

Supplementary Material

Experimental details of the V/K KIE measurements:

Competitive KIEs: All experiments were performed in MTEN Buffer (50 mM MES, 25 mM TRIS, 25 mM EtOH-NH₂ and 100 mM NaCl) at pH = 9.0, 70 μ M NADPH, and a 200 fold excess of H₂F. The pH was adjusted at the experimental temperature (with the relevant calibration buffers at that temperature). These conditions are identical to the conditions previously used for kinetic studies with this enzyme.^{1,2} The H-transfer step with this enzyme is irreversible by three orders of magnitude.¹ To further assure the irreversibility of the DHFR reaction it was carried out under aerobic conditions, including oxygen bubbling following quenching, for rapid oxidation of the product H₄F. Quenching of the enzyme was achieved with an excess of methotrexate ($K_d=1$ nM). Samples were stored at -80 °C prior to HPLC analysis as described in details elsewhere.^{3,4} The reactions were quenched at various time points and after completion, and then analyzed for depletion of tritium in product, which yields the V/K KIE.^{5,6} For 1° KIE (Table 1) all data points are from at least 3 independent experiments with at least 4 points from each experiment. The observed 2 β D/T KIE (only 1.058 \pm 0.003) is from eight independent experiments with at least 5 points from each experiment. The error propagation for the Swain-Schaad *exponent* was conducted as described before.⁷⁻⁹

Data analysis: The numerical solution for Northrop equations¹⁰ for intrinsic H/T, H/D, and D/T KIEs was carried out using a program that we now present on : <http://cricket.chem.uiowa.edu/~kohen/tools.html> under Intrinsic KIEs. Since errors cannot be simply propagated from the observed values (for the same reason a numerical solution is needed), all combinations of observed H/T and D/T KIEs were calculated and averaged at each temperature.

The Northrop procedure assumes that the reaction has no reverse commitment (or that EIE=1) and that the Swain-Schaad relationship holds for intrinsic 1° KIEs even if tunneling is involved.¹⁰ In this context, it is important to note that the DHFR reaction was irreversible (no reverse commitment), and that in systems demonstrated to proceed with tunneling, the experimental relationship between primary D/T and H/T KIEs is valued at, or very near, the semiclassical limit.^{7-9,11,12} Even if the magnitude of the exponent (3.34) was altered for a full tunneling model, it would not be expected to change within the temperature range of this study, and hence, would not affect the temperature dependence of intrinsic isotope effects.¹³

Figure 1 in the communication presents KIEs on a logarithmic scale vs. the reciprocal of the absolute temperature. Curve fitting was carried out as a least root-mean-square fit exponential regression for proper error analysis, yielding the isotope effects on the preexponential factor and on the energy of activation (cf., the exponential factor) of the Arrhenius equation.

Tables 1 and 2 below present the raw data:

Table 1: Observed V/K KIEs.

Temp. °C	observed D/T KIE	observed H/T KIE
45	1.65 \pm 0.02	4.82 \pm 0.11
35	1.66 \pm 0.01	4.80 \pm 0.12
25	1.66 \pm 0.03	4.85 \pm 0.09
15	1.63 \pm 0.01	4.76 \pm 0.04
10	1.57 \pm 0.03	3.69 \pm 0.03
5	1.51 \pm 0.02	3.06 \pm 0.10

Table 2: Intrinsic KIEs calculated using the Northrop method.¹⁰

Temp. °C	intrinsic KIE (H/T) ^a	intrinsic KIE (H/D) ^a	intrinsic KIE (D/T) ^a
45	6.09 – 0.36	3.58 – 0.15	1.72 – 0.03
35	6.29 – 0.37	3.63 – 0.15	1.73 – 0.03
25	6.10 – 0.42	3.55 – 0.17	1.72 – 0.04
15	5.90 – 0.29	3.47 – 0.12	1.70 – 0.02
10	5.91 – 0.51	3.72 – 0.21	1.75 – 0.04
5	6.18 – 0.39	3.54 – 0.16	1.72 – 0.03

^a Reported errors are from all combinations of H/T and D/T observed KIEs for each temperature point. Errors were calculated at the 95% confidence interval.

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