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Microbial Growth on Oxalate by a Route not Involving Glyoxylate Carboligase

BY MAUREEN A. BLACKMORE* AND J. R. QUAYLE
Department of Microbiology, University of Sheffield, Western Bank, Sheffield S10 2TN, U.K.

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1. The metabolism of oxalate by the pink-pigmented organisms, *Pseudomonas* AM 1, *Pseudomonas* AM 2, *Protaminobacter ruber* and *Pseudomonas extorquens* has been compared with that of the non-pigmented *Pseudomonas oxalaticus*. 2. During growth on oxalate, all the organisms contain oxalyl-CoA decarboxylase, formate dehydrogenase and oxalyl-CoA reductase. This is consistent with oxidation of oxalate to carbon dioxide taking place via oxalyl-CoA, formyl-CoA and formate as intermediates, and also reduction of oxalate to glyoxylate taking place via oxalyl-CoA. 3. The pink-pigmented organisms, when grown on oxalate, contain L-serine–glyoxylate aminotransferase and hydroxypyruvate reductase but do not contain glyoxylate carboligase. The converse of this obtains in oxalate-grown *Ps. oxalaticus*. This indicates that, in contrast with *Ps. oxalaticus*, synthesis of C_3 compounds from oxalate by the pink-pigmented organisms occurs by a variant of the 'serine pathway' used by *Pseudomonas* AM 1 during growth on C_1 compounds. 4. Evidence in favour of this scheme is provided by the finding that a mutant of *Pseudomonas* AM 1 that lacks hydroxypyruvate reductase is not able to grow on oxalate.

Synthesis of cell constituents from oxalate by *Pseudomonas oxalaticus* has been shown (Quayle, Keech & Taylor, 1961; Quayle & Taylor, 1961; Quayle, 1963*a*,*b*) to involve reduction of oxalate to glyoxylate by the following two reactions:

$$\text{Oxalate} + \text{succinyl-CoA} \rightarrow \text{oxalyl-CoA} + \text{succinate} \quad (1)$$

$$\text{Oxalyl-CoA} + \text{NADPH} + \text{H}^+ \rightarrow \text{glyoxylate} + \text{NADP}^+ + \text{CoA} \quad (2)$$

The glyoxylate is then converted into glycerate (and thence into other cell constituents) by the glycerate pathway, discovered by Kornberg and his colleagues (see Kornberg, 1966) in glycollate-grown *Escherichia coli* and species of *Pseudomonas*:

$$2\ \text{Glyoxylate} \rightarrow \text{tartronic acid semialdehyde} + \text{CO}_2 \quad (3)$$

$$\text{Tartronic acid semialdehyde} + \text{NADH} + \text{H}^+ \rightarrow \text{glycerate} + \text{NAD}^+ \quad (4)$$

The necessary energy for growth on oxalate is derived from the following series of catabolic reactions:

$$\text{Oxalyl-CoA} \rightarrow \text{formyl-CoA} + \text{CO}_2 \quad (5)$$

$$\text{Formyl-CoA} + \text{oxalate} \rightarrow \text{formate} + \text{oxalyl-CoA} \quad (6)$$

$$\text{Formate} + \text{NAD}^+ \rightarrow \text{CO}_2 + \text{NADH} + \text{H}^+ \quad (7)$$

$$\text{Sum: Oxalate} + \text{NAD}^+ \rightarrow 2\text{CO}_2 + \text{NADH} + \text{H}^+ \quad (8)$$

One of the key reactions in the anabolic sequence is reaction (3), which is catalysed by glyoxylate carboligase. The present paper records studies of the mode of utilization of oxalate by a group of related organisms that have been found not to contain glyoxylate carboligase when grown on oxalate.

MATERIALS AND METHODS

Isolation, maintenance and growth of organisms. During mutation studies with *Ps. oxalaticus*, growth of pink-pigmented colonies, presumably from an air-borne contaminant, were observed on agar plates that contained oxalate as the sole carbon source. The organism was isolated by repeated subculture in liquid oxalate growth medium (see below) and by selection of single colonies from oxalate–agar plates. The organism is a Gram-negative, motile rod, approx. $1.5\mu\text{m} \times 4\mu\text{m}$, with one polar flagellum. The organism has similar growth characteristics and morphology to *Pseudomonas* AM 1 (Peel & Quayle, 1961) and hence it was designated *Pseudomonas* AM 2. Stock cultures of *Pseudomonas* AM 2 and *Ps. oxalaticus* were maintained as described for *Ps. oxalaticus* (Blackmore, Quayle & Walker, 1968). Stock cultures of *Pseudomonas* AM 1, *Protaminobacter ruber* and *Pseudomonas extorquens* were maintained as

* Present address: Department of Biochemistry, University of Liverpool, Liverpool L59 38X, U.K.

described for *Pseudomonas* AM1 (Peel & Quayle, 1961). Mutant 20B-L of *Pseudomonas* AM1 was maintained on similar slopes except that 50mM-succinate was used as carbon source in place of methylamine. The medium used for liquid growth was as described by Blackmore & Quayle (1968). Carbon sources were used at a concentration of 0.05M, except methanol, which was used at a concn. of 0.5% (v/v). The organisms were grown in 600ml of medium contained in 2 litre conical flasks that were shaken on a rotary shaker at 30°C. *Micrococcus denitrificans* was maintained and grown as described by Kornberg & Morris (1965). Growth was followed by measurement of the increase in extinction at 540nm in a Unicam SP. 600 spectrophotometer.

Preparation of cell-free extracts. Bacteria, obtained from 600ml of growth medium by centrifugation, were suspended in 3–5ml of 0.05M-sodium phosphate buffer, pH7.0. Extracts were then prepared by disrupting the bacteria with an ultrasonic cell distintegrator (MSE 100W) for 2min at 0°C, followed by centrifugation at 18000*g* for 10min in a refrigerated centrifuge. The extracts contained 1–7mg of protein/ml.

Protein determination. Protein was determined by the Folin–Ciocalteu method as described by Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as standard.

Special chemicals. Purified enzymes, nicotinamide nucleotides, ATP, GSH and CoA were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The dibarium salt of ribulose diphosphate was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Lithium hydroxypyruvate was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Glyoxylic acid was obtained from British Drug Houses Ltd., Poole, Dorset, U.K.

Enzyme assays. (*a*) 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 the spectrophotometric measurement of the rate of reduction of NAD in the presence of formate. The reaction was followed at 340nm at 30°C in a Beckman model DB recording spectrophotometer. One unit of activity is defined as that amount of enzyme which catalyses the reduction of 1μmol of NAD in 1min under the assay conditions.

(*b*) 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°C. One unit of activity is defined as that amount of enzyme which catalyses the reduction of 1μmol of NADP in 1min under the assay conditions.

(*c*) Oxalyl-CoA decarboxylase (oxalyl-CoA carboxylyase, EC 4.1.1.8). This enzyme was assayed manometrically by measuring the rate of anaerobic evolution of CO_2 from oxalate in the presence of ATP, succinate, thiamin pyrophosphate and CoA, by a modified version of the method described by Quayle *et al.* (1961). Double-armed Warburg flasks contained 50μmol of sodium phosphate buffer, pH7.5, 20μmol of GSH, 10μmol of ATP, 0.5μmol of CoA, 0.5μmol of thiamin pyrophosphate, 10μmol of $MgCl_2$, 1μmol of succinate, bacterial extract and water to a final vol. of 2ml. The flasks were flushed with O_2-free N_2. After equilibration at 30°C, 0.2ml of sodium oxalate (0.2M) was added from the first side arm. The reaction was terminated after 60min by the addition of 0.2ml of 1M-H_2SO_4 from the second side arm. One unit of activity is defined as that amount of enzyme which catalyses the evolution of 1μmol of CO_2 in 1min under the assay conditions.

(*d*) Glyoxylate carboligase. This enzyme was assayed manometrically by the method described by Blackmore & Quayle (1968). This involved measurement of the rate of anaerobic evolution of CO_2 from glyoxylate. One unit of activity is defined as that amount of enzyme which catalyses the evolution of 1μmol of CO_2 in 1min under the assay conditions.

(*e*) Hydroxypyruvate reductase (D-glycerate–NAD oxidoreductase, EC 1.1.1.29). This enzyme was assayed by a modification of the method of Stafford, Magaldi & Vennesland (1954). Silica cuvettes (3ml, light-path 1cm) contained 100μmol of sodium acetate buffer, pH4.5, 0.4μmol of NADH and 2μmol of lithium hydroxypyruvate in a total vol. of 3ml. Extract was added and the decrease in extinction at 340nm was measured in a recording spectrophotometer against a blank containing all components except NADH. The rate was corrected for the oxidation of NADH by the extract in the absence of substrate. One unit is defined as that amount of enzyme which catalyses the oxidation of 1μmol of NADH in 1min under the assay conditions.

(*f*) L-Serine–glyoxylate aminotransferase. This enzyme was assayed by measuring the glyoxylate-dependent formation of hydroxypyruvate from L-serine. Cell-free extracts of the pink-pigmented organisms under study contain relatively high concentrations of an NADH-linked hydroxypyruvate reductase (Table 2). Consequently the aminotransferase activity could be measured by following NADH oxidation in the presence of glyoxylate and L-serine. The complete reaction mixture, contained in a 1.5ml silica cuvette (1cm light-path) at 18–21°C consisted of 50μmol of potassium phosphate buffer, pH7.1, 0.01μmol of pyridoxal phosphate, 0.15μmol of NADH, 5μmol of sodium glyoxylate, 5μmol of L-serine, extract (containing up to 200μg of protein) and water to 1ml. The blank cuvette lacked NADH. The rate of decrease in extinction at 340nm before the addition of L-serine (due to NADH oxidase and glyoxylate reductase activities) was subtracted from the new rate observed in its presence. One unit of activity is defined as that amount of enzyme that catalyses the oxidation of 1μmol of NADH in 1min under the assay conditions.

In the two cases where hydroxypyruvate reductase was absent or present in relatively low activity, namely succinate-grown *Pseudomonas* AM2 or oxalate-grown *Ps. oxalaticus*, a cell-free extract of succinate-grown *M. denitrificans* was added to the assay mixture to provide a source of NADPH-linked hydroxypyruvate reductase (Gibbs, 1966). NADPH was substituted for NADH in these two instances, otherwise the assays were performed in a fashion similar to that described above.

Aspartate and glutamate would not replace serine as amino group donors. This was tested for in the case of aspartate by seeking glyoxylate-dependent formation of oxaloacetate from aspartate. The formation of oxaloacetate was measured by following NADPH oxidation at 340nm in the presence of an excess of NADP-linked malate

dehydrogenase supplied by an extract of succinate-grown *M. denitrificans* (Kornberg & Morris, 1965). In the case of glutamate as donor, glyoxylate-dependent disappearance of glutamate was sought for, by using a discontinuous assay for glutamate (Wyngaarden & Ashton, 1959). In no case was any appreciable activity observed.

(*g*) *erythro*-β-Hydroxyaspartate aldolase. This enzyme was assayed by the method of Gibbs & Morris (1964), which involves measuring pyruvate formation from *erythro*-DL-β-methyl-β-hydroxyaspartate, the keto acid being assayed spectrophotometrically as its 2,4-dinitrophenylhydrazone. Since extracts of the pink-pigmented organisms contained no hydroxyaspartate dehydratase (see below), formation of glyoxylate from *erythro*-β-hydroxyaspartate was also tested in a way similar to that described above for the methyl analogue.

(*h*) *erythro*-β-Hydroxyaspartate dehydratase. This enzyme was assayed by measuring oxaloacetate formation from *erythro*-β-hydroxyaspartate by the methods described by Kornberg & Morris (1962). Oxaloacetate formation was measured spectrophotometrically either as its semicarbazide at 252 nm or by the oxidation of NADH consequent on the reduction of the oxaloacetate to malate catalysed by excess of NAD-linked malate dehydrogenase present in the extract. In some experiments, cell-free extracts of succinate-grown *M. denitrificans* were added to the test system as a source of NADP-linked malate dehydrogenase (Kornberg & Morris, 1965). This enables oxaloacetate formation from hydroxyaspartate to be measured in terms of NADPH oxidation. NADPH was not affected by the NADH oxidase activity present in the extracts of the organisms under study, and thus enabled a further, sensitive check to be made on the presence or absence of hydroxyaspartate dehydratase.

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

RESULTS AND DISCUSSION

Identity of the organisms. Throughout the work described in this paper, only slight difference in pigmentation and growth rate between *Pseudomonas* AM 2 and *Pseudomonas* AM 1 have been observed. It thus seems likely that they are closely similar strains of the same species.

Pseudomonas AM 1, *Pr. ruber* and *Ps. extorquens* are all pink-pigmented bacteria that are capable of growth on several C_1 compounds and also on a wide variety of other substrates, including oxalate (Stocks & McCleskey, 1964). Stocks & McCleskey (1964) consider that they are sufficiently alike to be considered one species and suggest that they be tentatively considered as strains of *Vibrio extorquens* (Bassalik). Although *Ps. oxalaticus* also can grow on formate or oxalate, it is quite distinct from any of the organisms considered to be strains of *V. extorquens*, e.g. it is non-pigmented and does not grow on methanol.

Growth rates of the organisms on different substrates. Accurate measurement of growth rates of the organisms is difficult owing to clumping of the bacterial suspension. For the pink-pigmented organisms this clumping varies with the growth substrate, increasing in the order oxalate < methanol < formate. Table 1 shows the approximate growth rates of *Pseudomonas* AM 2, *Pseudomonas* AM 1, *Pr. ruber*, *Ps. extorquens* and *Ps. oxalaticus* on methanol, formate and oxalate. The pink-pigmented organisms all grow much slower on formate or oxalate than does *Ps. oxalaticus*.

Oxidation of oxalate. All the pink-pigmented organisms, when grown on oxalate, contained oxalyl-CoA decarboxylase and formate dehydrogenase, the enzymes which catalyse reactions (5) and (7) respectively (Table 2). Oxalyl-CoA decarboxylase was either undetectable or present in decreased amount when *Pseudomonas* AM 2 was grown on succinate, methanol or formate. This indicates that the enzyme has a specific role during growth on oxalate. The fact that formate dehydrogenase is present in oxalate-grown cells at six times the specific activity of that in succinate-grown cells indicates that it too is involved in oxalate metabolism. These enzyme activities are thus consistent with oxalate being oxidized by the pink organisms in the same way as by *Ps. oxalaticus*, i.e. by reactions (5)–(8).

Reduction of oxalate to glyoxylate. The pink organisms, when grown on oxalate, contained oxalyl-CoA reductase, the enzyme that catalyses reaction (2) (Table 2). Although the specific activity was lower than that found in oxalate-grown *Ps. oxalaticus*, a specific role for the enzyme in oxalate

Table 1. *Growth rates of organisms growing on methanol, formate or oxalate as sole carbon source*

The bacteria were grown in 50 ml of oxalate growth medium contained in 250 ml Monod flasks. These were shaken on a rotary shaker at 30°C. Growth was followed by measurement of the increase in extinction at 540 nm in a Unicam SP. 600 spectrophotometer.

	Mean generation time (h)				
Substrate	*Pseudomonas* AM 2	*Pseudomonas* AM 1	*Pr. ruber*	*Ps. extorquens*	*Ps. oxalaticus*
Methanol	5–7	5	5	6	No growth
Formate	7	8	10	7	3½
Oxalate	10	13	18	10	4½

Table 2. *Enzyme activities of organisms grown on different carbon sources*

The organisms were grown for 24–48 h in 600 ml of medium containing carbon source at 50 mM, except for methanol, which was 0.5% (v/v). The cells were harvested, cell-free extracts prepared, and enzymes assayed as described in the Materials and Methods section. Values in parentheses are duplicate determinations on separate cultures.

Organism	Growth substrate	Specific activities (units/mg of protein)					
		Formate dehydrogenase	Oxalyl-CoA decarboxylase	Oxalyl-CoA reductase	Glyoxylate carboligase	Hydroxypyruvate reductase	Serine–glyoxylate aminotransferase
Pseudomonas AM 2	Formate	0.23	0.018	0.002	Undetectable	1.23	0.05
	Methanol	0.10	Undetectable	0.006	0.002	1.77	0.30
	Oxalate	0.44	0.22	0.07	Undetectable	1.14	0.05 (0.12)
	Succinate	0.07	Undetectable	Undetectable	Undetectable	0.26	Undetectable
Pseudomonas AM 1	Oxalate	0.44	0.17	0.06	Undetectable	1.19	0.07
Pr. ruber	Oxalate	0.30 (0.58)	0.22 (0.4)	0.07 (0.07)	Undetectable	2.4 (2.6)	0.11
Ps. extorquens	Oxalate	0.42	0.08	0.06	Undetectable	1.0	0.11
Ps. oxalaticus	Oxalate	1.40	0.33	1.60 (1.78)	0.24 (0.17)	Undetectable	Undetectable

metabolism is indicated by its decrease in specific activity consequent on growth of *Pseudomonas* AM 2 on substrates other than oxalate.

Conversion of glyoxylate into glycerate. In contrast with oxalate-grown *Ps. oxalaticus*, none of the pink organisms when grown on oxalate contained more than trace amounts of glyoxylate carboligase, catalysing reaction (3). This indicates that the glycerate pathway of biosynthesis cannot be operating during growth on oxalate. Two possible alternative pathways suggest themselves: (*a*) the hydroxyaspartate pathway, utilized by *M. denitrificans* during growth on glycollate (Kornberg & Morris, 1965); (*b*) the serine pathway, utilized by *Pseudomonas* AM 1 during growth on methanol or formate (Large & Quayle, 1963; Heptinstall & Quayle, 1970). The key biosynthetic reaction in pathway (*a*) is a condensation of glycine and glyoxylate to give hydroxyaspartate, catalysed by hydroxyaspartate aldolase:

$$HO_2C\cdot CH_2\cdot NH_2 + OHC\cdot CO_2H \rightarrow HO_2C\cdot CH(NH_2)\cdot CH(OH)\cdot CO_2H \quad (9)$$

Operation of this pathway during growth on oxalate by the pink organisms is however eliminated by the absence of either hydroxyaspartate aldolase or hydroxyaspartate dehydratase in cell-free extracts. All the different assay methods described in the Materials and Methods section gave completely negative results for the presence of either of these enzymes. This could not be due to the presence of inhibitors in the extracts as addition of the same quantities of extract to extracts of glycollate-grown *M. denitrificans* did not inhibit the activities of hydroxyaspartate aldolase or hydroxyaspartate dehydratase present therein.

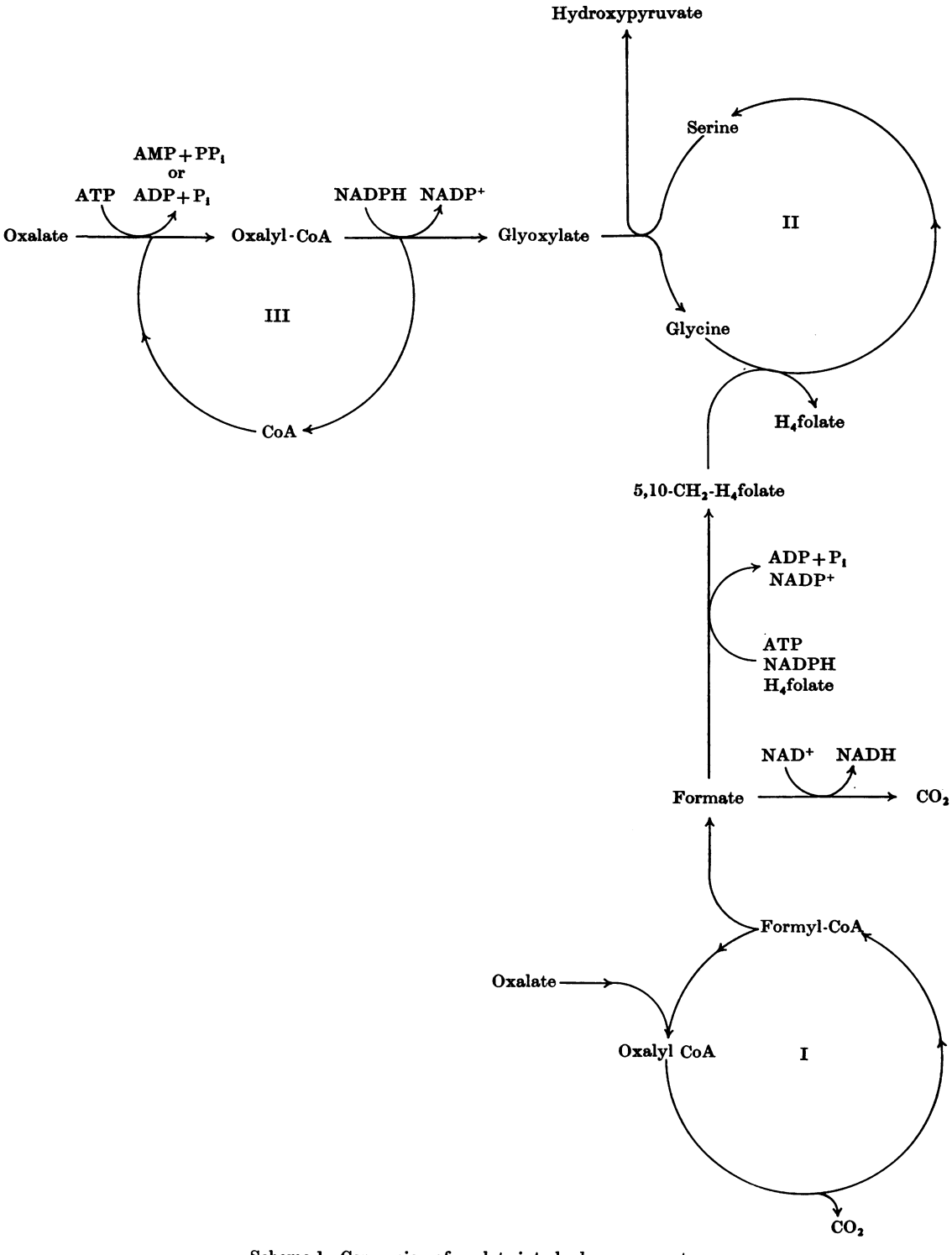
The conversion of a C_2 compound into glycerate by pathway (*b*) is thought to be carried out by the following reactions:

$$\text{Glycine} + 5,10\text{-}CH_2\text{-}H_4\text{folate} \rightarrow \text{serine} + H_4\text{folate} \quad (10)$$

$$\text{Serine} + \text{keto acid} \rightarrow \text{hydroxypyruvate} + \text{amino acid} \quad (11)$$

$$\text{Hydroxypyruvate} + NADH + H^+ \rightarrow \text{glycerate} + NAD^+ \quad (12)$$

A key enzyme of this sequence is hydroxypyruvate reductase catalysing reaction (12). Consistent with its key role during growth on C_1 compounds are the findings that (*a*) the specific activity of this enzyme is higher in methanol- and formate-grown *Pseudomonas* AM 1 by respective factors of four and two over that in succinate-grown *Pseudomonas* AM 1 (Large & Quayle, 1963); (*b*) lack of the enzyme in mutant 20B-L results in inability of *Pseudomonas* AM 1 to grow on C_1 compounds, growth on succinate remaining unimpaired (Heptinstall & Quayle,



Scheme 1. Conversion of oxalate into hydroxypyruvate.

1970). By these criteria, hydroxypyruvate reductase is also implicated in growth on oxalate by the pink organisms. (*a*) The enzyme is present at high specific activity in all the pink organisms when grown on oxalate (Table 2). Further, the specific activity of the enzyme is similar in oxalate-grown *Pseudomonas* AM 2 to that in the same organism grown on formate or methanol, and may be contrasted with the fourfold lower activity when the same organism is grown on succinate. (*b*) Mutant 20B-L, lacking hydroxypyruvate reductase, has now been found to be unable to grow on oxalate.

This indicates that the pink organisms may be using a variant of the serine pathway to synthesize glycerate from oxalate. In this variant, formate would be made from oxalate by reactions (1), (5) and (6), and activated to methylene tetrahydrofolate by reactions demonstrated in formate-grown *Pseudomonas* AM 1 (Large & Quayle, 1963). Oxalate would be reduced to glyoxylate by reactions (1) and (2), which might then provide, by transamination, the glycine necessary for reaction (10). To investigate this possibility, enzyme(s) were sought that could accomplish the transamination of glyoxylate to glycine.

Conversion of glyoxylate into glycine. Cell-free extracts of the pink organisms, when grown on oxalate, were found to contain a serine–glyoxylate aminotransferase:

$$\text{L-serine} + \text{glyoxylate} \rightleftharpoons \text{hydroxypyruvate} + \text{glycine} \quad (13)$$

No transamination of glyoxylate with aspartate in place of serine could be detected in any of the pink organisms grown on oxalate. Glutamate as an amino-group donor was tested with oxalate-grown *Pseudomonas* AM 2, and again, no activity could be detected.

The fact that serine–glyoxylate aminotransferase was present in *Pseudomonas* AM 2 when grown on oxalate, methanol or formate, but was undetectable in the succinate-grown organism (Table 2) suggests that it has a specific role during growth on the first three substrates but not on succinate.

Overall conversion of oxalate into glycerate. The above enzymic results are consistent with the operation, during growth on oxalate by the pink organisms, of Scheme 1. In cycle I oxalate is decarboxylated to formate, which is either oxidized to CO_2 to give energy in the form of NADH or is fed into cycle II after conversion into methylene tetrahydrofolate. A further molecule of oxalate is reduced to glyoxylate in cycle III and is also fed into cycle II. The input of one molecule of methylene tetrahydrofolate and one molecule of glyoxylate would result in a net conversion to one molecule of hydroxypyruvate via serine and glycine as intermediary metabolites. The hydroxypyruvate could then be reduced to glycerate by hydroxypyruvate reductase and thence assimilated into cell constituents.

It should be noted that no activities of either hydroxypyruvate reductase or serine–glyoxylate aminotransferase could be detected in oxalate-grown *Ps. oxalaticus* (Table 2). This is to be expected from the fact that, during growth on oxalate, *Ps. oxalaticus* possesses a glycerate pathway in which neither enzyme is implicated. The finding of the specific serine–glyoxylate aminotransferase in *Pseudomonas* AM 2 when grown on C_1 compounds or oxalate may be an important one. The mechanism of the net synthesis of glycine from C_1 units in *Pseudomonas* AM 1 remains an unsolved problem; the appearance of this specific transaminase during growth on C_1 compounds points to glyoxylate as being the precursor of glycine. If the pink organisms can indeed catalyse the net synthesis of glyoxylate from formate during growth on formate, then it raises the question as to how much glyoxylate might be made in this way by these organisms during growth on oxalate. In this case the formate would be derived from decarboxylation of the oxalate. This indirect conversion of oxalate into glyoxylate via formate might take place alongside the direct conversion of oxalate into glyoxylate by the reactions of cycle III in Scheme 1. The specific activity of oxalyl-CoA reductase in the oxalate-grown pink organisms is 20–30-fold lower than that in oxalate-grown *Ps. oxalaticus*. This might indicate that only a small amount of oxalate carbon is being directly reduced to glyoxylate by the pink organisms. However two other factors are relevant: (*a*) the growth rates of the pink-pigmented organisms on oxalate are one-half to one-quarter of that of *Ps. oxalaticus* on the same substrate; (*b*) synthesis of one molecule of glycerate from oxalate by the glycerate pathway demands reduction of two molecules of oxalate to two molecules of glyoxylate, as compared with one molecule of oxalate/molecule of glycerate formed by the serine pathway. Hence, in comparison with *Ps. oxalaticus*, operation of factors (*a*) and (*b*) could decrease the amount of oxalyl-CoA reductase necessary for the pink-pigmented organisms to grow on oxalate.

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