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Aspects of Glycine and Serine Biosynthesis during Growth of *Pseudomonas* AM1 on C_1 Compounds

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1. Methanol or formate can replace serine or glycine as supplements for growth on succinate of the auxotrophic mutants 20S and 82G of *Pseudomonas* AM1, showing that the organism can synthesize glycine and serine in net fashion from C_1 units. 2. Double mutants of *Pseudomonas* 20S and 82G have been prepared (20ST-1 and 82GT-1) that are unable to grow on succinate + 1 mM-glyoxylate, succinate + 2 mM-methanol or methanol alone. 3. Mutants 20ST-1 and 82GT-1 lacked serine–glyoxylate aminotransferase activity, and revertants to the phenotype of 20S and 82G regained serine–glyoxylate aminotransferase activity. A total revertant of 82GT-1 to wild-type phenotype regained activities of serine hydroxymethyltransferase and serine–glyoxylate aminotransferase. 4. The activity of serine–glyoxylate aminotransferase in methanol-grown *Pseudomonas* AM1 is eightfold higher than in the succinate-grown organism. 5. The combined results show that in *Pseudomonas* AM1 serine–glyoxylate aminotransferase is necessary for growth on C_1 compounds and is involved in the conversion of methanol into glycine via glyoxylate. 6. It is suggested that the phosphorylated pathway of serine biosynthesis from phosphoglycerate replenishes the supply of α-amino groups necessary for the flow of glyoxylate through the main assimilatory pathway during growth on C_1 compounds.

Studies by Large, Peel & Quayle (1961, 1962*a*,*b*), Large & Quayle (1963) and Heptinstall & Quayle (1970) 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:

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

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

$$\text{Serine} + R\text{–}CO\text{–}CO_2H \rightleftharpoons \text{hydroxypyruvate} + R\text{–}CH(NH_2)\text{–}CO_2H \quad (3)$$

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

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

The operation of this assimilatory pathway during synthesis of cell constituents from C_1 compounds requires the synthesis of one molecule of glycine for each molecule of phosphoglycerate formed; little is known of the pathway involved in the synthesis of this glycine molecule from C_1 compounds. On the basis of whole-cell radioisotope experiments (Large *et al.* 1962*a*) two alternative pathways have been suggested. One involves the direct synthesis of the glycine skeleton from a C_1 compound and CO_2, the other pathway involves carboxylation of a C_3 compound (Large *et al.* 1962*b*) and subsequent cleavage of the resulting C_4 skeleton into two C_2 compounds, one of which is channelled into the 'serine' pathway as glycine, the other being available for the net synthesis of cell constituents. There is, as yet, no enzymic evidence in support of either of these possibilities.

The isolation of mutants of *Pseudomonas* AM1 that are unable to synthesize serine or glycine from higher carbon compounds such as succinate (Harder & Quayle, 1971) has opened a new approach towards the problem of glycine synthesis from C_1 compounds. The present paper describes this approach with particular reference to the conversion of methanol into glycine and the involvement of glyoxylate as a precursor to the glycine.

MATERIALS AND METHODS

The methods used for the growth of the organisms, preparation of cell-free extracts, determinations of protein

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and preparation of (±)-tetrahydrofolic acid are described in Harder & Quayle (1971).

Chemicals. Purified nicotinamide nucleotides were obtained from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany. 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

Enzyme assays were performed at 30°C except for phosphoserine phosphatase, which was assayed at 37°C.

L-*Serine–glyoxylate aminotransferase.* This enzyme was assayed by measuring the glyoxylate-dependent formation of hydroxypyruvate from L-serine. Cell-free extracts of *Pseudomonas* AM1 contain relatively high concentrations of an NADH-linked hydroxypyruvate reductase (Table 3) which is normally in excess of L-serine–glyoxylate aminotransferase activity. Consequently the aminotransferase activity could be measured by following NADH oxidation in the presence of glyoxylate and L-serine. The assay was performed anaerobically in sodium phosphate–potassium phosphate buffer, pH 7.5. The reaction mixture was as described by Blackmore & Quayle (1970). The rate of decrease in extinction at 340nm before the addition of L-serine (due to glyoxylate reductase activity) was subtracted from the rate observed after its addition. In some cases where hydroxypyruvate reductase was present in relatively low activities, namely succinate-grown wild-type or mutant organisms, a cell-free extract of succinate-grown *Micrococcus denitrificans* was added to the reaction mixture to provide a source of NADPH-linked hydroxypyruvate reductase (Gibbs, 1966). NADPH was substituted for NADH in these cases, but otherwise the assays were performed in a fashion similar to that described above. The activities of the transaminase found in the presence or absence of a cell-free extract of *M. denitrificans* were actually similar, so that in later assays an extract of *M. denitrificans* was not included in the reaction mixtures and NADH was used.

L-*Serine–tetrahydrofolate 5,10-hydroxymethyltransferase (serine hydroxymethylase, EC* 2.1.2.1). The enzyme was assayed as described by Harder & Quayle (1971).

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).

D-*Glycerate–NAD oxidoreductase (hydroxypyruvate reductase, EC* 1.1.1.29). This was assayed anaerobically at pH 7.5 in sodium phosphate–potassium phosphate buffer as described by Large & Quayle (1963) by following the oxidation of NADH at 340nm consequent on reduction of hydroxypyruvate.

RESULTS

A key to the mutants used in the following work is given in Table 1.

Synthesis of glycine and serine from methanol. Harder & Quayle (1971) have reported the isolation and characterization of serine- and glycine-auxotrophs of *Pseudomonas* AM1. These mutants require supplemental amounts of serine or glycine for growth on succinate media and it was shown that the extent of growth of the mutants on succinate is proportional to the initial concentration of the respective supplement. If in *Pseudomonas* AM1 net synthesis of glycine from C_1 compounds is possible, either directly or indirectly, then low concentrations of methanol or formate might allow growth of the serine- and glycine-auxotrophs on succinate.

Two mutants, 20S and 82G, described by Harder & Quayle (1971), have been used in these studies. Mutant 20S requires serine or glycine for growth on succinate and lacks phosphoserine phosphatase. Mutant 82G requires glycine for growth on succinate and when grown on succinate+supplemental glycine lacks serine hydroxymethyltransferase. It has now been found that either methanol or formate, when added at 2mM final concentration to succinate media, allows growth of both the mutants (Table 2). This shows that in *Pseudomonas* AM1, when synthesis of serine or glycine from succinate is blocked, the organism is able to synthesize both these

Table 1. *Description of mutants of* Pseudomonas *AM*1

Mutant	Description
20BL	Lacks hydroxypyruvate reductase (Heptinstall & Quayle, 1970).
20S	Lacks phosphoserine phosphatase (Harder & Quayle, 1971).
20ST-1	Double mutant derived from 20S. Lacks phosphoserine phosphatase and serine–glyoxylate aminotransferase.
$20STR_p$	Partial revertant of 20ST-1. Lacks phosphoserine phosphatase but has regained serine–glyoxylate aminotransferase.
82G	Lacks serine hydroxymethyltransferase when grown on supplemented succinate media (Harder & Quayle, 1971).
82GT-1	Double mutant derived from 82G. Lacks serine hydroxymethyltransferase and serine–glyoxylate aminotransferase.
$82GTR_p$	Partial revertant of 82GT-1. Lacks serine hydroxymethyltransferase but has regained serine–glyoxylate aminotransferase.
$82GTR_t$	Total revertant of 82GT-1 to wild-type phenotype. Has regained serine hydroxymethyltransferase and serine–glyoxylate aminotransferase.

Table 2. *Growth requirements of a serine-requiring* (20S) *and a glycine-requiring* (82G) *mutant of* Pseudomonas *AM* 1

The growth medium was a salts solution containing 50mM-succinate or 0.5% methanol and supplemented as indicated. Methods are described in the Materials and Methods section. +, Growth; −, no growth.

Growth substrate	Supplement	Growth of organism: Wild-type	Mutant 20S	Mutant 82G
Succinate	None	+	−	−
	1mM-Serine	+	+	−
	1mM-Glycine	+	+	+
	2mM-Methanol or 2mM-formate	+	+	+
Methanol	None	+	+	+

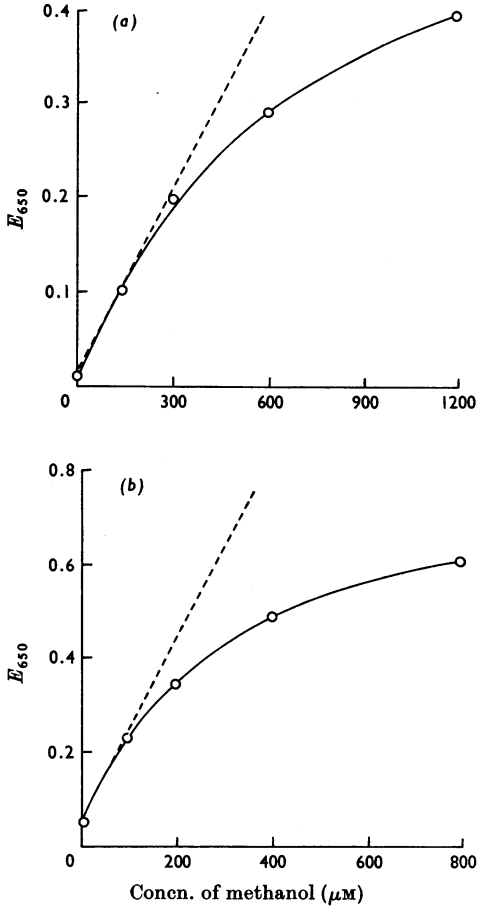


Fig. 1. Growth responses of a serine-requiring (20S) and a glycine-requiring (82G) mutant of *Pseudomonas* AM 1 to limiting concentrations of methanol. The growth medium was a salts solution containing 50mM-succinate and supplemented with methanol as indicated. Methods are described in the Materials and Methods section. (*a*) Mutant 20S; (*b*) mutant 82G.

amino acids from either methanol or formate. The extent of growth of both mutants in succinate media was studied as described by Harder & Quayle (1971) and found to depend on the initial concentration of methanol present. A linear relationship was previously found between growth of these mutants and the initial glycine concentration in the medium (Harder & Quayle, 1971). When methanol was used as a supplement, the growth response of both mutants was found to increase in a non-linear fashion with increasing methanol concentration (Figs. 1*a* and 1*b*). This behaviour is probably due to the fact that methanol is readily oxidized by the cells (both mutants are able to grow on methanol as sole carbon and energy source), whereas glycine is not. It seems therefore justified to calculate the growth response of the mutants as a function of growth-limiting methanol concentrations from the tangents to the curves (Figs. 1*a* and 1*b*) at very low methanol concentrations. These are shown as broken lines on the figures. The slopes of the graphs in Figs. 1(*a*) and 1(*b*) are 0.69 and 2.0, the units in both cases being ΔE_{650}/mmol of supplement. By using the same units, the growth response on succinate of the same two mutants, 20S and 82G, to growth-limiting concentrations of glycine may be calculated from the data of Harder & Quayle (1971) to be 0.75 and 1.9 respectively. Thus the relative molar amounts of the respective supplements, methanol or glycine, that allow a similar amount of growth of the mutants on succinate can be seen to be in the approximate ratio, methanol: glycine 1:1. The relative molar amounts of the supplements serine and glycine allowing a similar growth response of mutant 20S on succinate were found to be in the ratio 1:1.85 (Harder & Quayle, 1971). In the absence of quantitative data showing the relative amounts of methanol, glycine and serine that are directly incorporated into cell constituents it is not possible to deduce from the growth response curves the stoicheiometry of the overall conversion

of methanol and carbon dioxide into glycine and thence to serine.

The fact that glycine can be used as a supplement for growth of both the mutants on succinate shows that C_1 units can be made from glycine. Cleavage of the glycine skeleton according to the following overall reaction:

$$\text{Glycine} + H_4\text{folate} \rightarrow 5,10\text{-}CH_2\text{–}H_4\text{folate} + CO_2 + NH_3 + 2H \quad (6)$$

has been demonstrated in cell-free extracts of *Peptococcus glycinophilus* (Klein & Sagers, 1966) and *Arthrobacter globiformis* (Jones & Bridgeland, 1966; Kochi & Kikuchi, 1969). In the latter organism this reaction is to a minor extent reversible. It was decided to see whether this reaction could be demonstrated in cell-free extracts of *Pseudomonas* AM1, for if the generation of C_1 units from glycine in *Pseudomonas* AM1 arises from such a cleavage reaction, reversal of the reaction might accomplish glycine synthesis from C_1 units. However, all attempts to demonstrate cleavage of [1-^{14}C]glycine, [1-^{14}C]glyoxylate or [1-^{14}C]glycollate in anaerobic reaction mixtures as described by Klein & Sagers (1966) or Kochi & Kikuchi (1969) with cell-free extracts of methanol-grown *Pseudomonas* AM1 or mutant 20S grown on succinate + 1mM-glycine, have failed.

Under aerobic conditions a thiamin pyrophosphate-dependent cleavage of [1-^{14}C]glyoxylate has been found. The rate of this cleavage reaction was low but could just account for the growth rate of mutant 20S on succinate + 1mM-glycine on the basis of generation of the necessary C_1 units. It is probable that this cleavage of glyoxylate is catalysed by pyruvate oxidase, as the mammalian enzyme is known to decarboxylate glyoxylate at half the rate that it decarboxylates pyruvate (Kohlhaw, Deus & Holzer, 1965). No conditions could be found for reversing this cleavage reaction in extracts of methanol-grown *Pseudomonas* AM1.

Isolation and characterization of double mutants. Further work on the source of glycine during growth on C_1 compounds would be aided if it were known whether glycine is formed directly or from a precursor, e.g. another C_2 compound. The work described in the previous section offered an approach to this problem using mutants 20S and 82G. These mutants grow on succinate if provided with a source of glycine. Now methanol can provide a source of glycine and hence if a 'compound X' were an intermediate in the pathway leading from methanol to glycine, it could be pinpointed by its ability to substitute for the glycine supplement and by the isolation of double mutants of 20S or 82G, still able to grow on succinate+supplemental glycine, but no longer able to grow on succinate + 'compound X', succinate+supplemental methanol, or methanol itself.

The number of likely C_2 precursors of glycine is small and the report by Blackmore & Quayle (1970) that serine–glyoxylate aminotransferase is present at high activity in extracts of methanol-grown *Pseudomonas* AM2 but is undetectable in extracts of the succinate-grown organism, focuses attention on glyoxylate as a candidate. Glyoxylate can substitute for glycine as supplement for growth of mutants 20S and 82G on succinate (Table 3), and hence double mutants were sought in which 'compound X' was glyoxylate.

The preparation and isolation of the double mutants followed the general procedure described by Harder & Quayle (1971). The mutants were expressed in succinate + 1mM-glycine medium and the penicillin treatments were done in succinate + 1mM-glyoxylate medium, in which the desired mutants would not grow. Master plates were prepared from succinate + 1mM-glycine medium and these were replicated on to succinate + 1mM-glycine and succinate + 1mM-glyoxylate media. Two classes of mutants were obtained: one class derived from mutant 20S was called 20ST and the other, derived from mutant 82G, was called 82GT. Four representatives of each class of mutant were isolated in pure culture; as the results obtained with the different mutants of each class were essentially similar, only the results obtained with one of each, 20ST-1, and 82GT-1, are given.

The growth requirements of mutants 20ST-1 and 82GT-1 were studied both on plates and in liquid medium. The results (Table 3) show that both mutants 20ST-1 and 82GT-1 were no longer able to grow on succinate + 1mM-glyoxylate, succinate + 2mM-methanol or methanol itself, whereas growth on succinate + 1mM-glycine medium was unimpaired. In mutant 20ST-1 serine could substitute for glycine, indicating that the enzyme lesions present in mutants 20S and 82G were also present in the double mutants.

Cell-free extracts of wild-type *Pseudomonas* AM1 grown on succinate and methanol and of mutants 20S, 82G, 20ST-1 and 82GT-1 grown on succinate + 1mM-glycine + 1mM-formate were tested for the presence of serine–glyoxylate aminotransferase, hydroxypyruvate reductase, serine hydroxymethyltransferase and phosphoserine phosphatase. The specific activities of the first three enzymes in methanol-grown wild-type cells were higher than in succinate-grown wild-type cells (Table 4), in accord with these enzymes having a special function during growth of *Pseudomonas* AM1 on methanol. As reported by Harder & Quayle (1971) mutant 20S lacked phosphoserine phosphatase activity and mutant 82G lacked serine hydroxymethyltransferase activity. In addition to the lesion from the

Table 3. *Growth requirements of the double mutants (20ST-1 and 82GT-1) and revertants of these mutants (20STR$_p$, 82GTR$_p$ and 82GTR$_t$) of* Pseudomonas *AM 1*

Mutant 20ST-1 was derived from a serine-requiring mutant 20S, mutant 82GT-1 was derived from a glycine-requiring mutant 82G of *Pseudomonas* AM 1. 20STR$_p$ and 82GTR$_p$ are partial revertants of mutants 20ST-1 and 82GT-1 respectively, 82GTR$_t$ is a total revertant of mutant 82GT-1. The growth medium was a salts solution containing 50mM-succinate or 0.5% methanol and supplemented as indicated. Methods are as described in the Materials and Methods section. +, Growth; −, no growth.

Growth substrate	Supplement	Growth of organism							
		Wild-type	Mutant 20S	Mutant 82G	Mutant 20ST-1	Mutant 82GT-1	Revertant 20STR$_p$	Revertant 82GTR$_p$	Revertant 82GTR$_t$
Succinate	None	+	−	−	−	−	−	−	+
	1mM-serine	+	+	−	+	−	+	−	+
	1mM-Glycine	+	+	+	+	+	+	+	+
	1mM-Glyoxylate	+	+	+	−	−	+	+	+
	2mM-Methanol	+	+	+	−	−	+	+	+
Methanol	None	+	+	+	−	−	+	+	+

parent mutants, mutants 20ST-1 and 82GT-1 were found to lack serine-glyoxylate aminotransferase activity.

The physiological significance of the new mutations was further demonstrated by preparation of revertants. Since both double mutants reverted readily to the phenotype of the single mutants from which they were derived, it was not necessary to use a mutagen. Consequently all revertants obtained were 'spontaneous' revertants. Three classes of revertants were isolated: revertants called 20STR$_p$ which were again able to grow on succinate + 1mM-glyoxylate, succinate + 2mM-methanol and methanol media; revertants called 82GTR$_p$ which had similarly regained the ability to grow on succinate + glyoxylate, succinate + methanol and methanol media, and revertants called 82GTR$_t$ which were able to grow on succinate or methanol minimal media (Table 3). Batches of a representative of each class of these revertants were grown on succinate + 1mM-glycine + 1mM-formate medium and extracts prepared from them were assayed for the four enzymes mentioned above. Serine–glyoxylate aminotransferase activity was found to be present in every case, the specific activity being in the range 0.2–0.4 μmol/h per mg of protein (Table 4). Serine hydroxymethyltransferase activity was found to be present in an extract of revertant 82GTR$_t$. Since revertants 20STR$_p$, 82GTR$_p$ and 82GTR$_t$ had regained the ability to grow on succinate + 1mM-glyoxylate, succinate + 2mM-methanol and methanol growth media, these results show that in *Pseudomonas* AM 1: (i) serine–glyoxylate aminotransferase is necessary for growth on C_1 compounds; (ii) serine–glyoxylate aminotransferase is involved in the conversion of methanol into glycine; (iii) glyoxylate is the precursor of glycine during growth on C_1 compounds. The finding that mutant 82GTR$_t$ had regained serine hydroxymethyltransferase activity and was again able to grow on succinate minimal medium gives further support to the report by Harder & Quayle (1971) that this enzyme is essential for the synthesis of glycine during growth of *Pseudomonas* AM 1 on succinate and similar substrates.

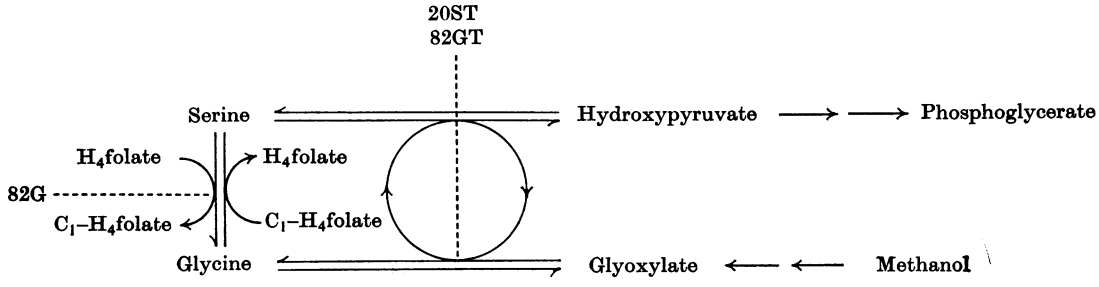
DISCUSSION

The finding that methanol can replace glycine as a supplement for growth of serine- and glycine-auxotrophs of *Pseudomonas* AM 1 on succinate demonstrates that in these mutants a pathway exists for the conversion of methanol into glycine. For the synthesis of serine, a further C_1 unit is necessary and this finding offers further support for the involvement of serine hydroxymethyltransferase (reaction 2) in the assimilatory pathway which operates during growth of *Pseudomonas*

Table 4. *Enzyme activities in wild-type and mutants of* Pseudomonas *AM* 1

The organisms were grown for 30–40 h in 400 ml of salts medium containing 50 mM-succinate, 0.5% methanol or 50 mM-succinate + 1 mM-glycine + 1 mM-formate (SGF) as indicated. The cells were harvested, cell-free extracts prepared and enzymes assayed as described in the Materials and Methods section. Duplicate values are taken from measurements on separately grown batches of cells. N.D. = Not detectable.

		Specific activity (μmol/h per mg of protein)			
Organism	Substrate and supplement	Serine–glyoxylate aminotransferase	Hydroxypyruvate reductase	Serine hydroxymethyltransferase	Phosphoserine phosphatase
Wild-type	Methanol	6.2; 5.3	61; 67	3.8; 4.2	1.4; 1.7
Wild-type	Succinate	0.6; 0.8	5.4; 6.3	0.7; 0.8	1.5; 1.6
20S	SGF	0.7; 0.5	3.8; 2.7	0.6; 0.9	N.D.
82G	SGF	0.9; 0.7	4.1; 5.1	N.D.	1.2; 1.4
20ST-1	SGF	N.D.	2.8; 3.7	0.5; 0.8	N.D.
82GT-1	SGF	N.D.	5.1; 4.3	N.D.	1.5; 1.2
$20STR_p$	SGF	0.3; 0.2	3.4; 2.8	0.4; 0.7	N.D.
$82GTR_p$	SGF	0.2; 0.4	3.8; 4.1	N.D.	1.6; 1.8
$82GTR_t$	SGF	0.3; 0.4	5.3; 5.1	0.4; 0.5	1.4; 1.9



Scheme 1. Conversion of glyoxylate into hydroxypyruvate in *Pseudomonas* AM 1. The broken lines represent enzyme lesions in the mutants.

AM 1 on C_1 compounds. These results do not enable a decision to be made between the two possibilities for net glycine synthesis, direct or cyclic.

The results obtained with the double mutants of *Pseudomonas* AM 1 clearly point to glyoxylate as an obligatory intermediate in the net conversion of C_1 compounds to glycine. The dependence of growth on C_1 compounds on the possession of a functional specific serine–glyoxylate aminotransferase, and the eightfold increase in specific activity of this enzyme in extracts of methanol-grown *Pseudomonas* AM 1 as compared with the succinate-grown organism, point to glyoxylate as the immediate precursor of glycine (see Scheme 1). The concerted action of serine hydroxymethyltransferase and serine–glyoxylate aminotransferase could constitute a cycle converting glyoxylate and methylene tetrahydrofolate into hydroxypyruvate that utilizes catalytic quantities of an α-amino group. The continued operation of this cycle would demand maintenance of the concentration of serine as amino-group donor necessary to keep glyoxylate flowing through the main assimilatory pathway of the cell. Withdrawal of glycine or serine for synthesis of purines or proteins might therefore check the main flow of carbon into the cell. In these circumstances a replenishment route for aminating the hydroxypyruvate skeleton could be of advantage. In the preceding paper (Harder & Quayle, 1971) evidence was presented suggesting that the phosphorylated pathway of serine biosynthesis plays some role during growth of *Pseudomonas* AM 1 on C_1 compounds. We now suggest that this role could be one of amino-group replenishment. In view of other aminotransferases in the cell, it might be expected that the phosphorylated pathway would play an ancillary role rather than an essential role in this respect. Control of the phosphorylated pathway by end-product inhibition rather than by enzyme repression (Harder & Quayle, 1971) would

be consistent with the postulated physiological role during growth on C_1 compounds.

The mechanism of biosynthesis of the C_2 skeleton of glycine from C_1 units still remains unsolved. The finding that serine can be synthesized from glycine in mutants 20S and 82G shows that a C_1 unit can be generated from glycine; reversal of such a cleavage might furnish an answer to this problem. However, work with cell-free extracts pointed to cleavage of glyoxylate, probably by action of pyruvate oxidase, rather than cleavage of glycine as the possible source of the C_1 unit. No significant reversal of glyoxylate cleavage could be observed under a variety of experimental conditions. Nevertheless, the work with mutants 20ST-1 and 82GT-1 has now clearly implicated glyoxylate as the precursor of glycine and directs further work towards synthesis of the former C_2 compound. Mutants 20ST-1 and 82GT-1, being unable to convert glyoxylate into glycine, may accumulate glyoxylate or precursors of glyoxylate from C_1 compounds. Isotopic experiments at the whole-cell level with these mutants now may offer a new experimental approach to this central problem.

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