose diphosphate carboxylase; □, concn. of oxalate; △, concn. of formate.

experiment. The results are shown in Fig. 2. The formate concentration dropped at 22 μmoles/ml. during the first generation and at 26 μmoles/ml. during the second generation. Hence, as before, formate utilization is the major type of metabolism adopted over two generations of growth. A switch-over to autotrophic metabolism is indicated by the synthesis of ribulose diphosphate carboxylase. Slowing down of oxalate utilization both as source of energy and source of carbon atoms for cell constituents is indicated by the decrease in specific activities of oxalyl-CoA decarboxylase and oxalyl-CoA reductase respectively.

In earlier work, Quayle & Keech (1960) detected synthesis of ribulose diphosphate carboxylase in *P. oxalaticus* when formate was added to a suspension of the organism growing on oxalate after the organism had grown for seven generations on oxalate after adaptation from growth on formate, but were unable to detect enzyme synthesis if the organism had not been growing previously on formate. Such behaviour would not be expected in view of the work now reported, in which ribulose diphosphate carboxylase synthesis was always observed in a growth medium containing both formate and oxalate. This apparent discrepancy may be resolved by comparing the experimental conditions used by Quayle & Keech (1960) with

Table 2. *Prolonged metabolism of a mixture of formate and oxalate by* P. oxalaticus

A drop of either formate-grown or oxalate-grown *P. oxalaticus* was used as inoculum for flasks containing 600 ml. of growth media containing formate, oxalate or mixtures of the two as carbon source. The flasks were incubated at 30° with shaking until the organism had grown to a visible cell density. The bacteria were harvested and assayed for enzyme activity and the concentrations of formate and oxalate in the growth media were measured where indicated. Details of the assays are given in the Methods and Materials section.

Inoculum	Final growth medium	Concn. of oxalate (mM)			Concn. of formate (mM)			Oxalyl-CoA reductase (unit/mg.)	Oxalyl-CoA decarboxylase (units/mg.)	Ribulose diphosphate carboxylase (unit/mg.)
		Before growth	After growth	Difference	Before growth	After growth	Difference			
Formate-grown	Formate (44 mM) + oxalate (56 mM)	56	54	2	44·4	17·3	27·1	Undetectable	1·08	0·01
Oxalate-grown	Formate (51 mM) + oxalate (56 mM)	56	55	1	51	42·8	8·2	Undetectable	3·1	0·01
Formate-grown	Formate (50 mM)							0·01	0·17	0·01–0·04*
Oxalate-grown	Oxalate (50 mM)							0·3–0·4*	2–4·5*	0·0002

* Values obtained in several separate experiments.

those shown in Fig. 2. Quayle & Keech (1960) added formate to a final concentration of 10mM (instead of 50mM, renewed at each generation of growth, as used in the present work) and assayed for synthesis of ribulose diphosphate carboxylase during a period of 2hr. after the addition of formate. From the data of Fig. 2 of the present paper it is clear that these are conditions in which detection of ribulose diphosphate carboxylase would be difficult and unreliable. In 2hr. (approximately one-half of a generation time) the specific activity of the enzyme would have risen little; also, the rate of utilization of formate (22μmoles/ml./generation under the conditions described in the present paper) is such that, at a concentration of 10mM, the formate might well have been used up within the 2hr. period.

Metabolism of a mixture of formate and oxalate over many generations of growth of P. oxalaticus. The above two experiments followed the first two generations of growth of either formate-grown or oxalate-grown *P. oxalaticus* in a mixture of formate and oxalate. The growth rate of the organism makes it technically difficult to extend this type of experiment over many generations of growth. The specific activities of the enzymes being measured were still changing at the end of the experiment; to find out the final pattern adopted by the organism after prolonged growth in the mixed substrate, two flasks containing 600ml. each of the mixed substrate were inoculated with a drop of a suspension of either formate-grown or oxalate-grown *P. oxalaticus*. The organisms were then grown at 30°, harvested, assayed for enzymic activity and the formate and oxalate contents of the supernatant solutions were estimated. The results are shown in Table 2. In the mixed media formate was utilized 8–13 times as fast as was oxalate. The presence in the organism of ribulose diphosphate carboxylase and the virtual absence of oxalyl-CoA reductase shows that formate carbon atoms (as carbon dioxide) were being used biosynthetically whereas oxalate was not. This is also consistent with a later observation that the organism when grown in a mixture of formate and oxalate does not contain appreciable activity of glyoxylate carboligase. Oxalyl-CoA decarboxylase, an indicator of the capacity of the cell to catabolize oxalate, was present at specific activities approaching, or within the range of, those normally encountered in the oxalate-grown organism.

Growth on mixtures of formate and 'slow'-growth substrates

P. oxalaticus grows on either malonate or glycerol as sole carbon source with respective mean generation times of 14 and 24hr. at 30°, i.e. at one-quarter and one-eighth of the rate on formate. In neither case could appreciable activities of either formate dehydrogenase or ribulose diphosphate carboxylase be detected (Table 3). The organism in an equimolar mixture of formate and either malonate or glycerol grew at the same rate as in formate alone, synthesized formate dehydrogenase and ribulose diphosphate carboxylase and utilized all the formate from the growth medium (Table 3).

These results show the occurrence of adaptation from slow heterotrophic growth on either malonate or glycerol to faster autotrophic growth on formate.

Growth on mixtures of formate and 'equi'-growth substrates

The growth rate of *P. oxalaticus* is difficult to measure accurately because of clumping in the bacterial suspensions. Within this experimental uncertainty, the growth rate on glycollate or glyoxylate as sole carbon source could not be distinguished from that on formate. Cells grown on glycollate or glyoxylate contained glyoxylate carboligase, indicating the involvement of the glycerate pathway of biosynthesis as has been found in *Escherichia coli* and several species of *Pseudomonas* (see Kornberg & Elsden, 1961). The type of

Table 3. *Metabolism of malonate, glycerol and formate by* P. oxalaticus

P. oxalaticus was grown for 24hr. at 30° in four separate liquid media (600ml.) containing respectively malonate, formate+malonate, glycerol and formate+glycerol (all at a concn. of 50mM). The cells were harvested and assayed for formate dehydrogenase and ribulose diphosphate carboxylase activity. The concentration of formate in the growth media was measured before and after growth as detailed in the Methods and Materials section.

Growth substrate	Formate dehydrogenase (unit/mg. of protein)	Ribulose diphosphate carboxylase (unit/mg. of protein)	Concn. of formate remaining after growth (mM)
Malonate	0·006	Undetectable	
Glycerol	0·007	Undetectable	
Formate+malonate	0·163	0·011	0·05
Formate+glycerol	0·15*	0·014*	0

* Separate cultures were used for each enzyme assay.

Table 4. *Metabolism of glycollate and formate by* P. oxalaticus

A sample of glycollate-grown *P. oxalaticus* was resuspended at a cell density of 0·15 mg. dry wt./ml. in 600 ml. of growth medium containing a mixture of 50 mM-sodium formate + 50 mM-sodium glycollate as carbon source. The suspension was shaken for 6 hr. at 30° and the bacteria were then harvested by centrifuging. The enzyme activities in the bacteria, and the formate and glycollate concentrations in the growth medium at the beginning and end of the growth period, were measured as described in the Methods and Materials section.

	Sp. activity of enzymes (unit/mg. of protein)			Utilization of substrate (μmoles/ml.)	
Growth substrate	Formate dehydrogenase	Ribulose diphosphate carboxylase	Glyoxylate carboligase	Glycollate	Formate
Glycollate	0·001	Undetectable	0·18		
Glycollate + formate	0·007	Undetectable	0·21	12·4	26·5

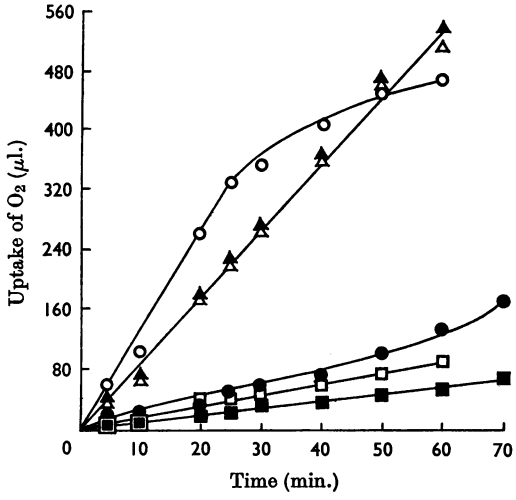

Fig. 3. Oxidation of formate and glycollate by washed suspensions of *P. oxalaticus* grown on glycollate and glycollate plus formate. The main compartments of Warburg vessels contained 150 μmoles of sodium phosphate buffer, pH 7·0, and bacteria (4 mg. dry wt.) washed with 10 mM-sodium phosphate buffer, pH 7·0; the centre wells contained 0·1 ml. of 4 N-KOH; the side arms contained 40 μmoles of either sodium formate or sodium glycollate. The final volume of the flask contents was 3 ml. The flasks were incubated at 30° for 1 hr. ■, Glycollate-grown, endogenous; ▲, glycollate-grown + glycollate; ●, glycollate-grown + formate; □, (glycollate + formate)-grown, endogenous; △, (glycollate + formate)-grown + glycollate; ○, (glycollate + formate)-grown + formate.

metabolism adopted by the organism in equimolar mixtures of formate plus glycollate or glyoxylate was studied by measuring (*a*) the relative rates of utilization of the substrates, (*b*) the activity of glyoxylate carboligase (indicative of heterotrophic utilization of the C_2 substrate), (*c*) the activity of formate dehydrogenase (indicative of formate catabolism), (*d*) the activity of ribulose diphosphate carboxylase (indicative of autotrophic utilization of formate carbon atoms). The results for glycollate and glycollate plus formate are shown in Table 4. The presence of glyoxylate carboligase and the absence of ribulose diphosphate carboxylase shows that the predominant mode of metabolism adopted in the mixed substrate is heterotrophic utilization of glycollate. In view of the very low activity of formate dehydrogenase detected in cell-free extracts of the organism when grown on the mixed substrate, it was surprising to find that formate was rapidly utilized. Repeated attempts to detect a nicotinamide nucleotide-linked formate dehydrogenase in such cells proved negative. These attempts included assay in anaerobic quartz cuvettes under an atmosphere of nitrogen so as to eliminate interference due to NADH oxidase activity. Nevertheless it was found manometrically that, whereas glycollate-grown cells will oxidize formate only after a lag of approximately 30 min., cells grown on a formate–glycollate medium will oxidize formate without lag (Fig. 3). Precisely similar behaviour is observed in growth on a mixture of succinate and formate (see below). It thus appears that the presence of extracellular formate in a medium containing another carbon source can induce the formation of some enzymic system resulting in formate oxidation, this system being quite distinct from the NAD-linked formate dehydrogenase associated with autotrophic utilization of formate. This unknown system has not been investigated further in the present work.

Table 5 shows the results obtained when *P. oxalaticus* is grown on glyoxylate and mixtures of glyoxylate and formate. The specific activity of formate dehydrogenase in the glyoxylate-grown organism is very low, whereas when the organism is grown on formate plus glyoxylate the enzyme is present in it at a specific activity of over half that found in the organism grown on formate alone. It thus seems likely that, in this mixed substrate, formate is functioning as a source of energy through

Table 5. *Metabolism of glyoxylate and formate by* P. oxalaticus

A sample of glyoxylate-grown *P. oxalaticus* was resuspended at a cell density of 0·13mg. dry wt./ml. in 600ml. of growth medium containing a mixture of 50mM-sodium glyoxylate+50mM-sodium formate as carbon source. The suspension was shaken for 6hr. at 30° and the bacteria were then harvested by centrifuging. The enzyme activities in cell-free extracts of the bacteria and the formate and glyoxylate concentrations in the medium at the beginning and end of the growth period were measured as described in the Methods and Materials section.

	Sp. activity of enzymes (unit/mg. of protein)			Utilization of substrate (μmoles/ml.)	
Growth substrate	Formate dehydrogenase	Ribulose diphosphate carboxylase	Glyoxylate carboligase	Glyoxylate	Formate
Glyoxylate	0·01	Undetectable	0·07		
Glyoxylate+formate	0·24	0·003	0·065	5·3	40·8

nicotinamide-nucleotide-linked dehydrogenation. Ribulose diphosphate carboxylase is absent, or present at a very low specific activity, in growth on glyoxylate or the mixed substrate, whereas glyoxylate carboligase is present at similar specific activities in both cases. The predominant mode of metabolism adopted by the organism in the mixed substrate thus appears to be heterotrophic assimilation of glyoxylate accompanied by some utilization of formate, via formate dehydrogenase, as ancillary energy source and possibly by a very limited autotrophic metabolism.

Growth on mixtures of formate and 'fast'-growth substrates

P. oxalaticus grows rapidly on succinate, lactate or citrate with mean generation times of approx. 2hr. Cells grown on any of these substrates alone or in equimolar admixture with formate possess negligible activities of formate dehydrogenase and ribulose diphosphate carboxylase. This shows that no adaptation to autotrophic utilization of formate takes place under these conditions. Despite this, in a growth mixture containing equimolar amounts of formate and one of the three other substrates, formate is utilized by the organism along with the other substrate. The rate of utilization was found to be similar in the three different mixed substrates and was quite rapid; when expressed as the amount of formate consumed/unit increase in the dry weight of the cells, the rate was approx. 60% of that found for formate utilization by the organism when growing in an equimolar mixture of formate and oxalate. Repeated attempts to detect nicotinamide nucleotide-linked formate dehydrogenase activity in cell-free extracts of the organism after growth on these mixed substrates proved negative. That formate from the mixed substrate is oxidized by the organism is indicated by the finding that cells grown on succinate plus formate will oxidize formate manometrically without lag. In contrast, succinate-grown cells will only oxidize formate manometrically after a lag of approx. 30min. This behaviour is thus very similar to that previously described in growth on glycollate and glycollate plus formate, and points to the existence of an inducible system, quite distinct from the nicotinamide nucleotide-linked formate dehydrogenase, that can lead to formate oxidation.

DISCUSSION

The results presented in this paper show that the enzymes formate dehydrogenase and ribulose diphosphate carboxylase, necessary for autotrophic growth on formate, are synthesized by *P. oxalaticus* when faced with either formate alone or a mixture of formate and a 'slow'-growth substrate, i.e. one on which the organism can only grow much more slowly than on formate alone. The response to the presence of formate suggests that this compound or a derivative of it may function as an inducer of these enzymes. However, the enzymes are not synthesized by the organism growing on a mixture of formate and a 'fast'-growth substrate such as succinate, citrate or lactate. This suggests that, in addition, control by some form of metabolite repression must operate. It has been suggested (Magasanik, 1961; Mandelstam, 1961) that metabolite repression may operate as a result of the increased pool size of some metabolite consequent on rapid growth on a rich substrate, e.g. glucose. In synthesis of β-galactosidase by *Escherichia coli* it was found that glucose was not a unique source of metabolite repressor; it differed only quantitatively from other substrates such as succinate and lactate (Mandelstam, 1962). The property of being a good growth substrate, i.e. capable of supporting rapid growth, was correlated with ability to repress the enzyme synthesis: the better the growth substrate, the more effective it was as a source of co-repressor. The analogy with the behaviour encountered in regulation of synthesis of the enzymes involved in autotrophic metabolism in *P. oxalaticus* is clear.

The mean generation time of *P. oxalaticus* on formate is 3½hr. It is thus reasonable that the

presence of 'fast'-growth substrates supporting doubling times of approx. 2hr. (e.g. succinate, citrate and lactate) should result in the repression of autotrophic enzymes, whereas 'slow'-growth substrates supporting doubling times of over 12hr. (e.g. glycerol and malonate) should not lead to repression.

A more complex situation exists when the organism is faced with a mixture of formate and a substrate permitting a closely similar doubling time to that on formate (e.g. glycollate or glyoxylate). On the one hand, the energy required to assimilate the C_2 substrate is very much less than that required to assimilate the C_1 substrate, and also the C_2 substrate is potentially a better energy source than is formate; hence, on energetic grounds, heterotrophy is preferable to autotrophy. On the other hand, the growth rates on the different substrates are closely similar and thus the pool sizes of the metabolite repressors may not be sufficient to overcome the inducing action of the formate; hence autotrophic enzymes might be synthesized. In the event, the metabolism adopted is predominantly a heterotrophic utilization of the C_2 substrate.

A gradation of metabolism occurs in mixtures of formate and each of the C_2 substrates glycollate, glyoxylate and oxalate: (*a*) with glycollate, heterotrophic utilization of the C_2 substrate; (*b*) with glyoxylate, predominantly heterotrophic utilization of the C_2 substrate, utilization of formate as an ancillary energy source via formate dehydrogenase and possibly as a substrate for a very limited autotrophic metabolism; (*c*) with oxalate, autotrophic utilization of formate as both carbon and energy source, utilization of oxalate as ancillary energy source. Thus as the oxidation level of the C_2 substrate rises, it becomes of diminishing importance as a substrate relative to formate. In fact, the C_2 and C_1 substrates interchange roles of being main carbon source with that of merely being ancillary energy source.

It is not known what metabolites actually function as inducers or co-repressors of synthesis of formate dehydrogenase and ribulose diphosphate carboxylase, although the apparent simplicity of the substrates and products of the former enzyme invites further study of this question. The fact that formate dehydrogenase can be synthesized when ribulose diphosphate carboxylase is not, e.g. in growth on mixtures of formate and oxalate, shows that these two enzymes are not under the control of a single regulon. Also, since formate is utilized by an unknown enzyme system to some extent in the presence of all substrates tested, it is clear that the repression exerted by 'fast'-growth substrates is not due to exclusion of formate from the cell. Hurlbert (1966) has suggested that synthesis of phosphoribulokinase is subject to catabolite repression during growth of *Chromatium* on a variety of organic compounds. McFadden & Tu (1967) have also suggested that growth of *Hydrogenomonas facilis* on some organic substrates results in repression of ribulose diphosphate carboxylase and phosphoribulokinase.

The ability of the organism to grow faster autotrophically on formate than heterotrophically on oxalate, despite the greater energy demands of the former type of metabolism, is of interest, especially since metabolism of the two substrates involves a common source of energy. This choice of autotrophy may be dictated by the much greater rate of formate catabolism that is possible compared with that of oxalate catabolism, the Q_{O_2} for formate being $2\frac{1}{2}$ times that for oxalate. This illustrates that maximal growth rate is the guiding principle in bacterial metabolism rather than maximal efficiency (Pardee, 1961). Also related to this problem is the fact that formate added to the growth medium causes induction of ribulose diphosphate carboxylase in the organism growing on oxalate, yet formate produced intracellularly from the catabolism of oxalate fails to induce the synthesis of the enzyme. The contrast in behaviour may be due to a lower intracellular concentration of formate during growth on oxalate compared with growth on formate, arising from the fact that the specific activity of the terminal enzyme of oxalate catabolism, formate dehydrogenase, is higher in an oxalate-grown cell than in a formate-grown cell; yet the overall rate of oxidation of oxalate by an oxalate-grown cell is lower than that of formate by a formate-grown cell.

The metabolism of *P. oxalaticus*, under certain conditions, instances combinations of features of both heterotrophic and autotrophic metabolism. Similar behaviour is found elsewhere, e.g. the utilization of organic matter by chemoautotrophic organisms (Butler & Umbreit, 1966; Borichewski & Umbreit, 1966; Delwiche & Finstein, 1965; Ida & Alexander, 1965; London & Rittenberg, 1966; Banerjee & Schlegel, 1966; Kelly, 1967; Smith & Hoare, 1968), the functioning of the ribulose diphosphate cycle during growth of photosynthetic bacteria on organic substrates (Lascelles, 1960; Hurlbert & Lascelles, 1963); the photoassimilation of acetate by blue–green algae (Pearce & Carr, 1966; Hoare, Hoare & Moore, 1967) or *Chromatium* (Fuller, Smillie, Sisler & Kornberg, 1961) and the presence of ribulose diphosphate carboxylase in *Hydrogenomonas facilis* (McFadden, Kuehn & Homann, 1967) or *Hydrogenomonas* H16 (Gottschalk, Eberhardt & Schlegel, 1964) during growth in the presence of some organic substrates. Such studies as these are now leading towards a re-evaluation of the classical distinction between heterotrophic and autotrophic metabolism, and a

revision of the older notion that the two modes of metabolism are mutually exclusive.

This work was supported in part by the U.S. Air Force under Grant no. AF-EOAR-64-8, through the European Office, Office of Aerospace Research, and was done during the tenure of a Science Research Council Studentship (M.A.B.).

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