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Boundary Conditions for the Swain−Schaad Relationship as a Criterion for Hydrogen Tunneling

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Abstract: Hydrogen quantum mechanical tunneling has been suggested to play a role in a wide variety of hydrogen-transfer reactions in chemistry and enzymology. An important experimental criterion for tunneling is based on the breakdown of the semiclassical prediction for the relationship among the rates of the three isotopes of hydrogen (hydrogen -H, deuterium -D, and tritium -T). This is denoted the Swain−Schaad relationship. This study examines the breakdown of the Swain−Schaad relationship as criterion for tunneling. The semiclassical (no tunneling) limit used hereto (e.g., 3.34, for H/T to D/T kinetic isotope effects), was based on simple theoretical considerations of a diatomic cleavage of a stable covalent bond, for example, a C−H bond. Yet, most experimental evidence for a tunneling contribution has come from breakdown of those relationship for a secondary hydrogen, that is, not the hydrogen whose bond is being cleaved but its geminal neighbor. Furthermore, many of the reported experiments have been mixed-labeling experiments, in which a secondary H/T kinetic isotope effect was measured for C−H cleavage, while the D/T secondary effect accompanied C−D cleavage. In experiments of this type, the breakdown of the Swain−Schaad relationship indicates both tunneling and the degree of coupled motion between the primary and secondary hydrogens. We found a new semiclassical limit (e.g., 4.8 for H/T to D/T kinetic isotope effects), whose breakdown can serve as a more reliable experimental evidence for tunneling in this common mixed-labeling experiment. We study the tunneling contribution to C−H bond activation, for which many relevant experimental and theoretical data are available. However, these studies can be applied to any hydrogen-transfer reaction. First, an extension of the original approach was applied, and then vibrational analysis studies were carried out for a model system (the enzyme alcohol dehydrogenase). Finally, the effect of complex kinetics on the observed Swain−Schaad relationship was examined. All three methods yield a new semiclassical limit (4.8), above which tunneling must be considered. Yet, it was found that for many cases the original, localized limit (3.34), holds fairly well. For experimental results that are between the original and new limits (within statistical errors), several methods are suggested that can support or exclude tunneling. These new and clearer criteria provide a basis for future applications of the Swain−Schaad relationship to demonstrate tunneling in complex systems.

Introduction

The kinetic relationship among the three isotopes of hydrogen has been used as a mechanistic tool in organic and physical chemistry for many years. One of the first investigations was carried out by Streitwieser and co-workers (1958) studying the acetolyses of cyclopentyl tosylates.[1] Several investigators examined these relationship under extreme temperature (20−1000 K) and as a probe for tunneling,[2−4] where tunneling is the phenomenon by which a particle transfers through a reaction energy barrier due to its wavelike property. Experimental and theoretical studies also used these isotopic relationships to suggest a coupled motion between primary and secondary hydrogens in hydride transfer and in elimination reactions in the gas phase and in organic solvents.[5−7] In recent years there has been a significant interest in the contribution of quantum mechanical tunneling of hydrogen to enzyme-catalyzed reactions. Several experimental findings were not in accordance with models neglecting H-tunneling (denoted below as semiclassical models).[8,9] In the last two years a growing number of theoretical papers have attempted to rationalize some of the experimental findings, while developing state of the art theoretical methods.[10−14]

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(1) Streitwieser, A.; Jagow, R. H.; Fahey, R. C.; Suzuki, F. *J. Am. Chem. Soc.* **1958**, *80*, 2326−2332.
(2) Bigeleisen, J. In *International Atomic Energy Agency*; Gerin, P.: Vienna, 1962; Vol. 1, p 161.
(3) Stern, M. J.; Vogel, P. C. *J. Am. Chem. Soc.* **1971**, *93*, 4664−4675.
(4) Stern, M. J.; Weston, R. E. *J. Chem. Phys.* **1974**, *60*, 2803, 2808, 2814.
(5) Huskey, W. P.; Schowen, R. L. *J. Am. Chem. Soc.* **1983**, *105*, 5704−5706.
(6) Saunders: W. H. *J. Am. Chem. Soc.* **1985**, *107*, 164−169.
(7) Amin, M.; Price, R. C.; Saunders: W. H. *J. Am. Chem. Soc.* **1990**, *112*, 4467−4471.
(8) Kohen, A.; Klinman, J. P. *Acc. Chem. Res.* **1998**, *31*, 397−404.
(9) Kohen, A.; Klinman, J. P. *Chem. Biol.* **1999**, *6*, R191−198.
(10) Alhambra, C.; Corchado, J. C.; Sánchez, M. L.; Gao, J.; Truhlar, D. J. *J. Am. Chem. Soc.* **2000**, *122*, 8197−8203.
(11) Agarwal, P. K.; Webb, S. P.; Hammes-Schiffer, S. *J. Am. Chem. Soc.* **2000**, *122*, 4803−4812.
(12) Webb, S. P.; Agarwal, P. K.; Hammes-Schiffer, S. *J. Phys. Chem. B* **2000**, *104*, 8884−8894.
(13) Billeter, S. R.; Webb, S. P.; Agarwal, P. K.; Iordanov, T.; Hammes-Schiffer, S. *J. Am. Chem. Soc.* **2001**, ASAP.

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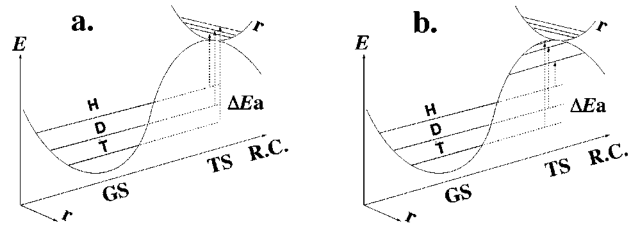

Figure 1. (a) Semiclassical model of KIEs. Different energies of activation (ΔE_a) for a light isotopic complex (H), a medium one (D), and a heavy one (T), result from their different zero-point energies (ZPE) at the ground state (GS) and transition state (TS). R.C. is the reaction coordinate, and r is orthogonal to the reaction coordinate. (b) Tunneling correction model. In addition to its higher ZPE, the lighter isotope tunnels at a lower energy under the top of the barrier, resulting in a smaller relative ΔE_a.

One of the most significant experimental results suggesting a contribution of H-tunneling to an enzyme-catalyzed reaction has come from the breakdown of the Swain−Schaad exponential relationship (*EXP*, as defined in eq 1 below). Those relationship are the semiclassical (no tunneling) correlations among the rates of the three isotopes of hydrogen, and were first defined by Swain et al. in 1958.[15] These relationship can be predicted from the masses of the isotopes under examination. The principle is that without tunneling this *EXP* results from zero-point energy (ZPE) differences between the ground state (GS) and the transition state (TS). Since H has a higher GS ZPE relative to D and T, it should react faster than D and T (Figure 1.a.). The semiclassical Swain−Schaad relationship will be altered in the case of tunneling contribution to reaction rate in the following way: Since H is lighter than D or T, the contribution of quantum mechanical tunneling to its transfer rate is much more significant than for D or T.[8,9,16] Tunneling deflates the effective energy of activation (ΔE_a) for H transfer relative to that of D and T, and its transfer becomes faster than that predicted semiclassically (Figure 1.b.). Such effects can be investigated experimentally by comparison of the ratio between the reaction rate constants of those isotopes using kinetic isotope effect (KIE) studies. KIE is the ratio between the reaction rates of a light isotope (*l*) and a heavy one (*h*) (*l*/*h* KIE).

The Swain−Schaad exponential relationships were originally defined for primary (1°) KIEs:[15]

$$\frac{k_\mathrm{H}}{k_\mathrm{T}} = \left(\frac{k_\mathrm{H}}{k_\mathrm{D}}\right)^{EXP} \text{ or } EXP = \frac{\ln(k_\mathrm{H}/k_\mathrm{T})}{\ln(k_\mathrm{H}/k_\mathrm{D})} \qquad (1)$$

where k_i is the reaction rate constant for isotope *i*. If tunneling does not contribute to the H-transfer, *EXP* can be calculated from:[17]

$$EXP = \frac{\ln(k_\mathrm{H}/k_\mathrm{T})}{\ln(k_\mathrm{H}/k_\mathrm{D})} = \left(\frac{1}{\sqrt{\mu_\mathrm{H}}} - \frac{1}{\sqrt{\mu_\mathrm{T}}}\right)\Big/\left(\frac{1}{\sqrt{\mu_\mathrm{H}}} - \frac{1}{\sqrt{\mu_\mathrm{D}}}\right) \qquad (2)$$

where μ_i is the reduced mass for isotope *i*. The original *EXP* was calculated for H/T versus H/D KIEs and yielded a value of 1.44 (using atomic masses). If tunneling contributes to the reaction rate, *EXP* would be smaller than 1.44. In the investigation of H-tunneling it is more common to use T as a frame of reference and to compare H and D in the following way:

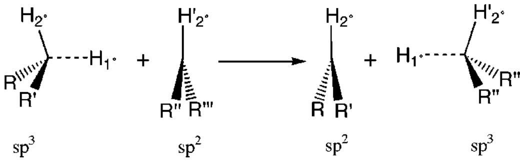

Figure 2. Principle reaction tested for H-tunneling using the Swain−Schaad relationship.

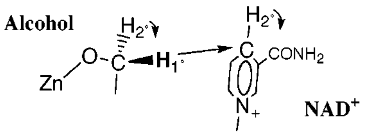

Figure 3. C−H bond cleavage step in the ADH-catalyzed reaction. A hydride (H_1°) is transferred from the alcoholate carbon to the C4 of the nicotinamide cofactor (NAD^+). Two 2° hydrogens are changing their bond orders as the reaction progresses from reactants to products.

$$EXP = \frac{\ln(k_\mathrm{H}/k_\mathrm{T})}{\ln(k_\mathrm{D}/k_\mathrm{T})} = \left(\frac{1}{\sqrt{\mu_\mathrm{H}}} - \frac{1}{\sqrt{\mu_\mathrm{T}}}\right)\Big/\left(\frac{1}{\sqrt{\mu_\mathrm{D}}} - \frac{1}{\sqrt{\mu_\mathrm{T}}}\right) \qquad (3)$$

Equation 3 defines the relationship of H/T to D/T KIEs, for which the semiclassical *EXP* is 3.26 (for atomic masses: $\mu_i = m_i$). Tunneling should result in an inflated H/T KIE and an inflated *EXP*.[8,9] The upper limit for this *EXP* with no tunneling contribution is critical, because only experimental values larger than this limit may be used as evidence for tunneling. The upper semiclassical limit used hitherto is 3.34 and was calculated following Streitwieser's suggestion to use the reduced masses of ^{12}C−H, ^{12}C−D, and ^{12}C−T.[1,6]

While studying organic or enzymatic reactions, the pattern of isotopic labeling of reactants is often more complex than the one considered in the original Swain−Schaad relationship. Several theoretical studies in the 1980s have suggested that mixed-labeling experiments (as described below) would be the most sensitive indicator of H-tunneling.[5,6] For many complex systems experimental evidence for tunneling results from a breakdown of these relationship for secondary kinetic isotope effects (2° KIEs) in a mixed-labeling competitive experiment.[8,9] Figure 2 defines the general type of reaction that has been studied experimentally and will serve as a general model throughout this report. In this reaction, the C−H bond is being cleaved, and the primary (1°) hydrogen is transferred while the secondary (2°) hydrogen changes its bond order from σ(s-sp^3) to σ(s-sp^2). A detailed example of such an experiment and its analysis in the context of the Swain−Schaad relationship is presented below.

The first enzyme that was studied using mixed-labeling experiments was yeast alcohol dehydrogenase (ADH).[18] The 1° hydrogen in that system is the pro-*R* hydrogen of benzyl alcohol which is transferred to the *re* face at the C4 position of a nicotinamide cofactor (Figure 3). In a mixed-labeling experiment, the 1° H/T KIE ($k_\mathrm{H}/k_\mathrm{T}$) is measured with H in the 2° position (the *S* benzylic hydrogen) and is denoted by $k_\mathrm{HH}/k_\mathrm{TH}$, where k_{ij} is the rate constant for H-transfer with isotope *i* in the 1° position and isotope *j* in the 2° position. The 2° H/T KIE is measured with H at the *R* position and is denoted by $k_\mathrm{HH}/k_\mathrm{HT}$.

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(14) Antoniou, D.; Schwartz, S. D. *J. Chem. Phys.* **2001**, *105*, 5553−5558.
(15) Swain, C. G.; Stivers, E. C.; Reuwer, J. F.; Schaad, L. J. *J. Am. Chem. Soc.* **1958**, *80*, 5885−5893.
(16) Berry, R. S.; Rice, S. A.; Ross, J. *Physical Chemistry*; John Wiley & Sons: New York, 1980.
(17) Melander, L.; Saunders: W. H. *Reaction Rates of Isotopic Molecules*; Krieger, R. E.: Malabar, FL, 1987.
(18) Cha, Y.; Murray, C. J.; Klinman, J. P. *Science* **1989**, *243*, 1325−1330.

Table 1. Experimental Examples for Inflated ^{M}EXPs from Mixed-Labeling Studies of Enzymes[a]

enzyme	1° ^{M}EXP	2° ^{M}EXP	2° H/T KIE[b]	ref
yeast ADH	3.58 (±0.08)	10.2 (±2.4)	1.35 (±0.015)	18
L57V liver ADH	3.14 (±0.05)	4.55 (±0.75)	1.332 (±0.007)	39
F93W liver ADH	3.31 (±0.02)	6.13 (±0.50)	1.333 (±0.004)	39
L57 F liver ADH	3.30 (±0.03)	8.50 (±0.99)	1.318 (±0.007)	39
ADH-hT (65 °C)[c]	3.50 (±0.05)	13.9 (±4.8)	1.231 (±0.009)	27
TIM[d]	NA	4.4 (±1.3)	1.27 (±0.03)	40

[a] All the data (excluding ADH-hT) are reported for experiments at 25 °C. [b] The semiclassical 2° H/T KIE values range from 1.0 to 1.35. Importantly, all the experimental values presented are significantly larger than 1.06 (see text). [c] ADH from *Bacillus stearothermophilus*. [d] Triose-phosphate isomerase.

The 1° and 2° D/T KIE measurements, on the other hand, are conducted with D in the geminal position, and are denoted by k_{DD}/k_{TD} and k_{DD}/k_{DT}, respectively. The exponential relationship calculated from such mixed-labeling experiments will be denoted below as ^{M}EXP. If the 1° and 2° hydrogens are independent of each other, the rule of geometrical mean (RGM[19]) imposes that the isotopic label at the geminal position should not affect the ^{M}EXP, so that:

$$1°\ EXP = \frac{\ln(k_{HH}/k_{TH})}{\ln(k_{DH}/k_{TH})} = \frac{\ln(k_{HH}/k_{TH})}{\ln(k_{DD}/k_{TD})} = 1°\ ^{M}EXP \quad (4)$$

and

$$2°\ EXP = \frac{\ln(k_{HH}/k_{HT})}{\ln(k_{HD}/k_{HT})} = \frac{\ln(k_{HH}/k_{HT})}{\ln(k_{DD}/k_{DT})} = 2°\ ^{M}EXP \quad (5)$$

By contrast, if the motions of the 1° and 2° hydrogens are coupled along the reaction coordinate, a breakdown of the RGM will result in an inflated 2° ^{M}EXP. The 1° KIE will have a secondary component and will be deflated, but since the 2° H/D KIE is very small (~1.2), the expected deflation of the 1° ^{M}EXP is very small. The 2° KIE, on the other hand, will have a primary component and will be significantly inflated. Tunneling of the 1° H will induce a large 2° H/T KIE (k_{HH}/k_{HT}) relative to the more semiclassical 2° D/T KIE (k_{DD}/k_{DT}), due to the reduced effect of tunneling from D in the primary position. In the mixed-labeling experiment, when there is coupled motion between the 1° and 2° hydrogens, tunneling along the reaction coordinate results in inflation of the 2° ^{M}EXP because H-tunneling is more significant than tunneling of D. A mathematically rigorous explanation of the high sensitivity of the mixed-labeling experiment to H-tunneling can be found in refs 20 and 21. Both Huskey[20] and Saunders[22,23] have independently shown that exceptionally large values for ^{M}EXP are computed only for 2° KIEs resulting from coupled motion and tunneling. They concluded that the extra isotopic substitution is an essential feature of the experimental design. These theoretical findings are in full accordance with the experimental results from mixed-labeling competitive experiments.[8,9] Table 1 summarizes several examples of such experimental findings for enzyme-catalyzed C−H bond activation reactions.

The currently used upper limit of 3.34 is valid for 2° H/T and D/T KIEs for systems where the 1° and 2° motions are independent of each other.[5,17,22] When the 1° and 2° hydrogens are coupled, measured ^{M}EXPs are affected by both the Swain−Schaad *EXP* (eqs 2 or 3) and the 1°−2° cross-labeling effect, namely the rule of geometrical mean (RGM).[24] In general, RGM can be defined as:

$$1°\ \mathrm{RGM} = \frac{\ln(k_{li}/k_{hi})}{\ln(k_{lj}/k_{hj})} \quad \text{and} \quad 2°\ \mathrm{RGM} = \frac{\ln(k_{il}/k_{ih})}{\ln(k_{jl}/k_{jh})} \quad (6)$$

where the *l*/*h* KIE is measured once with isotope *i* in the geminal position and once with isotope *j* in the geminal position. For simple semiclassical KIEs, RGM is unity, or in other words, there is no isotope effect on an isotope effect.[19]

Equation 3 can be redefined for the Swain−Schaad exponent (*s*):

$$1°\ s = \frac{\ln(k_{Hi}/k_{Ti})}{\ln(k_{Di}/k_{Ti})} \quad \text{and} \quad 2°\ s = \frac{\ln(k_{iH}/k_{iT})}{\ln(k_{iD}/k_{iT})} \quad (7)$$

where *i* is H or D, and for the RGM (*r*):

$$1°\ r = \frac{\ln(k_{iH}/k_{TH})}{\ln(k_{iD}/k_{DT})} \quad \text{and} \quad 2°\ r = \frac{\ln(k_{Hi}/k_{HT})}{\ln(k_{Di}/k_{DT})} \quad (8)$$

The observed ^{M}EXPs in the mixed-labeling experiment are then a product of *r* and *s*:[20,25]

$$1°\ r \cdot s = \frac{\ln(k_{HH}/k_{TH})}{\ln(k_{HD}/k_{TD})} \cdot \frac{\ln(k_{HD}/k_{TD})}{\ln(k_{DD}/k_{TD})} = \frac{\ln(k_{HH}/k_{TH})}{\ln(k_{DD}/k_{TD})} = 1°\ ^{M}EXP \quad (9)$$

and

$$2°\ r \cdot s = \frac{\ln(k_{HH}/k_{HT})}{\ln(k_{DH}/k_{DT})} \cdot \frac{\ln(k_{DH}/k_{DT})}{\ln(k_{DD}/k_{DT})} = \frac{\ln(k_{HH}/k_{HT})}{\ln(k_{DD}/k_{DT})} = 2°\ ^{M}EXP \quad (10)$$

In the current report, we examine whether the currently used upper limit for the semiclassical ^{M}EXP (SC ^{M}EXP), above which an experimental ^{M}EXP can indicate tunneling, is valid in the mixed-labeling experiment. We test the nature of the semiclassical relation for a 2° Swain−Schaad relationship whose breakdown serves as evidence for tunneling. Three different approaches are presented below. First, the effect of reduced mass on ^{M}EXP for systems with coupled motion is examined analytically. Second, inflated semiclassical ^{M}EXPs are investigated by a new vibrational analysis of a model system (ADH). Third, kinetic complexity that may lead to a false signature of tunneling is examined via numerical analysis. In examination of the last two issues, we use model systems to calculate and demonstrate the point in question, but the conclusions are of general importance. The first two approaches have general implications for organic, bioorganic, and enzymatic reactions such as the one described in Figure 2. The last approach is applicable to most enzymatic and other kinetically complex

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(19) Bigeleisen, J. *J. Chem. Phys.* **1955**, *23*, 2264−2267.
(20) Huskey, W. P. *J. Phys. Org. Chem.* **1991**, *4*, 361−366.
(21) Huskey, W. P. In *Enzyme Mechanism from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, Fl., 1991; pp 37−72.
(22) Saunders: W. H. *Croat. Chem. Acta* **1992**, *65*, 505−515.
(23) Lin, S.; Saunders: W. H. *J. Am. Chem. Soc.* **1994**, *116*, 6107−6110.
(24) Suhnel, J.; Schowen, R. L. In *Enzyme Mechanism from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, Fl., 1991; pp 3−36.
(25) Rucker, J.; Klinman, J. P. *J. Am. Chem. Soc.* **1999**, *121*, 1997−2006.

reactions. Importantly, all three methods result in a new limit above which experimental results may indicate tunneling.

Methods and Results

SC ^{M}EXP from Reduced Mass. A simple reduced mass consideration leads to the currently used upper limit of $\ln(k_H/k_T)/\ln(k_D/k_T) = 3.34$.[1,6,8,9] This limit was calculated from the reduced masses of only the two atoms whose covalent bond is being cleaved in the reaction. Thus, the stretching mode between these two atoms was considered as a pure, isolated vibrational mode. There is no straightforward reason this should hold for a system with 1°−2° coupled motion. If the motion of atoms other than the hydrogen and the carbon whose bond is being cleaved are part of the reaction coordinate, a different reduced mass has to be considered. We calculated ^{M}EXPs from eqs 9 and 10 using reduced masses of a variety of coupled modes for the general reaction illustrated in Figure 2. In the case of three nuclei with equal contributions to the reactive mode, a simple analytical solution may be applied. The reduced masses for nuclei moving in opposite directions can be calculated from $\mu = \Pi m_i/\Sigma m_i$ where m_i is the mass of an atom. The reduced mass for nuclei moving in the same direction is the sum of their masses. Not surprisingly, very large systems yield reduced masses close to the mass of the particle being transferred and therefore to ^{M}EXP values close to 3.3. The results for one atom and diatomic systems are 3.26 and 3.34, respectively.[1,15] Between those extremes, the ^{M}EXP values were larger than the 3.34 limit. The largest ^{M}EXP found was 4.25 for a system in which the carbon is moving in the opposite direction from both the 1° and 2° hydrogens.

In this extreme case, both hydrogens are moving with the same frequency and phase. This is not a high probability scenario, as the ground-state frequency for C−H stretch is double that of its out-of-plane bend. Nevertheless, it still constitutes an extreme maximum above which simple reduced-mass considerations cannot account for inflated ^{M}EXP.

It is possible to consider the coupling of another coordinate to the cleaved C−H bond analytically through the secular equation:[26]

$$|\mathbf{FG} - \lambda \mathbf{I}| = 0 \qquad (11)$$

Here **F** is the force constant matrix, **G** defines the reduced mass and coupling for each internal coordinate, and **I** is the identity matrix. The unknown (λ) is related to a vibrational frequency ($\lambda = 4\pi^2\nu^2$; if **F** is of dimension $N \times N$, there will be N values of λ that satisfy eq 11, i.e., N vibrational frequencies) of the system and can be used to calculate the ZPE and, hence, ^{M}EXP.

Of course, for the one-coordinate case one obtains Streitwieser's value of 3.34. However, eq 11 can also be used to consider a slightly more complex case, such as the coupling of the two bonds in a CH_2 group. In this case the frequencies are a function not only of the three masses but also of the H−C−H angle (φ), and it is easily shown that both the 1° and 2° $^{M}EXP(\varphi)$'s reach a maximum at $^{M}EXP(180°) = 3.34$.

A similar analysis of the coupling between the H−C−H bend and the C−H stretch yield 1° and 2° ^{M}EXPs that are functions of the C−H bond length, φ, and the ratio of the force constants for the stretch and bend. This approach leads to an inflated ^{M}EXP only for geometries that are hard to understand in the context of the reaction described in Figure 2 (e.g., a linear C−H- - -H system) and an equal degree of coupling where both coordinates have the same force constants. The maximum ^{M}EXP for those systems is 4.25.

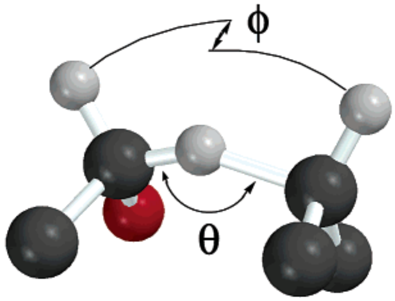

Figure 4. Truncated model for the ADH-catalyzed reaction. θ is the C−H−C angle, and ϕ is the dihedral angle between the two secondary hydrogens.

Semiclassical ^{M}EXP from Vibrational Analysis of a Model System. Since there is no analytical solution for a system in which more than two coordinates are coupled, a numerical simulation is employed. The goal, again, is to find whether the semiclassical component of a KIE measured by a mixed-labeling experiment can lead to an inflated 2° ^{M}EXP and to find a new maximum for a semiclassical ^{M}EXP (SC ^{M}EXP). Such a maximum, if greater than 3.34, will serve as a new upper limit above which tunneling must be invoked. Yeast ADH was chosen as a model for the general reaction in Figure 2, since it has been studied intensively and kinetic data, including data from mixed-labeling experiments, are available. The experimental findings of Cha et al.[19] for yeast ADH were 7.13 ± 0.07 and 1.73 ± 0.02 for 1° H/T and D/T KIEs, respectively, and 1.35 ± 0.015 and 1.030 ± 0.006 for 2° H/T and D/T, respectively. These KIEs lead to a 1° ^{M}EXP of 3.58 ± 0.08 and a 2° ^{M}EXP of 10.2 ± 2.4 (the error propagation was described elsewhere[27]). We found that those results are reproducible and reliable[28] and thus used them in constructing a model system based on the ADH reaction (Figure 3). Those data were used to parametrize the coupling constants of a truncated model (Figure 4) of the alcoholate substrate and the cofactor (NAD^+), and the contribution of semiclassical KIEs to inflated ^{M}EXPs was studied.

Rucker and Klinman[25] have used vibrational analysis and the Bigeleisen−Mayer equation to calculate isotope effects for the YADH-catalyzed oxidation of benzyl alcohol by NAD^+ (Figure 3). In the context of transition-state theory and normal statistical analysis, KIEs can be calculated from the Bigeleisen−Mayer equation:[29,30]

$$\mathrm{KIE} = \mathrm{MMI}\cdot\mathrm{EXC}\cdot\mathrm{ZPE} \qquad (12)$$

where MMI is a mass-moment of inertia term, EXC is a vibrational excitation term, and ZPE is a zero-point energy term. A truncated system was used with empirical force constants and geometric parameters for the cutoff model for reactants and proposed transition states. The Bell tunneling correction[31] was

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(26) Wilson, E. B.; Decius, J. C.; Cross, P. C. *Molecular Vibrations*; Dover Publications: New York, 1955.

(27) Kohen, A.; Cannio, R.; Bartolucci, S.; Klinman, J. P. *Nature* **1999**, *399*, 496−499.

(28) Chin, J. K.; Kohen, A.; Klinman, J. P. Unpublished results.

(29) Bigeleisen, J.; Mayer, M. G. *J. Chem. Phys.* **1947**, *15*, 261−267.

(30) Bigeleisen, J.; Wolfsberg, M. *Adv. Chem. Phys.* **1958**, *1*, 15−76.

(31) Bell, R. P. *The Tunnel Effect in Chemistry*; Chapman & Hall: London & New York., 1980.

applied to calculate KIEs, and the experimental results of Cha et al.[18] were used to parametrize the potential surface and coupling constants. Rucker and Klinman found that only a model that has both substantial H-tunneling and coupling between the reaction coordinate and a large number of vibrational modes could fit the experimental results. In accordance with previous studies, they also found that the 2° D/T KIE is expected to be the most sensitive parameter to changes in reaction coordinate properties. Rucker and Klinman studied a linear ($\theta = 180°$) C−H−C transfer and a dihedral angle between the two 2° hydrogens (ϕ) equal to 0° (i.e., 2° hydrogens and the C−H−C system were in one plane).

In this study we used the Rucker−Klinman model and methodology to examine the semiclassical (no tunneling) values of the 2° ^{M}EXP in the cross-labeling experiment. We repeated their calculation for a large conformational space ($240° > \theta > 120°$ every 20° and 360° for ϕ in 60° increments). θ and ϕ define the relative conformation of the reactants and were constrained while the vibrational frequencies and KIEs were calculated at each conformation. Other geometric parameters and the force constants, excluding the off-diagonal coupling constants, were set as described in ref 25. All calculations were done on a 500 MHz Macintosh G4 using the program BEBOVIB IV.[32] This program solves the vibrational secular equations for the molecules of interest using user-defined force fields. The program then computes isotope effects using the calculated vibrational frequencies and the Bigeleisen equation with, or without, tunneling correction. At each conformation, the coupling constants were adjusted while the calculated KIE was fitted to the experimental value reported in ref 18. The parameter space ϕ was fully swept through ($-180 < \phi < 180$), while ϕ covered a 60° deviation from linear C−H−C transfer ($120 < \theta < 240$) following the definition of near attack conformation (NAC).[33,34] Additionally, the enthalpy of formation for each conformation was calculated at the AM1 level of theory to avoid conformations that are not physically relevant (enthalpy of formation, ΔH_f, higher by 2 kcal/mol than the average: $\Delta\Delta H_f \geq \Delta H_f - \overline{\Delta H_f}$). No such conformation was found within the parameter space mentioned above. Conformations that exhibit $\Delta\Delta H_f$ close to 2 kcal/mol were further examined at the MP3 and AM1-SM2 levels of theory, but no significant $\Delta\Delta H_f$, that may suggest significant steric repulsion, were found. All calculations were done using the MacSpartan Plus program (Wavefunction, Inc.).

For each conformation, the adjustment of the coupling constants was terminated when the calculated KIEs (with the Bell tunneling correction) were within 3% of the experimental value. The semiclassical KIE (SC KIE) was then taken to be the semiclassical component of the observed KIE, and the semiclassical ^{M}EXP (SC ^{M}EXP) was calculated from eqs 9 and 10. Most of the calculated values were between 3.3 and 3.4 for 1° SC ^{M}EXP and 3.3 and 3.6 for 2° SC ^{M}EXPs. Table 2 summarizes SC ^{M}EXPs that exceeded those values (rows 1−6 and 8−14). Row 7 presents the SC ^{M}EXP for the geometry closest to the one measured for the horse liver ADH with a substrate analogue and NAD$^+$.[35] The structure of the yeast ADH, whose kinetic data are used here, is not available.

Table 2. A Partial Set of 1° and 2° SC ^{M}EXPs Calculated for Various Conformations

	ϕ (deg)	θ (deg)	1° SC ^{M}EXP	2° SC ^{M}EXP
1[a]	0	180	3.35	3.48
2	60	180	3.39	3.59
3	120	180	3.39	4.02
4	180	180	3.34	3.54
5	−120	180	3.31	3.48
6	−60	180	3.31	3.41
7[b]	20	120	3.38	3.69
8	120	240	3.31	4.10
9	120	220	3.35	4.13
10	120	200	3.37	4.60
11	120	180	3.39	4.02
12	120	160	3.39	3.92
13	120	140	3.38	3.88
14	120	120	3.39	4.09
15	100	200	3.35	4.25
16	110	200	3.36	4.42
17	130	200	3.37	4.49
18	140	200	3.35	4.31
19	120	195	3.38	4.48
20	120	205	3.38	4.50

[a] Reproduced from the model of Rucker and Klinman.[25] [b] From the crystal structure of HLADH with pentafluorobenzyl alcohol, NAD$^+$.[35]

The 2° SC ^{M}EXP reached a maximum value of 4.60 at $\theta = 200°$ and $\phi = 120°$ (row 10 in Table 2). Although this conformation is far from that determined by X-ray diffraction for horse liver ADH, it is a reasonable conformation for many other systems and thus a valid one in the context of this study. The conformation space near that geometry was "scanned" in 10° increments to ensure that this maximum is not a local artifact. The implication of this analysis to the interpretation of experimental data is addressed under Discussion.

SC ^{M}*EXP* with Kinetic Complexity. In the case of complex reaction kinetics (e.g., an enzymatic reaction) a different kind of inflated 2° ^{M}EXP may be found, and additional examination is required to prevent a false signature of tunneling. In such a case the intrinsic ^{M}EXP ($^{M}EXP_{\mathrm{int}}$) follows the Swain−Schaad prediction, but an inflated observed ^{M}EXP ($^{M}EXP_{\mathrm{obs}}$) can result from kinetic complexity. The KIE$_{\mathrm{obs}}$ may be different from the intrinsic one because of kinetic steps that are not isotopically sensitive, such as reactant binding, product release, and conformational rearrangement of the protein complex in the case of an enzymatic reaction. That kinetic complexity may alter the observed KIE as follows:[36]

$$\mathrm{KIE_{obs}} = \frac{\mathrm{KIE_{int}} + C_f + \mathrm{EIE}\cdot C_r}{1 + C_f + C_r} \tag{13}$$

where EIE is the equilibrium isotope effect, and C_f and C_r are the forward and reverse commitments to catalysis, respectively. It is apparent from eq 13 that KIE$_{\mathrm{obs}}$ can range from KIE$_{\mathrm{int}}$ to EIE as a function of C_r and from KIE$_{\mathrm{int}}$ to one as a function of C_f, but the effect of the commitment to catalysis on $^{M}EXP_{\mathrm{obs}}$ is not immediately apparent. Grant and Klinman[37] examined a case in which 2° H/T KIE$_{\mathrm{int}}$ = 1.1, and 2° EIE = 1.35. Both are common values for 2° hydrogen isotope effects when a C−H bond is changing its bond order from σ(s-sp^3) to σ(s-sp^2) (cf., Figure 2). The commitment values examined were $C_f = 0$, and C_r ranging from 0.05 to 1. The calculated $^{M}EXP_{\mathrm{obs}}$ at $C_r = 1$

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(32) Sims, L. B.; Burton, G.; Lewis, D. E. Program No. 337,; Quantum Chemistry Program Exchange; Department of Chemistry, Indiana University: Bloomington, IN, 1977; Vol. BeboVib IV.

(33) Bruice, T. C.; Lightstone, F. C. *Acc. Chem. Res.* **1999**, *32*, 127−136.

(34) Bruice, T. C.; Benkovic, S. J. *Biochemistry* **2000**, *39*, 6267−6273.

(35) Ramaswamy, S.; Eklund, H.; Plapp, B. V. *Biochemistry* **1994**, *33*, 5230−5237.

(36) Northrop, D. B. In *Enzyme Mechanism from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, Fl., 1991; pp 181−202.

(37) Grant, K. L.; Klinman, J. P. *Bioorg. Chem.* **1992**, *20*, 1−7.

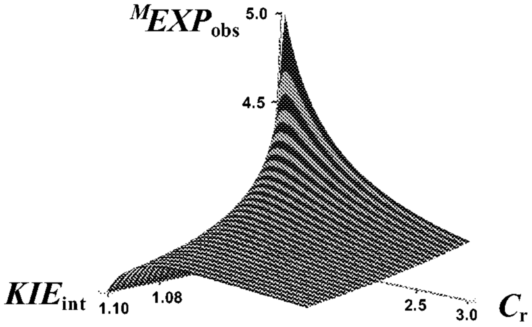

Figure 5. 2° $^{M}EXP_{obs}$ as a function of 2° H/T KIE_{int} and C_r, where $C_f = 0$ and 2° H/T EIE = 1.35 (eq 14).

was 4.78 which is significantly larger than 3.34. In the current work, we attempt to examine the general effect of kinetic complexity on the observed 2° ^{M}EXP. We used eq 13 to calculate the 2° $^{M}EXP_{obs}$ as a function of C_r and KIE_{int}:

$$^{M}EXP_{obs} = \frac{\ln(\text{H/T KIE}_{obs})}{\ln(\text{D/T KIE}_{obs})} = \frac{\ln((\text{KIE}_{int} + \text{EIE}\cdot C_r)/(1 + C_r))}{\ln((\text{KIE}_{int}{}^{1/3.3} + \text{EIE}^{1/3.3}\cdot C_r/(k_H/k_D)_r)/(1 + C_r/(k_H/k_D)_r))} \quad (14)$$

where $(k_H/k_D)_r$ is the 1° KIE for the reverse reaction and is used to calculate the reverse commitment (C_r) for D from that of H. The C_r is the ratio between product release and the reverse H-transfer. When using the mixed-labeling method, C_r for D is C_r for H divided by the 1° H/D KIE for the reverse reaction (e.g., 1.6 for the aldehyde to alcohol conversion for ADH).[38] Since this is a semiclassical model (no tunneling), all the parameters in the denominator are dependent on the corresponding parameters in the numerator. D/T KIE_{int} and EIE are related to the H/T isotope effects by the Swain−Schaad relationship. $C_f > 0$ will always decrease the KIE_{obs} and is insignificant in a search for maximal SC ^{M}EXP, so $C_f = 0$ was used in conjunction with eq 14 to calculate $^{M}EXP_{obs}$. Partial derivatives of this function do not result in maxima; therefore, a numerical method was employed to examine eq 14. For most of the parameter space the observed 2° ^{M}EXP is close to 3.3. Yet, inflated observed ^{M}EXPs result from a combination of small finite C_r, large 2° EIE, and small intrinsic 2° KIE. Figure 5 graphically illustrates eq 14 in this intriguing range. In this figure, $^{M}EXP_{obs}$ is presented as a function of both 2° H/T KIE_{int} and C_r at the narrow parameter range where inflated ^{M}EXP arises (EIE = 1.3, $0 < C_r < 3$, $1.01 < KIE_{int} < 1.1$, and $3.3 < {}^{M}EXP_{obs} < 5.0$). The $^{M}EXP_{obs}$ is again close to 3.3 for most of the range, but if $C_r \rightarrow 0.225$ and $KIE_{int} \rightarrow 1.01$ then $^{M}EXP_{obs} \rightarrow 4.8$. These isotope effects and commitment to catalysis are not unrealistic since a small 2° KIE_{int} may result in a large observed value. Although 1° KIE_{obs} is normally smaller than 1° KIE_{int}, this is not true for 2° KIEs since the 2° EIE is commonly larger than the 2° KIE, and because the 2° KIE_{obs} ranges from 2° KIE_{int} to 2° EIE as function of C_r. This results from eq 13 and is demonstrated in Figure 6. Figure 6 presents 2° H/T KIE_{obs} as function of C_r when $C_f = 0$ and $KIE_{int} = 1.01$. An observed 2° KIE as large as 1.06 (at $C_r = 0.225$) would normally be considered to be experimentally significant and may lead to a false signature of tunneling if not examined further. As demonstrated in Figure 5, at C_r close to zero, the $^{M}EXP_{obs}$ always goes to 3.3, but at very small KIE_{int}, there is a C_r-dependent ridge. The $^{M}EXP_{obs}$ along this ridge may reach an extremely large value (e.g., $^{M}EXP_{obs} = 5.4$ at $KIE_{int} = 1.001$ and $C_r = 0.071$). Importantly, such a value is not likely to be mistaken for a valid estimation of $^{M}EXP_{int}$ because it would result from a very small C_r, negligible 2° KIE_{int} with a large 1° KIE, large 2° EIE, and 2° $KIE_{obs} < 1.01$ (from eq 13). Such a case is unlikely to be misinterpreted as evidence for tunneling because the observed 2° H/T KIE will be close to unity within experimental error (for most experimental procedures). Yet, special attention should be paid to the values within the parameter range presented in Figure 5 for which the 2° KIE_{obs} is small (∼1.06) but statistically reliable.

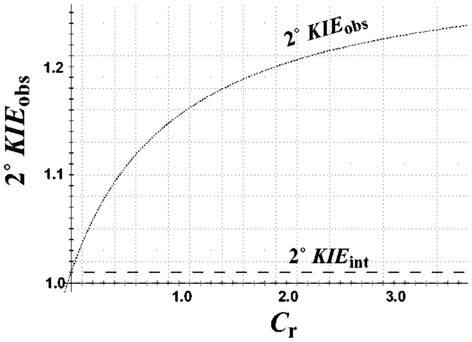

Figure 6. 2° H/T KIE_{obs} as a function of C_r for 2° H/T $KIE_{int} = 1.01$ where $C_f = 0$ and 2° H/T EIE = 1.35 (eq 13).

This analysis is valid for every H-transfer in a reaction that involves 1° and 2° hydrogens (Figure 2). The only "system specific" parameter used was the 1° H/T KIE on the reverse reaction (by which C_r for D is related to that for H in eq 14). This 1° KIE should be estimated specifically for each system under investigation, if inflated $^{M}EXP_{obs}$s are suspected to result from kinetic complexity (e.g., small 2° KIE_{obs}, together with small C_r as indicated from the ^{M}EXP relating primary isotope effects).

Discussion

The currently used semiclassical limit of the Swain−Schaad exponential relationship for C−H bond cleavage in a H/T versus D/T KIEs experiment is 3.34. This number results from eq 3 by using the reduced mass of ^{12}C and the hydrogen isotopes. Experimental *EXP* larger than 3.34 has been considered evidence for H-tunneling. Calculating the effect of altered reduced-mass in the mixed-labeling experiment on the semiclassical ^{M}EXP has not been examined before. At the extreme scenario for which the coupled atoms move as one, a maximum value for the ^{M}EXP is 4.25. An analytical solution for coupled normal modes is only available for one coordinate coupled to the reaction coordinate at a time. Such examination is indeed oversimplified but is the next reasonable step beyond the approach used hereto. Nevertheless, most coordinates whose coupling to the reaction coordinate (the C−H stretch) were calculated, have little effect on the predicted SC ^{M}EXP. The two most reasonable coordinates would be the H−C−H angle and the 2 °C−H stretch. Both result in ^{M}EXPs that are equal or smaller than 3.34. Only a few cases lead to 2° SC ^{M}EXPs as large as 4.25, but their coupling to the

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(38) Rubach, J. K.; Ramaswamy, S.; Plapp, B. V. *Biochemistry* **2001**, *40*, 12686−12694.

(39) Bahnson, B. J.; Park, D. H.; Kim, K.; Plapp, B. V.; Klinman, J. P. *Biochemistry* **1993**, *32*, 5503−5507.

(40) Alston, W. C.; Manska, M.; Murray, C. J. *Biochemistry* **1996**, *35*, 12873−12881.

reaction coordinate offers little physical sense (e.g., a linear C−H- - -H system). In summary, the extension of the Swain−Schaad approach to slightly more complicated H−C−H systems where both the 1° and 2° ${}^{M}EXP$ can be derived analytically, does not result in a new upper limit for a tunneling threshold. In this simple analytic approach, the ${}^{M}EXP$ reaches a maximum of 4.25 only for systems with extreme geometries, and extreme coupling constants.

For the more realistic multiple coupling case a numerical solution was calculated for a model system. The empirical vibrational analysis of the yeast ADH model system examined the maximal values of SC ${}^{M}EXP$ over a wide conformational range of H donor and acceptor. This simulation demonstrates that the general reaction described in Figure 2 may result in 2° ${}^{M}EXP$s as large as 4.6 with no tunneling contribution. Since calculated 2° SC ${}^{M}EXP$s larger than 3.6 were rare, most experimental 2° ${}^{M}EXP$s larger than that value would indicate a tunneling contribution. Only in some cases, where the 2° ${}^{M}EXP$ is close to 4, would a detailed analysis be needed. As demonstrated above, even simple vibrational analysis might indicate whether an inflated ${}^{M}EXP$ could be simulated with no tunneling correction. In some cases a more detailed analysis is possible, given the availability of relevant structure and computational power (e.g., refs 10, 11, and 14). Importantly, an intrinsic 2° ${}^{M}EXP$ larger than 4.6 could not be simulated without a tunneling contribution.

As for the effect of kinetic complexity on the observed 2° ${}^{M}EXP$, the kinetic analysis described above suggests that a 2° ${}^{M}EXP_{obs} > 4.8$, strongly supports H-tunneling within the context of the Swain−Schaad relationship. A 2° ${}^{M}EXP_{obs} > 3.3$ but smaller than 4.8, supports H-tunneling but additional information is required to exclude the combination of kinetic circumstances that could lead to the “false positive” as described above. Fortunately, part of this potential problem is often easy to solve in the mixed labeling experiment if a racemic mixture of labeled substrate is used. By analyzing the products of the reaction, both 1° and 2° KIEs result from each experiment. For each 2° ${}^{M}EXP_{obs}$ a 1° ${}^{M}EXP_{obs}$ can be calculated. Since kinetic complexity (e.g., C_f or C_r) can only deflate the primary value, a 1° ${}^{M}EXP_{obs}$ close to or larger than 3.3, is a good indication of small and insignificant commitment values.

Conclusions

While using the mixed labeling method (k_{HH}/k_{HT} vs k_{DD}/k_{DT}),[6,8,9] an experimental 2° ${}^{M}EXP$ larger than 4.8 (within statistical error) may serve as a reliable indication for H-tunneling. For observed 2° ${}^{M}EXP$ between 3.3 and 4.8 and 2° H/T KIEs < 1.06, care must be exercised, and additional evidence is needed to indicate H-tunneling. Such additional examinations consist of simple analytical or numerical solutions such as these described here. In some cases a higher level of calculation could be employed. For ADHs, for example, several state of the art theoretical examinations have recently supported the tunneling contribution suggested from the inflated 2° ${}^{M}EXP$s.[10,11,14]

For systems where kinetic complexity is suspected to play a role, additional examination may include estimation of commitment factors (C_f and C_r) and EIE values. As described above, this can be a rather straightforward process when the mixed-labeling experiment measures both 1° and 2° KIEs simultaneously.

The Swain−Schaad exponential relationships are very useful tools for studying tunneling in a wide variety of disciplines such as organic chemistry, physical chemistry, and enzymology. Thus, it is encouraging that the semiclassical limit appears, with rare and testable exceptions, to be generally valid. Our analysis suggests that these relationships can indeed be used with a high degree of confidence for the examination of 2° KIEs and 2° ${}^{M}EXP$s in the mixed-labeling experiment. This conclusion is of special significance because the mixed-labeling experiment is one of the most sensitive and useful experimental indicators of tunneling in studying enzymatic reactions and in the condensed phase in general.

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