The Biosynthesis of Serine and Glycine in *Pseudomonas* AM1 with Special Reference to Growth on Carbon Sources other than C₁ Compounds

By W. HARDER* AND J. R. QUAYLE

Department of Microbiology, University of Sheffield, Sheffield S10 2TN, U.K.

(Received 18 September 1970)

1. A mutant, 20S, of *Pseudomonas* AM1 was obtained that requires a supplement of serine to grow on succinate, lactate or ethanol. This mutant lacks phosphoserine phosphatase and revertants to wild-type phenotype regained this enzymic activity showing that the phosphorylated pathway of serine biosynthesis is necessary for growth on these three substrates. 2. The requirement for supplemental serine by mutant 20S could be met by glycine, suggesting that *Pseudomonas* AM1 can obtain C₁ units from glycine. 3. Mutant 20S grows on C₁ compounds at a lower rate compared with the wild type. Supplementation with serine stimulated the growth rate of the mutant suggesting that the phosphorylated pathway of serine biosynthesis plays some role, but not an essential role, during growth on C₁ compounds. 4. A mutant, 82G, was obtained that requires a supplement of glycine to grow on succinate, lactate or ethanol. When grown in such supplemented media, the mutant lacks serine hydroxymethyltransferase and revertants to wild-type phenotype regained enzymic activity showing that during growth on succinate, lactate or ethanol, glycine is made from serine via serine hydroxymethyltransferase, and that the organism can obtain C₁ units from glycine. 5. Mutant 82G grew on methanol and then contained serine hydroxymethyltransferase suggesting that this enzyme is necessary for growth on C₁ compounds and that *Pseudomonas* AM1 may synthesize two such enzymes, one used in growth on C₁ compounds, the other used in growth on other substrates. Mutant 82G might lack the latter enzyme. 6. Phosphoglycerate dehydrogenase is specifically inhibited by L-serine and the regulatory implications of this are discussed.

On the basis of enzyme lesions in nutritional mutants it has been shown that the biosynthesis of serine in *Escherichia coli* (Pizer, 1963, 1965; Pizer & Potochny, 1964; Umbarger, Umbarger & Siu, 1963), *Haemophilus influenzae* (Pizer, Ponce-De-Leon & Michalka, 1969) and *Salmonella typhimurium* (Umbarger & Umbarger, 1962; Umbarger *et al.* 1963) depends on a pathway involving the following reactions:

Glycine is synthesized from serine by the following reaction:

* Present address: Department of Microbiology, University of Groningen, Kerklaan 30, Haren (Gr), The Netherlands.

and since in *E. coli* a mutational loss of this enzyme produces a nutritional requirement for glycine that cannot be satisfied by serine (Pizer & Potochny, 1964) it has been concluded that this reaction is essential for glycine biosynthesis.

Enzymes catalysing reactions (1)–(4) have been found in cell-free extracts of *Pseudomonas* AM1 by Heptinstall & Quayle (1970), which led these authors to suggest that the above phosphorylated pathway operates to supply serine and glycine when the organism is grown on succinate or similar compounds. When *Pseudomonas* AM1 is grown on C₁ compounds, glycolytic intermediates are formed by a pathway in which the following reactions have been implicated (Large, Peel & Quayle, 1961, 1962*a,b*; Large & Quayle, 1963; Heptinstall & Quayle, 1970):

Scheme 1. Alternative routes of serine metabolism in *Pseudomonas* AM1. The numerals in parentheses refer to equations in the text; the dotted lines represent enzyme lesions in the respective mutants 82G, 20S and 20 BL. Mutant 20 BL is described by Heptinstall & Quayle (1970).

Reactions (1)–(3) and (6)–(8) interconnect phosphoglycerate and serine, resulting in a cyclic pathway for serine metabolism (Scheme 1).

It was decided to study the physiological significance of the phosphorylated pathway (reactions 1–3) for the biosynthesis of serine in *Pseudomonas* AM1 during growth on succinate or similar compounds. According to the above scheme, reaction (4) has a key function both in glycine synthesis when the organism is grown on succinate, and, as reaction (5), in serine synthesis during growth on C₁ compounds. This suggests a dual role for the enzyme in question and is thus of special interest. This paper records the isolation and properties of serine- and glycine-auxotrophs of *Pseudomonas* AM1 and their bearing on the physiological role of the phosphoryl-

ated pathway for the synthesis of serine, and of serine hydroxymethyltransferase (reaction 4) for the synthesis of glycine, during growth of the organism on succinate or similar carbon sources.

MATERIALS AND METHODS

Growth of the organism. *Pseudomonas* AM1 was maintained on an inorganic salts medium (Jayasuriya, 1955) containing 50 mM-methylamine hydrochloride and 1.5% (w/v) agar. The organism was transferred monthly; slopes were incubated at 30°C for 3 days and stored at 4°C. Serine- and glycine-requiring mutants of *Pseudomonas* AM1 were maintained similarly on the above medium and on media containing 50 mM-succinate + 1 mM-L-serine or 50 mM-succinate + 1 mM-glycine + 1 mM-formate respectively. Growth in liquid medium was as described by Heptinstall & Quayle (1970). Determinations of specific growth rate μ ($\mu = \frac{1}{x} \cdot \frac{dx}{dt}$, where x is a para-

meter of cell mass) were made by following extinction changes at 650 nm in 250 ml conical flasks fitted with optically matched side arms and containing 40 ml of medium. Cultures of *Pseudomonas* M27 and *Pseudomonas* PP were kindly provided by Dr L. J. Zatman (Department of Microbiology, University of Reading, U.K.) and were maintained as described for *Pseudomonas* AM1. Departmental stock cultures of *Protaminobacter ruber* and *Vibrio extorquens* were maintained similarly.

Throughout this paper the terms 'salts solutions' and 'salts medium' refer to the solution of Jayasuriya (1955).

Preparation of cell-free extracts. Bacteria were disrupted in an ultrasonic disintegrator (MSE model 60W) by treating a suspension of 1 g wet wt. of cells in 4 ml of 50 mM-potassium phosphate-sodium phosphate buffer, pH 7.0, for 2 min at 0°C (power output 60W at 25 kHz). Extracts were used after centrifugation at 15000g for 15 min.

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 tris(cyclohexylammonium) salt (Calbiochem, Los Angeles, Calif., U.S.A.). Preparation of the free acid and assay for hydrolysis of the phosphate group was performed as described by Heptinstall & Quayle (1970). (±)-Tetrahydrofolic acid was prepared by hydrogenating folic acid in the presence of Pt catalyst by the method used by Jones, Guest & Woods (1961) for reduction of pteroyltriglutamate. The concentration of the solution was determined spectrophotometrically and the solution was stored as described by Heptinstall & Quayle (1970), except that H₂ was used for filling the Thunberg tubes. DL-O-Phosphoserine was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Preparation of mutants. The procedure used for the isolation of mutants of *Pseudomonas* AM1 was a modification of that described by Heptinstall & Quayle (1970), employing a series of three or four successive penicillin enrichments (Schlegel, 1965). A culture (50 ml) of the wild-type organism was grown overnight on succinate medium. The cells were harvested by centrifugation, washed once and resuspended at a cell density of approx. 10⁹ cells/ml in a solution of inorganic salts (Jayasuriya, 1955) in which the phosphate buffer had been replaced by 50 mM-tris-maleate buffer, pH 6.0. To 1.0 ml of this suspension was added 1.0 ml of a solution (4 mg/ml) of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and the resulting suspension was incubated on a shaker at 30°C for 2 h. The cells were then centrifuged, washed twice with sterile water and resuspended in 10 ml of a supplemented growth medium (see the text) that would permit growth of the required mutants. The cells were incubated on a shaker at 30°C for 20–30 h to express mutations. The suspension was then centrifuged and the cell pellet washed twice with sterile water. Cells were resuspended in 40 ml of succinate growth medium and incubated on a shaker at 30°C for 4 h. Benzylpenicillin (Glaxo Laboratories Ltd., Greenford, Middx., U.K.) was then added to a final concentration of 250 units/ml and the suspension was incubated at 30°C with shaking for approx. 20 h. After this incubation the suspension was centrifuged, the cell pellet washed twice with sterile water and cells were resuspended in 10 ml of supplemented growth medium. After incubation of the

cells for another 20–30 h at 30°C on a shaker, the suspension was centrifuged, and the cells were washed twice with sterile water and resuspended in 40 ml of succinate growth medium. After incubation on a shaker at 30°C for 4 h, benzylpenicillin was added to a final concentration of 400 units/ml and the suspension incubated at 30°C with shaking for approx. 30 h. The suspension was then centrifuged, the cell pellet washed twice with sterile water and the cells were resuspended in 5 ml of salts medium. Of this suspension 1 ml was transferred to 10 ml of supplemented medium and the cells were taken through another cycle of penicillin enrichment in which the penicillin concentration was increased to 600 units/ml. After three or four enrichment cycles the final suspension was centrifuged and the cells were washed twice with sterile water and resuspended in 10 ml of salts medium. Serial tenfold dilutions of this suspension were then made and 0.1 ml samples of appropriate dilutions were spread on plates of 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 and supplemented media. The replica plates were incubated for 4–6 days and compared with each other. Presumptive mutants were picked off, resuspended in a drop of salts medium 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 two enzymes of the phosphorylated pathway of serine biosynthesis were performed at 37°C. The other assay was 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-O-phosphoserine as described by Heptinstall & Quayle (1970).

Phosphoglycerate dehydrogenase (phosphohydroxypyruvate reductase). This assay was performed in anaerobic cuvettes under N₂ to eliminate NADH oxidase activity. The reaction was performed as described by Heptinstall & Quayle (1970) by following the oxidation of NADH at 340 nm consequent on reduction of phosphohydroxypyruvate.

L-Serine-tetrahydrofolate 5,10-hydroxymethyltransferase (serine hydroxymethyltransferase, serine hydroxymethylase, EC 2.1.2.1). The enzyme was assayed by following the formation of 5,10-CH₂-H₄folate from serine measured spectrophotometrically as reduction of NADP⁺ consequent on the oxidation of 5,10-CH₂-H₄folate to 5,10-CH=H₄folate by methylene tetrahydrofolate dehydrogenase (Large & Quayle, 1963). The latter enzyme was present in excess over serine hydroxymethylase in extracts of *Pseudomonas* AM1.

RESULTS

Isolation and characterization of mutants

If the proposed pathway (reactions 1–4) for the biosynthesis of serine and glycine in *Pseudomonas* AM1 during growth on succinate is correct, a

Table 1. *Growth inhibition of Pseudomonas AM 1 by serine and glycine*

The organism was grown in mineral salts medium with 0.25% (w/v) anhydrous sodium succinate or 0.25% (v/v) methanol as carbon source and supplemented as indicated. Methods are described in the Materials and Methods section.

Growth substrate	Supplement			Lag time (h)	Specific growth rate (h ⁻¹)
	Glycine (mm)	Serine (mm)	Methanol (mm)		
Succinate	—	—	—	4	0.20
	1	—	—	4	0.20
	2.5	—	—	6	0.14
	5	—	—	12	0.10
	—	1	—	4	0.20
	—	5	—	4	0.20
	—	10	—	4	0.20
	1	—	1	4	0.19
	2.5	—	2.5	5	0.14
	5	—	5	11	0.10
Methanol	—	—	—	12	0.17
	1	—	—	12	0.17
	2.5	—	—	12	0.16
	5	—	—	16	0.14
	—	1	—	12	0.17
	—	5	—	11	0.16
	—	10	—	12	0.17

mutant lacking one of the enzymes (1)–(3) would be unable to grow on succinate unless supplemented with serine. Similarly, a mutant lacking enzyme (4) would only be able to grow on succinate when supplemented with glycine and a source of C₁ units. Hence such mutants, called type II and type III mutants respectively (Heptinstall & Quayle, 1970), could be isolated on the basis of their ability to grow on succinate+serine or succinate+glycine+methanol respectively but not on succinate alone. Heptinstall & Quayle (1970) reported that growth of wild-type *Pseudomonas* AM1 is inhibited by low concentrations of serine and glycine. As this finding obviously presents problems in the isolation of type II and type III mutants, it was decided to study the growth inhibition of *Pseudomonas* AM1 by these amino acids in more detail. This was done in 250ml conical flasks with fitted side arm, containing 40ml of salts medium enriched with 0.25% (w/v) anhydrous sodium succinate supplemented with L-serine or glycine or both. The flasks were inoculated with 5ml of a 24h culture grown in the above medium deficient in amino acids, and incubated on a shaker at 30°C. The extinction at 650nm was measured at regular time-intervals and the specific growth rate and lag time were calculated from semi-logarithmic plots of extinction against time.

Serine had no effect on growth rate or lag time when present in succinate media in concentrations up to 10mm (Table 1). However, glycine caused

both a lower growth rate and a longer lag time when succinate media were supplemented with concentrations above 1mm. When the concentration of glycine was increased to 5mm, a 50% inhibition of the growth rate was found and at 10mm concentration no growth occurred. A similar inhibition was found when the succinate medium was supplemented with glycine plus an equal amount of either methanol or formate.

To see whether this behaviour is general among other organisms capable of growth on methanol, a survey was made of the growth inhibition by low concentrations of glycine of 27 new isolates and a number of previously described C₁ utilizers including *Pr. ruber* (den Dooren de Jong, 1927), *V. extorquens* (Bassalik, 1913), *Pseudomonas* M27 (Anthony & Zatman, 1964) and *Pseudomonas* PP (Ladner & Zatman, 1969). It was found that growth of all organisms tested was inhibited by the presence of 5mm-glycine in the succinate growth medium to an extent similar to that observed in *Pseudomonas* AM1. This sensitivity to glycine seems to be commonly encountered among methanol-utilizing bacteria and may therefore be connected with the special metabolism involved during growth of such organisms on C₁ compounds.

On the basis of the above results obtained with *Pseudomonas* AM1, it was decided to use both serine and glycine at concentrations of 1–2mm in supplemented media for mutant isolations. Although higher concentrations (2.9mm-serine and

Table 2. *Growth requirements of a serine-auxotroph (20S) and a glycine-auxotroph (82G) of Pseudomonas AM1*

The medium was a salts solution containing 50 mM growth substrate and supplemented with 1 mM-serine or 1 mM-glycine + 1 mM-formate (+, growth; -, no growth).

Growth substrate	Supplement	Growth of organism		
		Wild-type	Mutant 20S	Mutant 82G
Succinate	None	+	-	-
	Serine	+	+	-
	Glycine + formate	+	+	+
Lactate	None	+	-	-
	Serine	+	+	-
	Glycine + formate	+	+	+
Ethanol	None	+	-	-
	Serine	+	+	-
	Glycine + formate	+	+	+
Methanol	None	+	+	+

4.9 mM-glycine) have been used by Pizer (1965) for growth of corresponding mutants of *E. coli* it can be estimated from the growth requirements of these and similar mutants (Dempsey, 1969) that 1 mM concentrations of these supplements would allow sufficient growth of serine- and glycine-auxotrophs of *Pseudomonas AM1* for isolation purposes.

Serine-auxotrophs of Pseudomonas AM1. These mutants were expressed in succinate + 1 mM-serine growth medium, and the penicillin treatment was done in succinate growth medium, in which the desired mutants would not grow. The original mutant isolation procedure of Heptinstall & Quayle (1970) was modified as described in the Materials and Methods section since several attempts to obtain serine-requiring mutants using the former procedure had failed. Master plates were prepared by using succinate + 1 mM-serine medium and these were replicated on to plates containing respectively succinate + 1 mM-serine and succinate alone. A total of 36 mutants was isolated and six of these, which differed somewhat in their growth characteristics on succinate + serine media, were studied in more detail. As the results obtained with the six mutants were essentially similar, only the results obtained with one of them, 20S, are given.

The growth requirements of mutant 20S were studied both on plates and in liquid medium. The mutant was grown for 24–30 h at 30°C in 50 ml of succinate + 1 mM-serine medium on a shaker. The culture was centrifuged and the cell pellet was washed twice with salts medium. The cells were resuspended in approx. 25 ml of salts medium and the suspension was streaked on plates containing a number of different solid media (Table 2). A further portion of the suspension was used to inoculate tubes. These tubes contained 5 ml of liquid media of similar composition and were inoculated with 0.5 ml of the mutant suspension. Plates and tubes were incubated at 30°C (the tubes on a shaker) and

examined after 3 and 6 days. The results (Table 2) showed that the mutant 20S was only able to grow on succinate, lactate or ethanol when these media were supplemented with 1 mM-serine or 1 mM-glycine + 1 mM-formate. The mutant showed good growth on methanol or formate media although the growth rate was lower than that of the wild-type. The growth of the mutant on supplemented succinate medium was found to depend on the initial concentration of supplement. This was studied as follows. Batches of 30 ml of succinate media containing different concentrations of serine were dispensed in 250 ml conical flasks fitted with optically matched side arms. After sterilization (121 b/in² for 20 min) the flasks were inoculated with 1 ml of a washed suspension of the mutant (see above) and incubated at 30°C on a shaker. The extinction of the cultures at 650 nm was measured at regular time-intervals and the difference between the maximum extinction achieved and the initial extinction was taken as the growth response at that particular serine concentration (Fig. 1). A linear relationship was found between the growth response and the initial serine concentration (Fig. 2a). These results are similar to those reported by Pizer & Potochny (1964) with a serine-requiring mutant of *E. coli*. From the slope of the graph relating growth response and serine concentration and by using the relationship between extinction and dry weight of the wild-type organism it was calculated that for the formation of 1 g (dry wt.) of cells 165 mg of serine is needed. This value is similar to that found for a serine-requiring mutant of *E. coli* (Dempsey, 1969).

Glycine + formate can substitute for serine as a supplement for growth of the mutant on succinate (Table 2). Later it was found to be unnecessary to include methanol or formate in the glycine-supplemented growth media, indicating that the mutant organism is able to obtain C₁ units either from

glycine or from succinate. The growth response of the mutant to limiting glycine was studied as described above for limiting serine, and was found

Fig. 1. Growth response to limiting serine concentrations of a serine-requiring mutant (20S) of *Pseudomonas* AM1. The growth medium was a salts solution containing 0.4% (w/v) anhydrous sodium succinate as carbon source. Methods are described in the text. $\square$, No serine; $\blacktriangle$, 50 μM -serine; $\triangle$, 100 μM -serine; $\circ$, 200 μM -serine; $\bullet$, 400 μM -serine.

Fig. 2. Growth response of a serine-requiring mutant (20S) of *Pseudomonas* AM1 to (a) limiting serine and (b) limiting glycine concentrations. The growth medium was a salts solution containing 0.4% (w/v) anhydrous sodium succinate as carbon source. Methods are described in the text.

to be a linear function of the glycine concentration (Fig. 2b). The slopes of the graphs in Figs. 2(a) and 2(b) are 1.39 and 0.75 respectively, the units in each case being growth (ΔE_{650})/mmol of amino acid supplement. These slopes show that the relative molar amount of serine and glycine that allowed a similar growth response in succinate medium were in the ratio serine:glycine = 1:1.85.

Cell-free extracts of mutant 20S grown on succinate + 1 mM-serine were tested for the presence of two enzymes of the phosphorylated pathway, phosphoglycerate dehydrogenase and phosphoserine phosphatase (reactions 1 and 3). The specific activity of phosphoglycerate dehydrogenase in 20S was similar to that in the wild-type organism, whereas no activity of phosphoserine phosphatase could be demonstrated in extracts prepared from the mutant (Table 3). The physiological significance of the mutation was further demonstrated by preparation of revertants by using the method described by Yanofsky (1963). A 50 ml culture of mutant 20S was grown for 24 h on succinate + 1 mM-serine. After being harvested and washed twice in sterile water, the cells were resuspended in sterile salts solution and 10^8 cells were then spread on each of several plates containing succinate-salts-agar medium. Ethylmethanesulphonate was placed on circles of filter paper in the centre of the plates and the plates were incubated for 4–6 days at 30°C. Two types of revertant colonies were picked off: colonies growing at the edge of the growth inhibition zone close to the filter paper, called 'induced revertants', and colonies growing at random over the plate even in the absence of mutagen, which were called 'spontaneous revertants'. Four of each type of revertant colony were purified by re-streaking on succinate-containing growth medium. Batches of the revertants were then grown on succinate and extracts prepared from them were assayed for phosphoserine phosphatase activity. The enzyme was found to be present in every case, the specific activity being in the range 0.9–2.1 μmol of P_i

Table 3. Activities of phosphoserine phosphatase and phosphoglycerate dehydrogenase in wild-type and mutants of *Pseudomonas* AM 1

Assay methods are reported in the Materials and Methods section. The duplicate values are taken from measurements made on batches of cells grown separately on succinate + 1 mM-serine.

Organism	Enzyme activity ($\mu\text{mol/h}$ per mg of protein)	
	Phosphoserine phosphatase	Phosphoglycerate dehydrogenase
Wild-type	1.4; 1.7	3.3; 4.2
20S (serine-auxotroph)	Not detectable	4.6; 6.4
20SR _i (induced revertant to wild-type)	1.1; 1.6	4.1; 3.2
20SR _{sp} (spontaneous revertant to wild-type)	0.9; 1.3	2.8; 3.7

Table 4. *Effect of serine on the specific growth rates of wild-type and mutants of Pseudomonas AM 1*

The organisms were grown in a salts medium containing 0.5% (v/v) methanol, supplemented with L-serine as indicated.

Organism	Specific growth rates of organisms grown in different media (h ⁻¹)		
	Methanol	Methanol + 1 mM-serine	Methanol + 5 mM-serine
Wild-type	0.17	0.17	0.17
20S (serine-auxotroph)	0.12	0.14	0.14
73S (serine-auxotroph)	0.08	0.10	0.10
20SR _i (induced revertant to wild-type)	0.09	0.09	0.09
20SR _{sp} (spontaneous revertant to wild-type)	0.08	0.08	0.08

formed/h per mg of protein (Table 3). Since these revertants also regained the ability to grow on lactate and ethanol, besides succinate, these results show that phosphoserine phosphatase is necessary for the biosynthesis of serine, and hence that the phosphorylated pathway is the major route for serine biosynthesis when *Pseudomonas* AM1 is grown on succinate, lactate or ethanol.

The possible significance of the phosphorylated pathway during growth of *Pseudomonas* AM1 on methanol was studied by comparing the growth rates of wild-type, mutant and revertant organisms on methanol, supplemented with low concentrations of L-serine. Cultures (50ml) of wild-type, mutant 20S and the revertants 20SR_i and 20SR_{sp} were grown on methanol for 24h. After harvesting and washing twice with sterile salts medium the cells were resuspended in 10ml of salts medium and 1ml of this suspension was used to inoculate 250ml conical flasks fitted with optically matched side arms. These flasks contained 40ml of methanol growth medium, supplemented with different concentrations of serine. Extinction at 650nm was measured at regular intervals and the specific growth rate of each organism was calculated from a semi-logarithmic plot relating extinction and time. The results (Table 4) show that the growth rates of wild-type and revertant organisms were not influenced by the addition of serine to the growth medium. However, the growth rates of both mutants tested (20S and 73S) were significantly increased by the addition of 1 mM-serine to the minimal methanol medium, indicating that the phosphorylated pathway for serine biosynthesis has a physiological function, although not a necessary one, during growth of *Pseudomonas* AM1 on methanol.

Glycine-auxotrophs of Pseudomonas AM1. These mutants were expressed in succinate + 1 mM-glycine + 1 mM-formate (SGF) growth medium and the penicillin treatment was done in succinate growth medium in which the desired mutants would not grow. Master plates were prepared from SGF growth medium and these were replicated on to SGF growth medium and succinate plates.

Fig. 3. Growth response to limiting glycine concentrations of a glycine-requiring mutant (82G) of *Pseudomonas* AM1. The growth medium was a salts solution containing 0.4% (w/v) anhydrous sodium succinate as carbon source. Methods are described in the text.

Although more than 100 presumptive mutants were picked off, only two reasonably stable mutants were obtained in pure culture. Results obtained with both mutants were similar and therefore results obtained with only one of them, 82G, are given. The growth requirements of mutant 82G were studied on plates and in liquid medium as described above for mutant 20S, except that SGF growth medium was used as complete growth medium. The results (Table 2) showed that the mutant was only able to grow on succinate, lactate or ethanol when these media were supplemented with 1 mM-glycine + 1 mM-formate. Serine could not substitute for glycine, indicating that the mutant is no longer able to synthesize glycine from serine. As with the serine-auxotrophs, it was found to be unnecessary to include methanol or formate as a source of C₁ units in the glycine-supplemented growth media. The growth of the mutant on succinate was found to depend on the initial concentration of glycine

Table 5. *Activity of serine hydroxymethyltransferase in wild-type and mutants of Pseudomonas AM 1*

The assay method is described in the Materials and Methods section. The duplicate values are taken from measurements made on batches of cells grown separately. SGF = succinate + 1 mM-glycine + 1 mM-formate medium.

Organism	Specific activity ($\mu\text{mol/h}$ per mg of protein)	
	Methanol-grown	SGF-grown
Wild-type	3.6; 4.1	0.8; 1.1
82 G (glycine-auxotroph)	7.1; 8.2	not detectable
82 GR _{sp} (spontaneous revertant to wild-type)	4.0; 3.4	0.5; 0.6

present. This was studied as described above for mutant 20S. A linear relationship was found between growth and initial glycine concentration in the medium (Fig. 3). The amount of glycine needed for the formation of 1 g (dry wt.) of cells was calculated as described above and found to be 79 mg. The ratio of serine and glycine necessary to give a similar growth response in serine- and glycine-auxotrophs of *Pseudomonas* AM 1 is similar to that found by Pizer & Potochny (1964) for corresponding mutants of *E. coli* and is in agreement with calculations of Newman & Magasanik (1963).

Cell-free extracts of the mutant grown on SGF growth medium or succinate + 1 mM-glycine were tested for the presence of serine hydroxymethyltransferase activity (enzyme 4). Whereas activity of the enzyme was detected in cell-free extracts of the succinate- or methanol-grown wild-type organism, no activity of serine hydroxymethyltransferase could be demonstrated in extracts prepared from the mutant grown on SGF medium (Table 5). The physiological significance of the mutation was further demonstrated by isolation of spontaneous revertants. It was not necessary to use a mutagen as the mutant readily reverted spontaneously to wild-type phenotype and a total of five such revertants were purified on succinate-containing growth medium. Batches of the revertants were grown on SGF medium and extracts prepared from them were assayed for serine hydroxymethyltransferase activity. The enzyme was found to be present in every case, the specific activity being in the range 0.5–1.1 μmol of NADPH formed/h per mg of protein (Table 5). Since these revertants also regained the ability to grow on lactate and ethanol, besides succinate, these results show that serine hydroxymethyltransferase is necessary for the biosynthesis of glycine in *Pseudomonas* AM 1 when the organism is grown on any of these substrates.

Since serine hydroxymethyltransferase is thought to play a key role during growth of *Pseudomonas* AM 1 on C₁ compounds, it is noteworthy that mutant 82 G was able to grow on methanol (Table 2).

The enzyme was found to be present in relatively high activity in methanol-grown mutant cells (Table 5), thus supporting the suggestion (Large & Quayle, 1963; Heptinstall & Quayle, 1970) that the enzyme is implicated in the assimilation of C₁ compounds. Our results could be explained by assuming that wild-type *Pseudomonas* AM 1 contains two serine hydroxymethyltransferases, one constitutive enzyme, the function of which is to supply glycine during growth of the organism on succinate or similar compounds, and the other which is induced during growth on C₁ substrates, the function of which is to convert glycine into serine as part of the pathway involved in the assimilation of C₁ compounds. Mutant 82 G might then lack the constitutive enzyme, thus explaining the requirement for glycine during growth on succinate. If this assumption is correct, it may be predicted that enzyme activity could be induced in mutant 82 G which had been grown on succinate + glycine by incubating these cells in a formate or methanol growth medium. Such an experiment was carried out as follows: mutant 82 G was grown in 21 conical flasks each containing 400 ml of SGF medium for 30 h at 30°C on a shaker. The cells were harvested, the cell pellet obtained from three flasks was resuspended in 380 ml of salts solution and the suspension was incubated at 30°C on a shaker. After 15 min incubation, 20 ml of salts solution containing formate or methanol was added to give a final concentration of 40 mM with respect to formate or methanol. To see whether any enzyme induction was the result of protein synthesis *de novo*, another flask, to which chloramphenicol (150 $\mu\text{g/ml}$) was added, was treated similarly. At different time-intervals after addition of the C₁ compound, 100 ml of the cell suspension was rapidly cooled in crushed ice, the cells were collected by centrifugation and washed twice with 50 mM-sodium potassium phosphate buffer, pH 7.0. Cell-free extracts were prepared as described in the Materials and Methods section, and the extracts were assayed for serine hydroxymethyltransferase activity. The results (Fig. 4) show that serine hydroxymethyltransferase

activity appeared in the cells during the first 6 h after addition of the formate and that this induction was inhibited by chloramphenicol. Similar results were obtained with methanol as inducer.

Fig. 4. Induction of serine hydroxymethyltransferase in mutant 82G. The organism was grown in succinate+1 mM-glycine+1 mM-formate medium, the cells were harvested, resuspended in salts solution at a cell density of 1 mg/ml and at time zero sodium formate was added to the suspension to a final concentration of 40 mM. Preparation of cell-free extracts and assay of serine hydroxymethyltransferase are described in the Materials and Methods section. ○, Without chloramphenicol; □, with 150 µg of chloramphenicol/ml.

Regulation of serine biosynthesis

In Enterobacteriaceae (Pizer, 1963; Umbarger & Umbarger, 1962) and in *H. influenzae* (Pizer *et al.* 1969) the biosynthesis of serine is apparently regulated by end-product inhibition of the first enzyme of the phosphorylated pathway rather than by enzyme repression. Heptinstall & Quayle (1970) could find no evidence for repression of the enzymes of serine biosynthesis (1–3) in *Pseudomonas* AM1 by low concentrations of extracellular serine in the growth medium. It was decided therefore to test for inhibition of phosphoglycerate dehydrogenase (reaction 1) by L-serine and other amino acids in crude extracts of *Pseudomonas* AM1. Both succinate- and methanol-grown wild-type cells were used: the results obtained with extracts prepared from either of these cells were similar.

Out of seven amino acids tested, the only one that showed inhibition at concentrations below 1 mM was L-serine (Table 6). It was found that the latter amino acid inhibited phosphoglycerate dehydrogenase by 24–62% over the concentration range 0.02–0.1 mM. Increasing the concentration of L-serine to 1 mM produced no further inhibition of enzyme activity (Table 6). Pizer *et al.* (1969) observed almost 100% inhibition of phosphoserine formation from phosphoglycerate in cell-free extracts of *H. influenzae* when L-serine was present at a concentration of 0.08 mM. The concentration range of L-serine resulting in inhibition of phosphoglycerate dehydrogenase in *Pseudomonas* AM1 and the specificity of the inhibition (Table 6) is similar to that observed in *Haemophilus* (Pizer *et al.* 1969). These results suggest that the phosphorylated pathway of serine synthesis in *Pseudomonas* AM1 is controlled in similar fashion to that in *E. coli* and *H. influenzae*.

Table 6. Inhibition by serine of phosphoglycerate dehydrogenase in extracts of *Pseudomonas* AM1

The assay method is described in the Materials and Methods section. Concentrations of amino acids are given as final concentrations in the reaction mixture.

Amino acid tested	Concentration (mM)	Enzyme activity (µmol/h per mg of protein)	Inhibition (%)
None	—	3.22	0
L-Serine	0.02	2.45	23.8
L-Serine	0.06	1.45	54.9
L-Serine	0.1	1.21	62.5
L-Serine	1.0	1.19	63.0
D-Serine	0.1	3.20	0
D-Serine	1.0	2.83	12.6
DL-O-Phosphoserine	1.0	3.14	2.0
Glycine	1.0	3.20	0
L-Threonine	1.0	3.19	0
L-Lysine	1.0	3.21	0
L-Alanine	1.0	3.20	0

DISCUSSION

The finding that mutational loss of phosphoserine phosphatase activity in *Pseudomonas* AM1 leads to inability to grow on succinate, lactate or ethanol, but does not affect growth when these media are supplemented with serine, offers strong support for the operation of the phosphorylated pathway (reactions 1-3) as a major route for serine biosynthesis during growth on such compounds. Some involvement of this pathway during growth on C₁ compounds is also indicated by the increase in maximum specific growth rate of serine-auxotrophs on methanol when supplemented with 1 mM-serine. Growth of *Pseudomonas* AM1 on C₁ compounds involves the 'serine' pathway and the results presented above, taken together with those of Large *et al.* (1962*a,b*), Large & Quayle (1963) and Heptinstall & Quayle (1970), offer further support for the operation, in *Pseudomonas* AM1, of two metabolic routes that interconnect phosphoglycerate and serine (Scheme 1). The fact that each route possesses an essentially irreversible step, i.e. reaction (3) or reaction (8) respectively, dictates the direction of any net flow through each route.

A mutational loss of serine hydroxymethyltransferase activity (enzyme 4) in *Pseudomonas* AM1 leads to a requirement for supplemental amounts of glycine for growth on succinate, lactate or ethanol. As in the corresponding mutant of *E. coli* (Pizer & Potochny, 1964), serine cannot satisfy this requirement, which offers strong support for the physiological significance of this enzyme in biosynthesis of glycine during growth of *Pseudomonas* AM1 on higher carbon compounds. The finding that a mutant lacking serine hydroxymethyltransferase when grown on succinate + glycine was nevertheless able to grow on methanol or formate was unexpected in view of the implication of this enzyme in growth on C₁ compounds. However, its role in growth on C₁ compounds is supported by the finding that incubation of the mutant with methanol or formate induces the synthesis of the enzyme in question. The corollary to these results is that there may be two serine hydroxymethyltransferases in *Pseudomonas* AM1: one of them, the enzyme supplying the cell with glycine and C₁ units during growth on succinate, is lacking in mutant 82G, whereas the other, which is inducible and whose function is to convert glycine into serine during growth on C₁ compounds, is

unaffected. Such enzyme multiplicity would have many precedents in other areas of microbial intermediary metabolism and further evidence for it now needs to be sought at the enzymological level.

The properties of both the mutants 20S and 82G suggest that *Pseudomonas* AM1 can generate a C₁ unit from glycine. Reversal of such a reaction could have obvious significance for growth on C₁ compounds and this aspect will be further examined in the following paper (Harder & Quayle, 1971).

We thank Mr A. A. Hancock for his skilled technical assistance and the Science Research Council for financial support under Grant no. B/SR/6038.

REFERENCES

- Anthony, C. & Zatman, L. J. (1964). *Biochem. J.* **92**, 609.
 Bassalik, K. (1913). *Jb. wiss. Bot.* **53**, 255.
 Dempsey, W. B. (1969). *J. Bact.* **100**, 1114.
 den Dooren de Jong, L. E. (1927). *Zentbl. Bakt. Parasitkde. Abt. I*, **71**, 193.
 Harder, W. & Quayle, J. R. (1971). *Biochem. J.* **121**, 763.
 Heptinstall, J. & Quayle, J. R. (1970). *Biochem. J.* **117**, 563.
 Jayasuriya, G. C. N. (1955). *J. gen. Microbiol.* **12**, 419.
 Jones, K. M., Guest, J. R. & Woods, D. D. (1961). *Biochem. J.* **79**, 566.
 Ladner, A. & Zatman, L. J. (1969). *J. gen. Microbiol.* **55**, xvi.
 Large, P. J., Peel, D. & Quayle, J. R. (1961). *Biochem. J.* **81**, 470.
 Large, P. J., Peel, D. & Quayle, J. R. (1962*a*). *Biochem. J.* **82**, 483.
 Large, P. J., Peel, D. & Quayle, J. R. (1962*b*). *Biochem. J.* **85**, 243.
 Large, P. J. & Quayle, J. R. (1963). *Biochem. J.* **87**, 386.
 Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
 Newman, E. B. & Magasanik, B. (1963). *Biochim. biophys. Acta*, **78**, 437.
 Pizer, L. I. (1963). *J. biol. Chem.* **238**, 3934.
 Pizer, L. I. (1965). *J. Bact.* **89**, 1145.
 Pizer, L. I. & Potochny, M. L. (1964). *J. Bact.* **88**, 611.
 Pizer, L. I., Ponce-De-Leon, M. & Michalka, J. (1969). *J. Bact.* **97**, 1357.
 Schlegel, H. G. (1965). In *Anreicherungskultur und Mutantenauslese*, p. 204. Ed. by Schlegel, H. G. & Kröger, E. Stuttgart: Gustav Fisher Verlag.
 Umbarger, H. E. & Umbarger, M. A. (1962). *Biochim. biophys. Acta*, **62**, 193.
 Umbarger, H. E., Umbarger, M. A. & Siu, P. M. L. (1963). *J. Bact.* **97**, 1357.
 Yanofsky, C. (1963). *Cold Spring Harb. Symp. quant. Biol.* **28**, 581.