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Pathways Leading to and from Serine during Growth of *Pseudomonas* AM1 on C_1 Compounds or Succinate

By J. HEPTINSTALL* and J. R. QUAYLE
Department of Microbiology, University of Sheffield, S10 2TN, U.K.

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1. The following enzymes of the phosphorylated pathway of serine biosynthesis have been found in methanol- and succinate-grown *Pseudomonas* AM1: phosphoglycerate dehydrogenase, phosphoserine-α-oxoglutarate aminotransferase and phosphoserine phosphohydrolase. Their specific activities were similar in the organism grown on either substrate. 2. A procedure for preparation of auxotrophic mutants of *Pseudomonas* AM1 is described involving *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as mutagen and a penicillin enrichment step. 3. A mutant, M-15A, has been isolated that is unable to grow on methanol and that lacks phenazine methosulphate-linked methanol dehydrogenase. The mutant is able to grow on methylamine, showing that the amine is not oxidized by way of methanol. 4. Loss of methanol dehydrogenase activity in mutant M-15A led to loss of phenazine methosulphate-linked formaldehyde dehydrogenase activity showing that the same enzyme is probably responsible for both activities. 5. A mutant, 20B-L, has been isolated that cannot grow on any C_1 compound tested but can grow on succinate. 6. Mutant 20B-L lacks hydroxypyruvate reductase, and revertants that regained the ability to grow on methanol, methylamine and formate contained hydroxypyruvate reductase activity at specific activities similar to that of the wild-type organism. This shows that hydroxypyruvate reductase is necessary for growth on methanol, methylamine and formate but not for growth on succinate. 7. The results suggest that during growth of *Pseudomonas* AM1 on C_1 compounds, serine is converted into 3-phosphoglycerate by a non-phosphorylated pathway, whereas during growth on succinate, phosphoglycerate is converted into serine by a phosphorylated pathway.

Previous studies (Large, Peel & Quayle, 1961, 1962*a*,*b*; Large & Quayle, 1963) have led to the suggestion that glycolytic intermediates are formed during growth of *Pseudomonas* AM1 on C_1 compounds by a pathway involving the following steps:

$$C_1 \text{ compound} + \text{cofactors} + H_4\text{folate} \rightarrow 5,10\text{-}CH_2\text{–}H_4\text{folate} \quad (1)$$

$$5,10\text{-}CH_2\text{–}H_4\text{folate} + \text{glycine} \leftrightharpoons H_4\text{folate} + \text{serine} \quad (2)$$

$$\text{Serine} + R\cdot CO\cdot CO_2H \leftrightharpoons \text{hydroxypyruvate} + R\cdot CHNH_2\cdot CO_2H \quad (3)$$

$$\text{Hydroxypyruvate} + \text{NADH} \leftrightharpoons \text{D-glycerate} + NAD^+ \quad (4)$$

$$\text{D-Glycerate} + \text{ATP} \rightarrow \text{phosphoglycerate} + \text{ADP} \quad (5)$$

* Present address: Department of Chemistry, Washington State University, Pullman, Wash. 99165, U.S.A.

In the case of growth on methanol, it was suggested (Large & Quayle, 1963; Johnson & Quayle, 1964) that reaction (1) proceeds by way of:

$$CH_3OH \rightarrow HCHO + 2H \quad (6)$$

$$HCHO + H_4\text{folate} \rightarrow 5,10\text{-}CH_2\text{–}H_4\text{folate} \quad (7)$$

Reaction (6), catalysed by methanol dehydrogenase, was discovered by Anthony & Zatman (1964) in *Pseudomonas* M27. It can be linked to PMS† and its presence in *Pseudomonas* AM1 was shown by Johnson & Quayle (1964).

In the case of growth on formate, it was suggested (Large & Quayle, 1963) that reaction (1) consists of:

$$\text{Formate} + \text{ATP} + H_4\text{folate} \leftrightharpoons 10\text{-}HCO\text{–}H_4\text{folate} + \text{ADP} + P_i \quad (8)$$

$$10\text{-}HCO\text{–}H_4\text{folate} + H^+ \leftrightharpoons 5,10\text{-}CH{=}H_4\text{folate} + H_2O \quad (9)$$

† Abbreviations: PMS, phenazine methosulphate; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; DCPIP, 2,6-dichlorophenol-indophenol.

$$5,10\text{-}CH{=}H_4\text{folate} + NADPH \leftrightharpoons 5,10\text{-}CH_2{-}H_4\text{folate} + NADP^+ \quad (10)$$

The presence of enzymes catalysing reactions (2)–(10), with the exception of (7) which proceeds non-enzymically, was demonstrated in cell-free extracts of appropriately grown *Pseudomonas* AM 1 and it was shown that the specific activities of those catalysing (2), (4), (8) and (10) were higher in the organism when grown on the appropriate C_1 substrate than when grown on succinate. This provides evidence in favour of these enzymes being specially involved in assimilation of C_1 substrates. Since this work was done, studies with serine-requiring mutants of *Salmonella typhimurium* and *Escherichia coli* (Umbarger & Umbarger, 1962; Umbarger, Umbarger & Siu, 1963; Pizer, 1963) have shown that serine is formed in these organisms from glycolytic intermediates via a phosphorylated pathway:

$$\text{D-3-Phosphoglycerate} + NAD^+ \leftrightharpoons 3\text{-phosphohydroxypyruvate} + NADH \quad (11)$$

$$3\text{-Phosphohydroxypyruvate} + \text{L-glutamate} \leftrightharpoons O\text{-L-phosphoserine} + \alpha\text{-oxoglutarate} \quad (12)$$

$$O\text{-L-Phosphoserine} + H_2O \rightarrow \text{serine} + P_i \quad (13)$$

It was decided to see if a similar set of enzymes was present in *Pseudomonas* AM 1, and if so, whether their specific activities vary when the organism is grown on substrates such as succinate or methanol. This paper records such a study and also describes work with mutants of *Pseudomonas* AM 1 which bears on the physiological roles of the above phosphorylated and non-phosphorylated pathways, on the related problem of net biosynthesis of glycine from C_1 compounds, and on the mechanism of oxidation of C_1 compounds in this organism.

MATERIALS AND METHODS

Growth of the organism. Pseudomonas AM 1 was grown on methanol as described by Peel & Quayle (1961) except that a rotary shaker was used. For growth on succinate or methylamine, 50mM-sodium succinate or 50mM-methylamine hydrochloride was substituted for 0.5% (v/v) methanol as the carbon source. In all cases the inorganic salts medium of Jayasuriya (1955) was used. Determinations of mean generation times were made by following extinction changes at 650nm in 250ml conical flasks fitted with optically matched side arms and containing 40ml of medium.

Manometric methods. Consumption of O_2 was measured in conventional Warburg manometers at 30°C with air as the gas phase. After harvesting, cells were washed in 50mM-sodium–potassium phosphate buffer, pH7.0, and resuspended in this buffer to a final concentration of about 4mg dry wt./ml; 1ml of this cell suspension was placed in each main compartment of the Warburg flasks, to each of which was added 100μmol of sodium–potassium phosphate buffer, pH7.2, and in some cases, 20μmol of $(NH_4)_2SO_4$. The centre well contained 400μmol of KOH and the side arm contained substrate. The total volume in each flask was 2.3ml.

Preparation of cell-free extracts. Bacteria were disrupted either in a Hughes press (Hughes, 1951) or in an ultrasonic oscillator (M.S.E. Model 60W). Suspensions (1g wet wt. of cells/4ml of 50mM-tris–HCl buffer, pH7.5) were crushed in a Hughes press which had been cooled to −25°C. A few crystals each of ribonuclease and deoxyribonuclease were then added and the crushed material was allowed to thaw. To prepare sonic extracts, 1g wet wt. of cells was suspended in 4ml of 50mM-phosphate buffer, pH7.0, and ultrasonically treated for 2min at 0°C (power output 60W at 25kHz). Extracts prepared by both methods were used after centrifugation at 15000*g* for 15min.

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

Chemicals. Phosphohydroxypyruvic acid was supplied as the dimethylketal (tricyclohexylammonium) salt (Calbiochem, Los Angeles, Calif., U.S.A.). The free acid was prepared by the method of Ballou & Hesse (1956) by treatment with Dowex 50 (H^+ form) and hydrolysis at 40°C for 4 days; the solution was then neutralized and used as such. To determine whether any hydrolysis of the phosphate group had occurred, the solution was assayed for total and inorganic phosphate by the method of Berenblum & Chain (1938). For total phosphate assay, a sample of the diluted solution was wet-ashed by the method of Hanes & Isherwood (1949). It was found that the contribution of inorganic phosphate to the total phosphate present was less than 1%. Calcium DL-glycerate (Sigma Chemical Co., St Louis, Mo., U.S.A.) was converted into the sodium salt by treatment with Dowex-50 (H^+ form) and neutralization with NaOH. (±)-Tetrahydrofolic acid (100mg), obtained from Sigma, was dissolved by the addition of 10ml of 50mM-sodium–potassium phosphate buffer, pH7.5, containing 10mM-mercaptoethanol. The resulting solution was distributed among several Thunberg tubes, each filled with O_2-free N_2, sealed and stored at −15°C. The concentration of this solution was assayed by adding 10μl to 10ml of the original solvent and measuring the extinction at the absorption maximum of 297nm; ϵ for H_4folate was assumed to be 2.2×10^4 (Hatefi, Talbert, Osborn & Huennekens, 1960). In other cases folic acid was hydrogenated in the presence of Pt catalyst by the method used by Jones, Guest & Woods (1961) for reduction of pteroyltriglutamate.

Preparation of mutants. Conditions were found for preparing mutants of *Pseudomonas* AM 1 by using MNNG as mutagen (Adelberg, Mandel & Chen, 1965) and employing a penicillin treatment for enriching the required mutants (Davis, 1948). A culture (50ml) of the wild-type organism was grown overnight on succinate. The cells were harvested by centrifugation, washed once and resuspended at a cell density of 10^9 cells/ml (E_{650} 2.6) in a solution of inorganic salts (Jayasuriya, 1955) in which the phosphate buffer had been replaced by 50mM-tris–maleate buffer, pH6.0. To 0.3ml of this suspension was

added 0.3ml of a solution (4mg/ml) of MNNG, and the resulting suspension was incubated at 30°C for 3h. The cells were then centrifuged, washed once with sterile water and resuspended in 2ml of a supplemented growth medium (see the text) that would permit growth of the required mutants. The cells were incubated with aeration at 30°C overnight, in order to express mutations. For optimum enrichment of mutants with penicillin, the suspension was centrifuged and the cell pellet washed twice with sterile water before being resuspended in 10ml of succinate growth medium, which was incubated with aeration at 30°C for 3–4h. Penicillin was then added to a final concentration of 200 units/ml and the suspension was incubated at 30°C with shaking overnight. (Preliminary experiments had shown that penicillin at this concentration killed growing cells of *Pseudomonas* AM1 but did not affect non-growing cells.) After this incubation, samples of appropriately diluted suspension were plated out on to suitably supplemented medium (see the text). The plates were incubated for 3–4 days at 30°C and the most suitable ones chosen for replication on to succinate minimal medium. The replica plates were incubated for 3–4 days at 30°C and compared with the master plates. Presumptive mutants were picked off, resuspended in a drop of salts medium (Jayasuriya, 1955) and streaked on to fresh plates for further examination and for single-colony isolation.

By using this general procedure, large numbers of mutants of various phenotypes could be obtained.

Enzyme Assays

The assays of the three enzymes of the phosphorylated pathway of serine biosynthesis were performed at 37°C. The remaining assays were performed at 30°C.

Phosphoserine phosphohydrolase (phosphoserine phosphatase, EC 3.1.3.3). The enzyme was assayed by measuring the P_i liberated from DL-phosphoserine. The assay system (Umbarger *et al.* 1963) consisted of 600μmol of tris–HCl buffer, pH7.5, 30μmol of $MgCl_2$, 60μmol of DL-phosphoserine, extract (2–8mg of protein) and water to a volume of 3.0ml. The reaction was started by the addition of substrate; samples (0.25ml) were withdrawn into 0.75ml of 10% (w/v) trichloroacetic acid over a period of 30min. Protein precipitates were removed by centrifugation and 0.5ml samples of the supernatants were assayed for P_i by the method of Allen (1940).

Phosphoserine-α-oxoglutarate aminotransferase. The assay used was essentially that of Umbarger & Umbarger (1962). The formation of glutamate was followed in a system containing (per ml): 25μmol of phosphate buffer, pH7.5, 20μg of pyridoxal phosphate, 20μmol of sodium α-oxoglutarate, 20μmol of DL-phosphoserine and extract (2–6mg of protein). The reaction was started by the addition of extract, and samples (1.0ml) were withdrawn at intervals and heated at 100°C for 2min. Samples (0.15ml) of the supernatants after centrifugation were assayed for glutamate by the glutamate dehydrogenase assay of Wyngaarden & Ashton (1959). Significant glutamate was also found in controls lacking phosphoserine. The difference in the amount of glutamate produced in the presence and absence of phosphoserine was taken to be the amount produced by the aminotransferase under study.

Phosphoglycerate dehydrogenase (phosphohydroxypyruvate reductase). This assay was performed in anaerobic cuvettes in order to eliminate NADH oxidase activity. The reaction mixture contained (in 3.0ml): 300μmol of sodium–potassium phosphate buffer, pH6.0, 4μmol of $MgCl_2$, 0.6μmol of phosphohydroxypyruvate, 0.4μmol of NADH and extract (1–3mg of protein). The oxidation of NADH was followed at 340nm. The reference cuvette contained all the reactants except NADH and the reaction was started by tipping the extract from the side arm of the cuvette.

ATP–D-glycerate phosphotransferase (glycerate kinase, EC 2.7.1.31). This enzyme was assayed spectrophotometrically at 340nm by following the formation of ADP consequent upon the phosphorylation of glycerate; the ADP production was coupled to oxidation of NADH by adding phosphoenolpyruvate and purified pyruvate kinase and lactate dehydrogenase (Ornston & Ornston, 1969). NADH oxidase activity was minimized by centrifuging the extract at 90000*g* for 1h and using the supernatant fraction. The complete assay system consisted of 50μmol of potassium phosphate buffer, pH7.3, 1μmol of EDTA, 2.5μmol of phosphoenolpyruvate, 0.10μmol of NADH, 0.5μmol of ATP (tetrasodium salt), 10μmol of $MgCl_2$, 1μmol of sodium DL-glycerate, 20μg of pyruvate kinase, 50μg of lactate dehydrogenase and extract in a total volume of 1.0ml. The reference cuvette contained buffer only. The rate of NADH oxidation was first measured in the absence of glycerate, this rate being due to the action of adenosine triphosphatase and residual NADH oxidase; glycerate was added to the test cell and the increase in rate of NADH oxidation was used to calculate the activity of glycerate kinase. The stimulation in rate of NADH oxidation was dependent on the presence of glycerate, ATP, phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase, and resulted in reaction rates of three to four times that due to the combined activities of NADH oxidase and adenosine triphosphatase.

D-Glycerate–NAD oxidoreductase (hydroxypyruvate reductase, EC 1.1.1.29). This was assayed either anaerobically at pH7.5 in sodium–potassium phosphate buffer, or aerobically at pH4.5 with sodium acetate buffer as described by Large & Quayle (1963) by following the oxidation of NADH at 340nm consequent upon reduction of hydroxypyruvate.

Glycollate–NAD oxidoreductase (glyoxylate reductase, EC 1.1.1.26). This was assayed anaerobically in sodium–potassium phosphate buffer at pH7.5, as described by Large & Quayle (1963) by following the oxidation of NADH at 340nm consequent upon reduction of glyoxylate.

Methanol dehydrogenase. This was assayed manometrically by a modified method of Anthony & Zatman (1964). The manometer cups contained: in the main compartment, 300μmol of tris–HCl buffer (pH9.0), 2.8μmol of PMS, 45μmol of NH_4Cl, 16μmol of methanol; in the centre well, 0.2ml of 20% (w/v) KOH; in the side arm, bacterial extract (2–4mg of protein); total volume, 3ml. O_2 consumption was measured at 30°C.

DCPIP-linked formaldehyde dehydrogenase. This was assayed anaerobically by measuring the rate of reduction of DCPIP at 600nm by formaldehyde. The assay system used was that of Johnson & Quayle (1964) scaled up to a reaction volume of 3.0ml.

Serine hydroxymethyltransferase (serine hydroxymethylase, EC 2.1.2.1). Three assay methods were used. (*a*) Serine, produced from glycine and formaldehyde in the presence of H_4 folate, was measured manometrically by periodate oxidation (Batt, Dickens & Williamson, 1960; Large & Quayle, 1963). (*b*) The glycine-dependent removal of formaldehyde in the presence of H_4 folate was followed by the method of Scrimgeour & Huennekens (1962). (*c*) The formation of 5,10-CH_2–H_4folate from serine was followed by measuring spectrophotometrically the reduction of $NADP^+$ consequent upon the oxidation of 5,10-CH_2–H_4folate to 5,10-CH=H_4folate by methylene tetrahydrofolate dehydrogenase (Large & Quayle, 1963). The latter enzyme was present in excess over serine hydroxymethylase in extracts of *Pseudomonas* AM 1.

RESULTS

Activities of enzymes involved in serine metabolism

The three enzymes of the phosphorylated pathway of serine biosynthesis were found to be present in extracts of methanol- or succinate-grown *Pseudomonas* AM 1 (Table 1). Previous work (Large & Quayle, 1963) established the presence of glycerate kinase in *Pseudomonas* AM 1 but measurements of specific activity were not made; these have now been made and are also reported in Table 1. These results, taken together with those reported previously by Large & Quayle (1963), show that in *Pseudomonas* AM 1 there exist two metabolic routes that interconnect phosphoglycerate and serine (Scheme 1). The fact that each route possesses an essentially irreversible step, i.e. reaction (5) or (13) respectively, dictates the direction of any flow through each route.

It should be noted that only low activities of phosphoserine-α-oxoglutarate transaminase were detected. Further work is needed to establish whether the low activity is due to assay of the enzyme in a non-physiological direction or whether there are better amino group donors than glutamate.

Isolation of mutants

Many features of the scheme outlined in Scheme 1 should be open to direct investigation if mutants lacking the appropriate enzymes could be isolated. For example, if the scheme is correct, a mutant lacking an enzyme of the non-phosphorylated pathway [reactions (3), (4) or (5)], or an enzyme involved in net glycine synthesis from C_1 units (dotted lines in Scheme 1), would be unable to grow on any C_1 compound, but would be able to grow on a compound such as succinate, which could be catabolized to phosphoglycerate. Hence such mutants, called type I mutants, could be isolated on the basis of their ability to grow on succinate but not on methanol. Type I mutants could also arise from defects in C_1 oxidative enzymes. Conversely, a mutant lacking an enzyme of the phosphorylated pathway [reactions (11), (12) or (13)] would be unable to grow on succinate unless supplemented with serine, but would be able to grow on C_1 compounds. Such mutants, called type II mutants, could be isolated on the basis of their inability to grow on succinate unless supplemented with serine.

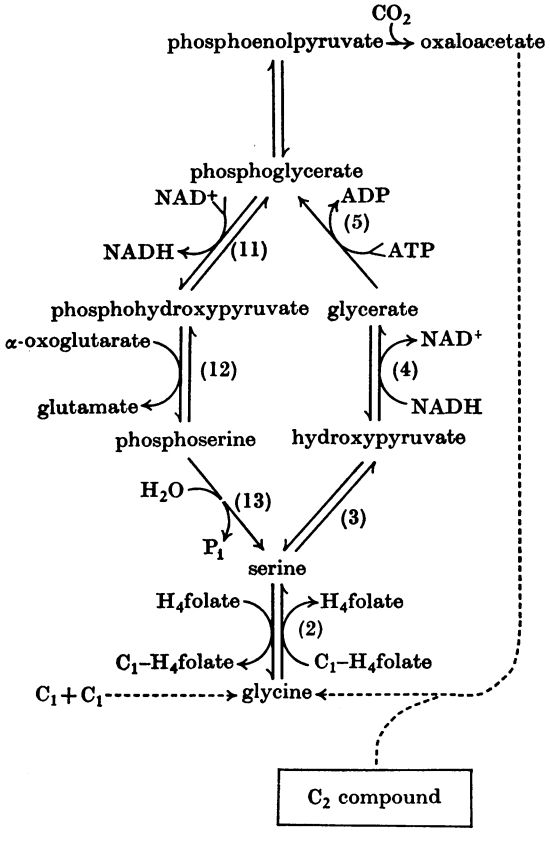
Mutants lacking serine hydroxymethylase would be unable to grow on succinate or on any C_1 compound. Supplementation of succinate with glycine and a source of C_1 units, e.g. methanol, should permit growth. If *Pseudomonas* AM 1 possesses, as postulated, an enzyme system that can catalyse net biosynthesis of glycine from C_1 units, and if this enzyme system were still sufficiently active during growth on succinate, then a serine hydroxymethylase-less mutant might require supplementation of succinate only with a C_1 compound instead of with glycine as well. Mutants lacking serine hydroxymethylase could be screened for on the basis of their inability to grow on succinate unless supplemented

Table 1. *Activities of enzymes of serine metabolism in* Pseudomonas *AM* 1

Assay methods are reported in the Materials and Methods section. The duplicate and triplicate values are taken from measurements made on separately grown batches of cells.

Enzyme	Specific activity (μmol/h per mg of protein)	
	Methanol-grown	Succinate-grown
Phosphoserine phosphatase	0.98; 2.1; 1.7	0.89; 2.1; 1.4
Phosphoserine α-oxoglutarate aminotransferase	0.04; 0.03	0.04; 0.05
Phosphoglycerate dehydrogenase	3.0; 2.9	2.1; 1.9
Glycerate kinase	2.1	0.44
Hydroxypyruvate reductase*	94.2	21.0

* From Large & Quayle (1963)



Scheme 1. Alternative routes of serine metabolism in *Pseudomonas* AM1. The numerals in parentheses refer to equations in the text; the dotted lines represent two hypothetical pathways leading to synthesis of the glycine skeleton.

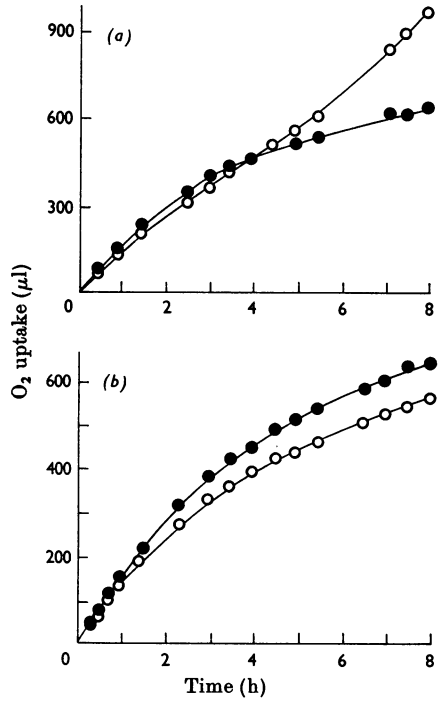


Fig. 1. Effect of methanol on washed suspensions of succinate-grown *Pseudomonas* AM1 and mutant M-15A. The manometric system contained 100 μmol of methanol where appropriate, as described in the Materials and Methods section. (*a*) Wild type. ○, +Methanol; ●, endogenous. (*b*) Mutant M-15A. ○, +Methanol; ●, endogenous.

with glycine and methanol; mutants with this phenotype are referred to as type III mutants.

Mutants were isolated by the general procedure described in the Materials and Methods section.

Type I mutants

These mutants were expressed in succinate growth medium, and the penicillin treatment was done in methanol growth medium, in which the desired mutants would not grow. Master plates were prepared from succinate medium and these were replicated on to methanol medium. A total of sixteen mutants was isolated. These were streaked out on to plates containing 100 mM-methylamine hydrochloride or 100 mM-sodium formate as carbon sources in order to see if they were deficient only in growth on methanol or in growth on other C_1 compounds as well. Two mutants grew on formate but not on either methanol or methylamine; ten mutants grew on either formate or methylamine but not on methanol; four mutants were unable to grow on any of the three C_1 compounds.

Tests were then made to see if methanol-oxidizing activity could be induced in the succinate-grown mutants. This was done by suspending the washed succinate-grown organism in Warburg flasks containing 50 mM-sodium–potassium phosphate buffer, pH 7.0, 20 μmol of ammonium sulphate (as a possible source of nitrogen for induced enzyme synthesis), and 100 μmol of methanol, as described in the Materials and Methods section. Control flasks lacked methanol. The flasks were incubated for 8 h and the oxygen consumption was measured. It was found with the wild-type organism that after 4 h of incubation the suspension containing methanol showed an increasing rate of oxygen uptake over the endogenous control (Fig. 1*a*). This increase coincided with appearance of methanol dehydrogenase activity [reaction (6)] in cell-free extracts. Each mutant was tested manometrically in this

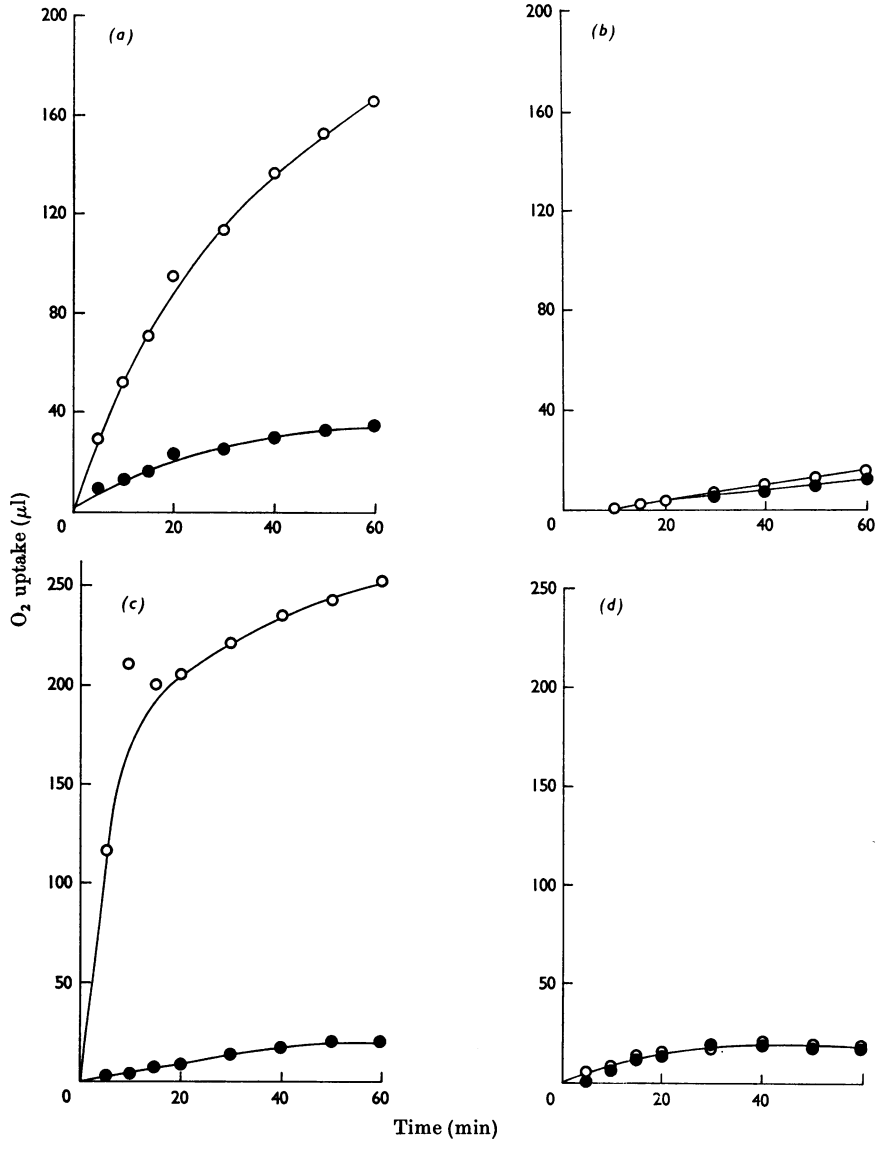
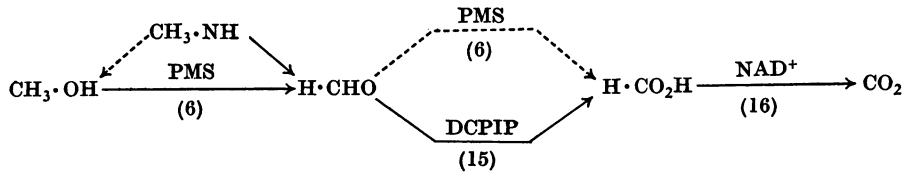


Fig. 2. PMS-dependent oxidation of methanol or formaldehyde by extracts of methylamine-grown *Pseudomonas* AM1 and mutant M-15A. The conditions of assay are described in the Materials and Methods section. ○, Presence of substrate; ●, endogenous. (*a*) Wild-type extract+formaldehyde. (*b*) Mutant M-15A extract+formaldehyde. (*c*) Wild-type extract+methanol. (*d*) Mutant M-15A extract+methanol.

way and larger batches of cells, exposed to methanol overnight under the same conditions, were tested for the presence of methanol dehydrogenase in cell-free extracts prepared from them, by the assay described in the Materials and Methods section. Such tests carried out on the ten mutants that grew on formate or methylamine but not on methanol gave a variety of results: in some of the mutants, methanol-oxidizing activity and methanol dehydrogenase activity could be induced, suggesting a possible lesion in the energy-coupling mechanism; in others, methanol-oxidizing activity in the whole cells could not be induced but the methanol dehydrogenase enzyme was induced, suggesting a lesion in



Scheme 2. Oxidation of C_1 compounds by *Pseudomonas* AM1. The numerals in parentheses refer to equations in the text. The dotted lines indicate steps whose possible involvement in methylamine oxidation has been eliminated by the behaviour of mutant M-15A.

the electron transport system; two mutants showed neither induction of methanol-oxidizing activity nor synthesis of the PMS-linked methanol dehydrogenase. The results for one of these two mutants, M-15A, are shown in Fig. 1*b*.

Mutant M-15A. Mutant M-15A, which grew on formate or methylamine but not on methanol, and which lacked methanol dehydrogenase, was further studied with respect to the mechanisms of oxidation of C_1 substrates.

Methylamine-grown *Pseudomonas* AM1 contains both the PMS-linked methanol dehydrogenase (6) see Fig. 2*c*, and a PMS-linked methylamine dehydrogenase (Eady & Large, 1968):

$$CH_3\cdot NH_3^+Cl^- + PMS + H_2O \rightarrow H\cdot CHO + PMSH_2 + NH_4^+Cl^- \quad (14)$$

The presence of the PMS-linked methanol dehydrogenase in the methylamine-grown organism raises the question whether, during growth on methylamine, the substrate is first converted into methanol and the methanol then oxidized to formaldehyde (Scheme 2). However, since methylamine-grown mutant M-15A lacks the methanol dehydrogenase (Fig. 2*d*), this enzyme cannot indeed be necessary for growth on methylamine.

In addition to the PMS-linked methanol dehydrogenase (6), methanol-grown *Pseudomonas* AM1 contains two further enzymes that catalyse the oxidation of C_1 compounds (Johnson & Quayle, 1964):

$$HC\cdot HO + H_2O + DCPIP \rightarrow H\cdot CO_2H + DCPIPH_2 \quad (15)$$

$$H\cdot CO_2H + NAD^+ \rightarrow CO_2 + NADH \quad (16)$$

These three enzymes together can thus catalyse the total oxidation of methanol to carbon dioxide (see Scheme 2). A further possibility has been raised by Ladner & Zatman (1969) who have proposed that the methanol dehydrogenase (6) may also catalyse the oxidation of formaldehyde to formate. If, in the assay for PMS-linked methanol dehydrogenase in extracts of methylamine-grown *Pseudomonas* AM1, 16 μmol of methanol-free formaldehyde is substituted for the methanol, the formaldehyde is oxidized at 26% of the rate of methanol oxidation (Figs. 2*a* and 2*c*). This may be compared with the behaviour of extracts of methylamine-grown mutant M-15A, lacking the PMS-linked methanol dehydrogenase, which show zero activity towards formaldehyde in this assay (Fig. 2*b*). These findings indicate that the oxidation of methanol and formaldehyde observed in extracts of the wild-type organism under the above experimental conditions is catalysed by one enzyme rather than two, and supports the suggestion of Ladner & Zatman (1969) that enzyme (6) possesses dual substrate specificity towards methanol and formaldehyde.

The ability of washed suspensions of mutant M-15A to oxidize formaldehyde was nevertheless unimpaired, as compared with wild-type *Pseudomonas* AM1. This was shown by incubating suspensions of the succinate-grown organisms overnight at 30°C in 50mM-phosphate buffer, pH 7.2, in the presence of 0.2% (v/v) methanol and 10mM-ammonium sulphate. The organisms were harvested and tested manometrically for oxidation of formaldehyde (15 μmol), as described in the Materials and Methods section. The wild-type organism and mutant M-15A oxidized formaldehyde at rates of 110 and 95 μl of O_2 consumed/h per mg dry wt. respectively. The succinate-grown organisms both contained similar activities of enzyme (15), i.e. 1.55 and 1.52 μmol of DCPIP reduced/h per mg of protein for the wild-type and mutant respectively. Since methylamine-grown mutant M-15A contains enzyme (15) but lacks enzyme (6) and yet can oxidize formaldehyde as well as can the wild-type organism, the former enzyme is implicated in

Table 2. *Enzyme activities in* Pseudomonas *AM* 1 *and mutant* 20*B-L*

Assay conditions are as described in the Materials and Methods section.

Organism and growth substrate	Specific activities (μmol/h per mg of protein)		
	Hydroxypyruvate reductase		Glyoxylate reductase
pH ...	4.5	7.5	7.5
Wild type (succinate)	16.6	7.8	1.06
Wild type (methanol)	125.0	52.2	
Mutant 20B-L (succinate)	0	0	0
Mutant 20B-L (succinate*)	0	0	

* Grown on succinate and incubated overnight with methanol.

formaldehyde oxidation *in vivo* rather than the latter (Scheme 2).

Mutant 20B-L. One of the four type I mutants, 20B-L, which would not grow on methanol, methylamine or formate, was able to oxidize methanol after incubation overnight in the presence of methanol, at rates similar to the wild-type organism. This indicated that its C_1-oxidative abilities were unimpaired and that its lesion might be in the carbon-assimilation pathway. Cell-free extracts of the succinate-grown organism were tested for the presence of the enzymes of the non-phosphorylated pathway [(3), (4), (5) and serine hydroxymethylase (2)]. The specific activities of enzymes (3), (5) and (2) in 20B-L were similar to those in the wild-type organism, whereas no activity of hydroxypyruvate reductase could be demonstrated at either pH 4.5 or pH 7.5 even in extracts prepared from the succinate-grown mutant, which had been incubated overnight in the presence of methanol (Table 2).

The physiological significance of the mutation was further demonstrated in reversion experiments by the method of Yanofsky (1963). A 50ml culture of mutant 20B-L was grown for 24h on succinate. After harvesting and washing twice in sterile water, the cells were resuspended in sterile Jayasuriya salts solution (Jayasuriya, 1955) and 10^8 cells were then spread on each of several plates containing methanol–salts–agar medium. Ethyl methanesulphonate was placed on circles of filter paper in the centre of the plates and the plates were incubated for 3–4 days at 30°C. Twelve revertant colonies were picked off and streaked out on to plates containing methanol growth medium. Batches of the revertants were then grown on methanol and extracts prepared from them were assayed for hydroxypyruvate reductase activity. The enzyme was found to be present in every case, the specific activities at pH 4.5 being in the range 72.9–136.5 μmol of NADH oxidized/h per mg of protein. Since these revertants also regained the ability to grow on methylamine or formate, these results show that hydroxypyruvate reductase is necessary for growth on these C_1 compounds but not for growth on succinate.

Large & Quayle (1963) concluded that hydroxypyruvate reductase was also responsible for the reduction of glyoxylate to glycollate that could be observed in extracts of *Pseudomonas* AM 1, since the ratio of the two activities remained the same after ammonium sulphate fractionation. In confirmation of this, an extract of succinate-grown mutant 20B-L was found to lack glyoxylate reductase activity when assayed at either pH 4.5 or 7.5 (Table 2). Hydroxypyruvate and glyoxylate reductase activities are also presumed to be common to the same enzyme in plant or animal tissue (see Sallach, 1966).

As mentioned above, incubation of suspensions of the succinate-grown mutant with methanol induced methanol-oxidizing activity. When suspensions of the mutant, which had been grown on succinate and incubated overnight with methanol, were incubated in the same manometric system containing 10 μmol of methanol it was found that the O_2 uptake was 55±5% of the theoretical maximum for total oxidation of the methanol to carbon dioxide. Similar experiments with wild-type organisms gave results of 65–85%. This suggested that the mutant might be either excreting some assimilatory product into the medium or only partially oxidizing the methanol. However, no evidence could be obtained for the presence of keto acids (in particular, hydroxypyruvate or glyoxylate), amino acids, methanol, formaldehyde or formate in the supernatants in the Warburg flasks after oxidation of methanol had stopped. Further, when 10 μmol of [^{14}C]methanol was oxidized by suspensions of either mutant 20B-L or wild-type bacteria, no significant difference could be found

between the two organisms with respect to the distribution of isotope between the aqueous supernatants, the 80%-ethanol extracts of the bacterial pellets and the bacterial pellets themselves after ethanolic extraction.

Mutants of types II and III

Mutants of types II and III are defined by their inability to grow on succinate unless supplemented with serine or with glycine plus methanol respectively. It was found that growth of *Pseudomonas* AM 1 was inhibited by low concentrations of some amino acids, e.g. no growth was observed after incubation for 6h at 30°C in succinate growth medium to which had been added 5mM-serine and 5mM-glycine. For this reason the concentration of the serine and glycine supplements was kept to 1mM and 1.3mM respectively, although higher concentrations (2.9mM-serine and 4.9mM-glycine) had been used by Pizer (1965) for growth of the corresponding mutants of *E. coli*. In case the glycine supplement might be growth-limiting, 5mM-glyoxylate was used as an additional supplement. It was considered that the glyoxylate might function as a precursor of glycine, particularly since the presence of 5mM-glyoxylate in methanol growth medium stimulates growth of *Pseudomonas* AM 1.

Several mutant isolations were tried in which the minimal growth medium contained 50mM-succinate as carbon source and the supplemented growth medium consisted of 50mM-succinate + 1mM-serine + 1.3mM-glycine + 5mM-glyoxylate (medium SSGG) or 50mM-succinate + 1.3mM-glycine + 5mM-glyoxylate + 5mM-methanol (medium SGGM). Many mutants of various phenotypes were obtained: none of them was able to grow on methanol, methylamine or formate; most of them grew more slowly on succinate than did the wild-type organism; growth on succinate was generally stimulated by the supplement. Two mutants, F and H 12, were selected for further study. Mutant F was isolated by using the supplemented medium SSGG. It grew on succinate at half the rate of wild-type *Pseudomonas* AM 1. The main stimulatory effect of the SSGG medium was due to glyoxylate, which when added singly to succinate minimal medium restored the growth rate of mutant F to 88% of that of the wild type; glycine stimulated growth to 70% of that of the wild type; serine was without effect. Mutant H 12 was isolated by using the supplemented medium SGGM. It was unable to grow on succinate unless supplemented with glyoxylate + methanol; addition of either supplement singly to succinate growth medium resulted in only slight growth. The growth requirements of these mutants are thus very close to those predicted for a type III mutant lacking serine hydroxymethylase. However, cell-free extracts of the organisms grown on supplemented succinate medium possessed serine hydroxymethylase of specific activity comparable with that of the similarly grown wild-type organism. Serine hydroxymethylase was assayed by the three methods described in the Materials and Methods section.

DISCUSSION

The finding that mutational loss of hydroxypyruvate reductase in *Pseudomonas* AM 1 leads to inability to grow on methanol, methylamine or formate, but does not affect growth on succinate, offers strong support for operation of the metabolic scheme shown in Scheme 1. The involvement of the non-phosphorylated pathway in growth on C_1 compounds is also indicated by the higher specific activities of enzymes (4) and (5) during growth on methanol compared with growth on succinate. The converse changes in specific activities of the enzymes of the phosphorylated pathway (11), (12) and (13) during growth on methanol or succinate, which would support a special involvement of the pathway in synthesis of serine from glycolytic intermediates, were not observed. Nor could any evidence be found for repression of phosphoglycerate dehydrogenase (11) when comparing the specific activity of the enzyme in extracts of wild-type cells grown on medium SSGG in place of unsupplemented succinate medium. These negative findings may, however, reflect a lack of control at the level of enzyme synthesis, as is observed with *E. coli*. Pizer & Potochny (1964) showed from competition experiments with *E. coli* that although exogenous serine inhibited endogenous synthesis of serine and glycine from glucose or fructose by more than 90%, the concentrations of phosphoglycerate dehydrogenase (11) and phosphoserine phosphatase (13) remained unchanged. Their experiments indicated that, instead, control was mainly being exerted by inhibition of the activity of enzyme (11). A decrease in the specific activity of enzyme (11), but not enzyme (13), was observed when the growth medium was enriched with a mixture of L-threonine, L-methionine, L-leucine and DL-isoleucine. The physiological significance of repression by these four amino acids is not understood. Clearly, isolation of a mutant of *Pseudomonas* AM 1 lacking an enzyme of the phosphorylated pathway is needed to establish unequivocally the role of this pathway in the latter organism.

The demonstration that hydroxypyruvate reductase is an enzyme necessary for growth on methanol, methylamine or formate by the 'serine pathway' [reactions (2), (3), (4) and (5)] adds further significance to the observation of Lawrence, Kemp &

Quayle (1970) that methane-grown *Methanomonas methanooxidans* contains this enzyme at high specific activity, whereas methane-grown *Pseudomonas methanica* and *Methylococcus capsulatus* do not. This fact, taken in conjunction with the results of whole cell radioisotope experiments and the absence from *Mtn. methanooxidans* of an enzyme system catalysing condensation of formaldehyde and ribose 5-phosphate, led to the conclusion that the serine pathway operated in the first organism but not in the other two.

One of the potentially most valuable mutants for further investigation of the serine pathway would be a type III mutant lacking serine hydroxymethylase. Study of such a mutant could establish whether serine hydroxymethylase is necessary for the major assimilation of carbon into cell constituents during growth on C_1 compounds, and for the synthesis of glycine and C_1 units during growth on glycolytic intermediates. In addition, such a mutant might accumulate glycine or precursors of glycine during metabolism of C_1 compounds and thus enable a direct approach to be made, at the whole-cell level, to the still unsolved problem of net synthesis of the glycine skeleton from C_1 compounds. No such mutant has yet been isolated, even though mutants F and H 12 show nutritional requirements close to those predicted for a mutant of this type. The nature of the lesion(s) in these mutants is not known but the simplest explanation might be that they are double mutants, affected in enzymes essential for growth on C_1 and C_4 substrates. No positive enzymic evidence for this is yet available.

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