Relationship between the Time-Dependence of a Transient-State Kinetic Isotope Effect and the Location of Complexes in a Reaction Sequence

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We prove here a new transient-state kinetic rule which states that the ratios of the first derivatives of kinetic isotope effect time courses, extrapolated to zero time, provide integral values which specify the precise step number in the reaction sequence in single isotope substituted reactions. This rule defines such absolute numbers even where the steps involved are too fast to provide measurable concentrations of intermediates and when the full reaction sequence is unknown.

Introduction

The measurement of kinetic isotope effects (KIEs) has provided a major tool for the elucidation of the mechanisms of chemical reactions providing both some degree of kinetic resolution as well as information on the nature of the transition state of the isotope-sensitive step. The intrinsic KIE is defined as $KIE_{int} = k_H/k_D$ (where k_H and k_D designate the forward rate constants for a given step for the unsubstituted and deuterio-substituted reactions). In the general case, however, the KIE observed from any kind of kinetic rate measurements does not equal KIE_{int} since, in a reaction of any degree of complexity, such an observed value must contain contributions from the rate constants of the other steps involved in the reaction. On occasion, the isotope-sensitive step may be rate-limiting to such an extent that the value of the observed KIE may approach that of KIE_{int} within the accuracy of the measurement. In the more usual case, however, the KIE_{int} must be resolved from the mathematical expression of the rate law for the given reaction. Most of the work applying this approach to the study of enzyme-catalyzed reactions has been based on steady-state kinetic analysis.[1] The strength of this approach is its rigorously derived mathematical basis. Its principal limitation has been that of the frequently severe kinetic masking of the intrinsic KIE by slower steps in the reaction, although the use of multiple isotopic substitutions as described by Northrop[2] and Cleland and co-workers[3] have considerably increased the mechanistic resolution of this approach. The transient-state kinetic approach, on the other hand, permits the direct observation of the time courses of individual reaction intermediates in real time and thus permits the determination of intrinsic KIE values in a more direct manner.[4] Its application, however, has up until recently been hampered by the lack of the comprehensive rigorously derived body of theory that is the prime feature of the steady-state approach. We have begun to develop the basic elements of such a theory. Before proceeding, we must note that, although a steady-state KIE is by definition independent of both time and the specific signal by which it is measured, its transient-state counterpart is strongly dependent on both time and signal. Recognizing these features, we denote an experimentally determined transient-state kinetic isotope effect as a "TKIE". Having observed that the effect of a single-step substituted transient-state KIE (TKIE) is manifested on every step of the entire reaction sequence, and defining the observed TKIE for a component "x" as $TKIE_X = d[X]_H/dt/d[X]_D/dt$, we proved a first rule of transient-state kinetic isotope effects: case I, the time dependence of the TKIE curve of any complex occurring after the isotopic sensitive step must equal the intrinsic KIE (KIE_{int}) at zero time and then its value must initially decrease with time; case II, the TKIE curve of any complex occurring prior to the isotope-sensitive step must have a value of unity at zero time and then must decrease with time.[5] More recently, we proved an additional rule of TKIEs, this one applying to multistep isotopically substituted reactions such as the measurement of D_2O solvent TKIEs.[6] That rule states that in such a reaction the zero-time intercept of the TKIE of any given component equals the algebraic product of the intrinsic KIEs of all of the forward rate constants which precede its formation. Here, we demonstrate the validity of a new rule; one which establishes a numerical relationship between the rates of decrease with time of the TKIE of a singly substituted isotope reaction component and the specific location of that component in the reaction sequence.

The impetus for the theoretical exploration described here was provided by the observation that the TKIEs of the successive complexes of the glutamate dehydrogenase reaction (Figure 5 of ref. 7) varied markedly in their time dependences, that of the last complex formed decreasing more slowly than those of the earlier complexes. A reexamination of these data showed that the TKIE time courses of the group of complexes obeyed the first rule, in that they all converged to a common value at $t = 0$. However, the rates of decrease of these TKIEs with time suggested an inequality of the form: $d(TKIE_D)/dt < d(TKIE_C)/dt < d(TKIE_B)/dt$

Theoretical Development for a Simple Case. The two-step reaction

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C \qquad (1)$$

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is the largest fully reversible sequential mechanism for which an algebraic integral exists. Although such a reaction is mechanistically insufficient for any realistic enzyme reaction, examination of its mathematical solution illuminates a fundamental property which is applicable to the general case which we consider below. The general solution for the time dependencies of components B and C in eq 1 is[8]

$$[\mathrm{B}](t) = k_1\left[\left(\frac{k_{-2}}{\lambda_1\lambda_2} + \frac{k_{-2}-\lambda_1}{\lambda_2(\lambda_1-\lambda_2)}\right)\mathrm{e}^{-\lambda_1 t} + \frac{\lambda_2 - k_{-2}}{\lambda_2(\lambda_1-\lambda_2)}\mathrm{e}^{-\lambda_2 t}\right] \quad (2)$$

$$[\mathrm{C}](t) = k_1k_2\left[\frac{1}{\lambda_1\lambda_2} + \frac{\mathrm{e}^{-\lambda_1 t}}{\lambda_1(\lambda_1-\lambda_2)} - \frac{\mathrm{e}^{-\lambda_2 t}}{\lambda_2(\lambda_1-\lambda_2)}\right] \quad (3)$$

where

$$\lambda_1 = \frac{1}{2}(p+q)$$

and

$$\lambda_2 = \frac{1}{2}(p-q)$$

and

$$p = k_1 + k_2 + k_3 + k_4;$$

$$q = \sqrt{p^2 - 4(k_1k_2 + k_{-1}k_{-2} + k_1k_{-2})}$$

A corresponding set of equations for the same reaction (assuming the first step to be isotopically sensitive) can be written by substituting $k_1/\mathrm{KIE_{int}}$ for k_1, and $k_2/\mathrm{KIE_{int}}$ for k_2

$$\text{Defining:}\quad \mathrm{TKIE_B} = \frac{\mathrm{d[B_H]}/\mathrm{d}t}{\mathrm{d[B_D]}/\mathrm{d}t} \text{ and } \mathrm{TKIE_C} = \frac{\mathrm{d[C_H]}/\mathrm{d}t}{\mathrm{d[C_D]}/\mathrm{d}t} \quad (4)$$

We now define a new function, n_S

$$n_S = \lim_{t\to 0}\frac{\mathrm{d(TKIE_B)}/\mathrm{d}t}{\mathrm{d(TKIE_C)}/\mathrm{d}t} \quad (5)$$

We proceed to evaluate this function using L'Hôpital's rule. As written, the function yields the indeterminate value of 0/0. Successive applications of L'Hôpital's rule, however, finally yield a finite value of $n_S = 2$. Thus, $\mathrm{TKIE_B}$ initially decreases at a rate precisely twice that of $\mathrm{TKIE_C}$ where B is the immediate product of the isotope-sensitive step in the forward direction of the reaction and C is the product of the following step. It is clear from the proof of eq 5 that the value of n_S is completely independent of all rate constants and of the value of $\mathrm{KIE_{int}}$ for this simple two-step case and that it reflects only the position of the isotope-sensitive step in the reaction.[9] The mathematical relationships involved in eqs 2−5 are more readily grasped by reference to their graphic portrayal in Figure 1.

General Case. We now consider the more realistic reaction scheme

$$\mathrm{A} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{B} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{C} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{D} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{E} \quad (6)$$

Formally integrated kinetic equations for such a scheme do not exist, as we have noted. The use of numerically evaluated equations, however, provides a means of establishing the relationships we require without any substantial loss of precision. Figure 1 shows the time courses of each of the substituted and unsubstituted species in the reaction sequence shown in eq 6 assuming that the isotopically sensitive step to be step one. Panel a of Figure 2 shows the time courses of the TKIEs for each species. It can be seen from the common intercept on the $t = 0$ axis that $\lim_{t\to 0}$ TKIE for each species is precisely equal to the assumed intrinsic KIE listed in the figure legend, as required by the first rule. Panel b of Figure 2 shows the time courses of the first derivatives of the various component TKIEs. Panel c of Figure 2 shows the time courses of the ratio of $\mathrm{d(TKIE_B)}/\mathrm{d}t$ to the $\mathrm{d(TKIE)}/\mathrm{d}t$ of each of the components C, D, and E. Inspection of these intercepts in panel c shows that the zero-time intercept for each post-isotope-sensitive step is an integer and that the value of that integer is identical to the sequence number of the step which produces that complex. Thus, we have now demonstrated (but, at this point, not formally proved) a new rule of transient-state kinetic isotope effects

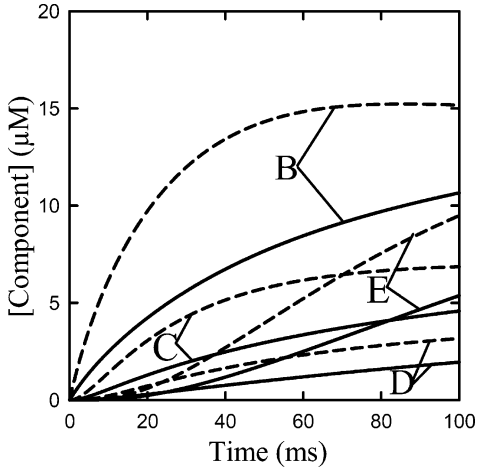

Figure 1. Concentration time dependences for H and D substituted components in the reaction shown in eq 6 assuming the isotope effect to be on step 1. Dashed lines indicate unsubstituted components and solid lines indicate isotope-substituted species of the component bracketed with it. Assumed values for the constants were $k_1 = 20$, $k_2 = 7$, $k_3 = 75$, $k_4 = 150$, $k_5 = 70$, $k_6 = 120$, $k_7 = 132$, $k_8 = 35$, $\mathrm{KIE_{int}} = 2.7$.

$$n_S = \frac{\lim_{t\to 0}\mathrm{d(TKIE_1)}/\mathrm{d}t}{\lim_{t\to 0}\mathrm{d(TKIE_{nS})}/\mathrm{d}t} \quad (7)$$

where n_S is the number of the step in a sequence such as that shown in eq 6. This relationship, which we will designate as the "second rule", applies to both normal and inverse KIEs but is not generally applicable to reactions in which isotope effects occur in multiple steps.

Effect of a Pre-Isotopic Transfer Step on the Rule. If one assumes the more realistic case where the isotope effect occurs in the second step of the sequence shown in eq 6 rather than in the first, the behavior of the KIEs of the components is somewhat altered. The component time courses for the unsubstituted and the substituted reactions under this assumption for the four-step case of eq 6 are shown in Figure 3. As seen in panel a of Figure 4, the zero time value of TKIE for B, which is now a pre-isotope affected entity, is unity as required by the first rule[6] described in the Introduction. Moreover, as can be

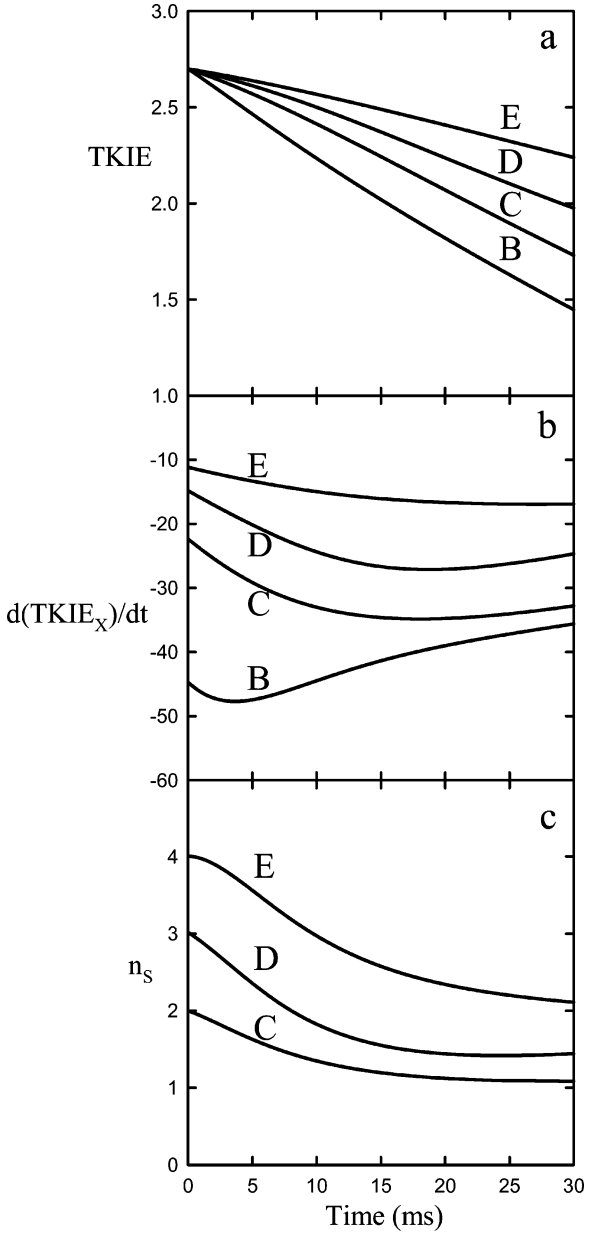

Figure 2. (a) Time courses of TKIEs for each product species of the reaction shown in eq 6; calculated from the curves shown in Figure 1 according to eq 4. (b) Time courses of $d(KIE_X)/dt$ for each product species. (c) Time courses of $d(KIE_B)/dt/d(KIE_X)/dt$ for each post isotope-sensitive step product complex. The intercept at zero-time for each complex provides its value of n_S as defined in eq 7 and identifies its reaction step number in the scheme shown in eq 6.

seen in panel c of Figure 4, the resulting intercepts of n_S no longer obey the predictions of eq 7; when n_S is calculated using the first species to exhibit an isotope effect (C in Figure 4), values of 1, 1.5, 2, and 2.5 are obtained instead of the integers 1, 2, 3, and 4. The discrepancy is due to an erroneous assignment of the initial step when computing n_S. The nonintegral series was obtained by assuming that the numbering system should begin with the isotopic-sensitive step (which is in fact step 2, not step 1). If the computed nonintegral values of n_S are multiplied by the step-number, 2, of the first isotopically sensitive reaction, then n_S is transformed into a series giving the correct step number for each reaction. This behavior and its mode of detection is a completely general feature of linear

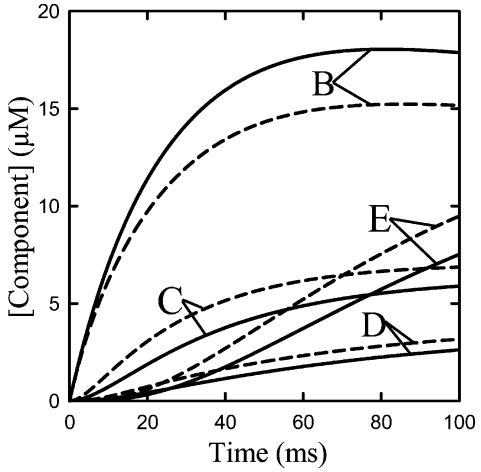

Figure 3. Concentration time dependences for the same reaction shown in Figure 1 using the same set of rate constants, but now assuming the isotope-sensitive step to be the second step in the scheme shown in eq 6.

singly substituted reactions. For example, if step 3 is the locus of the isotope effect, the series of integers required by eq 7 will result only if values obtained for n_S are multiplied by 3. The analytical proof, presented below, provides the mathematical basis for this operation. The potential for this property to detect reactions prior to the isotopically sensitive step should be evident.

Analytic Proof of the Second Rule for the General Case

The application of the numerical evaluation of the approach of TKIEs to their zero limit which we have described is very useful in several ways. First of all, it provides full component time courses which are not obtainable from the formal limiting value equations we will describe below in validating the rule and in obtaining values of the n_S function. Second, it may provide some conceptual insight into the nature of the rule from a viewpoint that is more directly related to the physical reaction as it would be observed experimentally than that of the formal mathematical treatment. Finally, it provides a facile means of exploring the applicability of the second rule to more complex reactions and varying numbers and loci of an isotopic substitution. If, in a given case, varying any rate constant or KIE value would have an effect on the $t = 0$ intercepts of n_S, then eq 5 is not applicable. However, even though we have tested eq 5 extensively using the numerical integration approach without ever encountering an exception, it remains anecdotal in nature and must be regarded as a demonstration rather than a formal proof. To fill this gap in our argument, we now provide an analytic proof of the second rule.

Proof that n_S Counts Reactions after an Isotopically Sensitive Step. *General Case.* Consider a species X_i which reacts (perhaps through many steps) to give a species X_j. The function $n_S(i, j)$ is defined as

$$n_S(i,j) = \frac{\lim_{t\to 0} \frac{dTKIE_i}{dt}}{\lim_{t\to 0} \frac{dTKIE_j}{dt}}$$

To evaluate this function, the limits of the derivatives in the

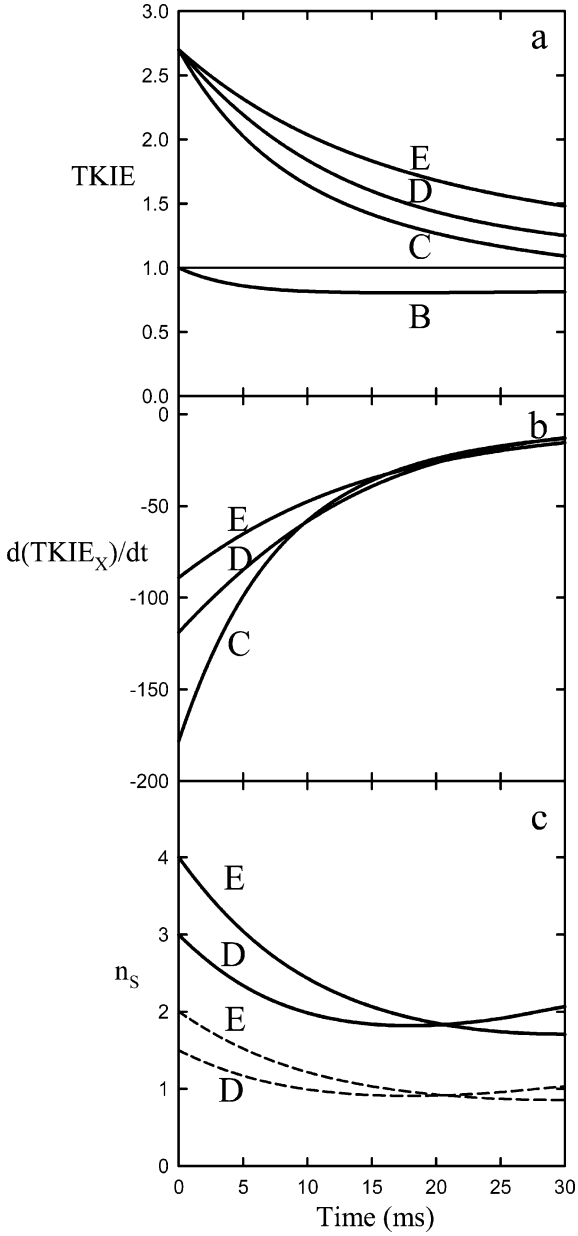

Figure 4. TKIE behavior for a reaction shown in Figure 3. The dashed lines in panel c are calculated on the basis that C is the first post-isotopic product. The solid lines are calculated based on the argument described in the text.

numerator and denominator need to be evaluated. Applying to the definition of TKIE gives

$$\lim_{t\to 0} \frac{\mathrm{dTKIE}_i}{\mathrm{d}t} = \lim_{t\to 0} \frac{\mathrm{d}}{\mathrm{d}t}\left[\left(\frac{\mathrm{d}X_i}{\mathrm{d}t}\right)_{\mathrm{H}}\left(\frac{\mathrm{d}X_i}{\mathrm{d}t}\right)_{\mathrm{D}}^{-1}\right] \tag{8}$$

Simplifying and recognizing the presence of TKIE in the result gives

$$\lim_{t\to 0} \frac{\mathrm{dTKIE}_i}{\mathrm{d}t} = \lim_{t\to 0} \frac{\left[\left(\frac{\mathrm{d}^2X_i}{\mathrm{d}t^2}\right)_{\mathrm{H}} - \mathrm{TKIE}_i\left(\frac{\mathrm{d}^2X_i}{\mathrm{d}t^2}\right)_{\mathrm{D}}\right]}{\left(\frac{\mathrm{d}X_i}{\mathrm{d}t}\right)_{\mathrm{D}}} \tag{9}$$

Evaluating this limit for any two species of interest allows n_S to be computed. The behavior of the components in this expression depends on the rate laws and the number and distribution of isotopically sensitive steps. A reversible sequence without branches and with only the first step being isotopically sensitive will be analyzed.

Conditions for a Limit that is Not 0/0. Evaluating the limit of the ratio above is approached by considering the limiting behavior of the numerator and denominator. If species far enough "downstream" are considered, the ratio has the form of 0/0 and L'Hôpital's rule will be applied, generating higher-order derivatives. Whether or not the numerator and denominator are zero depends on the rate laws and the value of the index *i*. For instance, the rate law for X_1 has a term with X_1 in it; it will extrapolate to a nonzero value at $t = 0$. In contrast, the rate law for X_5 does not have concentrations that extrapolate to nonzero values at $t = 0$. Similarly, the second derivatives in the numerator could be zero or nonzero. If they are nonzero, the numerator might still be zero because a difference is being computed.

Examining higher-order derivatives (computed from the rate laws describing the mechanism) and assuming that only the first species X_1 is present at the start of the reaction allows the following generalizations for a linear mechanism.

The *n*th-order time-derivative of the *i*th species extrapolates to a nonzero value when $n \geq i - 1$. Thus, in attempting to evaluate the limit with repetitive applications of L'Hôpital's rule, limits will go from zero to nonzero once the order of the derivative is high enough ($i - 1$). A few of the lowest-order derivatives that have nonzero limits are given in the Table 1.

Although the derivatives in the numerator are nonzero when the order is $i - 1$, the reaction mechanism imposed by the chemistry causes this difference to be zero as described below. As a result, a nonzero numerator is obtained for the *i*th species when the order of the derivatives in the difference equals the index *i*, as listed in the table.

Conditions for a Nonzero Numerator. Consider the first nonzero derivatives for the *i*th species (derivatives with order $i - 1$)

$$\begin{aligned}\text{numerator} &= \lim_{t\to 0}\left[\left(\frac{\mathrm{d}^{i-1}X_i}{\mathrm{d}t^{i-1}}\right)_{\mathrm{H}} - \mathrm{TKIE}_i\left(\frac{\mathrm{d}^{i-1}X_i}{\mathrm{d}t^{i-1}}\right)_{\mathrm{D}}\right] \\ &= [(\prod_{h=1}^{i-1} k_h^{\mathrm{H}})X_{1,\mathrm{H}}^0 - \mathrm{TKIE}_i^0(\prod_{h=1}^{i-1} k_h^{\mathrm{D}})X_{1,\mathrm{D}}^0]\end{aligned} \tag{10}$$

Assuming identical initial concentrations of the protio and deutero reactants allows the concentration to be factored from both terms. Because only the first reaction (index $h = 1$) is isotopically sensitive, all rate constants in the products will be identical except for the factor k_1, which experiences an intrinsic KIE of $\mathrm{KIE}_{1,\mathrm{int}}$. The limit of TKIE_i as $t \to 0$ is the product of all of the forward intrinsic KIEs, in this case $\mathrm{KIE}_{1,\mathrm{int}}$, which converts k_1^{D} to k_1^{H}, giving the numerator a value of zero

$$\begin{aligned}\text{numerator} &= [(\prod_{h=1}^{i-1} k_h^{\mathrm{H}})X_{1,\mathrm{H}}^0 - \mathrm{TKIE}_i(\prod_{h=1}^{i-1} k_h^{\mathrm{D}})X_{1,\mathrm{D}}^0] \\ &= (\prod_{h=2}^{i-1} k_h)[k_1^{\mathrm{H}} - \mathrm{KIE}_{1,\mathrm{int}}k_1^{\mathrm{D}}]X_1^0 \\ &= (\prod_{h=2}^{i-1} k_h)[k_1^{\mathrm{H}} - k_1^{\mathrm{H}}]X_1^0 = 0\end{aligned} \tag{11}$$

TABLE 1. Derivatives that Have Nonzero Values as $t \rightarrow 0$

species	$\lim_{t\to 0}\left(\frac{d^n X_i}{d^n t}\right) \neq 0$	$\lim_{t\to 0}\left[\left(\frac{d^n X_i}{d^n t}\right)_H - \mathrm{TKIE}\left(\frac{d^n X_i}{d^n t}\right)_D\right] \neq 0$
X_1	$\lim_{t\to 0}\left(\frac{dX_1}{dt}\right) = -k_1 X_1^0$	$\lim_{t\to 0}\left(\frac{d^2 X_1}{dt^2}\right) = k_1(k_1 + k_{-1})X_1^0$
X_2	$\lim_{t\to 0}\left(\frac{dX_2}{dt}\right) = k_1 X_1^0$	$\lim_{t\to 0}\left(\frac{d^2 X_2}{dt^2}\right) = -k_1(k_1 + k_{-1} + k_2)X_1^0$
X_3	$\lim_{t\to 0}\left(\frac{d^2 X_3}{dt^2}\right) = k_1 k_2 X_1^0$	$\lim_{t\to 0}\left(\frac{d^3 X_3}{dt^3}\right) = -k_1 k_2(k_1 + k_{-1} + k_2 + k_{-2} + k_3)X_1^0$
X_4	$\lim_{t\to 0}\left(\frac{d^2 X_4}{dt^3}\right) = k_1 k_2 k_3 X_1^0$	$\lim_{t\to 0}\left(\frac{d^4 X_4}{dt^4}\right) = -k_1 k_2 k_3(k_1 + k_{-1} + k_2 + k_{-2} + k_3 + k_{-3} + k_4)X_1^0$
X_i	$\lim_{t\to 0}\left(\frac{d^{i-1} X_i}{dt^{i-1}}\right) = (\prod_{h=1}^{i-1} k_h)X_1^0$	$\lim_{t\to 0}\left(\frac{d^i X_i}{dt^i}\right) = -(\prod_{h=1}^{i-1} k_h)(\sum_{h=1}^{i} k_h + \sum_{h=1}^{i-1} k_{-h})X_1^0$

Similar considerations applied to the next-higher order derivative give a nonzero result

$$\text{numerator} = \lim_{t\to 0}\left[\left(\frac{d^i X_i}{dt^i}\right)_H - \mathrm{TKIE}_i\left(\frac{d^i X_i}{dt^i}\right)_D\right] = -(\prod_{h=1}^{i-1} k_h)[(\sum_{h=1}^{i} k_h + \sum_{h=1}^{i-1} k_{-h})_H - (\sum_{h=1}^{i} k_h + \sum_{h=1}^{i-1} k_{-h})_D]X_1^0 \quad (12)$$

The rate constants in the sums are the same for both the protio and deutero reactions, except for those of the first step, where there is a KIE. Therefore, subtraction cancels all but the first rate constants, simplifying the expression to

$$\text{numerator} = -(\prod_{h=1}^{i-1} k_h)[k_1^H + k_{-1}^H - k_1^D - k_{-1}^D] \neq 0 \quad (13)$$

Calculating the Limit of the Derivatives of TKIE. The results listed in the Table 1 may be substituted into eq 9 to calculate limits of derivatives of TKIE_i. For $i = 1$

$$\lim_{t\to 0}\frac{d\mathrm{TKIE}_1}{dt} = \frac{k_1^H(k_1^H + k_{-1}^H - k_1^D - k_{-1}^D)X_1^0}{-k_1^D X_1^0} = -\mathrm{KIE}_{1,\mathrm{int}}(k_1^H + k_{-1}^H - k_1^D - k_{-1}^D) \quad (14)$$

For $i = 2$

$$\lim_{t\to 0}\frac{d\mathrm{TKIE}_2}{dt} = \frac{k_1^H(k_1^H + k_{-1}^H + k_2 - k_1^D - k_{-1}^D - k_2)X_1^0}{-k_1^D X_1^0} = -\mathrm{KIE}_{1,\mathrm{int}}(k_1^H + k_{-1}^H - k_1^D - k_{-1}^D) \quad (15)$$

For $i = 3$, L'Hôpital's rule will be needed. Its application to eq 9 gives

$$\lim_{t\to 0}\frac{d\mathrm{TKIE}_3}{dt} = \lim_{t\to 0}\frac{\left[\left(\frac{d^3 X_3}{dt^3}\right)_H - \mathrm{TKIE}_3\left(\frac{d^3 X_3}{dt^3}\right)_D\right]}{\left(\frac{d^2 X_3}{dt^2}\right)_D} - \lim_{t\to 0}\frac{d\mathrm{TKIE}_3}{dt} \quad (16)$$

This intermediate result is recursive, with the quantity we wish to find (left side) produced as part of the effort to find it (second term, right side). The first term on the right side, when analyzed by the criteria described above, gives a limit consisting of the ratio of nonzero numbers. Thus

$$\lim_{t\to 0}\frac{d\mathrm{TKIE}_3}{dt} = -\frac{1}{2}\mathrm{KIE}_{1,\mathrm{int}}(k_1^H + k_{-1}^H - k_1^D - k_{-1}^D) \quad (17)$$

When $i = 4$, two applications of L'Hôpital's rule to eq 9 are required, giving

$$3\lim_{t\to 0}\frac{d\mathrm{TKIE}_4}{dt} = \lim_{t\to 0}\frac{\left[\left(\frac{d^4 X_4}{dt^4}\right)_H - \mathrm{TKIE}_4\left(\frac{d^4 X_4}{dt^4}\right)_D\right]}{\left(\frac{d^3 X_4}{dt^3}\right)_D} \quad (18)$$

The right side of the equation is not 0/0, so that

$$\lim_{t\to 0}\frac{d\mathrm{TKIE}_4}{dt} = -\frac{1}{3}\mathrm{KIE}_{1,\mathrm{int}}(k_1^H + k_{-1}^H - k_1^D - k_{-1}^D) \quad (19)$$

A pattern can be seen. Each application of L'Hôpital's rule adds another "recursive" term which acts, in effect, as a counter. The counting stops when the limit can be evaluated. In the general case, for species i ($i > 2$), $i - 2$ applications of L'Hôpital's rule are needed, giving the general result

$$\lim_{t\to 0}\frac{d\mathrm{TKIE}_i}{dt} = -\left(\frac{1}{i-1}\right)\mathrm{KIE}_{1,\mathrm{int}}(k_1^H + k_{-1}^H - k_1^D - k_{-1}^D) \quad (20)$$

Calculating n_S. Having computed individual derivatives of TKIE, n_S may be calculated from their ratios. For example

$$n_S(2,3) = \lim_{t\to 0}\left[\frac{\dfrac{d\mathrm{TKIE}_2}{dt}}{\dfrac{d\mathrm{TKIE}_3}{dt}}\right] = \frac{-\mathrm{KIE}_{1,\mathrm{int}}(k_1^H + k_{-1}^H - k_1^D - k_{-1}^D)}{-\frac{1}{2}\mathrm{KIE}_{1,\mathrm{int}}(k_1^H + k_{-1}^H - k_1^D - k_{-1}^D)} = 2 \quad (21)$$

$$n_S(2,4) = \lim_{t\to 0}\left[\frac{\dfrac{d\mathrm{TKIE}_2}{dt}}{\dfrac{d\mathrm{TKIE}_4}{dt}}\right] = \frac{-\mathrm{KIE}_{1,\mathrm{int}}(k_1^H + k_{-1}^H - k_1^D - k_{-1}^D)}{-\frac{1}{3}\mathrm{KIE}_{1,\mathrm{int}}(k_1^H + k_{-1}^H - k_1^D - k_{-1}^D)} = 3 \quad (22)$$

In general, when the first step is isotopically sensitive, for i and $j \geq 2$

$$n_S(i,j) = \lim_{t\to 0}\left[\frac{\dfrac{d\mathrm{TKIE}_i}{dt}}{\dfrac{d\mathrm{TKIE}_j}{dt}}\right] = \frac{-\frac{1}{i-1}\mathrm{KIE}_{1,\mathrm{int}}(k_1^H + k_{-1}^H - k_1^D - k_{-1}^D)}{-\frac{1}{j-1}\mathrm{KIE}_{1,\mathrm{int}}(k_1^H + k_{-1}^H - k_1^D - k_{-1}^D)} = \frac{j-1}{i-1} \quad (23)$$

Therefore, when $i = 2$ (or 1), reactions from the isotopically sensitive first step are counted by n_S, in agreement with eq 7. Equation 23 provides an analytic proof of the behavior of n_S in cases where the isotope sensitive step is preceded by one or more earlier steps, producing a series of nonintegral values from eq 7. This formal proof provides a basis for the procedure deduced from the numerical integration examples described in an earlier section; only multiplication of the computed nonintegral values of n_S by the actual step number of the reaction sequence provides the unique series of integers expressed by eq 7.

The second rule has been explicitly proven above for linear reaction sequences. It can also be shown that when the reaction sequence has branch-points with alternative pathways that lead to product, the mathematical approach shown above is still valid and n_S will still represent the number of reaction steps leading to the species in question. In the case where there are an unequal number of steps in the alternative pathways, n_S will give the number of steps through the shortest pathway.

Discussion

It is clear that in the case of single-step isotope-substituted reaction sequences, equations of the form of (5) or (7) must apply and that the ratios of the derivatives of the limits of the TKIEs of successive reaction products must be related by a series of integral numbers. If application of either of these equations to experimental data produces a set of nonintegral values, the reaction sequence must be expanded with additional steps at the appropriate places until eq 7 is satisfied. If the observed values of n_S appear to be too high to satisfy eq 7 within experimental error, extra post-isotopic steps must be added to the reaction sequence; if they appear to be too low, then one or more pre-isotopic steps must be added until a set of integral values for n_S is obtained.

It is also clear that the mathematics of consecutive reversible single-step substituted reaction sequences counts all steps including those which provide no observable signal as well as those with such high commitment factors that their resulting complexes do not accumulate to any significant degree. This being so, it follows that if one can evaluate the TKIE function for a late step in a single-step isotopically substituted reaction as well as that for the initial isotope-sensitive step, the total number of intervening steps in the included portion of the reaction sequence can be determined unambiguously.

Both the derivation of the second rule provided here and the behavior of chemical reactions in the transient state predicted by it involve counter-intuitive aspects at several levels. The most obvious of such questions is that of how the values of TKIE_b and TKIE_c as defined in eq 4 and employed in eq 5 lead to finite limiting values at $t \to 0$ when the concentrations of all product species must equal 0 at $t = 0$. The answer here involves the precise definition of the limit of a function. Recalling that the limit of a function does not necessarily equal the actual value of the function at the limit, and acknowledging that at $t = 0$, [B] and [C] = 0, we take note of the fact that, as shown clearly in Figure 2, panels a and b, the concentrations and the functions derived from the time dependencies of those concentrations all approach the initial value of 0 at different rates and thus produce the nonzero limiting functions shown in Figure 2. The apparent anomaly lies in the fact that the product concentrations equal zero only at $t = 0$ itself. Such values, then, represent mathematical singularities. Since singularities, by definition, cannot exist for any finite period of time, they are never actually expressed in the behavior of physical systems.

A second concern, expressed by colleagues who have explored our formulation, is the occurrence of discontinuities observed at longer times in plots of $d(\mathrm{TKIE}_X)/dt$. Here it should be noted that the values of this and indeed of all TKIE functions used here lead to simple interpretations only when extrapolated to $t = 0$. The behavior of these entities at finite times are highly dependent on the values of the rate constants involved. For this reason, the curves in Figure 2 have been limited to the relatively early time span required for extrapolation to the $t = 0$ ordinate. It can be seen from Figure 1 in which a longer time span is portrayed that $d[B_H]/dt$ passes through a value of 0 at about 75 ms, while that of $d[B_D]/dt$ is still rising. It is clear that at some later time, as the slower isotope-substituted reaction catches up with the normal reaction, the value of $d[B_D]/dt$ will itself equal 0 and will thus produce indeterminate TKIE_B value and a resulting discontinuity. This (and other odd features appearing at later times) are simply due to the fact that, as time proceeds, the isotopic and normal reactions become increasingly out of phase and are no longer comparable.

Experimental Scope of the Rule. The practicality of application of the Second rule to a given reaction depends on both the kinetic properties of the reaction and on the availability of analytic tools capable of distinguishing individual intermediate species. In general it is potentially applicable to chemical reactions that may be sufficiently slow to be measurable by conventional continuous kinetic measurements. For very fast reactions such as enzymatic catalysis, the use of the stopped flow absorbance and fluorescence techniques are indicated. Where intermediate steps involve the release of a specific chemical entity, the quench flow technique, which does not

necessarily require unique spectroscopic identities, offers a somewhat broader applicability. This being said, the maximum attainable scope of the approach is defined by the mathematics of the theory itself, and it is appropriate to discuss those aspects here.

Since the rule is independent of the mass of the isotope, tritium and other isotopes can be employed as well as deuterium by use of the quench flow technique in some cases.

The ratio of the sensitivity of n_S to its uncertainty varies inversely as the step number increases. For the first few steps, the values of n_S for successive steps differ by a very substantial amount but the extrapolation to zero time of these rapidly decreasing functions is subject to a corresponding large error. Conversely, the slower rate of decrease of the later steps permits a more accurate determination of their individual n_s values but the degree of accuracy required to distinguish between them becomes more severe.

Although extrapolation of the multiexponential component time courses to zero time constitutes a major source of error in the application of the second rule, application of the first rule to the total data set of a reaction may reduce the degree of uncertainty in this extrapolation, particularly in the case of rapid early steps whose origin is lost in the instrumental dead-time as well as in cases where the accumulation of a given intermediate provides a very low signal-to-noise ratio. If it can be shown that the $TKIE_X$ time courses for at least two reactive species converge at a single point on the zero-time axis, then according to the first rule that point may be taken as $TKIE_{int}$ and assigned as the zero-time point for the TKIE curves for all other species.

Even where it is not possible to obtain accurate values of n_S for every step in a given reaction, useful information may be obtained using this approach. For example, determination of n_S for only one very early step and one much later step are most easily obtained and may clearly define the number of otherwise unknowable transition states between them. Such a pair of n_S values may also be sufficient to reveal the number of steps preceding the isotope sensitive step. A second special case where this approach may be useful is in a reaction where two products appear to arise at nearly the same point in time. The approach described here may constitute the method of choice for resolving the question of whether the two steps are truly concerted (involving only a single transition state) or, if not, what is the order of their formation.

The second rule of TKIEs developed here should be applicable to chemical reactions in general and may provide a unique new approach to the investigation of enzyme mechanisms. It is clear that the seemingly arcane relationships expressed in the first three rules of TKIEs must be implicit in the sets of simple differential equations that suffice to completely define the kinetic behavior of even the most complicated reaction schemes. This evolution of complexity from simple origins (exemplified by eqs 2 and 3) reflects the extensive coupling of exponential and preexponential terms of the equation for any given reaction species and the further extension of that coupling forced by the mechanism-dictated relationships between the terms of the various species involved. It is a commonplace event that the transient-state product time course of a five to eight step reaction can be fitted to a high degree of precision by a two or three term exponential equation. Differentiation, as required by expressions needed for TKIEs, tends to reveal evidence of the underlying complexity of the system. Further exploration of the nature of these mathematical relationships, particularly those which may find expression in physically measurable terms, may therefore be warranted.

Conclusion

We summarize here the formal expressions of the three rules of TKIEs which we have thus far established stated in a consistent nomenclature.

Consistent nomenclature defining the transient-state kinetic isotope effect on a given component, X

$$TKIE_X = \frac{d[X]_H/dt}{d[X]_D/dt}$$

The First Rule of Transient-State TKIEs. This rule states that for any postisotope substituted step in a single-step substituted linear reaction sequence[5]

$$\lim_{t\to 0} TKIE_X = KIE_{int}$$

For any postisotope substituted step in such a reaction

$$\lim_{t\to 0} TKIE_X = 1$$

In either case,

$$\lim_{t\to 0} dTKIE_x/dt$$

is negative for normal isotop effects but positive for inverse effects.

The Second Rule of Transient-State TKIEs. This rule states that for such reactions

$$n_S = \frac{\lim_{t\to 0} d(TKIE_1)/dt}{\lim_{t\to 0} d(TKIE_n)/dt}$$

where n_S is the number of the step in the reaction sequence whose product formation follows k_n.

The Third Rule of Transient-State TKIEs. This rule states that in a multistep isotope-sensitive linear reaction[6]

$$KIE_{n,int} = \frac{\prod_{i=1}^{n} \lim_{t\to 0} TKIE_i}{\prod_{i=1}^{n-1} \lim_{t\to 0} TKIE_i}$$

Simply stated, in a multistep substituted linear reaction sequence, the TKIE observed for a given species at $t = 0$ is the algebraic product of all of the KIE_{int}s for all steps preceding the formation of the product of the nth step.

It should be noted that the TKIEs for the reverse rate constants are absent from the expressions for all three of these rules.

We see no reason to presume that this list of rules of TKIEs is complete.

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(9) Although solutions to a set of differential equations obtained by numerical integration are by their nature anecdotal and do not provide a formal proof of a Rule, we have tested our solutions by varying the values of all rate constants and of both $KIE_{int.}$s over a wide range of values and have found no variation in the "TKIE" or "n_S" parameters we report here.