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Hydrogen isotope fractionation in lipids of the methane-oxidizing bacterium *Methylococcus capsulatus*

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Abstract—Hydrogen isotopic compositions of individual lipids from *Methylococcus capsulatus*, an aerobic, methane-oxidizing bacterium, were analyzed by hydrogen isotope-ratio-monitoring gas chromatography–mass spectrometry (GC-MS). The purposes of the study were to measure isotopic fractionation factors between methane, water, and lipids and to examine the biochemical processes that determine the hydrogen isotopic composition of lipids. *M. capsulatus* was grown in six replicate cultures in which the δD values of methane and water were varied independently. Measurement of concomitant changes in δD values of lipids allowed estimation of the proportion of hydrogen derived from each source and the isotopic fractionation associated with the utilization of each source.

All lipids examined, including fatty acids, sterols, and hopanols, derived $31.4 \pm 1.7\%$ of their hydrogen from methane. This was apparently true whether the cultures were harvested during exponential or stationary phase. Examination of the relevant biochemical pathways indicates that no hydrogen is transferred directly (with C-H bonds intact) from methane to lipids. Accordingly, we hypothesize that all methane H is oxidized to H₂O, which then serves as the H source for all biosynthesis, and that a balance between diffusion of oxygen and water across cell membranes controls the concentration of methane-derived H₂O at 31%. Values for $\alpha_{l/w}$, the isotopic fractionation between lipids and water, were 0.95 for fatty acids and 0.85 for isoprenoid lipids. These fractionations are significantly smaller than those measured in higher plants and algae. Values for $\alpha_{l/m}$, the isotopic fractionation between lipids and methane, were 0.94 for fatty acids and 0.79 for isoprenoid lipids. Based on these results, we predict that methanotrophs living in seawater and consuming methane with typical δD values will produce fatty acids with δD between -50 and -170% , and sterols and hopanols with δD between -150 and -270% . Copyright © 2002 Elsevier Science Ltd

1. INTRODUCTION

What little is known about fractionation of hydrogen isotopes in microorganisms pertains to the isotopic compositions of methane produced by methanogenic archaea (Balabane et al., 1987; Sugimoto and Wada, 1995; Waldron et al., 1999; Whiticar et al., 1986), of H₂ produced by photosynthetic cyanobacteria (Luo et al., 1991), and of bulk biomass produced by photosynthetic bacteria and algae (Estep and Hoering, 1981; Estep, 1984; Quandt et al., 1977; Stiller and Nissenbaum, 1980). Unfortunately, none of these products is directly preserved in the geologic record. Moreover, analyses of catabolic end products such as methane provide little information about isotopic fractionations associated with microbial production of lipids, the products that are preserved over geologic timescales. Bulk biomass contains a large proportion of *O*- and *N*-bound H that exchanges readily with water, greatly complicating interpretation of isotopic data. More detailed information regarding H fractionation in higher plants is available (see references in Sauer et al., 2001; Yakir, 1992), but applying those results to heterotrophic bacteria is problematic. Considering the important role prokaryotes play in the Earth's biogeochemical cycles, any technique that allows further identification and understanding of their contributions to the sedimentary record would be invaluable.

Pathways of H metabolism—and, therefore, the associated isotopic fractionations—can be complex. For most plants, water serves as the only hydrogen source. For all other organisms, H bound in substrates is an additional and evidently very important source (Estep and Dabrowski, 1980). In principle, each form of H within assimilated substrates constitutes a separate source (e.g., CHOH vs. CHO vs. CH₂). With these points in mind, we have begun studies of metabolic fractionation by focusing on sources of H used in the biosynthesis of lipids by methanotrophic bacteria. The question is attractive both in its simplicity and in its inclusiveness. There are only two possible sources of H: water or one of the four equivalent H atoms in CH₄. The results also provide a further view of the fractionation of H isotopes in lipid biosynthesis (cf. Sessions et al., 1999).

The lipids of methanotrophic bacteria are also interesting as components of sedimentary records. Preliminary results indicate that the D/H ratios of lipids can be preserved over geologic timescales (Andersen et al., 2001; Li et al., 2001). Strong depletion of D in both biogenic and thermogenic methane is well documented (Schoell, 1980; Whiticar et al., 1986), and it is frequently possible to distinguish the multiple sources of methane on the basis of isotopic ratios. In the analogous case of carbon isotopes, organisms consuming CH₄ produce ¹³C-depleted lipids that serve as diagnostic geochemical evidence for the presence of methane consumption (Hayes, 1994; Hinrichs et al., 1999). Currently, it is unknown whether the depletion of

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Table 1. Summary of experimental conditions.

Culture	H-source	δD (‰)	OD ^a	Cell density ^b
I	CH ₄	-150	0.273	70
	H ₂ O	-87		
II	CH ₄	111	0.230	66
	H ₂ O	-87		
III	CH ₄	111	0.163	48
	H ₂ O	104		
IV	CH ₄	-150	0.338	84
	H ₂ O	104		
V	CH ₄	-150	0.195	37
	H ₂ O	104		
VI	CH ₄	-150	0.401	400
	H ₂ O	-87		

^a Optical density at harvest, measured at 420 nm.

^b Measured at harvest, mg dry biomass/L.

D in methane is reflected in the δD values of lipids produced by methanotrophs.

In this study we systematically examine D/H ratios in lipids produced by the aerobic, methane-oxidizing bacterium *Methylococcus capsulatus* (strain "Bath"). Cultures were grown using the four possible combinations of "heavy" CH₄ ($\delta D = +111\%$) vs. Vienna standard mean ocean water [V-SMOW]) and H₂O (+104‰) and "light" CH₄ (-150‰) and H₂O (-87‰). Simultaneous solution of mass-balance equations provides a straightforward view of the relative importance of each source of H and associated isotopic fractionations.

2. MATERIAL AND METHODS

2.1. Cultures and Substrates

Culture techniques for *M. capsulatus* have been described by Jahnke (1992) and Jahnke and Nichols (1986). Briefly, *M. capsulatus* was grown in water-jacketed flasks at 40°C, continuously bubbled with 100 mL/min of a mixture of CH₄ (50%), O₂ (9.6%), CO₂ (0.8%), and N₂ (40%). Growth medium contained the mineral salts of Whittenbury and Dalton (1981) with CuSO₄ added (7.5 μ mol/L) to inhibit expression of the soluble form of methane monooxygenase (sMMO). Two isotopically distinct supplies of CH₄ and H₂O were used for the experiments (Table 1). Deuterium-enriched CH₄ was prepared by mixing 2.70 L-kPa of CH₃D (Cambridge Isotope Labs, Andover, MA) with 34.4 L-MPa of ultra-high-purity CH₄ (Scott Gas, Fremont, CA) in a standard high-pressure cylinder. Deuterium-enriched H₂O was prepared by mixing 300 μ L of 99.5% D₂O (Cambridge Isotope Labs) with 20 L of distilled H₂O. The δD value of each CH₄ and H₂O source was determined at Indiana University by offline combustion (of CH₄), reduction to H₂ over U turnings, and measurement by dual-inlet isotope ratio mass spectrometry following the method described by Schimmelmann et al. (1999).

Starter cultures used to inoculate each culture were prepared in nonenriched CH₄ and H₂O. For all experiments, these accounted for <0.5% of the final biomass. Cultures were harvested after 2 to 4 d based on optical density (OD) measured at 420 nm using a Milton-Roy 401 spectrophotometer. Exponential-phase cultures (I, II, III, V) were harvested at OD \approx 0.2, and stationary-phase cultures (IV, VI) were harvested at OD > 0.3 (Table 1). Culture VI was grown to a very high cell density of 400 mg/L. Harvested cells were immediately centrifuged then lyophilized.

Extraction and preparation of lipids was similar to that described by Summons et al. (1994). Briefly, Bligh and Dyer (1959) extraction followed by cold acetone precipitation produced an acetone-soluble fraction (supernatant) and a phospholipid fraction (precipitate; Summons and Jahnke, 1992). Solid-phase extraction of the acetone-soluble

lipids on amino-propyl stationary phase (0.5 g of Supelclean-NH₂; Supleco, Bellefonte, PA) produced two fractions: hydrocarbons and sterols eluted with 5 mL of 9:1 dichloromethane (DCM)/acetone, and fatty acids eluted with 8 mL of 50:1 DCM/formic acid. Fatty acids in this fraction are assumed to represent free fatty acids, although they may be derived in part from cleavage of phospholipids during sample preparation. Ten percent of the phospholipid fraction was set aside for analysis of fatty acids. Hopanol cleavage products were prepared from the remainder of the phospholipid fraction by periodate oxidation followed by NaBH₄ reduction (Rohmer et al., 1984), then separated from fatty acids by solid-phase extraction.

Both acetone-soluble and phospholipid fatty acid fractions were derivatized as methyl esters by heating with CH₃OH/acetyl chloride. The dimethylated derivative of phthalic acid was prepared in parallel with each group of samples to measure the δD value of H added by methylation. Phthalate provides a convenient means for correction of methyl-H, both because δD of the disodium salt of phthalate can be readily measured by offline combustion/reduction, and because of the favorable 6/4 ratio of methyl-H/phthalate-H in the dimethylated product. The δD of disodium phthalate was $-97.0 \pm 0.6\%$, and the δD of methyl H added by derivatization was $-102 \pm 3\%$. Sterol and hopanol fractions were derivatized as acetyl esters by heating with acetic anhydride/pyridine. δD of this batch of acetic anhydride was previously measured by offline combustion/reduction (Sauer et al., 2001).

2.2. Isotopic Analyses

Individual lipids were identified by gas chromatography-mass spectrometry (GC-MS) using a Hewlett Packard 5973 mass-selective detector, and their abundances were quantified by gas chromatography with flame ionization detection relative to an internal standard (pristane or epiandrosterone). Hydrogen isotope ratios were measured using a Finnigan-MAT Delta-Plus XL connected to an HP 6890 gas chromatograph via the Finnigan GC-III pyrolysis interface. The H₃-factor for this instrument was measured daily by injecting pulses of H₂ gas with constant δD and peak heights varying over a 10-fold range, and was typically <6 ppm mV⁻¹ with a daily variability of <0.2 ppm mV⁻¹. Mass-2 and -3 ion-current data were collected in 250-ms intervals and corrected for H₃⁺ contributions using the "pointwise" correction scheme of Sessions et al. (2001). Data were processed with Isodat NT software version 1.1 (Finnigan-MAT, Bremen). Considering the range of peak height and δD differences between sample and standard peaks, variability in the H₃-factor, and time constants associated with data collection and processing, the maximum systematic error expected due to H₃⁺ correction for these samples is <0.9‰ (Sessions et al., 2001).

Two types of isotopic standards were analyzed. A mixture of C₁₆-C₃₀ *n*-alkanes ("external standards," obtained from the Biogeochemical Laboratories, Indiana University; <http://php.indiana.edu/~aschimme/hc>), with concentrations varying over a fivefold range, was analyzed after every third sample analysis. Between three and five *n*-alkanes ("internal standards") were also coinjected with each unknown sample. One of these was used as the isotopic reference peak for each analysis (*n*-C₁₈ for fatty acids, $\delta D = -55\%$, *n*-C₃₂ for sterols and hopanols, $\delta D = -210\%$), while the remaining standards were used to assess accuracy.

3. RESULTS AND DISCUSSION

3.1. Precision and Accuracy

The analytical precision, as measured by the pooled standard deviation of replicate analyses of unknown samples, was 3.7‰. Pooled standard deviations for internal and external standards over the course of the analyses were 5.6 and 5.1‰, respectively. This disparity in precision probably reflects the differing timescales, ranging from hours (samples) to months (standards). Although cultures were not replicated, comparison of cultures harvested during exponential and stationary phase (below) indicates that δD values were reproducible between cultures to within $\pm 20\%$ (2σ).

Table 2. Concentrations of extracted lipids (μg lipid/mg dry biomass).

Compound ^a	Culture					
	I	II	III	IV	V	VI
14:0 fatty acid (AS)	2.2	0.10	0.14	2.8	0.10	1.2
14:0 fatty acid (PL)	0.56	2.1	2.0	0.49	1.5	3.8
16:1 fatty acid ^b (AS)	25	1.8	2.8	37	2.0	9.1
16:1 fatty acid ^b (PL)	9.1	29	31	5.8	26	18
16:0 fatty acid (AS)	21	1.8	2.5	29	2.3	7.9
16:0 fatty acid (PL)	12	30	31	10	29	30
squalene	3.5	1.6	1.1	1.4	0.79	0.56
4 α -methyl-5 α -cholest-8(14)-en-3 β -ol	3.8	1.4	1.4	1.7	1.1	2.5
diplopterol + 4 α -methyl-5 α -cholest-8(14),24-dien-3 β -ol	3.0	1.5	1.4	1.1	1.0	0.51
4,4-dimethyl-5 α -cholest-8(14)-en-3 β -ol	5.4	2.3	2.1	2.5	1.7	1.2
4,4-dimethyl-5 α -cholest-8(14),24-dien-3 β -ol	0.14	0.23	0.89	0.11	0.22	0.049
hopan-30-ol	2.1	2.3	3.6	4.3	12	3.1
3 β -methylhopan-30-ol	3.8	3.0	4.0	6.5	12	13

^a AS = acetone-soluble fraction; PL = phospholipid fraction.

^b Mixture of up to four positional isomers.

The arithmetic mean error for external standards was 0.7‰ and for internal standards was -0.9‰ , indicating that no significant systematic errors were present. The root-mean-square accuracy of external standards for *M. capsulatus* was 4.9‰, whereas for internal standards it was 8.5‰. This decrease in accuracy, with no substantial decrease in precision, is due primarily to systematic offsets in two of the internal standards. The other six coinjected *n*-alkanes revealed no systematic errors, so the offsets were probably caused by coelution of other peaks with the standard compounds. Considering all these factors, the accuracy of δD measurements probably approaches 5‰.

3.2. *M. capsulatus* Lipids

Concentrations of extracted lipids in *M. capsulatus* are summarized in Table 2, and δD values measured for those lipids are

summarized in Table 3. In general, fatty acids were about 10-fold more abundant in the phospholipid fraction than in the acetone-soluble fraction. Cultures I and IV were exceptions to this pattern. The increase in acetone-soluble fatty acids and concurrent decrease in phospholipids in cultures I and IV probably represents hydrolysis of the phospholipids during sample workup. *M. capsulatus* produces up to four positional isomers of 16:1 fatty acids (Δ^8 , Δ^9 , Δ^{10} , and Δ^{11} ; Jahnke and Diggs, 1989) which were not chromatographically separated in our analyses. Composite values are reported in Tables 2 and 3 for all 16:1 isomers.

M. capsulatus is unusual in producing both sterols, via the oxygen-dependent cyclization of squalene, and hopanols via oxygen-independent cyclization. Two sterols are abundant: 4 α -methyl-5 α (H)-cholest-8(14)-en-3 β -ol, and 4,4-dimethyl-5 α -

Table 3. δD values^a of extracted lipids.

Compound ^b	Culture					
	I	II	III	IV	V	VI
14:0 fatty acid (AS)	-157			-16		-157
14:0 fatty acid (PL)					-17	-177
16:1 fatty acid ^c (AS)	-172	-95	41	-59	-43	-169
16:1 fatty acid ^c (PL)	-171	-94	33	-50	-33	-172
16:0 fatty acid (AS)	-122	-55	88	-19	-4	-146
16:0 fatty acid (PL)	-130	-53	89	-3	23	-138
squalene	-258	-208	-103	-162	-142	-251
4 α -methyl-5 α -cholest-8(14)-en-3 β -ol + diplopterol	-255	-199	-92	-162	-149	-258
4 α -methyl-5 α -cholest-8(14),24-dien-3 β -ol	n.a. ^d	n.a.	-87	n.a.	n.a.	n.a.
4,4-dimethyl-5 α -cholest-8(14)-en-3 β -ol	-250	-188	-75	-152	-135	-256
4,4-dimethyl-5 α -cholest-8(14),24-dien-3 β -ol	n.a.	n.a.	-81	n.a.	n.a.	n.a.
hopan-30-ol	-256	-209	-99	-162	-160	-245
3 β -methylhopan-30-ol	-231	-168	-45	-132	-126	-233
CH ₄ ^e	-150	111	111	-150	-150	-150
H ₂ O ^e	-87	-87	104	104	104	-87

^a Values are the mean of three or more analyses, and are corrected for H added by derivatizing agents.

^b AS = acetone-soluble fraction; PL = phospholipid fraction.

^c Mixture of up to four positional isomers.

^d Not available, concentration was too low.

^e Values repeated from Table 1.

cholest-8(14)-en-3 β -ol (Summons et al., 1994). Their $\Delta^{8(14),24}$ diunsaturated analogs are produced in lower abundance, and quantities were sufficient for isotopic analysis only in culture III. Squalene and diploptene were also abundant in the acetone-soluble fraction. In GC-MS analyses using a DB-5ms analytical column (J&W Scientific), diploptene coeluted with 4-methylcholestadienol. In hydrogen isotope-ratio-monitoring GC-MS analyses using an HP-1 analytical column (Agilent Technologies), diploptene coeluted with 4-methylcholestenol, and a single combined δD value is reported for these compounds in Table 3. Diploptene was present at roughly the same abundance as 4-methylcholestenol in all cultures. Hopan-30-ol and 3 β -methylhopan-30-ol were abundant in the periodate cleavage products of bacteriohopanepolyol. Smaller amounts of C₃₁ hopanol and methylhopanol were also present, but quantities were not sufficient for isotopic measurement.

Values of δD in both fatty acids and triterpenols responded strongly to changes in δD of either methane or water. In general, lipids were depleted in D relative to water but enriched in D relative to methane. Cultures in which methane was enriched in D relative to the water, a situation almost never encountered in nature, are an exception to this pattern.

3.3. Calculation of Hydrogen Sources

Lipid hydrogen in *M. capsulatus* can derive from CH₄ or from H₂O. Allowing for fractionation associated with utilization of each hydrogen source, the hydrogen isotopic composition of a particular lipid will be described by

$$R_l = f_m R_m \alpha_{l/m} + (1 - f_m) R_w \alpha_{l/w} \quad (1)$$

where R is the D/H ratio of lipids, methane, and water, α is the fractionation factor defined as $\alpha_{a/b} = R_a/R_b$, and f_m is the fraction of hydrogen derived from methane. In this formulation, α represents the net fractionation between lipids and methane or water and results from many individual isotope effects, including those due to substrate assimilation, biosynthetic fractionations at branchpoints in the flow of metabolites, exchange, and perhaps others. Many of these individual effects may change with varying growth conditions, but at present such a level of detail cannot be discerned. Note also that $\alpha_{l/w}$ and $\alpha_{l/m}$ can have different values for each lipid.

Equation 1 is nearly exact,[†] and in theory a multivariate regression of R_l on R_m and R_w can lead to values of the coefficients f_m , $\alpha_{l/m}$, and $\alpha_{l/w}$. Regression—minimization of the sum of squared errors—is preferred to simultaneous solution of linear equations because multiple values of R are available for each set of f_m , $\alpha_{l/m}$, and $\alpha_{l/w}$ values, even assuming that values of f and both alphas differ between different lipids. To test this approach, we used a numerical minimization of errors for the six cultures, as follows. Values of δD were independent of growth phase (see section 3.5.5), so all cultures were treated equally in our analysis. For each compound, initial estimates of f_m , $\alpha_{l/m}$, and $\alpha_{l/w}$ were chosen. Values of R_m and R_w obtained from Table 1 were then used to calculate the expected values of

R_l in each of the six cultures. Comparing the predicted values of R_l to the measured values of R_l , we calculated the sum of

squared errors ($\Psi = \sum_{n=1}^6 [(R_{n,m} - R_{n,p})^2]$, where the subscripts m and p represent measured and predicted R_l values) and recorded that value. Systematic variation of f_m , $\alpha_{l/m}$, and $\alpha_{l/w}$ across a range of plausible values then produced an array of values for [Ψ , f_m , $\alpha_{l/m}$, $\alpha_{l/w}$].

Analysis of these data indicates that a minimum value for Ψ does exist, i.e., there is a unique combination of values for [f_m , $\alpha_{l/m}$, $\alpha_{l/w}$] which “best-fit” the measured δD data. Unfortunately, the minimum is only weakly determined—the four-dimensional equivalent of what appears in three dimensions as a broad, flat valley floor—so that small uncertainties in measured δD values lead to very large uncertainties in the best-fit values for f_m , $\alpha_{l/m}$, and $\alpha_{l/w}$. This is largely a consequence of the fact that there are three coefficients but only two independent variables (R_m and R_w) in Eqn. 1. If the coefficients are parameterized as $R_l = aR_m + bR_w$, where $a = f_m \alpha_{l/m}$ and $b = (1 - f_m) \alpha_{l/w}$, then best-fit values for the coefficients a and b can be determined with relatively small uncertainties (Table 4).

Using the definitions $\delta D \equiv 1000[(R/R_{VSMOW} - 1)]$ and $\epsilon \equiv 1000(\alpha - 1)$, Eqn. 1 can be rewritten as

$$\delta D_l = f_m \left(\delta D_m + \epsilon_{l/m} + \frac{\delta D_m \epsilon_{l/m}}{1000} \right) + (1 - f_m) \left(\delta D_w + \epsilon_{l/w} + \frac{\delta D_w \epsilon_{l/w}}{1000} \right). \quad (2)$$

Values of δD and ϵ typically have magnitudes on the order of 50 to 200‰, so the quantity $(\delta D \cdot \epsilon / 1000)$ is less than 10% of either δD or ϵ , and to a good approximation can be ignored to give the more familiar form

$$\delta D_l = f_m (\delta D_m + \epsilon_{l/m}) + (1 - f_m) (\delta D_w + \epsilon_{l/w}). \quad (3)$$

This approximation improves as the values of δD and ϵ approach zero. The terms in Eqn. 3 can be expanded and rearranged to yield

$$\delta D_l = f_m \delta D_m + (1 - f_m) \delta D_w + f_m \epsilon_{l/m} + (1 - f_m) \epsilon_{l/w}. \quad (4)$$

The values of δD_m and δD_w were varied in our cultures, while f_m , $\epsilon_{l/m}$, and $\epsilon_{l/w}$ are properties of the bacteria. Because growth conditions were identical between cultures, we assume that the values of f_m , $\epsilon_{l/m}$, and $\epsilon_{l/w}$ were constant for a given lipid in all cultures. The last two terms in Eqn. 4 are therefore constant, and can be combined giving

$$\delta D_l = f_m \delta D_m + (1 - f_m) \delta D_w + \bar{\epsilon}_l \quad (5)$$

where $\bar{\epsilon}_l = f_m \epsilon_{l/m} + (1 - f_m) \epsilon_{l/w}$ and represents the weighted-average isotopic fractionation between lipids and hydrogen sources.

Although Eqn. 5 is approximate, f_m is the only coefficient of the two experimental variables (δD_m and δD_w). Numerical minimization of errors using Eqn. 5, exactly analogous to that described above for Eqn. 1, leads to strongly determined best-fit values of f_m and $\bar{\epsilon}_l$ for each lipid (Table 4). Values of r^2 were greater than 0.98 in every case, indicating that all six cultures are fit very well by Eqn. 5 using a single set of values for f_m and

[†] The exact form of Eqn. 1 requires fractional abundances [D/(D+H)] rather than isotopic ratios (D/H). For a natural abundance of D (~150 ppm), these quantities differ by only 0.015%.

Table 4. Summary of parameters and uncertainties from multivariate regression calculations.

Compound	$f_m \alpha_{l/m}^a$	$(1 - f_m) \alpha_{l/w}^a$	$10^3(\bar{\alpha} - 1)^b$	$\bar{\varepsilon}_l^c$	f_m^d	σ_f^e	$\alpha_{l/m}^f$	σ_m^e	$\alpha_{l/w}^f$	σ_w^e	$\alpha_{w/m}^g$	σ_{wm}
squalene	0.220	0.601	-179	-175	0.29	0.011	0.76	0.05	0.85	0.02	0.90	0.06
4-methyl sterol + diplopterol	0.247	0.578	-175	-172	0.32	0.014	0.78	0.05	0.85	0.02	0.92	0.06
4,4-dimethyl sterol	0.253	0.582	-165	-162	0.32	0.014	0.80	0.05	0.85	0.02	0.94	0.07
hopanol	0.238	0.583	-179	-175	0.31	0.014	0.77	0.05	0.84	0.02	0.91	0.07
3-methyl hopanol	0.280	0.578	-142	-140	0.33	0.010	0.84	0.04	0.87	0.02	0.97	0.05
16:1 fatty acid (AS)	0.305	0.626	-69	-68	0.33	0.012	0.93	0.06	0.93	0.03	1.00	0.07
16:1 fatty acid (PL)	0.278	0.653	-69	-68	0.30	0.014	0.92	0.04	0.94	0.03	0.98	0.05
16:0 fatty acid (AS)	0.322	0.650	-28	-28	0.33	0.011	0.97	0.03	0.97	0.02	1.00	0.04
16:0 fatty acid (PL)	0.327	0.646	-27	-23	0.29	0.010	1.13	0.05	0.91	0.02	1.24	0.06

^a Calculated from Eq. 1 as described in text.

^b $\bar{\alpha} \equiv f_m \alpha_{l/m} + (1 - f_m) \alpha_{l/w}$ and is calculated from the coefficients obtained for Eq. 1, and the value of f_m obtained from Eq. 5.

^c $\bar{\varepsilon}_l \equiv f_m \varepsilon_{l/m} + (1 - f_m) \varepsilon_{l/w}$ and is calculated from Eq. 5.

^d Calculated from Eq. 5.

^e Uncertainty estimated from Monte Carlo calculations.

^f Calculated from Eq. 1, using the value of f_m obtained from Eq. 5.

^g $\alpha_{w/m} = \alpha_{l/m} / \alpha_{l/w}$

$\bar{\varepsilon}_l$. The estimates of f_m obtained in this way can then be used to calculate values for $\alpha_{l/m}$ and $\alpha_{l/w}$ (Table 4) from $f_m \alpha_{l/m}$ and $(1 - f_m) \alpha_{l/w}$, i.e., the parameterized coefficients of Eqn. 1.

Two important questions remain: how large are systematic errors, if any, introduced by the approximations inherent in Eqn. 5, and how sensitive are these solutions to uncertainties in measured values of δD ? To answer the former question, Table 4 compares values of $\bar{\varepsilon}_l$ obtained from the approximate Eqn. 5 to values of $1000[(f_m \alpha_{l/m} + (1 - f_m) \alpha_{l/w}) - 1]$ obtained from the exact Eqn. 1. Since $\varepsilon \equiv 1000(\alpha - 1)$, these quantities would be equal if Eqn. 5 were exact. Table 4 shows that they differ by <4%, and in most cases by <2%. These small differences suggest that errors due to approximations in Eqn. 5 are negligible. Because Eqn. 5 is exact when $\delta D = 0\text{‰}$, this lack of systematic errors can be attributed to the fact that all six experiments were considered simultaneously, and that δD values of both H_2O and CH_4 varied almost symmetrically around 0‰. Thus systematic errors present when $\delta D_w = -87\text{‰}$ are approximately cancelled by errors present when $\delta D_w = +104\text{‰}$. Future experiments should be designed to take full advantage of such symmetry.

The sensitivity of calculated values of f_m , $\alpha_{l/m}$, and $\alpha_{l/w}$ to uncertainties in measured δD values was examined by Monte Carlo analysis using the software @RISK (Palisade Corporation, Newfield, NY; Winston, 1996). Under computer control, random values were chosen for each δD measurement based on a normal probability distribution derived from the mean and standard deviation of the measured values ($\sigma = 5.5\text{‰}$ for lipids, $\sigma = 2.0\text{‰}$ for CH_4 and H_2O). The numerical minimization of errors for both Eqns. 1 and 5, described above, then yielded values of f_m , $\alpha_{l/m}$, and $\alpha_{l/w}$. These calculations were repeated $\sim 10,000$ times for each lipid, and the standard deviations for values of f_m , $\alpha_{l/m}$, and $\alpha_{l/w}$ were calculated from the results of the multiple trials (Table 4). Uncertainties calculated in this way for f_m are between 1.0% and 1.4%. In comparison, the standard deviation of f_m for all fatty acids is 2.0%, and for all isoprenoid lipids is 1.5%, in good agreement with the Monte Carlo estimates. Similarly, Monte Carlo estimates of uncertainty are $\sim 50\text{‰}$ for $\alpha_{l/m}$ and $\sim 20\text{‰}$ for $\alpha_{l/w}$, and are only

slightly smaller than the variation observed within the two lipid classes.

3.4. Pathways of Hydrogen in Biosynthesis

The value of f_m is virtually constant across all compounds at $31.4 \pm 1.7\%$ (Table 4), indicating that $\sim 70\%$ of the hydrogen in all *M. capsulatus* lipids is derived from water in the culture medium. Other workers have grown methylotrophic organisms on deuterated water or methanol as sources of D-labeled biomolecules. The metabolism of methanol in those organisms is similar to that of methane in *M. capsulatus*, so they serve as a basis for comparison with our results. Mosin et al. (1996) grew the bacterium *Methylobacillus flagellatum* in 98% D_2O , and found that the deuterium content of amino acids obtained from protein hydrolysis (and after accounting for exchangeable *N*- and *O*-bound H) ranged from 44% to 96%. Batey et al. (1996) grew the bacterium *Methylophilus methylotrophis* in 52:48 $D_2O:H_2O$, and determined that the deuterium content of RNA hydrogen was identical to that of supplied water. Massou et al. (1999) grew the methylotrophic yeast *Pichia pastoris* and *Pichia angusta* in both D_2O/CH_3OH and H_2O/CD_3OD , and found that a constant $\sim 20\%$ of the hydrogen in lipids and amino acids derived from methanol, but that $\sim 40\%$ of the hydrogen in carbohydrates derived from methanol. These differences were interpreted as reflecting differing amounts of H-exchange between organic substrates and water during biosynthesis. Qualitatively, these results confirm our finding that water, rather than methane or methanol, is the primary source of lipid hydrogen in aerobic heterotrophs growing on C-1 compounds.

To quantitatively discuss the significance of these results, it is first necessary to consider the pathways through which hydrogen flows from CH_4 and H_2O to lipids (Fig. 1) in *M. capsulatus*. During lipid biosynthesis, there are three immediate sources for organic hydrogen: C-bound hydrogen in biosynthetic precursors such as acetate, hydrogen added during reduction by NADPH, and hydrogen that exchanges with or is obtained from cellular water (Sessions et al., 1999). There are

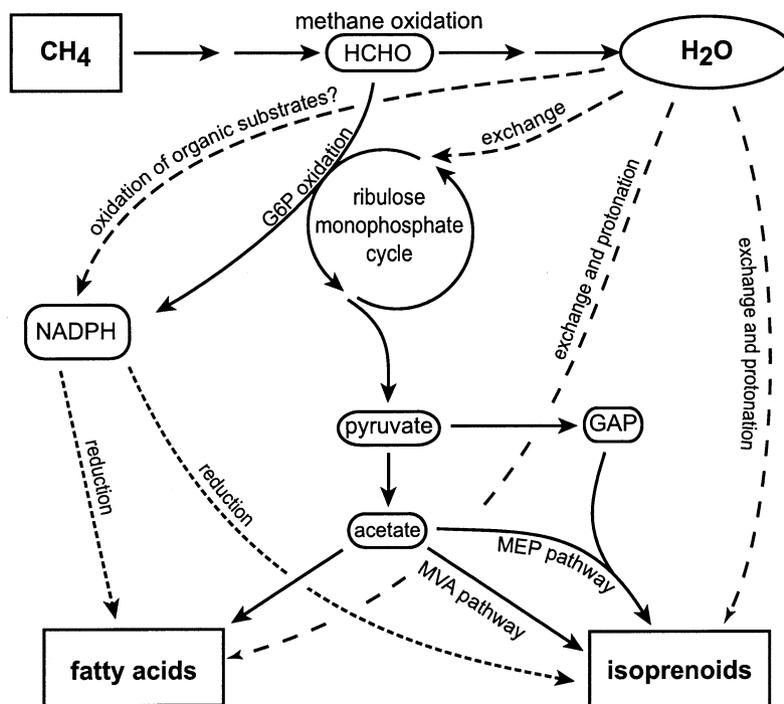


Fig. 1. Generalized flow of hydrogen in *M. capsulatus* metabolism. Arrows indicate reactions of hydrogen, rather than carbon, so for example CH_4 is oxidized to H_2O . Carbon from methane is incorporated into biomass by the RuMP (ribulose monophosphate) cycle, but hydrogen from methane is not. Solid lines indicate carbon-bound hydrogen carried on organic substrates; dotted lines indicate hydrogen carried by NADPH; dashed lines indicate hydrogen in water. It is expected that either the MVA or MEP pathway, but not both, operates in *M. capsulatus*. GAP is glyceraldehyde phosphate.

also three different biosynthetic pathways which must be considered: synthesis of *n*-alkyl lipids from acetate and synthesis of isoprenoid lipids from either mevalonic acid (MVA) or methylerythritol phosphate (MEP). The importance of each hydrogen source can vary between these pathways.

Fatty acid synthesis proceeds similarly in both eukaryotes and bacteria (Fig. 2), with the head-to-tail linkage of acetate molecules to form a chain that grows in 2-carbon increments (Wakil et al., 1983). After the addition of acetate to the chain, the carboxyl carbon of the previous acetate is reduced by addition of H^- from NADPH. Dehydration removes the resulting hydroxyl group and one hydrogen from the adjacent methylene (former methyl) group. Addition of a second H^- from

NADPH, plus H^+ from water, hydrogenates the double bond. The net result of this process is that roughly one-quarter of lipid hydrogen is inherited from the methyl group of acetate, one-quarter is obtained directly from water, and one-half is supplied by NADPH. The extent of hydrogen exchange accompanying this process is currently unknown. Up to 75% of C-bound hydrogen is potentially subject to exchange with water via keto-enol tautomerization, although the importance of this exchange probably depends on whether intermediate molecules remain bound to enzymes. In *Escherichia coli* for example, the fatty acid biosynthetic enzymes exist as separate enzymes in the cytosol, whereas in eukaryotes the enzymes exist as a single multienzyme complex (White, 1995).

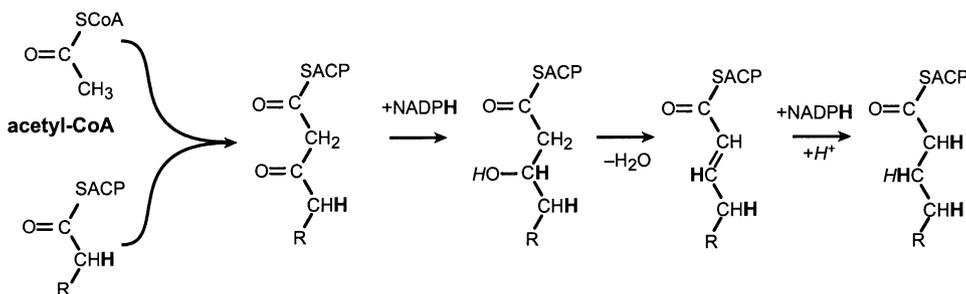


Fig. 2. Sources of hydrogen during fatty acid biosynthesis (after White, 1995). Hydrogen shown in bold is supplied by NADPH, that shown in italics is obtained from water, that shown in plain type is inherited from organic substrates. The initial condensation of acetyl-CoA with the fatty acid chain occurs in several steps which do not affect hydrogen budgets and have been omitted.

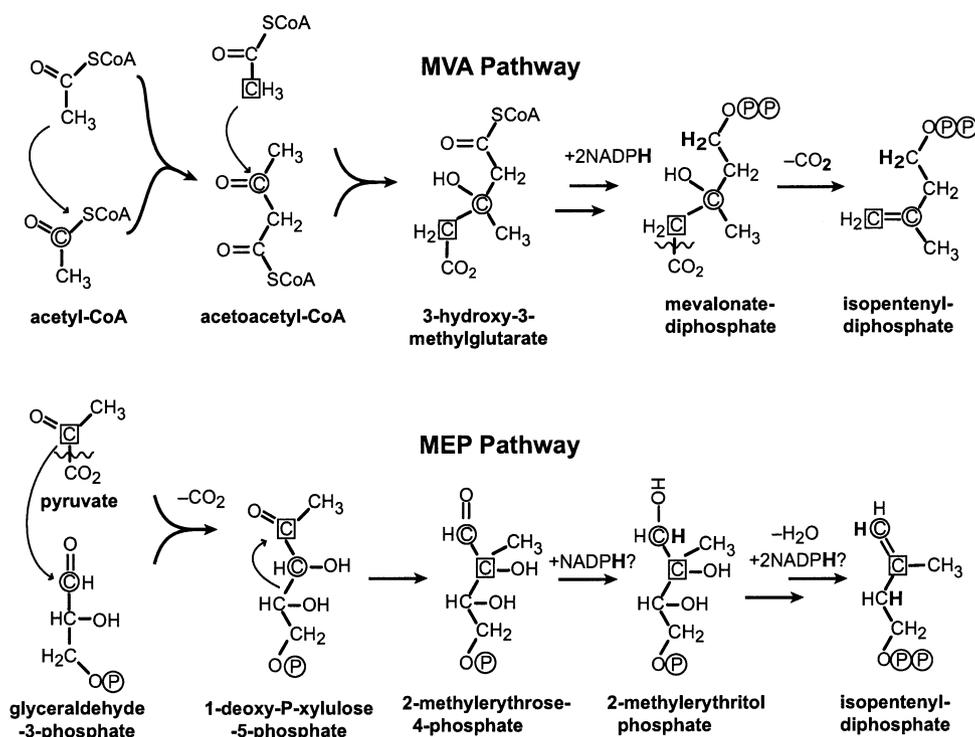


Fig. 3. Sources of hydrogen during MVA and MEP pathways of isoprenoid biosynthesis (after Lange et al., 2000). Hydrogens are shown in plain and bold type as in Figure 2. Two arrows indicate multiple steps which have been combined in this illustration.

The isoprenoid biosynthetic pathways used by *M. capsulatus* have not been studied, so both the MVA and MEP pathways are summarized in Figure 3. In general, bacteria use only one isoprenoid pathway, not both (Lange et al., 2000). In the MVA pathway, three acetyl-coenzyme A (CoA) molecules are condensed and reduced to form the 6-carbon intermediate mevalonate, using two NADPH molecules in the process. Concerted loss of the carboxyl carbon at C-6 and the hydroxyl group at C-3 results in the fully reduced product isopentenyl diphosphate, in which seven out of nine hydrogens are inherited directly from the methyl groups of acetate and the remaining two are from NADPH. The MEP pathway begins with the condensation and decarboxylation of glyceraldehyde phosphate and pyruvate (Schwender et al., 1996) to give the 5-carbon intermediate deoxyxylulose phosphate. A carbon-skeletal rearrangement, followed by a series of unknown steps that must involve three reductions and one dehydration, then lead to isopentenyl diphosphate. The MEP pathway has only recently been recognized, and the reducing cofactors are assumed to be NADPH, although this has not yet been demonstrated. Considering what is known, three of nine hydrogens in isopentenyl diphosphate are inherited directly from the methyl group of pyruvate, and two to four are inherited directly from glyceraldehyde phosphate. The remaining hydrogens (2 to 4) are supplied either by a reductant, presumably NADPH, or by water.

To make the connection between these immediate sources of hydrogen (NADPH and organic substrates) and the ultimate sources of hydrogen (CH_4 and H_2O), it is necessary to trace in turn the sources of the hydrogen on NADPH and in the organic

substrates. In *M. capsulatus*, carbon is assimilated through the ribulose-monophosphate (RuMP) cycle (Fig. 4). Carbon enters the pathway as formaldehyde, and is condensed with the 5-carbon sugar ribulose-5-phosphate (Anthony, 1982). A series of isomerizations produces glucose-6-phosphate, which is then oxidized to 6-phosphogluconate. The carbon inherited from formaldehyde is at C-1, the oxidized position. Accordingly, no hydrogen can be transferred directly (i.e., with the C-H bond remaining intact) from methane to biomass. It is possible that methane hydrogen is transferred from C-1 to other carbon positions during isomerization reactions, but here we assume that this is not the case.

A major source of NADPH is the oxidation of glucose-6-phosphate during the RuMP cycle of carbon assimilation (Fig. 4). Although *M. capsulatus* has not been examined specifically, in many other NADP-reducing enzyme systems, the H at the oxidized position is quantitatively transferred to NADP^+ (Ables et al., 1992). If this occurs in *M. capsulatus*, the methane-derived H at C-1 of glucose will be transferred to NADPH and could thus be used in biosynthesis. This would provide an indirect, but presumably conservative, pathway for the transfer of methane-H to lipids. Unfortunately, there is little information about alternative sources of NADPH, such as the pentose-phosphate pathway, in *M. capsulatus*. Each C assimilated by the RuMP cycle yields one NADPH and thus provides more than enough reducing power to account for the elemental composition of biomass, so it is possible that no other sources of NADPH exist. The only firm conclusion is that NADPH

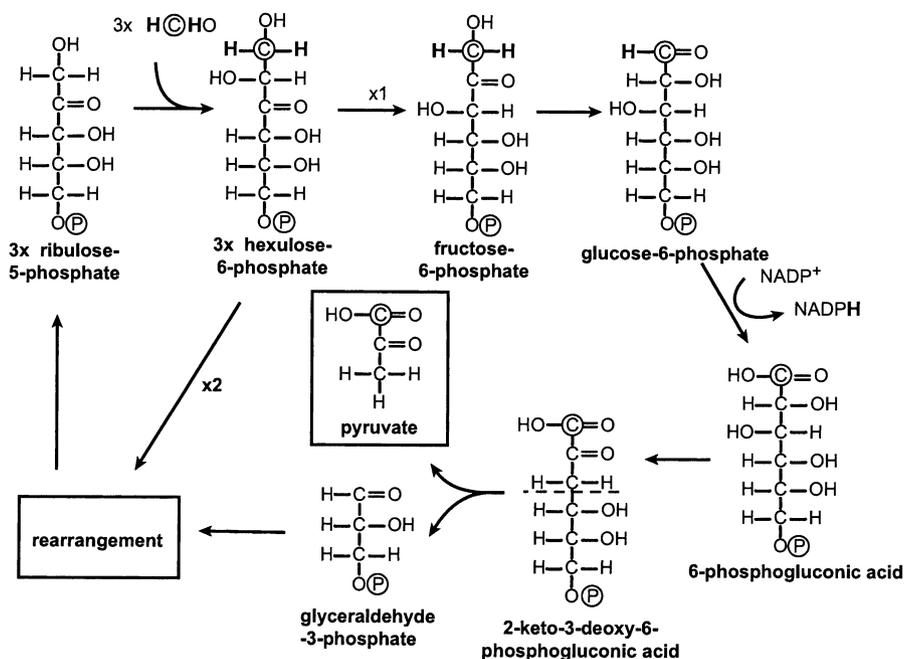


Fig. 4. RuMP cycle for assimilation of carbon in *M. capsulatus* (after Anthony, 1982). Hydrogen shown in bold is inherited from methane.

provides a *potential* pathway for transferring hydrogen from methane to lipids.

With the details of these biosynthetic processes in hand, we now turn to the significance of $f_m = 31\%$ in *M. capsulatus* lipids. Three possible explanations can be considered: (1) all methane-derived H is transferred via NADPH derived from the oxidation of glucose-6-phosphate in the RuMP cycle; (2) methane-derived H is transferred both by NADPH and by H₂O derived from the oxidation of methane; or (3) all methane-derived H is transferred via H₂O. In the first case, the proportion of NADPH hydrogen deriving from CH₄ vs. H₂O is not known, but should be constant for all lipids. In fatty acids, ~50% of hydrogen comes from NADPH, whereas in isoprenoid lipids this proportion is 22% (MVA pathway) or 33% (MEP pathway). Regardless of which biosynthetic pathway is used, isoprenoid lipids and fatty acids obtain different proportions of their hydrogen from NADPH. Different values of f_m should therefore arise in these two lipid classes, but no such difference is observed. Exchange of C-bound H in lipids with cellular water could alter the proportion of H contributed by NADPH, but considerable coincidence would be required for exchange to produce a constant value of f_m .

The second alternative is that the proportions of methane-derived H in the three hydrogen sources (NADPH, H₂O, and organic substrates) are such that they precisely counterbalance the differing inputs from these sources to fatty acids and isoprenoid lipids. In this case, there are three unknown quantities (the fraction of methane-H in each hydrogen source) that are constrained by only two isotopic mass balance equations. There is, consequently, an infinite number of possible combinations of methane-H in the three sources that would lead to a constant f_m in all lipids. Nevertheless, all of the possibilities

would require a surprising level of coincidence to produce an invariant f_m in all lipids.

The third alternative is that 31% of the intracellular water in *M. capsulatus* derives from CH₄ and that this controls the hydrogen isotopic composition of all biosynthetic products. This explanation is attractive for its simplicity in explaining a constant f_m in all types of lipids, but requires a mechanism for regulating the fraction of methane-derived H₂O. The amounts of CH₄ oxidized (roughly 1 g of CH₄ per 5-L culture based on growth-yield estimates of Anthony, 1982) are small in comparison to H in the growth medium, so the oxidation of CH₄ in cells would have to continuously make up for the loss of methane-derived H₂O to the medium.

The required counterbalancing of water diffusion by methane oxidation might be accomplished by the following mechanism. Given the 2:1 stoichiometry (O₂:CH₄) required for methane oxidation, and the proportions in which CH₄ and O₂ were supplied (50% and 10%, respectively) we assume that the rate of methane oxidation in our cultures is limited by the supply of O₂. The flux of O₂ diffusing across a membrane into a cell is given by

$$F_o = \frac{D_o}{L} ([O_2]_{out} - [O_2]_{in})A \quad (6)$$

where D_o is the diffusion coefficient for O₂ in a lipid membrane, L is the thickness of the cell wall, A is the surface area of the cell, and brackets indicate concentration (Crank, 1975). If methane oxidation is indeed limited by O₂ supply, [O₂]_{in} will be approximately zero. Similarly, the flux of methane-derived water out of the cell is

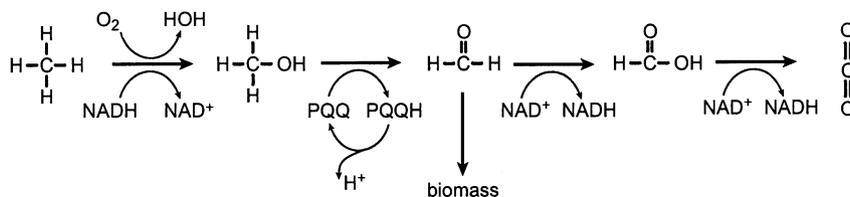


Fig. 5. The oxidation of CH_4 to CO_2 in *M. capsulatus* (after Anthony, 1982). An intermolecular kinetic isotope effect leads to assimilation of methane that is depleted in D relative to supplied methane. An intramolecular isotope effect leads to enrichment of D in each subsequent oxidation product (Wilkins et al., 1994). PQQ is the prosthetic group of methanol dehydrogenase, and transfers only electrons from methanol to electron-transport chains.

$$F_w = \frac{D_w}{L} ([\text{H}_2\text{O}^*]_{\text{in}} - [\text{H}_2\text{O}^*]_{\text{out}}) A \quad (7)$$

where D_w is the diffusion coefficient for water, and H_2O^* indicates water derived from the oxidation of methane. As described above, the 5-L cultures are virtually infinite reservoirs of water, so $[\text{H}_2\text{O}^*]_{\text{out}}$ is zero.

Conservation of mass requires that the rate at which methane hydrogen is oxidized in the cell must equal the flux of water hydrogen leaving the cell plus the flux of hydrogen diverted to biosynthesis ($F_o = F_w + F_b$). In *M. capsulatus*, F_b is less than 8% of F_o (50% of methane C converted to biomass \times 50% of methane H remaining in $\text{CH}_2\text{O} \times$ 31% of lipid H derived from methane) and so can be ignored. By substituting Eqns. 6 and 7 into $F_o \approx F_w$ and rearranging, we obtain:

$$\frac{D_o}{D_w} \approx \frac{[\text{H}_2\text{O}^*]_{\text{in}}}{[\text{O}_2]_{\text{out}}} \quad (8)$$

D_o and D_w are physical properties, so the left side of Eqn. 8 must be constant. The partial pressure of O_2 in cultures— $[\text{O}_2]_{\text{out}}$ —was held constant by equilibration with the supplied gas. The concentration of methane-derived water inside the cell, and hence f_m , should therefore be approximately constant. Because the effects of membrane thickness and surface area cancel in Eqn. 8, $[\text{H}_2\text{O}^*]_{\text{in}}$ should remain constant as a cell grows and changes size. We note here, and discuss in more detail below, that an identical argument applies to the case when methane oxidation is limited by the diffusion of CH_4 rather than O_2 into the cell.

Molecular-dynamical calculations of diffusion through model lipid bilayer membranes suggest that the ratio of D_o and D_w should be between 10^4 and 10^2 (Marrink and Berendsen, 1996). Assuming $[\text{O}_2]_{\text{out}}$ is 20 ppm based on the solubility of O_2 in H_2O (Lide, 1994) gives an estimate of $[\text{H}_2\text{O}^*]_{\text{in}} / [\text{O}_2]_{\text{out}}$ of 1.5×10^4 . Equation 8 is therefore consistent with available experimental data.

The preceding analysis oversimplifies a number of aspects of methane metabolism in *M. capsulatus*. First, in addition to diffusion through lipid membranes, water can enter some—or perhaps all—cells by diffusion through membrane-bound transport proteins and through specialized “water channel proteins,” or aquaporins (Haines, 1994). In fact, this pathway may account for >90% of water flow in some types of cells (Verkman, 1994). Second, *M. capsulatus* is not a well-mixed, hollow sphere. Particulate methane monooxygenase (pMMO), and hence the site of initial methane oxidation, is localized within extensive internal membranes (Anthony, 1982). These mem-

branes are folded into stacks, and water within those stacks may not rapidly mix with the cytoplasm. Furthermore, at least one step in methane oxidation (the oxidation of methanol to formaldehyde; Fig. 5) probably occurs in the periplasm rather than in the cytosol (Hooper and DiSpirito, 1985). Finally, the rate of methane oxidation is unlikely to be limited solely by the supply of oxygen (or methane) into the cell, particularly as individual cells reach senescence. Below we present evidence that, once synthesized, lipids in *M. capsulatus* do not turn over. All that is required, then, is that methane oxidation is diffusion-limited while the cells are actively growing.

All of these details suggest that the true explanation for a constant f_m is more complicated than our simple diffusion model implies. Nevertheless, none of them exclude our central proposal, which is that the transfer of substrate hydrogen into biomass occurs via water and is regulated by a balancing of physical transport, rather than biochemical, processes. We have no direct evidence that this is the case, so our proposal must remain a hypothesis.

3.5. Fractionation Factors

If methane H is transferred to lipids via water, then the isotopic fractionation between lipids and methane should be the product of the fractionations between lipids and water and between water and methane ($\alpha_{l/m} = \alpha_{l/w} \times \alpha_{w/m}$). In isoprenoid lipids, $\alpha_{l/m}$ averages 0.79 while $\alpha_{l/w}$ averages 0.85, implying that $\alpha_{w/m}$ is 0.93 (Table 4). For fatty acids, the values of $\alpha_{l/m}$ and $\alpha_{l/w}$ are statistically indistinguishable at 0.94 and 0.95, respectively, and so $\alpha_{w/m}$ is approximately 1.0. These latter averages exclude the results for 16:0 phospholipid fatty acids. Those data, which appear anomalous (Table 4), represent single-compound analyses from six separate cultures. Such a repeated systematic error could result from the coelution of some other compound with the 16:0 fatty acid. We cannot explain why $\alpha_{w/m}$ differs between isoprenoids and fatty acids. The differences could represent the localization of different steps in both lipid biosynthesis and methane oxidation in different parts of the cell. More simply, they could indicate that methane is not quantitatively oxidized to water.

Isotope effects during oxidation of methane by soluble methane monooxygenase (sMMO) have been measured in vitro (Nesheim and Lipscomb, 1996; Wilkins et al., 1994). The binding of methane to sMMO is accompanied by an intermolecular isotope effect with a magnitude of 1.75, so methane entering the oxidation pathway will be depleted in D relative to the supplied methane. Subsequent oxidation steps (Fig. 5) are

Table 5. Comparison of average fractionation factors^a from *M. capsulatus*, plants, microalgae, and sedimentary lipids.

	Fatty acids		Isoprenoids	
	$\alpha_{l/m}$	$\alpha_{l/w}$	$\alpha_{l/m}$	$\alpha_{l/w}$
<i>M. capsulatus</i>	0.94 (.03)	0.95 (.02)	0.79 (.03)	0.85 (.01)
Macrophytes ^b		0.81 (.02)		0.78 (.02)
Phytoplankton ^c		0.79 (.02)		0.70 (.03)
Macrophytes ^d		0.85 (.03)		0.78 (.04)
Sediments ^e		0.87 (.01)		0.81 (.02)

^a Values are the mean and (1 σ).

^b Two higher plants and two brown algae reported by Sessions et al. (1999).

^c Two phytoplankton cultures reported by Sessions et al. (1999).

^d Eleven higher plants and three algae reported by Estep and Hoering (1980). Data for "total saponifiable lipids" are assumed to represent fatty acids, and "total nonsaponifiable lipids" are assumed to represent isoprenoids.

^e Lipids extracted from surface sediments in five lakes and two shallow marine sites by Sauer et al. (2001).

accompanied by large intramolecular isotope effects in which H is removed preferentially, leading to progressive enrichment of D in the resulting methanol, formaldehyde, and formate. Provided the intermolecular effect associated with assimilation of methane is dominant, the water derived from these oxidative steps will be depleted in D relative to the supplied methane, as indicated by our results. However, quantitative comparison of our results to those in vitro experiments is hampered by several uncertainties: (1) large discrepancies in the magnitude of isotope effects measured by workers using different types of in vitro experiments, (2) the extent to which methane entering a cell is consumed, (3) differences between the soluble and particulate forms of MMO, and (4) the possibility of isotope effects accompanying diffusion of methane across cell membranes.

Coleman et al. (1981) measured changes in methane δD accompanying the growth of two enrichment cultures of unidentified, methane-oxidizing bacteria at 26°C. Methane/water fractionation factors calculated from this data were 1.297 and 1.325 for the two cultures. These data agree with the direction of fractionation we observe, but indicate much smaller values of $\alpha_{w/m}$ of 0.75 to 0.77. Coleman et al. (1981) note that the measured fractionations increased more than twofold when comparing experiments at 11° and 26°C, contrary to the expected decrease in magnitude of kinetic isotope effects with increasing temperature. Such an effect could be due to changes in species composition with increasing temperature.

3.5.1. Comparison of Fractionation to Photoautotrophs

In photoautotrophs, where H₂O represents the only potential source for organic hydrogen, the value of $\alpha_{l/w}$ can be measured directly. In higher plants and the few algae examined to date, $\alpha_{l/w}$ has a value of 0.79 to 0.87 for fatty acids (Table 5), whereas in *M. capsulatus* the mean value is 0.95. Similarly, $\alpha_{l/w}$ for isoprenoid lipids is 0.70 to 0.81 in plants and algae, and is 0.85 in *M. capsulatus*.

There are several plausible explanations for the smaller fractionations observed in *M. capsulatus*. Because $\alpha_{l/w}$ represents

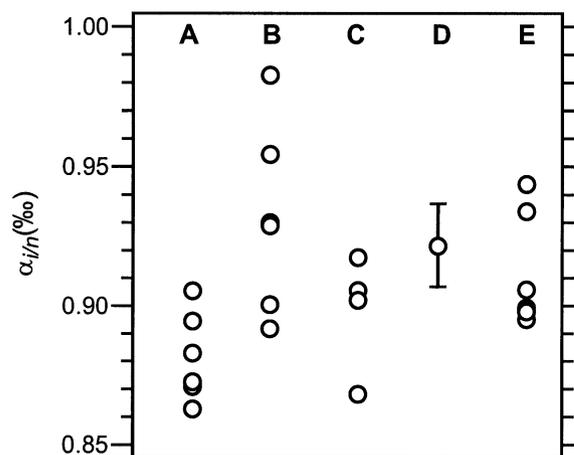


Fig. 6. Hydrogen isotopic fractionation between isoprenoid and *n*-alkyl lipids. Except for groups D and E, each point represents the fractionation ($\alpha_{l/n}$) between the mean δD value for isoprenoid lipids and the mean for *n*-alkyl lipids in a single plant or culture. Marker for group D indicates the mean and 1 σ range for *n*-alkyl and isoprenoid lipids extracted from lake sediments. Markers for group E indicate *n*-C₂₂ alkane and 5 α -cholestane from different sediment horizons. Group A—current *M. capsulatus* experiments; B—aquatic higher plants, brown algae, diatoms, dinoflagellates, Sessions et al. (1999); C—terrestrial and aquatic higher plants, Estep and Hoering (1980); D—extractable lipids in Great Pond sediments, Sauer et al. (2001); E—6 million-year-old Mediterranean Sea sediments (Andersen et al., 2001).

the net result of multiple processes relating water to lipids, the value of $\alpha_{l/w}$ must include fractionations due to exchange with water, uptake of H⁺ during the reduction of double bonds, and (potentially) reduction by NADPH carrying water-derived hydrogen. An enrichment in D of approximately 160‰ accompanies exchange between carbohydrates and water in plants (Yakir and DeNiro, 1990), so increased exchange between lipids and water in *M. capsulatus* might plausibly produce larger values of $\alpha_{l/w}$. Alternately, a decreased supply of NADPH derived from water (as opposed to methane via glucose-6-phosphate, see Fig. 4) would also result in larger $\alpha_{l/w}$, because NADPH is thought to be strongly depleted in D relative to water (Estep and Hoering, 1981; Yakir and DeNiro, 1990).

3.5.2. Fractionation Between *n*-Alkyl and Isoprenoid Lipids

The average isotopic fractionation between isoprenoids and *n*-alkyl lipids ($\alpha_{i/n}$) from a single culture of *M. capsulatus* is 0.881 ± 0.016 (Fig. 6). Using the data of Sessions et al. (1999) and Estep and Hoering (1980), comparison of δD values for sterols and fatty acids in individual plants and algae indicates the range of $\alpha_{i/n}$ values is 0.982 to 0.868, with a mean of 0.918. The average isotopic fractionation between *n*-alkanes and sterols extracted from pond sediments, and from the same presumed biologic source, was 0.922 ± 0.015 (Sauer et al., 2001). The average fractionation between *n*-C₂₂ alkane and 5 α -cholestane extracted from 6-million-year-old Mediterranean Sea sediments was 0.90 ± 0.04 (Andersen et al., 2001). Several explanations have been suggested for the hydrogen isotopic

fractionation between *n*-alkyl and isoprenoid lipids, including fractionation in biosynthetic pathways, differences in time or location of biosynthesis, and differences in the amount of H derived from NADPH vs. substrates (Estep and Hoering, 1980; Sessions et al., 1999). Our results do not distinguish between these possibilities.

The distribution of the MVA and MEP pathways for isoprenoid biosynthesis has been well established in higher plants (Lichtenthaler, 1999), where the MVA pathway is used to produce cytosolic isoprenoid compounds such as sterols and pentacyclic triterpenols, and the MEP pathway is used to synthesize plastidic isoprenoids, including phytol and carotenoids. In two higher plants and a dinoflagellate culture examined by Sessions et al. (1999), phytol was consistently depleted in D relative to sterols by 50 to 100‰. This difference was tentatively attributed to fractionations characteristic of the MVA and MEP pathways. The isoprenoid pathways used by *M. capsulatus* are unknown, but nearly all other Proteobacteria that have been examined carry genes for enzymes of the MEP pathway exclusively (Lange et al., 2000). If it is confirmed that *M. capsulatus* uses the MEP pathway, then our observation that $\alpha_{D,H}$ in *M. capsulatus* is similar to that in many plants (Fig. 6) would imply that there is no significant difference in D/H fractionation between the two pathways. Isotopic fractionation between phytol and sterols in plants (Sessions et al., 1999) must then be due to fractionation of water, NADPH, or organic substrates between the chloroplast and cytoplasm.

3.5.3. Fractionation in Triterpenol Biosynthesis

Hopan-30-ol derives from squalene, the precursor of all triterpenols (Fig. 7). In the four cultures harvested during exponential growth, δD values of hopan-30-ol were identical to those of squalene. This suggests that little or no hydrogen isotopic fractionation is associated with the branching of squalene between sterol and hopanol biosynthetic pathways. To facilitate comparison of sterols and hopanols between cultures in which δD values varied widely, we therefore normalized δD values of all triterpenols to those of squalene from the same culture (Fig. 8).

A significant deuterium enrichment, averaging 32‰, was measured in 3-methylhopan-30-ol relative to both hopan-30-ol and squalene (Fig. 8). Similarly, 3-methylhopanols were ^{13}C -enriched relative to coexisting hopanols by 2 to 6‰ in cultures grown under conditions similar to ours (Jahnke et al., 1999; Summons et al., 1994). Squalene is converted to 3-methyldiplopterol, the precursor of 3-methylhopanoids, by the intact transfer of a methyl group from *S*-adenosyl-methionine to diplopterol (Summons et al., 1994). This methyl group represents only ~6% of hydrogen and ~3% of carbon in the methylhopanol, so the observed isotopic shifts would seem to require that the methyl group has δD ~500‰ enriched and $\delta^{13}C$ ~200‰ enriched relative to the parent hopanol. As noted by Summons et al., the required carbon isotopic contrast is practically impossible. Exchange of H with H_2O could explain the hydrogen isotopic enrichment of 3-methylhopanol, but not the carbon isotopic enrichment. Differences in the timing of biosynthesis of the two hopanoid end products could be responsible, but δD values for cultures harvested in different phases of growth are equivalent, implying that this is not the

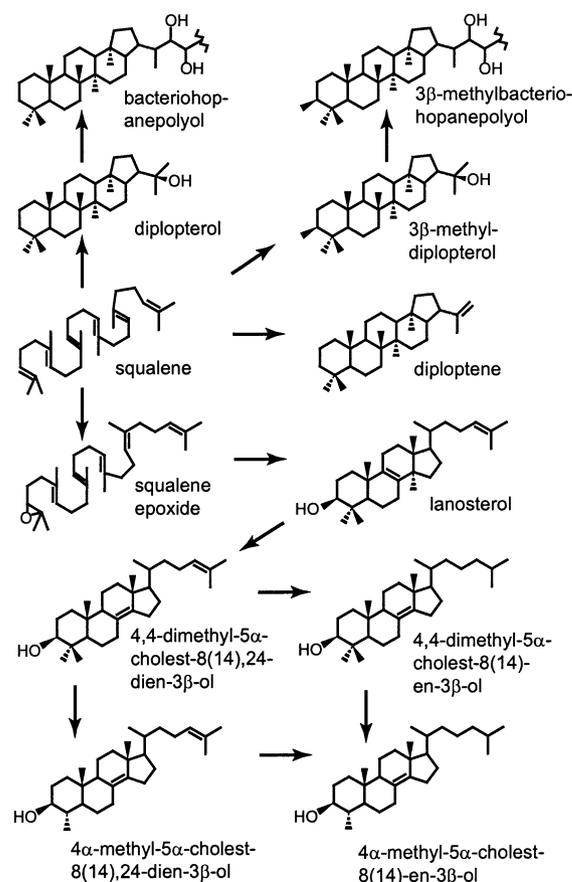


Fig. 7. Proposed pathways for sterol and hopanol biosynthesis in *M. capsulatus* (after Summons et al., 1994).

case. Our results add support to the suggestion (Summons et al., 1994) that the biosyntheses of these two products may differ fundamentally.

Comparison of δD values for the monoene vs. diene sterols in culture III indicates that there is no significant shift in δD associated with hydrogenation of the Δ^{24} double bond (Table 3). Presumably this hydrogenation is the result of H^- supplied by NADPH, and H^+ from water. The average δD of these two hydrogens must therefore be close to the average δD for lipid hydrogen, although the measurement is not particularly sensitive because the added hydrogen represents only ~4% of total lipid hydrogen.

3.5.4. Fractionation in Fatty Acid Biosynthesis

Hydrogen isotopic compositions of fatty acids obtained from the acetone-soluble and phospholipid fractions are compared in Figure 9. The average difference in δD of equivalent lipids (e.g., 16:0 fatty acid) between the two fractions was 3 ± 12 ‰. There is, accordingly, no systematic difference between these two functionally defined fractions.

The hydrogen isotopic compositions of the saturated and monounsaturated C_{16} fatty acids differ significantly (Fig. 9). Considering both acetone-soluble and phospholipid fractions, this difference averages 43 ± 9 ‰, with 16:0 enriched in D

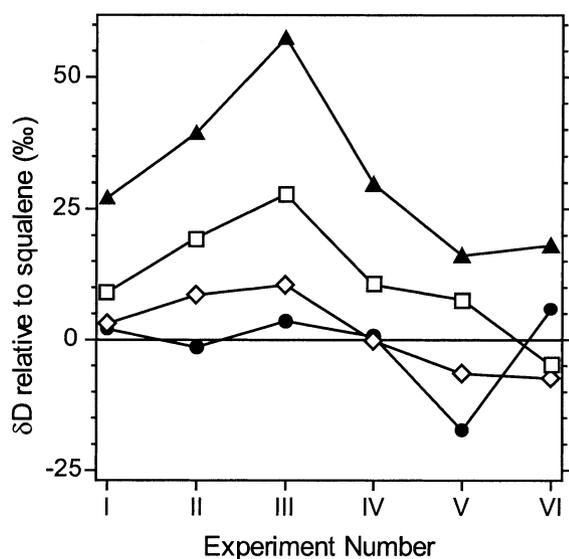


Fig. 8. Hydrogen isotopic compositions of *M. capsulatus* triterpenols: hopan-30-ol (solid circles), 3-methylhopan-30-ol (solid triangles), 4-methylcholest-8(14)-enol (open diamonds), and 4,4-dimethylcholest-8(14)-enol (open squares). All δD values are relative to that of squalene from the same culture.

relative to 16:1. δD values for saturated C_{14} fatty acids fall between those of the 16:0 and 16:1 fatty acids. *M. capsulatus* is known to synthesize unsaturated fatty acids via the anaerobic mechanism (Jahnke and Diggs, 1989), in which the carbon-carbon double bond created by dehydration (Fig. 2) is preserved during continued chain elongation. The net difference

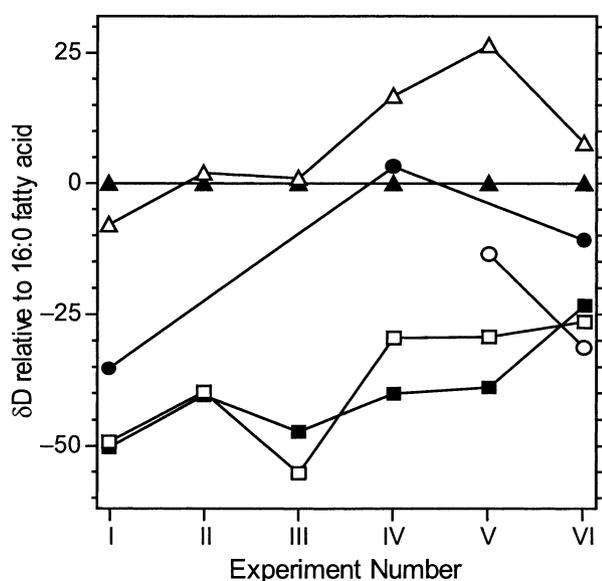


Fig. 9. Hydrogen isotopic compositions of *M. capsulatus* fatty acids: 14:0 (circles), 16:1 (squares), and 16:0 (triangles). Filled symbols represent fatty acids from the acetone-soluble fraction, open symbols represent those from the phospholipid fraction. All δD values are relative to that of 16:0 fatty acid from the acetone-soluble fraction of the same culture.

between hydrogen in 16:0 and 16:1 fatty acids is therefore the two hydrogens added during one double-bond hydrogenation. For these two hydrogen atoms alone to explain the isotopic difference would require that their combined δD value was 667‰ more enriched than the average δD of the 16:1 fatty acid, an improbable level of enrichment. By comparison, hydrogenation of the Δ^{24} position in sterols does not produce any measurable change in δD . Given the similarities between the two fatty acids, the most plausible explanation is that there is a more fundamental difference in the biosynthesis of the two compounds. For example, their synthesis may be mediated by different isozymes or cofactors, with consequent differences in associated fractionation.

3.5.5. Fractionation and Growth Rate

Hydrogen isotopic compositions of lipids did not vary significantly between cultures harvested during exponential or stationary phases of growth (compare cultures I and VI, and IV and V in Table 3). Tritium and radiocarbon-labeling experiments with *E. coli* have shown that, although significant turnover of membrane phospholipids does occur, this process involves the transfer of intact fatty acids between different polar head groups (Cronan, 1968; Cronan and Vagelos, 1972). The membrane fatty acids themselves appear highly resistant to degradation by the host organism, despite the fact that *E. coli* possesses an active β -oxidation system for degrading fatty acids. Based on our D/H data, and by analogy with *E. coli*, we speculate that this lack of turnover of membrane lipids may be a general feature of bacteria, and that δD values of bacterial lipids accordingly reflect hydrogen isotopic budgets during growth of the host organisms.

In contrast, Yakir and DeNiro (1990) showed that, during heterotrophic processing of carbohydrates in plants, transformations of carbohydrate carbon skeletons led to the exchange of C-bound H in carbohydrates with cellular water, even though the net inventory of carbohydrate hydrogen did not change. Associated isotopic fractionations led to a strong increase in δD of carbohydrates during this processing. Yakir (1992) accordingly argued that δD of plant carbohydrates should be correlated with metabolic status. During periods of low photosynthetic activity, the utilization and reprocessing of existing biochemical stores should increase, leading to a net increase in δD . Sessions et al. (1999) found indirect evidence that this situation is also true in lipids from higher plants. Sterols from dormant plants were D-enriched relative to actively growing cultures by 50 to 100‰, whereas fatty acids were enriched in D by 0 to 30‰. These two points of view—conservation of lipid H in bacteria, and isotopic alteration of lipid H in plants—need not be mutually exclusive, though some discrepancies remain to be resolved.

3.6. δD Values of Lipids from Native Methanotrophs

The data in Table 4 can be used to predict the δD values of lipids from methanotrophs living in natural environments, but with two significant sources of uncertainty. The first reflects the unknown variability of f_m under natural conditions. If the magnitude of f_m is controlled by the proportion of lipid H derived from NADPH vs. water, it should remain constant. On

Table 6. Predicted δD values^a for lipids from methanotrophic bacteria living under natural conditions.

	δD of water		
	+50‰	0‰	-50‰
δD of methane			
-100‰	-6 to -65 -105 to -180	-53 to -94 -148 to -205	-100 to -122 -191 to -231
-200‰	-6 to -103 -105 to -211	-53 to -131 -148 to -237	-100 to -160 -191 to -262
-300‰	-6 to -140 -105 to -243	-53 to -169 -148 to -268	-100 to -197 -191 to -294

^a Two ranges are given for each combination of methane and water: the top represents the predicted range for fatty acids, the bottom that for isoprenoid lipids. Each range was calculated by assuming that f_m varies between 0% and 40%, and that $\alpha_{l/m}$ and $\alpha_{l/w}$ remain constant.

the other hand, if our hypothesis regarding diffusive balances is correct, then f_m will vary with the supply of limiting substrate as shown by Eqn. 8. The lower limit for f_m is zero, attained if the concentration of limiting substrate (O_2 or CH_4) was nearly zero. The upper limit for f_m depends on the partial pressures of both gases that can be maintained in the water, and f_m could theoretically be greater than 70%. As a practical matter, it is unlikely for natural waters to contain both O_2 and CH_4 in high abundance, nor can *M. capsulatus* necessarily support the high methane-oxidation rates required to reach that theoretical value. Given these uncertainties, we choose 40% as our estimate of the upper limit for f_m .

The second point of uncertainty is whether the magnitude of fractionations can vary between the nutrient replete, methane- and oxygen-rich conditions of our experiments and those encountered in natural waters. For example, Summons et al. (1994) observed a modest change in carbon-isotopic fractionation between cultures in which sMMO was inhibited by addition of Cu versus those without added Cu. We cannot assess this latter source of variations from the available data, and so proceed under the assumption that the fractionations remain the same.

Given these assumptions, the predicted ranges of δD values for lipids from native methanotrophs are shown in Table 6. For each range, the larger δD value corresponds to $f_m = 0\%$, while the smaller (more negative) δD value corresponds to $f_m = 40\%$. Given typical values for seawater $\delta D = 0\%$ and thermogenic "dry" methane $\delta D = -150\%$ (Schoell, 1980), δD values for both fatty acids and isoprenoids would be enriched in D by up to 100‰ relative to equivalent lipids produced by marine plants (Sessions et al., 1999). This is counterintuitive, because the methanotrophs consume a relatively D-depleted food source, and is opposite to the case for carbon isotopes where methane-consumers are ^{13}C -depleted relative to primary producers. Values of δD for biogenic methane as low as -300% are known (Whiticar et al., 1986), and in those cases δD values of lipids from methanotrophs would be indistinguishable from those of primary producers. We conclude that methanotroph lipids in the sedimentary record will be difficult to identify solely on the basis of D/H ratios.

4. CONCLUSIONS

1. In our cultures, $31 \pm 2\%$ of all lipid hydrogen in *M. capsulatus* was derived from methane, despite the fact that no hydrogen is transferred with the C-H bond intact from methane to lipids. This is apparently true for both *n*-alkyl and isoprenoid lipids, and for cultures in both exponential and stationary phase. The simplest explanation for this experimental observation is that all methane H is first oxidized to H_2O , which then serves as the hydrogen source for all biosynthesis.

2. A balance of diffusional processes provides a possible mechanism for regulating the content of cellular water derived from methane oxidation, and for producing a constant fraction of methane-derived H in all lipids. Because this mechanism does not rely on specific biochemical pathways, it could be a general feature of many organotrophs and/or hydrogenotrophs.

3. Isotopic fractionation between lipids and water ($\alpha_{l/w}$) in *M. capsulatus* averages 0.95 in fatty acids, and 0.85 in isoprenoid lipids. These fractionations are significantly smaller than those observed in photosynthetic organisms. Isotopic fractionation between lipids and methane ($\alpha_{l/m}$) averages 0.94 in fatty acids and 0.79 in isoprenoids.

4. Given the relationships derived from our cultures, native methanotrophs living in seawater are predicted to produce fatty acids with δD values of approximately -50 to -170% , and sterols and hopanols with δD values of approximately -150 to -270% . These values are similar or D-enriched relative to those of photoautotrophs. In contrast, methanotrophs commonly are strongly depleted in ^{13}C .

5. 3-Methylhopanol is enriched in D relative to coexisting hopanol by an average of 32‰. 16:0 fatty acids are enriched in D relative to coexisting 16:1 fatty acids by an average of 43‰. In both cases, differences in isotopic compositions cannot readily be explained by the small structural differences between the pairs of compounds. The differences therefore suggest some fundamental separation of biosynthetic processes between otherwise similar lipids.

6. δD values of lipids did not change when cultures were harvested during exponential vs. stationary phase growth. We view this as additional evidence that bacterial lipids do not turn over once they are synthesized, and preserve isotopic ratios prevalent during the time of growth.

Many uncertainties remain about the processes that transform hydrogen from methane and water into lipid hydrogen. Although the specific biochemical pathways present in *M. capsulatus* are unique in many ways, the fundamental processes involved in producing lipids are common to all organisms. Predictions about the flow of hydrogen to lipids based solely on the known details of biosynthetic pathways do not satisfactorily explain all of our data. It follows that attempts to understand isotopic fractionation in organotrophs must focus on the flow of reducing power (H^-) to lipids, and on cellular water budgets, rather than solely on the flow of C-bound H through biochemical pathways.

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