Minimal Kinetic Mechanism and General Equation for Deuterium Isotope Effects on Enzymic Reactions: Uncertainty in Detecting a Rate-Limiting Step†

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ABSTRACT: A general equation is proposed for representing the kinetic functions which govern the expression of an isotope effect on the maximal velocity of an enzyme-catalyzed reaction. The origin and form of the functions are illustrated by examining a series of enzymatic mechanisms of progressively increasing complexity. The number of functions similarly increase, reaching a limit of three, with differing thermodynamic and kinetic properties. Further expansion of mechanisms causes an orderly and predictable algebraic expansion of each function, making it possible to write out, by simple inspection, the kinetic equation describing an isotope effect expressed on the maximal velocity for any enzymatic mechanism in which the isotope perturbs a single reactive step. The functions are interactive and allow for the possibility that an isotope effect on V_{max} may be independent of the rate of a second, isotopically insensitive step, be it infinitely fast or slow. This allowance leads to an uncertainty of the ability of an isotope effect to detect a rate-limiting step, and the unequal distribution of kinetic and thermodynamic properties among three functions leads to an inadequacy of the singular concept of a rate-limiting step to serve as a basis for interpreting isotope effects on enzyme-catalyzed reactions. A minimal mechanism for consideration of isotope effects is proposed in order to embrace all three functions. It consists of a single catalytic step which is isotopically sensitive and reversible, two reversible precatalytic steps, and one reversible postcatalytic step, plus steps for binding and release of substrates and products.

The purpose of measuring isotope effects on enzyme-catalyzed reactions has generally been to determine whether or not the isotopically sensitive step is rate determining (Walsh, 1979). That competitive measurements of isotopic discrimination cannot give this information was noted by Abeles et al. (1960), elaborated by Simon & Palm (1966), and reiterated by Northrop (1975). Because isotopic discrimination measures effects only on V/K, it is therefore sensitive only to that portion of a reaction mechanism up to and including the first irreversible step. This "caveat of isotopic discrimination" applies to all studies employing competitive measurements of trace-labeled substrates, including tritium and heavy atom isotope effects (O'Leary, 1978). Noncompetitive measurements employ near-total labeling of substrates and, being responsive to all steps in an enzymatic reaction, provide the only means for determining isotope effects on maximal velocities. The latter, in turn, are the only isotopic data which are presently acceptable for interpretations concerning rate-determining steps and, in practice, are limited to isotope effects of deuterium.

An attempt to understand the kinetic functions governing the expression of isotope effects led Northrop (1975) to formulate general equations based on newly defined kinetic parameters representing three components of any enzymatic mechanism: a substrate release component, a catalytic component, and a product release component. These proved to be unwieldly and impractical, but the equations did reveal a range of uncertainty in the interpretations of V isotope effects on an absolute scale. To narrow the range of uncertainty, it was proposed that quantitative measures of the percent of partial rate limitation might be obtained through comparisons to intrinsic isotope effects. A second form of uncertainty was revealed in an alternative formulation of isotopic rate equations (Northrop, 1977) when it was found that a group of rate constants could be factored out which was common to isotopic equations for V/K effects. Termed the "reverse commitment to catalysis", this kinetic function depends upon the reversibility of catalysis and can reduce the expression of an isotope effect below that expected by a second partially rate-limiting step acting alone.

Now, as a result of a search for the algebraic series governing the remaining rate constants, a third and final kinetic function has been identified and factored out. Unlike the earlier uncertainties, neither limits nor direction can be assigned to the effects of this function on the expression of a kinetic isotope effect.

Theory

The origin and form of the kinetic functions governing the expression of deuterium isotope effects on the maximal velocity can be illustrated by examining a series of enzymatic mechanisms of progressively increasing complexity. For each mechanism, a normal rate equation is divided by an analogous equation containing deuterium-sensitive rate constants. Ratios of individual rate constants of similar form are arranged in groups (by trial and error) to find the algebraic series which expand in parallel to the expanding mechanism. The nomenclature used is that proposed by Northrop (1977) in which the conventional expression for an isotope effect, k_H/k_D, is written ${}^Dk$; similarly, the deuterium isotope effect on the maximal velocity is written ${}^DV$, an equilibrium effect, ${}^DK_{eq}$, and so forth.

For a simple three-step kinetic mechanism

$$E_1 + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_4 \xrightarrow{k_7^*} E_5 \xrightarrow{k_9} E_1 + P \qquad (1)$$

the expression of a deuterium isotope effect on V is governed by a single, simple function consisting of a ratio of rate constants called the forward "ratio of catalysis", R_f

$${}^DV = \frac{{}^Dk_7 + \frac{k_7}{k_9}}{1 + \frac{k_7}{k_9}} = \frac{{}^Dk + R_f}{1 + R_f} \qquad (2)$$

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If catalysis is reversible, a second function appears in the expression, the reverse "commitment to catalysis", C_r

$$E_1 + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_4 \underset{k_8^*}{\overset{k_7^*}{\rightleftharpoons}} E_5 \xrightarrow{k_9} E_1 + P \tag{3}$$

$$^{D}V = \frac{^{D}k_7 + \frac{k_7}{k_9} + \frac{k_8}{k_9}}{1 + \frac{k_7}{k_9} + \frac{k_8}{k_9}} = \frac{^{D}k + R_f + C_r}{1 + R_f + C_r} \tag{4}$$

When an equilibrium isotope effect is present, it is found multiplied by the reverse commitment to catalysis only and then only in the numerators of these and subsequent expressions (see eq 18 and 22 below). If additional but irreversible steps are added to the mechanism, no new functions appear; the commitment factor retains its earlier definition, but a more general form for the ratio of catalysis is revealed as being a sum of individual ratios of catalysis to each additional step, regardless of whether these are pre- or postcatalytic steps.

$$E_1 + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_2 \xrightarrow{k_3} E_3 \xrightarrow{k_5} E_4 \underset{k_8^*}{\overset{k_7^*}{\rightleftharpoons}} E_5 \xrightarrow{k_9} E_6 \xrightarrow{k_{11}} E_7 \xrightarrow{k_{13}} E_1 + P \tag{5}$$

$$^{D}V = \frac{^{D}k_7 + \left(\frac{k_7}{k_3} + \frac{k_7}{k_5} + \frac{k_7}{k_9} + \frac{k_7}{k_{11}} + \frac{k_7}{k_{13}}\right) + \frac{k_8}{k_9}}{1 + \left(\frac{k_7}{k_3} + \frac{k_7}{k_5} + \frac{k_7}{k_7} + \frac{k_7}{k_{11}} + \frac{k_7}{k_{13}}\right) + \frac{k_8}{k_9}} = \frac{^{D}k + R_f + C_r}{1 + R_f + C_r} \tag{6}$$

Making the postcatalytic steps reversible adds no new functions but does increase the complexity of both the reverse commitment to catalysis and the ratio of catalysis. The first obeys a pattern of sums of individual commitment factors for each form of enzyme, while the complexities of the second can be resolved into the summation form of eq 6 if expressed in terms of the net rate constants (indicated by primes) as defined by Cleland (1975)

$$E_1 + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_2 \xrightarrow{k_3} E_3 \xrightarrow{k_5} E_4 \underset{k_8^*}{\overset{k_7^*}{\rightleftharpoons}} E_5 \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E_6 \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E_7 \xrightarrow{k_{13}} E_1 + P \tag{7}$$

The general (bottom) portion of eq 6 applies to this mechanism where

$$C_r = \frac{k_8}{k_9} + \frac{k_8}{k_9}\frac{k_{10}}{k_{11}} + \frac{k_8}{k_9}\frac{k_{10}}{k_{11}}\frac{k_{12}}{k_{13}} \tag{8}$$

$$R_f = \frac{k_7}{k_3} + \frac{k_7}{k_5} + \frac{k_7}{k_9'} + \frac{k_7}{k_{11}'} + \frac{k_7}{k_{13}'} \tag{9}$$

with net rate constants

$$k_{13}' = k_{13} \tag{10}$$

$$k_{11}' = \frac{k_{11}k_{13}'}{k_{12} + k_{13}'} \tag{11}$$

$$k_9' = \frac{k_9 k_{11}'}{k_{10} + k_{11}'} \tag{12}$$

Making a precatalytic step reversible increases the complexity of what was the ratio of catalysis. In order to group rate constants in a manner similar to eq 9, something is left over. Furthermore, this "something" is unusual and thus represents a new function controlling the expression of isotope effects.

The previous functions all consist of separate ratios of rate constants from *different* steps of a mechanism, but the ratio(s) now "left over" consist of rate constants from the *same* step

$$E_1 + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_2 \xrightarrow{k_3} E_3 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_4 \underset{k_8^*}{\overset{k_7^*}{\rightleftharpoons}} E_5 \xrightarrow{k_9} E_6 \xrightarrow{k_{11}} E_7 \xrightarrow{k_{13}} E_1 + P \tag{13}$$

$$(R_f)_{\text{apparent}} = \left(\frac{k_7}{k_3} + \frac{k_7}{k_5} + \frac{k_7}{k_9} + \frac{k_7}{k_{11}} + \frac{k_7}{k_{13}}\right)\left(\frac{k_5}{k_5 + k_6}\right) \tag{14}$$

Making the remaining precatalytic step reversible increases the complexity of the forward commitment to catalysis in a manner similar to the effect of postcatalytic reversibility on the reverse commitment factor. Also, the ratio of catalysis expands in a similar manner with both pre- or postcatalytic reversibility, provided that Cleland's definition of net rate constants treats the step immediately preceding the catalytic step as if it were irreversible (indicated by double primes, i.e., $k_5'' = k_5$ and $k_3'' = k_3k_5/(k_4 + k_5)$). Finally, the general form of the "something left over" emerges as a pattern of sums of reciprocals of individual equilibrium constants for each step of the mechanism separating substrate binding from the catalytic step. For that reason, it is designated E_f and its function is identified as the forward "equilibration preceding catalysis"

$$E_1 + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_3 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_4 \underset{k_8^*}{\overset{k_7^*}{\rightleftharpoons}} E_5 \xrightarrow{k_9} E_6 \xrightarrow{k_{11}} E_7 \xrightarrow{k_{13}} E_1 + P \tag{15}$$

$$^{D}V = \frac{^{D}k_7 + \frac{\frac{k_7}{k_3''} + \frac{k_7}{k_5''} + \frac{k_7}{k_9} + \frac{k_7}{k_{11}} + \frac{k_7}{k_{13}}}{1 + \frac{k_6}{k_5} + \frac{k_6}{k_5}\frac{k_4}{k_3}} + \frac{k_8}{k_9}}{1 + \frac{\frac{k_7}{k_3''} + \frac{k_7}{k_5''} + \frac{k_7}{k_9} + \frac{k_7}{k_{11}} + \frac{k_7}{k_{13}}}{1 + \frac{k_6}{k_5} + \frac{k_6}{k_5}\frac{k_4}{k_3}} + \frac{k_8}{k_9}} = \frac{^{D}k + R_f/E_f + C_r}{1 + R_f/E_f + C_r} \tag{16}$$

The addition of more steps to the linear reaction sequence causes a simple expansion of the kinetic functions C_f, C_r, R_f, and E_f in the manner indicated by the examples given. No new ratios or functions appear. Therefore the general case can be defined: for a mechanism having an unknown number of steps preceding or following an isotopically sensitive ith step

$$\ldots E_{i-1} \underset{k_{-(i-1)}}{\overset{k_{i-1}}{\rightleftharpoons}} E_i \underset{k_{-i}^*}{\overset{k_i^*}{\rightleftharpoons}} E_{i+1} \underset{k_{-(i+1)}}{\overset{k_{i+1}}{\rightleftharpoons}} E_{i+2} \ldots \tag{17}$$

the general equation for the isotope effect expressed on V is

$$^{D}V = \frac{^{D}k_i + R_f/E_f + C_r{}^{D}K_{eq}}{1 + R_f/E_f + C_r} \tag{18}$$

where

$$R_f = \ldots + \frac{k_i}{k_{i-2}''} + \frac{k_i}{k_{i-1}''} + \frac{k_i}{k_{i+1}'} + \frac{k_i}{k_{i+2}'} + \ldots \tag{19}$$

$$E_f = 1 + \frac{1}{(K_{eq})_{i-1}}\left[1 + \frac{1}{(K_{eq})_{i-2}}\left(1 + \frac{1}{(K_{eq})_{i-3}} \ldots\right)\right] \tag{20}$$

$$C_r = \frac{k_{-i}}{k_{i+1}}\left[1 + \frac{k_{-(i+1)}}{k_{i+2}}\left(1 + \frac{k_{-(i+2)}}{k_{i+3}} \ldots\right)\right] \quad (21)$$

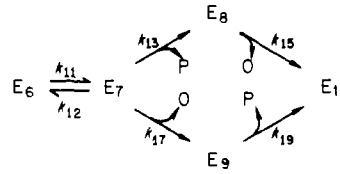
The general equation is analogous to the expression of isotope effects on V/K described previously (Northrop, 1977)

$$^{D}V/K = \frac{^{D}k_i + C_f + C_r{}^{D}K_{eq}}{1 + C_f + C_r} \quad (22)$$

where the forward commitment to catalysis, C_f, is determined by

$$C_f = \frac{k_i}{k_{-(i-1)}}\left[1 + \frac{k_{i-1}}{k_{-(i-2)}}\left(1 + \frac{k_{i-2}}{k_{-(i-3)}} \ldots\right)\right] \quad (23)$$

Branch points arising from random addition of substrates and release of products are readily accommodated within these general forms by replacing the rate constant for the release of a reactant by the appropriate net rate constants. For example, consider the following random segment as a variant of the mechanism of eq 7



(24)

The breakdown of E_7 is $k_{13} + k_{17}$, which is substituted for k_{13} in eq 8 to obtain the appropriate commitment to catalysis. The net rate constants replacing those defined in eq 10 and 11 are

$$k_{13,17}' = \frac{k_{13} + k_{17}}{1 + \frac{k_{13}}{k_{15}} + \frac{k_{17}}{k_{19}}} \quad (25)$$

$$k_{11}' = \frac{k_{11}(k_{13} + k_{17})}{k_{12} + k_{13} + k_{17}} \quad (26)$$

These net rate constants substitute for k_{13}' and k_{11}', respectively, in eq 9 to obtain the appropriate ratio of catalysis. Branching pathways within the catalytic sequence can similarly be accommodated by net rate constant substitutions if the branching excludes the isotopically sensitive step; if inclusive, then additional complexities arise from having two isotopically sensitive steps that are not covered by the present equations. Consequently, this system makes it possible to write out, by simple inspection, the kinetic equation describing an isotope effect expressed on the maximal velocity for any enzymatic mechanism in which the isotope perturbs a single reactive step.

Results of Simulations

The validity of the general equation as an accurate representation of isotope effects in more complex mechanisms is difficult to prove directly. An inductive method was chosen whereby a variety of isotope effects on V and V/K were computed from assumed rate constants by using eq 23–28 and compared to values obtained from accepted steady-state rate equations; that is, V_H and V_D were calculated separately, and isotope effects were obtained from their ratios. Initially, all rate constants were assigned a value of 1, and then each rate constant was systematically varied from 10-fold less to 10-fold greater than unity by an iterative computer operation. Mechanisms were extended to include four pre- and four postcatalytic, reversible, steps. An exact agreement was obtained in all cases.

This exercise produced an unexpected result: increasing a rate constant for a step preceding catalysis did not always have the same effect on ^{D}V as a similar change following catalysis. Table I illustrates this phenomenon within the mechanism

$$E_1 + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_3 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_4 \underset{k_8^*}{\overset{k_7^*}{\rightleftharpoons}} E_5 \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E_6 \xrightarrow{k_{11}} E_1 + P \quad (27)$$

In case 1, the deuterium isotope effect on the maximal velocity decreases (as expected) as the relative rate of a postcatalytic step, governed by k_9, decreases and becomes rate limiting. In case 2, a similar decrease in ^{D}V follows a decrease in the relative rate of a precatalytic step, governed by k_3. However, in case 3, decreasing the rate of this same precatalytic step results in an *increase* in ^{D}V. More importantly, in case 4, changes in the rate of this forward step have no effect on ^{D}V.

This exercise also provided an opportunity to consider extremes of expression and suppression of isotope effects on ^{D}V, namely, the maximal and minimal possible values for ^{D}V when the isotopically sensitive step is represented by either the largest or smallest rate constant by a factor of 2. Two orders of magnitude of variation were again arbitrarily chosen as the total range for relative values of rate constants. Case 5 in Table I shows ^{D}V ranging from an undetectable level to 6.82 or 97% of the full effect when catalysis is "slow". Similarly, in case 6, ^{D}V varies again from an undetectable level to a respectable 2.96 or 33% of the full effect when catalysis is "fast". Surprisingly, maximizing all forward steps other than catalysis (and similarly minimizing reverse steps) does not produce the maximal expression of the isotope effect but may be close, with $^{D}V = 6.66$ in case 5b, but only 1.67 in case 6b.

Discussion

Components of General Equations. Understanding kinetic isotope effects on enzyme-catalyzed reactions has been difficult due to a lack of clear concepts of what it is that governs the expression of the effect on measurable kinetic parameters. The concept of a rate-limiting step originates in the study of chemical reactions and treats the expression of an isotope effect as if it were governed by a single kinetic function: a large isotope effect means the step is rate limiting; a small effect means it is not. For an enzymatic reaction consisting of a series of irreversible steps, this would appear to be a sufficient treatment. For example, setting k_8 equal to 0 in eq 5 and 6 does result in a single function governing the expression of ^{D}k in ^{D}V. This function, the ratio of catalysis, is the easiest to understand because it is most clearly a kinetic function. Being a sum of separate ratios, it shows that one alternate slow step can abolish the expression of the isotope effect by causing one of the separate ratios to become large. On the other hand, a moderate rate constant for the isotopically sensitive step causes all the ratios to become significant and thus collectively abolish the expression of the isotope effect. The two are not the same but will appear the same within the steady-state kinetic measurements.

Allowing catalysis to be reversible brings in the second function, the reverse commitment to catalysis. It is more difficult to understand, because it is as much a thermodynamic as a kinetic function. If the postcatalytic steps favor a return to the isotopically sensitive step, this function will abolish the expression of the isotope effect by allowing the sensitive step to approach equilibrium, and what ultimately will be expressed is the equilibrium isotope effect, having a value near unity (Cleland, 1980). Because V/K isotope effects are governed only by commitment factors (see eq 22), illustrations of the thermodynamic rather than kinetic function of these factors

Table I: Simulation of Deuterium Isotope Effects in a Minimal Mechanism Using the General Equation[a]

	k_3	k_4	k_5	k_6	k_7*	k_8*	k_9	k_{10}	k_{11}	^DV
case 1	1	1	1	1	1	1	10	1	1	3.31
	1	1	1	1	1	1	1	1	1	2.20
	1	1	1	1	1	1	0.1	1	1	1.21
case 2	10	0.1	1	1	1	1	1	1	1	2.19
	1	0.1	1	1	1	1	1	1	1	2.11
	0.1	0.1	1	1	1	1	1	1	1	1.75
case 3	10	10	1	1	1	1	1	1	1	2.28
	1	10	1	1	1	1	1	1	1	2.41
	0.1	10	1	1	1	1	1	1	1	2.46
case 4	10	1	1	1	1	1	1	1	1	2.20
	1	1	1	1	1	1	1	1	1	2.20
	0.1	1	1	1	1	1	1	1	1	2.20
case 5										
a	10	0.2	10	0.2	0.1	10	0.2	10	0.2	1.00
	(0.097)		(0.002)		(<0.001)		(0.004)		(0.200)	
b	10	0.2	10	0.2	0.1	0.2	10	0.2	10	6.66
	(9.427)		(3.289)		(0.098)		(9.804)		(10.000)	
c	0.2	10	0.2	10	0.1	0.2	10	0.2	10	6.82
	(<0.001)		(0.002)		(0.098)		(9.804)		(10.000)	
case 6										
a	5	0.1	5	0.1	10	5	0.1	5	0.1	1.00
	(3.268)		(0.189)		(0.004)		(0.002)		(0.100)	
b	5	0.1	5	0.1	10	0.1	5	0.1	5	1.67
	(4.901)		(4.950)		(9.800)		(4.902)		(5.000)	
c	0.1	5	0.1	5	10	0.1	5	0.1	5	2.96
	(0.001)		(0.066)		(9.800)		(4.902)		(5.000)	

[a] Simulations assumed a deuterium isotope effect of $^Dk_7 = {}^Dk_8 = 7$ of the mechanism of eq 27. Because all rate constants enter into the general equation as ratios, units cancel out, leaving only relative values of rate constants as significant. Net rate constants (k') are given in parentheses.

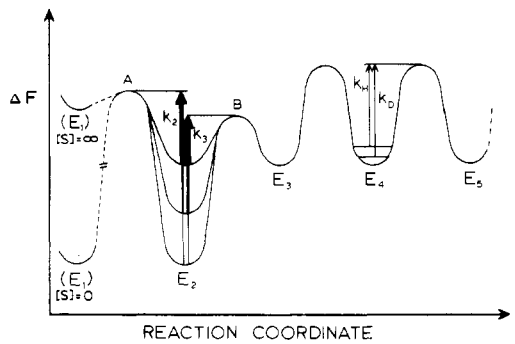


FIGURE 1: Reaction-coordinate diagram illustrating stabilization of a Michaelis complex (E_2). The early portion of case 3 of Table I is portrayed, in which stabilization of the Michaelis complex causes an increase in ^DV but has no effect on $^DV/K$.

can more readily be made with $^DV/K$ than ^DV. One illustration is given in the reaction-coordinate diagram of Figure 1. Stabilization of the Michaelis complex will have no effect on the expression of an isotope effect on V/K, because the relative tendency of E_2 to cross energy barrier A vs. barrier B will not have changed. Both k_3 and k_2 will have been decreased, but not their ratio, and this ratio is the commitment factor for E_2. Thus, like a thermodynamic component which is independent of the route of a reaction, commitments to catalysis are independent of the energy levels of reaction intermediates. In contrast, the decreasing k_3 will have a direct effect of lowering the maximal velocity and raising the ratio of catalysis, which may in turn depress ^DV—observations which would appear to be evidence for a shift in the rate-limiting step.

The present study now reveals that multiple reversible steps preceding catalysis bring in yet a third function, the equilibration preceding catalysis. This is the most difficult function to understand as a kinetic determinant, because its algebraic form suggests it is a purely thermodynamic function. Being the sum of equilibrium constants, it shows that an unfavorable preequilibration can enhance the expression of an isotope effect by elevating the isotopically sensitive step to the top of an energy barrier where the step can act as a "gate" to the remaining reaction sequence. Thus, despite slower steps further on (see below and case 6c, Table I), the decision at the gate giving access to these steps may still be expressed. This concept will explain, in part, the increase in ^DV of case 3, illustrated in Figure 1: stabilization of the Michaelis complex places the isotopically sensitive step nearer the top of the free energy profile for this segment of the enzymatic mechanism.

As a further complication, the first and third functions are interactive. Extracting R_f/E_f from eq 16 and factoring out k_3, one obtains

$$\frac{R_f}{E_f} = \frac{\left(\frac{k_7}{k_5} + \frac{k_7}{k_9} + \frac{k_7}{k_{11}}\right)k_3 + \frac{k_7(k_4 + k_5)}{k_5}}{\left(\frac{k_5 + k_6}{k_5}\right)k_3 + \frac{k_4k_6}{k_5}} \quad (28)$$

or, in coefficient form

$$\frac{R_f}{E_f} = \frac{a(k_3) + b}{c(k_3) + d} \quad (29)$$

If coefficients $a/c = b/d$, then both R_f/E_f and, subsequently, ^DV will be independent of k_3. The concepts of a gate and catalytic ratio will be in balance, which is the kinetic relationship simulated in case 4 of Table I. Alternatively, if $a/c > b/d$, then the importance of the gate outweighs the catalytic ratio and the decreasing k_3 decreases R_f/E_f, causing the otherwise incongruous *increase* in the expressed isotope effect of case 3. Only when $a/c < b/d$, will the relationship between k_3 and ^DV appear "normal" as in case 2. Therefore, the uncertain relationship between the rate constants comprising coefficient ratios a/c and b/d determines whether fast or slow steps preceding catalysis enhance or suppress the isotope effect

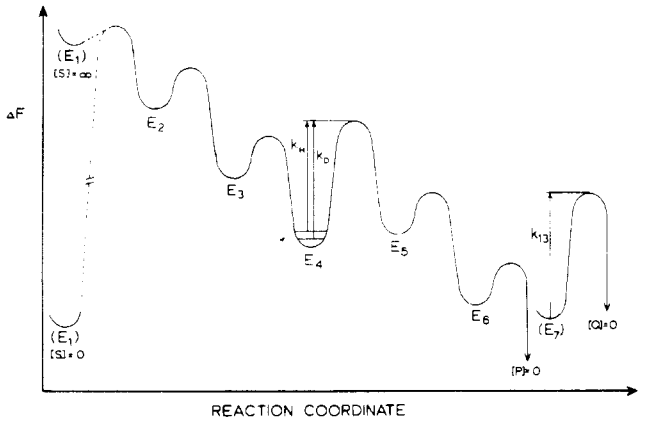


FIGURE 2: Reaction-coordinate diagram illustrating an enhanced expression of an isotope effect by minimizing the ratio of catalysis. Shown at left is case 5b of Table I, where $C_r = 0.02$, $R_f = 0.04$, $E_f = 1.02$, $^Dk = 7$, $^DV = 6.66$, $^DV/K = 1.00$, and $k_2 = 1$. Shown at right is the addition of a second product release step governed by k_{13}, assigned a relative value of 0.1 in this illustration.

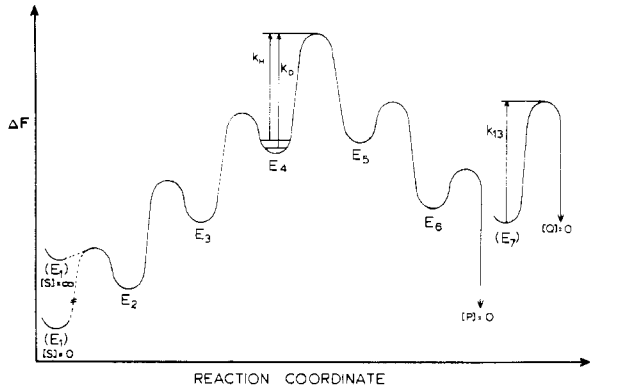


FIGURE 3: Reaction-coordinate diagram illustrating an enhanced expression of an isotope effect by maximizing the equilibration preceding catalysis. Shown at left is case 5c of Table I, where $C_r = 0.02$, $R_f = 26.02$, $E_f = 2551$, $^Dk = 7$, $^DV = 6.82$, $^DV/K = 6.82$, and $k_2 = 10$. Shown at right is the addition of a second product release step governed by k_{13}, assigned a relative value of 0.1 in this illustration.

and not how rate limiting that particular step, or the isotopically sensitive step, might be. Equations similar in coefficient form to eq 29 can be obtained for k_4 and k_5 as well as k_3, but not for k_6. Nor can an equation similar to eq 29 be derived for a reverse (even numbered) rate constant if preequilibration is represented by only one step. Thus, two steps of equilibration are required to fully encompass this uncertainty.

The interactivity of functions must also be considered in understanding extremes of expression of isotope effects. For maximization of ^DV, the *ratio* R_f/E_f must be minimized, and this minimum does not coincide with the minimum of R_f. Case 5b of Table I was obtained by minimizing the ratio of catalysis within the set limits. A large isotope effect on V results, but it is not as large as that in case 5c, obtained by maximizing the equilibration preceding catalysis within the same set limits. In both cases, changes in the rate constants governing the isotopically sensitive step cause directly proportionate changes in calculated maximal velocities (not shown). Both cases are thus candidates for having detected an isotopically sensitive rate-limiting step, but the two cases are very different. The free-energy profiles of Figures 2 and 3 emphasize these differences.

Kinetic Interpretations Based on General Equations. The common usage of the concept of a rate-limiting step (or partially rate-limiting steps) seems adequately contained in the kinetic function defined as the ratio of catalysis, which relates barrier heights of forward steps to each other. But as discussed above, this first function fails to encompass the expression of an isotope effect on an enzyme-catalyzed reaction. Jencks (1969) stressed the importance of distinguishing between a *rate constant* and an absolute *rate* for a particular step in a chemical reaction, because while the first reflects only the barrier height, the second takes into account both the energy level of intermediates and the barrier height for the step. Thus, two properties contribute to identifying a rate-limiting step, one kinetic (barrier height) and one thermodynamic (energy level of intermediates). Jencks further identified the rate-limiting step as the conversion crossing the highest point on the free-energy profile and not simply the step with the highest barrier. Both the kinetic and thermodynamic properties are thus accounted for in this singular criterion. Extending this point of view to enzyme-catalyzed reactions, it would appear that the isotopically sensitive step is rate limiting in case 5c, but not in case 5b. If so, then how do we account for the large isotope effect associated with case 5b in the presence of other steps superior to it on the energy diagram (Figure 2), and how is it possible that *lowering* the energy level of some intermediates while leaving the barrier heights and superior energy levels of other intermediates alone (Figure 1) can sometimes cause changes in the expression of the isotope effect on V, either decreases (case 2) or increases (case 3), but cause no change in $^DV/K$?

On the other hand, Cleland (1975) noted that the rate of a step is the product of the concentration of the intermediate form of enzyme times its net rate constant (which is the rate constant that would produce the same flux through the step if it were irreversible) and, more importantly, that all steps will yield the same product at steady state, that is, $k_1'[E_1] = k_3'[E_2] = k_5'[E_3]$, etc. With this view, it would appear that all steps have the same absolute rate and how rate limiting a step is would be proportional to the steady-state level of the enzyme form undertaking the step (analogous to the energy level) or inversely proportional to its net rate constant (a measure of the effective barrier height). Both the kinetic and thermodynamic properties are thus again accounted for, but this criterion argues the opposite of the one above, namely, for case 5b and against case 5c.[1] Furthermore, if so, then how do we account for the absence of an effect in case 5a, with its lower net rate constant, or the significant effect in case 6c, with its larger net rate constant for the isotopically sensitive step, as compared to the net rate constants for other steps of the same example?

The answers lie in the distribution of kinetic and thermodynamic properties between three functions in an enzymatic reaction, as opposed to their combination in a single function in a chemical reaction: one kinetic (R_f), one thermodynamic (E_f), and one a mixture of the two (C_r). In case 5b (Figure

[1] One reviewer proposed a variant of this approach, arguing that Jencks' criterion remains valid if one identified the "true reactant", defined as that intermediate form of enzyme present in highest concentration prior to the most rate-determining step, such a step being identified as governed by the transition state, lying along the most favorable pathway for converting the true reactant into products, which is present in lowest concentration. However, this view is refuted by cases 2–4, where the relative concentraions of transition states remain the same within each case, yet the isotope effects respectively decrease, increase, or are unchanged as k_3 decreases, which in turn increases the qualification of E_2 as the true reactant. Furthermore, in case 4 where all transition states were set at the same concentration (but need not have been in order to satisfy the condition that $a/c = b/d$ of eq 29) decreasing k_3 below 10^{-4} causes E_2 to constitute more than 99.9% of E_t, and V/E_t to equal 99.9% of k_3, yet the expression of the isotope effect remains unchanged at 2.2.

2), it *is* the step with the highest barrier that will express the isotope effect despite the superior position of E_2 on the reaction-coordinate diagram, because this example was made maximally dependent upon the ratio of catalysis and therefore insensitive to the relative energy level of intermediates. The other extreme, case 5c (Figure 3), while it appears to detect the rate-limiting step, is equally misleading because it is maximally dependent upon the equilibration preceding catalysis and thus highly insensitive to barrier heights and to events following the isotopically sensitive step. This can be illustrated by considering an additional variant in mechanism: a slow, ordered release of a second product, governed by k_{13}, shown on the right of the reaction-coordinate diagrams. Because the release of the first product is irreversible (i.e., initial velocity conditions), the reaction-coordinate diagrams are discontinuous; also, the new step contributes only to the ratio of catalysis function. Assigning values to k_{13} within the limits of Table I has no detectable effect on ^{D}V for case 5c. Decreasing k_{13} yet another order of magnitude, to 0.01, decreases ^{D}V only from 6.82 to 6.80; even at $k_{13} = 0.001$, $^{D}V = 5.22$. In contrast, k_{13} at 0.1 decreases ^{D}V of case 5b from 6.66 to 3.94. The very different response of these two cases to an additional step following an irreversible process verifies the unequal distribution of kinetic and thermodynamic properties of the enzymatic reaction among different functions, because the new step is purely kinetic: it lies on a different energy profile diagram, thus negating the thermodynamic contribution, and contributes only to recycling the catalyst. For a chemical reaction, no such recycling is necessary, and the rate of disappearance of a reactant is normally independent of events following the first irreversible step.

Regarding $^{D}V/K$, it too is independent of events following the first irreversible step and thus often fails to detect a rate-limiting step by failing to encompass a complete enzymatic turnover. But even for a reaction mechanism with a single irreversible step, $^{D}V/K$ fails because V/K represents an apparent *rate constant* at zero substrate concentration, conditions under which there is no *rate*, so it fails to meet the criterion of Jencks. As an apparent but singular rate constant, V/K has the properties of a single ground state and single barrier height. In $^{D}V/K$, the common ground state cancels out with k_1, leaving a function that relates only the relative position of the barrier peaks to each other on the reaction-coordinate diagram, hence, the insensitivity to the pertubation depicted in Figure 1 and absence of an effect in Figure 2. Also V/K contains k_2 which is absent from V; thus, even if catalysis were rate limiting in V, it is possible for V/K to be devoid of an isotope effect when k_2 is small.

What is left is an uncertain relationship between the expression of isotope effects on the maximal velocity of enzyme-catalyzed reactions and rate-limiting steps. The nature of this uncertainty can be portrayed by an analogy to boxes. For a chemical reaction, the width of a box (rate limitation) can be calculated from a knowledge of the volume (isotope effect) because the three sides of the box are of equal length (kinetic and thermodynamic properties are in a constant relationship). For an enzymatic reaction, calculating the width of a box from a measurement of the volume is very hazardous, because the three sides may be of unequal length (kinetic and thermodynamic properties are unequally distributed between R_f, E_f, and C_f). The volume measured may be the third power of the shortest side (case 5a) or the longest (case 6c).

Minimal Mechanism for Considering Isotope Effects. Occam's razor has long been employed in steady-state kinetics in the form of minimal representation of the number and sequence of catalytic and conformational events occurring within the central complex. Indeed, Cleland (1963) dispensed with these events altogether in his classic papers, because their number and sequence are not expressed when reaction velocities vary as a function of substrate product, or dead-end inhibitor concentrations. But when only one of these events is perturbed, as with an isotope effect, the same employment of Occam's razor cuts too fine. For full representation of the kinetic and thermodynamic properties governing the expression of isotope effects, possible reaction mechanisms and related rate equations must include at least two precatalytic reversible steps and one postcatalytic reversible step, in addition to substrate binding and product release steps. Two steps before catalysis are necessary because the full uncertainty expressed in eq 29 and cases 2–4 requires at least two steps. An additional step after catalysis is needed to allow for variety in the reverse commitment to catalysis independent of the rate of the reverse catalytic step. Finally, the isotopically sensitive step itself must be included and considered reversible, even if only microscopically so. An example of a minimal mechanism for a single substrate–single product enzyme-catalyzed reaction is eq 27.[2]

It would also appear prudent to require a similar minimal mechanism for other kinetic perturbations that may alter separate events within the central complex differently, for example, changes in temperature and pH or comparisons between alternate substrates, isozymes, or modified enzymes. The kinetic complexities described here may be expected to be worse in these examples because of the possibilities of perturbing more than one step. In fact, because of the unique property of the isotope effect to specifically alter a single step that can be identified, the value of isotope effect measurements may be in sorting out the meaning of these other, more complex, perturbations by providing a reference point. It also follows that interpretations of these other kinetic perturbations are inadequately served by the singular (and now outmoded) concept of a rate-limiting step.

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[2] Obviously, if a reaction is fully reversible and studied in *both* forward and reverse directions, then *two* reversible steps on each side of the isotopically sensitive step are necessary.