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Hydrogen and carbon isotope fractionation during experimental production of bacterial methane

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Abstract—This paper presents C and H isotope compositions of compounds involved in methane production by pure cultures of *Methanobacterium formicicum*. The C isotope compositions of the methane produced and of the residual CO_2 are compared to data observed in natural conditions in marine sediments. This comparison leads to further evidence that CO_2 reduction is an important mechanism for microbial generation of methane in deep marine sediments. The H isotope compositions show involvment of the water hydrogen into methane as well as hydrogen exchange between water and molecular hydrogen in the course of CO_2 reduction. A mechanism is proposed as a possible explanation for the data obtained involving conjugated reactions of CO_2-reduction and enzymatic reduction of water.

Key words: ^{13}C, deuterium, methane, *Methanobacterium formicicum*

INTRODUCTION

During the last decade, an increasing number of studies have been carried out on methane formation, migration and occurrences. A better understanding and characterization of the mechanisms leading to bacterial or thermogenic methane production has indeed proved to be of great interest in hydrocarbon exploration (Fuex, 1977).

Rosenfeld and Silverman (1959) first showed that an important carbon isotope fractionation occurs when methane is produced by microbial processes from methanol. Carbon isotope composition of methane has since been widely used in field studies to distinguish between thermal and biogenic gas, the latter being more enriched in ^{12}C (Colombo *et al.*, 1964; Galimov, 1973; Claypool *et al.*, 1983). In addition, experiments with pure bacteria cultures were performed to further investigate isotope fractionations through methane production by different methanogen species growing at different temperatures and with different substrates (Games and Hayes, 1976; Fuchs *et al.*, 1979).

A combination of C and H isotope composition analyses was later used to investigate mechanisms after microbial oxidation of methane was shown to enrich the residual methane in ^{13}C with carbon isotope composition approaching that of thermogenic gas (Coleman *et al.*, 1981; Barker and Fritz, 1981).

Works on hydrogen isotope composition of methane, in addition to thermal vs microbial gas distinction, dealt with different pathways for biogenic gas production namely CO_2 reduction and acetate fermentation (Schoell, 1980, 1983; Woltemate *et al.*, 1984; Whiticar *et al.*, 1986). Different pathways for methane production involve different sources for methane hydrogen which may lead to characteristic correlations between the hydrogen isotope composition of methane and that of the formation water.

There is, however, no data in the literature on the isotope fractionation between molecular hydrogen, which is believed to act as an electron donor during microbiological reduction of CO_2 (Abram and Nedwell, 1978; Bryant, 1979) and methane. This lack of data may be explained by the very low concentration of H_2 present in the natural methanogenesis areas (Mah *et al.*, 1977).

We present in this paper hydrogen and carbon isotopic data of products and reactants as they change during methane production under controlled laboratory conditions by *Methanobacterium formicicum*, a mesophilic autotroph methanogen that utilizes H_2/CO_2 in strict anaeroby as an energy and carbon source for growth. The experiment described was designed to identify the hydrogen source during methane generation. For this purpose, water of a different isotope composition was used in parallel experiments.

EXPERIMENTAL

Culture of methanogens

Pure cultures of *Methanobacterium formicicum* was supplied by Professor R. S. Wolfe (Department of Microbiology, Urbana, University of Illinois). The

cultures were performed in the Biotechnologie-Environnement Laboratory of Institut Français du Pétrole.

Basal medium that was used for growth and for maintenance of stock cultures of *M. formicicum* was identical to medium 1 described by Balch *et al.* (1979). Media preparation was performed according to the specifications of Balch and Wolfe (1976). Anaerobic medium was prepared by boiling the complete medium lacking cysteine and sulfide under a stream of N_2:CO_2 (80:20) gas. Cysteine was then added, the flask was stoppered, and the medium was dispensed in an anaerobic hood, 5 ml/tube (Bellco Glass, Inc., Vineland, N.S.). Before autoclaving, the gas phase in each tube was exchanged for the substrate, hydrogen–carbon dioxide, by means of gasing manifold (Balch and Wolfe, 1976). The volume of the gas phase was 23 cm^3. Just before inoculation, 0.1 ml of sterile 2.5% $Na_2S \cdot 9H_2O$ was added to each tube of the medium. Cultures were grown for 3 days at 34°C without shaking under a pressurized atmosphere (3 atm) containing H_2 and CO_2 in proportion 80:20. Methane production was routinely used for the measurement of growth.

Five sets of cultures were prepared according to this procedure, inoculated with *M. formicicum* and supplied with aliquots of the same initial H_2/CO_2 gas mixture. The culture medium of each set was prepared with water of different isotopic composition by mixing in different amounts of D_2O. Sets A, B, C and D are composed of 4 cultured tubes each and set E of 2 cultured tubes.

In addition, five references were prepared consisting of a H_2/CO_2 gas mixture and an aliquot of water media.

Isotopic analyses

Initial CO_2, H_2 and water used in the experiments were analyzed for their deuterium and carbon-13 contents. In the culture tubes, isotope analyses were performed on residual CO_2 and H_2, the water medium, the methane as well as the cell material.

These analyses were carried out on a 602D siamese VG Micromass spectrometer. The isotopic composition is reported in the usual $\delta^{13}C$ and δD notation in per mil relative to PDB and SMOW standards respectively.

Gas phase compounds ($CO_2/H_2/CH_4$) separation and methane oxidation: we have developed a technique for quantitative separation and isotopic analysis of $CH_4/H_2/CO_2$ gas mixtures, using gas chromatography separation principles. The procedure is as follows: the gas mixture contained in the cultured tubes is admitted into a vacuum line through a microsyringe. It is allowed to pass first through a trap cooled down to dry-ice temperature (where water vapor is retained) and then through a glass-coil tubing at liquid nitrogen temperature where the total amount of CO_2 and part of the CH_4 is trapped. A glass tube filled with silica gel at liquid nitrogen temperature (previously degassed at 250°C) acts like a pump on the CH_4/H_2 mixture. After 20 min, separation is completed between CO_2 (ready for mass spectrometer analysis) and $CH_4 + H_2$. Partial release of H_2 is achieved by bringing the silica gel tube to freezing pentane temperature (≈ -120°C). An aliquot of the H_2 released is used for isotope analysis, the remaining H_2 being pumped away. Methane is then released by bringing silica gel to room temperature, and converted to CO_2 and H_2O by passing over copper oxide at 800°C. The evolved CO_2 is used for $\delta^{13}C$ analysis of methane and the water is reduced to H_2 as is described below.

Hydrogen isotope fractionation during separation of H_2 by the technique used was found to be negligible for our purposes. A detailed report on isotopic and quantitative data for H_2 separation following this procedure is given in Balabane and Letolle (1986).

Overall reproducibility for values obtained with this procedure is $\pm 1‰ \delta^{13}C$ (CO_2 and CH_4) and $\pm 4‰ \delta D$ (H_2 and CH_4).

Once the gas phase is removed, the cells are harvested by centrifugation and rinsed twice. The water is reduced on hot uranium (Bigeleisen *et al.*, 1952). Water is introduced as a vapor into the reduction system, which prevents most dissolved unknown organic species from interfering with the isotopic composition of water. The hydrogen evolved is recovered through the intermediate formation of uranium hydride (Friedman and Hardcastle, 1970) and analyzed for relative D content. The cell material is dried (48 hr pumping at 50°C through liquid nitrogen trap) before combustion in an O_2 atmosphere to CO_2 and H_2O. Water is then reduced to H_2. Standard deviation is $\pm 1‰ \delta D$ (water), $\pm 2‰ \delta D$ and $\pm 0.1 \delta^{13}C$ (cell material).

RESULTS AND DICUSSION

The isotopic compositions of products and reactants, as well as the amount of methane produced are listed in Table 1.

Carbon isotopes

The C isotope composition of initial CO_2 as measured in the reference tubes is $-30‰$. Residual gas analyzed after reaction in the cultured tubes is enriched in ^{13}C ($\delta^{13}C = -5$ to $+9‰$). Progressive enrichment in ^{13}C of the residual CO_2 reservoir is due to preferential uptake of ^{12}C by the bacteria in the course of cell material building and CH_4 production ($\delta^{13}C$ cells $= -33$ to $-35‰$; $\delta^{13}C$ $CH_4 = -42$ to $-51‰$).

Isotope fractionation cannot be accurately calculated because of lack of data on the relative carbon distribution between CO_2, CH_4 and cell material.

Figure 1 shows the difference in C isotope composition between CH_4 and CO_2 (ΔCH_4–$CO_2 \approx 48‰$).

Table 1. Isotopic data for methane production by *M. formicicum*

Set		δ^2H‰ (SMOW)				δ^{13}C‰ (PDB)			CH_4 yield (mmol)
		H_2O	H_2	Cells[3]	CH_4	CO_2	Cells[3]	CH_4	
A	Ref.	−43	−647						
	Cult.	−44	−753		−441				
		−42	−748		−452		(1)		
		−42	−748	(2)	−451				
		−40	−746		−456				
B	Ref.	−9	−653			−28.4			
	Cult.	−16			−388	−4.2		−47.9	0.05
		−21			−392	+0.2		−48.2	0.08
		−18	−747	−115	−386	−4.4	−35.5	−50.6	0.05
		−23	−738		−396	+5.8		−44.5	0.09
C	Ref.	+55	−655			−30.9			
	Cult.	+46			−375	+2.5		−45.3	0.13
		+39	−728		−385			−44.8	0.20
		+39		−92	−377	+8.9	−33.2	−43.4	0.20
		+46	−725			+0.3		−45.0	0.32
D	Ref.	+132	−654			−30.6			
	Cult.	+121	−712		−325	−2.1		−49.7	0.05
		+117	−708		−331			−42.7	0.27
		+111	−699	−73	−318	+0.3	−34.4	−45.5	0.13
		+110	−711		−323			−43.7	0.32
E	Ref.	+208	−656			−31.1			
	Cult.	+189	−694	(2)	−276	+0.4	(2)	−47.6	0.22
		+187	−698		−279	+3.6		−45.8	0.10

Ref. = reference tubes containing initial $CO_2 + H_2$ gas mixture and water medium. Cult. = cultured tubes containing residual $CO_2 + H_2$, water medium, cell material and methane. (1) In set A, the amount of CO_2 supplied was too small which led to total consumption of CO_2 and a small yield of cell material. (2) Insufficient amount of cell material for isotopic analysis. (3) Isotopic composition is performed on the cell material harvested in the four culture tubes and mixed together as to obtain required organic matter amounts for combustion.

Isotopic compositions are plotted vs the methane yield which corresponds to a certain degree to the advancement of the process.

Our experimental data are compared with data obtained in natural conditions by Galimov and Kvenvolden (1983) for coexisting CH_4 and CO_2 in the deep-sea sediments. $\delta^{13}CO_2$ changed from ≈ -24‰ in subsurface sediments ($T = 2$–$4°C$) *to* ≈ -4‰ at depths of 350–400 m ($T = 15$–$19°C$). Changes in C isotope composition of coexisting methane are from ≈ -90‰ to ≈ -66‰.

ΔCH_4–CO_2 observed at the depth 350–400 m and shown on Fig. 1 is larger than that observed in the experiment. This may be due, however, to higher temperature (34°C) maintained during the experiment. With the appropriate corrections (see footnote

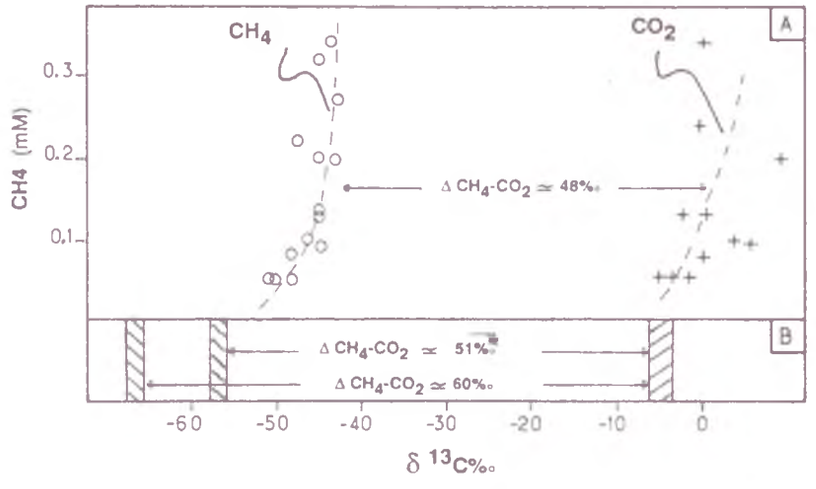

Fig. 1. Comparison of difference in C isotope composition between CH_4 and CO_2 (ΔCH_4–CO_2) derived from our measured data (A) and ΔCH_4–CO_2 measured by Galimov and Kvenvolden (1983) in sediments of the Blake Outer Ridge, DSDP Leg 76, at a depth of 350–400 m (B: ▨ $\delta^{13}C_{CO_2}$; ▧ $\delta^{13}C_{CH_4}$). *A correction of about 9‰ is applied to account for the difference between the temperature at the 350–400 m depth in field conditions ($\simeq 17°C$) and the temperature of the experiment ($\simeq 34°C$). This correction is derived from the ΔCH_4–CO_2 obtained by Galimov and Kvenvolden in subsurface ($T \simeq 2°C$; ΔCH_4–$CO_2 \simeq 69$‰) and at 350–400 m ($T \simeq 17°C$; ΔCH_4–$CO_2 \simeq 60$‰).

in Fig. 1), one can conclude that isotope fractionation during experimental microbial reduction of CO_2 corresponds fairly well to the values recorded in natural condition. This is further evidence that CO_2 reduction is an important mechanism of microbial generation of methane in deep marine sediments.

Hydrogen isotopes

The initial isotope compositions of water and molecular hydrogen are those measured in the reference tubes, i.e. before inoculation with *M. formicicum*. The results in Table 1 show relative deuterium concentration of the water provided for each set of experiments (from -43 to $+208$ δD‰) and the isotope composition of the molecular hydrogen which remains unchanged (≈ -653 δD‰) from one set to another. It is important to note that, in the reference tubes, the isotope composition of H_2 is not affected by the different deuterium concentrations of the water which means that no contamination happens in the course of the experimental procedure described above for compounds separation, or isotope exchange occurs between both compounds upon preparation of the culture medium before inoculation.

In the cultured tubes, the isotopic composition of water show slightly lower δD-values in comparison with water in the reference tubes. This is due to the addition, in the course of inoculation, of about 0.3 ml of water with $\delta D = -45$‰ (tap water) to the initial water medium (of about 5 ml).

δD of the methane is independent of the amount of methane produced contrary to what is observed for carbon-13 methane composition (Fig. 1). A parallel observation was made by Fuchs *et al.* (1979) who found $\delta^{13}C$ of *M. thermoautotrophicum* cell organic material as well as that of the methane produced to be dependent on the gassing rate of CO_2/H_2 supply whereas δD of the cells was independent of the gassing rate.

In Fig. 2, the isotopic composition of methane, molecular hydrogen and cell organic material is plotted relative to that of the water medium for the corresponding set of experiments. All of the components exhibit a dependence on the isotopic composition of the water. The systems H_2O—CH_4, H_2O—H_2 and H_2—CH_4 are not in isotopic equilibrium. This is inferred from comparison between fractionation factors calculated from our measured values and those calculated by Bottinga (1969) for systems in equilibrium. Different isotopic composition of methane in the different sets of the experiment indicates involvement of water hydrogen into methane formation as already settled by many authors (Nakai *et al.*, 1974; Schoell, 1980; Daniels *et al.*, 1980). The new finding that evolves from our experiments is that the isotopic composition of water affects that of the molecular hydrogen. This means that there is, in the course of methanogenesis process, an isotope exchange between water and molecular hydrogen, i.e. microbiological consumption of molecular hydrogen is not an irreversible process as is believed.

To account for the observed interdependence of hydrogen isotope composition of water, molecular hydrogen and methane, a mechanism may be proposed where CO_2-reduction would include some kind of enzymatic reduction of water with protons being transferred into the methane formation chain. A conjugated pair of the reactions may be proposed to explain the mechanism, for example:

$$R—CHO + H_2O \rightarrow H_2 + R—COOH$$

$$\tfrac{1}{4}CO_2 + H_2 \rightarrow \tfrac{1}{2}H_2O + \tfrac{1}{4}CH_4$$

One explanation for the observed dependence of δD_{H_2} on δD_{water} would be mixing of intracellular H_2 of the biochemical reactions with outside H_2-reservoir.

CONCLUSION

Production of methane via the CO_2-reduction

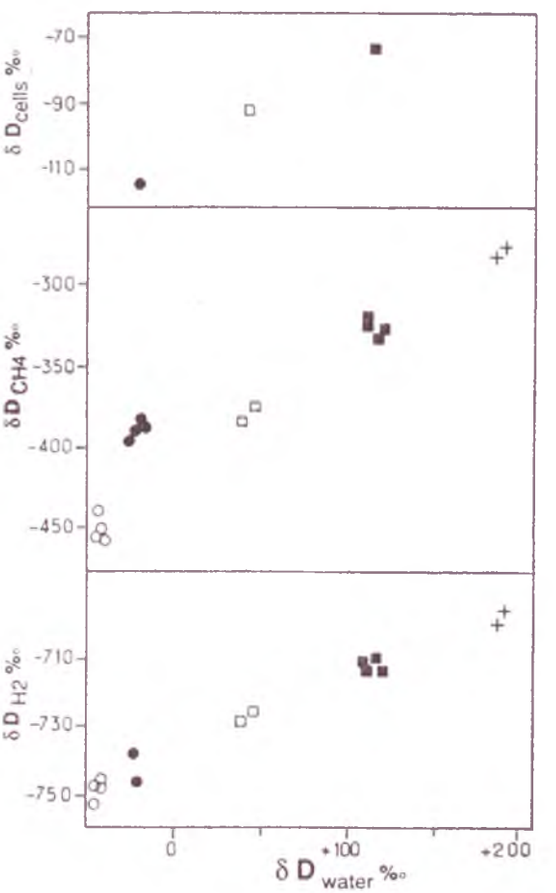

Fig. 2. Variations in the deuterium relative content of residual H_2, methane and cell material as related to the isotopic composition of the water medium in sets A, B, C, D and E (○, ●, □, ■, +).

pathway was performed by pure culture of *Methanobacterium formicicum*. The C and H isotope composition of compounds was measured before reaction (initial substrate) and after reaction (residual substrates and methane produced). Components were separated by a new method described allowing a satisfactory precision for isotopic composition analyses.

The hydrogen isotope composition of all the hydrogenated compounds of the medium (H_2, cell organic material and CH_4) shows dependence on the δD of water. This agrees with previous works for δD_{CH_4}–δD_{water} dependence describing involvement of water hydrogen into methane formation. Our results show that the residual H_2 reservoir isotope composition is affected by δD of the water medium. Hydrogen exchange between water and H_2 in the course of methane production may be inferred directly from the data obtained. Conjugated reactions of CO_2-reduction and enzymatic reduction of water with proton being transferred into the methane formation chain are proposed as a possible explanation for the experimentally observed data.

The C isotope composition of residual CO_2 and coexisting CH_4 shows a fairly good correspondence with field data for deep marine sediments.

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