Pergamon

Geochimica et Cosmochimica Acta, Vol. 68, No. 3, pp. 433–442, 2004

0016-7037/04 $30.00 + .00

doi:10.1016/S0016-7037(03)00459-9

Optimal methods for estimating kinetic isotope effects from different forms of the Rayleigh distillation equation

K. M. SCOTT,[1,*] X. LU,[2] C. M. CAVANAUGH,[1] and J. S. LIU*[2]

[1]Department of Organismic and Evolutionary Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA
[2]Department of Statistics, Harvard University, 1 Oxford Street, Cambridge, MA 02138, USA

(*Received December* 10, 2002; *accepted in revised form June* 17, 2003)

Abstract—The interpretation of stable isotope values hinges on precise, accurate estimates of kinetic isotope effects (α), which are equal to $^1k/^2k$, where ^1k and ^2k are the reaction rate constants for the two isotopes. Kinetic isotope effects are commonly determined by monitoring the reactant concentration (C) and isotope ratio (R) relative to their initial values (C_1 and R_1, respectively). Values of α are estimated from the C and R values by using the Rayleigh distillation equation (RDE).

$$(R/R_1) = (C/C_1)^{1/\alpha-1} \quad \text{(A)}$$

We conducted simulation experiments to evaluate the precision of the many different published approaches of estimating α from linearized versions of the RDE and reached the following conclusions: (a) kinetic isotope effects estimated from the slope of the line ln(R) vs. ln(C) were accurate and precise; (b) regressing ln(R_i/R_j) on ln(C_i/C_j), where all i datapoints were compared to all preceding datapoints j resulted in inflated 95% confidence intervals; (c) forcing the regression of ln(R/R_1) on ln(C/C_1) through the origin resulted in 95% confidence intervals for α that covered the true value less than 90% of the time; and (d) regression methods that compensate for errors in both x and y values need to be used with caution.

When combining multiple datasets, values of α were sensitive to the form of the equation and the level of error. If all datasets had the same level of error, the optimal estimate of α was achieved by a linear regression with dummy variables. However, when the three datasets had different levels of error, the optimal estimate of α and much narrower 95% confidence intervals were obtained by using the Pitman estimator. Our study demonstrates that some of the other methods jeopardize the accuracy and precision of empirically determined kinetic isotope effects, thus confounding the interpretation of stable isotope values in the environment.

1. INTRODUCTION

Kinetic isotope effects, which are the degree to which a reaction discriminates between two isotopes, have been determined for a variety of biologic, chemical, and physical processes. For example, kinetic isotope effects have been used to model the formation of carbonaceous chondrite asteroids (Wang et al., 2001; Richter et al., 2002), to explain the isotopic composition of stratospheric N_2O (Rahn et al., 1998), to determine the mechanism of CO_2 fixation by the enzyme RubisCO (Roeske and O'Leary, 1984; Roeske and O'Leary, 1985; Robinson et al., 2003), and to model sulfate reduction by thermophilic bacteria in deep sea pore fluids (Rudnicki et al., 2001). Kinetic isotope effects (α) are equal to $^1k/^2k$, where ^1k and ^2k are the reaction rate constants for the lighter and heavier isotopes, respectively (Table 1; O'Leary et al., 1992). Because different isotopes react with different rates, the isotopic composition of a material bears the "fingerprints" of the kinetic isotope effects of the various processes that have affected it. Accurate determinations of kinetic isotope effects, then, are key to the interpretation of the isotopic compositions of materials ranging from asteroids to biomass, and to their use in predictive models.

Values of α for a particular reaction or irreversible process are commonly determined using a reactant-depletion method, in which the reaction takes place with a nonreplenished reactant (O'Leary, 1980). Paired measurements of reactant concentration (C) and reactant isotope ratio (R) are taken over time. The change in the isotopic composition of the reactant as it is consumed by the reaction is described by the Rayleigh distillation equation (RDE):

$$(R/R_1) = (C/C_1)^{1/\alpha - 1} \quad (1)$$

where R and C are the isotope ratio and concentration, respectively, of the reactant at time t, and R_1 and C_1 are the corresponding quantities initially present (Broecker and Oversby, 1971; Mariotti et al., 1981). In Eqn. 1, α is equal to R_r/R_p, where R_r is the isotope ratio of the available reactant, and R_p is the isotope ratio of the product formed at this R_r. Some researchers have also defined α as the inverse (R_p/R_r; e.g., Mariotti et al., 1981).

One can rewrite the RDE to estimate α from R and C values as

$$\ln(R) = (1/\alpha - 1)\ln(C) + \ln(R_1/C_1^{(1/\alpha - 1)}). \quad (2)$$

(Fig. 1a). The slope of the line is equal to $1/\alpha - 1$, which can be estimated by the simple linear regression with the y value being ln(R) and the x value being ln(C). Despite the simplicity of Eqn. 2, another linear form of the RDE,

* Authors to whom correspondence should be addressed (kscott@cas.usf.edu and jliu@stat.harvard.edu).

Table 1. Symbols and abbreviations.

Symbol	Definition
Isotope terminology	
α	kinetic isotope effect, $= R_s/R_p$
k_1, k_2	reaction rate constants for two isotopes
R	isotope ratio
R_1	initial value of R
R_r	isotope ratio of the reactant
R_p	isotope ratio of the product of a reaction
RDE	Rayleigh distillation equation
C	reactant concentration
C_1	initial value of C
Statistics terminology	
β	true value of the slope
$\hat{\beta}$	slope of a line from a dataset, $= \alpha^{-1} - 1$
$\bar{\beta}$	weighted or unweighted average of the slopes from three datasets
$\tilde{\beta}$	average of the slopes from three datasets, weighted by the slopes' variances
I_i	upper or lower limit for a 95% confidence interval
CI	confidence interval
D	dummy variable for a line that includes several datasets
δ	error in x
ε	error in y
γ	y-intercept of a line
f	frequency
LSCE	linear squares cubic equation
p	probability
$R_{i.samp}$	isotope ratio of a sample
$R_{i.std}$	isotope ratio of a standard
$R_{c.samp}$	ratio of ion currents generated from a sample by a mass spectrometer
$R_{c.std}$	ratio of ion currents generated from a sample by a mass spectrometer
s	sample standard deviation
s^2	sample variance
$s_{\hat{\beta}}^2$	variance of a slope
$s_{\bar{\beta}}^2$	variance of an unweighted or weighted average slope
U_i	$x_i - \bar{x}$
V_i	$y_i - \bar{y}$
v	degrees of freedom
W_i	$\dfrac{\omega(x_i)\omega(y_i)}{\beta^2\omega(y_i) + \omega(x_i)}$
$\omega(x_i)$	weight of the x observations
$\omega(y_i)$	weight of the y observations
$\bar{x}$	mean value of x
$\bar{y}$	mean value of y
$\hat{y}$	value of y predicted from a regression line

$$\ln(R/R_1) = (1/\alpha - 1)\ln(C/C_1), \quad (3)$$

is more frequently utilized (Fig. 1b). With $y = \ln(R/R_1)$ and $x = \ln(C/C_1)$, one can again estimate the slope $1/\alpha - 1$ by linear regression (Pennock et al., 1996; Hunkeler and Aravena, 2000; Wang et al., 2001). In theory, the y-intercept of this line should be zero; accordingly, some force the regression through the origin (Mariotti et al., 1981; Weger et al., 1990; Guy et al., 1993). One difficulty with this approach is that regressing $\ln(R/R_1)$ on $\ln(C/C_1)$, and forcing it through the origin places an added emphasis on the initial timepoint (Henry et al., 1999). To ameliorate this overemphasis, some investigators regress $\ln(R_i/R_j)$ on $\ln(C_i/C_j)$ based on the equation

$$\ln(R_i/R_j) = (1/\alpha - 1)\ln(C_i/C_j), \quad (4)$$

where the R and C values of all i timepoints are divided by the R and C values of all j timepoints preceding them (Fig. 1c; Weger et al., 1990; Guy et al., 1993).

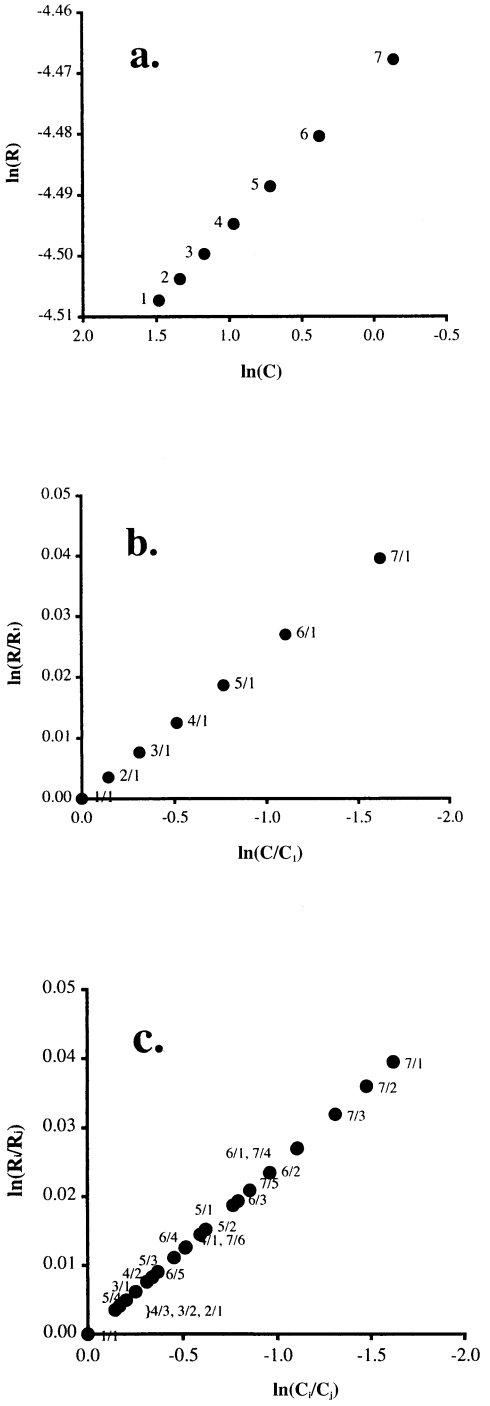

Fig. 1. Data from a reactant-depletion experiment, plotted using three different linearizations of the Rayleigh distillation equation. The numbers next to the points on the lines indicate the corresponding timepoints. The slope of all lines $= (1/\alpha - 1)$. a) $x = \ln(C)$ and $y = \ln(R)$. b) $x = \ln(C/C_1)$ and $y = \ln(R/R_1)$. c) $x = \ln(C_i/C_j)$ and $y = \ln(R_i/R_j)$.

Several different regression methods have also been used for estimating α. Standard linear regression assumes that all errors are associated with the y values and derives the least-square

estimate of the slope by minimizing $\Sigma(y_i\text{-}\hat{y}_i)^2$, where y_i is the observed y values and $\hat{y}_i$ is predicted y values from the regression line (Sokal and Rohlf, 1995). To accommodate measurement error in the x and y values, one can estimate the slope by solving a "Least Squares Cubic Equation" (LSCE; York, 1966; York, 1967):

$$\beta^3\sum_i\frac{W_i^2U_i^2}{\omega(x_i)} - 2\beta^2\sum_i\frac{W_i^2U_iV_i}{\omega(x_i)} - \beta\left(\sum_i W_i^2U_i^2 - \sum_i\frac{W_i^2V_i^2}{\omega(x_i)}\right) + \sum_i W_iU_iV_i = 0 \quad (5)$$

where $U_i = x_i - \bar{x}$, $V_i = y_i - \bar{y}$, $W_i = \dfrac{\omega(x_i)\omega(y_i)}{\beta^2\omega(y_i) + \omega(x_i)}$, and $\omega(x_i)$ and $\omega(y_i)$ are the weights of each observation. This equation has several solutions that vary based on the weights of the x and y values ($\omega(x_i)$ and $\omega(y_i)$, respectively). When there is no error in the x values, $\omega(x_i)$ is set to infinity and $\omega(y_i)$ is set to one so that Eqn. 5 gives rise to the standard least-squares solution:

$$\hat{\beta} = \frac{\sum(y_i - \bar{y})(x_i - \bar{x})}{\sum(x_i - \bar{x})^2} \quad (6)$$

Further, methods for combining data from replicated experiments have not been rigorously compared. Some investigators average the estimated α values (Robinson et al., 2003) while others combine data from replicated experiments into a single linear regression (Guy et al., 1993). It is not obvious which method of assigning x and y values, weighting observations, and combining datasets is best.

To test the many approaches to estimating α values, simulated datasets were used to compare the accuracy and precision of α values generated from commonly used forms of the RDE (Berges et al., 1994; Ritchie and Prvan, 1996). We generated 1000 datasets with a true value of 1.025 for α (intermediate for RubisCO enzymes; Roeske and O'Leary, 1984; Roeske and O'Leary, 1985; Guy et al., 1993; Robinson et al., 2003) and measurement errors mimicking those present in a typical dataset. Each dataset was regressed using the different linear forms of the RDE and different methods of combining replicated experiments. The population of α values generated by each regression was assessed for precision and accuracy.

2. MATERIAL AND METHODS

2.1. Simulated Datasets

The investigation that instigated this study was the determination of the kinetic isotope effect for CO_2 fixation by the Calvin cycle enzyme ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO). Depending on the source of the enzyme, RubisCO has a kinetic isotope effect ($^{12}k/^{13}_{k}$) from 1.018 to 1.030 (Roeske and O'Leary, 1984; Roeske and O'Leary, 1985; Guy et al., 1993; Robinson et al., 2003). In these measurements of α, five to seven datapoints are collected over the time course of the reaction as CO_2 is consumed, with values of R ($^{13}CO_2/^{12}CO_2$) rising from ~0.0110 to 0.0115 as values of C (the concentration of dissolved inorganic carbon) fall from ~4.4 to 0.87 mM. The C and R values collected from these experiments are used to calculate α values using a linearized version of the RDE.

Using the determination of RubisCO α values as a model, an idealized, error-free set of seven datapoints of reactant concentration and isotope ratio was generated by using C values typical for the initial and final reactant concentrations (C_1 and C_7) and dividing the interval between them by five evenly spaced C values (C_2 to C_6). A value of R_1 typical for the initial isotope ratio was chosen and, with an α of 1.025, Eqn. 1 was used to calculate R_2 to R_7. From this idealized dataset, 1000 simulated datasets were generated by introducing normally distributed error in R and C to mimic measurement error (Fig. 2). For the "low" level of error, which was similar to what is typically observed in RubisCO kinetic isotope effect experiments (KMS and CMC, pers. obs.), the standard deviation of the R values was fixed at 0.015% of the maximum value of R; for C, the standard deviation was 3% of the maximum value of C. Datasets with "medium" and "high" levels of error were used to test the robustness of the estimates, and to extend the results to experiments with larger levels of error (Table 2).

2.2. Estimating α from a Single Dataset

2.2.1. Methods for minimizing least squares

Simulated datasets were fitted with lines using four versions of the LSCE: 1) $\omega(x_i) = (\infty)$ and $\omega(y_i) = 1$, in which all error was associated with the y values, 2) reduced major axis regression, with $\omega(x_i)/\omega(y_i) = \sigma^2_y/\sigma^2_x$, where $\sigma^2_y = \Sigma(y_i - \hat{y})^2$ and σ^2_x is the corresponding term for the x values, 3) $\omega(x_i)/\omega(y_i) = 1$, and 4) $\omega(x_i)/\omega(yi) = s^2[\ln(R)]/s^2[\ln(C)]$. The α values generated using these regression methods with the lines described in section 2.2.2 were compared.

2.2.2. Methods for assigning x and y values

The three commonly used forms of the RDE described in Eqn. 2–4 were compared. The regressions based on Eqn. 3 and 4 were run twice: once forced through the origin, and once with an unconstrained y-intercept. To compensate for multiple use of each data point in Eqn. 4, the number of degrees of freedom was calculated by subtracting the number of experiments conducted from the total number of time points taken (Weger et al., 1990).

2.2.3. Simulation evaluation

From the slope $\hat{\beta}$ of each regression line, we computed α as

$$\alpha = \frac{1}{\hat{\beta} + 1}. \quad (7)$$

The limits of the 95% confidence interval ($I_{1\alpha}$, $I_{2\alpha}$) were calculated from the limits of the 95% confidence interval of the slope of the line ($I_{1\hat{\beta}}$,$I_{2\hat{\beta}}$) according to

$$I_{1\alpha} = \frac{1}{I_{2\hat{\beta}} + 1} \text{ and } I_{2\alpha} = \frac{1}{I_{1\hat{\beta}} + 1} \quad (8)$$

The frequency with which this interval covered the true value among the 1000 simulated datasets was determined. From the population of 1000 α values, the mean and standard deviation were calculated. Three criteria were used to evaluate the results from each method: (a) the width of the 95% confidence intervals for the α values estimated from each dataset, (b) the frequency with which these confidence intervals covered the true value of α, and (c) whether the mean value of α was equal to the true value of 1.025.

2.3. Estimating α from Multiple Datasets

When replicating an experiment, it is necessary to combine datasets to generate an overall "average" α value. Four different methods for combining datasets were evaluated to determine which was optimal if the datasets had similar or widely divergent levels of error. The methods described in 2.3.1 through 2.3.3 combine the datasets by averaging the slopes calculated independently from three different datasets, while 2.3.4 describes the use of dummy variables. In each case, 1000 sets of three datasets, either with the same level of error or each with a different level (one low, one medium, and one high) were generated to test each method, and the values of α were evaluated using the criteria described in section 2.2.3.

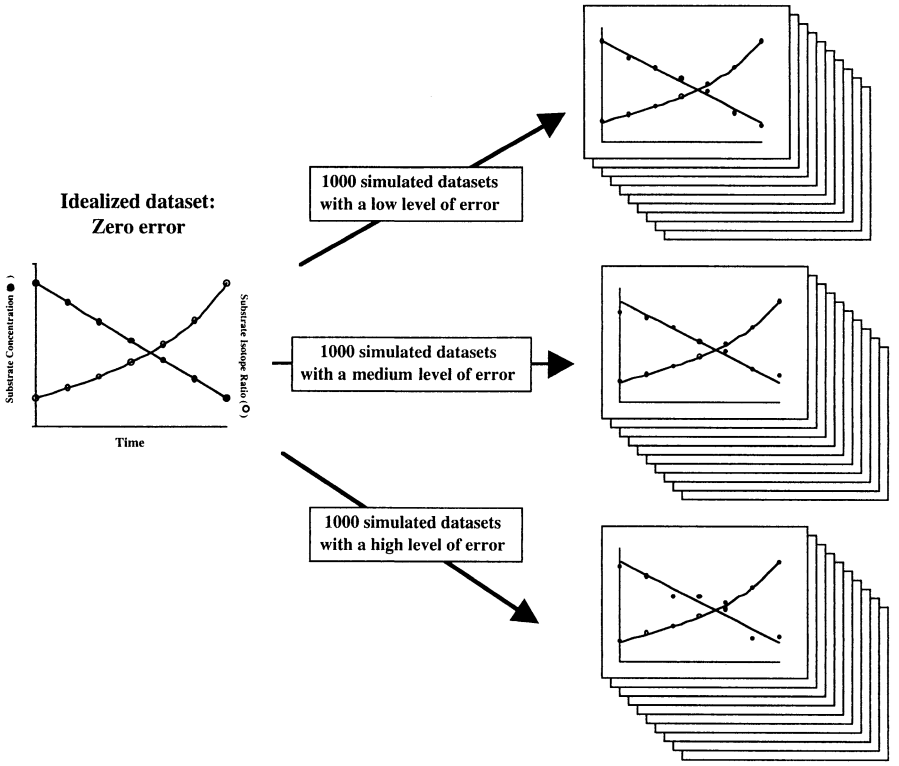

Fig. 2. Generation of simulated datasets with different levels of error. Beginning with an idealized Rayleigh distillation with an α of 1.025, where measurements of the reactant concentration and isotope ratio have zero error, three sets of 1000 simulated datasets were generated with low, medium, and high levels of random error.

2.3.1. Averaging the slopes from the three datasets

The simplest averaging method was to generate slopes $\hat{\beta}_1,\hat{\beta}_2,\hat{\beta}_3$ from three datasets by regressing them as described in section 2.2, and to calculate an unweighted average slope ($\bar{\beta}$). Values of α were calculated from $\bar{\beta}$ values (Eqn. 7). This procedure was repeated to generate 1000 α values from 1000 groups of three datasets. The standard deviation of this population of α values was calculated as for single-run experiments. The variance of $\bar{\beta}$ ($=s_{\beta}^2$) was determined from the variance for $\hat{\beta}_1,\hat{\beta}_2,\hat{\beta}_3$ as $s_{\bar{\beta}}^2 = (s_{\hat{\beta}1}{}^2 + s_{\hat{\beta}2}{}^2 + s_{\hat{\beta}}{}^2)/9$.

2.3.2. Weighted average of the slopes from the three datasets

The second averaging method was to weight the slope from each dataset by its variance. In this case, the weighted average slope was calculated as

$$\tilde{\beta} = \frac{\hat{\beta}_1 s_{\hat{\beta}1}{}^{-2} + \hat{\beta}_2 s_{\hat{\beta}2}{}^{-2} + \hat{\beta}_3 s_{\hat{\beta}3}{}^{-2}}{S_{\hat{\beta}1}{}^{-2} + S_{\hat{\beta}2}{}^{-2} + s_{\hat{\beta}}{}^{-2}} \tag{9}$$

Table 2. Levels of Error Incorporated into the Simulated Datasets.

Level of error[a]	% of the maximum value of C	% of the maximum value of R
Low	3	0.015
Medium	5	0.1
High	5	1

[a] "Level of error" refers to the standard deviations for C and R, which were fixed at the indicated percent of the maximum values of C and R.

Values of α were calculated from these weighted average slope values as described in Eqn. 7. The variance of $\tilde{\beta}$ can be computed as

$$s_{\tilde{\beta}}{}^2 = \frac{1}{s_{\hat{\beta}1}{}^{-2} + s_{\hat{\beta}2}{}^{-2} + s_{\hat{\beta}3}{}^{-2}} \tag{10}$$

Unfortunately, there is no theoretical basis to determine what the number of degrees of freedom should be when calculating the standard deviation of the average and weighted average slopes. For the purposes of this study, the number of degrees of freedom for the averaging methods was estimated to be 15 (=21 datapoints –3 slopes –3 intercepts). Using the weighted average method is also problematic when the number of data points per experiment was small (<10), because it results in imprecise estimates of the variances, which adds to the imprecision of the slope estimate (see Eqn. 9).

2.3.3. Using a Pitman estimator

Since the three slope estimates are mutually independent and follow t-distributions (with possibly different degrees of freedom), all centering at the true slope β, it is most efficient to use the "Pitman estimator" to combine the three experiments:

$$\hat{\beta} = \frac{\int \beta f_{v1}\left(\frac{\hat{\beta}_1 - \beta}{s_{\hat{\beta}1}}\right) f_{v2}\left(\frac{\hat{\beta}_2 - \beta}{s_{\hat{\beta}2}}\right) f_{v3}\left(\frac{\hat{\beta}_3 - \beta}{s_{\hat{\beta}3}}\right) d\beta}{\int f_{v1}\left(\frac{\hat{\beta}_1 - \beta}{s_{\hat{\beta}1}}\right) f_{v2}\left(\frac{\hat{\beta}_2 - \beta}{s_{\hat{\beta}2}}\right) f_{v3}\left(\frac{\hat{\beta}_3 - \beta}{S_{\hat{\beta}3}}\right) d\beta} \tag{11}$$

(Pitman, 1939; Cox and Hinkley 1974), where $f_v(\bullet)$ is the probability density function of the t-distribution with ν degrees of freedom, and the ν_i are the degrees of freedom of the $\hat{\beta}_i$ estimated from each line, respectively. The Pitman estimator is identical in form to a Bayes estimator with a uniform prior distribution. In Bayesian analyses, prior

Table 3. Precision and Accuracy of Kinetic Isotope Effects (α) Calculated from Single Simulated Datasets with Regressions Assigning all Error to the y Values[a].

Regression method	Level of error[b]	Mean value of α[c]	Std. dev. of α[c]	Mean 95% CI ± std. dev.[d]	Cover frequency[e]
$\ln(R)$ vs. $\ln(C)$					
	low	1.02499	0.0003	0.0013 ± 0.0004	94.4%
	medium	1.02497	0.0009	0.0043 ± 0.0013	95.2%
	high	1.02505	0.0075	0.0376 ± 0.0122	94.8%
$\ln(R/R_1)$ vs. $\ln(C/C_1)$					
Unconstrained	low	1.02499	0.0003	0.0016 ± 0.0006	94.4%
	medium	1.02496	0.0010	0.0052 ± 0.0019	95.8%
	high	1.02526	0.0088	0.0458 ± 0.0168	95.2%
Forced through origin	low	1.02501	0.0004	0.0012 ± 0.0004	85.8%
	medium	1.02500	0.0013	0.0032 ± 0.0011	76.3%
	high	1.02526	0.0110	0.0339 ± 0.0121	87.8%
$\ln(R_i/R_j)$ vs $\ln(C_i/C_j)$					
Unconstrained	low	1.02495	0.0003	0.0022 ± 0.0007	98.6%
	medium	1.02488	0.0010	0.0080 ± 0.0025	99.6%
	high	1.02505	0.0090	0.0641 ± 0.0208	99.4%
Forced through origin	low	1.02499	0.0003	0.0012 ± 0.0004	92.7%
	medium	1.02497	0.0009	0.0043 ± 0.0013	95.2%
	high	1.02505	0.0075	0.0343 ± 0.0112	93.1%

[a] These values were calculated from 1000 simulated datasets, each with seven timepoints.
[b] "Level of error" refers to the standard deviations for C and R, as defined in Table 2.
[c] Calculated from the population of 1000 α values generated by the 1000 simulated datasets.
[d] Mean and standard deviation of the 95% confidence intervals (CI) for α from the simulated datasets.
[e] Percent of simulated datasets whose 95% confidence interval covered the true value of 1.025.

distributions are based on information available before data collection; posterior distributions are generated by modifying the prior distributions based on the data. When a noninformative (uniform) prior distribution of beta is used, the resulting posterior distribution of β is:

$$P(\beta|\text{data}) = \frac{f_{v1}\left(\frac{\hat{\beta}_1 - \beta}{s_{\hat{\beta}1}}\right) f_{v2}\left(\frac{\hat{\beta}_2 - \beta}{s_{\hat{\beta}2}}\right) f_{v3}\left(\frac{\hat{\beta}_3 - \beta}{S_{\hat{\beta}3}}\right)}{\int f_{v1}\left(\frac{\hat{\beta}_1 - \beta}{s_{\hat{\beta}1}}\right) f_{v2}\left(\frac{\hat{\beta}_2 - \beta}{S_{\hat{\beta}}}\right) f_{v3}\left(\frac{\hat{\beta}_3 - \beta}{s_{\hat{\beta}3}}\right) d\beta} \tag{12}$$

whose mean value is exactly the same as the Pitman estimator (Eqn. 11).

The 95% confidence interval (I_1, I_2) of β can be calculated from the integrals:

$$\int_{-\infty}^{I1} p(\beta|\text{data})d\beta = 0.025 \int_{-\infty}^{I2} p(\beta|\text{data})d\beta = 0.975 \tag{13}$$

Different from a standard Bayesian interval, however, it can be proven that interval (I_1,I_2) covers the true β value 95% of the time if there is no error in x and the same experiments are repeated indefinitely (Cox and Hinkley 1974). Since these integrals do not have an explicit solution, it is necessary to use a numerical approximation method to tackle the problem. These methods approximate the area beneath a curve by dividing a specified range on the x-axis of the distribution (which is the range in $\hat{\beta}_i$ values here) into a number of intervals (discretization steps). The value of the distribution at the beginning of each discretization step was calculated, and multiplied by the width of the interval. The area beneath the curve was estimated from the sum of these approximated areas. For this study, the discretization was centered at the average of the $\hat{\beta}_i$, and ranged over ten times the average standard deviation or 5 times the range of the $\hat{\beta}_i$ (whichever was greater). Then, 4000 equally spaced grid points were taken within this region. The Pitman estimate approaches the estimate resulting from a variance-weighted average of the three slopes when the degrees of the freedom become large.

2.3.4. *Regressing with dummy variables*

For this method of combining datasets, a single regression equation was generated that included the data from all three datasets, with dummy variables to account for differences in the y-intercept between datasets (Neter et al., 1990). This approach is particularly attractive if the error variances are the same and the number of datapoints and the spread of the x values are not the same between experiments, because the datasets are weighted by their respective "informativeness." The regression line for this method is:

$$y = \hat{\beta}x + \gamma_1 + \gamma_2 D_2 + \gamma_3 D_3 \tag{14}$$

where γ_1 is the y-intercept term for the first dataset, γ_2 and γ_3 are the difference between γ_1 and the y-intercept terms for the second and third datasets, respectively, and D_2 and D_3 are dummy variables. The dummy variable D_2 is equal to 1 if x and y are from the second dataset, and is equal to zero if x and y are from the first or third datasets. Likewise, D_3 is equal to 1 if x and y are from the third dataset, and is equal to zero otherwise. For example, if x and y are from the first dataset, Eqn. 14 reduces to $y = \hat{\beta}x + \gamma_1$. If x and y are from the second dataset, $y = \hat{\beta}x + \gamma_1 + \gamma_2 D_2$. To calculate α from the slopes of these lines, Eqn. 7 was used. The 95% confidence interval of α from each combined regression equation was calculated from the 95% confidence interval of the slope as described in Eqn. 8. The number of degrees of freedom used to calculate the standard deviation of the slope was 17 (21 datapoints −3 y-intercepts −1 slope).

3. RESULTS

3.1. Estimating α from a Single Dataset

3.1.1. *Methods for minimizing least squares*

At a low level of error, all methods for minimizing least squares resulted in estimates of α that had similar standard

Table 4. Precision and Accuracy of Kinetic Isotope Effects (α) Calculated from Single Simulated Datasets with Reduced Major Axis Regression[a].

Regression method	Level of error[b]	Mean value of α[c]	Std. dev. of α[c]	Mean 95% CI ± std. Dev.[d]	Cover frequency[e]
1n(R) vs. 1n(C)					
	low	1.0250	0.0001	0.0007 ± 0.0002	96.1%
	medium	1.0251	0.0009	0.0043 ± 0.0014	93.5%
	high	1.0304	0.0070	0.0404 ± 0.0143	95.3%
1n(R/R_1) vs. 1n(C/C_1)					
Unconstrained	low	1.0250	0.0002	0.0008 ± 0.0003	95.8%
	medium	1.0251	0.0010	0.0052 ± 0.0019	94.3%
	high	1.0308	0.0082	0.0497 ± 0.0199	96.2%
1n(R_i/R_j) vs 1n(C_i/C_j)					
Unconstrained	low	1.0250	0.0002	0.0012 ± 0.0004	99.2%
	medium	1.0252	0.0010	0.0079 ± 0.0026	99.5%
	high	1.0405	0.0100	0.0751 ± 0.0284	99.3%

[a] These values were calculated from 1000 simulated datasets, each with seven timepoints.
[b] "Level of error" refers to the standard deviations for C and R, as defined in Table 2.
[c] Calculated from the population of 1000 α values generated by the 1000 simulated datasets.
[d] Mean and standard deviation of the 95% confidence intervals (CI) for α from the simulated datasets.
[e] Percent of simulated datasets whose 95% confidence interval covered the true value of 1.025.

deviations and 95% confidence intervals (Tables 3–6). At medium and high levels of error, most generated mean α values of 1.025, the "true" α value used to generate the datasets (Tables 3–6). However, using reduced major axis regression for datasets with higher levels of error in both R and C resulted in mean values of α substantially greater than the true value used to generate the simulated datasets (Table 4). Given the sensitivity of the accuracy of the estimates of α generated from reduced major axis regressions to error in R and C, this method should not be used to analyze experimental data, when the actual level of error is unknown. Further, weighting the x and y values as in Tables 5 and 6 did not result in any improvement in the α value estimates. Accordingly, all subsequent results presented here use "typical" least-squares regression, which assumes all error is in the y-value.

3.1.2. Methods for assigning x and y values

The precision and accuracy of the α values from regressing ln(R) on ln(C) were high (Table 3). The 95% confidence intervals were reasonably narrow, covering the true value approximately 95% of the time. The mean of the α values was equal to the true value at all levels of error.

Regressing ln(R/R_1) on ln(C/C_1) with an unconstrained y-intercept resulted in unbiased estimates of α values, and 95% confidence intervals that covered the true value ~95% of the time (Table 3). However, the widths of the confidence intervals were slightly greater than those from regressing ln(R) on ln(C), which is primarily due to the fact that the information in the first data point (R_1, C_1) is not utilized by the regression. However, if the regression was forced through the origin, regressing ln(R/R_1) on ln(C/C_1) resulted in 95% confidence

Table 5. Precision and Accuracy of Kinetic Isotope Effects (α) Calculated from Single Simulated Datasets Regressed Using the Least Squares Cubic Method, with $\omega(X_i)/\omega(Y_i) = 1$[a].

Regression method	Level of error[b]	Mean value of α[c]	Std. dev. of α[c]	Mean 95% CI ± std. dev.[d]	Cover frequency[e]
1n(R) vs. 1n(C)					
	low	1.0250	0.0003	0.0013 ± 0.0004	95.2%
	medium	1.0249	0.0009	0.0042 ± 0.0013	94.4%
	high	1.0251	0.0077	0.0376 ± 0.0122	94.3%
1n(R/R_1) vs. 1n(C/C_1)					
Unconstrained	low	1.0250	0.0003	0.0016 ± 0.0006	94.8%
	medium	1.0250	0.0010	0.0052 ± 0.0019	94.3%
	high	1.0250	0.0088	0.0459 ± 0.0167	95.5%
1n(R_i/R_j) vs 1n(C_i/C_j)					
Unconstrained	low	1.0250	0.0003	0.0024 ± 0.0008	99.5%
	medium	1.0249	0.0010	0.0079 ± 0.0025	99.6%
	high	1.0249	0.0091	0.0701 ± 0.0227	99.2%

[a] These values were calculated from 1000 simulated datasets, each with seven timepoints.
[b] "Level of error" refers to the standard deviations for C and R, as defined in Table 2.
[c] Calculated from the population of 1000 α values generated by the 1000 simulated datasets.
[d] Mean and standard deviation of the 95% confidence intervals (CI) for α from the simulated datasets.
[e] Percent of simulated datasets whose 95% confidence interval covered the true value of 1.025.

Table 6. Precision and Accuracy of Kinetic Isotope Effects (α) Calculated from Single Simulated Datasets Regressed Using the Least Squares Cubic Method, with $\omega(X_i)/\omega(Y_i)$ Estimated by Repeated Measurements[a].

Regression method	Level of error[b]	Mean value of α[c]	Std. dev. of α[c]	Mean 95% CI ± std. dev.[d]	Cover frequency[e]
1n(R) vs. 1n(C)					
	low	1.0250	0.0003	0.0013 ± 0.0004	94.6%
	medium	1.0250	0.0009	0.0043 ± 0.0014	95.1%
	high	1.0253	0.0077	0.0377 ± 0.0124	95.2%
1n(R/R_1) vs. 1n(C/C_1)					
Unconstrained	low	1.0250	0.0003	0.0016 ± 0.0006	96.4%
	medium	1.0250	0.0010	0.0052 ± 0.0018	95.9%
	high	1.0254	0.0088	0.0461 ± 0.0169	93.7%
1n(R_i/R_j) vs 1n(C_i/C_j)					
Unconstrained	low	1.0250	0.0003	0.0025 ± 0.0008	99.4%
	medium	1.0250	0.0010	0.0080 ± 0.0025	99.3%
	high	1.0256	0.0088	0.0706 ± 0.0234	99.3%

[a] These values were calculated from 1000 simulated datasets, each with seven timepoints.
[b] "Level of error" refers to the standard deviations for C and R, as defined in Table 2.
[c] Calculated from the population of 1000 α values generated by the 1000 simulated datasets.
[d] Mean and standard deviation of the 95% confidence intervals (CI) for α from the simulated datasets.
[e] Percent of simulated datasets whose 95% confidence interval covered the true value of 1.025.

intervals that covered the true value less than 90% of the time. This may be due to the susceptibility of the slopes from this regression to error in R_1 and C_1.

When regressing $\ln(R_i/R_j)$ on $\ln(C_i/C_j)$ with an unconstrained y-intercept, the 95% confidence intervals were sometimes twice as broad as those generated using the other methods, and covered the true value of α >98% of the time (Table 3), which may indicate that the correction in the number of degrees of freedom for using each datapoint more than once (Section 2.2.2) overcompensated somewhat and inflated the width of the 95% confidence interval. Forcing this line through the origin narrowed the 95% confidence intervals to values similar to those obtained when regressing $\ln(R)$ on $\ln(C)$, though the frequency with which they covered the true value was still a bit low (Table 3). The increased frequency with which the 95% confidence intervals included the true value of α, relative to regressing $\ln(R/R_1)$ on $\ln(C/C_1)$ is likely due to the removal of some of the added emphasis on R_1 and C_1, even when forcing the regression through the origin.

Further analyses were not conducted for regressions forced through the origin, as the 95% confidence intervals for the α values from these regressions did not reliably cover the true value 95% of the time.

3.2. Estimating α from Multiple Datasets

The regression of $\ln(R)$ on $\ln(C)$ was used to examine the effects of different methods to combine the results of three simulated datasets. Two scenarios were explored: (i) the level of error for the three datasets was the same, and (ii), the level of error for the three datasets was different: one had a low level, one had a medium level, and one had a high level of error. In case (i), averaging the slopes from the three datasets resulted in average α values with 95% confidence intervals that covered the true value ~95% of the time (Table 7). However, when the three datasets had different levels of error, this frequency dropped to ~91%. Values of α calculated from a weighted average of the slopes had 95% confidence intervals that covered the true value less than 95% of the time, whether the level of error of the three datasets was the same or not (Table 7). This is probably due to inaccuracies introduced by variance estimates from small sample sizes (see Eqn. 9).

When a Pitman estimator was used to estimate values of α from datasets with unequal levels of error, the 95% confidence intervals covered the true value ~95% of the time. Further, using a Pitman estimator substantially increased the precision of the estimated α values, with substantially smaller standard deviations for the population of 1000 α values and narrower 95% confidence intervals (Table 7).

When three datasets with similar levels of error were combined into a single regression with dummy variables to generate a mean α value, the standard deviations for α were smaller, and the 95% confidence intervals were narrow (Table 7). However, when the levels of error for the three datasets were different, the 95% confidence interval was substantially broader than that from the Pitman procedure.

Regression with dummy variables, determined to be a suitable method for combining datasets with similar levels of error (Table 7), was used to evaluate different forms of the RDE when generating α values from three datasets. Similar to when only a single dataset was used, regressing $\ln(R)$ on $\ln(C)$ resulted in accurate α values (=1.025) and 95% confidence intervals that covered the true value ~95% of the time (Table 8). When regressing $\ln(R/R_1)$ on $\ln(C/C_1)$ with an unconstrained y-intercept, the standard deviation of the population of 1000 α values was similar to those generated when regressing $\ln(R)$ on $\ln(C)$ (Table 8). However, the 95% confidence intervals for individual α values determined from each set of three datasets were broader, and they covered the true value >97% of the time. Regressing $\ln(R_i/R_j)$ on $\ln(C_i/C_j)$ with an unconstrained y-intercept and calculating α values from three lines, similar to when only one line was used (Table 3), generated wider 95% confidence intervals which included the true value

Table 7. Precision and Accuracy of Kinetic Isotope Effects (α) Calculated from Three Simulated Datasets with Varying Levels of Error Using Different Methods to Combine the Datasets[a].

Method of combining the datasets	Level of error[b]	Mean value of α[c]	Std. dev. of α[c]	Mean 95% CI ± std. dev.[d]	Cover frequency[e]
Datasets with same level of error					
Average	medium	1.0250	0.0005	0.0021 ± 0.0004	95.7%
Weighted average	medium	1.0250	0.0005	0.0019 ± 0.0004	92.0%
Dummy variables	medium	1.0250	0.0005	0.0021 ± 0.0004	95.9%
Datasets with different levels of error					
Average	low, medium, and high	1.0250	0.0026	0.0106 ± 0.0034	90.7%
Weighted average	low, medium, and high	1.0250	0.0003	0.0010 ± 0.0003	90.5%
Pitman estimator	low, medium, and high	1.0250	0.0003	0.0012 ± 0.0004	94.4%
Dummy variables	low, medium, and high	1.0250	0.0026	0.0106 ± 0.0031	95.8%

[a] These values were calculated from 1000 simulated datasets, each with seven timepoints, with a regression assigning all error to the *y* values. For all datasets, 1n*R* was regressed on 1n*C*.
[b] "Level of error" refers to the standard deviations for *C* and *R*, as defined in Table 2. "All" indicates that each dataset had a different level of error: one had a low level, one had a medium level, and one had a high level.
[c] Calculated from the population of 1000 α values generated by 1000 groups of three simulated datasets. The mean value of α was 1.025 for all regressions included in this table.
[d] Mean and standard deviation of the 95% confidence intervals (CI) for α from the simulated datasets.
[e] Percent of simulated datasets whose 95% confidence interval covered the true value of 1.025.

>99% of the time (Table 8), further indicating that the number of degrees of freedom was inappropriate.

4. DISCUSSION

4.1. Suggested Form of the RDE for Estimating α

Overall, the results of our study show that kinetic isotope effects are most accurately estimated from the RDE by regressing ln(R) on ln(C) using linear regression with error assigned to the *y* values. Each datapoint is used only once, the precision and accuracy of the α estimates are high at realistic levels of experimental error, and the 95% confidence intervals for α cover the true value approximately 95% of the time and are the narrowest among all methods that result in correct coverage.

In contrast, regressing ln(R/R_1) on ln(C/C_1) is problematic: forcing the line through the origin places too much emphasis on the first data point, and not forcing this line through origin is equivalent to throwing away an observation, as the degrees of freedom decrease by 1 by adding an unconstrained *y*-intercept. Regressing all ln(R_i/R_j) on all ln(C_i/C_j) makes the determination of the degrees of freedom unnecessarily complicated and results in inflated 95% confidence intervals (Tables 3 and 8). However, when the level of precision of the R values approaches the level of uncertainty with which the absolute isotope ratio of the standard is known (e.g., when measuring permeg rather than permil differences), it is more appropriate to use the regression described in Eqn. 3 for the following reasons. During the calibration of a mass spectrometer, one uses the presumed isotope ratio of the standard to calibrate the ratio of ion currents corresponding to the isotopic forms of the compound of interest. The isotope ratio of the sample ($R_{i.samp}$) is calculated as $R_{i.samp}=R_{c.samp}*R_{i.std}/R_{c.std}$, where $R_{c.samp}$ and

Table 8. Precision and Accuracy of Kinetic Isotope Effects (α) Calculated from Three Simulated Datasets Using Different Linear Versions of the RDE.[a]

Regression method	Level of error[b]	Mean value of α[c]	Std. dev. of α[c]	Mean 95% CI ± std. dev.[d]	Cover frequency[e]
1n(R) vs. 1n(C)					
	low	1.0250	0.0002	0.0006 ± 0.0001	94.8%
	medium	1.0250	0.0005	0.0019 ± 0.0003	95.8%
	high	1.0249	0.0044	0.0183 ± 0.0033	95.0%
1n(R/R_1) vs. 1n(C/C_1)					
Unconstrained	low	1.0250	0.0002	0.0010 ± 0.0002	97.4%
	medium	1.0249	0.0006	0.0031 ± 0.0008	97.5%
	high	1.0250	0.0050	0.0278 ± 0.0072	98.6%
1n(R_i/R_j) vs 1n(C_i/C_j)					
Unconstrained	low	1.0250	0.0002	0.0012 ± 0.0002	99.8%
	medium	1.0249	0.0006	0.0042 ± 0.0007	99.8%
	high	1.0249	0.0052	0.0339 ± 0.0061	99.9%

[a] These values were calculated from 1000 sets of three simulated datasets of seven timepoints apiece, with a regression assigning all error to the *y* values. For all regression methods, the three datasets were combined by regressing them in a single line with dummy variables.
[b] "Level of error" refers to the standard deviations for *C* and *R*, as defined in Table 2.
[c] Calculated from the population of 1000 α values generated by 1000 sets of three simulated datasets.
[d] Mean and standard deviation of the 95% confidence intervals (CI) for α from the simulated datasets.
[e] Percent of simulated datasets whose 95% confidence interval covered the true value of 1.025.

$R_{c.std}$ are the ratio of ion currents generated from the sample and standard, respectively, and $R_{i.std}$ is the isotope ratio of the standard (Santrock et al., 1985). Calculating α values using R/R_1 values has the advantage of canceling out $R_{i.std}$. In this case, using the regression described in Eqn. 3 without forcing it through the origin would be optimal.

Regression methods that incorporate error in x-values did not give more accurate or precise estimates of α; further, reduced major axis regression generated mean α values consistently higher than the true value at higher levels of error (Tables 4–6). This is due to the sensitivity of reduced major axis regression slope estimates to the size of the error in y. Since

$$y = \beta z + \gamma \tag{15}$$

where z is the true value of x ($x=z+\delta$, and δ is the error in x), the slope of the line in reduced major axis regression is

$$\hat{\beta} = \sqrt{\frac{\sum(y_i-\bar{y})^2}{\sum(x_i-\bar{x})^2}} = \sqrt{\frac{\beta^2\sum(z_i-\bar{z})^2 + \sum(\varepsilon_i-\bar{\varepsilon})^2}{\sum(z_i-\bar{z})^2 + \sum(\delta_i-\bar{\delta})^2}} \tag{16}$$

In order for Eqn. 16 to give a consistent estimate of β,

$$\sum(\varepsilon_i-\bar{\varepsilon})^2 = \beta^2\sum(\delta_i-\bar{\delta})^2 \tag{17}$$

implying that the error variance in y needs to be about β^2 times that in x. Datasets with medium and high levels of error in y thus resulted in an overestimate of β.

Measurement errors in x values induce an inflation of the sum of squares (denominator in Eqn. 6) approximately equal to $(n-1)s^2$, where s^2 is the variance of the measurement. This effect can be neglected if s is much smaller than the range of $\ln(C)$ values. On the other hand, if the variance of the measurement errors in $\ln(C)$ can be determined, the slope estimates from Eqn. 6 can be adjusted to

$$\hat{\beta} = \frac{\sum(y_i-\bar{y})(x_i-\bar{x})}{\sum(x_i-\bar{x})^2 - (n-1)s^2} \tag{18}$$

4.2. Suggested Methods for Combining Datasets

When the results of replicated experiments with similar levels of error are combined to give a mean α, a single regression line with dummy variables is a reliable way to obtain accurate and precise estimates of α. Unweighted averages gave similar results here only because all three of the datasets combined had the same number of observations and a similar spread in x values. Otherwise, taking the unweighted average has the undesirable consequence of weighting less informative datasets (fewer datapoints or a smaller spread in x values) equally to more informative ones. The weighted average method is not very reliable when small samples sizes are involved and uncertainties in variance estimates are not negligible. Further, there is no theoretical method to estimate the appropriate degrees of freedom for these averaging approaches.

If the level of error among the three datasets varies substantially, we recommend using the Pitman estimator under these circumstances, as it substantially increases the precision of the α estimates. Given that Pitman estimators are a novelty to non-statisticians, to facilitate the application of this method by other researchers, a Matlab program to calculate α values with Pitman estimators is available as an electronic annex from http://www.fas.harvard.edu/~junliu/isotope or from the Elsevier website (http://www.elsevier.com/inca/publications/store/2/1/2/). When all datasets have a similar level of error, using a Pitman estimator will yield results similar to those obtained when taking the average or using the dummy variable method. In general, the Pitman estimation approach is preferable as a way to combine information from replicated experiments in all situations although it is theoretically more involving and computationally more challenging.

4.3. Extension of the Results to Other Systems and Experimental Designs

Although this study was instigated by measurements of enzymatic kinetic isotope effects, the results are applicable to any Rayleigh distillation and to measurements of α with any true value. Measured values of α span a broad range, from $\alpha = 1.0007$ for the diffusion of $^{12}CO_2$ and $^{13}CO_2$ through water (O'Leary, 1984) to $\alpha=1.3$ for the hydrogenation of methylated substrates by methanogens (Whiticar, 1999). Since the standard deviation of the 1000 α estimates is equal to

$$s_{sample} = \sqrt{\frac{\sum(\alpha_i-\bar{\alpha})^2)}{n-1}} \tag{19}$$

it will scale with the value of α. Likewise, since the standard deviation of the slope estimate is

$$s_{\hat{\beta}} = \sqrt{\frac{\sum(y_i-\hat{y})^2/(n-2)}{\sum(x_i-\bar{x})^2}} \tag{20}$$

it will also scale with the value of α. Accordingly, the 95% confidence intervals will include the true value of α with frequencies virtually identical to the ones presented here.

The conclusions from this study are also applicable to different experimental designs. For instance, the results would have been the same if the simulated datasets had a number of datapoints other than seven. With fewer datapoints, the slope estimates for the lines (and therefore the α values) will be more sensitive to error in the individual datapoints, which will increase both the standard deviation for the population of α values and the standard deviation of the slope estimates. Since the width of the 95% confidence intervals will therefore also increase (Eqn. 20), the frequency with which they cover the true value should be similar to the frequencies reported here, for seven datapoints. It is perhaps more profitable to use the Pitman estimator in this situation, as this would narrow the widths of the 95% confidence intervals. The same discussion, adjusted accordingly, holds true for datasets larger than seven. Better estimates of α values will substantially improve the interpretation of the isotopic compositions of materials of terrestrial and extraterrestrial origin, thus enhancing our understanding of the biologic, chemical, or physical alterations affecting them.

Acknowledgments—We are most thankful for the suggestions of John Hayes and two anonymous reviewers. We gratefully acknowledge support from the National Science Foundation (NSF-OCE # 0002460 to CMC and KMS; NSF-DMS # 0094613 and # 0204674 to JSL) and the National Undersea Research Program (UAF-WA # 98-06 to CMC and KMS).

Associate editor: J. Horita

REFERENCES

Berges J. A., Montagnes D. J. S., Hurd C. L., and Harrison P. J. (1994) Fitting ecological and physiological data to rectangular hyperbolae: A comparison of methods using Monte Carlo simulations. *Mar. Ecol. Prog. Ser.* **114,** 175–183.

Broecker W. S. and Oversby V. M. (1971) Rayleigh distillation. In *Chemical Equilibria in the Earth* (ed. F. Press), pp. 165–167. McGraw-Hill Book Company, New York.

Cox D. R. and Hinkley D. V. (1974) Theoretical Statistics. Chapman & Hall, London, pp. 221–222 and 444.

Guy R. D., Fogel M. L., and Berry J. A. (1993) Photosynthetic fractionation of the stable isotopes of oxygen and carbon. *Plant Physiol.* **101,** 37–47.

Henry B. K., Atkin O. K., Day D. A., Millar A. H., Menz R. I., and Farquhar G. D. (1999) Calculation of the oxygen isotope discrimination factor for studying plant respiration. *Aust. J. Plant Physiol.* **26,** 773–780.

Hunkeler D. and Aravena R. (2000) Evidence of substantial carbon isotope fractionation among substrate, inorganic carbon, and biomass during aerobic mineralization of 1,2-dichloroethane by. *Xanthobacter autotrophicus. Appl. Environ. Microbiol.* **66,** 4870–4876.

Mariotti A., Germon J. C., Hubert P., Kaiser P., Letolle R., Tardieux A., and Tardieux P. (1981) Experimental determination of nitrogen kinetic isotope fractionation: Some principles; illustration for the denitrification and nitrification process. *Plant Soil.* **62,** 413–430.

Neter J., Wasserman W., and Kutner M. H. (1990) Qualitative independent variables. In *Applied linear statistical models,* Chap. 10, pp. 349–385. Irwin, Inc. Boston.

O'Leary M. H. (1980) Determination of heavy-atom isotope effects on enzyme-catalyzed reactions. *Meth. Enzymol.* **64,** 83–104.

O'Leary M. H. (1984) Measurement of the isotopic fractionation associated with diffusion of carbon dioxide in aqueous solution. *J. Phys. Chem.* **88,** 823–825.

O'Leary M. H., Madhavan S., and Paneth P. (1992) Physical and chemical basis of carbon isotope fractionation in plants. *Plant, Cell, Env.* **15,** 1099–1104.

Pennock J. R., Velinsky D. J., Ludlam J. M., and Sharp J. H. (1996) Isotopic fractionation of ammonium and nitrate during uptake by *Skeletonema costatum*: Implications for $\delta^{15}N$ dynamics under bloom conditions. *Limnol. Oceanogr.* **41,** 451–459.

Pitman E. J. G. (1939) The estimation of the location and scale parameters of a continuous population of any given form. *Biometrika* **30,** 391–421.

Rahn T., Zhang H., Wahlen M., and Blake G. A. (1998) Stable isotope fractionation during ultraviolet photolysis of N_2O. *Geophys. Res. Lett.* **25,** 4489–4492.

Richter F. M., Davis A. M., Ebel D. S., and Hashimoto A. (2002) Elemental and isotopic fractionation of Type B calcium-, aluminum-rich inclusions: Experiments, theoretical considerations, and constraints on their thermal evolution. *Geochim. Cosmochim. Acta* **66,** 521–540.

Ritchie R. J. and Prvan T. (1996) A simulation study on designing experiments to measure the K_m of Michaelis-Menten kinetics curves. *J. Theor. Biol.* **178,** 239–254.

Robinson J. J., Scott K. M., Swanson S. T., O'Leary M. H., Horken K., Tabita F. R., and Cavanaugh C. M. (2003) Kinetic isotope effects and characterization of form II RubisCO from the chemoautotrophic endosymbionts of the hydrothermal vent tubeworm. *Riftia pachyptila. Limnol. Oceanogr.* **48,** 48–54.

Roeske C. A. and O'Leary M. H. (1984) Carbon isotope effects on the enzyme-catalyzed carboxylation of ribulose bisphosphate. *Biochem.* **23,** 6275–6284.

Roeske C. A. and O'Leary M. H. (1985) Carbon isotope effect on carboxylation of ribulose bisphosphate catalyzed by ribulosebisphosphate carboxylase from *Rhodospirillum rubrum*. *Biochem.* **24,** 1603–1607.

Rudnicki M. D., Elderfield H., and Spiro B. (2001) Fractionation of sulfur isotopes during bacterial sulfate reduction in deep ocean sediments at elevated temperatures. *Geochim. Cosmochim. Acta* **65,** 777–789.

Santrock J., Studley S. A., and Hayes J. M. (1985) Isotopic analyses based on the mass spectrum of carbon dioxide. *Anal. Chem.* **57,** 1444–1448.

Sokal R. R. and Rohlf F. J. (1995) Biometry: the principles and practice of statistics in biological research. W. H. Freeman and Company, New York.

Wang J., Davis A. M., Clayton R. N., Mayeda T. K., and Hashimoto A. (2001) Chemical and isotopic fractionation during the evaporation of the FeO-MgO-SiO_2-CaO-Al_20_3-TiO_2 rare earth element melt system. *Geochim. Cosmochim. Acta* **65,** 479–494.

Weger H. G., Guy R. D., and Turpin D. H. (1990) Cytochrome and alternative pathway respiration in green algae. *Plant Physiol.* **93,** 356–360.

Whiticar M. J. (1999) Carbon and hydrogen isotope systematics of bacterial formation and oxidation of methane. *Chem. Geol.* **161,** 291–314.

York D. (1966) Least-squares fitting of a straight line. *Can J Phys.* **44,** 1079–1086.

York D. (1967) The best isochron. *Earth Planet. Sci. Lett.* **2,** 479–482.