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Microbial Growth on C_1 Compounds

3. DISTRIBUTION OF RADIOACTIVITY IN METABOLITES OF METHANOL-GROWN *PSEUDOMONAS* AM1 AFTER INCUBATION WITH [^{14}C]METHANOL AND [^{14}C]BICARBONATE*

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The pathway by which carbon is incorporated into cell constituents during growth of *Pseudomonas* AM1 on methanol or formate has been investigated by chromatographic analysis of the metabolites labelled during incubation of the organism with ^{14}C-labelled substrates (Large, Peel & Quayle, 1961). The results of this study indicated that substantial quantities of carbon dioxide and carbon in a more reduced form are assimilated by a pathway which leads to rapid labelling of serine, glycine, malate and aspartate.

It has been suggested that growth of *Pseudomonas* AM1 on methanol or formate, as with growth of *Pseudomonas* PRL-W4 on methanol (Kaneda & Roxburgh, 1959), involved the condensation of glycine with a C_1 unit, derived from methanol or formate, to give serine by the action of serine hydroxymethylase. The mechanism of the prior synthesis of glycine was not known. A study has now been made of the distribution of radioactivity in glycine, serine, malic acid and phosphoglyceric acid, isolated from methanol-grown *Pseudomonas* AM1 after incubation with [^{14}C]methanol or [^{14}C]bicarbonate (hydrogen [^{14}C]carbonate).

* Part 2: Large, Peel & Quayle (1961).

METHODS AND MATERIALS

Maintenance and growth of the organism. The growth conditions have been described by Peel & Quayle (1961).

Isotopic materials. Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks., and handled as described by Large *et al.* (1961).

Brief incubation of cells with [^{14}C]*methanol or* [^{14}C]*bicarbonate.* Cultures of methanol-grown bacteria were harvested by centrifuging while in the logarithmic-growth phase. The cells were resuspended in a solution containing 10 mM-KH_2PO_4 adjusted to pH 7·0 with 2N-NaOH, 10 mM-ammonium chloride, and methanol (see below) to a cell density of 6 mg. dry wt. of bacteria/ml. The bacterial suspensions (50 ml. or 25 ml., see Tables 1–3) were placed in a sintered-glass funnel (G = 1; 8 cm. diameter × 5 cm. depth) through which was blown either air or air–carbon dioxide (99:1, v/v). After 5 min. incubation at 30°, [^{14}C]methanol or [^{14}C]bicarbonate was added to the suspensions and 1 min. later the entire contents of the funnel were tipped into 4 vol. of boiling ethanol. Each resulting extract was cooled and centrifuged, and the insoluble residue was further extracted with two 5 ml. portions of aq. 20% (v/v) ethanol. These extracts were combined and evaporated at 30° under nitrogen at reduced pressure to a volume of approx. one-fiftieth of the original. Samples (0·15 ml.) of the concentrated supernatant solutions were chromatographed two-dimensionally on paper (Whatman no. 4,

18¼ in. × 22 in.) with the solvent systems: 500 g. of phenol–13 ml. of 90% (w/v) formic acid–167 ml. of water (Kornberg, 1958) and butan-1-ol–propionic acid–water (47:22:31, by vol.; Benson *et al.* 1950). The radioactive compounds were located by radioautography (Large *et al.* 1961) and eluted from the paper with water. Mixtures of radioactive serine and glycine thus obtained were separated by chromatography in the solvent system butan-1-ol–acetone–water–diethylamine (10:10:5:2, by vol.; Hardy, Holland & Nayler, 1955).

Phosphate esters were dephosphorylated by incubation with Polidase-S (Schwarz Laboratories Inc., New York, U.S.A.) according to the procedure of Benson, Bassham & Calvin (1951), and the glyceric acid was separated from the resulting mixture by two-dimensional chromatography in the phenol–formic acid and butanol–propionic acid–water systems described above.

Long-term incubation of cells with [^{14}C]*methanol.* Growth medium (400 ml.) containing [^{14}C]methanol (100 m-moles, 1 mc of ^{14}C) as carbon source was inoculated with *Pseudomonas* AM1. The suspension was divided equally between two conical culture flasks, one of which was flushed with carbon dioxide-free air and the other with air–carbon dioxide (99:1, v/v). The flasks were shaken at 30°, and the bacteria were grown to a cell density of about 0·3 mg. dry wt./ml. and then were harvested by centrifuging. The organisms were treated with 15 ml. of boiling ethanol, the resulting suspension was centrifuged, and the solid residue was washed with 3 ml. of boiling aq. 20% (v/v) ethanol. The washed solid material from each culture was heated with 5 ml. of 6 N-hydrochloric acid in sealed tubes at 110° overnight to hydrolyse the protein. The tubes were then opened and their contents evaporated to dryness at 60° under a stream of nitrogen. Residual acid was removed by adding 2 ml. of water to each tube and again evaporating to dryness. These hydrolysates were dissolved in 1 ml. of water and analysed chromatographically by the methods described in the preceding section.

Preparation of samples for degradation. The purified radioactive compounds eluted from the final chromatograms were mixed with about 300 mg. of authentic unlabelled compounds and then crystallized to constant specific activity. For glyceric acid, the calcium salt was used. The solvent used for crystallizing serine, glycine and calcium glycerate was aqueous ethanol, and for malic acid a mixture of benzene and acetic acid. The samples were dried in high vacuum at 80°.

Degradation of labelled compounds. The specific activity of the labelled compounds was determined by total oxidation to carbon dioxide with Van Slyke–Folch reagent as described by Sakami (1955).

Glycine was degraded by the method described by Sakami (1955). This involved decarboxylation with ninhydrin to yield formaldehyde and carbon dioxide. The formaldehyde was steam-distilled and oxidized by sodium hypoiodite to formic acid, which was then oxidized by mercuric acetate to carbon dioxide.

Serine and glyceric acid were degraded by the procedure described by Sakami (1955). This involved periodate oxidation which yielded carbon dioxide, formic acid and formaldehyde from C-1, C-2 and C-3 respectively. The formic acid was oxidized by mercuric acetate to carbon dioxide; the formaldehyde remaining in the solution was oxidized in two steps to carbon dioxide as described above for glycine.

Malic acid was degraded according to the following reactions:

$$\underset{4}{HO_2C}\cdot\underset{3}{CH_2}\cdot\underset{2}{CH(OH)}\cdot\underset{1}{CO_2H}\xrightarrow{MnO_2}\underset{3}{CH_3}\cdot\underset{2}{CHO}+\underset{1+4}{2CO_2} \quad (1)$$

$$\underset{3}{CH_3}\cdot\underset{2}{CHO}\xrightarrow{CrO_3}\underset{3}{CH_3}\cdot\underset{2}{CO_2H} \quad (2)$$

$$\underset{3}{CH_3}\cdot\underset{2}{CO_2H}\xrightarrow{HN_3}\underset{3}{CH_3}\cdot\underset{2}{NH_2}+\underset{2}{CO_2} \quad (3)$$

$$\underset{3}{CH_3}\cdot\underset{}{NH_2}\xrightarrow{\text{Combustion}}\underset{3}{CO_2} \quad (4)$$

$$\underset{4}{HO_2C}\cdot\underset{3}{CH_2}\cdot\underset{2}{CH(OH)}\cdot\underset{1}{CO_2H}\xrightarrow{H_2SO_4}\underset{1}{CO} \quad (5)$$

$$\underset{1}{CO}\xrightarrow{\text{Iodic sulphate}}\underset{1}{CO_2} \quad (6)$$

This scheme has been used previously by Wood, Stjernholm & Leaver (1956).

In the present work, conditions for reaction (1) were similar to those given for lactate degradation by Sakami (1955). Reaction (2) was also carried out under the conditions given by this author. Reaction (3) was that of Phares (1951); methylamine was trapped in sulphuric acid and the resulting methylamine sulphate oxidized to carbon dioxide by combustion with Van Slyke–Folch reagent. The conditions for reactions (5) and (6) were those given by Utter (1951); the preparation of iodic sulphate used in reaction (6) has been described by Sakami (1955).

Assay of $^{14}CO_2$. $^{14}CO_2$ was converted into barium [^{14}C]-carbonate by precipitation with barium chloride (5%, w/v), and was then plated in triplicate on paper disks (2·69 cm.^{2}) as described by Sakami (1955). The samples were assayed for radioactivity in a gas-flow counter (Model D-47, Nuclear Instrument and Chemical Corp., Chicago, U.S.A.) and corrected for self-absorption.

RESULTS

The labelling patterns of glycine, serine, phosphoglyceric acid and malic acid, obtained from cells incubated for 1 min. with [^{14}C]methanol or [^{14}C]bicarbonate, are given in Tables 1 and 2 respectively. A striking feature is the uniform distribution of radioactivity in glycine after incubation with [^{14}C]methanol (Table 1). The incorporation of [^{14}C]bicarbonate under similar conditions leads to the formation of glycine labelled predominantly in the carboxyl group (Table 2). It is therefore possible that the fixation of radioactivity from [^{14}C]methanol into the carboxyl group of glycine is due to the fixation of $^{14}CO_2$ derived from [^{14}C]methanol by rapid oxidation within the cell. In order to examine this possibility, a culture of methanol-grown cells was briefly incubated with [^{14}C]methanol in the presence of air–carbon dioxide to lower the specific activity of the endogenous $^{14}CO_2$, and the labelling pattern of glycine from these cells was determined. The uniform labelling pattern observed when the flushing gas was air

(Table 1) changed to one in which the carboxyl group contained only 24% of the total radioactivity (Table 3). The same effect was observed in the labelling pattern of glycine obtained from protein hydrolysates of cells grown for several generations on [^{14}C]methanol. The glycine obtained from cells which had been grown in a stream of carbon dioxide-free air was uniformly labelled (Table 4). This is to be expected where the cells were being allowed to assimilate their endogenous $^{14}CO_2$, which would be

Table 1. *Distribution of radioactive carbon in glycine, serine, phosphoglyceric acid and malic acid derived from* Pseudomonas AM1 *growing on* [^{14}C]*methanol in air*

[^{14}C]Methanol (92·5 μmoles; 500 μc of ^{14}C) was added at zero time to an aerated suspension of bacteria (300 mg. dry wt.) in 50 ml. of 10 mM-phosphate buffer (pH 7·0), 10 mM-NH_4Cl and 10 mM-methanol. After incubation for 1 min. the suspension was poured into 200 ml. of boiling ethanol. The isolation of radioactive compounds from the resulting solution is described under Methods.

Compound	Carbon atom	Specific radioactivity of $Ba^{14}CO_3$ derived from each C atom (counts/min./mg. of $BaCO_3$)	Sum of specific activities of individual C atoms	Specific activity of $Ba^{14}CO_3$ obtained from combustion × no. of C atoms in molecule	Percentage of total radioactivity in molecule contributed by each C atom
Glycine	CO_2H (1)	13·3	27·0	28·7	49·1
	$CH_2 \cdot NH_2$ (2)	13·7			50·9
Serine	CO_2H (1)	5·46	22·7	22·7	24·1
	$CH \cdot NH_2$ (2)	6·03			26·6
	$CH_2 \cdot OH$ (3)	11·2			49·4
Phosphoglyceric acid	CO_2H (1)	8·66	29·2	34·0	29·7
	$CH \cdot OH$ (2)	8·32			28·5
	$CH_2 \cdot O \cdot PO_3H_2$ (3)	12·2			41·8
Malic acid	CO_2H (1)	18·5	80·1	84·4	23·1
	$CH \cdot OH$ (2)	16·1			20·1
	CH_2 (3)	24·2			30·2
	CO_2H (4)	21·3*			26·6*

* Obtained by difference between C-1 and C-4 together and C-1.

Table 2. *Distribution of radioactive carbon in glycine, serine and malic acid derived from* Pseudomonas AM1 *growing on methanol in the presence of air and sodium* [^{14}C]*bicarbonate*

Sodium [^{14}C]bicarbonate (20 μmoles; 500 μc of ^{14}C) was added at zero time to an aerated suspension of bacteria (300 mg. dry wt.) in 50 ml. of 10 mM-phosphate buffer (pH 7·0), 10 mM-NH_4Cl and 157 mM-methanol. After incubation for 1 min. the suspension was poured into 200 ml. of boiling ethanol. The isolation of radioactive compounds from the resulting solution is described under Methods.

Compound	Carbon atom	Specific radioactivity of $Ba^{14}CO_3$ derived from each C atom (counts/min./mg. of $BaCO_3$)	Sum of specific activities of individual C atoms	Specific activity of $Ba^{14}CO_3$ obtained from combustion × no. of C atoms in molecule	Percentage of total radioactivity in molecule contributed by each C atom
Glycine	(1)	6·50	7·66	7·08	84·7
	(2)	1·16			15·3
Serine	(1)	6·84	8·31	7·98	82·3
	(2)	1·28			15·4
	(3)	0·19			2·3
Malic acid	(1)	19·2	50·3	46·0	38·1
	(2)	3·23			6·4
	(3)	2·87			5·8
	(4)	25·0*			49·7*

* Obtained by difference between C-1 and C-4 together and C-1.

of the same specific radioactivity as the [^{14}C]-methanol from which it was derived. When the endogenous $^{14}CO_2$ was diluted by flushing with air–carbon dioxide, the specific radioactivity of the carboxyl group of the glycine was lowered to 7·5% of the total radioactivity in the molecule. This effect on the labelling pattern is greater than that observed in the brief incubation, probably because the flushing process was more efficient at a cell density reduced twenty-fold. The results of these experiments thus show that the carboxyl group of glycine is derived mainly from carbon dioxide, and the methylene group from some C_1 unit derived from methanol but not in rapid equilibrium with carbon dioxide.

The similarity in the distribution of radioactivity between C-1 and C-2 of glycine and C-1 and C-2 of serine under all experimental conditions (Tables 1–3) suggests that glycine and serine are interconvertible by addition of a C_1 unit to C-2 of glycine, or by removal of this C_1 unit from serine. A comparison of the serine-labelling patterns resulting from the incorporation of [^{14}C]methanol (Table 1) and [^{14}C]bicarbonate (Table 2) shows that C-3 of serine is derived from methanol rather than from carbon dioxide. This conclusion is supported by the observation that in cells incubated briefly with [^{14}C]methanol the percentage of radioactivity contained in C-3 of serine was increased from 49·4 to 61·4 when the culture was supplied with non-radioactive carbon dioxide.

Comparison of the labelling patterns of serine and malate produced in short-term incubations with [^{14}C]methanol or [^{14}C]bicarbonate shows that the C_3 carbon skeleton of serine cannot arise solely from malate after decarboxylation at C-4. Such a decarboxylation would yield the following patterns in serine, the percentages of labelling being given for C-1, C-2 and C-3 respectively: from [^{14}C]methanol, 31·4:27·4:41·2 (Found: 24·1:26·6:49·4); from $^{14}CO_2$, 75·8:12·7:11·5 (Found: 82·3:15·4:2·3). It is, however, possible that serine is a

Table 3. *Distribution of radioactive carbon in glycine and serine derived from* Pseudomonas AM 1 *growing on* [^{14}C]*methanol in the presence of air–carbon dioxide*

[^{14}C]Methanol (46·9 μmoles; 250 μc of ^{14}C) was added at zero time to a suspension of bacteria (140 mg. dry wt.) in 25 ml. of 10 mM-phosphate buffer (pH 7·0), 10 mM-NH_4Cl and 10 mM-methanol. The suspension was flushed with air–carbon dioxide (99:1, v/v). After incubation for 1 min. the suspension was poured into 100 ml. of boiling ethanol. The isolation of radioactive compounds from the resulting solution is described under Methods.

Compound	Carbon atom	Specific radioactivity of $Ba^{14}CO_3$ derived from each C atom (counts/min./mg. of $BaCO_3$)	Sum of specific activities of individual C atoms	Specific activity of $Ba^{14}CO_3$ obtained from combustion × no. of C atoms in molecule	Percentage of total radioactivity in molecule contributed by each C atom
Glycine	(1)	3·85	16·1	17·7	23·8
	(2)	12·3			76·2
Serine	(1)	4·10	49·3	48·7	8·3
	(2)	14·9			30·3
	(3)	30·3			61·4

Table 4. *Distribution of radioactive carbon in glycine obtained from protein hydrolysates of* Pseudomonas AM 1 *growing on* [^{14}C]*methanol in carbon dioxide-free and -enriched air*

Two cultures of *Pseudomonas* AM 1 were grown in conical culture flasks each containing 200 ml. of growth medium with [^{14}C]methanol (50 m-moles, 0·5 mc of ^{14}C) as carbon source. One flask was flushed with air and the other with air–carbon dioxide (99:1, v/v). The bacteria were grown to a cell density of 0·3 mg. dry wt./ml., harvested by centrifuging and killed with boiling ethanol. The precipitated protein was washed with aq. ethanol, hydrolysed with 6 N-HCl, and the labelled glycine was isolated from the hydrolysates as described under Methods.

Flushing gas	Carbon atom	Specific radioactivity of $Ba^{14}CO_3$ derived from each C atom (counts/min./mg. of $BaCO_3$)	Sum of specific activities of individual C atoms	Specific activity of $Ba^{14}CO_3$ obtained from combustion × no. of C atoms in molecule	Percentage of total radioactivity in molecule contributed by each C atom
CO_2-free air	(1)	18·0	37·6	42·8	48·0
	(2)	19·6			52·0
Air–CO_2 (99:1, v/v)	(1)	2·6	34·5	34·2	7·5
	(2)	31·9			92·5

precursor of malate, since the carboxylation of a C_3 compound derived from serine to give oxaloacetate or malate, whose carbon atoms are partially randomized by reversible conversion into fumarate, could result in labelling patterns similar to those observed in malate.

The distribution of radioactivity in phosphoglycerate produced by cells incubated with [^{14}C]-methanol (Table 1) is closely similar to that which would be obtained by decarboxylation of malate at C-4 and different from that observed in serine (see above). Hence, if serine is a precursor of phosphoglycerate, there must be an irreversible step in the reaction sequence which prevents the equilibration of the C_3 skeleton of phosphoglycerate with that of serine.

DISCUSSION

Kaneda & Roxburgh (1959) found that, when methanol-grown *Pseudomonas* PRL-W4 was incubated with [^{14}C]methanol or [^{14}C]bicarbonate, one of the first stable intermediates to be labelled was serine. It was suggested that the organism fixed carbon through the agency of serine hydroxymethylase:

$$CH_2(NH_2)\cdot CO_2H + `CH_2\cdot OH'\text{-tetrahydrofolate} \rightleftharpoons CH_2(OH)\cdot CH(NH_2)\cdot CO_2H + \text{tetrahydrofolate} \quad (7)$$

Analysis of the rates at which metabolites were labelled during short-term incubations of *Pseudomonas* AM1 with [^{14}C]methanol and [^{14}C]bicarbonate also led to the suggestion that, in this organism, hydroxymethylation of glycine to give serine might serve as a main step in the synthesis of C_3 compounds during growth on methanol (Large *et al.* 1961).

The labelling patterns now observed in serine and glycine under all conditions tested are consistent with such an interrelationship, provided that there exists a mechanism for deriving hydroxymethyltetrahydrofolate from methanol. Methanol has been shown to be a precursor of the hydroxymethyl group of serine and the methyl groups of choline in mammalian tissue (for review, see Bach, 1955).

The fact that the labelling patterns of serine and malate are in accord with the formation of malate by carboxylation of a C_3 fragment derived from serine, rather than the reverse reaction, is an important one. It lends support to the conclusion (Large *et al.* 1961) that the serine, which is observed, together with malate, at the earliest times of incubation of *Pseudomonas* AM1 with [^{14}C]-methanol, is itself a primary product of methanol incorporation and is not merely derived from the malate. The transformation of serine into either pyruvate or phosphoenolpyruvate, which could be carboxylated to give oxaloacetate or malate, might occur through the action of serine dehydrase giving rise to pyruvate:

$$CH_2(OH)\cdot CH(NH_2)\cdot CO_2H \rightarrow CH_3\cdot CO\cdot CO_2H + NH_3 \quad (8)$$

or by transamination of serine to hydroxypyruvate followed by reduction, phosphorylation and dehydration to give phosphoenolpyruvate.

Since both serine dehydrase and glycerate kinase catalyse essentially irreversible reactions, reversal of either scheme for transformation of serine into pyruvate or phosphoenolpyruvate would not be expected to occur. Hence the labelling pattern of C-1, C-2 and C-3 in malate would not appear in serine.

If serine synthesis from glycine plays a major role in the formation of C_3 compounds, then a mechanism must exist for the prior synthesis of glycine, or its precursors, from C_1 units. The labelling data show that such a mechanism would require the derivation of the aminomethyl carbon atom mainly from methanol and the carboxyl group from carbon dioxide. Direct condensation of carbon dioxide and a reduced C_1 unit is one possibility, although the quantitatively low incorporation of ^{14}C into glycine in whole cells of *Pseudomonas* AM1 does not support this idea (Large *et al.* 1961). Splitting of a C_4 compound to give two C_2 compounds, one or both of which could form glycine, is an alternative mechanism. Evidence in favour of this is the rapidity with which malate is labelled on incubation of whole cells with either [^{14}C]methanol or [^{14}C]bicarbonate, and also the fact that the labelling patterns in malate and glycine are such that glycine might be produced from C-1 and C-2 or C-3 and C-4 of malate.

Two alternative pathways for the synthesis of cell constituents from methanol by *Pseudomonas* AM1 which are in accordance with the data are summarized in Fig. 1. In one scheme, condensation of a C_1 unit and carbon dioxide would yield glycine directly (Fig. 1*a*). In the second scheme, regeneration of glycine by cleavage of a C_4 compound would result in the net synthesis of a C_2 compound (Fig. 1*b*). The organism would then in effect be synthesizing its cell constituents from this C_2 compound.

SUMMARY

1. The distribution of radioactivity in glycine, serine, phosphoglyceric acid and malic acid isolated from methanol-grown *Pseudomonas* AM1 after incubation with [^{14}C]methanol or [^{14}C]bicarbonate has been investigated.

2. The data indicate that the carboxyl group of glycine is mainly derived from carbon dioxide, and the methylene carbon from methanol.

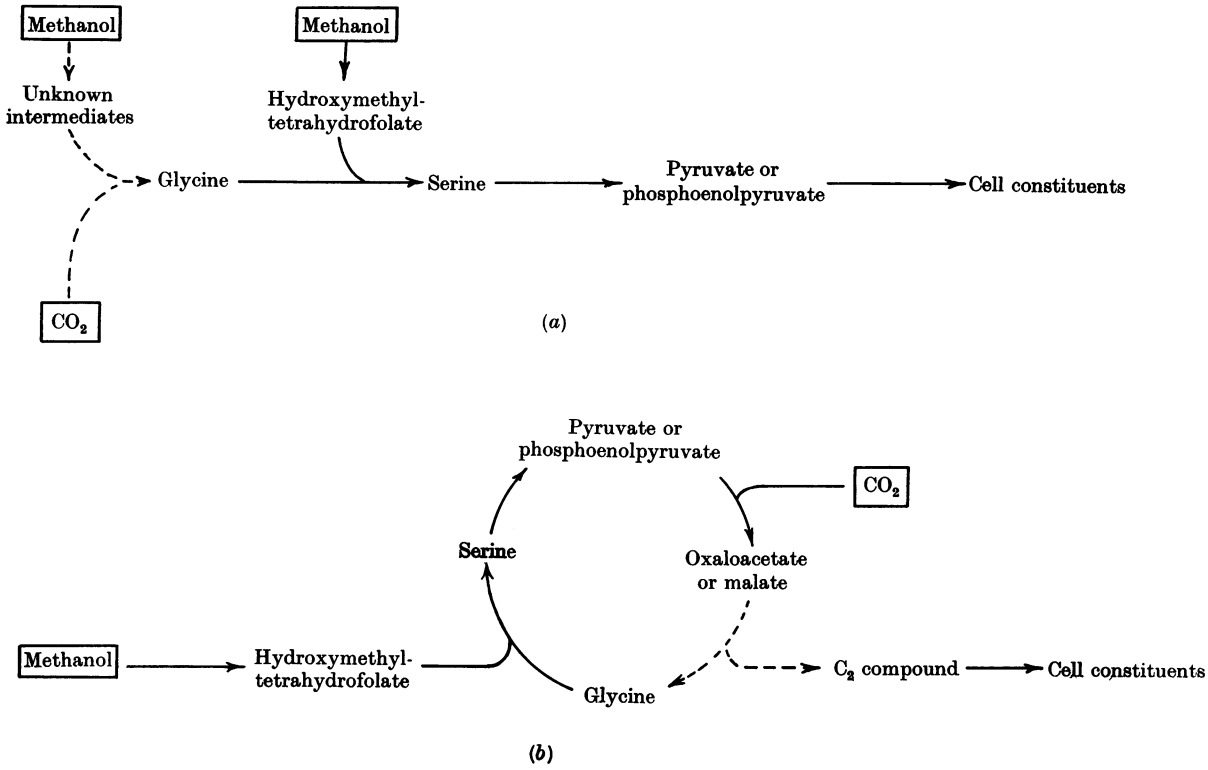

Fig. 1. Possible routes for synthesis of cell constituents from methanol by *Pseudomonas* AM1. The broken lines represent the hypothetical schemes for the crucial step in the biosynthesis, namely the net synthesis of glycine from C_1 units. (*a*), Direct synthesis of glycine; (*b*), cyclic synthesis of glycine.

3. The hydroxymethyl group of serine is derived mainly from methanol, whereas the distribution of radioactivity between C-1 and C-2 of serine is the same under all conditions as that between C-1 and C-2 of glycine.

4. The labelling patterns of serine and malate are consistent with the formation of malate by carboxylation of a C_3 fragment derived from serine, rather than with the reverse reaction.

5. Two possible schemes for synthesis of cell constituents from methanol are outlined. Both schemes implicate hydroxymethylation of glycine to give serine as the major step in the synthesis of C_3 compounds. Alternative means whereby the glycine necessary for such a reaction might be made from methanol are discussed.

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