Proc. Natl. Acad. Sci. USA
Vol. 76, No. 1, pp. 494–498, January 1979
Microbiology

Utilization of trimethylamine and other *N*-methyl compounds for growth and methane formation by *Methanosarcina barkeri*

(methanogenesis/choline and betaine fermentation/creatine/sarcosine/growth yields)

H. HIPPE*, D. CASPARI†, K. FIEBIG†, AND G. GOTTSCHALK†

†Institut für Mikrobiologie der Universität Göttingen and *Deutsche Sammlung von Mikroorganismen der Gesellschaft für Strahlen- und Umweltforschung mbH München, Grisebachstrasse 8, D-3400 Göttingen, West Germany

Communicated by H. A. Barker, October 2, 1978

ABSTRACT **A number of *N*-methyl compounds, including several methylamines, creatine, sarcosine, choline, and betaine, were readily fermented by enrichment cultures yielding methane as a major product. Methylamine, dimethylamine, trimethylamine, and ethyldimethylamine were fermented by pure cultures of *Methanosarcina barkeri*; except for ethyldimethylamine, these amines are considered important substrates of this methanogenic microorganism. Creatine, sarcosine, choline, and betaine were fermented to methane only by mixed cultures. During growth of *M. barkeri* on methyl-, dimethyl-, or trimethylamine, methanol was not excreted into the medium. The fermentation of trimethylamine gave rise to an intermediary accumulation of methyl- and dimethylamine in the medium. An accumulation of methylamine during the fermentation of dimethylamine was not observed. Methane and ammonia were produced from the three methylamines by *M. barkeri* in amounts expected on the basis of the appropriate fermentation equations. The growth yield was 5.8 mg of cells (dry weight) per mmol of methane and was not dependent on the kind of methyl compound used as substrate.**

The methanogenic bacteria are very restricted with respect to the nature of the substrates that can be used for methane formation. All species described are able to produce methane from molecular hydrogen and carbon dioxide. In addition, some species can use carbon monoxide, formate, acetate, or methanol as a methanogenic substrate (1–5). Among this group of highly specialized organisms *Methanosarcina barkeri* seems to be most versatile with respect to the number of substrates utilized. It is able to use all the compounds mentioned above, except formate, for methane formation (1, 6, 7).

In addition to methanol, a rather large group of compounds exists containing methyl groups which can be transferred easily to other acceptors or carriers: the *N*-methyl compounds. Little is known about their role in methanogenesis. Zhilina and Zavarzin (8) reported growth on methylamine of a mixed culture of *Methanosarcina* and *Desulfovibrio*. Recently, it has been demonstrated that the methyl groups of choline are converted to methane via trimethylamine in the rumen (9). Methyl- and dimethylamine are naturally occurring compounds (10, 11) but probably trimethylamine is more abundant in anaerobic habitats. It is formed from choline by organisms such as *Desulfovibrio desulfuricans* (12, 13). Furthermore, the trimethylamine-*N*-oxide present in marine fish is reduced by bacteria to trimethylamine after the fish have died (14). Several bacteria, especially enterobacteria and phototrophic bacteria, have been shown to carry out this reduction under anaerobic conditions (15–17). Another potential precursor of trimethylamine is betaine, the fate of which under anaerobic conditions is unknown.

Trimethylamine and a number of other *N*-methyl compounds including the monomethylamine compounds sarcosine and creatine were tested for their ability to serve as substrates in methanogenesis. The results reported here demonstrate that the methylamines are used for methane formation by pure cultures of *M. barkeri* and that other *N*-methyl compounds are readily fermented to methane by mixed cultures.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

METHODS AND MATERIALS

Organisms. Cultures of *M. barkeri* strain MS (neotype strain) (DSM 800), Fusaro (DSM 804), 3 (DSM 805), and "Zhilina" (DSM 1232) were obtained from the German Collection of Microorganisms, Göttingen. Strain Kolksee was isolated from a methanogenic enrichment culture of Lake Kolksee, Germany, mud and calcium acetate. Strain G-1 was obtained from M. P. Bryant, Urbana, IL, and has been isolated from anaerobic sewage sludge from the sewage plant at Göttingen, Germany. Cultures of *Methanobacterium ruminantium* PS (DSM 861) and *Mb.* strain MOH(DSM 863) were received from K. Braun, Institute of Microbiology, Göttingen.

Growth Media. The Hungate technique (18, 19) for media preparation and cultivation, as modified by Bryant (20), was used. The media were prepared under an atmosphere of 80% nitrogen/20% carbon dioxide; traces of oxygen were removed by passing the gas mixture through a heated copper column.

The *Methanosarcina* strains were cultivated in a medium (pH 6.5–6.8) that contained the following (per liter): K_2HPO_4, 0.348 g; KH_2PO_4, 0.227 g; NH_4Cl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.25 g; NaCl, 2.25 g; $FeSO_4 \cdot 7H_2O$, 2 mg; resazurin, 1 mg; vitamin solution (21), 10 ml; trace elements solution (22) without Na_2 EDTA and $FeSO_4$, 3 ml; yeast extract (Difco), 2 g; casitone (Difco), 2 g; $NaHCO_3$, 0.85 g; methanol, 10 ml; cysteine hydrochloride, 0.3 g; and $Na_2S \cdot 9H_2O$, 0.3 g. Methanol (50% vol/vol) and the reducing agents were heat-sterilized separately under N_2 as concentrated aqueous solutions. Neutral stock solutions of amino compounds were filter-sterilized and freed from oxygen by flushing with 80% N_2/20% CO_2. The substrate solutions were injected into the autoclaved medium with a hypodermic syringe prior to the addition of the reducing agents. The final concentration of the amino compounds in the medium was 50 or 100 mM (see *Results*).

For enrichments, the same medium but without yeast extract, casitone, and cysteine was used. For some enrichments (see Table 1), the medium of Barker (23) was used, which contained per liter of tap water the following minerals: K_2HPO_4, 0.4 g (pH 6.8); NH_4Cl, 1.0 g; $MgCl_2$, 0.1 g; and $Na_2S \cdot 9H_2O$, 0.3 g.

Methanobacterium ruminantium and *Mb.* strain MOH were grown in the following medium (grams per liter): KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; NaCl, 0.4 g; NH_4Cl, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; $FeSO_4 \cdot 7H_2O$, 2 mg; trace elements solution (21), 2 ml; vitamin solution (21), 10 ml; resazurin, 1 mg; sodium acetate, 1 g; yeast extract (Difco), 2 g; cysteine hydrochloride, 0.5 g; $Na_2S \cdot 9H_2O$, 0.5 g; and $NaHCO_3$, 4.0 g (pH 7.0).

The gas atmosphere was 80% H_2/20% CO_2. For testing utilization of methylamines, the medium was supplemented by 25 mM amine, and a gas mixture of 80% N_2 and 20% CO_2 was used.

Cultivation and Enrichment Methods. Enrichments were made in tubes (Bellco 18 × 142 mm or anaerobic culture tubes, Hungate type) that contained 10 ml of medium. They were inoculated with 1 ml of anaerobic mud samples and incubated at 30°C. Pure cultures of *Methanosarcina* were grown in 5 ml of medium in similar tubes at 30°C; they were maintained by transfer of 5% inocula. Stock cultures were kept on methanol medium at room temperature in order to extend the interval between transfers. We frequently checked cultures for purity by inoculating CMC medium (24), medium for sulfate reducers (25), and AC medium (Difco) and by microscopic observation.

For growth studies on methylamines, 100 ml of medium was prepared in heavy-walled 1-liter bottles closed with black rubber stoppers, which were held in place by a wire closure during autoclaving and culturing.

Analytic Methods. Methylamines and methanol were determined by gas chromatography using a Perkin Elmer Model 900, equipped with a 6 m × 2 mm glass column, packed with Pennwalt 231 GC-Packing (Applied Science Laboratories, Inc., State College, PA), and with a flame ionization detector. The injector temperature was 225°C, the oven temperature was 55°C, and the detector temperature was 250°C. Nitrogen was used as carrier gas at a flow rate of 20 ml·min^{-1}. Before injection, 25 μl of 6 M KOH was added to 200 μl of cell-free culture medium in serum-stoppered 2-ml glass vials to release the free amines from their salts. After shaking vigorously for about 30 sec, 2 μl of the liquid sample was used for analysis.

Total gas production of cultures was followed by measuring the gas formed using hypodermic syringes as described by Chung (26). The methane content of the gas phase of the cultures was determined by gas chromatography with the instrument mentioned above. A glass column (2 m × 2 mm), packed with Porapak QS (Riedel de Haën, Selze, Germany) was used. The carrier gas was nitrogen at a flow rate of 23.5 ml·min^{-1}. All other conditions were as described above.

Protein was measured by a modification of the method of Bradford (27) as follows: 1 ml of cell suspension was anaerobically centrifuged, washed twice with 1 ml of 0.9% NaCl, and resuspended in 0.5 ml of 1 M NaOH. After 10 min at room temperature, the alkaline suspension was heated for 15 sec at 100°C and thereafter quickly cooled in an ice bath. The sample was centrifuged and 20–100 μl of the supernatant was used for protein determination. The aliquots were made up to 100 μl final volume with 1 M NaOH when necessary, and 5 ml of Coomassie brilliant blue (0.01%) was added. After 5 min at room temperature the absorption at 595 nm was measured, and the protein concentration was read from a calibration curve made from standard solutions of bovine serum albumin in 1 M NaOH. Linearity was found in the range from 5 to 45 μg of protein per 100-μl sample.

Determination of cell dry weight was performed by filtering 10- to 20-ml culture samples through preweighed membrane filters (SM 11303, 1.2-μm pore size, Sartorius, Göttingen, Germany). After they were washed with 0.9% NaCl, the filters were dried to constant weight at 95°C and reweighed against controls. Ammonia was determined enzymatically with glutamate dehydrogenase (28).

Enzymes and Chemicals. Glutamate dehydrogenase (EC 1.4.1.3), ADP, and NADPH were obtained from Boehringer Mannheim, Germany. 2-Keto-glutaric acid was from Schuchardt, München; Coomassie brilliant blue G-250 and bovine serum albumin were from Serva, Heidelberg, Germany. Methylamine hydrochloride, dimethylamine hydrochloride, trimethylamine hydrochloride, betaine, choline hydrochloride, creatine, sarcosine, *N,N*-dimethylformamide, and *N,N*-dimethylurea were purchased from Merck, Darmstadt, Germany. *N*-methylbutylamine and *N,N,N'*-trimethylethylenediamine were from Riedel de Haën, Selze, Germany. Diethylmethylamine, dimethylethylamine, *N,N*-dimethylethylenediamine, *N,N*-dimethylglycine, *N,N*-dimethyl-1,3-propanediamine, and tetramethylammonium chloride were obtained from Fluka, Buchs, Switzerland.

RESULTS

Methane Formation from *N*-Methyl Compounds by Enrichment Cultures. Media containing various *N*-methyl compounds at 50 mM were prepared, inoculated with mud samples, and incubated at 30°C under strictly anaerobic conditions. The production of gas was measured, and the methane content of the gas mixture was determined until there were only minimal changes in intervals of 5 days. Five different mud

Table 1. Production of gas and methane from *N*-methyl compounds by enrichment cultures

Substrate*	Enrichment I		Enrichment II		Methane,† mol	
	Gas (total), μmol	Methane, μmol	Gas (total), μmol	Methane, μmol	Per mol of substrate	Per mol of *N*-methyl group
Methanol	414	302	446	302	0.60	(0.60)
Methylamine	490	391	462	342	0.68	0.68
Dimethylamine	1000	785	981	771	1.54	0.77
Trimethylamine	1471	1158	1166	934	1.87	0.62
N-Ethyldimethylamine	731	583	675	540	1.08	0.54
Creatine	653	694	724	796	1.59	1.59
Sarcosine	910	713	872	702	1.40	1.40
N,N-Dimethylglycine	1386	1048	1363	1032	2.06	1.03
Choline	2074	1482	2018	1579	3.16	1.05
Betaine	1888	1351	1624	1287	2.57	0.86
N,N-Diethylmethylamine‡	0	0	0	0	—	—

Enrichment I was made with mud from the Leine river and enrichment II, with black mud from a pond (Burgteich). For enrichment I the medium of Barker (23) was used.

* The enrichment cultures contained 500 μmol of substrate per 10 ml.

† Values for methane were taken from enrichment I.

‡ The following compounds also showed no gas and methane production: *N*-methylbutylamine, *N,N*-dimethylethylenediamine, *N,N,N'*-trimethylethylenediamine, *N,N*-dimethyl-1,3-propanediamine, phenyltrimethylammonium chloride, *N,N*-dimethylformamide, and *N,N*-dimethylurea.

samples were used. The results of two series of experiments are given in Table 1; those obtained in the other three series were comparable. It can be seen that most of the substrates were highly methanogenic. Approximately 75% of the gas produced was methane. Depending on the substrate used, up to 3.16 mol of methane was formed per mol of substrate. Methanol, methylamine, dimethylamine, trimethylamine, and ethyldimethylamine yielded 0.54–0.77 mol of methane per mol of *N*-methyl group, indicating that the amount of methane formed was proportional to the methyl group content of these substrates. All other methanogenic substrates, notably creatine and sarcosine, produced more methane than could be expected from the number of methyl groups present. Apparently, the demethylated products were acted upon by the mixed population of the enrichment cultures and were also partially converted to methane. Some of the compounds tested were nonmethanogenic. Diethylmethylamine, for instance, was inert under the conditions used, whereas ethyldimethylamine was readily fermented. *N*-methylated urea was apparently not used by the organisms, and neither were a number of diamino compounds.

The time course of methane formation from some of the substrates of Table 1 is shown in Fig. 1. When substrates such as dimethylglycine and sarcosine were present in the cultures, methane production started only after a considerable lag period and continued up to 50 days. Trimethyl- and dimethylamine, on the other hand, were fermented readily.

Methane Formation from Methylamines by *M. barkeri*. Microscopic examination of the enrichments revealed the presence of *Methanosarcina* in practically all cultures. Therefore, growth of six strains of *M. barkeri* on the methanogenic substrates of Table 1 was tested. All six strains produced methane and grew on methylamine, dimethylamine, trimethylamine, and ethyldimethylamine, as well as on methanol. The other methanogenic substrates listed in Table 1 did not support growth of *M. barkeri*, indicating that additional organisms have to act upon them in order to make them utilizable to the methane bacteria. Accordingly, methane was not produced by *M. barkeri* from tetramethylammonium chloride.

Growth of *Methanobacterium ruminantium* and of *Mb.* strain MOH on methylamine, dimethylamine, and trimethylamine was also tested. In no case could growth or methane formation be observed.

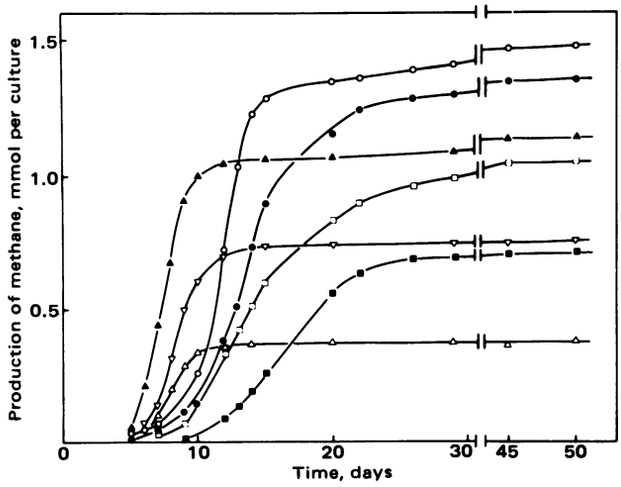

FIG. 1. Course of methane production from various *N*-methyl compounds by enrichment cultures. Each culture (10 ml) contained 500 μmol of substrate and was inoculated with 1 ml of mud from the Leine river. The production of gas and its methane content were determined. △, Methylamine; ■, sarcosine; ▽, dimethylamine; □, *N,N*-dimethylglycine; ▲, trimethylamine; ●, betaine; ○, choline.

Growth of *M. barkeri* on Methylamines. *M. barkeri* was grown with methanol, methylamine, dimethylamine, or trimethylamine, and the formation of methane and ammonia and the increase of the protein content of the cultures were determined. Furthermore, the consumption of the substrate during growth was measured. The results obtained with 100-ml cultures are summarized in Fig. 2. Growth on methylamine was somewhat slower than on methanol. Substrate consumption was connected with the formation of ammonia (8.6 mmol of ammonia per 9.6 mmol of methylamine); 6.2 mmol of methane was produced. Considering that some methylamine is used for biosynthetic purposes and that theoretically only 75% of the methyl groups can be converted to methane, the values were in the range expected. This was also observed for the fermentation of dimethylamine, which yielded 8.9 mmol of ammonia and 14.5 mmol of methane per 11 mmol of dimethylamine. The consumption of 10.7 mmol of trimethylamine by *M. barkeri* was connected with the formation of 19.6 mmol of methane and 8.8 mmol of ammonia. Cell protein increased parallel to methane formation up to 0.45 mg per ml and decreased after the exhaustion of trimethylamine.

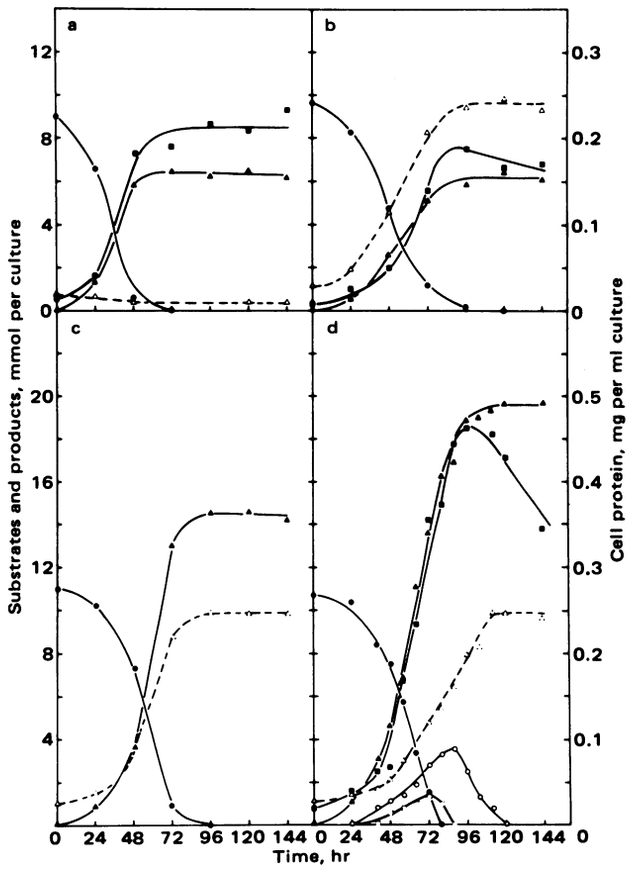

FIG. 2. Growth and methanogenesis by *M. barkeri* strain Fusaro with methanol (*a*), methylamine (*b*), dimethylamine (*c*), and trimethylamine (*d*). The experiments were carried out with 1000-ml bottles which contained 100 ml of medium. The inoculum (5.0 ml) was grown on the corresponding substrate. Samples (1.0 ml) were withdrawn and analyzed for their methanol, amine, ammonia, and protein contents. In addition, the production of gas and its methane content were determined. Values given are corrected for the decrease in culture volume caused by the withdrawal of samples and are expressed as amounts per 100-ml culture. The protein content of the dimethylamine culture was not determined. ●, Growth substrate; ○ and □, methyl- and dimethylamine as intermediary products in the fermentation of trimethylamine; ▲, methane; △, ammonia; ■, cell protein.

Table 2. Growth yields of *M. barkeri* with methanol and with methyl-, dimethyl-, and trimethylamine

Substrate	Methane produced, mmol/culture	Increase in dry weight, mg/culture	Yield*
Methanol	6.32	35.00	5.5
Methanol†	14.04	86.00	6.1
Methylamine	6.35	37.00	5.8
Dimethylamine	13.28	82.00	6.2
Dimethylamine	14.75	76.00	5.2
Trimethylamine	21.59	130.00	6.0
Trimethylamine	22.71	130.00	5.7

The experiments were carried out in 1000-ml bottles containing 100 ml of medium. The substrate concentration was 100 mM except when indicated.
* Milligrams of dry weight per millimole of methane.
† 200 mM.

Samples withdrawn from the cultures growing on methyl-, dimethyl-, or trimethylamine were also assayed for their methanol content, which in all cases was below the limits of detection by gas chromatography (below 10 μmol per 100-ml culture). However, the fermentation of trimethylamine was connected with the intermediary appearence of methyl- and dimethylamine in the culture medium (Fig. 2*d*). An analogous excretion of methylamine during the fermentation of dimethylamine was not observed.

The growth yields of *M. barkeri* with methanol and the methylamines were determined. The data that are summarized in Table 2 indicate that the methyl groups of the four substrates were equally effective in furnishing the organisms with metabolic energy for growth. The variations observed might partly result from differences in the amounts of lysed cells present in the cell aggregates of *M. barkeri*.

DISCUSSION

Methanol is fermented by *M. barkeri* according to the following equation (6):

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

On the basis of the results obtained in this study, it can be concluded that methyl-, dimethyl-, and trimethylamine are fermented according to analogous equations.

$$4\ CH_3NH_3Cl + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_4Cl$$
$$2\ (CH_3)_2NH_2Cl + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ NH_4Cl$$
$$4\ (CH_3)_3NHCl + 6\ H_2O \rightarrow 9\ CH_4 + 3\ CO_2 + 4\ NH_4Cl$$

Methanogenesis of these amines is carried out by pure cultures of *M. barkeri* so that the short list of substrates for methanogenic bacteria can be extended with these compounds. In addition, ethyldimethylamine has been shown to function as a substrate for *M. barkeri*. This, however, is probably not of any biological significance.

It is apparent from the course of growth and of methane production that the methylamines are utilized by *M. barkeri* strain Fusaro at rates comparable to the rate of methanol utilization. Because these amines are produced in the anaerobic decomposition of various *N*-methyl compounds, it is conceivable that they serve as important natural substrates for *Methanosarcina* and, therefore, may also be used for enrichment and isolation of these organisms.

Methanobacterium ruminantium and *Mb.* strain MOH did not show any growth or methane formation with methyl-, dimethyl-, or trimethylamine. These species and all others hitherto known are also unable to use methanol, and it seems that *Methanosarcina* has specialized in the methanogenesis of compounds containing methyl groups. In addition to methanol and the methylamines, acetate is an important substrate for *Methanosarcina* (4, 5, 23), its methyl group serving as a direct precursor of methane (29, 30). Other possible substrates for *Methanosarcina* are *S*-methyl compounds. Recently, Zinder and Brock (31, 32) have reported methane production from methionine, methane thiol, and dimethylsulfide by anaerobic lake sediments. The organisms involved have not been characterized, but it would not be surprising if they were identified as *Methanosarcina* strains.

The conversion of the methylamines to methane, carbon dioxide, and ammonia was not connected with an intermediary excretion of methanol. In no case did methanol appear in the culture medium during amine degradation. The fermentation of trimethylamine, however, resulted in the intermediary excretion of some methyl- and dimethylamine. Detectable amounts of methylamine could not be encountered when dimethylamine was used as substrate. Similar observations have been made by Meiberg and Harder (33) when they studied the anaerobic growth of *Hyphomicrobium* strain X on dimethyl- or trimethylamine and nitrate. These authors provided evidence that trimethylamine functions as an inhibitor for the further degradation of dimethyl- and methylamine. Such an effect of trimethylamine might also account for the results obtained with *M. barkeri*.

The growth yields for methanol and the three amines did not show significant differences, indicating that the methyl groups of these substrates were energetically similar. The yields were slightly higher than those estimated by Stadtman (34) for growth of *M. barkeri* on methanol (4.4 g dry weight of cells per mol of methane produced). The corresponding values for growth of methanogenic bacteria on molecular hydrogen and carbon dioxide are lower (approximately 2.5 g dry weight of cells per mol of methane produced; refs. 35 and 36). This difference is probably due to the formation of carbon dioxide in the course of the fermentation of methanol and of the amines, which may allow ATP synthesis by substrate-level phosphorylation in the N^{10}-formyl tetrahydrofolate synthetase reaction.

The ratio, mol of methane produced per mol of *N*-methyl compound consumed, has been found very useful in the interpretion of the results obtained in studies on the decomposition of methanogenic amines in enrichment cultures. Values above 0.75 indicate that not only the *N*-methyl groups but also other groups of a particular substrate molecule contribute to methane production. Substrates that gave values above 0.75 (e.g., choline, betaine, creatine, and sarcosine) were not attacked by pure cultures of *M. barkeri*.

We greatly appreciate the technical assistance of Ingrid Tilly. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

1. Kluyver, A. J. & Schnellen, C. G. T. P. (1947) *Arch. Biochem.* **14**, 57–70.
2. Barker, H. A. (1956) *Bacterial Fermentations* (Wiley, New York), pp. 1–27.
3. Wolfe, R. S. (1971) in *Advances in Microbial Physiology*, eds. Rose, A. H. & Wilkinson, J. F. (Academic, New York), Vol 6, pp. 107–146.
4. Zeikus, J. G. (1977) *Bacteriol. Rev.* **41**, 514–541.
5. Mah, R. A., Ward, D. M., Baresi, L. & Glass, T. L. (1977) *Annu. Rev. Microbiol.* **31**, 309–341.
6. Schnellen, C. G. T. P. (1947) Dissertation (Technical University of Delft, De Maasstad, Rotterdam).
7. Stadtman, T. C. & Barker, H. A. (1951) *J. Bacteriol.* **61**, 81–86.

8. Zhilina, T. N. & Zavarzin, G. A. (1973) *Microbiologija* **42,** 266–273.
9. Neill, A. R., Grime, D. W. & Dawson, R. M. C. (1978) *Biochem. J.* **170,** 529–535.
10. Miller, M. W. (1961) *The Pfizer Handbook of Microbial Metabolites* (McGraw-Hill, New York).
11. Asatoor, A. M. & Simenhoff, M. L. (1965) *Biochim. Biophys. Acta* **111,** 384–392.
12. Hayward, H. R. & Stadtman, T. C. (1959) *J. Bacteriol.* **78,** 557–561.
13. Baker, F. D., Papiska, H. R. & Campbell, L. L. (1962) *J. Bacteriol.* **84,** 973–978.
14. Dyer, W. J. (1959) *J. Assoc. Off. Anal. Chem.* **42,** 292–294.
15. Kim, K. E. & Chang, G. W. (1974) *Can. J. Microbiol.* **20,** 1745–1748.
16. Yamamoto, J. & Ishimoto, M. (1977) *Z. Allg. Mikrobiol.* **17,** 235–242.
17. Madigan, M. T. & Gest, H. (1978) *Bacteriol. Proc.* 133 (abstr.).
18. Hungate, R. E. (1950) *Bacteriol. Rev.* **14,** 1–49.
19. Hungate, R. E. (1969) in *Methods in Microbiology,* eds. Norris, J. R. & Ribbons, D. W. (Academic, New York), Vol. 3B, pp. 117–132.
20. Bryant, M. P. (1972) *Am. J. Clin. Nutr.* **25,** 1324–1328.
21. Wolin, E. A., Wolin, M. J. & Wolfe, R. S. (1963) *J. Biol. Chem.* **238,** 2882–2886.
22. Pfennig, N. & Lippert, K. D. (1966) *Arch. Mikrobiol.* **55,** 245–256.
23. Barker, H. A. (1936) *Arch. Mikrobiol.* **11,** 265–267.
24. Holdemann, L. V. & Moore, W. F. C. (1972) *Anaerobe Laboratory Manual* (Virginia Polytechnic Institute and State University, Blacksburg, VA).
25. Postgate, J. R. (1963) *Appl. Microbiol.* **11,** 265–267.
26. Chung, K. T. (1976) *Appl. Environ. Microbiol.* **31,** 342–348.
27. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
28. Da Fonseca-Wollheim, F., Bergmeyer, H. U. & Gutman, J. (1974) in *Methoden der Enzymattschen Analyse,* ed. Bergmeyer, H. U. (Verlag Chemie, Weinheim, Germany), pp. 1850–1853.
29. Stadtman, T. C. & Barker, H. A. (1949) *Arch. Biochem.* **21,** 256–264.
30. Pine, M. J. & Barker, H. A. (1956) *J. Bacteriol.* **95,** 1943–1951.
31. Zinder, S. H. & Brock, T. D. (1978) *Appl. Environ. Microbiol.* **35,** 344–352.
32. Zinder, S. H. & Brock, T. D. (1978) *Nature (London)* **273,** 226–228.
33. Meiberg, J. B. M. & Harder, W. (1978) *J. Gen. Microbiol.* **106,** 265–276.
34. Stadtman, T. C. (1967) *Annu. Rev. Microbiol.* **21,** 121–142.
35. Roberton, A. M. & Wolfe, R. S. (1970) *J. Bacteriol.* **102,** 43–51.
36. Zehnder, A. J. B. & Wuhrmann, K. (1977) *Arch. Microbiol.* **111,** 199–205.