Supplemental Information: Solvent Deuterium Isotope Effects of Substrate Reduction by Nitrogenase from Azotobacter vinelandii

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Nitrogenase Purification

Figure S1: Representative chromatography traces and denaturing gels from purification of nitrogenase enzyme from *Azotobacter vinelandii*. Av1 and Av2 purified using ion exchange chromatography (left) followed by size exclusion chromatography (middle). Details of purification described in *Methods*. Av1 collected from ion exchange column at 19-22.5% Elution Buffer (312-341 mM NaCl) and Av2 collected from ion exchange column at 37.5-40.8% Elution Buffer (469-497 mM NaCl). Av1 collected from size exclusion at 1671-1703 mL and Av2 collected from size exclusion column at 1940-1971 mL. To run gel (BioRad Mini-PROTEAN TGX Stain Free Gels), 15 µL sample was combined with 5 µL of loading dye and 11 µL of the mixture was loaded into each well. Gel ran at 200 V for 20 minutes then rinsed with milliQ water, stained with Coomassie blue, and destained with a 40% methanol 10% acetic acid solution.
Gas Chromatographs of Headspace of Acetylene Reduction Assays

Figure S2: Representative gas chromatogram of headspace of acetylene reduction assay of nitrogenase in 100% H₂ (black) or 51% D₂O (green). Inset is zoom of region where ethylene peak elutes (1.8 min). Assay and GC-MS quantification of ethylene isotopologues described in Methods. Headspace (50 µL) injected using a Hamilton gastight syringe. Column: GASPRO PLOT-Q (Restek), column temperature: 80 °C (isothermal).
Mass Spectra of Ethylene Peak of Headspace of Acetylene Reduction Assays

Figure S3: Mass spectra of ethylene peaks in the headspace of acetylene reduction assays in 100% H$_2$O, 25% D$_2$O, 51% D$_2$O, and 73% D$_2$O. Abundance normalized to peak of greatest abundance. Mass spectra generated by integrating under ethylene peak of extracted ion chromatograms of m/z = 23-32. As the mole fraction of D$_2$O in buffer increases, greater abundance seen at m/z = 29 and m/z = 30, corresponding to increase in C$_2$H$_3$D and C$_2$H$_2$D$_2$ in the headspace.
Deconvolution of Overlapping Mass Spectra of Ethylene Isotopologues

Mass spectra of the headspace of acetylene reduction assays in deuterated solvent show abundance at m/z = 28 from C$_2$H$_4$, m/z = 29 from C$_2$H$_3$D, and m/z = 30 from C$_2$H$_2$D$_2$. Quantifying the relative amounts of these species is not as straightforward as simply comparing the abundance at m/z = 28 vs. m/z = 29 vs. m/z = 30 because of the fragmentation that ethylene undergoes upon mass spectrometry analysis at 70 eV. The abundance in the mass spectrum at m/z = 29 is due to the molecular ion of C$_2$H$_3$D and also the fragmentation of C$_2$H$_2$D$_2$ in which it has lost one atomic mass unit (amu). Similarly, the abundance at m/z = 28 is due to the molecular ion of C$_2$H$_4$, the fragments of C$_2$H$_3$D that have lost one amu, and the fragments of C$_2$H$_2$D$_2$ that have lost two amu. Therefore, these overlapping mass spectra need to be deconvoluted in order to quantify relative amounts of C$_2$H$_4$, C$_2$H$_3$D, and C$_2$H$_2$D$_2$. The method used for this deconvolution is described here.

With each assay, a non-deuterated ethylene standard was used to measure the fragmentation of C$_2$H$_4$ and the other ethylene isotopologues were assumed to have the same fragmentation pattern, as has been assumed in previous reports. All abundance values were obtained by integrating under the extracted ion chromatogram of each ion.

First, the abundance at each m/z value for the mass spectra of C$_2$H$_4$ was normalized to the molecular ion at m/z = 28 to generate the fraction of the abundance of the molecular ion at that particular m/z value. For example $F(27, C_2H_4)$ is equal to the abundance at m/z = 27 divided by the abundance at m/z = 28 of the same spectra:

$$F(28, C_2H_4) = \frac{A_{C_2H_4}(28)}{A_{C_2H_4}(28)} = 1$$

$$F(27, C_2H_4) = \frac{A_{C_2H_4}(27)}{A_{C_2H_4}(28)}$$

$$F(26, C_2H_4) = \frac{A_{C_2H_4}(26)}{A_{C_2H_4}(28)}$$
\[
F(25, C_2H_4) = \frac{A_{C_2H_4}(25)}{A_{C_2H_4}(28)}
\]

where \(A_{C_2H_4}(m/z)\) is the observed abundance at that \(m/z\) value from the ethylene standard analyzed using the same method as the assay samples. For the deuterated ethylene isotopologues, \(F(27, C_2H_4)\) is the probability that a molecule of ethylene will fragment with the loss of one H/D relative to the molecular ion. \(F(26, C_2H_4)\) is the probability that the ethylene molecule will fragment by the loss of two H/Ds and \(F(25, C_2H_4)\) is the probability that ethylene will fragment by losing 3 H/Ds.

Next, the abundance values from the mass spectra of the headspace samples of the acetylene reduction assays were deconvoluted by calculating the contribution at each \(m/z\) from each isotopologue (written as \(C(m/z, C_2H_2L_2)\)). To do this, each isotopologue was considered independently. For the dideuterated isotopologue, the molecular ion is \(m/z = 30\) and the only contribution to the abundance at \(m/z = 30\) is the dideuterated ethylene. The fragments of \(C_2H_2D_2\) in which one H atom is lost will contribute to the abundance at \(m/z = 29\). Because \(C_2H_2D_2\) has 2 H atoms and 2 D atoms, the probability that it will lose one H atom (when it fragments by the loss of one H or D) is 1/2. Therefore, the contribution from \(C_2H_2D_2\) at \(m/z = 29\) \((C(29, C_2H_2D_2))\) is 1/2 multiplied by the probability of losing one H/D \((F(27, C_2H_4))\), thus \(C(29, C_2H_2D_2) = A(30) \times \frac{1}{2} F(27, C_2H_4)\). This analysis was repeated for each \(m/z\) from the molecular ion \((m/z = 30)\) to \(m/z = 25\). Then this analysis was repeated for the singly-deuterated ethylene.

**Contribution from \(C_2H_2D_2\):**

\[
C(30, C_2H_2D_2) = A(30)
\]

\[
C(29, C_2H_2D_2) = A(30) \times \frac{1}{2} F(27, C_2H_4)
\]

\[
C(28, C_2H_2D_2) = A(30) \times \frac{1}{2} F(27, C_2H_4) + A(30) \times \frac{1}{6} F(26, C_2H_4)
\]
\[ C(27, C_2H_2D_2) = A(30) \times \frac{4}{6} F(26, C_2H_4) \]
\[ C(26, C_2H_2D_2) = A(30) \times \frac{1}{6} F(26, C_2H_4) + A(30) \times \frac{1}{2} F(25, C_2H_4) \]
\[ C(25, C_2H_2D_2) = A(30) \times \frac{1}{2} F(25, C_2H_4) \]

**Contribution from \( C_2H_3D \):**

\[ C(29, C_2H_3D) = A(29) - C(29, C_2H_2D_2) \]
\[ C(28, C_2H_3D) = C(29, C_2H_3D) \times \frac{3}{4} F(27, C_2H_4) \]
\[ C(27, C_2H_3D) = C(29, C_2H_3D) \times \frac{1}{4} F(27, C_2H_4) + C(29, C_2H_3D) \times \frac{1}{2} F(26, C_2H_4) \]
\[ C(26, C_2H_3D) = C(29, C_2H_3D) \times \frac{1}{2} F(26, C_2H_4) + C(29, C_2H_3D) \times \frac{1}{4} F(25, C_2H_4) \]
\[ C(25, C_2H_3D) = C(29, C_2H_3D) \times \frac{3}{4} F(25, C_2H_4) \]

**Contribution from \( C_2H_4 \):**

\[ C(28, C_2H_4) = A(28) - C(28, C_2H_2D_2) - C(28, C_2H_3D) \]
\[ C(27, C_2H_4) = A(27) - C(27, C_2H_2D_2) - C(27, C_2H_3D) \]
\[ C(26, C_2H_4) = A(26) - C(26, C_2H_2D_2) - C(26, C_2H_3D) \]
\[ C(25, C_2H_4) = A(25) - C(25, C_2H_2D_2) - C(25, C_2H_3D) \]

The relative amounts of \( C_2H_4 \), \( C_2H_3D \), and \( C_2H_2D_2 \) were determined by comparing the
molecular ion peak of the spectra of each isotopologue after subtracting the contribution from the fragments of the other isotopologues (\( C(28, C_2H_4) \), \( C(29, C_2H_3D) \), and \( C(30, C_2H_2D_2) \), respectively). The results are summarized in Table S1.

Table S1: Relative Amounts of Ethylene Isotopologue Products of Acetylene Reduction Assays Quantified by GC-MS

| Deuterium Enrichment | \( C_2H_4 \)      | \( C_2H_3D \)   | \( C_2H_2D_2 \) |
|----------------------|-------------------|----------------|-----------------|
| 25%                  | 65 ± 1\%          | 30.6 ± 0.9\%   | 3.9 ± 0.2\%     |
| 51%                  | 28 ± 2\%          | 52 ± 2\%       | 20 ± 1\%        |
| 73%                  | 13 ± 3\%          | 49 ± 2\%       | 37 ± 2\%        |

FTIR Standard Curves of Deuterated Ethylenes

The FTIR spectra of the headspace of acetylene reduction assays contained sharp stretches in the 840 -1000 cm\(^{-1}\) region, corresponding to the wagging mode of the H-C-H or H-C-D moieties.\(^2\) The peak heights of these stretches were used to quantify the relative amounts of ethylene isotopologue products from acetylene reduction assays. It is well reported that the molar absorptivity of the \( C_2H_4 \) stretch (949 cm\(^{-1}\)) and the \( C_2H_2D_2 \) stretch (843 cm\(^{-1}\)) are equivalent. However, there is some discrepancy as to whether the \( C_2H_3D \) stretch (943 cm\(^{-1}\)) has a molar absorptivity that is half of that of \( C_2H_4 \) and \( C_2H_2D_2 \) or equivalent to these species. In quantifying the products of nitrogenase reduction of \( C_2D_2 \), Han and Newton\(^3\) report that the molar absorptivity of the \( C_2H_3D \) peak is half of that of the \( C_2H_4 \) peak so they doubled the \( C_2H_3D \) peak height for their analysis. In similar measurements, Benton et al.\(^4\) and Fisher et al.\(^5\) report that the molar absorptivity of the \textit{trans}-\( C_2H_2D_2 \) peak is half of that of the \( C_2H_4 \) and \textit{cis}-\( C_2H_2D_2 \) peaks, but they don’t explicitly address the molar absorptivity of the peak from the singly deuterated species, \( C_2H_3D_1 \) at 943 cm\(^{-1}\). Due to this uncertainty in the literature, we performed a standard curve with \( C_2H_4 \) and \( C_2H_3D_1 \) to
determine their molar absorptivities: C$_2$H$_3$D was quantitatively transferred to a 1 L Schlenk flask to a final pressure of 1 atm and a separate flask was purged with C$_2$H$_4$ for 10 minutes and vented to 1 atm. FTIR spectra were obtained using the same procedure that was used when analyzing the headspace of nitrogenase acetylene reduction assays except known quantities (50 – 100 µL) of 1 atm C$_2$H$_4$/C$_2$H$_3$D were transferred to the degassed sample chamber and FTIR spectra were obtained. The peak heights of the C$_2$H$_4$ (949 cm$^{-1}$) and C$_2$H$_3$D (943 cm$^{-1}$) stretches were plotted against the moles of these species in the sample chamber to obtain a standard curve. The absorbance from these stretches was calculated from the percent transmittance and this was plotted against the known amount of moles of the ethylene isotopologue in the FTIR gas cell (Figure S4). Trendlines were generated in Excel and the slope was the molar absorptivity of the species. In agreement with the report by Han and Newton, the molar absorptivity of C$_2$H$_3$D was about half of that of C$_2$H$_4$. We also simulated the molecular spectra of C$_2$H$_4$, C$_2$H$_3$D, and C$_2$H$_2$D$_2$ using TheoReTS$^6$ and obtained similar values for the molar absorptivities: 46.7 cm$^{-1}$ for C$_2$H$_2$D$_2$, 24.3 cm$^{-1}$ for C$_2$H$_3$D, and 41.7 cm$^{-1}$ for C$_2$H$_4$. To our knowledge, this is the only reported standard curve of C$_2$H$_4$ and C$_2$H$_3$D by FTIR and is necessary for quantifying ethylene isotopologues by FTIR.
Figure S4: FTIR standard curves of ethylene isotopologues $\text{C}_2\text{H}_4$ and $\text{C}_2\text{H}_3\text{D}$. Peak transmittance values of the $\text{C}_2\text{H}_4$ stretch at 949 cm$^{-1}$ (black) and the $\text{C}_2\text{H}_3\text{D}$ stretch at 943 cm$^{-1}$ (red) were converted to absorbance ($\text{Abs} = 2\log(\% \text{ Transmittance})$) and plotted against the amount of ethylene present in the FTIR gas cell (µmol). Linear least squares fit trendlines were fit to the data in Excel and the slope of the line was the molar absorptivity of the species.

$y = 0.0510x + 0.0845$
$R^2 = 0.9915$

$y = 0.0238x + 0.0355$
$R^2 = 0.9938$
FTIR Spectra of Headspace of Acetylene Reduction Assays

Figure S5: FTIR spectra of the headspace of acetylene reduction assays performed in varying mole fraction of deuterium in the solvent. In 100% H$_2$O, the only observed stretch is at 949 cm$^{-1}$ due to the presence of C$_2$H$_4$. Rotational stretches are observed on either side of the main C$_2$H$_4$ peak. In 22% D$_2$O, a peak emerges at 943 cm$^{-1}$ due to C$_2$H$_3$D in the headspace. At 51% D$_2$O, the C$_2$H$_3$D peak at 943 cm$^{-1}$ increases and a peak emerges at 843 cm$^{-1}$ due to the presence of cis-C$_2$H$_2$D$_2$. 
| Deuterium Enrichment | C$_2$H$_4$ | C$_2$H$_3$D | C$_2$H$_2$D$_2$ |
|---------------------|----------|----------|--------------|
| 22%*               | 54.9%    | 41.7%    | 3.4%         |
| 51%                | 31 ± 1%  | 54 ± 1%  | 14 ± 2%      |
| 73%                | 16 ± 1%  | 53.0 ± 0.9% | 30.9 ± 0.7% |

* only one trial performed
Figure S6: H$_2$/HD region of $^1$H NMR spectra from headspace of proton reduction assay. Without deuterium decoupling (top), HD signal is a 1:1:1 triplet. Deuterium decoupling (bottom) condenses triplet into single peak. NMR tube filled with 1 mL CDCl$_3$ and capped with PTFE septa. Headspace from proton reduction assay (3 mL) transferred to a NMR tube with bubbling though CDCl$_3$. NMR performed on a Varian 600 MHz Spectrometer with 5 mm triple resonance inverse probe (parameters: 512 scans, 16 sec relaxation delay, pulse angle 90 °C). $^1$H NMR (CDCl$_3$, 600 MHz) H$_2$: δ ppm 4.63 (s, 2H); HD: δ ppm 4.59 (t, J=48 Hz, 1H)

Note: We first attempted to quantify the products of proton reduction assays (H$_2$, HD, and D$_2$) in H$_2$O/D$_2$O with GC-MS. Modifications to the GC-MS system were made to optimize quantification of the hydrogen isotopologues: installation of 5 Å molecular sieve column for sufficient separation of H$_2$ from background argon; installation of external power supply to manually decrease the ionization energy to a level below that of the carrier gas,
He, which would interfere with the D$_2$ signal (both produce m/z = 4 ions). Despite these optimizations, GC-MS was not suitable for our system because of the considerable mass bias among D$_2$, HD, and H$_2$: the error in the standard curve of D$_2$ was a significant portion of the H$_2$ signal. When the headspace of nitrogenase proton reduction assays was analyzed with these GC-MS conditions, the results agreed with the quantification by $^1$H NMR reported below; however, the precision was not satisfactory.
Proton Inventory Analysis of Nitrogenase Reduction
Figure S7: Proton inventory analysis of acetylene, cyanide, and proton reduction by nitrogenase. Results of all assays at 25% D$_2$O (top), 51% D$_2$O (middle) and 75% D$_2$O (bottom) are shown as markers. Solid lines are plots of Equations 1 - 3 (left axis; blue, green, and orange, respectively) and Equation 4 (right axis; black), the model expressions for the mole fraction of ethylene isotopologue product or H$_2$ to HD ratio as a function of the isotope effect. The dashed lines are plots of Equations (left axis; light blue, orange, brown, dark blue, teal, respectively), the model expressions for the mole fraction of methane isotopologue product as a function of the isotope effect. Markers are experimental data from the reduction assays with triangles as the results of GC-MS quantification of acetylene reduction assays, squares as the FTIR quantification of acetylene reduction assays, dotted circles as the GC-MS quantification of cyanide reduction assays and circles as the $^1$H NMR quantification of proton reduction assays. The black circles are the experimental data from each proton reduction assay trial. All data plotted for cyanide and proton reduction data. Mean values plotted for acetylene reduction data and error bars are smaller than marker size.

**Derivation of Isotope Effect Model Curves**

The reduction of acetylene to ethylene and protons to H$_2$ involves the incorporation of 2 H/D atoms into the product. With each H/D addition, there is a probability, $p$, that a hydrogen atom is added and a probability, $q$, that a deuterium atom is added. The probability that a hydrogen is added is a function of the rate of this transfer and the mole fraction of hydrogen in the solution:  

\[
p = \frac{k_H f_H}{k_H f_H + k_D f_D}
\]

where $k_H$ and $k_D$ are the rates of hydrogen and deuterium addition, respectively, and $f_H$ and $f_D$ are the mole fractions of hydrogen and deuterium in the solvent, respectively. The isotope effect (IE) is defined as $\frac{k_H}{k_D}$ and the expression for $p$ can be rewritten as:

\[
p = \frac{IE f_H}{IE f_H + f_D}
\]
It follows that the probability of adding a deuterium (instead of a hydrogen) can be written as:

\[ q = 1 - p = \frac{f_D}{IEf_H + f_D} \]

To generate H\(_2\) and C\(_2\)H\(_4\), two hydrogens are added. For HD and C\(_2\)H\(_3\)D, one hydrogen and one deuterium are added; and for D\(_2\) and C\(_2\)H\(_2\)D\(_2\), two deuteriums are added. Making the assumption that \( p \) and \( q \) are the same for the first and second proton addition, the mole fraction of dihydrogen and ethylene isotopologue products can be written as the product of the appropriate probabilities of adding H vs. D:

\[ X_{C_2H_4} = X_{H_2} = p^2 = \frac{IE^2f_H^2}{(IEf_H + f_D)^2} \]  
\[ X_{C_2H_3D} = X_{HD} = 2pq = \frac{2IEf_Df_H}{(IEf_H + f_D)^2} \]  
\[ X_{C_2H_2D_2} = X_{HD} = q^2 = \frac{f_D^2}{(IEf_H + f_D)^2} \]

For the results of the proton reduction assays, only the ratio of H\(_2\) to HD could be obtained so the isotope effect was calculated by the following equation:

\[ \frac{X_{H_2}}{X_{HD}} = \frac{IE^2f_H^2}{(IEf_H + f_D)^2} \cdot \frac{(IEf_H + f_D)^2}{2IEf_Df_H} = \frac{IEf_H}{2f_D} \]

In order to explicitly calculate the isotope effect from the relative amounts of isotopologue products, equations 1-4 were solved for the isotope effect:

\[ IE_{C_2H_4} = \frac{f_D\sqrt{X_{C_2H_4}}}{f_H - f_H\sqrt{X_{C_2H_4}}} \]

\[ IE_{C_2H_3D} = \frac{\sqrt{-f_D^2f_H^2(2X_{C_2H_3D} - 1) - X_{C_2H_3D}p_Df_Df_H + p_Df_H}}{f_H^2X_{C_2H_3D}} \]
\[ IE_{C_2H_2D_2} = \frac{f_D \left( \frac{1}{\sqrt{X_{C_2H_2D_2}}} - 1 \right)}{f_H} \] (7)

\[ IE_{H_2/HD} = \frac{2f_D}{f_H} \cdot \frac{X_{H_2}}{X_{HD}} \] (8)
Least Square Fit of Acetylene and Proton Reduction Assay Results

A least squares fit was performed on the acetylene reduction data across all levels of deuterium enrichment for each ethylene isotopologue (Figure S8). The relative amount of each ethylene isotopologue (percentage of total ethylene produced) was plotted against the mole fraction of deuterium in the solvent. Then, the scipy.optimize.curve_fit function was used in Jupyter notebooks to perform a non-linear least squares fit of the data to Equations 1-3. The isotope effects obtained from these fits are $1.28 \pm 0.04$, $1.4 \pm 0.1$, and $1.59 \pm 0.05$ for C$_2$H$_4$, C$_2$H$_3$D, and C$_2$H$_2$D$_2$, respectively.

Figure S8: Least squares fits (solid lines) of Equations 1-3 to experimental acetylene reduction assay results (points). Relative amounts of C$_2$H$_4$(red), C$_2$H$_3$D(black), and C$_2$H$_2$D$_2$(blue) plotted against the percentage of deuterium enrichment in the buffer. Plots and least squares fit (scipy.optimize.curve_fit) generated in Jupyter Notebooks.
The results of the proton reduction assay were fit to Equation 7 using the same method as described above (Figure S9). The isotope effect value obtained from this fit was 4.2 ± 0.1.

Figure S9: Least squares fit of experimental proton reduction assay results to Equation 7. Ratio of \( \text{H}_2 \) to HD plotted against the percentage of deuterium enrichment in the buffer. Experimental data are black circles and least squares fit is dotted black line. Plots and least squares fit (scipy.optimize.curve_fit) generated in Jupyter Notebooks.
Figure S10: Least squares fits (dashed lines) of Equations to experimental cyanide reduction assay results (points). Mole fraction of CH$_4$ (light blue), CH$_3$D (orange), CH$_2$D$_2$ (brown), CHD$_3$ (dark blue), CD$_4$ (teal), plotted against the mole fraction of deuterium enrichment in the buffer. Plots and least squares fit (scipy.optimize.curve_fit) generated in Jupyter Notebooks.
Estimation of Fractionation Factor of H₂ Production by Nitrogenase

The level of incorporation of heavy isotopes into enzyme products are also of interest in the field of geochemistry and these results are termed fractionation factors instead of isotope effects. The fractionation of a heavy isotope such as deuterium is related to the ratio of the amount of deuterium in the product (in parts per million) to the natural abundance of deuterium in a standard water sample. Mechanistic conclusions are rarely made from these fractionation factors and instead, they provide clues as to the microbial sources of important chemicals such as NH₃ and CH₄ in the environment.⁹⁻¹³ Upon inspection of the definitions of isotope effect and fractionation factor, in the case of solvent isotope effect experiments where protons are sourced from the solvent as in reduction by nitrogenase, the fractionation factor is the reciprocal of the isotope effect. In this work, we calculate isotope effects of nitrogenase reduction assays; however, the results can also easily be expressed as fractionation factors and are relevant to studies of geological nitrogen fixation. In reports measuring deuterium fractionation during dihydrogen production by cyanobacteria¹⁴ or purified hydrogenases¹⁰ significant depletion of deuterium was also observed. When we convert our isotope effect values to fractionation factors (Table S3) our results are comparable to these studies on hydrogenases, which could indicate that deuterium depletion is a common feature of H₂ production. In fact, these levels of deuterium fractionation are similar to that of H₂ production by the electrolysis of water.¹⁴

The fractionation factor (α) is defined as the ratio of deuterium to hydrogen in the products divided by the ratio of deuterium to hydrogen in the solvent:

\[ \alpha_{\text{H₂evolution}} = \frac{R_{\text{H₂}}}{R_{\text{H₂O}}} \]  

(9)

where \( R_{\text{H₂}} \) is the ratio of D to H in the \( L₂ \) products and \( R_{\text{H₂O}} \) is the ratio of D to H in the...
solvent ($L$ is the nomenclature for either H or D). In order to calculate the D to H ratio of the $L_2$, we need to know the relative amount of $D_2$ produced, which we weren’t able to quantify by $^1$H NMR. However, we can calculate an estimate of the relative amounts of H$_2$, HD and D$_2$ using the isotope effect that we calculated from equation 5, by inserting the calculated isotope effect into equations 1-3. These calculations were performed with our proton reduction data and the results are shown in Table S3. The fractionation factor was estimated to be 0.24 ± 0.03, 0.25 ± 0.05, and 0.20 ± 0.02 from assays performed in 25% D$_2$O, 51% D$_2$O, and 73% D$_2$O, respectively. All of the values are within error of each other.
Table S3: Calculating Fractionation Factor of Proton Reduction

| sample       | H2/HD     | IE        | calc_H2   | calc_HD   | calc_D2   | R_H2     | α        |
|--------------|-----------|-----------|-----------|-----------|-----------|----------|----------|
| 25% D₂O trial 1 | 6.790778  | 4.527186  | 0.867544  | 0.127753  | 0.004703  | 0.073629 | 0.220888 |
| 25% D₂O trial 2 | 5.600473  | 3.733649  | 0.842796  | 0.150487  | 0.006718  | 0.089278 | 0.267835 |
| 25% D₂O trial 3 | 6.600526  | 4.400351  | 0.864124  | 0.130917  | 0.004959  | 0.075752 | 0.227255 |
| average      | 6.330593  | 4.220395  | 0.858154  | 0.136386  | 0.005460  | 0.079553 | 0.238659 |
| stdev        | 0.639417  | 0.426278  | 0.013410  | 0.001097  | 0.008489  |          | 0.025467 |
| 51% D₂O trial 1 | 2.105380  | 4.382628  | 0.653009  | 0.310162  | 0.036830  | 0.237487 | 0.228174 |
| 51% D₂O trial 2 | 2.044485  | 4.255867  | 0.645607  | 0.315780  | 0.038614  | 0.244560 | 0.234970 |
| 51% D₂O trial 3 | 1.942120  | 4.042781  | 0.632438  | 0.325643  | 0.041919  | 0.257451 | 0.247355 |
| 51% D₂O trial 4 | 2.400800  | 4.997584  | 0.684978  | 0.285312  | 0.029710  | 0.208264 | 0.200097 |
| 51% D₂O trial 5 | 1.539298  | 3.204253  | 0.569750  | 0.370136  | 0.060114  | 0.324823 | 0.312085 |
| 51% D₂O trial 6 | 1.775673  | 3.696300  | 0.608844  | 0.342881  | 0.048275  | 0.281583 | 0.270541 |
| 51% D₂O trial 7 | 2.071615  | 4.312341  | 0.648942  | 0.313254  | 0.037803  | 0.241358 | 0.231893 |
| 51% D₂O trial 8 | 1.943430  | 4.045508  | 0.632613  | 0.325514  | 0.041892  | 0.256600 | 0.246538 |
| average      | 1.977850  | 4.117158  | 0.634523  | 0.323585  | 0.041892  | 0.256600 | 0.246538 |
| stdev        | 0.251864  | 0.524288  | 0.033977  | 0.009058  | 0.034556  |          | 0.033201 |
| 73% D₂O trial 1 | 0.986996  | 5.283329  | 0.437567  | 0.447843  | 0.114590  | 0.511742 | 0.189275 |
| 73% D₂O trial 2 | 0.965335  | 5.167379  | 0.430994  | 0.451014  | 0.117991  | 0.523225 | 0.193522 |
| 73% D₂O trial 3 | 0.839624  | 4.494459  | 0.389863  | 0.469054  | 0.141083  | 0.601564 | 0.222496 |
| 73% D₂O trial 4 | 0.728799  | 3.940912  | 0.351766  | 0.482665  | 0.165569  | 0.686060 | 0.253748 |
| 73% D₂O trial 5 | 0.965839  | 5.222683  | 0.434147  | 0.449503  | 0.116350  | 0.517685 | 0.191472 |
| 73% D₂O trial 6 | 0.942058  | 5.094090  | 0.426766  | 0.453015  | 0.120219  | 0.530753 | 0.196306 |
| average      | 0.904775  | 4.867142  | 0.411850  | 0.458849  | 0.129300  | 0.561838 | 0.207803 |
| stdev        | 0.100756  | 0.536295  | 0.034179  | 0.013966  | 0.020242  | 0.069175 | 0.025585 |
Time Course of NaCN reduction assays in H$_2$O and D$_2$O

Figure S11: Time course of NaCN reduction assays in H$_2$O (grey) and 72% D$_2$O (green). Dashed lines are linear least squares fit to trial 2 from assays performed in H$_2$O (grey dashed, rate = 7.861 nmol methane produced min$^{-1}$) or 72% D$_2$O (green, rate = 7.095 nmol methane produced min$^{-1}$).
Mass Spectra of Methane Peak of Headspace of NaCN Reduction Assays

Figure S12: Mass spectra of methane peaks of headspace of acetylene reduction assays in 100% H$_2$O, 25% D$_2$O, 51% D$_2$O, and 75% D$_2$O. Abundance normalized to peak of greatest abundance. Mass spectra generated by integrating under methane peak of extracted ion chromatograms of m/z = 14-20. As mole fraction of D$_2$O in buffer increases, greater abundance seen at m/z = 17, m/z = 18, m/z = 19, and m/z = 20, corresponding to increase in CH$_3$D, CH$_2$D$_2$, CHD$_3$ and CD$_4$ in the headspace.
Table S4: Relative Amounts of Methane Isotopologue Products of Cyanide and Methyl Isocyanide Reduction Assays Quantified by GC-MS

|                  | NaCN Reduction |         |         |         |         |
|------------------|----------------|---------|---------|---------|---------|
|                  | CH<sub>4</sub> | CH<sub>3</sub>D | CH<sub>2</sub>D<sub>2</sub> | CHD<sub>3</sub> | CD<sub>4</sub> |
| 25% D<sub>2</sub>O | 74 ± 0.3       | 25 ± 0.3 | 0       | 0       | 0       |
| 51% D<sub>2</sub>O | 41 ± 1         | 45 ± 2   | 12 ± 3  | 2.13 ± 0.04 | 0       |
| 75% D<sub>2</sub>O | 9 ± 3          | 41 ± 3   | 34 ± 4  | 14 ± 2  | 2.3 ± 0.6 |

|                  | CH₃NC Reduction |         |         |         |         |
|------------------|-----------------|---------|---------|---------|---------|
| 51% D<sub>2</sub>O | 37 ± 1          | 45 ± 2  | 14 ± 2  | 2.6 ± 0.1 | 0       |
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