

Calculations of Intrinsic Isotope Effects and the Detection of Tunneling in Enzyme-Catalyzed Reactions

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The equation of Northrop [1975, *Biochemistry* 14, 2644] for calculating intrinsic isotope effects from observed deuterium and tritium isotope effects on V/K , in which hydrogen is the reference isotope, has been extended to experimental designs using either deuterium or tritium as a reference. Partial derivatives of the intrinsic equations allow calculation of the relative precision of the three referenced isotope effects and these favor the order deuterium > tritium > hydrogen. In comparisons of observed and calculated isotope effects when hydrogen tunneling is present, both the precision and the magnitude of the difference was greater for intrinsic calculations than for exponentiations based upon a breakdown in the Swain-Schaad relationship. © 1990 Academic Press, Inc.

Observed kinetic isotope effects on kinetic parameters (i.e., $(V/K)_H/(V/K)_D$ and (V_H/V_D)) of enzyme-catalyzed reactions are usually smaller than intrinsic isotope effects (k_H/k_D) because of the presence of multiple and reversible steps preceding and following an isotopically-sensitive step (I). Values for intrinsic isotope effects may sometimes be calculated from observed deuterium and tritium isotope effects on V/K by use of the following equation (2):

$$\frac{(V/K)_H/(V/K)_D - 1}{(V/K)_H/(V/K)_T - 1} = \frac{k_H/k_D - 1}{k_H/k_T - 1} = \frac{k_H/k_D - 1}{(k_H/k_D)^{1.442} - 1} \quad [1]$$

The functional basis of Eq. [1] is the Swain-Schaad relationship (3),

$$k_H/k_T = (k_H/k_D)^{1.442}, \quad [2]$$

which has been described as undergoing a "breakdown" when applied to observed isotope effects which are less than intrinsic values (4).

Alternative forms of the Swain-Schaad relationship are possible, with either deuterium as a reference,

$$k_D/k_H = (k_D/k_T)^{-2.263} \quad [3]$$

or tritium,

$$k_T/k_H = (k_T/k_D)^{3.263}. \quad [4]$$

It follows that two alternative forms of Eq. [1] must also exist, one with deuterium as reference,

$$\frac{(V/K)_D/(V/K)_H - 1}{(V/K)_D/(V/K)_T - 1} = \frac{k_D/k_H - 1}{(k_D/k_H)^{-2.263} - 1} = \frac{(k_H/k_D)^{-1} - 1}{(k_H/k_D)^{0.442} - 1}, \quad [5]$$

and one with tritium as reference (5),

$$\frac{(V/K)_T/(V/K)_D - 1}{(V/K)_T/(V/K)_H - 1} = \frac{k_T/k_D - 1}{(k_T/k_D)^{3.263} - 1} = \frac{(k_H/k_D)^{-0.442} - 1}{(k_H/k_D)^{-1.442} - 1}. \quad [6]$$

An important question in the use of intrinsic equations is the precision to which intrinsic isotope effects can be calculated from experimental effects (6). To answer this question, one needs to define the propagation of error, which obeys the first derivative of the algebraic function describing changes of the dependent variable in terms of changes in the independent variables (7). Writing Eqs. [1], [5], and [6] in the general form

$$\frac{(V/K)_a/(V/K)_b - 1}{(V/K)_a/(V/K)_c - 1} = \frac{ab - 1}{ac - 1} = \frac{(k_H/k_D)^n - 1}{(k_H/k_D)^m - 1}, \quad [7]$$

the partial differential equations are

$$\frac{\partial(k_H/k_D)}{\partial(ab)} = + \frac{(k_H/k_D)^m - 1}{n(k_H/k_D)^{n-1}(ac - 1) - m(k_H/k_D)^{m-1}(ab - 1)} \quad [8]$$

and

$$\frac{\partial(k_H/k_D)}{\partial(ac)} = - \frac{(k_H/k_D)^n - 1}{n(k_H/k_D)^{n-1}(ac - 1) - m(k_H/k_D)^{m-1}(ab - 1)} \quad [9]$$

The variance of the intrinsic isotope effect is determined by

$$\text{Variance } (k_H/k_D) = \text{Variance } (ab) \left[\frac{\partial(k_H/k_D)}{\partial(ab)} \right]^2 + \text{Variance } (ac) \left[\frac{\partial(k_H/k_D)}{\partial(ac)} \right]^2. \quad [10]$$

Finally, the standard errors of a calculated intrinsic isotope effect is obtained by taking the square route of the variance of k_H/k_D .

The practical significance of Eqs. [8]–[10] is illustrated in Fig. 1, which shows that the propagation of error expands when $(V/K)_H/(V/K)_D$ approaches values of one, and is smallest when $(V/K)_H/(V/K)_D$ approaches k_H/k_D . Experimental designs with protium as the reference propagate the largest relative error and deuterium the least. A tritium-referenced design falls in between, with asymptotic approaches to the protium curve at low isotope effects and to the deuterium curve at high effects.

The minimal error found near intrinsic values, shown on the right of Fig. 1, was not anticipated as it runs contrary to what is generally believed (8). The conflict arises from misinterpreting the conclusions of Albery and Knowles (4), who asked a similar question but answered it differently than is done here. They questioned

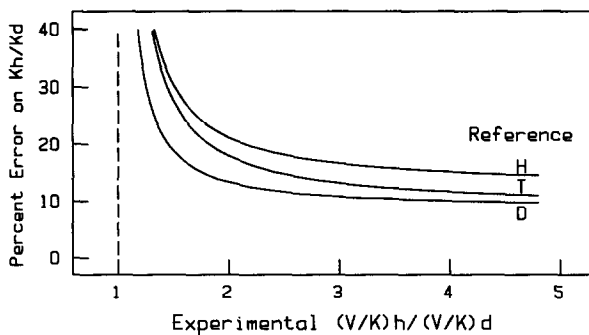


FIG. 1. Propagation of experimental error into calculated intrinsic isotope effects. The curves were simulated by assuming $\pm 3\%$ experimental error in values of measured isotope effects on V/K , originating from an intrinsic deuterium isotope effect of 5. Only the positive or upper half of the error envelop is portrayed.

what would happen to experimental errors when subjected to an exponential calculation, and whether this might preclude obtaining useful information. They answered in terms of a contour diagram of the breakdown of the Swain–Schaad relationship, leaving useful information undefined. The breakdown portrayed in the contour diagram referred to percentage changes in the exponent of Eq. [2] as a function of deuterium isotope effects, which is governed by a partial differential equation very different from Eq. [8].

In practice, the precise magnitude of the intrinsic isotope effect is more useful than the operative exponent. For example, intrinsic isotope effects of enzyme-catalyzed reactions are necessary in order to deduce transition state structures (9). An important exception, however, is in the detection of hydrogen tunneling, which has a long history in chemical reactions (10) and has recently been extended to enzymatic reactions (11). The traditional approach used exponentiation, and involves a comparison of deuterium and tritium isotope effects,

$$(k_H/k_D)^{1.442} = (k_H/k_T)_{\text{calc}} > (k_H/k_T)_{\text{obs}}. \quad [11]$$

Alternatively, Saunders (12) has proposed that the tritium-referenced Swain–Schaad relationship,

$$(k_D/k_T)^{3.263} = (k_H/k_T)_{\text{calc}} < (k_H/k_T)_{\text{obs}}, \quad [12]$$

was more sensitive to tunneling than the form in Eq. [11]. If tunneling occurs, then substituting an observed value for k_H/k_D in Eq. [11] leads to a calculated value for k_H/k_T that is larger than the observed tritium isotope effect. Similarly, substitution of an observed value for k_D/k_T in Eq. [12] leads to a calculated value for k_H/k_T that is smaller than the observed tritium isotope effect. Sometimes new exponents which will bring the values of $(k_H/k_T)_{\text{calc}}$ and $(k_H/k_T)_{\text{obs}}$ into agreement are calculated and reported, which is the type of numerical analysis addressed by the equations of Albery and Knowles (4).

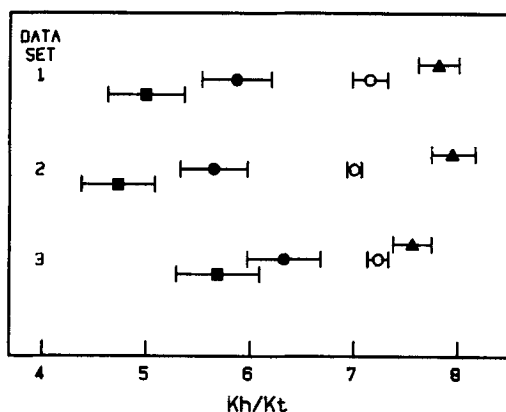


FIG. 2. Detection of hydrogen tunneling by intrinsic equations and by exponential. Observed tritium isotope effects of Cha *et al.* (11) are shown as open circles, exponentiating by 3.26 are solid circles, exponentiating by 1.44 are solid triangles, and intrinsic isotope effects are solid squares. Intrinsic tritium isotope effects and error bars were calculated from an equation similar to Eq. [6], with appropriate exponential substitutions in Eqs. [8] and [9], formulated to express tritium rather than deuterium isotope effects.

The relative sensitivities of Eqs. [11] and [12] are illustrated in Fig. 2, using primary isotope effect data from Table 2 of Cha *et al.* (11). Comparisons between observed values of the tritium isotope effect on V/K for benzyl alcohol (average, 7.15) and exponentiated values calculated from $(V/K)_D/(V/K)_T$ according to Eq. [12] (average, 5.95) were used to detect hydrogen tunneling in the oxidation reaction catalyzed by yeast alcohol dehydrogenase. Also illustrated are values calculated from $(V/K)_H/(V/K)_D$, which in turn were derived from the data using a comparable level of experimental error, in order to contrast exponentiation according to Eq. [11] (average, 7.78). As projected by Saunders, the comparison based on Eq. [12] does generate a larger difference than does Eq. [11]. However, much of the advantage is lost by its greater propagation of error, represented by the horizontal error bars.

As a new proposal, the use of the intrinsic equations as a means to detect tunneling is also illustrated in Fig. 2. The isotope effects of Cha *et al.* (11) were entered into Eq. [6] to yield values for the intrinsic deuterium isotope effect. These values were converted to intrinsic tritium isotope effects and found to be much lower than observed (average, 5.14), but without a proportionate increase in the propagation of error. While all three approaches similarly generate significant differences between observed and calculated isotope effects sufficient to support a contribution from hydrogen tunneling to the reaction rate, the three calculations differ in precision. The signal-to-noise ratios in Fig. 2 are 1.4, 1.8, and 2.7 for Eqs. [11], [12], and [6], respectively. Hence, intrinsic equations have a statistical advantage over exponentiation as a means of detecting tunneling, or other origins of breakdown of the Swain-Schaad relationship, such as low magnetic fields (13).

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