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Activation and Inactivation of Methanol: 2-Mercaptoethanesulfonic Acid Methyltransferase from *Methanosarcina barkeri*

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Methanol is converted to methane by crude extracts of *Methanosarcina barkeri*. The first reaction involved in this process, is catalyzed by methanol:2-mercaptoethanesulfonic acid methyltransferase (EC 2.1.1.-). The methyltransferase has an optimum at pH 6.5 and is not inhibited by 2-bromoethanesulfonic acid. Pyridoxal-5'-phosphate acts as an inhibitor (K_i = 0.30 mM). The methyltransferase was tested in the presence of 2-bromoethanesulfonic acid, which inhibits the conversion of 2-(methylthio)ethanesulfonic acid to methane. The reaction is subject to activation and inactivation. Inactivation is brought about by the presence of oxygen, flavin mononucleotide, flavin adenine dinucleotide, and 2-(methylthio)ethanesulfonic acid, the product of the reaction. Activation of the system requires the presence of ATP and Mg^{2+} and of hydrogen. Hydrogen can be replaced by enzymatic systems, such as pyruvate dehydrogenase, which deliver free hydrogen.

Methanosarcina barkeri is one of the most versatile methanogenic bacteria. It converts CO_2 plus H_2, acetate, various methylamines, CO plus H_2, and methanol to CH_4 (12, 13, 18, 22, 27). Methanol is converted to CH_4 and CO_2 according to the following equation (13):

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O.$$

In the presence of H_2 methanol is completely converted to CH_4.

The reduction of methanol to CH_4 was the subject of previous studies (2, 7, 23; T. J. Hutten, Ph.D. thesis, University of Nijmegen, 1982). Blaylock and Stadtman (2, 5) suggested that the methyl moiety of methanol was transferred to a cobalamin to form methylcobalamin which was subsequently reduced to CH_4 (3, 4). They reported the resolution of a methanol: cob(I)alamin methyltransferase system into four components, including a red corrinoid protein, a ferredoxin, and a heat-stable, dialyzable cofactor (2, 6). The reaction required ATP and H_2.

2-Mercaptoethanesulfonic acid (coenzyme M, HS-CoM), a unique coenzyme of methanogens (1, 19, 24), participates in methanogenesis from methanol as well as from CO_2 (7, 9, 10, 14). HS-CoM is methylated, and the product formed is reduced by 2-(methylthio)ethanesulfonic acid (CH_3S-CoM) reductase (10) that contains the Ni-containing cofactor F_{430} (8).

The isolation and purification of a methylcobalamin:HS-CoM methyltransferase from *Methanobacterium bryantii* was described by Taylor and Wolfe (25).

Previously, Shapiro and Wolfe (21) described the presence of a methanol:HS-CoM methyltransferase from *M. barkeri*. The enzyme requires catalytic amounts of ATP and Mg^{2+}; the lag phase, which was observed on incubation of cell-free extracts with methanol and HS-CoM, was shortened when a preincubation with ATP and Mg^{2+} was performed.

Here we report on properties of this enzyme in crude extracts and on the activation and inactivation reactions which occur on incubation of the enzyme under various conditions.

MATERIALS AND METHODS

Culture methods and preparation of cell-free extracts. Cells of *M. barkeri* strain MS (DSM800) were mass cultured in a 350-liter fermentor in a mineral medium with methanol as substrate and under a gas atmosphere of N_2-CO_2 (80:20, vol/vol) as described before (14). Cells were harvested in the late exponential phase and stored under H_2 at −80°C.

Cell-free extracts were prepared after washing the cells twice with 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (pH 7.2). A suspension of 40% wet cells was made in the same buffer containing 15 mM $MgCl_2$, and the cells were broken by passage through a French pressure cell (14). After centrifugation for 30 min at 25,000 × *g* and 4°C the

cell-free extract was stored under H_2 at −80°C in 10-ml serum bottles closed with black butyl rubber stoppers and crimped aluminum seal caps.

Enzyme assays. Incubation mixtures were prepared in an anaerobic glove box (14) containing 97.5% N_2, 2.5% H_2, and oxygen at a concentration below 1 ppm (1 μl/l). Incubations, supply of the gas mixtures, and measurements of concentrations in the gas and liquid phase took place outside the anaerobic glove box.

A typical reaction mixture (final volume, 100 μl) contained 12.5 mM methanol, 7.0 mM 2-propanol, 9.38 mM ATP, 6.25 mM $MgCl_2$, and 50 μM bromoethanesulfonic acid (BrES) in 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (pH 7.2). The amount of protein varied between 0.4 and 1 mg. The reaction was performed in 10-ml serum vials which were closed with red rubber stoppers and crimped aluminum seal caps. The vials were kept on ice, and the appropriate gas or gas mixture was added. Incubation mixtures under N_2 were prepared by a threefold cycle of evacuation and gassing of an assay mixture which did not contain methanol and 2-propanol. These compounds were then added with a gas-tight Hamilton syringe. The incubation was started by placing the vials in a water bath at 37°C. The course of the reaction was followed by incubation of a series of reaction vials; after each time interval, one vial was placed on ice and opened to stop the reaction.

Activation of the enzyme was performed by preincubation of the cell-free extract together with ATP and Mg^{2+} under H_2–N_2 (50:50, vol/vol) for 10 to 30 min. After preincubation the gas phase was replaced by N_2, and the reaction was started by the anaerobic addition of methanol, 2-propanol, HS-CoM, and BrES.

Inactivation experiments were performed in a similar way. The compounds tested for their inactivating effect were present during a second preincubation under N_2.

The pH optimum of the enzymic reaction was measured in a mixture of 300 μl of cell-free extract and 100 μl of 250 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.5) containing ATP, Mg^{2+}, BrES, HS-CoM, methanol, and 2-propanol at concentrations that resulted in final values as given above and small amounts of HCl and KOH to adjust the pH of the mixture. The final volume of the assay mixture was 450 μl. The mixture was incubated for 6 min under H_2–N_2 (50:50, vol/vol), the reaction was stopped, and the pH and the final concentration of methanol were measured.

Analytical procedures. Methanol was measured with a Pye Unicam GCD gas chromatograph. The following conditions were used: SS column (6 ft by ⅛ in; ca. 1.83 m by 3.2 mm); 0.2% Carbowax 1500 on Carbopack (80/100); column temperature, 130°C; injection temperature, 170°C; flame ionization detector at 170°C; N_2 as carrier gas, 25 ml/min; and sample size, 0.1 μl.

Together with methanol (final concentration, 12.5 mM), 2-propanol (final concentration, 7.0 mM) was routinely added to the final incubation mixture as an internal standard. 2-Propanol did not affect the reactions studied. When an incubation was performed under 100% N_2, methanol and 2-propanol were added with a gas-tight Hamilton syringe. To define the exact assay volume for gas chromatography, 10 μl of 125 mM 1-propanol was added at the end of the incubation to serve as a second internal standard.

Methane was measured on a Poropack Q (80/100) column as described by Hutten et al. (14). Ethane was used as an internal standard in the gas phase of the incubation mixture.

HS-CoM derivatives were determined by isotachophoresis by the method of Hermans et al. (11).

Protein was determined with the Coomassie brilliant blue G-250 method (20) with bovine serum albumin as a standard. The values obtained are about 0.7 times lower than those obtained by the method of Lowry et al. (17).

Materials. Black butyl rubber stoppers were obtained from Rubber B.V., Hilversum, The Netherlands, and were used for anaerobic storage of cells, cell-free extracts, and stock solutions. Red rubber stoppers were purchased from Helvoet B.V., Belgium, and were used in incubation experiments.

The biochemicals were purchased from Boehringer, Mannheim, West Germany. Serva Blau G was obtained from Serva Feinbiochemica, Heidelberg, West Germany. BrES was obtained from Aldrich Europe, Beerse, Belgium. HS-CoM was purchased from Merck-Schuchardt A.G., Darmstadt, Germany. CH_3S-CoM was prepared by methylation of HS-CoM with dimethyl sulfate; the preparation was pure as judged by isotachophoretic analysis and ^1H nuclear magnetic resonance, and it was active in the CH_3S-CoM reductase system. Coenzyme F_{420} was a gift of A. Pol from our department.

Gases were obtained from Hoek Loos, Schiedam, The Netherlands. To remove traces of oxygen, H_2 was passed over a catalyst (BASF R0-20) at room temperature, and N_2 was passed over a prereduced catalyst (BASF R3-11) at 150°C. Both catalysts were gifts of BASF Aktienge-sellschaft, Ludwigshafen, West Germany.

RESULTS

Effect of BrES. Methane formation is known to be inhibited completely by small amounts of BrES (19), an analog of 2-mercaptoethanesulfonic acid (HS-CoM). This effect is obvious in the tests of methane production from methanol by crude cell-free extracts (Fig. 1), but BrES does not affect the methanol conversion and CH_3S-CoM formation by the methanol:HS-CoM methyltransferase reaction. These experiments provide a method to measure the methyltransferase activity separate from the methanogenic CH_3S-CoM reductase activity. In previous studies (21; R. P. Gunsalus, Ph.D. thesis, University of Illinois, Urbana, 1977) either N_2 was applied as a gas phase or polyphosphate was added to stop the latter activity. However, these methods did not allow a study on the effect of H_2, and the use of polyphosphates interferes with the need of Mg^{2+} ions in the conversion of methanol to CH_3S-CoM (21).

In the experiments described below the methanol:HS-CoM methyltransferase reaction was studied in the presence of BrES at a concentration five times higher than the K_i (11 μM), which we measured for the CH_3S-CoM reductase system.

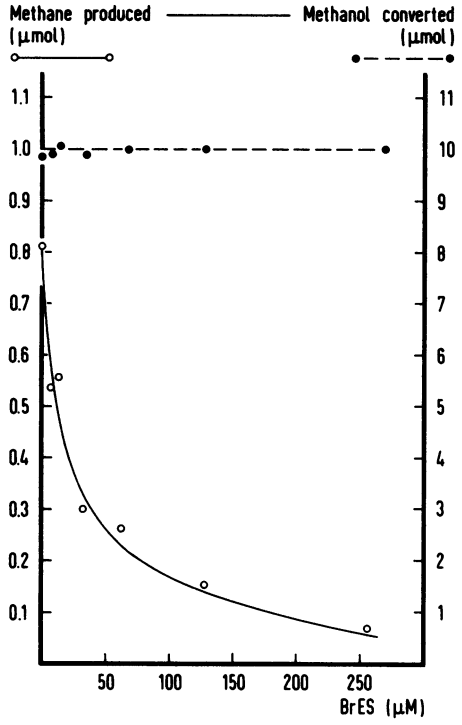

FIG. 1. Effect of BrES on methane formation and methanol conversion by a cell-free extract of *M. barkeri* under H_2–N_2 (50:50, vol/vol). The reaction mixtures (final volume, 0.4 ml) contained the following: 7.5 mM ATP, 5 mM $MgCl_2$, 25 mM HS-CoM, 25 mM methanol, 10 mM 2-propanol, 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (pH 7.2), 2.2 mg of protein, and BrES as indicated. The gas phase was H_2–N_2 together with 100 μl of ethane in a total volume of 12 ml. Since H_2, ATP, and Mg^{2+} were present during the incubation, no separate activation step was performed. Incubation was for 30 min at 37°C. Methane production (○) and methanol conversion (●) were measured.

pH dependence. The methyltransferase reaction shows an optimum at pH 6.5, and 50% activity was found at pH 5.6 and 7.5. Most experiments were carried out at pH 7.2, at which the activity was 70% of the optimal value, but buffering capacity was optimal.

Influence of different gas phases. The methyltransferase was tested with various H_2–N_2 mixtures as the gas phase. At low H_2 concentrations (from 0 up to 2.5%) the specific activity was 30 nmol of methanol converted per min per mg of protein. A 10-fold higher value was found in tests performed with 50 to 100% H_2 in the gas phase.

The enzymatic activity was lost by exposure to air for 30 min; on subsequent incubation under an atmosphere of N_2, after thorough removal of the air, no activity was found. However, when the reaction was performed with H_2 as the gas phase the specific activity was 50 nmol/min per mg.

Effect of preincubation. H_2 was important in the enhancement of the specific activity of the methyltransferase, but the activation was optimal only when ATP and $MgCl_2$ were present (Fig. 2). The activation of the methyltransferase

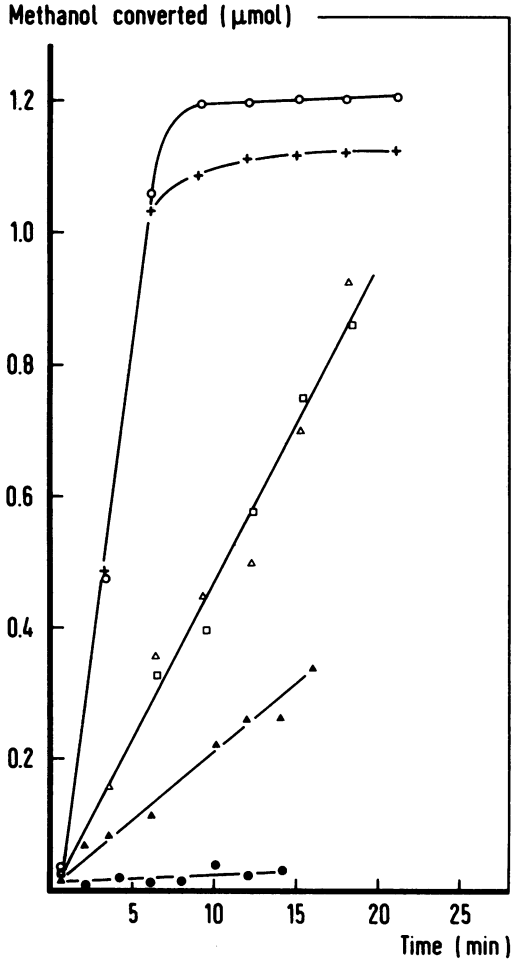

FIG. 2. Effect of various preincubation conditions on the activity of the methyltransferase in cell-free extract of *M. barkeri*. Reaction mixtures, containing 0.7 mg of protein, were preincubated for 15 min at 37°C in the presence of the following: 11.5 mM ATP and 7.7 mM $MgCl_2$ (○); 15.4 mM HS-CoM (□); 10.7 mM ATP, 7.15 mM $MgCl_2$, and 14.3 mM HS-CoM (+); no additions (△). These preincubations took place under H_2–N_2 (50:50, vol/vol). In a second series N_2 was used as the gas phase, and the reaction mixtures contained the following: 11.5 mM ATP and 7.7 mM $MgCl_2$ (▲) and no additions (●). The incubation at 37°C was started after the reaction mixture was placed under N_2 and completed to the standard composition as described in the text.

required a period of about 15 to 30 min and occurred in the absence of HS-CoM (Fig. 2). Once the extract is activated in this way, the methyltransferase reaction does not require the further presence of H_2 (data not shown).

Inactivation with electron acceptors other than oxygen. Some oxidized electron carriers were added under N_2 to the extract, in which the methyltransferase had been activated under H_2, to check for possible inactivation. The addition of F_{420}, NAD, and NADP (all tested at a final concentration of 1 mM) did not affect the activity, but the addition of flavin adenine dinucleotide and flavin mononucleotide (final concentration, 1 mM) resulted in a reduction of the activity to 30%; the observed inactivation was small when H_2 was present in the final incubation mixture (Table 1). The presence of CH_3S-CoM, the product of the transmethylation reaction, also caused inactivation. This inactivation was not observed when BrES, which could inhibit the reduction of CH_3S-CoM, was present simultaneously, or when the final incubation was performed in the presence of H_2 (Table 1).

Activation by other reducing systems. A number of reducing systems was tested for the ability to replace H_2 in the activation process of the methyltransferase (Table 2). The reducing systems were incubated under N_2 as the gas phase for 15 min with the methyltransferase which had been inactivated by incubation of the extract with CH_3S-CoM and ATP and $MgCl_2$ under N_2. The methyltransferase was activated in the presence of pyruvate and coenzyme A, which together may deliver electrons to coenzyme F_{420} through the pyruvate dehydrogenase system as described previously for *Methanobacterium thermoautotrophicum* (29). NADH and NADPH had only low activities in the activation process.

TABLE 1. Influence of some electron acceptors on activated methyltransferase[a]

Addition (mM)[b]	% Activity	
	N_2[c]	H_2[c]
None	100[d]	100[d]
Flavin adenine dinucleotide (1)	30	90
Flavin mononucleotide (1)	30	90
CH_3S-CoM (0.5)	50	ND[e]
CH_3-S-CoM (1)	10	ND
CH_3S-CoM (1) + BrES (0.05)	100	ND

[a] Cell-free extract was activated under H_2–N_2 (50:50, vol/vol) in the presence of 11.5 mM ATP and 7.7 mM $MgCl_2$ for 15 min at 37°C. Additions were made under N_2 as described in the text. The mixtures were incubated for another 15 min before the reaction was started by the addition of the substrates.

[b] Final concentration in the incubation mixture.

[c] The gas phase of the final incubation mixture.

[d] Equivalent to a specific activity of 0.26 μmol of methanol converted per min per mg of protein.

[e] ND, Not determined.

TABLE 2. Influence of various H_2 donating systems on inactivated methyltransferase[a]

Addition (mM)[b]	% Activity
None	0
H_2[c] (2 atm)	100[d]
NADH (2)	10
NADPH (2)	10
Pyruvate (2.5)	0
CoA[e] (1.25)	0
Pyruvate (2.5) + CoA (1.25)	90

[a] Cell-free extract was inactivated by preincubation for 15 min at 37°C in the presence of 1.4 mM CH_3S-CoM, 10.7 mM ATP, and 7.1 mM $MgCl_2$ under N_2 as a gas phase. After this preincubation, a second preincubation was performed for 15 min at 37°C in the presence of the above-mentioned additions under N_2 as a gas phase. Thereafter, enzymic activity was tested as described in the text with N_2 as the gas phase.

[b] Final concentration in the incubation mixture.

[c] H_2 was added after inactivation. After 15 min, H_2 was replaced by N_2 and enzymic activity was tested.

[d] Equivalent to a specific activity of 0.18 μmol of methanol converted per min per mg of protein under N_2.

[e] CoA, Coenzyme A.

Catalytic role of ATP. The transmethylase requires the presence of catalytic amounts of ATP, as was shown by Shapiro and Wolfe (21).

The catalytic effect was counteracted by the presence of small amounts of air, which was injected in the gas phase of the incubation mixture (Fig. 3). The presence of oxygen (500 ppm) in the gas phase required about a threefold higher amount of ATP to obtain a similar conversion of methanol.

Role of ATP in the transmethylation reaction. H_2 and ATP were required to obtain full activity, but once activation had proceeded H_2 was no longer required. To study the function of ATP, the extract was activated first, and ATP was removed by the addition of a mixture of hexokinase, glucose, and adenylate kinase in a second preincubation (Fig. 4). It appeared that ATP was needed in the activation process, but once the enzyme was brought into the activated form, the presence of ATP was less essential.

Inhibition of methyltransferase. Various compounds were tested on the ability to inhibit the enzyme. Pyridoxal-5′-phosphate inhibited (K_i of 0.30 mM) the reaction when added before or after preincubation and both under N_2 and H_2. Cyanide (2 mM), sodium azide (2 mM), and sodium dithionite (1 mM) inhibited the reaction 10, 10, and 90%, respectively.

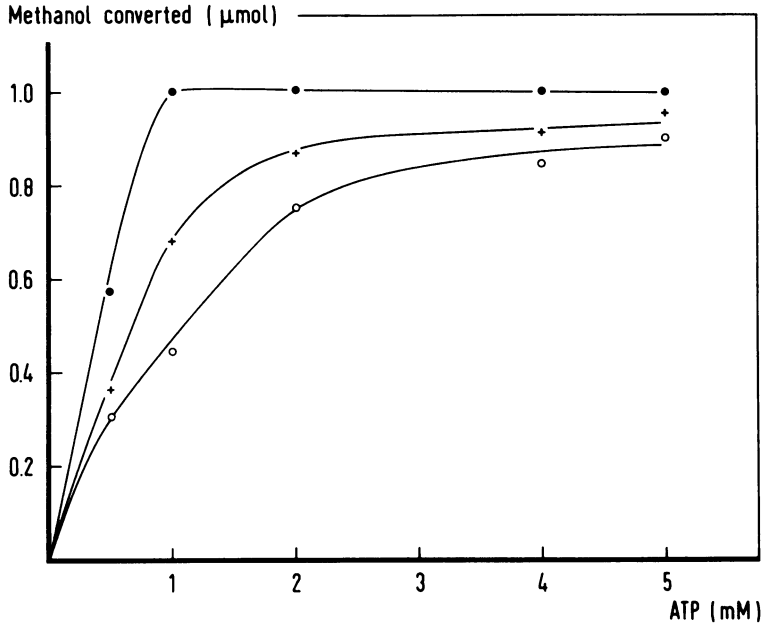

FIG. 3. Effect of oxygen on the ATP requirement of the methyltransferase reaction in cell-free extract of *M. barkeri*. Standard incubation mixtures (see text) containing 0.6 mg of protein with increasing amounts of ATP were incubated under H_2–N_2 (50:50, vol/vol) without O_2 (●), with 250 ppm O_2 (+), and with 500 ppm O_2 (○). The incubation was performed for 15 min at 37°C.

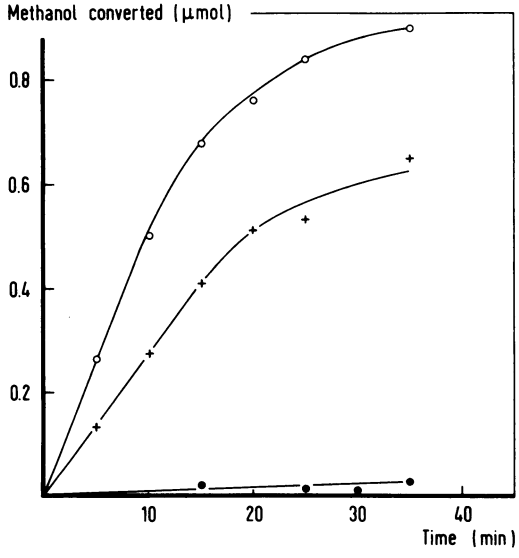

FIG. 4. Effect of the removal of ATP on the activity of activated methyltransferase in cell-free extract of *M. barkeri*. A mixture of hexokinase (0.8 U), adenylate kinase (0.4 U), and glucose (50 mM) was added to extracts, containing 0.7 mg of protein, before activation (●) and after activation (+) with ATP and Mg^{2+} (see text). The resulting mixture was then preincubated for 15 min at 37°C. In a separate experiment *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (pH 7.2) was added to the activated extract (○). After the preincubation the reaction was started by completing the reaction mixture to the composition as given in the text.

DISCUSSION

Methanol:HS-CoM methyltransferase of *M. barkeri* was found to be insensitive to the powerful inhibitor of methanogenesis, BrES, whereas CH_3S-CoM reductase was very sensitive to it. This demonstrates that BrES is not an inhibitor in all reactions which involve HS-CoM. The inhibiting effect may be restricted to those reactions in which HS-CoM functions in an activated state (14), viz., in a form in which it is bound to factor F_{430} (15, 26).

Catalytic amounts of ATP are required in the conversion of methanol to CH_3S-CoM (21). The present results demonstrate that ATP is required in an activation process.

The activation process requires the presence of H_2, which is most probably involved in a reduction. A 5- to 100-fold enhancement of the activity was obtained, depending on the quality of the extract tested. The beneficial effect of H_2 was brought about also by pyruvate in the presence of coenzyme A, but NADH and NADPH, together with F_{420}, were unable to activate the methyltransferase.

Inactivation probably involves an oxidation. This oxidation occurs when CH_3S-CoM, the product of the transmethylation reaction, is present and is reduced to methane in the absence of a potent electron donor like H_2. The inactivation by the presence of CH_3S-CoM does not occur when BrES is present in the reaction mixture. Also flavin adenine dinucleotide and flavin

mononucleotide, but not NAD or NADP, inactivate the system; however, in the presence of H_2 the inactivation by flavin adenine dinucleotide and flavin mononucleotide was greatly diminished.

The inhibiting effect of pyridoxal-5'-phosphate requires further study; the compound appears to interact in the transmethylation reaction. The methyltransferase was not inhibited by cyanide or azide. This may indicate that no metal ions participate in the enzymic reaction.

Recently Wood et al. (28) reported the production of methane from a chemically methylated B_{12} protein from *M. barkeri*. Preliminary results indicate a possible role of a B_{12} (factor III) protein in the methyltransferase reaction (unpublished results). This may explain the inhibiting effect of pyridoxal-5'-phosphate, which is known to inhibit the dioldehydrase reaction in which a B_{12} protein participates (16).

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