Hydrogen isotope fractionation in algae: III. Theoretical interpretations

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Hydrogen isotope measurements of lipid biomarkers preserved in sediments are most commonly interpreted as qualitative, rather than quantitative indicators of paleoprecipitation owing to an imperfect knowledge of all factors controlling the isotopic fractionation occurring during biosynthesis. Here, we first offer a brief review of appropriate procedures for preparing enriched isotope substrates for use in tracer studies and outline the approximate δD threshold at which this transition occurs. We then present new interpretations to explain deviations from common stable isotope effects observed in our previous culture experiments and other studies. We draw particular attention to the disagreement between intercept and slope for product–substrate relationships from those predicted for isotope systems, even when δ2H values are high, and attribute it to kinetic isotope fractionation. We demonstrate that reconstructing paleoenvironmental water δD values by simply adding a δD to measured biomarkers δD values will result in a bias toward deuterium enriched values. This applies even to implicit reconstructions in the form of qualitative interpretations of measured lipid δD values as indicators of past hydroclimate. We therefore recommend reconstructing water δD values from lipid δD values using fractionation factor (α).

We also discuss the apparently contradictory increase in D/H fractionation observed at elevated temperature and suggest that this may be the result of the unique wave-particle duality of hydrogen isotopes, which permits isotopologues to avoid surmounting the activation energy barrier that is necessary in traditional kinetic reactions.

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1. Introduction

Improved understanding of the factors that control the hydrogen isotopic composition of algal lipid biomarkers in lacustrine and marine sediments holds great potential for direct reconstruction of water δD values, a long-standing goal of paleoclimatology. All hydrogen in phytoplankton derives from the water in their immediate environment and algae are not affected by processes that alter δD values in land plants (e.g. transpiration). Lipid biomarkers are generally well preserved in sediments and have thus become attractive targets for studies aimed at reconstructing paleoprecipitation (e.g. Sauer et al., 2001; Huang et al., 2002; Englebrecht and Sachs, 2005; van der Meer et al., 2007; Sachs et al., 2009; Smittenberg et al., 2011).

As in previous papers (Zhang and Sachs, 2007; Zhang et al., 2009), the defined reference standard for hydrogen isotope ratios, described as the relative abundances of deuterium (D; ²H) and protium (H; ¹H) is Vienna Standard Mean Ocean Water (VSMOW). D/H is used interchangeably with R, the raw isotope ratio, and δD is defined as: δD = (Rsample/VSMOW – 1) × 1000‰. The fractionation factor, α, is defined as:  αlipid-water = Rlipid/Rwater = (δDlipid + 1000)/(δDwater + 1000) and the enrichment factor, ε, is: ε lipid-water = (δDlipid − δDwater)/(δDwater − 1) × 1000. The hydrogen isotopic difference between a biomarker and water is defined as: Δ lipid-water = δDlipid − δDwater.

The large mass difference between H and D results in exceptionally large fractionation during hydrogen isotope reactions. In an extreme case, the rate constants for dissociation of H₂ → 2H and D₂ → 2D differ by 31 times, i.e. kH/kD ≈ 31 (Chang, 2005). Although for reactions where most hydrogen is bound to carbon atoms, the observed ratio of kC-D/kC-H is smaller (≈ 5) but still appreciable (Chang, 2005). This leads to a large range of δD values and accentuates nonlinearities of δ notation that can often be neglected for other stable isotope systems.

In this study we examine the disagreement between product and substrate δD values when viewed in the context of established stable isotope fractionation theory, as well as inexplicably larger fractionation at higher growth temperature. We illustrate these issues using results from two previous papers (Zhang and Sachs,
2. Isotope calculations in tracer-level studies

As shown through decades of research using stable isotopes at artificially high concentrations in tracer studies, as \( \delta \) values and D/H ratios reach extremes the approximate nature of this terminology becomes inadequate to accurately predict the isotopic composition of mixtures using a mass balance approach (e.g. Brenna et al., 1997). We offer the following brief review of this subject in the context of our batch cultures as a reminder of the need to use atom fraction, or F notation in such work, and illustrate the threshold to which this transition occurs.

We compared the difference between linear mixing model calculations based on D/H ratios and F notation by preparing two water samples with each approach. They predicted \( \delta \) values of 33‰ and 357‰, a difference of 324‰, but the measured difference of samples prepared based on the D/H calculation was only 1‰. This illustrates the difference between the F notation vs. F notation samples was 313‰, and therefore within the intended range.

The range over which \( \delta \)D notation is appropriate is illustrated in Fig. 1A. We plot the \( \delta \)D in log scale to define the applicable range to use \( \delta \)D notation in linear mixing calculations (Fig. 1A). Log (\( \delta \)D) vs. D/(D + H) ratio (Fig. 1A) begins to curve after log (\( \delta \)D) > 4, i.e., \( \delta \)D > 10,000 (Fig. 1A). The range for linear mixing calculations should thus be limited to \( \delta \)D < 10,000‰ where D/(D + H) > 0.1710 or D/H = 0.1713 (Fig. 1B), which more than covers the natural range of \( \delta \)D values.

3. Disagreement between slope and intercept on regression lines

In organic geochemistry, hydrogen isotope fractionation is typically treated in the same manner as carbon and oxygen isotope fractionations. In this traditional view, the slope and intercept in linear regression equations from field observations or laboratory cultures are considered as the fractionation factor (\( \alpha \), slope) and the enrichment factor (\( c \), intercept). Isotopic fractionation is described by a fractionation factor, \( \alpha \) where

\[
\alpha = \frac{R_{\text{product}}}{R_{\text{substrate}}}.
\]

By this definition, \( \alpha \) can be determined from any single pair of product and substrate \( \delta \)D measurements. From the definition \( \delta D_{\text{product}} = \alpha \times \delta D_{\text{substrate}} \), it follows that

\[
\delta D_{\text{product}} = \alpha \times \delta D_{\text{substrate}} \times (\alpha - 1) \times 1000.
\]

A concrete example of this phenomenon, we consider the fractionation of hydrogen isotopes by photoautotrophs (algae or higher plants) during photosynthesis. Because water is the source of all hydrogen in photoautotrophs, the isotopic compositions of their lipids and environmental water are expected to be related by \( \delta D_{\text{product}} = \alpha \times \delta D_{\text{substrate}} \times (\alpha - 1) \times 1000 \), in which a single fractionation factor represents the net effect of all biosynthetic processes. If multiple lipid–water pairs are measured for the same isotope reaction over a range of substrate \( \delta D \) values they define a regression line and the slope and intercept of this line gives two additional estimations of the fractionation factor (Table 1). The slope, \( \alpha \), and the intercept (\( \alpha - 1 \)) 1000 (also referred to as \( c \)) should give the same estimate for the value of \( \alpha \) using the stated equations (Sessions and Hayes, 2005), and both should equal \( \alpha \) as calculated from any individual product–substrate pair.

For well constrained isotope reactions, such as equilibrium fractionation between water and vapor, the expectation that the intercept, \( c \), is exactly equal to (\( \alpha - 1 \)) 1000, for slope-derived \( \alpha \), holds even for multiple consecutive isotope reactions (Craig and Gordon, 1965). Either \( \alpha \) or \( c \) may be used to describe the isotopic effect of a reaction or sequence of reactions. Several authors have attempted to calibrate the magnitude of D/H fractionation in freshwater algae by measuring values of \( \delta D \) for lipids and lake water from sites covering a latitudinal range. Results are summarized in Table 2. These studies show that for biomarker hydrogen isotope data, estimates of fractionation derived from the slope compared to the estimates from the intercept of a regression line are not equal.

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**Fig. 1.** The applicable range of a binary linear mixing equation using \( \delta \)D notation. (A) The relation between log (\( \delta \)D) and D/(D + H) ratio (F). When log (\( \delta \)D) > 4, i.e., \( \delta \)D > 10,000‰ or D/(D + H) > 0.001710, the line begins to curve, i.e. any further small change of D/(D + H) ratio would lead to much larger variation in \( \delta \)D notation. (B) The range of applicability of \( \delta \)D or D/H ratio for binary linear mixing calculation. Mixing that involves an end member with very large \( \delta \)D values should not use \( \delta \)D notation for the linear mixing calculation, but rather D/(D + H) or F notation, which is always correct.
alkadiene is related to growth water

\[ \frac{\Delta D_{\text{alkadiene}}}{\Delta D_{\text{water}}} = 0.708 \]

Calibrations between biomarker and water

The relations among biomarker and water values calculated from individual measurement pairs falling between these two extremes. We refer to this issue as the "slope/intercept disagreement". The fractionations should be equivalent, and the fact that they are not implies that the relationship is not as simple as has been assumed. Sessions and Hayes (2005) suggested that slope/intercept disagreement might largely be attributable to the relatively low \( R^2 \) values in the data sets that they analyzed, but our data demonstrate the slope/intercept disagreement despite \( R^2 \) values that are in excess of 0.99 (Fig. 2). This suggests that the issue is a real and unexplained phenomenon and requires explanations.

Lipid biosynthesis reactions are complex and typically involve multiple equilibrium and kinetic isotope effects (Hayes, 2001). Because it is usually not possible to measure all precursor compounds and intermediate products, intermediate fractionation steps are commonly grouped into one "apparent fractionation factor" that is defined as a single \( \alpha \) or \( \varepsilon \) value. Use of this convention to describe the isotope effects in lipid biosynthesis might therefore be a possible cause of the slope/intercept disagreement.

If all hydrogen in photoautotrophic lipids is fundamentally derived from water, one view of biosynthesis is to treat it as a black box with H in water as input and H in the biomarkers as output. For algae, if such fractionation is in thermodynamic equilibrium, the pairs of biomarkers and water would fall on a line: \( \Delta D_{\text{lipid}} = \Delta D_{\text{lipid-water}} \times \Delta D_{\text{water}} + (\Delta D_{\text{lipid-water}} - 1) \times 1000 \). However, taking this view results in the previously discussed slope/intercept disagreement.

As an alternative approach to address the hydrogen isotope ratios of lipids during biosynthesis, consideration in the context of a kinetic isotope model (e.g., Criss, 1999) may more adequately capture the complexity and allow room for a plausible explanation. Generalized isotope fractionation during algal growth can be written as

\[ AD + WH \xrightarrow{\frac{s}{k}} AH + WD \]

where \( A \) and \( W \) stand for a particular algal biomarker and water, respectively, and \( H \) and \( D \) for hydrogen and deuterium, respectively. The biosynthesis involves multiple steps from water and here we treat the whole process as one box with input and output shown only. The forward and the reverse reactions don’t proceed at identical rates, but rather at rates indicated by the quantities \( s \) and \( k \), respectively, suggested by the arrows (\( k \) is a first-order rate constant), multiplied

\[ \Delta D_{\text{of water}} = \Delta D_{\text{water}} \times \Delta D_{\text{lipid-water}} + (\Delta D_{\text{lipid-water}} - 1) \times 1000 \]

**Table 1**

<table>
<thead>
<tr>
<th>Cultures</th>
<th>Water ( \Delta D ) at harvest</th>
<th>( C_{18:1} ) fatty acid ( \Delta D )</th>
<th>( C_{29:2} ) alkadiene ( \Delta D )</th>
<th>( A_{\text{lipid-water}} )</th>
<th>( A_{\text{alkadiene-FA}} )</th>
<th>( A_{\text{FA-W}} )</th>
<th>( A_{\text{alkadiene-W}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2W1</td>
<td>-26</td>
<td>-206</td>
<td>-245</td>
<td>-180</td>
<td>-39</td>
<td>0.816</td>
<td>0.775</td>
</tr>
<tr>
<td>A2W2</td>
<td>129</td>
<td>-76</td>
<td>-120</td>
<td>-205</td>
<td>-44</td>
<td>0.819</td>
<td>0.799</td>
</tr>
<tr>
<td>A2W3</td>
<td>221</td>
<td>-7</td>
<td>-58</td>
<td>-228</td>
<td>-51</td>
<td>0.813</td>
<td>0.772</td>
</tr>
<tr>
<td>A2W4</td>
<td>321</td>
<td>7</td>
<td>7</td>
<td>-249</td>
<td>-65</td>
<td>0.812</td>
<td>0.762</td>
</tr>
<tr>
<td>A2W5</td>
<td>500</td>
<td>207</td>
<td>131</td>
<td>-293</td>
<td>-76</td>
<td>0.805</td>
<td>0.754</td>
</tr>
</tbody>
</table>

\( \Delta D_{\text{alkadiene}} \) vs. water regression line:

- \( \Delta D_{\text{alkadiene-W}} = 0.783 \times \Delta D_{\text{water}} - 181 \)
- \( \Delta D_{\text{alkadiene,FA}} = 0.708 \times \Delta D_{\text{water}} - 219 \)
- \( \Delta D_{\text{alkadiene}} = 0.905 \times \Delta D_{\text{FA}} - 55 \)

**Table 2**

Calibrations between \( \Delta D \) values of biomarkers and environmental water.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Derived relationship</th>
<th>( \Delta D_{\text{lipid}} )</th>
<th>( \Delta D_{\text{water}} )</th>
<th>( R^2 )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk lipids</td>
<td>( \Delta D_{\text{lipid}} = 0.546 \times \Delta D_{\text{water}} - 141 )</td>
<td>0.546</td>
<td>0.859</td>
<td>0.618</td>
<td>Sternberg (1988)</td>
</tr>
<tr>
<td>Phytoplankton sterols</td>
<td>( \Delta D_{\text{lipid}} = 0.748 \times \Delta D_{\text{water}} - 199 )</td>
<td>0.748</td>
<td>0.801</td>
<td>0.965</td>
<td>Sauer et al. (2001)</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>( \Delta D_{\text{lipid}} = 0.939 \times \Delta D_{\text{water}} - 167 )</td>
<td>0.939</td>
<td>0.833</td>
<td>0.894</td>
<td>Huang et al. (2002)</td>
</tr>
<tr>
<td>( C_{18:1} ) fatty acid</td>
<td>( \Delta D_{\text{lipid}} = 0.783 \times \Delta D_{\text{water}} - 181 )</td>
<td>0.783</td>
<td>0.819</td>
<td>0.999</td>
<td>Zhang and Sachs (2007)</td>
</tr>
<tr>
<td>( C_{29:2} ) alkadiene</td>
<td>( \Delta D_{\text{lipid}} = 0.708 \times \Delta D_{\text{water}} - 219 )</td>
<td>0.708</td>
<td>0.781</td>
<td>0.998</td>
<td>Zhang and Sachs (2007)</td>
</tr>
</tbody>
</table>

\( a \) Fractionation factor calculated from the slope of the derived relationship.

\( b \) Fractionation factor calculated from the intercept of the derived relationship.
by the concentration terms. Given the fact that the amount of hydrogen atoms in the water was approximately 1000 times greater than that in the algal biomass, $R_w$ can be considered a constant.

Adapting from Criss (1999), the δD value of an algal lipid is:

$$\delta D_a = e^{-\text{kt}} \times \delta D_k + 1000 \times (x_{A-W} - 1) \times (1 - e^{-\text{kt}}) + x_{A-W} \times (1 - e^{-\text{kt}}) \times \delta D_w$$

where $t$ is time, and $\delta D_k$ is the initial lipid δD value at $t = 0$, i.e. when the inoculant was brought into culturing. This equation contains three terms. The first two are constants for five batch cultures for a given strain, but the third term is a function of the water δD values (i.e. the heavier the water δD value, the larger $A_{A-W}$). Since the five cultures for each species in our experiments (Zhang and Sachs, 2007) started at the same time and were grown under the same cultures for each species in our experiments (Zhang and Sachs, 2007) started at the same time and were grown under the same growing conditions, $e^{-\text{kt}}$ can be treated as a constant. In this case, the slope of the linear regression is $x_{A-W} \times (1 - e^{-\text{kt}})$, and the intercept is $e^{-\text{kt}} \times \delta D_k + 1000 \times (x_{A-W} - 1) \times (1 - e^{-\text{kt}})$. The terms $x_{A-W}$ and 1000 ($x_{A-W} - 1$), which describe the slope and intercept under thermodynamic equilibrium are still included in this equation, but additional terms are included to describe the kinetic effects. If, after infinite time equilibrium is attained, i.e. $t \rightarrow \infty$, then, $e^{-\text{kt}} \rightarrow 0$ and as a result, the equation can be simplified to $\delta D_a = 1000 \times (x_{A-W} - 1) + x_{A-W} \times \delta D_w$, equivalent to the equilibrium condition, and under such circumstances $x$ and $e$ will be consistent with the observed relation between $\delta D_a$ and $\delta D_w$.

Although our experiments were conducted with D-enriched material and therefore include a range of δD values that are outside the range of available international reference materials (Sternberg, 1988; Sauer et al., 2001; Huang et al., 2002; Sessions and Hayes, 2005), we consider the fact that previous investigations also observed the slope/intercept disagreement to be an argument against the idea that our results are a referencing artifact. Similarly it could be argued that our results might be driven by slight differences in the growth curves of the algae in our batch cultures, but this would require a systematic effect as a function of the substrate δD values, which we also consider unlikely. Instead, we suggest that the disagreements in spite of high $R^2$ values in our studies and previous observations in other studies most likely stem from the fact that although hydrogen isotope fractionation in sedimentary organic geochemistry is most often considered in the context of thermodynamic equilibrium (e.g. Huang et al., 2002; Sessions and Hayes, 2005), in reality such equilibrium is likely never attained during biosynthetic reactions and they should be considered kinetic.

### 4. Possible bias in paleoprecipitation reconstruction when using $A$

Although most hydrogen isotope applications do not make direct use of $A$ notation ($\delta D_{\text{product}} - \delta D_{\text{substrate}}$), paleoclimatic studies that consider lipid δD values as a direct indicator of hydrology without the consideration of the magnitude of D/H fractionation are effectively doing so implicitly (Polissar and D’Andrea, 2014).

Fig. 2 demonstrates how $A$ values between lipid biomarkers and water are related with water δD values in cultures of $B$. braunii A race, Titicaca strain. δD values of both $C_{18:1}$ fatty acids and alkadienes in five cultures were linearly correlated with water δD values at harvest (Table 1), with $R^2 = 0.998$ and 0.999, respectively (Fig. 2). $A_{A-W}$ values of $-180\%\text{e}$, $-205\%\text{e}$, $-228\%\text{e}$, $-249\%\text{e}$ and $-293\%\text{e}$ in five cultures corresponded to water δD values of $-26\%\text{e}$, $129\%\text{e}$, $221\%\text{e}$, $321\%\text{e}$ and $500\%\text{e}$, respectively (Table 1; Fig. 2). The magnitude of $A_{A-W}$ therefore changes as water δD values change ($A_{A-W}$ represented by dotted arrows); the larger δD, the bigger magnitude of $A_{A-W}$. The same effect occurs for the $C_{29}$ alkadiene, but the slope of the regression line for $C_{29}$ alkadiene δD–water δD (0.708) is smaller than that for $C_{18:1}$ fatty acid (0.783) (Fig. 2). $C_{29}$ alkadiene is the product of $C_{18:1}$ fatty acid decarboxylation (Templier et al., 1984, 1991), so the progressive decrease in slope with each subsequent measured biosynthetic product reflects the $A$ change at each stage and not just for the earliest measured product. Presumably, if another lipid were synthesized from $C_{29}$ alkadiene, its δD value would show analogous substrate-δD dependence on the $C_{29}$ alkadiene, resulting in an even smaller slope. This illustrates how different compounds would record different δD value changes in response to identical changes in the δD value of source water and therefore why it is important to consider the fractionation factor in interpreting hydrogen isotope data. In addition, this might also serve as a good example how multiple fractionation processes involved in a “net” fractionation result in stronger slope/intercept disagreement. We therefore recommend reconstructing water δD values from lipid δD values using $A$, which accounts for the D/H discrimination between lipid and water, using the equation: $\delta D_w = (\delta D_{\text{lipid}} + 1000)/z - 1000$ (Zhang and Sachs, 2007).

The fractionation factor, $z$, can be obtained through culture experiments or core-top studies. In our case, despite the increased $A_{A-W}$ values with increased water δD values, the $x$ values calculated from individual product–substrate pairs in these five cultures are nearly constant, 0.816, 0.819, 0.813, 0.812 and 0.805 (average = 0.813, standard deviation 0.005) (Table 1). Similarly, the $x$ values of alkadienes calculated this way in these five cultures are 0.775, 0.779, 0.772, 0.762 and 0.754 (average = 0.769, standard deviation = 0.01) (Table 1). This suggests that the fractionation calculated from any one of these pairs would give a reasonable approximation of $z$, and therefore provide a more reliable means of comparing the change in source water δD values that is driving each lipid δD value. Interestingly, the $x$ values calculated from product–substrate pairs agree much more closely with the intercept-derived fractionation factor from the regression equations than with those calculated from the slope. Converting each of the average $x$ values above to $\epsilon$ values for comparison, $-187\%\text{e}$ and $-231\%\text{e}$ for $C_{18:1}$ fatty acid and $C_{29}$ alkadiene agree much more closely with the intercepts of $-181\%\text{e}$ and $-219\%\text{e}$, respectively, than they do with the slope-derived $\epsilon$ values of $-217\%\text{e}$ and $-292\%\text{e}$, respectively (Table 1). This again highlights the disagreement between the slope and intercept.

### 5. Kinetic hydrogen isotope effect in enzyme-catalyzed reactions: rate independence

In the energy profile of an uncatalyzed reaction (Fig. 3A), $y$ is the free energy difference for the reaction ($y = -RT \ln K_{eq}$) and $x$ is the activation energy for the forward direction, with $x + y$ representing the activation energy for the reverse (Cleland and Northrop, 1999). Lipid synthesis reactions are enzyme catalyzed and therefore involve the formation of an enzymatic substrate complex, ES, between the substrate and the enzyme, and it is assumed that reactants are always in equilibrium with ES. Following formation, the ES complex isomerizes to an enzymatic product, followed by dissociation of the product to give a free enzyme again (Fig. 3B). In such a profile for an enzyme catalyzed reaction with the same $K_{eq}$ (Fig. 3B), the activation energy is reduced to that required for the conversion of ES to EP (Z).

A kinetic isotope effect (KIE) results from activation energy differences for the different isotopologue reactants, and much of its magnitude is due to the differences of zero-point energy (ZPE) between the ground state and the transition state of the reaction. KIE is the ratio of rates of the two isotopologue reactants (molecules that only differ in their isotopic compositions), e.g. KIE = $k_{18}/k_{20}$. In biochemistry, $x$ is defined as $x = R_{\text{react}}/R_{\text{product}} = K_{\text{cat}}/k_0$ (Valentine et al., 2004). In this paper, we define $x$ as $R_{\text{product}}/R_{\text{water}}$ so that $x = 1[1]/(k_{18}/k_{20})$. For proton transfer
reactions, one of the most fundamental and prevalent processes in biology, this ratio of rates between H and D isotope is characteristic of the reaction coordinate and the nature of the transition state (Kohen, 2006):

\[
\ln(k_{H}/k_{D}) \approx -\frac{(\Delta G_{H} - \Delta G_{D})}{RT}, \; \text{i.e.} \; k_{H}/k_{D} \approx \exp\left[-\frac{(\Delta G_{H} - \Delta G_{D})}{RT}\right]
\]

where \(\Delta G_{H} - \Delta G_{D} \approx \Delta G_{ZPE}^{H} - \Delta G_{ZPE}^{D}\), where \(T\) is temperature, and \(R\) is the gas constant (Melander and Saunder, 1980; Kiefer and Hynes, 2006; Kohen, 2006). Kinetic fractionation is therefore only dependent on the differences in activation energy for the different isotopologue reactants and temperature, and is independent of substrate concentration. Accordingly, we observed that hydrogen isotope fractionation in fatty acids changed as a function of temperature in cultures of \(V. \) aureus grown at 25 \(^\circ\)C exhibiting substantially larger D/H fractionation than those grown at 15 \(^\circ\)C (Zhang et al., 2009), which is against the classic view that mass-dependent isotope fractionation decreases with increased temperatures. For example, the carbon isotope fractionation associated with malate synthesis in CAM plant \(Kalanchoe daigremontiana\) is temperature dependent. The fractionation is approximately –4‰ at low temperatures and approaches zero at higher temperatures (Deleens et al., 1985). Within the range of algal physiological temperatures in which our temperature experiments were conducted, isotope fractionation theory would predict a decrease in D/H fractionation with increasing temperature. Our observation of a temperature effect of opposite sign therefore requires an alternative explanation.

6. Hydrogen tunneling, a possible mechanism underlying inexplicable hydrogen isotope fractionation phenomena

The algae species \(E. \) unicoeca and \(V. \) aureus grown at 25 \(^\circ\)C exhibited substantially larger D/H fractionation than those grown at 15 \(^\circ\)C (Zhang et al., 2009), which is against the classic view that mass-dependent isotope fractionation decreases with increased temperatures. For example, the carbon isotope fractionation associated with malate synthesis in CAM plant \(Kalanchoe daigremontiana\) is temperature dependent. The fractionation is approximately –4‰ at low temperatures and approaches zero at higher temperatures (Deleens et al., 1985). Within the range of algal physiological temperatures in which our temperature experiments were conducted, isotope fractionation theory would predict a decrease in D/H fractionation with increasing temperature. Our observation of a temperature effect of opposite sign therefore requires an alternative explanation.

6.1. Review on hydrogen tunneling

Hydrogen isotopes are distinguished from other stable isotopes due to the wave-particle duality of matter. Hydrogen atoms and protons can transfer not only by classical over-barrier processes as described above, but also by quantum mechanical through-barrier processes, depending on their thermal energy in relation to the obstructing barrier (Smedarchina et al., 2006). Transition-state theory (TST) considers only the particle-like properties of matter. Quantum tunneling is the penetration of a particle into a region that is excluded in classical mechanics (due to it having insufficient energy to overcome the potential-energy barrier). In some cases tunneling is thought to be the dominant process by which hydrogen transfer is accomplished (Knapp and Klinman, 2002).

The wave nature of particles allows for the possibility that a particle penetrates a thin barrier even if the particle energy is less than the height of the barrier. From a classical mechanics point of view, tunneling cannot easily be explained since it would be the equivalent of a ball going through a wall without damaging the wall. A measure of this uncertainty in the position of a given hydrogen atom is the de Broglie wavelength, \(\lambda = h/(2mE)^{1/2}\), in which \(h\) is Planck’s constant, \(m\) the mass of the particle, and \(E\) its energy. \(\lambda^2\) is the probability that a particle will be in a given region of space (Fig. 3C). Assuming \(E = 20 \text{ kJ/mol} \approx 5 \text{ kcal/mol}\) the de Broglie wavelengths are 0.63 Å and 0.45 Å for protium (H) and deuterium (D), respectively (Knapp and Klinman, 2002). As hydrogen is typically transferred over a similar distance (<1 Å), that is close enough to the hydrogen wavelength for tunneling to occur.

In this dynamic view of enzyme catalysis it is the width, and not the height (as with TST), of an energy barrier that controls the reaction rate (Sutcliffe and Scruutton, 2000; Fig. 3C). The separation distance and symmetry of the S (substrate) and P (product) wells control tunneling probability (Fig. 3C). All else being equal, hydrogen has a higher tunneling probability than deuterium since a heavy isotope has a lower ZPE and its probability function is more localized in its well (Kohen, 2006). Additionally, all regions of the enzyme (not just the active site) likely contribute to the vibrations that drive quantum tunneling, thus providing a possible reason why enzymes are much larger than the active site alone (Sutcliffe and Scruutton, 2002). Quantum tunneling is thus an attractive means of transferring hydrogen from reactant to product in enzyme-catalyzed reactions where classical over-the-barrier energy requirements are large.

6.2. Temperature dependence of enzyme vibration in hydrogen tunneling and the effect on biomarker isotope fractionation

Increased hydrogen isotope fractionation at higher growth temperature is not explained by classic isotope theory. Basran et al.
(2001) observed a similar temperature-dependence and concluded that hydrogen tunneling may be significant at physiological temperatures, or even the sole means by which enzymes catalyze hydrogen transfer during C–H bond breakage. Kohen et al. (1999) measured thermophilic alcohol dehydrogenase (ADH) in the bacterium *Bacillus stearothermophilus* over a very wide temperature range (5–65 °C) and showed that tunneling made a significant contribution at 65 °C where the thermophilic enzyme was optimally functional. Decreased tunneling was observed below room temperature, indicating a transition for the protein at approximately 30 °C, analogous to previous findings with mesophilic ADH at 25 °C (Cha et al., 1989). This decrease in tunneling at reduced temperature was ascribed to reduced protein mobility (Kohen et al., 1999).

Kohen (2006) put forth a model in which an enzyme’s vibrations facilitated tunneling by reducing the distance over which hydrogen must travel between source and target molecules. In theoretical calculations, tunneling distance in a reaction catalyzed by the soybean enzyme lipoxygenase seems to be shorter than the distance between the source and target molecules (Hatcher et al., 2004). This suggests that whole-enzyme vibrations are needed to bring the two molecules closer when tunneling occurs. At reduced temperatures the amplitude of promoting vibrations may decrease below a level required for effective tunneling and the reaction begins to approximate more classical behavior with minimal tunneling (Kohen et al., 1999), i.e. fractionation observed will be mostly from normal KIE.

We suggest that hydrogen tunneling effects may account for the observed increased D/H fractionation at higher temperatures in lipids from *E. unicocca* and *V. aureus* (Zhang et al., 2009). Hydrogen tunneling effects may also explain seemingly anomalous temperature effects in methane oxidizing bacteria, where a more than a two fold increase in D/H fractionation was observed for cultures grown at 26 °C as compared to those grown at 11 °C, opposite the expected decrease in the magnitude of kinetic isotope effects as temperature increases (Coleman et al., 1981).

7. Discussion and future studies

We discussed possibilities responsible for the disagreement between slope and intercept in our culture experiments and previous studies. We suggest that kinetic fractionation can offer some explanation because biosynthesis most likely does not reach thermodynamic equilibrium. One way to test the kinetic hypothesis is to set up a series of culture experiments with different water δD values but grown under the same conditions, and to frequently sample the algae and measure its biomarker δD values. As kinetic fractionation evolves with time, we shall see the change of algal biomarker δD values with time. Correspondingly the disagreement between slope and intercept shall shrink.

Though hydrogen tunneling could explain the substantially larger fractionation at higher growth temperature, this hypothesis requires more investigation. A set of experiments involving different temperature gradients, a wider range (within the physiological temperature) and more biomarkers would be essential. Thus far there have been very few studies on the effects of temperature effect on hydrogen isotope fractionation in algal biosynthesis. Nevertheless this hypothesis could stimulate organic geochemists to design experiments and better understand the mechanism under neglected or inexplicable hydrogen isotope phenomena.

8. Conclusions

We have attempted to address unexplained aspects of hydrogen isotope systematics in algal culture experiments. The slope (a)–intercept (c) disagreement in spite of high R² that we observe in our δD_{lipid} – δD_{water} regressions and previous studies is not explained by thermodynamic isotope fractionation often incurred in organic geochemistry studies. We attribute it to kinetic isotope fractionation.

We reviewed how a (δD_{biomarker} – δD_{water}) is related to water δD values, and that this difference is especially apparent in hydrogen isotope studies due to the large isotopic fractionation that occurs. Therefore, reconstructing paleoenvironmental water δD values by simply adding a a to measured δD values of biomarkers will result in a bias toward deuterium-enriched values. We therefore recommend reconstructing water δD values from lipid δD values using α, which could be obtained through culture experiments or core-top studies. We also show that the kinetic isotope effect in an enzyme catalyzed reaction is dependent on temperature and the biosynthesis pathway, but is seemingly rate independent.

The substantially larger hydrogen isotope fractionation at higher growth temperature is not explained by traditional isotope theory. We offer an alternative hypothesis of hydrogen tunneling, which may contribute to the temperature dependence of D/H fractionation in algal lipid synthesis, with increased hydrogen tunneling at elevated temperature resulting in increased fractionation.

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