THE FORMATION OF CH_4 FROM N^5-METHYLTETRAHYDROFOLATE MONOGLUTAMATE BY CELL-FREE EXTRACTS OF *METHANOBACILLUS OMELIANSKII*

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The formation of CH_4 by cell-free extracts of *Methanobacillus omelianskii* from CO_2, pyruvate or serine was first demonstrated by Wolin, Wolin and Wolfe (1963). Studies by Blaylock and Stadtman (1963, 1964), using cell-free extracts of *Methanosarcina barkeri*, have demonstrated that methylcobalamin is a possible intermediate in the formation of CH_4 from methanol. With the discovery that CH_4 and $B_{12}r$ were the products of methylcobalamin reduction by crude extracts of *M. omelianskii* (Wolin *et al*, 1964), it was anticipated that methylcobalamin also may be an intermediate in the formation of CH_4 from CO_2, pyruvate or serine. However, recent studies in this laboratory suggest that N^5-methyltetrahydrofolate is an important intermediate in the formation of CH_4 from these substrates, and that methylcobalamin may not be an obligatory intermediate.

METHODS

N^5N^{10}-methylenetetrahydrofolate monoglutamate and N^5-methyltetrahydrofolate monoglutamate were synthesized by the method outlined by Guest et al (1964). Mercaptoethanol was omitted from all preparations since it was shown to inhibit strongly CH_4 formation. C^{14}-methylcobalamin was synthesized by the method of Müller and Müller (1962). Cell-free extracts were prepared by sonic disintegration, and CH_4 formation was assayed by the method previously described by Wolin et al (1963). N^5N^{10}-methylenetetrahydrofolate was stored as a solid under H_2, and solutions of N^5-methyltetrahydrofolate routinely were prepared by the reduction of the N^5N^{10}-methylene derivative with sodium borohydride in 0.05 M potassium phosphate buffer at pH 7.8. The product of this reduction gave λ max 290 mμ at pH 6.1, and at pH 1.2 there was a decrease in extinction at 290 mμ with the appearance of a second peak at 269 mμ. These solutions of N^5-methyltetrahydrofolate were standardized spectrophotometrically at 290 mμ by assuming a molar extinction coefficient of 25,000 cm^2/mole.

RESULTS

Crude extracts of M. omelianskii readily reduced N^5-methyltetrahydrofolate in the presence of ATP with the concomitant evolution of CH_4 (Fig. 1). The optimum substrate concentration was shown to be 6 to 12 μmoles with inhibition in the presence of higher concentrations of N^5-methyltetrahydrofolate (Fig. 2). Values presented have been corrected for an endogenous CH_4 formation of 0.6 to 0.8 μmole.

A similar ATP requirement has been demonstrated for the formation of CH_4 from CO_2, pyruvate, serine or methylcobala-

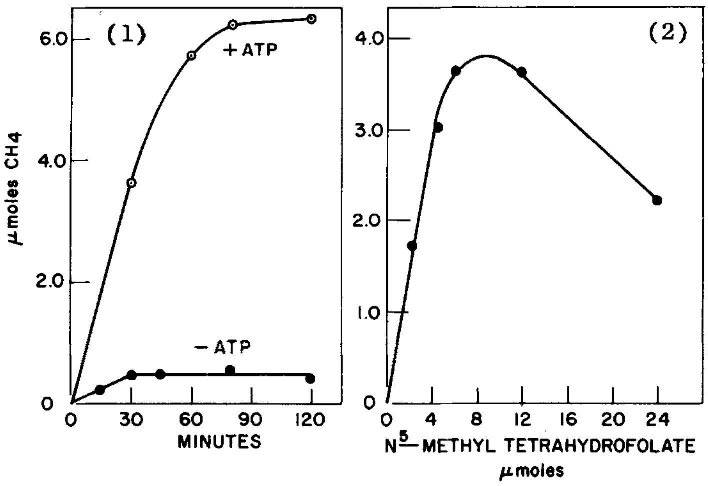

Fig. 1. Formation of methane from N^5-methyltetrahydrofolate. The reaction mixture contained: crude extract (97.6 mg of protein), 6.0 μmoles of N^5-methyltetrahydrofolate, 760 μmoles of potassium phosphate buffer pH 7.0, and 10.0 μmoles of ATP where indicated. Total reaction volume of 1.8 ml was incubated at 40°C under H_2.

Fig. 2. Effect of substrate concentration on the formation of methane. The reaction mixture was as described for Fig. 1 with N^5-methyltetrahydrofolate added as indicated. Reaction time, 30 min.

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min by extracts of M. omelianskii. Blaylock and Stadtman (1964) also have reported an ATP requirement for the formation of CH_4 from methylcobalamin by extracts of M. barkeri. Studies on the function of ATP in CH_4 formation by extracts of M. omelianskii have been complicated by the presence of competing ATP-ase activity.

When 2.0 μmoles of $B_{12}r$ were included in a reaction mixture with 6.0 μmoles of N^5-methyltetrahydrofolate, CH_4 was evolved only in the presence of ATP, and the rate of CH_4 formation was somewhat diminished, being 70% of that in the absence of $B_{12}r$. Extraction of the reaction products revealed the presence of methylcobalamin in all reaction flasks, including those which contained boiled extract. This result suggests that $B_{12}r$ (SH), under the conditions of the reaction, would serve as methyl acceptor in the non-enzymic transfer from the powerful alkylating agent N^5-methyltetrahydrofolate. If methylcobalamin were an obligatory intermediate a much more dramatic inhibition might have been expected in the presence of 2 µmoles of $B_{12}r$.

When C^{14}-methylcobalamin (0.4 µmole; 4,567 cpm) was incubated under H_2 with tetrahydrofolic acid (1.0 µmole in 2.7 µmoles of mercaptoethanol) and ATP (1.0 µmole), the rapid appearance of only one radioactive product was demonstrated by using chromatography and radioautography. This product was identified as methionine, following elution, two dimensional chromatography, and radioautography with standard dl-methionine. After 30 minutes at 37°C the reaction was complete with 36% incorporation in methionine (1,612 cpm). No methionine was formed when boiled extracts were substituted for crude extracts (2.7 mg of protein) in the reaction mixture.

The results of this experiment suggest that methionine is formed by the methylation of endogenous homocysteine, present in crude extracts, via the enzyme N^5-methyltetrahydrofolate: 1. homocysteine transferase, thus indicating a transfer of the methyl group from methylcobalamin to tetrahydrofolate to give the N^5-methyl derivative.

Extracts which had been passed down a G-25 Sephadex column were shown to contain N^5N^{10}-methylenetetrahydrofolate reductase activity (Fig. 3). This reductase is dependent on $NADH_2$, and the product is presumably N^5-methyltetrahydrofolate, since crude extracts readily evolved CH_4 from the N^5N^{10}-methylene derivative (Fig. 4).

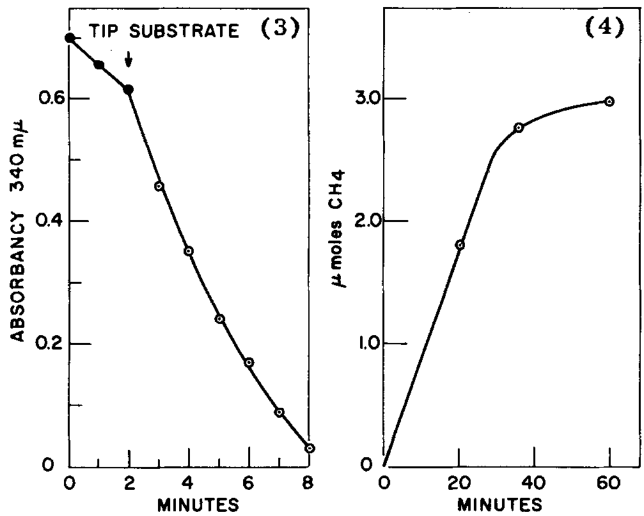

Fig. 3. Reduction of N^5N^{10}-methylenetetrahydrofolate by $NADH_2$. The anaerobic cuvette contained: sephadex-treated extract (0.72 mg of protein), 300 μmoles of potassium phosphate buffer at pH 7.0, and 0.5 μmole $NADH_2$. The reaction was started by tipping 2.0 μmoles of N^5N^{10}-methylenetetrahydrofolate from the sidearm. The total reaction volume was 3.0 ml and the gas phase was argon.

Fig. 4. Formation of methane from N^5N^{10}-methylene tetrahydrofolate. The reaction contained: crude extract (91.5 mg of protein), 760 μmoles of potassium phosphate buffer at pH 7.0, 3.0 μmoles of N^5N^{10}-methylenetetrahydrofolate, and 10.0 μmoles of ATP. The total reaction volume was 1.8 ml, the gas phase H_2, temperature 40°C.

Tetrahydrofolate derivatives are apparently involved in the formation of CH_4 as well as in the formation of methionine by cell-free extracts of *M. omelianskii*. These systems are at present under investigation.

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