Published in Applied and Environmental Microbiology 68, issue 10, 5205-5207, 2002
which should be used for any reference to this work

Quantification of Isotope Fractionation in Experiments with Deuterium-Labeled Substrate

Daniel Hunkeler
Center for Hydrogeology, University of Neuchatel, CH-2000 Neuchatel, Switzerland
Phone: (032) 718 25 60
Fax: (032) 718 26 03
E-mail: daniel.hunkeler@unine.ch

Isotope analysis is a potentially sensitive method to trace in situ degradation of organic contaminants. In a recent paper, Morasch et al. (3) investigated the mechanism of isotope fractionation during toluene biodegradation using deuterium-labeled toluene. The authors overlooked that the Rayleigh equation that is normally used to evaluate isotope fractionation at natural abundance level (2) is not applicable to studies with labeled substrate, particularly if large isotope fractionation occurs. For several of their experiments they obtained negative hydrogen isotope fractionation factors (see Table 1 in reference 3), which contradict the definition of the fractionation factor (see below). Since labeled compound will likely be used in further investigations to study isotope fractionation, it is important to demonstrate why the commonly used Rayleigh equation is usually not applicable in such studies and to provide an alternative method to quantify isotope fractionation.

The magnitude of isotope fractionation is normally characterized by the fractionation factor, which is defined as follows for kinetic isotope fractionation:

$$\alpha = \frac{H/L}{dH_p/dL_p} \tag{1}$$

where H and L are the concentrations of the substrate with heavy and light isotopes, respectively, at a given time and dH_p and dL_p are increments of product with heavy or light isotopes, respectively, that appear in an infinitely short time (instantaneous product). In some studies, the fractionation factor is defined by the inverse ratio (2). Since all terms in equation 1 are positive, α has to be positive. For mass balance reasons,

$$dH_p = -dH$$
$$dL_p = -dL \tag{2}$$

Combining equations 1 and 2 and rearrangement leads to

$$\frac{dL}{L} = \alpha \cdot \frac{dH}{H} \tag{3}$$

Integration of equation 3 from L_0 to L and H_0 to H gives

$$\ln \frac{L}{L_0} = \alpha \cdot \ln \frac{H}{H_0} \text{ or}$$
$$\frac{H}{H_0} = \left(\frac{L}{L_0}\right)^{1/\alpha} \tag{4}$$

Dividing both sides by L/L_0 yields

$$\frac{R}{R_0} = \left(\frac{L}{L_0}\right)^{(1/\alpha - 1)} \tag{5}$$

where R and R_0 are the isotope ratios (H/L) at a given time t and at time zero, respectively. The fraction of substrate that has not reacted yet, f, at time t is given by

$$f = \frac{L + H}{L_0 + H_0} = \frac{L \cdot (1 + R)}{L_0 \cdot (1 + R_0)} \tag{6}$$

Equations 5 and 6 are analogous to those given by Bigeleisen and Wolfsberg (1), except that here they were derived without any specific assumption about the reaction kinetics and using a different definition of α and f.

The crucial point is that L/L_0 in equation 5 can only be approximated by f if either (i) the concentrations of the heavy isotopes, H and H_0, are small, as common for studies at natural abundance level, or (ii) $1 + R \approx 1 + R_0$. In the first case, the first expression for f in equation 6 approaches L/L_0; in the second case, the second expression can be approximated by L/L_0. If one of these two conditions is fulfilled, equation 5 can be simplified to

$$\frac{R}{R_0} = f^{(1/\alpha - 1)} \tag{7}$$

which corresponds to the Rayleigh equation as used by the authors of the study (3). However, in the experiments with labeled compound presented in the study, condition i is not fulfilled since the compound with deuterium accounts for 50% of the total toluene concentration. Condition ii is not fulfilled either. For example, for the experiment illustrated in Fig. 1 in reference 3, R_0 is 1 and R varies between 1 and about 12 and thus, the assumption that $1 + R \approx 1 + R_0$ holds true is not valid. In other experiments, even higher R values of up to about 54 were observed (see Fig. 2 in reference 3).

By combining equations 5 and 6, an accurate equation is obtained that relates R, R_0, f, and α:

$$\ln \frac{R}{R_0} = \left(\frac{1}{\alpha} - 1\right) \cdot \ln \frac{f}{(1 + R)/(1 + R_0)} \tag{8}$$

This equation can be used to determine α by plotting $\ln(R/R_0)$ versus $\ln\{f/[(1 + R)/(1 + R_0)]\}$. Applying this approach to the data of the experiment with *Desulfobacterium cetonicum* (as given in Fig. 1 in reference 3), an α value of approximately 2.7 is obtained instead of -5.09. The value of 2.7 is only an approximation, since the data for the calculation were estimated from Fig. 1 in reference 3. The calculated value is in the typical range for primary hydrogen isotope effects. Using the correct equation, the introduction of an uncommon parameter to characterize isotope fractionation becomes unnecessary and the data can be discussed in a framework consistent with a large number of studies on isotope fractionation during enzymatic reactions.

REFERENCES

1. **Bigeleisen, J., and M. Wolfsberg.** 1958. Theoretical and experimental aspects of isotope effects in chemical kinetics. Adv. Chem. Phys. **1:** 15–76.
2. **Mariotti, A., J. C. Germon, P. Hubert, P. Kaiser, T. Letolle, A. Tardieux, and P. Tardieux.** 1981. Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. Plant Soil **62:**413–430.
3. **Morasch, B., H. H. Richnow, B. Schink, and R. U. Meckenstock.** 2001. Stable hydrogen and carbon isotope fractionation during microbial toluene degradation: mechanistic and environmental aspects. Appl. Environ. Microbiol. **67:** 4842–4849.

Authors' Reply

The comments by Dr. Hunkeler provide a valuable extension of our results on basic features of isotope fractionation. Indeed, the fractionation factor α_n (n for natural abundance) calculated in Morasch et al. (3) is valid to describe isotope fractionation of carbon and hydrogen isotopes only at low abundance of the heavier isotopes (^{13}C and D, respectively) as presented in the manuscript. For experiments at elevated abundances of the heavier isotope, the isotope fractionation factor α_l (l for labeled compounds) should be calculated using equation 3 as given by Bigeleisen and Wolfsberg (1) and mentioned by Dr. Hunkeler.

In Morasch et al. (3), we used the slope b of a linear regression of the data in a double logarithmic plot of $\ln(R_t/R_0)$ versus $\ln f$ ($f = C_t/C_0$, fraction of substrate remaining [C_t, substrate concentration at time A; C_0, substrate concentration at time zero]) to evaluate the extent of isotope fractionation (equation 2). In experiments with substrates of natural isotope composition, b can be converted directly to the fractionation factor α_n or the enrichment factor ε with $b = 1/\alpha - 1$ or $\varepsilon = b \times 1{,}000$, because equations 2 and 1 approximate equation 3 at low abundances of the heavier isotope (1, 2).

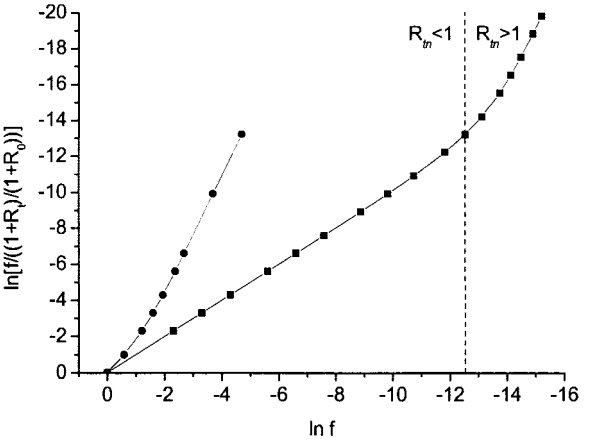

FIG. 1. Simulated hydrogen isotope fractionation experiment for toluene degradation by strain TRM1 as calculated with equation 3 versus equation 1 and $\alpha_l = \alpha_n = 3.3$. The calculations start with $R_{0n} = 0.0001$ for the simulation of an experiment with natural abundance of deuterium (■) and with $R_{0l} = 1$ for the simulation of an experiment with labeled compounds (●). R_t runs from R_0 to infinity. At natural abundance of the heavier isotope $\ln\{f/[(1 + R_t)/(1 + R_0)]\}$ approximates $\ln f$ because R_t and R_0 are very small and equation 3 approximates equation 1. Therefore, the slopes of the curves in the range of $\ln f > -12$ show the deviation in the description of isotope fractionation simulated with equation 1 or 3 for natural abundance (■) and labeled compounds (●). The dashed line depicts the isotope ratio of $R_{tn} = 1$.

What is the consequence of using equation 2 instead of equation 3 also in experiments with deuterium-labeled compounds at elevated abundance? In this case, the slope b calculated by equation 2 becomes a fitting parameter of the data

TABLE 1. D/H isotope fractionation factors α or ε and fitting parameter b obtained from studies with nonlabeled (α_n) or labeled toluene (α_l)[a]

Strain	Substrate mixture	α_n	α_l	ε_l	b
Desulfobacterium cetonicum	Toluene	1.247	1.247	−198.1	−0.198
	Toluene-d_8 and toluene-d_3		0.996 ± 0.005	4.016 ± 5.04	−0.002 ± 0.003
	Toluene-d_3 and nonlabeled toluene		3.772 ± 1.084	−734.9 ± 76.19	−1.251 ± 0.034
	Toluene-d_8 and toluene-d_5		2.058 ± 0.090	−514.1 ± 21.25	−0.679 ± 0.115
	Toluene-d_5 and nonlabeled toluene		1.009 ± 0.017	−8.920 ± 16.69	−0.005 ± 0.004
	Toluene-d_8 and nonlabeled toluene		3.244 ± 0.261	−691.7 ± 24.80	−1.196 ± 0.075
TRM1	Toluene	3.672	3.650	−726.0	−0.728
	Toluene-d_8 and toluene-d_3		0.885 ± 0.142	129.9 ± 181.3	0.167 ± 0.219
	Toluene-d_3 and nonlabeled toluene		3.384 ± 0.170	−704.5 ± 14.85	−1.280 ± 0.080
	Toluene-d_8 and toluene-d_5		2.070 ± 0.233	−516.9 ± 54.38	−0.917 ± 0.336
	Toluene-d_5 and nonlabeled toluene		1.014 ± 0.012	−13.81 ± 11.67	−0.012 ± 0.005
	Toluene-d_8 and nonlabeled toluene		3.276 ± 0.281	−694.7 ± 26.18	−1.219 ± 0.254
Thauera aromatica	Toluene-d_8 and nonlabeled toluene		2.543 ± 0.567	−606.8 ± 87.68	−0.816 ± 0.133
Geobacter metallireducens	Toluene-d_8 and nonlabeled toluene		2.550 ± 0.187	−607.8 ± 28.76	−1.004 ± 0.077
Pseudomonas putida strain mt-2	Toluene-d_8 and toluene-d_3		1.005 ± 0.0004	−4.98 ± 0.40	−0.016 ± 0.003
	Toluene-d_3 and nonlabeled toluene		22.96 ± 4.368	−956.4 ± 8.29	−4.218 ± 0.125
	Toluene-d_8 and toluene-d_5		13.65 ± 2.452	−926.7 ± 13.16	−2.696 ± 0163
	Toluene-d_5 and nonlabeled toluene		1.098 ± 0.031	−89.25 ± 25.71	−0.079 ± 0.041
	Toluene-d_8 and nonlabeled toluene		17.78 ± 13.46	−943.8 ± 42.58	−2.667 ± 0.163

[a] α_n was calculated with equation 1, α_l and ε were calculated with equation 3, and b was calculated with equation 2. ε is calculated as $\varepsilon = (1/\alpha_l - 1) \times 1{,}000$. Average isotope fractionation factors for experiments with mixtures of labeled toluene species α_l, the respective fitting parameter b, and the standard deviations result from three independent growth experiments. Original data were taken from reference 3. Fractionation at natural deuterium abundance was obtained only from a single growth experiment and is not given with a standard deviation.

which differs by a constant value from the slope b' calculated via equation 3, where $\ln(R_t/R_0)$ is plotted versus $\ln\{f/[(1 + R_t)/(1 + R_0)]\}$. The difference in using the two equations is depicted in Fig. 1 for a hypothetical experiment where the isotope ratio R_t runs from R_0 to infinity and ln$f$ is plotted versus $\ln\{f/[(1 + R_t)/(1 + R_0)]\}$. For natural abundance of deuterium, the slope of ln$f$ versus $\ln\{f/[(1 + R_t)/(1 + R_0)]\}$ equals 1, indicating that the two terms are almost identical as long as R_t is smaller than 1. If R_t is larger than 1, the slope of the curve changes and approximates another constant value. Note that the slope of the curve for $R_t > 1$ is similar to the slope of a simulated experiment using labeled compounds with $R_0 = 1$ if the same fractionation factor is applied (Fig. 1). The extent of fractionation is only hypothetical since one could hardly run a real degradation experiment over such enormous concentration ranges. Nevertheless, it shows that the difference in the calculations using equations 1 and 2 or 3 depends on the isotope ratio R_t being larger or smaller than 1. This property of the calculations becomes especially important if isotope fractionation of elements such as chlorine is studied, where the natural abundances of the heavier and lighter isotopes are almost equal ($R_0 = 1$).

In our experiments the concentrations were usually in the range of $0 < \ln f < -4$. Here, the difference of the slopes for labeled compounds and for natural abundance reveals the systematic difference between the two ways of calculation. Figure 1 also shows that the curve for the labeled compounds is not exactly a straight line. However, the experimental error of the isotope analysis is usually much larger than the error by fitting the data with a linear regression, but the description of the data set with equation 3 would certainly improve the interpretation.

With respect to the data produced by Morasch et al., the systematic difference in the description of the data set with equation 2 or 3 results in the same interpretation of the isotope fractionation experiments with deuterium-labeled compounds. The direct comparison of the obtained isotope fractionations of b and α clearly shows the relation of isotope fractionation and enzyme mechanisms. The major difference in the use of the two equations is that the absolute value of the commonly used isotope fractionation factor α can only be calculated from equation 3.

We have recalculated the isotope fractionation factors of the experiments with labeled compounds published in Morasch et al. (3) using equation 3 (Table 1). The recalculated data may provide the reader with fractionation factors comparable to those published in other studies. However, the data show also that an α_n obtained at a natural abundance of the heavier isotope is not necessarily identical with the α_l obtained in labeling experiments.

In summary, for experiments with defined conditions the use of deuterium-labeled compounds is an elegant way to overcome the problem of limited availability of isotope mass spectrometers for D/H analysis and to reduce analysis costs for basic studies of isotope fractionation. Isotope fractionation factor α should be calculated from labeling experiments with equation 3 but are difficult to relate to isotope fractionation occurring at natural abundance of hydrogen isotopes (3).

$$\ln(R_t/R_0) = (1/\alpha_n - 1) \times \ln f \quad (1)$$

$$\ln(R_t/R_0) = b \times \ln f \quad (2)$$

$$\ln(R_t/R_0) = (1/\alpha_l - 1) \times \ln\{f/[(1 + R_t)/(1 + R_0)]\} \quad (3)$$

REFERENCES

1. **Bigeleisen, J., and M. Wolfsberg.** 1959. Theoretical and experimental aspects of isotope effects in chemical kinetics. Adv. Chem. Phys. **1:**15–76.
2. **Mariotti, A., C. Germon, P. Hubert, P. Kaiser, R. Letolle, A. Tardieux, and P. Tardieux.** 1981. Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. Plant Soil **62:**413–430.
3. **Morasch, B., H. H. Richnow, B. Schink, and R. U. Meckenstock.** 2001. Stable carbon and hydrogen isotope fractionation during microbial toluene degradation: mechanistic and environmental aspects. Appl. Environ. Microbiol. **67:**4842–4849.

Rainer Meckenstock*
Hans H. Richnow
Center for Applied Geosciences
Eberhard-Karls-University of Tübingen
Wilhelmstr. 56
72076 Tübingen
Germany

*Phone: 49-7071-2973150
Fax: 49-7071-295139
E-mail: rainer.meckenstock@uni-tuebingen.de