Metabolic Processes Account for the Majority of the Intracellular Water in Log-Phase *Escherichia coli* Cells As Revealed by Hydrogen Isotopes†

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ABSTRACT: It is generally believed that water transport across biological membranes is essentially a near-instantaneous process, with water molecules diffusing directly across the membrane as well as through pores such as aquaporins. As a result of these processes by which water can equilibrate across a membrane, a common assumption is that intracellular water is isotopically indistinguishable from extracellular water. To test this assumption directly, we measured the hydrogen isotope ratio of intracellular water in *Escherichia coli* cells. Our results demonstrate that more than 50% of the intracellular water hydrogen atoms in log-phase *E. coli* cells are isotopically distinct from the growth medium water and that these isotopically distinct hydrogen atoms are derived from metabolic processes. As expected, the $^2H/^1H$ isotope ratio of intracellular water from log-phase cells showed an appreciably larger contribution from metabolic water than did intracellular water from stationary-phase cells (53 ± 12 and $23 \pm 5\%$, respectively). The $^2H/^1H$ isotope ratio of intracellular water was also monitored indirectly by measuring the isotope ratio of fatty acids, metabolites that are known to incorporate hydrogen atoms from water during biosynthesis. Significantly, the difference in the isotopic composition of intracellular water from log- to stationary-phase *E. coli* cells was reflected in the hydrogen isotope ratio of individual fatty acids harvested at the two different times, indicating that the isotope ratio of metabolites can be used as an indirect probe of metabolic activity. Together, these results demonstrate that contrary to the common assumption that intracellular water is isotopically identical to extracellular water, these two pools of water can actually be quite distinct.

Water is known to move into and out of cells via at least two different mechanisms (*1*−*4*). Until relatively recently, it was thought that the principal mechanism by which water entered and exited the cell was via diffusion through the membrane (*5*). Although polar molecules are generally unable to diffuse across biological membranes, the small size of water allows it to move through defects in the membrane as the lipids diffuse laterally (*1*−*4*). With the discovery of aquaporins, however, it was realized that water could also be transported through channels at essentially diffusion-controlled rates (*6*, *7*). The rate at which these two processes can theoretically occur has led to the generally accepted assumption that intracellular water is isotopically indistinguishable from extracellular water.

The apparent simplicity of water diffusion belies the true complexity of the situation. The cell is constantly undergoing numerous metabolic processes, many of which alter the intracellular water composition either directly by generating new water molecules (e.g., dehydration reactions, respiration, etc.) or indirectly through the generation of CO_2 [whose oxygen atoms can rapidly exchange with water due to the activity of carbonic anhydrase (*8*)] and other biomolecules such as carbohydrates capable of exchanging with water. Thus, it is possible that intracellular water could be quite distinct from extracellular water. In fact, a number of studies have demonstrated that the equilibration of water in plants and animals is a time-dependent process with the existence of different pools of water (*9*−*11*), one of which may be an intracellular pool (*12*−*14*). These conclusions are consistent with other recent data suggesting that different pools of water within cells have different diffusion rates (*15*−*18*).

We began to question the assumption that intracellular water is isotopically indistinguishable from extracellular water when using oxygen as a tracer to follow the biosyn-

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thesis of heme O (*19*). According to the accepted mechanisms of heme O biosynthesis, the oxygen atom of the 17-hydroxyethylfarnesyl moiety is derived from water (*20*). When we grew *Escherichia coli* cells in 95% $H_2{}^{18}O$, however, a significant fraction of the heme O molecules did not contain a labeled oxygen atom (*19*), suggesting that the isotope ratio of intracellular water can be different from that of extracellular water. Our subsequent experiments using isotope ratio mass spectrometry (IRMS)[1] to measure directly the intracellular water $^{18}O/^{16}O$ ratio confirmed that the isotope ratio of intracellular water can be isotopically distinct (*21*). In fact, our data demonstrate that approximately 70% of the intracellular water oxygen atoms extracted from log-phase *E. coli* cells grown in 2× LB are derived from metabolic processes and can therefore be isotopically distinct from extracellular water (*21*).

Given that a large percentage of the oxygen atoms in intracellular water can be derived from metabolic processes, we hypothesized that the same would also be true for hydrogen atoms. However, because protons can diffuse across membranes in a manner independent of oxygen atoms (e.g., through proton channels) (*22*), we predicted that the percentage of hydrogen atoms in intracellular water that are derived from metabolism might be lower than the percentage of oxygen atoms. To test our hypothesis, we performed a variety of studies using IRMS to measure the $^2H/^1H$ isotope ratio of intracellular water directly as well as indirectly using the $^2H/^1H$ ratio of fatty acids (biomolecules known to incorporate hydrogen atoms from solvent) (*1*, *23*−*26*).

In this study, we report that the hydrogen isotope ratio of intracellular water in *E. coli* can also be distinct from that of growth medium water and that it, too, appears to be a function of metabolic activity of the cell. These results, which provide important and independent confirmation of our oxygen isotope studies, reinforce the notion that the widely held assumption that intracellular water is isotopically identical to extracellular or growth medium water is not necessarily true. In fact, in rapidly metabolizing organisms, intracellular water can be significantly different from extracellular water, and this realization could have a profound impact on the fields of biochemistry, cellular biology, and biogeochemistry.

EXPERIMENTAL PROCEDURES

Cell Cultures. E. coli BL21(DE3) cultures were grown in 2× Miller Luria-Bertani (LB) broth (EMD Chemicals) at 37 °C to either mid-log or stationary phase as previously described (*21*). The cells were then collected via filtration and transferred to a vial; the vial was sealed, and the contents were frozen. Water was then extracted cryogenically from the cell pellets and spent medium samples (*21*). The desiccated cell pellets were stored at room temperature prior to lipid extraction.

Fatty Acid Extraction and Analysis. Total fatty acids were extracted from desiccated cell pellets by saponification and then converted to methyl esters for structural analysis by gas chromatography−quadrupole mass spectrometry (GC−MS) and for isotope ratio measurements by gas chromatography−isotope ratio mass spectrometry (GC−IRMS) (*27*). All steps of the extraction and methylation were performed in glassware that had been baked at 500 °C for 8 h to remove any organic contamination. All aqueous solutions were extracted five times with hexane prior to use, and organic solvents were of the highest grade and used without further purification. Control blank extractions showed no contamination when analyzed by GC−MS.

To prepare methyl esters of fatty acids, desiccated cell pellets were first saponified in 5 mL of 0.5 M NaOH for 2 h at 70 °C in 16 mm × 125 mm test tubes with Teflon-lined caps. The solution was then acidified to a pH of 3−6 by the dropwise addition of 4 M HCl; 2.5 mL of an aqueous 5% NaCl solution was added, and the mixture was extracted three times with methyl *tert*-butyl ether (MTBE). The organic layers were combined in a pear-shaped flask, and the majority of the MTBE was removed by rotary evaporation. The remaining solution was transferred to a borosilicate glass vial and evaporated to dryness under a stream of N_2. To methylate the isolated fatty acids, 1 mL of approximately 3% BF_3 in anhydrous methanol (Burdick and Jackson, Muskegan, MI) was added to the vial, which was capped with a Teflon-lined cap and sealed with Teflon tape. Methylation reaction mixtures were incubated for 2 h at 100 °C. The reaction mixture was transferred to a 16 mm × 125 mm test tube. The vial was rinsed three times with methanol and three times with hexane, with the rinse solutions added to the test tube; 2 mL of an aqueous 5% NaCl solution was added to the tube, and the mixture was extracted three times with 3 mL of hexane. The volume of the combined organic layers was reduced to approximately 100−200 μL by evaporation under a stream of N_2. The identity of major components of the mixtures was determined by GC−MS of 1 μL samples on a ThermoFinnigan Trace GC−MS equipped with a 30 m DB5 column.

Stable-Isotope Ratio Measurements. Stable-isotope ratio measurements were made at the Stable-Isotope Ratio Facility for Environmental Research at the University of Utah. Stable-isotope ratios are measured relative to internationally recognized standards (*28*). We calibrated laboratory standards to the international standards and then included the laboratory standards as internal standards in every run. Stable-isotope contents are expressed in "delta" notation as δ values in parts per thousand (‰), where $\delta‰ = [(R_A/R_{Std}) - 1] \times 1000‰$, and R_A and R_{Std} are the molar ratios of the rare to abundant isotope (e.g., $^2H/^1H$) in the sample and the standard, respectively. The standard used for both oxygen and hydrogen is Vienna Standard Mean Ocean Water (VSMOW) (*28*). The δ notation is nonlinear with respect to isotopic abundances, which can lead to large errors in calculation based on δ values if the range in isotope ratios is large, as is often the case with H (*29*). Although we report our water results in δ values, we made all calculations using R values, which is the exact form of the mass balance equations. There was no difference in the slopes calculated on the basis of R or δ values at the level they are reported in this paper (two decimal places).

The hydrogen stable-isotope ratios of water samples were determined on a ThermoFinnigan-MAT Delta Plus XL

[1] Abbreviations: GC−MS, gas chromatography−quadrupole mass spectrometry; FAME, fatty acid methyl ester; IRMS, isotope ratio mass spectrometry; LB, Luria-Bertini broth; MTBE, methyl *tert*-butyl ether; OD_{600}, optical density at 600 nm; R_A, molar ratio of the rare to the abundant isotope in sample A; TCEA, thermal conversion elemental analyzer; VSMOW, Vienna Standard Mean Ocean Water.

isotope ratio mass spectrometer (IRMS) (ThermoFinnigan-MAT, Bremen, Germany) equipped with a thermal conversion elemental analyzer (TCEA, ThermoFinnigan-MAT) and a GC-PAL autosampler (CTC Analytics, AG, Zwingen, Switzerland) (*30*). The injection volume was 0.5 μL. Water samples were analyzed in duplicate and the results averaged. The average standard deviation of repeated measurements of water standards was 2‰.

Stable hydrogen isotope ratios of lipids were measured on a ThermoFinnigan-MAT Delta Plus XL IRMS equipped with a Hewlett-Packard GC with a 30 m DB1 column coupled to a GC-TCIII interface. In this instrumental configuration, samples are injected into the GC and components of the mixture are separated on the GC column. The separated components then enter the pyrolysis reactor sequentially, and their hydrogen atoms are converted to H_2 gas. Each peak of H_2 enters the IRMS where its isotope ratio is determined; thus, the hydrogen isotope ratio of every well-separated compound present in sufficient quantity can be measured (*27*).

To characterize our system, we performed 24 injections over a two-week period of the standard alkane Mixture B prepared by Schimmelmann at the University of Indiana (Bloomington, IN) (*31*). The instrumental H_3 correction factor was determined daily. We used the data from two to three daily injections of Mixture B to validate the H_3 factor correction as described previously (*32*). The standard alkanes and fatty acid isotope ratio values were standardized against pulses of reference hydrogen gas ($\delta^2H = -202.45$‰) injected at the beginning and end of every run. The average absolute error of our measurements of the isotope ratio values of the individual standard alkane peaks was 4.5‰, with a standard deviation of 4.2.

We determined the correction factor for the three hydrogen atoms added to the fatty acids during the methylation step by measuring the hydrogen isotope ratio of a 9:0 fatty acid purchased from Alltech (Deerfield, IL) by direct injection into the TCEA, as described above for water. We then methylated the fatty acid using the procedure described above and measured the hydrogen isotope ratio of the FAME by GC−pyrolysis mass spectrometry. By comparing the hydrogen isotope ratios of the methylated and unmethylated forms of the fatty acid, we calculated that the ‰ ^2H value of the three hydrogen atoms added during methylation was −100‰. This calculation ignored the hydrogen atom on the carboxylic acid group, the isotope ratio of which we could not measure separately because it would have been lost during the methylation procedure. We assumed that the δ^2H value of the fatty acid was a function of its 17 alkyl hydrogen atoms, which contributed to the value of the ester. In so doing, we insert a potential inaccuracy into our calculation of the correction factor. As the ignored hydrogen atom was one of 18, the error is likely to be small and would not alter the correlation between the isotope ratios of the fatty acids and growth medium.

RESULTS

The Hydrogen Isotope Ratio of Intracellular Water Differs from That of Growth Medium Water. Water molecules can enter a cell via diffusion from the culture medium water or be generated during metabolic reactions. We showed previously that an isotopic gradient of water can be maintained during harvesting of cells by filtration (*21*) and that the water extracted from the filtered cell cake can be modeled as a two-component mixture of medium and metabolic water in which

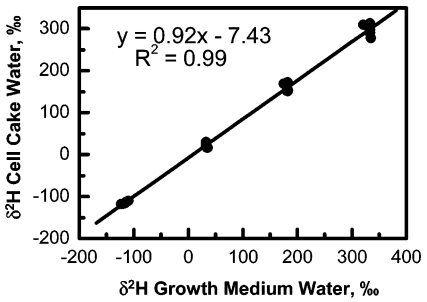

FIGURE 1: Regression of the hydrogen isotope ratio of extracted cell cake water vs that of growth medium water. Data are pooled from five experiments in which the cells were grown at 37 °C in 2× LB and harvested during mid-log phase.

$$\delta_{\text{cell cake}} = f(\delta_{\text{medium}}) + (1 - f)(\delta_{\text{metabolic}}) \qquad (1)$$

In these experiments, $\delta_{\text{cell cake}}$, δ_{medium}, and $\delta_{\text{metabolic}}$ are the hydrogen isotope ratios of the water extracted from the cell cake and the culture medium and of the metabolic water, respectively, and f is the fraction of the cell cake water that is identical to the culture medium water. If δ_{medium} is manipulated and $\delta_{\text{cell cake}}$ measured, eq 1 becomes the equation of a straight line where the slope is equal to f.

We grew four cultures of *E. coli* to mid-log phase in 2× LB medium made with isotopically varying water and harvested the cells on filters. The cell cakes were then scraped from the filters, sealed in vials, and frozen. Samples of the spent medium were also collected. Water was extracted from both the cell cakes and the spent medium, and the hydrogen isotope ratios were determined. This experiment was conducted five times.

The regressions of the plots of $\delta_{\text{cell cake}}$ versus δ_{medium} obtained in the five experiments were not significantly different ($F = 0.19$, where $F_{0.05} = 3.48$) (*33*), and the data from the five experiments were therefore combined (Figure 1). The slope of the regression line of the pooled data is 0.92 with a 95% confidence interval of 0.03. This result suggests that approximately 8% of the hydrogen atoms in extracted cell cake water were isotopically distinct from extracellular water. It is consistent with our earlier finding that ∼10% of the oxygen atoms in similar samples were metabolic (*21*). The average slope of the oxygen experiments was 0.90 with a 95% confidence interval of 0.04. The average slopes of the oxygen and hydrogen regressions are not significantly different ($F = 0.06$, where $F_{0.05} = 4.1$).

To test whether the hydrogen isotope ratio of intracellular water was correlated with metabolic activity, we repeated the experiment but instead harvested the cells after they had entered stationary phase, ∼12 h post-inoculation. The slope of the pooled data from two trials was 0.965, significantly different from the log-phase slope ($F = 16.8$, where $F_{0.01} = 7.8$). The results of this experiment indicate that when *E. coli* cells were harvested in stationary phase, 3.5% of the hydrogen atoms in the extracted cell cake water were isotopically distinct from growth medium water instead of 8%. These results are again very similar to our data with

Table 1: Regression Statistics from Washing Experiments of Extracted Cell Cake Water versus Wash Water

growth phase of cells	δ^2H for culture water (‰)	slope of δ^2H for extracted cell cake water vs wash water	R^2 of regression	*y*-intercept	calculated δ^2H of intracellular water (‰)[c,d]
log	−115	0.80	0.99	−18.1	−90.5
log	32	0.85	0.99	−10.6	−70.7
log	187	0.90	1.0	−0.3	−3.0
log	342	0.86	0.99	7.8	55.7
average[a]		0.85 (SE[e] = 0.21)			
stationary[b]	−120	0.88	0.99	−17.4	ND[f]
stationary[b]	−119	0.87	1.0	−14.3	ND[f]

[a] Average slope of log-phase experiments. The slopes of the individual log-phase experiments are not statistically different [$F = 0.88$, where $F_{0.05} = 4.35$ (*33*)]. [b] The slopes of the stationary-phase experiments were not statistically different from those of the log-phase experiments ($F = 0.56$, where $F_{0.05} = 4.1$). [c] The δ ^{2}H/^{1}H value of intracellular water = (*y*-intercept)/(1 − slope) in eq 2. Intracellular water is itself a combination of growth medium water and metabolic water as described by eq 3. [d] The average and standard error are not presented because the *y*-intercept values and the δ^2H/^{1}H values of the intracellular water are expected to be different due to the different δ^2H/^{1}H values of the growth medium waters. [e] Standard error. [f] Not determined.

oxygen isotope ratios, where the average slope of the stationary-phase experiments was 0.961.

In an additional experiment, the effect of metabolic rate was assessed by comparing intracellular water from cells grown at different temperatures. Two identical cultures were prepared; one was incubated at the standard temperature of 37 °C, while the second was incubated at 18 °C. The cells were harvested at log phase, and the water was cryogenically extracted in an identical fashion; the only difference between the two cultures was the incubation temperature and, therefore, the metabolic rate. As expected, a plot of cell cake water versus growth medium water yielded a slope that was significantly larger for the 18 °C cells than for the 37 °C cells (data not shown), indicating that a substantially smaller fraction of intracellular water is isotopically distinct from growth medium water when the cells are incubated at a reduced temperature. Together, these data are consistent with the hypothesis that the istopically distinct hydrogen atoms are derived from metabolism.

Approximately 50% of the Hydrogen Atoms in Intracellular Water in Log-Phase E. coli Cells Are a Product of Metabolism. To determine the percentage of intracellular water that is isotopically distinct from growth medium water and presumably derived from metabolism, it is necessary to account for the fact that water extracted from a cell cake contains both intracellular and extracellular water. To do this, we model the extracted cell cake water as a two-component mixture of intracellular and extracellular water:

$$\delta_{\text{cell cake}} = g(\delta_{\text{extracellular}}) + (1 - g)(\delta_{\text{intracellular}}) \quad (2)$$

where g is the fraction of the cell cake water that is extracellular water and $\delta_{\text{extracellular}}$ and $\delta_{\text{intracellular}}$ are the hydrogen isotope ratios of the extracellular and intracellular water, respectively. If $\delta_{\text{extracellular}}$ is manipulated and $\delta_{\text{cell cake}}$ measured, eq 2 becomes the equation of a straight line where the slope is equal to g.

We grew a culture of *E. coli* to mid-log phase in 2× LB, split the culture into four aliquots, and immediately harvested them on separate filters. As soon as the cell cakes appeared dry on the filters, they were washed with fresh 2× LB made with isotopically distinct water. This washing procedure replaced the extracellular water in the cake, and the isotope ratios of the water in the 2× LB used to wash the cell cakes ($\delta_{\text{wash solution}}$) were therefore equal to $\delta_{\text{extracellular}}$ in eq 2. We extracted water from the washed cell cakes and the wash solutions, measured their δ^2H values, and regressed the cell cake water values onto the wash water (Table 1). This experiment was conducted four times, varying the isotopic composition of the growth medium water. An *F* test showed that the slopes of the regression lines were not significantly different.

The average slope was 0.85 (Table 1), indicating that 15% of the total cell cake water was intracellular. This result was also very similar to that of our oxygen experiments (*21*), where the average slope of washing experiments was 0.86. An *F* test showed that the slopes of the oxygen and hydrogen regressions of wash water onto extracted cell cake water were not statistically different ($F = 0.005$, where $F_{0.05} = 4.1$). Two experiments with stationary-phase cells yielded slopes that were statistically indistinguishable from the slopes obtained with log-phase cells, indicating that the same percentage of cell cake water was intracellular when the cells were harvested at stationary phase ($F = 0.56$, where $F_{0.05} = 4.1$).

Using these data, we can calculate the fraction of hydrogen atoms in intracellular water that was derived from metabolism in log-phase cells as 0.08 (the fraction of hydrogen atoms that were distinct from medium water; Figure 1)/0.15 (the fraction of hydrogen atoms that were intracellular; Table 1) = 0.53 (or 53%). The total error in this estimation is 12% when the standard errors of the two slopes (0.014 for metabolic water and 0.021 for intracellular water) are used in a propagation-of-errors calculation (*34*). Our previous work showed that ∼71% of the oxygen atoms in intracellular water were derived from metabolism during log-phase growth, with a total error in the estimate of 19% (*21*).

At stationary phase, the slope of the extracted cell cake water versus medium water was 0.965. Thus, the fraction of cell cake water derived from metabolism is 0.035/0.15, or 23%. The total error in this estimation is 5% when the standard errors of the two slopes (0.007 for the stationary-phase metabolic water and 0.014 for the intracellular water) are used in a propagation-of-errors calculation (*34*). This compares very well with our previous data that indicated only ∼29% of the oxygen atoms in intracellular water were derived from metabolism in stationary-phase *E. coli* cells (*21*).

Calculating the Hydrogen Isotope Ratio of Metabolic Water. There are two independent methods of calculating the hydrogen isotope ratio of intracellular water. The first method is derived from eq 1 [$\delta_{\text{cell cake}} = f(\delta_{\text{medium}}) + (1 -$

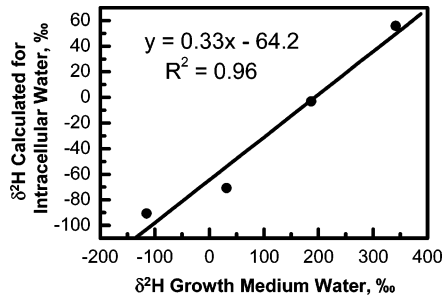

FIGURE 2: Regression of the calculated value of the hydrogen isotope ratio of intracellular water as determined from the washing experiments vs the hydrogen isotope ratio of the growth medium water. The slope of 0.33 (with a 95% confidence interval of 0.19) indicates that 48−86% of the water was generated during metabolism, in agreement with the value calculated independently using the regression in Figure 1.

$f)(\delta_{metabolic})]$. In our growth experiments, we manipulated δ_{medium} so that the slope of the graph in Figure 1 is equal to f and the intercept is equal to $(1 - f)(\delta_{metabolic})$. Therefore, we can calculate $\delta_{metabolic}$ by dividing the intercept value, −7.43, by $1 - f$, giving a result of approximately −93‰. The total error in this estimate is ∼40‰ as determined in a propagation-of-errors calculation (*34*) using the standard errors of measurement of the slope (0.014) and the intercept (2.8).

The second method for estimating $\delta_{metabolic}$ uses data from the wash experiments. In this approach, we must first calculate the δ^2H value of intracellular water. In eq 2, the y-intercept from the washing experiments is equal to $(1 - g)(\delta_{intracellular})$, where g is the slope of the line. Thus, dividing the intercept value by $1 - g$ yields an estimate of the δ^2H value of the intracellular water (Table 1). According to our model, the isotope ratio of the intracellular water can be represented as

$$\delta_{intracellular} = h\delta_{growth\ medium} + (1 - h)\delta_{metabolic} \quad (3)$$

where h is the fraction of intracellular water that originated from the growth medium. A plot of the calculated $\delta_{intracellular}$ values versus the measured $\delta_{growth\ medium}$ values yielded a regression slope of 0.33, representing h (Figure 2). The δ^2H value of the metabolic water is equal to the y-intercept value divided by $1 - h$. This value is −96‰, almost identical to the value estimated from the data in Figure 1, but derived using independent data.

The data in Figure 2 are also consistent with our previous estimate of the fraction of intracellular water that is derived from metabolism. From eq 3, that fraction of intracellular water is equal to $1 - h$, or 0.67. The 95% confidence interval for the slope shown in Figure 2 is 0.19, giving a range of values for $1 - h$ of 0.48−0.86, consistent with our previous estimate of 0.53 ± 0.11. When we used data from the stationary-phase growth experiments (slope = 0.965; y-intercept = 6.38), we calculated a value of −179‰ for the δ^2H value of metabolic water at stationary phase. The total error in this calculation is 54.5‰ (standard error of slope = 0.007 and of intercept = 1.4) as determined by a propagation-of-errors calculation (*34*).

The Isotope Ratio of Hydrogen Atoms in Fatty Acids Correlates with Intracellular Water. According to our model, if approximately 53% of the hydrogen atoms in log-phase intracellular water originate from metabolic activity, then the remaining 47% are equivalent to the culture medium water. Likewise, in stationary phase, ∼23% of the hydrogen atoms originate from metabolic activity, and the remaining 77% are equivalent to culture medium water. Presumably, the percentage of intracellular water that is isotopically equivalent to culture medium water increases as the culture progresses from log to stationary phase. We accordingly hypothesized that the difference in the contribution from culture medium water to intracellular water would be reflected in the hydrogen isotope ratios of fatty acids biosynthesized during log phase or later in the life of the culture.

The isotopic relationship between culture water, nutrients, and lipids can be expressed precisely in the equation

$$R_{fa} = f_{water}\alpha_{water}R_{water} + (1 - f_{water})\alpha_{nutrients}R_{nutrients} \quad (4)$$

where R_{fa}, R_{water}, and $R_{nutrients}$ represent the hydrogen isotope ratios (R values) of the fatty acid, culture water, and nutrients, respectively, f_{water} is the fraction of the fatty acid hydrogens that is derived from water, and α_{water} and $\alpha_{nutrients}$ (defined as R_{fa}/R_{water} and $R_{fa}/R_{nutrients}$, respectively) are the isotope fractionation factors between water and the fatty acid and between nutrients and the fatty acid, respectively. A regression of R_{fa} versus R_{water} should therefore yield a line with a slope of $f_{water}\alpha_{water}$ with an intercept of $(1 - f_{water})\alpha_{nutrients}R_{nutrients}$. If α_{water} can be assumed to be relatively constant between log and stationary phases (see the Discussion), then any change in the slope of the regression using the R values of fatty acids harvested from log- or stationary-phase cells would be ascribable to a change in f_{water}.

We prepared and methylated total fatty acids from cell pellets we had saved from two of the log-phase experiments and two of the stationary-phase experiments described above. These samples comprised two independent sets of four log-phase and four stationary-phase cultures produced in 2× LB made with isotopically varying water (16 total cultures). The hydrogen isotope ratio of the growth medium water at the time the cells were harvested had previously been determined.

The identity of various fatty acid methyl ester peaks was established by GC−MS. We determined the hydrogen isotope ratios of individual fatty acids by GC−IRMS, making a minimum of three independent measurements of each preparation. The average standard deviation of the triplicate measures of 14:0 and 16:0 fatty acid methyl esters from all four preparations was 3.4‰. A comparison of the slopes of the regressions of R_{fa} versus R_{water} of 14:0 and 16:0 fatty acids isolated from log- and stationary-phase cells (Table 2) shows that the slope of the regression of R_{fa} onto R_{water} is significantly greater in stationary phase. In other words, a greater percentage of the hydrogen atoms in 14:0 and 16:0 fatty acids are derived from extracellular water when the cells are in stationary phase, or conversely, fatty acids in log-phase cells contain more hydrogen atoms from metabolic water.

DISCUSSION

Metabolic Water. Using stable hydrogen isotopes as a probe, we have demonstrated that intracellular water can be isotopically distinct from extracellular water. Our results

Table 2: Regression Data of $R_{\text{fatty acid}}$ versus $R_{\text{medium water}}$ of 14:0 and 16:0 Fatty Acids Prepared from Two Independent Sets Each of Log-Phase and Stationary-Phase Cells[a]

	set A		set B		A and B	
fatty acid/growth phase	slope	SE[b]	slope	SE[b]	slope	SE[b]
14:0/log phase	0.58	0.03	0.54	0.01	0.56	0.02
14:0/stationary phase	0.76	0.01	0.70	0.02	0.73	0.02
16:0/log phase	0.61	0.03	0.55	0.02	0.58	0.03
16:0/stationary phase	0.75	0.00	0.69	0.01	0.72	0.01

[a] Column "A and B" shows the slopes of the relationships when the data from the two sets of cultures were pooled. The R values were calculated from the δ values according to the equation $\delta = [(R_{\text{sample}}/R_{\text{Std}}) - 1] \times 1000$, where $R_{\text{Std}} = R_{\text{VSMOW}} = 0.0001558$. [b] Standard error.

demonstrate that approximately 53% of the hydrogen atoms from intracellular water in log-phase *E. coli* cells are isotopically distinct from extracellular water and that these isotopically distinct hydrogen atoms are formed during metabolic processes. When the cells reach stationary phase, however, only 23% of the intracellular water hydrogen atoms are derived from metabolism, indicating that the ability of cells to maintain a large isotope gradient depends on their metabolic rate.

Our data allow us to calculate the isotopic ratio of metabolically-derived hydrogen atoms in intracellular water. Interestingly, the hydrogen isotope ratio of this metabolically formed water is −96‰ in log-phase cells, but δ^2H is −179‰ in stationary-phase cells. It is important to note that metabolic water consists of individual hydrogen and oxygen atoms within the pool of intracellular water molecules that did not originate as culture water but rather were derived from metabolic reactions. The source of the hydrogen atoms in metabolic water detected in these experiments is the hydrogen atoms in the nutrient molecules of the yeast extract and tryptone (an enzymatic hydrolysate of casein) supplied in the LB medium. Over the life cycle of a culture growing in LB, pools of specific substrate molecules could be depleted so that the bacteria would be metabolizing different mixtures of molecules with potentially differing hydrogen isotope ratios at different times. Thus, one possible factor contributing to the difference in the δ^2H values of metabolic water at log and stationary phases could be the changing substrate pools and accompanying changes in metabolic pathways.

Another factor that could contribute to the difference in the apparent δ^2H values of metabolic water at log and stationary phases is proton pumping (*35*), which could alter the hydrogen isotope ratio of intracellular water. The pK_a for H_2O is 14.00, while that of D_2O is 14.9, indicating that D_2O tends to dissociate almost 10 times less than H_2O (*36*). Thus, proton pumping might serve to enrich intracellular water by removing proportionally more protons than deuterons. If proton pumping is more active in log phase than in stationary phase, the isotope ratio of intracellular water in log-phase cells would be more enriched than that of stationary-phase cells, consistent with our data.

Isotopic Signature of Intracellular Water in Fatty Acids. The biosynthesis of saturated acyl fatty acids consists of repeated cycles of a four-step process in which (1) an acetate unit is added to the growing acyl chain in a transacylation reaction, (2) the carboxyl group of the acetate moiety is reduced, (3) the resulting hydroxyl and a hydrogen from the adjacent carbon are removed to generate a double bond, and (4) the double bond is then reduced (*24*). Steps 2−4 of this process comprise reactions in which hydrogen atoms are either added (steps 2 and 4) or removed (step 3) from the intermediate, and it is therefore expected that the hydrogen isotope ratio of fatty acids will be affected by the isotope ratio of the intracellular water at the time of biosynthesis (*37*). A complicating issue, however, is that hydrogen isotope fractionation at steps 2−4 will each contribute to the value of α_{water}, which represents the cumulative isotopic fractionation between culture water and the resulting fatty acid.

Our interpretation of the regression coefficients in Table 2 is that they reflect a larger contribution of hydrogen atoms from culture water to the hydrogen atoms of fatty acids in stationary phase than in log phase (*37*). This interpretation assumes that the differences are not caused by differences in α_{water} between log and stationary phases. Isotopic fractionation in biochemical processes arises from unequal zero-point energies of bonds to heavy and to light isotopes resulting in different activation energies (*38*). Thus, fractionation factors (α) are a function of both temperature and the energetics of the individual enzyme-catalyzed reactions that comprise the pathway. The culture growth temperature was held constant throughout our experiments, and consequently, the value of α_{water} should not change if the enzymology of fatty acid biosynthesis remains constant. No evidence suggests that the enzymology of fatty acid biosynthesis differs between log and stationary phases (*26*, *39*, *40*), and it is therefore reasonable to presume that there is no difference in α_{water} between log and stationary phases.

Our data indicate that the contribution of culture medium water to intracellular water increases as the culture progresses from log phase to stationary phase. On the basis of this information, we would predict that hydrogen atoms in fatty acids from cells harvested during stationary phase would have a greater contribution from culture medium water than fatty acids harvested from cells in log phase. This prediction is consistent with our observations (Table 2). Unfortunately, because of the number of unknown variables in eq 4, we cannot use the data in Table 2 to calculate directly the fraction of intracellular water derived from metabolism (*29*). Nevertheless, these data are very important because they confirm that the isotope ratios of metabolites can be used as indirect probes of metabolic rate in living cells.

Comparing Oxygen and Hydrogen Isotope Data. It is interesting to compare the hydrogen isotope results reported herein to our previously published results for oxygen isotopes (*21*). As noted above, our data indicate that 53 ± 12% of the hydrogen atoms found in intracellular water extracted from log-phase *E. coli* cells grown in 2× LB are isotopically distinct from extracellular water. Measuring the $^{18}O/^{16}O$ ratio of intracellular water from *E. coli* cells grown under the same conditions, however, we calculated that 71 ± 19% of the oxygen atoms were isotopically distinct from growth medium water and were generated during metabolic processes (*21*). Both sets of data indicate that metabolically generated water is an important and substantial component of intracellular water in *E. coli*.

Two possible explanations can account for the different percentage of hydrogen and oxygen atoms in intracellular water that is derived from metabolism. The first trivial explanation is that within experimental errors [which were calculated from a propagation of error in the slopes of the

regressions (*34*)], these numbers are not different at all. The second explanation is that hydrogen atoms and oxygen atoms in metabolic water exchange with extracellular water at different rates. When water diffuses into or out of a cell either directly across the membrane or through aquaporin channels, both hydrogen and oxygen atoms are exchanged. These processes would therefore be expected to maintain parity between calculated percentages of intracellular water that is derived from metabolic processes using either hydrogen isotopes or oxygen isotopes. In addition to transport as water, however, hydrogen ions can also pass through membranes in a manner independent of oxygen atoms (*22*). The mechanisms by which protons can be transported across membranes include, but are not limited to, (1) proton permeation through membranes, (2) active transport via proton-pumping enzymes (e.g., cytochrome *c* oxidase), (3) diffusion via voltage-gated proton channels, and (4) diffusion via proton-permeable ion channels (e.g., gramicidin) (*22*). Thus, there are clearly a variety of pathways by which protons in metabolic water can exchange with extracellular water, many of which are not available to oxygen ions, and therefore, there is no requirement that the percent of metabolic water calculated using these two different isotopes be equivalent. Nevertheless, the key conclusion that the majority of intracellular water in log-phase *E. coli* cells is generated during metabolic processes is supported by both sets of data.

Sources of Hydrogen and Oxygen in Metabolic Water. An important issue in the generation of metabolic water is the initial source of the hydrogen and oxygen atoms. As discussed above, the source of the protons in metabolic water is the LB growth medium. The oxygen atoms, however, have more than one potential source. In addition to the LB medium, oxygen atoms in metabolic water can also come from O_2 during respiration as the O_2 is reduced to water. While it might be tempting to conclude that atmospheric oxygen is the source of a large fraction of the oxygen atoms in metabolic water, this may not be the case. Recent evidence suggests that a significant fraction of the water generated by the action of cytochrome *c* oxidase in *Rhodobacter sphaeroides* is released into the periplasmic space (*41*), which would presumably rapidly equilibrate with extracellular water. However, even if cytochrome bo_3 of *E. coli* also releases water toward the "outside" of the cell, like *R. sphaeroides*, it is still theoretically possible that the isotopically distinct intracellular water we extracted resided in the periplasmic space. It is also possible that water released in this way diffuses or is transported back into the cytoplasm. Currently, our data do not allow us to address whether O_2 contributes significantly to the oxygen ions in metabolic water, and experiments are underway to address this question.

Another potential source of oxygen atoms in metabolic water is the LB medium. While both hydrogen and oxygen atoms in LB growth media can be released as water or otherwise solvent exchangeable atoms during biochemical processing, many of the "organic" oxygen atoms found in nutrients are released as CO_2. The oxygen atoms in CO_2 can then exchange with intracellular water as a result of the enzymatic activity of carbonic anhydrase before the CO_2 diffuses into the atmosphere (*8*). The best way to determine if the organic oxygen in the growth medium is a major component of the oxygen atoms in metabolic water is to grow *E. coli* in a medium in which the $^{18}O/^{16}O$ ratio is varied while all the other variables are kept constant. We are currently conducting these experiments.

Potential Sources of Error. We observe that both hydrogen and oxygen isotope ratios of water extracted from cell cakes are distinct from growth medium water. Is it possible that this observation can be caused by some phenomenon other than the presence of metabolic water inside of the cell? A trivial mechanism that could produce isotopic changes in cell cake water is evaporation. However, evaporation usually results in an increase in the concentration of heavy isotopes in the residual water, and our cell cakes were not uniformly isotopically enriched. While some samples were isotopically enriched relative to growth medium water, others sample were isotopically depleted. In addition, the slopes calculated from eq 2 using both hydrogen and oxygen isotope data (see Table 1 herein and Table 2 in ref *21*, respectively) are essentially identical. This provides additional evidence that evaporation is not a major artifact in our experiments because hydrogen and oxygen have different evaporative fractionations (*42*), and the two data sets would have had different slopes if evaporation was the major driving force. Nevertheless, while we can rule out evaporative enrichment as a primary source of error, it is possible that evaporation could be modulating the magnitude of our final results.

The proton pumping mentioned above would also cause the isotope ratio of intracellular water to be different from that of extracellular water (*35*). Again, however, the hypothesized isotopic enrichment due to proton pumping should cause the isotope ratio of intracellular water to be enriched compared to that of growth medium water in every sample, which is not what we observed. In addition, proton pumping cannot explain the data we obtained for the oxygen isotope ratio of water extracted from cell cakes, which is consistent with our hydrogen isotope ratio data (*21*).

Another possible mechanism that would cause the isotope ratios of our extracted cell cake water to be different from the growth medium water is incomplete extraction of water from the cell cakes. Extraction of water from our samples was accomplished by distillation in which the samples were heated under vacuum and the water vapor was collected in a cold finger. Distillation follows Rayleigh kinetics, and if the process is incomplete, the remaining water is isotopically enriched while the distillate is isotopically depleted relative to the initial pool. The extracted cell cake water in our samples was not uniformly isotopically depleted relative to growth medium water. Furthermore, if a pool of nonexchangeable and unextractable water does exist in the cell, it would have to be isotopically distinct from growth medium water to account for our data.

Our data are consistent with a two-component mixture, and our hypothesis is that this second component is metabolically-derived water. Is it possible that the second component is something other than metabolic water? One possible source of a second, nonmetabolic component is condensation. The cell cakes are stored at −20 °C, and it is theoretically possible that sufficient condensation forms on the samples and/or tubes prior to extraction to alter the isotopic ratio of the cell cake water. However, the calculated isotope ratio of this second component [$\delta^{18}O = -3.5‰$ (*21*); $\delta^{2}H = -96‰$] is not consistent with condensation of

atmospheric water vapor (*43*).[2] In addition, when the cells were chilled to 6 °C for 90 min prior to being harvested, the fraction of extracted cell cake water that was isotopically distinct from growth medium water was significantly reduced relative to that in cells that were not chilled (*21*). Because both the chilled and unchilled samples were the same size and were frozen and treated in an identical manner, it is expected that the amount of condensation that would form on the samples would be equal. The fact that the chilled cell cakes contained a significantly smaller fraction of isotopically distinct water and the fact that the isotope ratios of this metabolically distinct water were inconsistent with meteoric water provide evidence that conflicts with the hypothesis that condensation forms the second water component.

Could there be components other than water that are altering the isotopic ratio of the extracted cell cake water? The H/O ratios of the cell cake water as determined by TCEA indicate that if there are multiple components, they must be cellular components that are volatile at physiological pH and either have the same H/O ratio as water or are present in relatively low abundance. In addition, a variety of our data suggest that intracellular water truly is isotopically distinct from extracellular water, and it therefore seems unlikely that this is a significant source of error. Nevertheless, it is a possibility that cannot be completely ruled out.

It is important to note that both the hydrogen isotope ratio of extracted fatty acids described herein and the oxygen isotope ratio of isolated heme O (*19*) suggest that intracellular water can be isotopically distinct from growth medium water, and our data suggest that the origin of this isotopically distinct water is metabolism. The notion that metabolic water is present inside the cell is not surprising, as water is one of the catabolic end products of aerobic respiration. What is perhaps surprising, however, is that the apparent fraction of metabolically-derived hydrogen atoms in intracellular water is so large. Our calculation of the apparent fraction depends on the slopes in eqs 1 and 2. The slopes were determined by data regression, and we subtracted those values from 1.0 to yield the fraction of water in the cell cakes that is a product of metabolism (isotopically distinct from growth water, eq 1) and the fraction of cell cake water that is intracellular water (isotopically distinct from wash water, eq 2). The calculated values are small and therefore very sensitive to the slopes determined in the regressions. Factors that have a small effect on the data could have a significant effect on the calculated values. For example, if metabolic activity continued for the several seconds between the time the cell cake appeared dry on the filter and the time that it became frozen, and this water was exported out of the cell, it would alter both the calculated ratio of intracellular to extracellular water as well as the percentage of intracellular water that is metabolically derived. While this phenomenon would not affect our overall conclusions, it could alter the magnitude of our values, although it is difficult to predict the extent to which the calculations would be affected.

Finally, it should be stressed that none of the potential sources of error discussed above can, by itself, completely account for our data. While it is possible that any or all of them influence the absolute values reported here, the simplest explanation for our data is that metabolic activity does indeed change the isotope ratio of intracellular water and that these changes are reflected in the isotope ratios of metabolites that derive oxygen and/or hydrogen atoms from water. Thus, despite the fact that various sources of error (or some combination of errors) cannot be ruled out, the available data support our hypothesis that a significant fraction of intracellular water in log-phase *E. coli* cells is isotopically distinct from growth medium water and is probably derived from metabolism.

[2] The isotope ratios of meteoric water are described well by the Global Meteoric Water Line where $\delta^2H \approx (8\delta^{18}O + 10)‰$. The oxygen and hydrogen isotope ratios of local Salt Lake City water are approximately −16 and −120‰, respectively.

CONCLUSION

In summary, the data presented herein demonstrate that a significant fraction of the intracellular water in log-phase *E. coli* cells grown in 2× LB is derived from metabolic processes. Specifically, analysis of the hydrogen isotopes indicates that ∼53% of the hydrogen atoms in intracellular water in log-phase cells are derived from metabolism. As expected, this percentage decreases in stationary-phase cells, dropping to ∼23%. The percentage of intracellular water that is derived from metabolism is slightly lower when calculated using hydrogen isotopes (53 ± 12%) instead of oxygen isotopes (71 ± 19%). These differences may be due to inherent error in the calculations or to the more rapid exchange of protons with extracellular water. Significantly, the isotope ratio of intracellular water is reflected in the isotope ratios of fatty acids, indicating that the hydrogen and oxygen isotope ratios of metabolites can be used as an indirect probe for metabolic activity in living cells.

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REFERENCES

1. García-Martín, M. L., Ballesteros, P., and Cerdan, S. (2001) The Metabolism of Water in Cells and Tissues as Detected by NMR Methods, *Prog. Nucl. Magn. Reson. Spectrosc. 39*, 41−77.
2. Haines, T. H. (1994) Water Transport Across Biological Membranes, *FEBS Lett. 346*, 115−122.
3. Voet, D., and Voet, J. G. (2004) *Biochemistry*, 3rd ed., John Wiley & Sons, Hoboken, NJ.
4. Devlin, T. M. (2005) *Textbook of Biochemistry with Clinical Correlations*, 6th ed., John Wiley & Sons, Hoboken, NJ.
5. Booth, I. R., and Louis, P. (1999) Managing Hypoosmotic Stress: Aquaporins and Mechanosensitive Channels in *Escherichia coli*, *Curr. Opin. Microbiol. 2*, 166−169.
6. Stroud, R. M., Nollert, P., and Miercke, L. (2003) The Glycerol Facilitator GlpF, Its Aquaporin Family of Channels, and Their Selectivity, *Adv. Protein Chem. 63*, 291−316.
7. Calamita, G., Kempf, B., Bonhivers, M., Bishai, W. R., and Bremer, E. (1998) Regulation of the *Escherichia coli* Water Channel Gene *aqpZ*, *Proc. Natl. Acad. Sci. U.S.A. 95*, 3627−3631.
8. Silverman, D. N., and Lindskog, S. (1988) The Catalytic Mechanism of Carbonic Anhydrase: Implications of a Rate-Limiting Protolysis of Water, *Acc. Chem. Res. 21*, 30−36.
9. Speakman, J. R., Visser, G. H., Ward, S., and Król, E. (2001) The Isotope Dilution Method for the Evaluation of Body Composition, in *Body Composition Analysis of Animals* (Speakman, J. R., Ed.) pp 56−98, Cambridge University Press, Cambridge, U.K.

10. Nagy, K. A. (1989) Doubly-Labeled Water Studies of Vertebrate Physiological Ecology, in *Stable Isotopes in Ecological Research* (Rundel, P. W., Ehleringer, J. R., and Nagy, K. A., Eds.) pp 270−286, Springer-Verlag, New York.
11. Farquhar, G. D., and Lloyd, J. (1993) Carbon and Oxygen Isotope Effects in the Exchange of Carbon Dioxide between Terrestrial Plants and the Atmosphere, in *Stable Isotopes and Plant Carbon-Water Relations* (Ehleringer, J. R., Hall, A. E., and Farquhar, G. D., Eds.) pp 47−70, Academic Press, San Diego.
12. Zhu, X.-H., Zhang, N., Zhang, Y., Zhang, X., Ugurbil, K., and Chen, W. (2005) In vivo ^{17}O NMR Approaches for Brain Study at High Field, *NMR Biomed. 18*, 83−103.
13. Yakir, D. (1992) Water Compartmentatin in Plant Tissue: Isotopic Evidence, in *Water and Life: Comparative Analysis of Water Relationships at the Organismic, Cellular, and Molecular Levels* (Somero, G. N., Osmond, C. B., and Bolis, C. L., Eds.) pp 205−222, Springer-Verlag, Berlin.
14. Yakir, D. (1992) Variations in the Natural Abundance of Oxygen-18 and Deuterium in Plant Carbohydrates, *Plant, Cell Environ. 15*, 1005−1020.
15. Zhu, X.-H., Lei, H., Zhang, Y., Zhang, X., Zhang, N., Ugurbil, K., and Chen, W. (2002) *Proc. Int. Soc. Magn. Res. Med.*, 1094.
16. Potma, E. O., de Boeij, W. P., van Haastert, P. J. M., and Wiersma, D. A. (2001) Real-Time Visualiztion of Intracellular Hydrodynamics in Single Living Cells, *Proc. Natl. Acad. Sci. U.S.A. 98*, 1577−1582.
17. Wiggins, P. M. (2002) Water in Complex Environments Such as Living Systems, *Physica A 314*, 485−491.
18. Wiggins, P. M. (2001) High and Low Density Intracellular Water, *Cell. Mol. Biol. 47*, 735−744.
19. Brown, K. R., Brown, B. M., Hoagland, E., Manye, C. L., and Hegg, E. L. (2004) Heme A Synthase Does Not Incorporate Molecular Oxygen into the Formyl Group of Heme A, *Biochemistry 43*, 8616−8624.
20. Mogi, T. (2003) Biosynthesis and Role of Heme O and Heme A, in *The Iron and Cobalt Pigments: Biosynthesis, Structure, and Degradation* (Kadish, K. M., Smith, K. M., and Guilard, R., Eds.) pp 157−181, Academic Press, Amsterdam.
21. Kreuzer-Martin, H. W., Ehleringer, J. R., and Hegg, E. L. (2005) Oxygen Isotopes Indicate Most Intracellular Water in Log-Phase *Escherichia coli* Is Derived from Metabolism, *Proc. Natl. Acad. Sci. U.S.A. 102*, 17337−17341.
22. DeCoursey, T. E. (2003) Voltage-Gated Proton Channels and Other Proton Transfer Pathways, *Physiol. Rev. 83*, 475−579.
23. Schweizer, E. (1989) Biosynthesis of Fatty Acids and Related Compounds, in *Microbial Lipids* (Ratledge, C., and Wilkinson, S. G., Eds.) pp 3−50, Harcourt Brace Jovanovich, London.
24. Rock, C. O., and Cronan, J. E. (1996) *Escherichia coli* as a Model for the Regulation of Dissociable (Type II) Fatty Acid Biosynthesis, *Biochim. Biophys. Acta 1302*, 1−16.
25. Sessions, A. L., Jahnke, L. L., Schimmelmann, A., and Hayes, J. M. (2002) Hydrogen Isotope Fractionation in Lipids of the Methane-Oxidizing Bacterium *Methylococcus capsulatus*, *Geochim. Cosmochim. Acta 66*, 3955−3969.
26. Rock, C. O., and Jackowski, S. (2002) Forty Years of Bacterial Fatty Acid Synthesis, *Biochem. Biophys. Res. Commun. 292*, 1155−1166.
27. Burgoyne, T. W., and Hayes, J. M. (1998) Quantitative Production of H-2 by Pyrolysis of Gas Chromatographic Effluents, *Anal. Chem. 70*, 5136−5141.
28. Coplen, T. B. (1996) New Guidelines for Reporting Stable Hydrogen, Carbon and Oxygen Isotope-Ratio Data, *Geochim. Cosmochim. Acta 60*, 3359−3360.
29. Sessions, A. L., and Hayes, J. M. (2005) Calculation of Hydrogen Isotopic Fractionations in Biogeochemical Systems, *Geochim. Cosmochim. Acta. 69*, 593−597.
30. Gehre, M., Geilmann, H., Richter, J., Werner, R. A., and Brand, W. A. (2004) Continuous Flow ^{2}H/^{1}H and ^{18}O/^{16}O Analysis of Water Samples with Dual Inlet Precision, *Rapid Commun. Mass Spectrom. 18*, 2650−2660.
31. Sessions, A. L., Burgoyne, T. W., and Hayes, J. M. (2001) Determination of the H-3 Factor in Hydrogen Isotope Ratio Monitoring Mass Spectrometry, *Anal. Chem. 73*, 200−207.
32. Sessions, A. L., Burgoyne, T. W., and Hayes, J. M. (2001) Correction of H-3(+) Contributions in Hydrogen Isotope Ratio Monitoring Mass Spectrometry, *Anal. Chem. 73*, 192−199.
33. Sokal, R. R., and Rohlf, F. J. (1995) *Biometry: The Principles and Practice of Statistics in Biological Research*, 3rd ed., W. H. Freeman, New York.
34. Shoemaker, D. P., Garland, C. W., and Nibler, J. W. (1989) *Experiments in Physical Chemistry*, 5th ed., McGraw-Hill, New York.
35. Luo, Y.-H., Sternberg, L., Suda, S., Kumazawa, S., and Mitsui, A. (1991) Extremely Low D/H Ratios of Photoproduced Hydrogen by Cyanobacteria, *Plant Cell Physiol. 32*, 897−900.
36. Weast, R. C., Lide, D. R., Astle, M. J., and Beyer, W. H., Eds. (1990) *Handbook of Chemistry and Physics*, 70th ed., p D-166, CRC Press, Boca Raton, FL.
37. Sessions, A. L., Burgoyne, T. W., Schimmelmann, A., and Hayes, J. M. (1999) Fractionation of Hydrogen Isotopes in Lipid Biosynthesis, *Org. Geochem. 30*, 1193−1200.
38. Jencks, W. P. (1969) *Catalysis in Chemistry and Enzymology*, Dover Publications, New York.
39. Heath, R. J., and Rock, C. O. (1996) Roles of the FabA and FabZ β-Hydroxyacyl-Acyl Carrier Protein Dehydratases in *Escherichia coli* Fatty Acid Biosynthesis, *J. Biol. Chem. 271*, 27795−27801.
40. Rock, C. O. (2006) St. Jude Children's Research Hospital, Memphis, TN, private communication.
41. Schmidt, B., McCracken, J., and Ferguson-Miller, S. (2003) A Discrete Water Exit Pathway in the Membrane Protein Cytochrome *c* Oxidase, *Proc. Natl. Acad. Sci. U.S.A. 100*, 15539−15542.
42. Kendall, C., and Caldwell, E. (1998) Fundamentals of Isotope Geochemistry, in *Isotope Tracers in Catchment Hydrology* (Kendall, C., and McDonnell, J. J., Eds.) pp 51−86, Elsevier Science, New York.
43. Craig, H. (1961) Isotopic Variations in Meteoric Waters, *Science 133*, 1702−1703.

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