Formation of Methane from Serine by Cell-free Extracts of Methanobacillus omelianskii*


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Precursors of methane formation by cell-free extracts of the obligate anaerobe, Methanobacillus omelianskii, include carbon dioxide, pyruvate, and serine (1). Recently, methylcobalamin, CH$_3$-B$_12$, (2-4), and N$^x$-methyltetrahydrofolate, 5-CH$_3$-H$_4$-folate, have been shown to be substrates for cell-free methane formation (5). This communication is concerned with the role of tetrahydrofolate, H$_4$-folate, derivatives in the transfer of carbon 3 of 3-[^14]C-L-serine to give $[^14]$CH$_4$ by extracts of M. omelianskii and with the position of CH$_3$-B$_12$ in this metabolic pathway.

EXPERIMENTAL PROCEDURE

Culture Methods and Preparation of Extracts—M. omelianskii was provided by Dr. H. A. Barker; cells were mass cultured, harvested, and washed as described by Johns and Barker (6) with modifications by Wolin, Wolin, and Wolfe (1). Washed cells were stored at 4° under hydrogen to preserve their ability to form CH$_4$.

Cell-free extracts were prepared by exposing these bacteria, 1 g of cells (wet weight) per ml of 0.5 M KH$_2$PO$_4$ buffer at pH 7.0, to the maximum frequency output of a Branson sonic probe for 2 min at 0°. Cell debris was removed by centrifugation at 23,000 x g for 20 min at 0°. Extracts prepared by the above procedure contained 55 to 60 mg of protein per ml as determined by the biuret procedure (7). When CH$_4$ formation was being studied, extracts were used immediately after preparation.

Preparation of $[^14]$C-H$_4$-folate Derivatives—The method of Hatefi et al. (8) was used to prepare dH$_4$-folate; all folate derivatives were made with d-folate. The white crystalline product was freed from glacial acetic acid by lyophilization in the dark for 18 hours. N$_3$.N$_{10}$[^14]C-Methylene tetrahydrofolate (5,10-[^14]CH$_2$-H$_4$-folate) was prepared from equimolar amounts of ^14C-formaldehyde and H$_4$-folate by the method described by Guest, Foster, and Woods (9). N$_3$.5-CH$_3$-H$_4$-folate (5-[^14]CH$_3$-H$_4$-folate) was prepared by the chemical reduction of the freshly prepared 5,10-[^14]CH$_2$-H$_4$-derivative with sodium borohydride in 0.05 M KH$_2$PO$_4$ at pH 7.8. The purity of the 5-[^14]CH$_3$-H$_4$-folate was determined by observing the ratio of its absorbance at 290 and 245 mp at pH 6.1. Only preparations which gave an ~A$_{290}$/A$_{245}$ ratio of 3.5 were used, since ratios less than 3.5 indicated that some oxidation to 5-[^14]CH$_2$-H$_4$-folate (ratio 1.3) had occurred. A Cary model 14 spectrophotometer was used for measurements of absorbance. The purity of the 5-[^14]CH$_3$-H$_4$-folate was also established (see "Results," and Fig. 3) by following its elution with 0.3 M ammonium acetate buffer at pH 6.1 from a TEAE-cellulose$^1$ column generated in the phosphate form (9). To prevent possible oxidation to the H$_2$-folate derivative on the column, 50 mM 2-mercaptoethanol was included in the eluting buffer. Preparations of 5-CH$_3$-H$_4$-folate containing 2-mercaptoethanol were used as standards and were not used in enzymic reactions since 2-mercaptoethanol inhibited methane formation. The final concentration of 5-[^14]CH$_3$-H$_4$-folate was determined by assuming a molar extinction coefficient of 25,000 cm$^2$ per mole at 290 mp (9). The radioactivity present in a sample from each of the 10.0 ml-fractions eluted from the TEAE-cellulose column was counted in a Packard Tri-Carb scintillation spectrometer with a scintillation fluid composed of 0.1 g of 1.4-bis-2-(4-CH$_3$-5-phenyloxazolyl)benzene and 4.0 g of 2,5-diphenyloxazole per liter of toluene.

[^14]CH$_4$ and $[^14]$CO$_2$ were determined with a Packard gas chromatograph which contained a silica gel column connected to a stream splitter from which a portion of the sample was directed to a hydrogen flame ionization detector, and a portion was directed to a Packard combustion furnace. The total counts per sample injected were recorded in a scintillation spectrometer after passage through the combustion furnace. All of the assays were performed with the technique developed by Wolin, Wolin, and Wolfe (1), and data presented represent total $[^14]$CH$_4$ or $[^14]$CO$_2$ formed.

Other Chemicals—[^14]CH$_3$-B$_12$ was prepared by the alkylation of B$_12$ (SH)$_2$ with $[^14]$CH$_2$I by the method of Müller and Müller (10) as modified by Dolphin and Johnson (11). This material was purified, after phenol extraction, by elution with water from a carboxymethyl cellulose column followed by lyophilization. The above procedures were conducted in subdued light.

[^14]C-Formate (4.40 mC per mmole), 3-[^14]C-DL-serine (1 mC per mmole), and $[^14]$CH$_4$I were obtained from the New England Nuclear Corporation. Uniformly labeled 4-[^14]C-L-serine (120 mC per mmole) was obtained from Schwarz BioResearch, and 4-[^14]C-formaldehyde (10 mC per mmole) was obtained from Nichern, Inc.

RESULTS

Serine Transhydroxymethylase and 5,10-[^14]CH$_2$-H$_4$-folate Dehydrogenase—Serine transhydroxymethylase (12) was demonstrated conveniently by following the dehydrogenation of one of

$^1$Cellex T from Bio-Rad Laboratories.

$^2$B$_12$-(SH) is too unstable to isolate, but it is prepared by allowing reduced B$_12$ (B$_{12}$-F) to react with sulfide ions. Dolphin and Johnson have called it sulfitocobalamin (B$_{12}$-(SH)).
**Table I**

**Cofactor requirements for l-serine transhydroxymethylase and 5,10-CH₂-H₄-folate dehydrogenase**

Complete system: fractionated extract, 5.04 mg of protein; H₄-folate, 1.2 μmoles; B₆, 0.5 μmole; MgSO₄, 0.5 μmole; l-serine, 15.0 μmoles; KH₂PO₄ buffer at pH 7.5, 350 μmoles. In an anaerobic cuvette, reactions of Experiment A were started by tipping 1.0 μmole of NADP; reaction of Experiment B was started by tipping 1.0 μmole of NAD. Reaction mixture was incubated 5 min at room temperature under argon.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Δ Absorbance at 340 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A</td>
<td></td>
</tr>
<tr>
<td>Complete system + NADP</td>
<td>0.74</td>
</tr>
<tr>
<td>- Mg²⁺</td>
<td>0.04</td>
</tr>
<tr>
<td>- B₆</td>
<td>0</td>
</tr>
<tr>
<td>+ H₄-folate</td>
<td>0.012</td>
</tr>
<tr>
<td>- l-Serine</td>
<td>0.023</td>
</tr>
<tr>
<td>Experiment B</td>
<td></td>
</tr>
<tr>
<td>Complete system + NAD</td>
<td>0.11</td>
</tr>
</tbody>
</table>

The products of the reaction, 5,10-CH₂-H₄-folate, by the enzyme 5,10-CH₂-H₄-folate dehydrogenase to give N⁵,N¹⁰-methenyl-H₄-folate (5,10 - CH₂-H₄-folate) as the ultimate product (1). Crude sonicated extracts were fractionated with a saturated solution of ammonium sulfate at pH 7.0. The 40 to 65% fraction was freed from ammonium sulfate by passage down a Sephadex G-25 column. Extracts treated in this way were shown to contain both serine transhydroxymethylase and 5,10-CH₂-H₄-folate dehydrogenase activities. The latter enzyme was found to be NADP specific; only slight activity was observed when NAD was substituted for NADP in reaction mixtures (Table I). The combined activities of the two enzymes were assayed by following the reduction of NADP at 340 μm in an anaerobic cuvette. The reactions were completely dependent on the addition of H₄-folate; the results suggest that one isomer is favored in C₂ transfer (Fig. 1), followed by a much slower conversion of the other isomer, since the absorbance of NADPH obtained was about 65% of that expected. Serine transhydroxymethylase also requires Mg²⁺ and pyridoxal phosphate (B₆) for full activity (Table I).

Glycine was detected as the C₂ product of serine transhydroxymethylase activity. A reaction mixture was used which contained: extract, 2.53 mg of protein; H₄-folate, 2.0 μmoles; B₆, 0.5 μmole; MgSO₄, 0.5 μmole; NADP, 2.0 μmoles; uniformly labeled ¹⁴C-L-serine (5.0 μC), 1.0 μmole. The total reaction volume was 1.0 ml, and the reaction was incubated at 40° for 10 min in an atmosphere of argon. The protein was removed by centrifugation after the addition of 0.2 ml of 5 N HCl, and 0.02 ml of the supernatant was subjected to high voltage electrophoresis on Whatman No. 3MM paper in 4% formic acid for 1 hour at a potential of 4200 volts and a current of 200 ma. Results of radioautography showed the formation of a radioactive product which had similar electrophoretic mobility to glycine. This product was identified as glycine after elution and two-dimensional chromatography by the ascending method in phenol-formic acid-water (500 g of phenol in 167 ml of water + 13 ml of formic acid) and in 1-butanol-acetic acid-water (12:3:5), followed by radioautography.

5,10-CH₂-H₄-folate Reductase—The presence of 5,10-CH₂-H₄-folate reductase in crude extracts of *M. omelianskii* has been reported previously by Wood and Wolfe (5). Crude extracts were fractionated with a saturated solution of ammonium sulfate at pH 7.0, and the reductase activity was found in the 50 to 70% fraction. These extracts were freed from ammonium sulfate by passage down a Sephadex G-25 column, and the enzyme was assayed by following the decrease in absorbance of NADH at 340 μm (Fig. 2). This reductase was shown to be specific for NADH;
little activity was demonstrated when NADPH was substituted for NADH in reaction mixtures.

The product of this reduction was identified by comparing its elution from a TEAE-cellulose column to that of authentic 5-14CH3-H4-folate prepared by the chemical reduction of the 5,10-14CH2-derivative (Fig. 3). Ammonium acetate buffer at pH 6.1 was used as eluting buffer, and both the products of the enzymic and chemical reductions of 5,10-14CH2-folate were eluted at 0.3 M. The procedure adopted was essentially the same as that reported by Guest, Foster, and Woods (9), except that 20.0 μmoles of NADH were added dropwise during the course of the enzymic reaction instead of 8.0 μmoles of FADH2; the reaction mixture contained 23.0 mg of protein.

The identity of the product of the enzymic reduction of 5,10-14CH2-folate was confirmed by comparing its absorption spectra at pH 6.1 (Fig. 4A) and at pH 1.2 (Fig. 4B) to that of chemically prepared 5-14CH3-H4-folate.

Formation of 14CH4 from P4C-m-Serine, 5,10-14CH2-folate, and 5-14CH3-H4-folate-14CH4 was formed from all three substrates only in the presence of freshly prepared crude extract and ATP. The formation of 14CH4 from carbon 3 of serine and from 5-14CH2-folate was demonstrated in Fig. 5. When 5,10-14CH2-folate was the substrate, it was observed that after an initial lag, 14CO2 was evolved in the reactions in which ATP had been omitted, but in the presence of ATP, 14CH4 was evolved rapidly (Fig. 6). This result suggested that in the absence of ATP 5,10-14CH2-folate was oxidized via N10,14C-formyl-H4-
FIG. 5. Formation of $^{14}$CH$_4$ from 5-$^{14}$CH$_3$-H$_4$-folate and 3-$^{14}$C-DL-serine by crude extracts of *M. omelianskii*. Each reaction contained: extract, 88.5 mg of protein; ATP, 10.0 pmoles; KH$_2$PO$_4$ buffer at pH 7.0, 760 pmoles. Reactions were started by tipping in either (○) 5-$^{14}$CH$_3$-H$_4$-folate, 6.0 pmoles (0.125 μC per pmole) or (●) 3-$^{14}$C-DL-serine, 5.0 pmoles (0.10 μC per pmole); total liquid volume, 1.65 ml; gas atmosphere, hydrogen; temperature, 40°C.

FIG. 6. Formation of $^{14}$CH$_4$ and $^{14}$CO$_2$ from 5,10-$^{14}$CH$_2$-H$_4$-folate by crude extracts of *M. omelianskii* in the presence and absence of ATP. Reaction mixtures contained: extract, 89.6 mg of protein; ATP, 10.0 pmoles (where indicated); KH$_2$PO$_4$ buffer at pH 7.0, 760 pmoles. Each reaction was started by tipping in 6.0 pmoles (0.125 μC per pmole) of 5,10-$^{14}$CH$_2$-H$_4$-folate; total volume, 1.65 ml; gas atmosphere, hydrogen; temperature, 40°C.

FIG. 7. Formation of $^{14}$CH$_4$ and $^{14}$CO$_2$ from $^{14}$C-formate by crude extracts of *M. omelianskii* in the presence and absence of ATP. Reaction mixture contained: extract, 71.2 mg of protein; ATP, 10.0 pmoles (where indicated); KH$_2$PO$_4$ at pH 7.0, 760 pmoles. Reaction was started by tipping in 3.0 μmoles (0.225 μC per pmole) of $^{14}$C-formate; total volume, 2.10 ml; gas atmosphere, hydrogen; temperature, 40°C.

FIG. 8. Formation of $^{14}$CH$_4$ by crude extracts of *M. omelianskii* from 5-$^{14}$CH$_3$-H$_4$-folate (A) in the absence of CH$_3$-B$_12$, and (B) after the addition of 5.0 μmoles of CH$_3$-B$_12$. Reaction mixture contained: extract, 88.5 mg of protein; ATP, 10.0 pmoles; 5-$^{14}$CH$_3$-H$_4$-folate, 6.0 pmoles (0.125 μC per pmole); KH$_2$PO$_4$ buffer at pH 7.0, 760 pmoles; total volume, 1.7 ml; gas atmosphere, hydrogen; temperature, 40°C.
for $^{14}$CO$_2$ evolution under these experimental conditions. The evolution of $^{14}$CO$_2$ from $^4$C-formate is presented in Fig. 7. It seems likely that free formate is not a precursor of CH$_4$ formation and that the small, increasing amount of CH$_4$ formed in this experiment arises from $^{14}$CO$_2$ reduction (1).

**Position of CH$_3$-B$_12$**

CH$_3$-B$_12$ has been shown to be a good substrate for CH$_4$ formation by extracts of *M. omelianskii* (3), and the following experiments were designed to determine whether free CH$_3$-B$_12$ could serve as an intermediate in the formation of CH$_4$ from 5-CH$_3$-H$_4$-folate. Preliminary experiments with equimolar mixtures of CH$_3$-B$_12$ and 5-CH$_3$-H$_4$-folate showed that the total formation of CH$_4$ from these substrates, in the presence of one another, was additive. It would be anticipated that if free CH$_3$-B$_12$ were an intermediate after 5-CH$_3$-H$_4$-folate, then the addition of unlabeled CH$_3$-B$_12$ to a reaction which was already evolving $^{14}$CH$_4$ from 5-$^{14}$CH$_3$-

![Diagram](image)

**Scheme 1**

For CH$_3$-B$_12$ to its natural substrate for CH$_4$ formation, whereas CH$_3$-B$_12$ may be an artificial substrate for the same reaction.

**DISCUSSION**

Cell-free extracts of *M. omelianskii* contain L-serine transhydroxymethylase which, in the presence of H$_4$-folate, catalyzes the cleavage of L-serine to give glycine and 5,10-CH$_2$-H$_4$-folate. The cofactor requirements for this enzyme are identical with those reported for the same enzyme in mammalian systems (12, 13), and in microbial systems (15); the enzyme is only active in the presence of B$_6$ and Mg$^{++}$. The importance of this enzyme in the methane fermentation by *M. omelianskii* cannot be over emphasized since it is one of the key enzymes in C$_1$ transfer leading to the ultimate formation of methane.

The second enzyme in this folate sequence, 5,10-CH$_2$-H$_4$-folate reductase, has been partially purified and is specific for NADH. Katzen and Buchanan (16) have recently purified a similar enzyme from *Escherichia coli* and have shown that it is a flavoprotein; FADH$_2$ is the true cofactor for this reductase. Further purification of the enzyme from *M. omelianskii* will be necessary before it can be said with any certainty that NADH is the true cofactor for the reduction. However, if the enzyme is a flavoprotein, then the flavin moiety does not dissociate under prolonged dialysis.

The formation of $^{14}$CH$_4$ from $^4$C-serine, 5,10-$^{14}$CH$_2$-H$_4$-folate, 5-$^{14}$CH$_3$-H$_4$-folate, or $^{14}$CH$_3$-B$_12$ is completely dependent on ATP. The role of ATP in this reaction is not yet understood, but the presence or absence of this cofactor determines the direction of C$_1$ transfer in extracts of *M. omelianskii*. In the absence of ATP, 5,10-CH$_2$-H$_4$-folate is apparently oxidized via N$^{15}$-formyl-H$_4$-folate to give formate and H$_4$-folate as the products. This cycle may generate ATP which could then be utilized for further methane formation. Routes for C$_1$ transfer in the presence and absence of ATP are postulated in Scheme 1.

![Scheme 1](image)
coenzyme) constitute the majority of naturally occurring B12 compounds in this organism. Therefore, it appears that CH₃-cobalt 5,6-dimethylbenzimidazoylcobamide (CH₃-B₁₂) is not likely to be a true intermediate for methane formation in *M. omelianskii*. However, it may compete with 5-CH₃-H₄-folate for an active site of the methane-forming enzyme. The position of free CH₃-B₁₂ in the formation of methane seems to be analogous to that reported for the transfer of the methyl group in the biosynthesis of methionine in *E. coli* (18). The possibility that a cobamide moiety is bound in the enzyme protein forming the active site for methane formation cannot be ruled out. The purification and properties of this methane-forming enzyme are at present under investigation.

**SUMMARY**

Extracts of *Methanobacillus omelianskii* catalyze the C₁ transfer of carbon 3 of L-serine via N₅,N₁₀-methylenetetrahydrofolate, and N₇-methyltetrahydrofolate to give methane. The methane-forming enzyme only functions in the presence of adenosine triphosphate, and although the role of adenosine triphosphate in this reaction is not understood, this cofactor has been shown to play an important part in the regulation of C₁ transfer in this organism. The position of methylcobalamin in methane formation is discussed.

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**REFERENCES**

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