Analysis of the Kinetic Isotope Effects on Initial Rates in Transient Kinetics†

Bruce A. Palfey* and Rebecca L. Fagan

Department of Biological Chemistry, University of Michigan Medical School, 1301 Catherine Road, Ann Arbor, Michigan 48109-0606

Received June 19, 2006; Revised Manuscript Received July 31, 2006

ABSTRACT: A method was described recently for circumventing the difficulties in determining intrinsic kinetic isotope effects from eigenvalues obtained in transient kinetic experiments (Maniscalco, Tally, and Fisher (2004) *Arch. Biochem. Biophys. 425*, 165−172). The method, based on the isotope effects on initial rates of formation of intermediates, was validated by numerical simulation for only a few linear reaction sequences. A general analytical proof of the validity of the method is given in this work. The mathematical approach, using rate laws and L'Hôpital's rule, allows more complex reaction schemes to be analyzed. Several are analyzed in this article, illustrating the broad applicability and possible complications in this approach for determining intrinsic isotope effects. Some possible applications are noted, with particular attention being paid to nonlinear reaction schemes, the effect of measuring signals rather than concentration, and the ability to distinguish stepwise from concerted reactions.

Kinetic isotope effects (KIEs[1]) are invaluable in studies of enzyme reaction mechanisms and in probing the structure of transition states. However, intrinsic isotope effects are often difficult to measure directly because of the challenge in determining microscopic rate constants in complex kinetic schemes. Procedures for their determination by steady-state kinetic methods are well-established, and a large body of literature documents a generalized theory for extracting chemical information from experimental data (*1*−*3*). Transient kinetic methods provide a more direct view of enzyme action than steady-state methods, but a generalized theoretical framework for interpreting kinetic isotope effects had been lacking until recently, when Maniscalco, Tally, and Fisher analyzed the kinetic isotope effects on initial reaction rates for transient kinetic experiments (*4*). Their work showed that in sequential reaction schemes, intrinsic isotope effects on forward rate constants may be obtained from ratios of the time-derivatives of concentrations for protio and deutero species obtained in transient kinetic analysis (Fisher's third rule of transient-state KIEs). This should facilitate the analysis of substrate and solvent isotope effect data obtained in stopped-flow and rapid-quench studies, where several steps in a reaction sequence could be isotopically sensitive.

The validity of the analysis of transient kinetic schemes was demonstrated by numerical simulations of reaction time courses (*4*) except for two simple reaction schemes, which had analytical solutions. In all cases simulated, the new analytical formalism predicted the results obtained in digital experiments. Nonetheless, numerical simulations are inherently anecdotal, requiring the choice of a reaction scheme and values for the rate constants within that scheme. It cannot be concluded with absolute certainty that rules based on numerical simulations apply to all possible mechanisms and all possible values of rate constants. To address this possible uncertainty, a general mathematical proof of the result of Maniscalco, Tally, and Fisher is provided here, confirming their insightful analysis of linear reaction schemes. Moreover, this new mathematical analysis provides insights into measuring kinetic isotope effects in reaction schemes that had not yet been considered, extending the utility of this new analytical formalism to reaction schemes more complex than a linear series, and giving a general recipe that can be applied to situations not covered here. Some possible applications are noted, with particular attention being paid to nonlinear reaction schemes, the effect of measuring signals rather than concentration, and the ability to distinguish stepwise from concerted reactions.

METHODS

Nomenclature and Definitions. The notation and definitions of Maniscalco, Tally, and Fisher (*4*) are used throughout with only minor modifications. The authors define the time-dependent kinetic isotope effect (TKIE) for the transient rate of a reaction as

$$TKIE_{X_i} = \frac{\left(\frac{dX_i}{dt}\right)_H}{\left(\frac{dX_i}{dt}\right)_D} \quad (1)$$

where X_i is the concentration of the *i*-th species. The value of the TKIE at the start of the reaction is given by the limit of this expression as *t* approaches zero, defining the key parameter in this formalism.

† This work was supported by NIH Grant GM61087. R.L.F. was supported by NIGMS training grant GM07767.

* Corresponding author. Phone: (734) 615-2452. Fax: (734) 763-4581. E-mail: brupalf@umich.edu.

[1] Abbreviations: KIE, kinetic isotope effect; TKIE, time-dependent kinetic isotope effect; MTF, Maniscalco, Tally, and Fisher.

10.1021/bi061214h CCC: $33.50
Published on Web 10/25/2006

$$TKIE^0_{X_i} = \lim_{t\to 0} TKIE_{X_i} = \lim_{t\to 0} \frac{\left(\frac{dX_i}{dt}\right)_H}{\left(\frac{dX_i}{dt}\right)_D} \quad (2)$$

The intrinsic deuterium isotope effect on a reaction step is denoted as KIE_{int}, which is defined as the rate constant of the reaction of a protonated species (sometimes indicated by k^H) divided by the rate constant for the deuterated species (sometimes indicated by k^D). It will often be necessary to distinguish rate expressions referring to protio-reactants from expressions referring to deutero-reactants; this will be indicated by placing a vertical line after the expression with a subscript indicating the isotope.

Numerical Methods. Reaction mechanisms were simulated numerically using the fourth-order Runge−Kutta algorithm implemented in Berkeley Madonna 8.3. Simulations were generally run with time steps of 10^{-3} s or less. Simulated traces were imported into Microsoft Excel, where derivatives were calculated by dividing the change in consecutive concentration or absorbance values by the corresponding change in time. TKIE was calculated as the ratio of derivatives for traces simulated with protio or deutero rate constants. Extrapolations to $t = 0$ were made visually in Kaleidagraph (Synergy Software).

RESULTS

Summary of the MTF Analysis. Analyses using either integrated rate laws in simple mechanisms or numerical solutions of rate laws for a four-step mechanism demonstrated two important results (*4*). The first (eq 3) states that the limit as *t* approaches zero of the TKIE measured after a sequence of *n* reactions on the $n + 1$th species is the product of all the intrinsic isotope effects on the *n* forward reaction steps leading to the formation of that species.

$$TKIE^0_{n+1} = \prod_{i=1}^{n} KIE_{i,\text{int}} \quad (3)$$

The second result, a consequence of applying eq 3 to the formation of two consecutive species, allows the intrinsic isotope effect on the reaction separating them to be calculated.

$$KIE_{n,\text{int}} = \frac{TKIE^0_n}{TKIE^0_{n-1}} \quad (4)$$

Only intrinsic KIEs on forward reaction steps enter into this analysis. The mathematical simplicity suggests that these relationships could be extremely useful.

Mathematical Proof of Equation 3. Equation 3 was not rigorously proven mathematically for general reaction schemes, but it was shown to be valid for one-step and two-step reactions, where the limit of the ratios of the time-derivatives were computed analytically from the integrated rate laws, and for a four-step reaction, which was examined by numerically evaluating the limits after numerically solving the differential equations (*4*). A general analytical approach is presented here that proves the validity of eq 3 for linear reaction schemes and will later be extended to other mechanisms. This approach is based on (1) the realization that relatively simple algebraic expressions for the derivatives in eq 2 are available from the differential rate laws describing a mechanism, and (2) the limits may be evaluated in a straightforward fashion by considering initial conditions.

Scheme 1

Consider the linear sequence of four reversible reactions that was analyzed numerically (*4*) (Scheme 1). This mechanism is described by the following rate laws:

$$\frac{dX_1}{dt} = -k_1X_1 + k_{-1}X_2 \quad (5)$$

$$\frac{dX_2}{dt} = k_1X_1 - (k_{-1} + k_2)X_2 + k_{-2}X_3 \quad (6)$$

$$\frac{dX_3}{dt} = k_2X_2 - (k_{-2} + k_3)X_3 + k_{-3}X_4 \quad (7)$$

$$\frac{dX_4}{dt} = k_3X_3 - (k_{-3} + k_4)X_4 + k_{-4}X_5 \quad (8)$$

$$\frac{dX_5}{dt} = k_4X_4 - k_{-4}X_5 \quad (9)$$

The $TKIE^0$ values will be calculated for X_2, X_3, X_4, and X_5. Applying the definition of $TKIE^0$ and the rate law for species X_2 gives

$$TKIE^0_{X_2} = \lim_{t\to 0} \frac{\left(\frac{dX_2}{dt}\right)_H}{\left(\frac{dX_2}{dt}\right)_D} = \lim_{t\to 0} \frac{k_1X_1 - (k_{-1} + k_2)X_2 + k_{-2}X_3\Big|_H}{\frac{k_1}{KIE_{1,\text{int}}}X_1 - \left(\frac{k_{-1}}{KIE_{-1,\text{int}}} + \frac{k_2}{KIE_{2,\text{int}}}\right)X_2 + \frac{k_{-2}}{KIE_{-2,\text{int}}}X_3\Big|_D} \quad (10)$$

where the vertical lines with the subscript *H* or *D* indicate evaluation for protium or deuterium reactants, and the rate constants with deuterium are given by the rate constants for protium divided by the intrinsic isotope effects. The limit of this quantity as *t* approaches zero is obtained by realizing that at the start of the reaction, all concentrations are zero except for X_1, which has a value of X_1^0. If the initial value of X_1 is the same for the labeled and unlabelled reactions[2], it cancels from the numerator and denominator, as does k_1, leaving only the intrinsic isotope effect on the first step:

[2] The analysis is completely general for higher order reactions, including the common case in which the binding of substrate by enzyme initiates the reaction sequence. Isotope effects on binding are generally small for C−H/D bonds but may be sizable in the case of solvent isotope effects. Strictly speaking, such isotope effects do not alter the analysis presented here; the $TKIE^0$ contains contributions from every forward reaction step (emphasized in the section on rapid equilibria). However, because substrate binding is often too fast to observe, the initial concentration of the first *observable* species might vary with isotopic substitution because the binding has reached equilibrium before data are collected.

$$TKIE^0_{X_2} = KIE_{1,\text{int}}. \tag{11}$$

The calculation of $TKIE^0_{X_3}$ may be started in a similar fashion but requires another step to evaluate the limit. Because the initial concentrations of all the intermediates and products are zero, the limit as t approaches zero appears to be 0/0 and cannot be evaluated directly.

$$TKIE^0_{X_3} = \lim_{t\to 0} \frac{k_2X_2 - (k_{-2} + k_3)X_3 + k_{-3}X_4\Big|_H}{\dfrac{k_2}{KIE_{2,\text{int}}}X_2 - \left(\dfrac{k_{-2}}{KIE_{-2,\text{int}}} + \dfrac{k_3}{KIE_{3,\text{int}}}\right)X_3 + \dfrac{k_{-3}}{KIE_{-3,\text{int}}}X_4\Big|_D} = \frac{0-0+0}{0-0+0} \tag{12}$$

Limits of indeterminate forms may be evaluated by applying L'Hôpital's rule (*5*), which states that the limit of the ratio of two continuous zero-valued functions equals the limit of the ratio of the derivatives of the functions. By applying L'Hôpital's rule, eq 12 becomes

$$TKIE^0_{X_3} = \lim_{t\to 0} \frac{k_2\dfrac{dX_2}{dt} - (k_{-2} + k_3)\dfrac{dX_3}{dt} + k_{-3}\dfrac{dX_4}{dt}\Big|_H}{\dfrac{k_2}{KIE_{2,\text{int}}}\dfrac{dX_2}{dt} - \left(\dfrac{k_{-2}}{KIE_{-2,\text{int}}} + \dfrac{k_3}{KIE_{3,\text{int}}}\right)\dfrac{dX_3}{dt} + \dfrac{k_{-3}}{KIE_{-3,\text{int}}}\dfrac{dX_4}{dt}\Big|_D} \tag{13}$$

Algebraic expressions for each of the derivatives are available from the rate laws, giving an expression that is too long to be conveniently represented here. However, the numerator and denominator are sums of terms with only one term in each, that containing $X^0{}_1$, that does not extrapolate to zero as t approaches zero. Therefore the limit is

$$TKIE^0_{X_3} = \frac{k_1k_2{X_1}^0 + 0 + 0 + ...\Big|_H}{\dfrac{k_1k_2}{KIE_{1,\text{int}}KIE_{2,\text{int}}}{X_1}^0 + 0 + 0 + ...\Big|_D} = KIE_{1,\text{int}}KIE_{2,\text{int}} \tag{14}$$

This result is identical to that deduced previously from numerical simulations (*4*).

The analysis for X_4 requires sequential applications of L'Hôpital's rule. Applying the definition of $TKIE^0$ (eq 2) to X_4 gives an expression that initially evaluates as 0/0.

$$TKIE^0_{X_4} = \lim_{t\to 0} \frac{k_3X_3 - (k_{-3} + k_4)X_4 + k_{-4}X_5\Big|_H}{\dfrac{k_3}{KIE_{3,\text{int}}}X_3 - \left(\dfrac{k_{-3}}{KIE_{-3,\text{int}}} + \dfrac{k_4}{KIE_{4,\text{int}}}\right)X_4 + \dfrac{k_{-4}}{KIE_{-4,\text{int}}}X_5\Big|_D} \tag{15}$$

Applying L'Hôpital's rule gives an expression in terms of derivatives.

$$TKIE^0_{X_4} = \lim_{t\to 0} \frac{k_3\dfrac{dX_3}{dt} - (k_{-3} + k_4)\dfrac{dX_4}{dt} + k_{-4}\dfrac{dX_5}{dt}\Big|_H}{\dfrac{k_3}{KIE_{3,\text{int}}}\dfrac{dX_3}{dt} - \left(\dfrac{k_{-3}}{KIE_{-3,\text{int}}} + \dfrac{k_4}{KIE_{4,\text{int}}}\right)\dfrac{dX_4}{dt} + \dfrac{k_{-4}}{KIE_{-4,\text{int}}}\dfrac{dX_5}{dt}\Big|_D} \tag{16}$$

Expressions for the derivatives are obtained from the rate laws, giving eq 17. L'Hôpital's rule does not generate a proper form but instead gives a fraction whose limit still appears to be 0/0.

$$TKIE^0_{X_4} = \lim_{t\to 0} \frac{k_3[k_2X_2 - (k_{-2} + k_3)X_3 + k_{-3}X_4] + ... + ...\Big|_H}{\dfrac{k_3}{KIE_{3,\text{int}}}\left[\dfrac{k_2}{KIE_{2,\text{int}}}X_2 - \left(\dfrac{k_{-2}}{KIE_{-2,\text{int}}} + \dfrac{k_3}{KIE_{3,\text{int}}}\right)X_3 + \dfrac{k_{-3}}{KIE_{-3,\text{int}}}X_4\right] + ... + ...\Big|_D} \tag{17}$$

Therefore, L'Hôpital's rule is applied a second time, and after the derivatives are replaced by their rate-laws, a nonzero term divided by a nonzero term is obtained, allowing the limit to be evaluated.

$$TKIE^0_{X_4} = \lim_{t\to 0} \frac{k_3\left[k_2\dfrac{dX_2}{dt} - (k_{-2} + k_3)\dfrac{dX_3}{dt} + k_{-3}\dfrac{dX_4}{dt}\right] + ... + ...\Big|_H}{\dfrac{k_3}{KIE_{3,\text{int}}}\left[\dfrac{k_2}{KIE_{2,\text{int}}}\dfrac{dX_2}{dt} - \left(\dfrac{k_{-2}}{KIE_{-2,\text{int}}} + \dfrac{k_3}{KIE_{3,\text{int}}}\right)\dfrac{dX_3}{dt} + \dfrac{k_{-3}}{KIE_{-3,\text{int}}}\dfrac{dX_4}{dt}\right] + ... + ...\Big|_D} \tag{18}$$

$$TKIE^0_{X_4} = \lim_{t\to 0} \frac{k_3[k_2[k_1X_1 - (k_{-1} + k_2)X_2 + k_{-2}X_3] + ... + ...] + ... + ...\Big|_H}{\dfrac{k_3}{KIE_{3,\text{int}}}\left[\dfrac{k_2}{KIE_{2,\text{int}}}\left[\dfrac{k_1}{KIE_{1,\text{int}}}X_1 - \left(\dfrac{k_{-1}}{KIE_{-1,\text{int}}} + \dfrac{k_2}{KIE_{2,\text{int}}}\right)X_2 + \dfrac{k_{-2}}{KIE_{-2,\text{int}}}X_3\right] + ... + ...\right] + ... + ...\Big|_D} \tag{19}$$

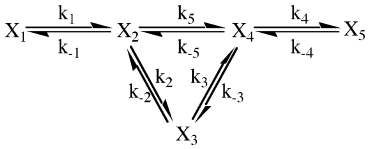
$$TKIE^0_{X_4} = \frac{k_3k_2k_1{X_1}^0\Big|_H}{\left(\dfrac{k_3}{KIE_{3,\text{int}}}\right)\left(\dfrac{k_2}{KIE_{2,\text{int}}}\right)\left(\dfrac{k_1}{KIE_{1,\text{int}}}\right){X_1}^0\Big|_D} \tag{20}$$

$$TKIE^0_{X_4} = KIE_{3,\text{int}}KIE_{2,\text{int}}KIE_{1,\text{int}} \tag{21}$$

Note that most of the terms generated by these manipulations (eqs 18 and 19) are products of rate constants and intermediate or product concentrations, which will go to zero in the limit of initial conditions (eq 20); therefore, many of these terms have not been explicitly written in the derivation above (+...). The final result (eq 21), identical to that deduced previously by numerical simulation, is obtained when the initial value of X_1 is identical for both isotopes.

These examples illustrate a general pattern. If one considers species far enough downstream from the starting species, applying the definition of $TKIE^0$ gives an indeterminate form (0/0) because none of the species in the rate expression (intermediates and products) are present at $t = 0$. Applying L'Hôpital's rule and substituting the rate laws for the derivatives generates more concentration terms, both upstream and downstream from the species under consideration. The concentrations of the downstream species will be zero at $t = 0$, thus removing these terms from consideration,

Scheme 2



whereas the upstream species will (eventually) generate a term whose value is not zero at $t = 0$. Once terms are generated whose values are not zero at the start of the reaction, L'Hôpital's rule can no longer be applied, and the limit (now a constant divided by a constant) is obtained. The original rate term that eventually generates the constant term is the rate for forming the species under consideration from an upstream reactant. This repetitive application of L'Hôpital's rule generates the products of all the forward rate constants for H and also for D from the most upstream term, giving the product of the intrinsic KIEs as postulated (*4*). Therefore, in a linear sequence of *n*-1 reversible reactions starting with X_1, calculating the TKIE^0 for the formation of the *n*-th species X_n (eq 22) will require *n*-1 applications of L'Hôpital's rule, giving the result in eq 23 reported previously (assuming equal concentrations for both isotopic reactions[2]) (*4*).

$$TKIE^0_{X_n} = \lim_{t\to 0} TKIE_{X_n} = \lim_{t\to 0} \frac{\left(\frac{dX_n}{dt}\right)_H}{\left(\frac{dX_n}{dt}\right)_D} = \lim_{t\to 0} \frac{k_{n-1}X_{n-1} - (k_{-(n-1)} + k_n)X_n + k_{-n}X_{n+1}\big|_H}{k_{n-1}X_{n-1} - (k_{-(n-1)} + k_n)X_n + k_{-n}X_{n+1}\big|_D} \quad (22)$$

$$TKIE^0_{X_n} = \lim_{t\to 0} \frac{\left(\frac{d^{n-2}X_n}{dt^{n-2}}\right)_H}{\left(\frac{d^{n-2}X_n}{dt^{n-2}}\right)_D} = \prod_{j=1}^{n-1} KIE_{j,\text{int}},\ n \geq 3 \quad (23)$$

The analysis presented above is general for sequential reaction schemes and proves the validity of the insightful numerical analysis already reported (*4*). These mathematical methods can be applied to other kinetic problems; the results for several other reaction schemes are presented below.

Pathways with Loops. When computing TKIE^0, the sequential application of L'Hôpital's rule in evaluating limits stops once a nonzero term is generated, which occurs when a reactant is reached whose starting concentration is nonzero. This probes the topology of a reaction network by finding the fewest number of reaction steps from the species of interest back to the initial (nonzero) reactant. If one considers a reaction scheme with loops, then the TKIE^0 of a species will be the product of the intrinsic KIEs on the shortest pathway back to the start so that if a loop has an unequal number of steps from the entrance to the exit, one side of the loop will not contribute. For example, consider Scheme 2. Applications of L'Hôpital's rule to the expressions for TKIE^0 show that $KIE_{2,\text{int}}$ and $KIE_{3,\text{int}}$ do not contribute to $TKIE^0_{X_4}$ or $TKIE^0_{X_5}$, although $KIE_{2,\text{int}}$ will contribute to $TKIE^0_{X_3}$.

$$TKIE^0_{X_5} = KIE_{1,\text{int}}KIE_{5,\text{int}}KIE_{4,\text{int}} \quad (24)$$

$$TKIE^0_{X_4} = KIE_{1,\text{int}}KIE_{5,\text{int}} \quad (25)$$

$$TKIE^0_{X3} = KIE_{1,\text{int}}KIE_{2,\text{int}} \quad (26)$$

$$TKIE^0_{X_2} = KIE_{1,\text{int}} \quad (27)$$

Note that it is possible for the longer branch in a looped pathway to be faster than the shorter branch. Nonetheless, the reactions of the faster but longer reaction pathway will still be excluded from the TKIE^0 for species beyond this loop because the TKIE^0 is determined by extrapolating to zero time. However, in this scenario, the amplitudes of the reaction phases required to determine TKIE^0 will be significantly diminished, making TKIE^0 difficult to determine accurately.

If both paths from the entrance to the exit of a loop have the same number of steps, then both branches of the loop contribute to TKIE^0 for species beyond the loop, and the KIEs contributed by the loop will not be intrinsic but instead will be a weighted average, as in the example in Scheme 3.

$$TKIE^0_{X_6} = KIE_{1,int}\left[\frac{k_2k_3 + k_5k_6}{\frac{k_2k_3}{KIE_{2,int}KIE_{3,int}} + \frac{k_5k_6}{KIE_{5,int}KIE_{6,int}}}\right]KIE_{4,int} \quad (28)$$

Note that if the flux through one branch is much higher than through the other, the KIEs contributed by the whole loop will approach the product of the intrinsic KIEs of the fast branch. For instance, if $k_2k_3 \gg k_5k_6$, then the flux will predominately be through the upper branch of Scheme 3, and

$$TKIE^0_6 \approx KIE_{1,int}KIE_{2,int}KIE_{3,int}KIE_{4,int} \quad (29)$$

Branched Pathways. Bifurcating pathways are often encountered in enzyme reactions. A simple reaction scheme is presented in Scheme 4. The TKIE^0 for *X*, *Y*, and *Z* are easily computed by applying the definition of TKIE^0 to the rate laws describing the system, giving the following results:

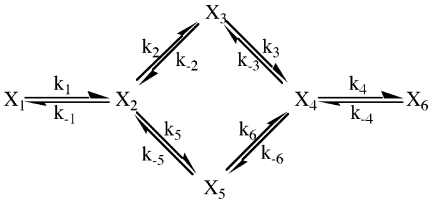
$$TKIE^0_X = \frac{k_1 + k_2}{\frac{k_1}{KIE_{1,\text{int}}} + \frac{k_2}{KIE_{2,\text{int}}}} \quad (30)$$

$$TKIE^0_Y = KIE_{1,\text{int}} \quad (31)$$

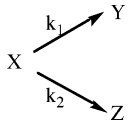
$$TKIE_Z = KIE_{2,\text{int}} \quad (32)$$

Interestingly, intrinsic isotope effects are obtained when either product is analyzed but not when the reactant is analyzed. In comparison, the observed rate constant for this reaction, obtained from the exponential describing concentration as a function of time, would be $k_1 + k_2$, regardless of whether *X*, *Y*, or *Z* were monitored, and the apparent isotope effect on this observed rate constant would be the same as that found for the TKIE^0 of *X* (eq 30).

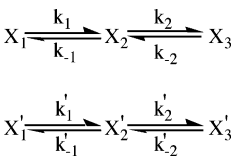
Scheme 3



Scheme 4



Scheme 5



Parallel Reactions. If two (or more) reaction pathways operate in parallel, for example, the reaction of different conformations of an enzyme that interconvert slowly but the two (or more) forms are experimentally indistinguishable, then intrinsic isotope effects are not obtained. Instead, an average weighted by the distribution of enzyme forms is obtained, as shown for the reactions in Scheme 5. In this example, each molecule adopts two forms, designated by the presence or absence of a prime, and these forms are indistinguishable experimentally so that measurements give the total, that is, $X_{2,TOT} = X_2 + X'_2$. In this case, the experimental TKIE^0 values will be measured for the total concentration of the different forms of a species.

$$TKIE^0_{X_2,obs} = \lim_{t\to 0}\frac{\left(\frac{dX_{2,TOT}}{dt}\right)_H}{\left(\frac{dX_{2,TOT}}{dt}\right)_D} = \lim_{t\to 0}\frac{\frac{d}{dt}[X_2 + X_2']\Big|_H}{\frac{d}{dt}[X_2 + X_2']\Big|_D} \quad (33)$$

$$TKIE^0_{X_2,obs} = \lim_{t\to 0}\frac{\frac{dX_2}{dt} + \frac{dX_2'}{dt}\Big|_H}{\frac{dX_2}{dt} + \frac{dX_2'}{dt}\Big|_D} \quad (34)$$

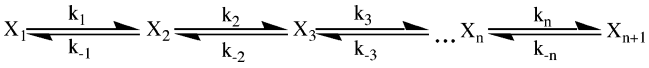
This limit can be evaluated by the methods described earlier in this article, giving

$$TKIE^0_{X_2,obs} = \frac{k_1 X_1{}^0 + k_1' X_1'^0\big|_H}{k_1 X_1{}^0 + k_1' X_1'^0\big|_D} \quad (35)$$

This can be expressed in terms of the experimentally observable total starting concentrations by assuming that X_1 comprises a fraction, f, of the total, and therefore, X'_1 is a fraction, $1 - f$ of the total.

$$TKIE^0_{X_2,obs} = \frac{fk_1 + (1-f)k_1'}{\frac{fk_1}{KIE_{1,\mathrm{int}}} + \frac{(1-f)k_1'}{KIE_{1',\mathrm{int}}}}\left(\frac{X^0_{1,TOT}\big|_H}{X^0_{1,TOT}\big|_D}\right) \quad (36)$$

Scheme 6



If identical concentrations are used in the protio and deutero reactions[2], the total initial concentrations cancel, giving the observed TKIE^0 in eq 37.

$$TKIE^0_{X_2,obs} = \frac{fk_1 + (1-f)k_1'}{\frac{fk_1}{KIE_{1,\mathrm{int}}} + \frac{(1-f)k_1'}{KIE_{1,\mathrm{int}}'}} \quad (37)$$

An analogous approach gives the observed TKIE^0 for X_3.

$$TKIE^0_{X_3,obs} = \frac{fk_1k_2 + (1-f)k_1'k_2'}{\frac{fk_1k_2}{KIE_{1,\mathrm{int}}KIE_{2,\mathrm{int}}} + \frac{(1-f)k_1'k_2'}{KIE_{1,\mathrm{int}}'KIE_{2,\mathrm{int}}'}} \quad (38)$$

Note that eqs 37 and 38 implicitly assume that there is no isotope effect on f, which is expected if substrate isotope effects are being considered. However, solvent isotope effects are possible on f. In that case, the values of f in the numerators and denominators of eqs 37 and 38 would differ.

Analysis of Isotope Effects on Signals. If a signal such as absorbance[3] is analyzed as a function of time, then the analysis for TKIE^0 must be modified. Analogous to the analysis of concentrations, the isotope effect on the transient rate of change of the signal, $TKIE_A$, is defined in eq 39.

$$TKIE_A = \frac{\left(\frac{dA}{dt}\right)_H}{\left(\frac{dA}{dt}\right)_D} \quad (39)$$

The following analysis will consider the limit of this quantity at the start of the reaction.

$$TKIE^0_A = \lim_{t\to 0} TKIE_A \quad (40)$$

It will be shown that by including a signal, there is no longer a guarantee that intrinsic isotope effects can be obtained for more than the first reaction. Two examples, a linear reaction sequence and a branching reaction, will be described to illustrate the influence of a signal.

First, consider the linear sequence of n-reversible reactions starting with X_1 and ending with X_{n+1} (Scheme 6), where each species X_i has an extinction coefficient ϵ_i. The absorbance of the solution at any time depends on the contribution of each species.

$$A = \sum_{i=1}^{n+1} \epsilon_i X_i \quad (41)$$

Equation 41 may be substituted into the definition of TKIE^0_A (eq 40), giving

[3] Absorbance is discussed for the sake of treating a specific example. However, the treatment is generally applicable to any signal that is directly proportional to concentration, such as fluorescence or circular dichroism.

$$TKIE_A^0 = \lim_{t\to 0}\frac{\left(\frac{dA}{dt}\right)_H}{\left(\frac{dA}{dt}\right)_D} = \lim_{t\to 0}\frac{\frac{d}{dt}\sum_{i=1}^{n+1}\epsilon_i X_i\Big|_H}{\frac{d}{dt}\sum_{i=1}^{n+1}\epsilon_i X_i\Big|_D} = \lim_{t\to 0}\frac{\sum_{i=1}^{n+1}\epsilon_i\frac{dX_i}{dt}\Big|_H}{\sum_{i=1}^{n+1}\epsilon_i\frac{dX_i}{dt}\Big|_D} \quad (42)$$

Each derivative is defined by the rate laws, describing Scheme 6 in terms of concentrations and rate constants. Substitution and grouping terms gives the following.

$$TKIE_A^0 = \lim_{t\to 0}\Big\{\{(\epsilon_2-\epsilon_1)k_1X_1 + [\epsilon_1k_{-1} - \epsilon_2(k_{-1}+k_2) + \epsilon_3k_2]X_2 + [\epsilon_2k_{-2} - \epsilon_3(k_{-2}+k_3) + \epsilon_4k_3]X_3 + ...\big|_H\}\Big/ \{(\epsilon_2-\epsilon_1)k_1X_1 + [\epsilon_1k_{-1} - \epsilon_2(k_{-1}+k_2) + \epsilon_3k_2]X_2 + [\epsilon_2k_{-2} - \epsilon_3(k_{-2}+k_3) + \epsilon_4k_3]X_3 + ...\big|_D\}\Big\} \quad (43)$$

All terms except those containing X_1 extrapolate to zero at the start of the experiment, giving eq 44.

$$TKIE_A^0 = \frac{(\epsilon_2-\epsilon_1)k_1{X_1}^0\Big|_H}{(\epsilon_2-\epsilon_1)\frac{k_1}{KIE_{1,int}}{X_1}^0\Big|_D} \quad (44)$$

If ϵ_1 and ϵ_2 are not identical, then the numerator and denominator are nonzero, and the limit can be evaluated directly. The condition of identical starting concentrations of protio and deutero X_1 gives the following.

$$TKIE_A^0 = KIE_{1,int} \quad (45)$$

No assumptions were made in obtaining eq 45 except that the reaction of X_1 to X_2 be accompanied by a spectral change; when this is so, only the intrinsic isotope effect on the conversion of X_1 to X_2 can be obtained from $TKIE_A^0$, because the first terms in the numerator and denominator of eq 43 each contain X_1 and extrapolate to nonzero values at the start of the reaction. If the X_1 terms were eliminated from eq 43, reactions further along the reaction pathway could contribute intrinsic isotope effects to $TKIE_A^0$. The term containing X_1 as a factor will be zero if $\epsilon_1 = \epsilon_2$, for example, the wavelength of observation is an isosbestic point for the first reaction. If this is so, then no terms in the expression for $TKIE_A^0$ (eq 43) will be nonzero, and L'Hôpital's rule must be used to evaluate the limit. Differentiating will never eliminate the factor of zero originating from the original X_1 term; therefore, this term may be ignored in subsequent manipulations. Applying L'Hôpital's rule generates the derivative of X_2, which may be replaced with the rate law for the reactions in Scheme 6. A term containing X_1 is generated, and because this extrapolates to a nonzero concentration at the start of the reaction, the limit may be computed, giving the result in eq 46.

$$TKIE_A^0 = \frac{\epsilon_1k_{-1} - \epsilon_2(k_{-1}+k_2) + \epsilon_3k_2}{\epsilon_1\frac{k_{-1}}{KIE_{-1,int}} - \epsilon_2\left(\frac{k_{-1}}{KIE_{-1,int}} + \frac{k_2}{KIE_{2,int}}\right) + \epsilon_3\frac{k_2}{KIE_{2,int}}}KIE_{1,int} \quad (46)$$

Note that eq 46 was derived under the condition that $\epsilon_1 = \epsilon_2$. Substituting that into eq 46 leads to the simplified result in eq 47.

$$TKIE_A^0 = KIE_{1,int}KIE_{2,int} \quad (47)$$

Intrinsic isotope effects for the next reaction can contribute only if the coefficients of both the X_1 and the X_2 terms in eq 43 are zero. This will occur if $\epsilon_1 = \epsilon_2 = \epsilon_3$. Note that an alternative condition could cause the coefficient of X_2 in eq 43 to be zero; if the ratio of the rate constants for the reactions of X_2 in the reverse and forward directions equals the ratio of the changes in extinction for these reactions (eq 48), then the coefficient of X_2 will be zero.

$$\frac{k_{-1}}{k_2} = \frac{\epsilon_2-\epsilon_3}{\epsilon_1-\epsilon_2} \quad (48)$$

However, eq 48 requires $\epsilon_1 \neq \epsilon_2$. In that circumstance, $TKIE_A^0$ will be determined by the X_1 term of eq 43 to give the result in eq 45, and the behavior of subsequent species will be irrelevant. Therefore, we need only consider the case when the reactions of X_1 to X_2 and X_2 to X_3 are spectrally silent, causing the coefficients of the X_1 and X_2 terms in eq 43 to both be zero. L'Hôpital's rule allows the evaluation of the limit. Because the coefficients of the original X_1 and X_2 terms are zero, their derivatives will always be multiplied by zero; therefore, the first nonzero term will be generated from X_3 after two rounds of differentiation. The result, given in eq 49, has features that are similar to those of eq 46.

$$TKIE_A^0 = \frac{\epsilon_2k_{-2} - \epsilon_3(k_{-2}+k_3) + \epsilon_4k_3}{\epsilon_2\frac{k_{-2}}{KIE_{-2,int}} - \epsilon_3\left(\frac{k_{-2}}{KIE_{-2,int}} + \frac{k_3}{KIE_{3,int}}\right) + \epsilon_4\frac{k_3}{KIE_{3,int}}}KIE_{2,int}KIE_{1,int} \quad (49)$$

Because ϵ_2 must be identical to ϵ_3 in order to obtain eq 49 from eq 43, eq 49 can be simplified to eq 50.

$$TKIE_A^0 = KIE_{3,int}KIE_{2,int}KIE_{1,int} \quad (50)$$

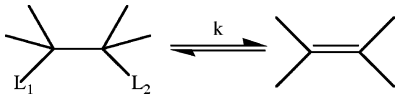
From these analyses, it is apparent that $TKIE_A^0$ in a linear reaction sequence will be the product of the intrinsic isotope effects leading up to the first step to give a signal change. Reactions after the first step to produce a signal change will not contribute to $TKIE_A^0$, regardless of the occurrence of subsequent isosbestic points.

The procedure for writing expressions for $TKIE_A^0$ can be applied to other reaction mechanisms. For instance, when applied to the branching pathway shown in Scheme 4, the result in eq 51 is obtained.

$$TKIE_A^0 = \frac{(\epsilon_Y-\epsilon_X)k_1 - (\epsilon_Z-\epsilon_X)k_2}{(\epsilon_Y-\epsilon_X)\frac{k_1}{KIE_{1,int}} - (\epsilon_Z-\epsilon_X)\frac{k_2}{KIE_{2,int}}} \quad (51)$$

Note that at wavelengths where one of the reactions does not produce a spectral change (either $\epsilon_X = \epsilon_Y$ or $\epsilon_X = \epsilon_Z$), intrinsic isotope effects will be obtained; otherwise, $TKIE_A^0$ will be wavelength-dependent and contain contributions from both intrinsic isotope effects. If the spectra of X, Y, and Z

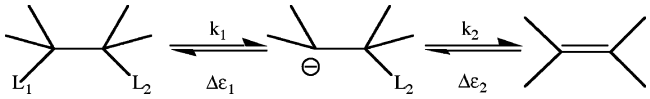
Table 1: $TKIE^0$ Values for a Concerted Reaction[a]



		concerted			
		semi-classical		tunneling	
L_1	L_2	$TKIE^0_{prod}$	$TKIE^0_A$	$TKIE^0_{prod}$	$TKIE^0_A$
D	H	$KIE_{1,int}$	$KIE_{1,int}$	$KIE_{1,int}$	$KIE_{1,int}$
H	D	$KIE_{2,int}$	$KIE_{2,int}$	$KIE_{2,int}$	$KIE_{2,int}$
D	D	$KIE_{1,int}KIE_{2,int}$	$KIE_{1,int}KIE_{2,int}$	$<KIE_{1,int}KIE_{2,int}$	$<KIE_{1,int}KIE_{2,int}$

[a] The $TKIE^0$ values expected for the concerted transfer of two hydrogens (L_1 and L_2) are tabulated assuming either semi-classical conditions, in which the Rule of the Geometric Mean applies (*6*), or assuming significant hydrogen tunneling, which causes the Rule of the Geometric Mean to be disobeyed. The values of $TKIE^0$ obtained either from direct product analysis ($TKIE^0_{prod}$) or from a signal ($TKIE^0_A$) are given for both semi-classical and tunneling transition states. The intrinsic isotope effect for transfer of L_1 and L_2 are $KIE_{1,int}$ and $KIE_{2,int}$, respectively. The reaction from substrate to product is assumed to be the first reaction in a possible sequence.

Table 2: $TKIE^0$ Values for a Stepwise Reaction[a]



		stepwise					
		semi-classical			tunneling		
L_1	L_2	$TKIE^0_{prod}$	$TKIE^0_A$, $\Delta\epsilon_1 \neq 0$	$TKIE^0_A$, $\Delta\epsilon_1 = 0$	$TKIE^0_{prod}$	$TKIE^0_A$, $\Delta\epsilon_1 \neq 0$	$TKIE^0_A$, $\Delta\epsilon_1 = 0$
D	H	$KIE_{1,int}$	$KIE_{1,int}$	$KIE_{1,int}$	$KIE_{1,int}$	$KIE_{1,int}$	$KIE_{1,int}$
H	D	$KIE_{2,int}$	1	$KIE_{2,int}$	$KIE_{2,int}$	1	$KIE_{2,int}$
D	D	$KIE_{1,int}KIE_{2,int}$	$KIE_{1,int}$	$KIE_{1,int}KIE_{2,int}$	$KIE_{1,int}KIE_{2,int}$	$KIE_{1,int}$	$KIE_{1,int}KIE_{2,int}$

[a] The $TKIE^0$ values expected for the stepwise transfer of two hydrogens (L_1 and L_2) are tabulated assuming either semi-classical conditions or significant hydrogen tunneling. However, for the stepwise reaction, tunneling does not alter the observable $TKIE^0$. The values of $TKIE^0$ obtained either from direct product analysis ($TKIE^0_{prod}$) or from a signal ($TKIE^0_A$) are given for both semi-classical and tunneling transition states. For $TKIE^0_A$, cases are considered where the formation of the intermediate is accompanied by a signal change ($\Delta\epsilon_1 \neq 0$) and where the formation of the intermediate is not accompanied by a signal change ($\Delta\epsilon_1 = 0$). The intrinsic isotope effect for transfer of L_1 and L_2 are $KIE_{1,int}$ and $KIE_{2,int}$, respectively. The reaction from substrate to product is assumed to be the first reaction in a possible sequence.

are known, then it should be possible to calculate the kinetic parameters in eq 51 from the variation of $TKIE^0_A$ with the wavelength of observation.

Multiple Deuterium Isotope Effects. There are many enzymatic reactions in which bonds to two hydrogens are broken, but only one kinetic phase is observable. In such situations, it is not clear *a priori* whether both bonds are broken in the same step (a concerted reaction) or in different steps with an intermediate that is too unstable to observe (a stepwise reaction). When the conversion of the reactant to the product can be observed directly as a single kinetic phase, multiple kinetic isotope effects can sometimes be used to determine whether the reaction is concerted (and the observed rate constant represents the actual rate constant) or whether the observed rate constant represents a net rate constant for the overall stepwise process. In semi-classical concerted hydrogen transfers (i.e., quantum tunneling is negligible), the Rule of the Geometric Mean (*6*) predicts that the product of intrinsic isotope effects for reactants individually labeled at two sites will equal the intrinsic isotope effect measured for the doubly labeled molecule. In contrast, for a stepwise reaction, the single and double isotope effects determined for the formation of the product from observed rate constants (the eigenvalues obtained from an exponent) will not obey the simple multiplicative relationship that concerted reactions obey.

Interestingly, $TKIE^0$ values obtained for concentrations cannot distinguish between concerted and stepwise mechanisms (Tables 1 and 2). The Rule of the Geometric Mean predicts that the isotope effects controlling a concerted reaction will be multiplicative, and because these are expressed in a single step, the $TKIE^0$ values will also be multiplicative. However, a stepwise reaction will also give a $TKIE^0$ containing the product of the intrinsic isotope effects of the two steps leading to the reaction product because the $TKIE^0$ value for a species formed by a linear reaction sequence is the product of the intrinsic isotope effects leading to that species. Therefore, a stepwise reaction will have the same $TKIE^0$ that a concerted reaction would in semi-classical hydrogen transfers. If quantum mechanical tunneling is significant in the concerted reaction, then the Rule of the Geometric Mean will not be obeyed, and the product of the intrinsic single isotope effects for a concerted reaction will not equal the double isotope effect determined from $TKIE^0$ values. Curiously, the equality would still hold for stepwise reactions involving tunneling, leading to a reversal of the classic diagnostic criterion.

The possible outcomes of double isotope effect experiments are slightly more complex with $TKIE^0_A$ owing to the dependence of $TKIE^0_A$ on extinction changes. A concerted reaction will give a $TKIE^0_A$ equal to the intrinsic isotope

effect, and the products of the effects observed for singly labeled substrates will equal the effect obtained with the doubly labeled substrate (in the absence of tunneling), similar to $TKIE^0$ for the concentration of the reaction product. For a stepwise pathway, two outcomes are possible. If the first reaction occurs with an extinction change, then $TKIE_A^0$ will only contain the intrinsic isotope effect for the first reaction. Therefore, isotopomers that transfer the label in the first step will produce a $TKIE_A^0$ equal to the intrinsic isotope effect for that bond cleavage, whereas the label that reacts in the second reaction will not contribute to $TKIE_A^0$. If the first reaction produces no extinction change, then $TKIE_A^0$ will be the product of the intrinsic isotope effects of both steps.

Rapid Equilibria and Unfavorable Rate-Determining Steps. Often there are interconversions in reaction pathways that are much faster than the reaction steps leading to or proceeding from them. In that case, the interconverting species maintain a concentration ratio determined by the equilibrium constant, that is, the ratio of the forward and reverse rate constants, governing the interconversion. Rate constants are not available from standard kinetic experiments for such rapid equilibrium steps because by definition, other steps are rate-determining. The inability to determine individual rate constants prevents the determination of KIEs by traditional kinetic means on reactions in a rapid equilibrium step.

Remarkably, kinetic isotope effects for steps in a rapid equilibrium can be obtained from the $TKIE^0$. For instance, the analysis given in eqs 18−21 shows that in a linear reaction sequence, all rate constants for forward reactions contribute to the value of $TKIE^0$, regardless of their magnitudes, and reverse rate constants are absent, regardless of their magnitudes. Thus, $TKIE^0$ will contain as factors the intrinsic isotope effects for forward reactions in rapid equilibria, which are not directly observable in kinetic traces.

This analysis was verified in numerical simulations in which a rapid equilibrium was included. In all cases examined, the $TKIE^0$ value obtained was that predicted from the forward rate constants, regardless of how fast the rapid equilibrium was relative to that of other reactions in the scheme and regardless of whether the rapidly equilibrating reaction occurred first in a sequence (simulating substrate binding to an enzyme) or in the middle of a reaction scheme. This behavior has powerful implications. In favorable experimental situations, it should be possible to obtain intrinsic kinetic isotope effects for reaction that are otherwise kinetically invisible because they are rapid equilibria. However, this sword is double-edged; a reaction whose presence is unsuspected because it is kinetically invisible due to a rapid equilibrium will still contribute its intrinsic isotope effect to $TKIE^0$. This reasoning is more general than rapid equilibria. Reactions are often invisible due to unfavorably located rate-determining steps. Nonetheless, their intrinsic isotope effects will contribute to $TKIE^0$. For instance, if an intermediate is formed at 1 s^{-1} and reacts in the forward direction at 1000 s^{-1}, it is unlikely to be detected. Nonetheless, the intrinsic isotope effect on the fast reaction will contribute to $TKIE^0$ for species later in the reaction sequence.

Experimental Limitations. Although the analyses presented in previous sections clearly show that the $TKIE^0$ can be a rich source of kinetic information, an important experimental limitation must be considered. The determination of $TKIE^0$ requires that experimental time-courses be extrapolated accurately to $t = 0$. The observation of reactions started by mixing are limited to times greater than the dead-time of the stopped-flow or quenching instrument, typically about a millisecond on most commercial rapid-reaction instruments. Reactions with rate constants on the order of 1000 s^{-1} will have proceeded to a significant extent before being observed, whereas much faster reactions will be over before data collection can begin. Consequently, it might be anticipated that when there are reactions that are rapid relative to the dead-time, their intrinsic KIEs might not be available in the experimentally determined $TKIE^0$. This was investigated through numerical simulation.

Figures 1 and 2 show simulations of concentration and absorbance as a function of time for a two-step reaction (panel A), the TKIE for the products (panel B), and the $TKIE_A$ (panel C). In Figure 1, all rate constants are less than or equal to 10 s^{-1}, values which are easily accessible in most rapid-mixing experiments. Two important features are worth noting in the change of TKIE with time. First, changes in direction are possible, as seen in the trace for the di-deutero reaction. Second, if data earlier than 10^{-3} seconds are ignored, extrapolation back to the correct $TKIE^0$ values is accurate. In contrast, when a large value for a rate constant is used (Figure 2), accurate extrapolation is not possible. The TKIE traces in Figure 2 undergo critical changes in direction at times earlier than one millisecond so that real experimental data would not reveal the true $TKIE^0$. Instead, experimental data would extrapolate to an erroneous $TKIE^0$.

DISCUSSION

The analysis presented previously (*4*) and developed further here provides the theoretical basis for a general method of determining intrinsic kinetic isotope effects from transient kinetic data. This approach relies on determining the initial velocity of the reaction of a species or in determining the initial rate of a signal change. Under favorable circumstances, delineated in Results, the calculation of $TKIE^0$ allows the convenient determination of intrinsic kinetic isotope effects. It is worth emphasizing that because reverse rate constants do not enter into expressions for $TKIE^0$, useful values can be measured even when there is a very high reverse commitment to catalysis. This is in contrast to steady-state studies of isotope effects, where high commitments mask intrinsic isotope effects.

It is anticipated that determining $TKIE^0$ will be more generally useful for rapid-quench data, where the various species can be analyzed individually, than for stopped-flow data, where all species often contribute to a signal. The determination of intrinsic tritium isotope effects by rapid-quench experiments may be particularly convenient by $TKIE^0$ analyses. Generally, the proportion of tritium in a labeled substrate is fairly small, with the vast excess being an unlabeled carrier. If methods other than counting tritium are available for quantifying the species present in quenched reaction mixtures, these will report on the time-course for the reaction of the whole sample, which is essentially the tritium-free carrier. The time-course of the reactions of the tritiated species can be determined separately by independent scintillation counting of the same quenched samples, allowing the labeled and unlabeled reactions to be performed in the same enzyme reaction.

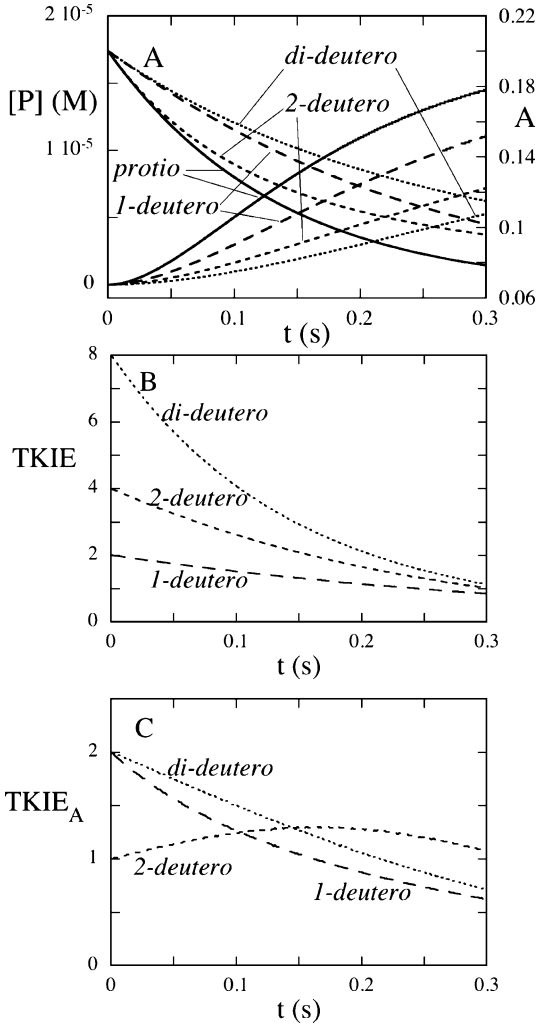


FIGURE 1: Simulation of a two-step reaction with favorable kinetics. A two-step reversible reaction scheme was simulated with values (for the fully protiated reactant) of the forward and reverse rate constants for the first step of 10 and 1 s^{-1}, respectively, and values of the forward and reverse rate constants for the second step of 10 and 1 s^{-1}, respectively. Two-fold isotope effects were used on both the forward and reverse rate constants for the first reaction, and 4-fold effects were used on the forward and reverse rate constants of the second reaction. Panel A shows the simulated concentration curves for the product (rising curves) and absorbance traces simulated for the same reactions (decreasing curves) using an extinction coefficient of 10 000 M^{-1} cm^{-1} for the reactant, 5000 M^{-1} cm^{-1} for the intermediate, and 3000 M^{-1} cm^{-1} for the product. The reaction having an isotope effect on the first step is marked as *1-deutero*, that with the isotope effect on the second step as *2-deutero*, and that with isotope effects on both steps as *di-deutero*. The time-courses of the TKIEs of the products are shown in B. These traces extrapolate to values at $t = 0$, which are predicted by the analysis of TKIE^0 in the text. Note that accurate extrapolations would be possible in real experiments because no sudden changes in direction occur near the practical cutoff for observability in mixing experiments (~1 ms). The time-courses of the TKIE$_A$'s for the absorbance traces are shown in C. As in B, accurate extrapolation is possible. Also note that because the extinction change in the first reaction is not zero, the TKIE$_A^0$ for the reaction with an isotope effect on the second step is 1, whereas that for the di-deutero case is the same as that for the reaction in which there is an isotope effect on the first step.

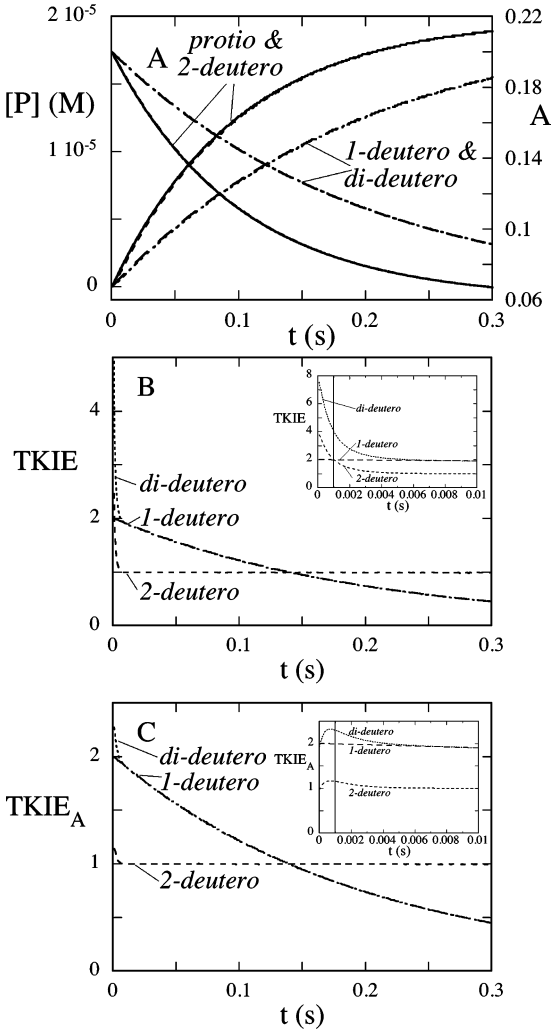


FIGURE 2: Simulation of a two-step reaction with unfavorable kinetics. A two-step reaction was simulated as in Figure 1, with the only differences being that the values of the forward and reverse rate constants for the last reaction (with protium) were now 2500 and 10 s^{-1}, respectively. Simulated traces for the product concentration and absorbance are shown in A. The TKIE traces for products are shown in B. The inset in B shows the earliest time range and has a vertical line drawn at 1 ms, which is roughly the earliest time that may be observed in mixing experiments. Note that although the TKIE values extrapolate to the values of TKIE^0 predicted by theory, these values may be difficult to determine for data starting at 1 ms. The TKIE$_A$ values are shown in C, with a close-up of the earliest times in the inset. Note that the curves for the 2-deutero and di-deutero reactions change direction much earlier than 1 ms, making accurate extrapolation impossible in real mixing experiments.

The analysis of signals in Results demonstrates that the intrinsic isotope effect of only the first reaction is guaranteed to contribute to the TKIE$_A^0$ of a sequential reaction, whereas sequences whose signal change is zero for initial steps will have contributions of intrinsic isotope effects from more reactions. The occurrence of two or more identical isosbestic points for sequential reactions may seem unlikely, but is not without precedent (*7*). More likely is the situation where a colorless reactant is converted, possibly after several steps, to a chromophore. In that case, the TKIE$_A^0$ could contain the intrinsic isotope effects of several reactions. Clearly, comparing values obtained at different wavelengths, or comparing different spectral signals, such as absorbance and fluorescence, will prove highly profitable.

Although the use of TKIE^0 was originally conceived as a way of determining solvent isotope effects, where the rates of several steps in a reaction pathway can be altered (*4*), its use, in principle, is completely general. Therefore, this

formalism could also be applied to measuring substrate isotope effects, where only one or a few steps are expected to be isotopically sensitive. These considerations could have particular relevance in the oxidation of substrates by flavin-utilizing enzymes. Frequently, molecules containing H-C-X-H substructures (where X can be carbon, nitrogen, oxygen, or sulfur) are oxidized to the corresponding double-bonded systems containing C═X, and the question of concerted versus stepwise is central to understanding the mechanism. For a stepwise mechanism, the initial transfer of a hydrogen to the flavin would cause a large spectral change, whereas the alternative, deprotonation of the substrate, could be spectrally silent.

The use of $TKIE^0$ offers potential advantages over determining intrinsic isotope effects from observed rate constants obtained by transient kinetics. The $TKIE^0$ generally has a mathematical form that allows the simple extraction of intrinsic isotope effects. In contrast, observed rate constants for the phases in reversible sequential reactions are the eigenvalues of systems of differential equations, which are the roots of polynomials of rate constants. Finding unique and reliable values for individual rate constants so that intrinsic isotope effects can be computed can be an arduous task. The difficulty is very system-dependent, becoming less difficult when the reaction phases are well-resolved because simplifications in the expressions for eigenvalues become possible. Also, when reactions are irreversible, as is often the case in highly exergonic redox reactions, the observed rate constants equal rate constants, diminishing the analytical advantage of the $TKIE^0$. However, when reactions are not well-resolved or irreversible, determining $TKIE^0$ is likely to be advantageous. Ultimately, an analysis strategy simultaneously combining both approaches is likely to be best.

The accuracy of experimental data puts limits on the usefulness of all methods for determining intrinsic isotope effects. Extrapolations of concentrations back to the start of a reaction, required for the calculation of $TKIE^0$, will be based on lower and lower concentrations as species further along a multistep reaction pathway are analyzed, causing the accuracy to diminish. Also, the accuracy of a $TKIE^0$ value is compromised by very fast reactions. Such reactions also present a challenge to traditional exponential fits. The extent to which these effects will be important will depend on the sensitivity and accuracy of the experimental method, which is system-dependent. Thus, the potential of using $TKIE^0$ values to determine intrinsic isotope effects is likely to vary from enzyme to enzyme. Its application to the reduction of the flavin of *N*-methyltryptophan oxidase has recently been reported (*8*). The $TKIE^0_A$ values obtained in that study agreed well with the isotope effects calculated from the ratios of the observed rate constants. The large value for $TKIE^0_A$ is clearly consistent with Ralph and Fitzpatrick's conclusion that the flavin is reduced by a hydride-transfer and rules out mechanisms invoking initial reactions that are isotope-insensitive and accompanied by a spectral change such as a single-electron transfer to the flavin.

It is hoped that the analyses presented here will stimulate the application of this potentially powerful new tool and assist in the proper interpretation of results. Other properties of the TKIE function are coming to light, including the curious ability to experimentally count the number of reactions between two species by the rate of variation of the TKIE (*9*).

ACKNOWLEDGMENT

We gratefully acknowledge the helpful comments from Professor Harvey Fisher (Veteran's Affairs Medical Center, Kansas City, MO) and Professor Sandro Ghisla (University of Konstanz, Germany).

REFERENCES

1. *Enzyme Mechanism from Isotope Effects* (1991) (Cook, P. F., Ed.) CRC Press, Boca Raton, FL.
2. Northrop, D. B. (1981) The expression of isotope effects on enzyme-catalyzed reactions, *Annu. Rev. Biochem. 50*, 103−131.
3. Northrop, D. B. (1981) Minimal kinetic mechanism and general equation for deuterium isotope effects on enzymic reactions: Uncertainty of detecting a rate-limiting step, *Biochemistry 20*, 4056−4061.
4. Maniscalco, S. J., Tally, J. F., and Fisher, H. F. (2004) The interpretation of multiple-step transient-state kinetic isotope effects, *Arch. Biochem. Biophys. 425*, 165−172.
5. Riddle, D. F. (1979) *Calculus and Analytic Geometry*, 3rd ed., pp 579−587,Wadsworth Publishing Company, Inc., Belmont, CA.
6. Bigeleisen, J. (1955) Statistical mechanics of isotope systems with small quantum corrections. I. General considerations and the rule of the geometric mean, *J. Chem. Phys. 23*, 2264−2267.
7. Entsch, B., and Ballou, D. P. (1989) Purification, properties, and oxygen reactivity of *p*-hydroxybenzoate hydroxylase from *Pseudomonas aeruginosa*, *Biochim. Biophys. Acta. 999*, 313−322.
8. Ralph, E. C., and Fitzpatrick, P. F. (2005) pH and kinetic isotope effects on sarcosine oxidation by *N*-methyltryptophan oxidase, *Biochemistry 44*, 3074−3081.
9. Fisher, H. F., Palfey, B. A., Maniscalco, S. J., and Indyk, L. (2006) Relationship between the time-dependence of a transient-state kinetic isotope effect and the location of complexes in a reaction sequence, *J. Phys. Chem. A 110*, 4465−4472.

BI061214H