Origin of Hydrogen in Methane Produced by
Methanobacterium thermoautotrophicum

LACY DANIELS,† GAIL FULTON, ROBIN W. SPENCER,+ AND WILLIAM H. ORME-JOHNSON† *

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

The production of deuterated methane by Methanobacterium thermoautotrophicum in H$_2$O-D$_2$O mixtures was examined by high-resolution mass spectrometry. The hydrogen in the methane arose solely from water and not from hydrogen gas. Hydrogen gas served only as an electron source in methanogenesis. A whole-cell product isotope discrimination of 1.5 favoring hydrogen over deuterium was observed in methane production in 81 atom% deuterated water. The distribution of deuterated methane species is described by a simple model of the overall reaction.

The bacterium Methanobacterium thermoautotrophicum uses carbon dioxide as both sole carbon source and catabolic electron acceptor. Some 95% of its total carbon flux is catabolic in the formal reaction of equation 1, the net reduction of carbon dioxide to methane by four equivalents of hydrogen gas (23, 25):

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$$

(1)

Although this carbon must pass through the oxidation states equivalent to formate, formaldehyde, and methanol, only the methanol level intermediate has been identified, as methyl coenzyme M, by Wolfe and colleagues (14, 22, 25). To begin our studies of the sequence of enzymatic reactions that must comprise equation 1, we wish to establish the origin of the hydrogen in the product methane as either water or hydrogen gas, or both. ("Protium" and "deuterium" and H and D are used to refer to specific isotopes of hydrogen; "hydrogen" refers to H or D regardless of molecular attachment, "hydrogen gas" and "water" refer to H$_2$, HD, or D$_2$, and H$_2$O or D$_2$O and any mixtures thereof.) Both origins have been proposed. Pine and Barker (16) reported (no data shown) that a mixed culture grown on ethanol in D$_2$O produced CH$_4$. Pine and Vishniac (17) showed that enrichment cultures from San Francisco Bay mud produced CHD$_3$ from CD$_3$COOH, suggesting that one hydrogen on acetate-derived methane comes from water. Neither of the above experiments excludes direct hydrogen incorporation from hydrogen gas. Penley and Wood (15) have shown that in methane production from methylcobalamin by extracts of M. bryantii (4), the hydrogens on the methyl ligand of methylcobalamin are retained and the fourth hydrogen comes from water. More recently, Sauer et al. (19) have suggested that hydrogen gas rather than water is the source of hydrogen in methane produced by M. ruminantium based on experiments in which H$_2$ and H$_2$O were employed. Fuchs et al. (10) have measured the deuterium enrichment of Methanobacterium thermoautotrophicum total cell material compared with natural abundance in water and hydrogen gas and concluded that most cellular hydrogen is derived from water; deuterium enrichment in methane was not measured.

The reactions comprising equation 1 are likely to begin with one or more hydrogenases which, by analogy to the hydrogenases in other genera, (18) are likely to catalyze the exchange to H$_2$ and D$_2$O to produce HD and D$_2$ that we report here. If a hydrogenase catalyzed the heterolytic scission of hydrogen gas to a proton and enzyme-bound hydride (11, 13, 18) and then transferred the hydride directly to a nonexchangeable redox coenzyme such as the $\delta$-hydroxy-5-deazaflavin factor 420 (F$_{420}$) (2, 9, 26) or nicotinamides, it is reasonable that that hydrogen might be again transferred directly in one or more of the reductive steps of methanogenesis.

We have addressed these questions with high-resolution mass spectroscopy of the methane and hydrogen gas species formed during methane production by Methanobacterium thermoautotrophicum from H$_2$ and CO$_2$ in D$_2$O. We found that the hydrogens in methane clearly arise ultimately from water and not hydrogen gas. A model predicting the distribution of deuterated methane species as a function of the isotope discrimination and deuterium enrichment in water is presented.

**MATERIALS AND METHODS**

**Chemicals and gases.** All chemicals used were of reagent grade. Matheson Scientific, Inc. supplied H$_2$
CO₂ (80:20, vol/vol, premixed) and prepurified N₂. Deuterium oxide (99.7 atom% D) was purchased from Achira.

**Growth of cells.** *M. thermoautotrophicum* strain ΔH (<27), kindly provided by J. G. Zeikus, was cultivated at 60°C as described previously (6) by the technique of Balch and Wolfe (5) with pressurized 60-ml Wheaton serum vials.

**Determination of methanogenesis.** Total methane in cultures and experiments was determined with a model 8600 gas chromatograph (Carle Instruments) with a 1-m Porapak R aluminum column at 88°C, nitrogen as the carrier gas, and a flame ionization detector. Gas samples of 0.3 ml were removed from vials and injected with a 1-ml Glash Pac syringe with Teflon pressure lock device as described previously (6).

**Isotopic analysis.** Deuterium enrichment in methane and hydrogen gas was determined with an AEI MS-9 high-resolution mass spectrometer. Each sample (1 to 6 ml) was introduced through a septum to the 2-liter reservoir; the ionization potential was set at 70 eV. Resolution was greater than 4,000, which was sufficient to resolve peaks at nominal m/e 20 differing in 0.005 m/e. This resolution was necessary to separate the methane species and their fragments from H₂O⁺, D₂O⁺, and other background ions when small samples were introduced. The distribution of methane species was calculated by sequentially subtracting from the high-mass end the parent ion and expected fragments from the total observed mass spectrum. (A detailed algorithm including the data from references [1] and [5] is available from the authors on request.) The fragmentation patterns of Diebler and Mohler (8) were used; other reported values (1) indicate an inherent variability in the fragmentation patterns even at the same ionization potential. This variability is the main factor limiting our accuracy in determining the distribution of isotopic species of methane, the accuracy decreasing for less deuterated methane as errors accumulate upon repeated subtraction of fragment intensities proportional to parent ion intensities.

The deuterium enrichment of water samples was determined by proton nuclear magnetic resonance: the concentration of H⁺ in the samples (at approximately 4.5 ppm from tetramethylsilane) was measured by comparison to known concentrations of added dioxane (at 3.55 ppm) in a Varian T-60 spectrometer.

**Experiments.** For the experiment of Fig. 1 and 2, 12 ml of a culture at mid-log phase (0.5 to 1 g [wet weight] of cells per liter) was centrifuged anaerobically in a 20-ml tube at 5,000 × g for 10 min, washed once with 5 ml of anaerobic D₂O containing 1 mM Na₂S, and resuspended in 5 ml of the same. The suspension was transferred to a 60-ml serum-stoppered vial, flushed, and then pressurized to 20 lb/in² with H₂-CO₂ and incubated with vigorous shaking at 60°C. Gas samples for gas chromatography and mass spectral analysis were removed at intervals with syringes equipped with pressure lock devices.

For the experiment of Table 1, seven vials of 20 ml of culture each at mid-log phase were centrifuged at 5,000 × g for 15 min under N₂ and resuspended in a total volume of 100 ml of buffer. This consisted of 98 ml of D₂O (99.7 atom% D) and 2 ml of a concentrated solution in water (containing per 100 ml of water: KH₂PO₄, 1.5 g; NH₄Cl, 6.0 g; Na₂CO₃, 0.84 g; pH 6.8), and was washed twice with buffer. The suspension was evacuated and flushed with N₂, and 30 ml was added to a 60-ml vial under N₂. The deuterium enrichment was decreased by the addition of 3 ml of H₂O. This vial was flushed, pressurized, and incubated as before; after 4 h at 60°C, samples were transferred to 12-ml evacuated vials for later analysis.

**Calculations and mathematical modeling.** The symbols used in this discussion are defined as follows: n, atom fraction D in water; p, atom fraction D in total methane; q, atom fraction D in total methane; a, product isotope discrimination; XCH₄, XCHD, XCD₃, and XCD₄, mole fraction of the subscript methane species.

The distribution of product species in a reaction that involves the acquisition of a proton or deuteron from water is a function of both the inherent preference of that reaction for H or D, called α, and the a priori probability of acquiring H or D, which is simply measured by the enrichment of the H₂O-D₂O mixture, n. For a simple reaction, α is the ratio of reaction rates in 100% H₂O and 100% D₂O. In H₂O-D₂O mixtures the product distribution is given by the following: p = mole fraction proto product = a/[a + (n/1 − n)]; q = mole fraction deutero product = 1 − p = 1/[a/(1/n − 1) + 1]. In the production of methane from CO₂, which involves four formal hydrogen transfers, if all four transfers have the same α the final distribution of methane species is given by (p + q), or CH₃ = p; XCH₃,D = 4p²q; XCH₂D = 6p²q²; XCHD = 4pq³; XCD = q. The atom fraction H in total methane is given by 1/4(4XCH₃ + 3XCH₂D + 2XCHD + XCHD₃), and the atom fraction D in total methane is given by 1/4(4XCH₃ + 3XCH₂D + 2XCHD + XCHD₃). It is readily shown that these atom fractions in total methane are identically equal to p and q as defined above, respectively.

The same distribution of methane species, (p + q), is given by a scheme in which there is no discrimination between H and D, that is α = 1, in any of the reactions that place hydrogen on the methane carbon, but there is a discrimination of α > 1 in the formation of nonexchangeable hydrogen donors, e.g., dihydro-F₄M, or NADPH, that are subsequently involved in direct hydrogen transfer to the methane carbon.

**RESULTS**

**Origin of hydrogen in methane.** Cells washed and resuspended in water (98.7 final atom% D) produced methane after a lag of approximately 1 h, and methane production ceased after 5 h (Fig. 1A). During this period, there was exchange of deuterium from D₂O into the H₂ gas phase, producing nearly equal amounts of HD and D₂ (Fig. 1B). Figure 2 shows that despite the variation in the isotope content of the gas phase, the deuterium enrichment in methane was sufficiently high and invariant to prove that all four hydrogens in methane have their ultimate origins in water and not hydrogen gas. This is particularly evident at the earlier time points.
Deuterium isotope discrimination in methane production. The difference in deuterium enrichment between methane and water shown in Fig. 2 indicates a preference for protons over deuterons in methanogenesis; however, the error in quantitatively determining the isotope effect was large at the high water enrichment used and low levels of methane produced. Thus, a more favorable water enrichment of 81 atom% D was used, and conditions were varied to maximize methane production: more cells were used than in the experiment of Fig. 2 and the cells were not previously washed and centrifuged in D₂O. Excessive manipulation of these extreme anaerobes increases the lag time and decreases the final methane production rates. The resuspension medium was buffered and contained ammonium salts. Deuterium enrichments in methane, water, and hydrogen gas were assayed after 4 h of incubation.

Table 1 contains the results of this experiment, from which the total methane deuterium enrichment $q$ is calculated. The product isotope

![Fig. 1. Production of methane, HD, and D₂ by M. thermoautotrophicum in 88.7 atom% deuterated water. (A) Methane production as percent (vol/vol) of gas phase. (B) Distribution of $H_2$ ($\bigcirc$), HD ($\Delta$), and $D_2$ ($\bigcirc$) as percent of total hydrogen gas.](image1)

![Fig. 2. Deuterium enrichment in methane, water, and hydrogen gas during methanogenesis by M. thermoautotrophicum. Symbols: $\bigcirc$, water; $\Delta$, methane; and $\bigcirc$, hydrogen gas. The errors associated with the measurement of hydrogen gas and water enrichments fall within the symbols. The water enrichment extrapolation to zero time reflects the hydrogen gas exchange reaction.](image2)

![Table 1. Distribution of deuterated methane species from 81 atom% deuterated medium.](table1)
discrimination $\alpha = 1.5 \pm 0.2$ is then calculated from $q = 0.73 \pm 0.02$ and the atom fraction D in water, $n = 0.81 \pm 0.01$. With this value for $\alpha$, the model distribution of methane species, $(p + q)^2$, yields the predicted mole fraction of each species also given in Table 1.

**DISCUSSION**

Since the hydrogens of methane originate in water, equation 1 can be separated into distinct oxidative and reductive halves:

$$4 \text{H}_2 \rightleftharpoons 8 \text{H}^+ + 8 e^-$$

(2)

$$8e^- + 8 \text{H}^+ + \text{CO}_2 \rightarrow \text{CH}_4 + 2 \text{H}_2\text{O}$$

(3)

Equation 2 is catalyzed by one or more hydrogenases, with the electrons initially reducing such cofactors as F$_{420}$, nicotinamides, flavins, molybdenum cofactors, or iron-sulfur centers. In D$_2$O, the reactions of equation 2 also exchange the H$_2$ to HD and D$_2$. Equation 3, the reductive half of methanogenesis, represents a sequence of reactions involving methyl coenzyme M and probably at least one other currently unknown carbon-carrying species (7, 14, 22, 25). In our experiments the H$^+$ produced in equation 2 is diluted more than 50-fold by D$^+$, since the mole fraction of hydrogen in hydrogen gas versus that in water is less than 0.02. Deuterated methane species could not arise from direct transfer from HD or D$_2$ produced via equation 2, since the deuterium enrichment in hydrogen gas is always much less than that in water or methane, particularly at early times in the experiment (Fig. 2). This essential measurement has not been made in previous studies (16, 17, 19). The results of the experiment in 81 atom% deuterated water (Table 1) also support this conclusion, since the deuterium enrichment in methane is again significantly greater than that of the hydrogen gas at the end of the incubation.

Our conclusions corroborate those of Pine and co-workers (16, 17) but are in sharp contrast to those of Sauer et al. (19). Sauer et al. based their conclusion that methane hydrogen arises from hydrogen gas on the total radioactivity observed in water, tritium gas, and methane, neglecting to take into account the vast molar excess of hydrogen in water over that in hydrogen gas in such an experiment. Using reasonable assumptions from their experimental description, the specific activities of tritium in methane vs. water and tritium gas support our conclusion that water, not hydrogen gas, is the ultimate source of the hydrogen in biogenic methane. (Assuming a liquid volume of 1 ml and 5 $\mu$mol of methane produced, the following specific activities [in microcuries per milligram-atom of H] were calculated from Sauer et al. [19], experiment 2, Table 2: 1,370 and 37 for the tracer $^3$H - H$_2$ and methane, respectively, and 1.0 and 0.22 for the tracer $^1$H - H$_2$O and methane, respectively. The specific activity of methane relative to that of the label source is clearly much greater when water is the label source [0.22] than when hydrogen gas is the label source [0.03].)

Since reductions by reduced F$_{420}$, like those of its chemical relatives dihydro-5-deazaflavins (21) and dihydronicotinamides (12), are likely to involve direct and complete hydrogen transfer from position 5, our conclusion is strongly supported by the recent observations of F. Jacobson and C. Walsh (personal communication) that tritium from tritium gas is not incorporated into reduced F$_{420}$ by partially purified H$_2$-F$_{420}$ oxidoreductase from *M. thermoautotrophicum* $\Delta$H and by the references of Fuchs et al. (10) to unpublished observations that hydrogenase from *M. thermoautotrophicum* does not catalyze a hydride transfer. It will be of interest to see the extent to which the methane produced in cell extracts is labeled after the addition of synthetically deuterated or tritiated dihydro-F$_{420}$, NADH, and NADPH.

Our experiments were conducted only with *M. thermoautotrophicum*, but the cohesiveness of methanogens as a group (4, 23, 25), especially with respect to their unique cofactors coenzyme M and F$_{420}$, suggests that the same source of hydrogen will be found in all methanogens that reduce CO$_2$ to methane. The same origin of methane hydrogen is expected in formate-utilizing methanogens, since formate is likely to be oxidized to CO$_2$ and H$_2$ and this carbon dioxide subsequently reduced to methane (24). However, when methanol, methylamine, or acetate is used to produce methane by *Methanosarcina barkeri*, the methyl hydrogens are probably retained in the product methane and only a single hydrogen is acquired from water (16, 17).

The apparent product isotope discrimination of 1.5 calculated from the data of Table 1 is larger than expected from an equilibrium isotope effect (20); there are at least four ways in which such an apparent isotope effect could arise in this system. First, there may be true, large product solvent isotope effects in one or more of the steps in methanogenesis that attaches a nonexchangeable hydrogen to the methanogenic carbon. Second, there may be a single true isotope effect in the production of a hydride-transferring cofactor such as dihydro-F$_{420}$ or NADPH. Third, the apparent effect may be due to a higher protium concentration (i.e., lower water atom% D) in intracellular water than in the extracellular water (from which $\alpha$ is calculated). This could arise from (i) a significant isotope effect in H$^+$. 


D* influx through the cell membrane, or (ii) a significant internal production of H* from H2 and hydrogenase inside the cell, or both. These interesting and very different possibilities are currently under investigation. The agreement between the observed distribution of deuterated methane species and that predicted by our simple model (Table 1) suggests that this problem may not be prohibitively complex.

ACKNOWLEDGMENTS

L.D. is supported by a Public Health Service postdoctoral fellowship from the National Institutes of Health; R.S. is a fellow of the Helen Hay Whitney Foundation. This work was supported by the College of Agriculture and Life Sciences, University of Wisconsin, and by Public Health Service research grant GM 17170 from the National Institute of General Medical Science.

We thank J. G. Zeikus for providing us with a culture of M. thermoautotrophicum and Mel Micke for expert technical assistance in obtaining the mass spectra, as well as C. Walsh and F. Jacobson for permission to quote results in advance of publication.

LITERATURE CITED