Nemerson, Y., Silverberg, S. A., and Jesty, J. (1974), *Thromb. Diath. Haemorrh. 32,* 57–64.
Owen, W. G., Esmon, C. T., and Jackson, C. M. (1974), *J. Biol. Chem. 249,* 594–605.
Papahadjopoulos, D., and Hanahan, D. J. (1964), *Biochim. Biophys. Acta 90,* 436–439.
Radcliffe, R. D., and Nemerson, Y. (1975), *J. Biol. Chem. 250,* 388–395.
Rapaport, S. I., Schiffman, S., Patch, M. J., and Ames, S. B. (1963), *Blood 21,* 221–235.
Reid, M. S., and Bieleski, R. L. (1968), *Anal. Biochem. 22,* 374–381.
Shulman, N. R., and Hearon, J. Z. (1963), *J. Biol. Chem. 238,* 155–164.
Silverberg, S. A., and Nemerson, Y. (1974), *Circulation 50,* 292.
Stenn, K. S., and Blout, E. R. (1972), *Biochemistry 11,* 4502–4514.
Ware, A. G., and Seegers, W. H. (1948), *Am. J. Physiol. 152,* 567–579.
Weber, K., Pringle, J. R., and Osborn, M. (1972), *Methods Enzymol. 27,* 3–27.

Steady-State Analysis of Kinetic Isotope Effects in Enzymic Reactions†

Dexter B. Northrop

ABSTRACT: The decrease in the rate of bond cleavage resulting from the presence of heavy isotopes is expressed to a greater or lesser extent as an isotope effect on steady-state kinetic parameters of enzyme-catalyzed reactions, depending upon complex relationships between individual rate constants. This paper describes these relationships and derives general kinetic expressions which allow the determination of the limits of the relative contribution of different reaction components to V_{max} and V_{max}/K_m. The value of the true isotope effect on a carbon–hydrogen bond breaking step, the rate of this step, and its percentage of partial rate limitation of the overall reaction, plus the dissociation constant of the enzyme–substrate complex, can be determined from the derived expressions by comparing deuterium and tritium isotope effects on V_{max}/K_m. In the absence of a measure of the true isotope effect, the lower limit of primary isotope effects of deuterium which may be interpreted as identifying the slowest or "rate-determining" step in an enzyme-catalyzed reaction is found to be $k_H/k_D = 8$, and not $k_H/k_D = 2$ as is currently supposed.

The major reason for determining kinetic isotope effects in enzymology has been to determine whether the maximal velocity is a measure of the rate of the step in which covalent change takes place (Jencks, 1969). Such a step is normally identified as the "rate-determining step" of the enzymatic reaction. The results to date and the steady-state concepts of current kinetic theory suggest, however, that the domination of maximal velocities of enzymatic reactions by a single covalent step is a rare event. It now appears likely that the maximal velocity of most enzymes is dependent upon several "rate-contributing" or "partially rate-limiting" steps. In this latter situation, apparent isotope effects have meaning only in terms of a comparison to the true isotope effects exerted on the catalytic step of covalent change. However, since true isotope effects could not previously be determined, the usual procedure has been to evaluate apparent effects on an absolute scale, based upon an implied comparison with apparent effects for other enzymes and results obtained in chemical reactions. Such an approach ignores the possible wide variation of the true isotope effects in enzyme-catalyzed reactions, which are, in addition, probably much greater than the current standards of comparison.

The primary purpose of the present study is to examine the origins and degrees of expression of isotope effects within the steady-state behavior of enzymes, in order to develop a theoretical basis for evaluating apparent isotope effects and to define the relative contributions of various partially rate-limiting steps to maximal velocities. A method for determining true isotope effects was discovered which clearly obviates the limitations and uncertainties previously encountered in the interpretation of isotopic data. The thermodynamics of bond-breaking processes which give rise to the presence and magnitude of kinetic isotope effects has been reviewed (Jencks, 1969) and is not a subject in this discussion. In addition, the present study is restricted quantitatively to primary isotope effects arising from isotopic substitutions of hydrogen in substrates displaying Michaelis–Menten kinetics, although extensions of the theory to other isotopes and secondary effects are possible in many instances.

Theoretical Analysis

The steady-state kinetic behavior of enzymes as a function of the concentration of one substrate generally obeys the equation

$$v = VS/(K + S) \quad (1)$$

where V is the maximal velocity and K the Michaelis constant. The kinetically independent constants are V and V/K, both complex functions consisting of several rate constants.

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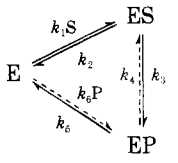
Consequently, a reduction in the magnitude of one rate constant as a result of isotopic substitution will be expressed to a greater or lesser extent on either V or V/K, depending upon the relationships between rate constants within these complex functions. In order to define these relationships it is useful to examine first the kinetic functions describing a simple reaction mechanism, and then turn to a more complex mechanism from which general equations may be derived.

Scheme I is a simplified reaction mechanism consisting

SCHEME I



of a minimal number of components. The first component is the binding of substrate (S) to enzyme, governed by the rate constants, k_1 and k_2. The second component is the catalytic step, governed by first-order rate constants, k_3 and k_4. For the purpose of isolating kinetic functions in this discussion, k_4 is set equal to zero, indicating irreversible catalysis. The third component is the dissociation of product from the enzyme, governed by the first-order rate constant, k_5. Since initial velocity conditions are assumed, the concentration of product (P) is assumed to be zero and thus the rate of the reaction governed by k_6 is also zero.

The steady-state rate equation for this mechanism is given in eq 2, from which kinetic expressions for V, K, and V/K may be derived (eq 3-5).

$$v = \frac{k_1k_3k_5[S]}{k_5(k_2 + k_3) + k_1(k_3 + k_5)[S]} \quad (2)$$

$$V = \frac{k_3k_5}{k_3 + k_5} \quad (3)$$

$$K = \frac{k_5(k_2 + k_3)}{k_1(k_3 + k_5)} \quad (4)$$

$$\frac{V}{K} = \frac{k_1k_3}{k_2 + k_3} \quad (5)$$

Assuming a primary deuterium isotope effect on catalysis, and no significant secondary isotope effects elsewhere, the degree to which this effect is expressed on V is dependent upon the ratio of k_3 to k_5. An expession for this function may be obtained by dividing eq 3, expressed in terms of the substrate containing protium, by the same equation expressed in terms of the substrate containing deuterium. Solving for the apparent isotope effect on V one obtains

$$\frac{V_H}{V_D} = \frac{k_{3H}/k_{3D} + (k_3/k_5)_H}{(k_3/k_5)_H + 1} \quad (6)$$

The apparent isotope effect on V approaches the value of the true isotope effect when the ratio k_3/k_5 is small, and approaches a value of one when the ratio is large.

Similarly, the degree to which an isotope effect on catalysis is expressed on V/K is dependent upon the ratio of k_3 to k_2. An expression for this function may be obtained by dividing eq 5, expressed in terms of the substrate containing protium, by the same equation expressed in terms of the substrate containing deuterium. Solving for the apparent isotope effect on V/K yields

$$\frac{(V/K)_H}{(V/K)_D} = \frac{k_{3H}/k_{3D} + (k_3/k_2)_H}{(k_3/k_2)_H + 1} \quad (7)$$

The apparent isotope effect on V/K varies inversely with the ratio of k_3 to k_2 to give values between one and the true isotope effect. Subtracting a value of one from both sides of eq 7 gives

$$\frac{(V/K)_H}{(V/K)_D} - 1 = \frac{k_{3H}/k_{3D} - 1}{(k_3/k_2)_H + 1} \quad (8)$$

which is a useful form of the equation for comparing deuterium and tritium isotope effects.

The expression of tritium isotope effects is governed by equations analogous to 6, 7, and 8. However, the determination of an isotope effect on V requires a total isotopic substitution while tritium can only be employed as a trace label. Hence, only V/K isotope effects of tritium may be experimentally determined,[1] and in practice, only eq 7 and 8 are applicable. Since the ratio k_3/k_2 is independent of the isotopic substitution, a comparison of deuterium and tritium isotope effects may be obtained by dividing eq 8 by the same equation expressed in terms of tritium. Their common denominators cancel out leaving

$$\frac{(V/K)_H/(V/K)_D - 1}{(V/K)_H/(V/K)_T - 1} = \frac{k_{3H}/k_{3D} - 1}{k_{3H}/k_{3T} - 1} \quad (9)$$

Equation 9 thus provides a means of comparing apparent isotope effects on V/K to the true isotope effects.

Rate equations for more elaborate enzyme mechanisms often appear hopelessly complex in terms of individual rate constants, and the expression of an isotope effect on a single rate constant, which appears numerous times in both the numerator and denominator of the rate equation, seems unintelligible. It is with some surprise, therefore, that close examination of a wide variety of rate equations reveals a possible reduction to simple kinetic expressions not significantly different in form or content to those already presented.

Scheme II illustrates a mechanism of greater complexity than Scheme I, sufficient to illustrate the form of kinetic

[1] Isotope effects of tritium, or any other isotope present at only trace levels, are determined by a procedure variously described as a discrimination, enrichment, competition, or enhancement experiment. The isotopic ratio (or specific radioactivity) of the substrate at the start of a reaction is compared to that of the substrate or product at some later time in the reaction. Simon and Palm (1966) have shown that competitive isotope effects are V/K effects, governed by the equation

$$\frac{V_H}{V_T} = \frac{(V/K)_H S_H}{(V/K)_T S_T}$$

The integrated form of this equation provides the function relating initial concentrations to those present at time t, namely

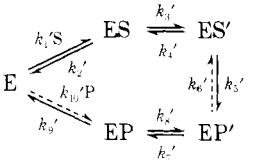
$$\left(\frac{S_H^0}{S_H^t}\right)^{(V/K)_T} = \left(\frac{S_T^0}{S_T^t}\right)^{(V/K)_H}$$

The following rearrangements (Cleland, personal communication) provide a form more compatible with experimental data as usually obtained:

$$\frac{(V/K)_H}{(V/K)_T} = \frac{\ln(1 - f)}{\ln\left((1 - f)\frac{R_p^t}{R_s^0}\right)}$$

where f is the fractional conversion of substrate to products, and R_s^0 and R_p' either the isotropic ratio or specific radioactivity of the substrates at time zero and the products at time t, respectively. Since the limit of $\ln(1 - x)$ as $x \to 0$ is $-x$, the equation approximates at low levels of conversion (10-20%) to $(V/K)_H/(V/K)_D = R_s^0/R_p'$. Therefore, in determining an isotope effect by a competitive experiment, it is important to either take note of the extent of conversion, or maintain low levels of conversion, in addition to measuring changes in the isotopic ratio. It can also be shown that comparisons between R_s^0 and R_s' are considerably less accurate than R_s^0 and R_p'. For further discussion of the assumptions, sensitivity, and errors of the method, see Melander (1960) or Bigeleisen and Wolfsberg (1958).

SCHEME II



expressions relevant to various specific but more elaborate enzyme mechanisms.

A reversible binding step (k_1',k_2'), an irreversible intermediate step (k_5'), and an irreversible product release (k_9') are again assumed. Two new features are added, a pre- and a post-equilibration step, governed by k_3', k_4' and k_7', k_8', respectively. These additional steps may represent substrate or product isomerization, enzyme conformational changes, additional but reversible catalytic steps, or the binding of a second substrate held at a fixed concentration.

The steady-state rate equation for the mechanism in Scheme II is

$$v = \frac{k_1'k_3'k_5'k_7'k_9'[S]}{(k_1'k_3'k_5'k_7' + k_1'k_3'k_5'k_8' + k_1'k_3'k_7'k_9' + k_1'k_4'k_7'k_9' + k_1'k_5'k_7'k_9' + k_1'k_3'k_5'k_9')[S] + k_3'k_5'k_7'k_9' + k_2'k_5'k_7'k_9' + k_2'k_4'k_7'k_9'} \quad (10)$$

The general form of kinetic isotope expression may be discerned from eq 10 by defining three new kinetic terms, each relating to one of the three components discussed for Scheme I.

$$k_a = \frac{k_2'}{[k_3'/(k_4' + k_5')] + 1} \quad (11)$$

$$k_b = \frac{k_3'k_5'}{k_3' + k_4' + k_5'} \quad (12)$$

$$k_c = \frac{k_7'k_9'}{k_7' + k_8' + k_9'} \quad (13)$$

The first term, k_a, is an apparent first-order rate constant for the breakdown of the enzyme–substrate complexes ES and ES′ to free enzyme and substrate. It is analogous to k_2 of Scheme I, but differs as a result of the preequilibration step which produces the ES′ complex that cannot directly dissociate without isomerization to ES. The second term, k_b, is the apparent first-order rate constant for the conversion of the first enzyme complex following substrate binding to the first enzyme complex immediately following the first irreversible step of the reaction, i.e.

$$ES \xrightarrow{k_b} EP'$$

It is analogous to k_3 of Scheme I. The third term, k_c, is the apparent first-order rate constant for the conversion of the enzyme complex immediately following the first irreversible step to free enzyme, i.e.

$$EP' \xrightarrow{k_c} E + P$$

It is analogous to k_5 of Scheme I. Equations 11, 12, and 13 will differ for different reaction mechanisms, but their form and the meaning of the kinetic terms k_a, k_b, and k_c will be the same for all enzymes displaying Michaelis–Menten kinetics.[2]

Grouping appropriate rate constants in eq 10, followed by substitution of the expressions given in eq 11–13, yields the following general kinetic equations.

$$v = \frac{k_1'k_bk_c[S]}{k_c(k_a + k_b) + k_1'(k_b + k_c)[S]} \quad (14)$$

$$V = \frac{k_bk_c}{k_b + k_c} \quad (15)$$

$$K = \frac{k_c(k_a + k_b)}{k_1'(k_b + k_c)} \quad (16)$$

$$\frac{V}{K} = \frac{k_1'k_b}{k_a + k_b} \quad (17)$$

From eq 15 and 17, the following general equations may be obtained to describe the factors governing the expression of an isotope effect (k_{bH}/k_{bD}) on V and V/K.

$$\frac{V_H}{V_D} = \frac{k_{bH}/k_{bD} + (k_b/k_c)_H}{(k_b/k_c)_H + 1} \quad (18)$$

$$\frac{(V/K)_H}{(V/K)_D} = \frac{\frac{k_{bH}/k_{bD}}{k_{aH}/k_{aD}} + (k_b/k_a)_H}{(k_b/k_a)_H + 1} \quad (19)$$

$$\frac{\frac{(V/K)_H}{(V/K)_D} - 1}{\frac{(V/K)_H}{(V/K)_T} - 1} = \frac{\frac{k_{bH}/k_{bD}}{k_{aH}/k_{aD}} - 1}{\frac{k_{bH}/k_{bT}}{k_{aH}/k_{aT}} - 1} \quad (20)$$

Equations 14–20, applicable to Scheme II, are analogous to eq 2–9, derived for Scheme I, but take into account possibilities of a multitude of separate steps, either known or unknown, contributing to each of the three kinetic components of enzymatic mechanisms. What remains to be described is the influence of these additional steps on the expression of isotope effects in the kinetic terms k_a, k_b, and k_c, in order to ultimately relate apparent effects to the true isotope effect.

In most instances k_a will not express an isotope effect. Conditions which allow k_{aH}/k_{aD} to be significantly different from one tend to result in suppression of the effect overall. Hence, for the practical purposes of comparing V and V/K effects only (see below), eq 19 may be reduced to eq 21, which is also more analogous to eq 7. For k_b and k_c, a

$$\frac{(V/K)_H}{(V/K)_D} = \frac{k_{bH}/k_{bD} + (k_b/k_a)_H}{(k_b/k_a)_H + 1} \quad (21)$$

simple proportion between the rate of the irreversible step and the sum of the forward and reverse rates of the equilibration step governs the degree of expression. For example, if deuterium substitution affects the irreversible step contributing to k_b, the expression of k_{5H}'/k_{5D}' is

$$\frac{k_{bH}}{k_{bD}} = \frac{k_{5H}'/k_{5D}' + \left(\frac{k_5'}{k_3' + k_4'}\right)_H}{\left(\frac{k_5'}{k_3' + k_4'}\right)_H + 1} \quad (22)$$

Similarly, if deuterium substitution affects the reversible equilibration step contributing to k_b, the expression of k_{3H}'/k_{3D}' in k_{bH}/k_{bD} is

$$\frac{k_{bH}}{k_{bD}} = \frac{k_{3H}'/k_{3D}' + \left(\frac{k_3' + k_4'}{k_5'}\right)_H}{\left(\frac{k_3' + k_4'}{k_5'}\right) + 1} \quad (23)$$

Isotope effects occurring on reversible steps are complicated by the probable presence of an effect on both the forward and reverse rates of the step. Equation 23 was derived assuming the forward and reverse effects were equal, i.e., $k_{3H}'/k_{3D}' = k_{4H}'/k_{4D}$. When the two effects are not equal,

[2] In the special case of a freely reversible enzymatic reaction producing only a single product, the release of product is the only irreversible step. Consequently k_c is equal to infinity and V equals k_b.

their ratio is the same as the ratio of the equilibrium constants of the overall reaction, and eq 23 increases in complexity to a less useful form:

$$\frac{k_{bH}}{k_{bD}} = \frac{k_{3H}'/k_{3D}' + \left(\frac{k_3' + k_4'(K_{eqH}/K_{eqD})}{k_5'}\right)}{\left(\frac{k_3' + k_4'}{k_5'}\right)_H + 1} \quad (24)$$

The relationship between the true isotope effect and effects on V/K in complex mechanisms can now be summarized by the following equations. Given an isotope effect on a reversible step (i.e., k_3 of Scheme II), eq 20 becomes

$$\frac{\frac{(V/K)_H}{(V/K)_D} - 1}{\frac{(V/K)_H}{(V/K)_T} - 1} = \frac{\left(\frac{k_{3H}'}{k_{3D}'} - 1\right)\frac{k_5'}{k_4'} + \frac{K_{eqD}}{K_{eqH}} - 1}{\left(\frac{k_{3H}'}{k_{3T}'} - 1\right)\frac{k_5'}{k_4'} + \frac{K_{eqT}}{K_{eqH}} - 1} \quad (25)$$

In the absence of an isotope effect on the equilibrium constant,[3] this reduces to

$$\frac{\frac{(V/K)_H}{(V/K)_D} - 1}{\frac{(V/K)_H}{(V/K)_T} - 1} = \frac{k_{3H}'/k_{3D}' - 1}{k_{3H}'/k_{3T}' - 1} \quad (26)$$

Similarly, given an isotope effect on an irreversible step (i.e., k_5' of Scheme II), eq 20 becomes

$$\frac{\frac{(V/K)_H}{(V/K)_D} - 1}{\frac{(V/K)_H}{(V/K)_T} - 1} = \frac{k_{5H}'/k_{5D}' - 1}{k_{5H}'/k_{5T}' - 1} \quad (27)$$

Equations 26 and 27 reveal the same fundamental relationship between true and apparent isotope effects in complex mechanisms as shown in eq 9 for a simple mechanism. It is important to note that these expressions are independent of the presence and number of equilibration steps either preceding or following the bond breaking step.

Discussion

Calculation of True Isotope Effects. Swain et al. (1958) have examined the relationship between primary isotope effects of deuterium and tritium, and found that for temperatures of 0–100°

$$\frac{k_H}{k_T} = \left(\frac{k_H}{k_D}\right)^{1.442} \quad (28)$$

This relationship has been observed experimentally in several chemical reactions where the true isotope effect can be directly observed, including reactions displaying very large isotope effects (k_H/k_D of 16 and 23), ascribed to the influence of tunneling (Lewis and Robinson, 1968). Combining eq 28 with eq 9, 26, or 27 yields the general function:

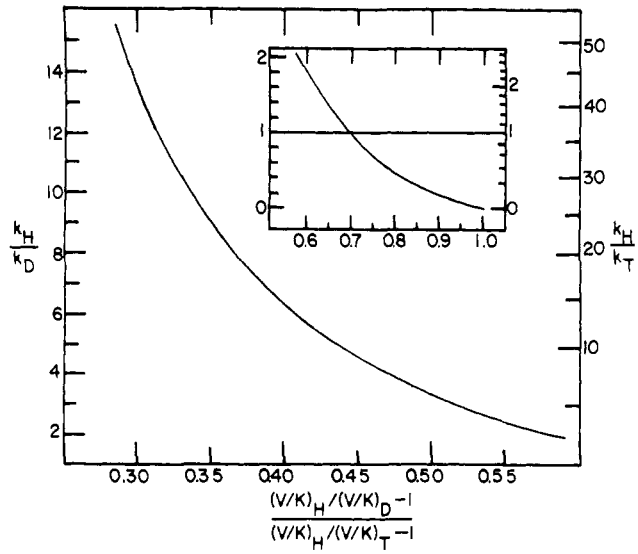


FIGURE 1: Values of true isotope effects as a function of apparent isotope effects on V/K. The curves were generated by substituting assumed values in eq 29. The vertical axis is scaled for both deuterium and tritium, but the latter is nonlinear. Consequently, values are more accurately obtained on the deuterium scale and converted to tritium by application of eq 28. The major curve extends from $k_H/k_D = 2$–15, although higher values are theoretically possible. The insert extends the function to the lower limit of zero, and therefore includes inverse isotope effects. The crossover point from normal to inverse isotope effects is found by L'Hospital's rule to be: limit $x \rightarrow 1$ $[(x-1)/(x^{1.442}-1)] = 1/1.442 = 0.6935$.

$$\frac{\frac{(V/K)_H}{(V/K)_D} - 1}{\frac{(V/K)_H}{(V/K)_T} - 1} = \frac{k_H/k_D - 1}{(k_H/k_D)^{1.442} - 1} \quad (29)$$

which can be solved for the true deuterium isotope effect. This is most readily accomplished by graphical analysis, illustrated in Figure 1, since eq 29 is a transcendental function. Thus, regardless of the complexity of the reaction mechanism, the true isotope effect can be determined from experimental measurement of the apparent deuterium and tritium isotope effects on V/K, followed by a simple reading of the true value on the graph of Figure 1. The only qualifications are that there is no equilibrium[3] or secondary isotope effect. Both of these effects are probably insignificant in the dehydrogenase enzymes, the hydrogen-transfer enzymes of greatest interest, since the transferable hydrogen is bonded to a tetrahedral carbon in both the substrates and coenzymes.

Once the true isotope effect has been determined, a comparison of this value with the apparent deuterium effect on V suggests a qualitative estimate of the overall rate contribution of the bond breaking step. Such a comparison can be misleading, however, particularly when the apparent isotope effect is small. The desired estimate is actually the difference between the observed maximal velocity and the velocity one would expect if the bond breaking step were not partially rate limiting. Repeating the earlier derivations and incorporating a condition where the bond breaking step is not rate limiting, it can be shown

$$f_V = \frac{V_H/V_D - 1}{k_H/k_D - 1} \quad (30)$$

where f_v is the fractional reduction of the maximal velocity of reaction with normal substrates attributed to the bond breaking step, and $100f_v$ is the percentage of partial rate

[3] Isotope effects on the equilibrium constant may be measured directly when the enzymatic reaction is freely reversible in the presence of substrates and products. For the purpose of comparing primary deuterium and tritium effects, only one need be determined, since

$$\frac{K_{eqH}}{K_{eqT}} = \left(\frac{K_{eqH}}{K_{eqD}}\right)^{1.442}$$

For example, a comparison of the specific activity of the tritium-containing substrate and product at equilibrium yields the tritium equilibrium effect, from which the deuterium equilibrium effect may be calculated.

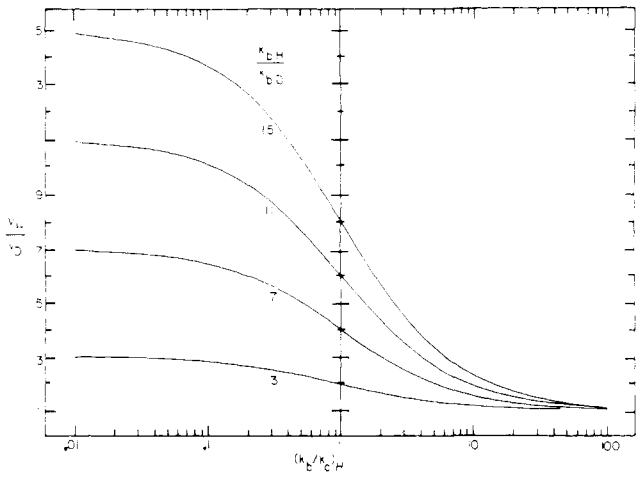


FIGURE 2: The expression of an isotope effect on the maximal velocity as a function of the ratio of k_b to k_c at different levels of expression of the effect on k_b. The curves were calculated through use of eq 18. Since eq 6, 7, 18, 21, 22, and 23 are all of the same general form, the figure may be utilized to evaluate isotopic expression of other kinetic functions by appropriate substitutions for V_H/V_D, k_b/k_c, and k_{bH}/k_{bD}.

limitation.[4] The value of f_v varies from 0 to 1, representing enzymatic reactions in which the bond breaking step does not contribute to the maximal velocity, to reactions in which it is totally rate limiting. Similarly

$$f_{v/k} = \frac{\frac{(V/K)_H}{(V/K)_D} - 1}{k_H/k_D - 1} \tag{31}$$

where $f_{v/k}$ is the fractional reduction of V/K.

Having determined the percentage of partial rate determination, and knowing the maximal velocity and Michaelis constant, it is also possible to determine the rate of the bond breaking step and the true dissociation constant of the enzyme-substrate complex. By appropriate substitutions, it can be shown that

$$\frac{V}{f_V} = k_H \tag{32}$$

and

$$\frac{V/K}{f_{v/k}} = k_H k_1/k_2 \tag{33}$$

Combining 32 and 33 gives the substrate dissociation constant, k_2/k_1.[5]

The Magnitude of Isotope Effects. A value of 15 for k_H/k_D is normally assumed to be the upper limit for deuterium substitutions on the rate of cleavage of carbon–hydrogen bonds (Richards, 1970; Simon and Palm, 1966). Most enzymes display lower values than this and have been described by Jencks (1969) as either "normal", displaying deuterium isotope effects in the range k_H/k_D = 6–10, or "abnormal", displaying even lower values. Three areas of consideration can account for lowered values. The first are special thermodynamic considerations surrounding the transition state of the bond-breaking process itself, which have been described by Westheimer (1961) and Jencks (1969), and give rise to the magnitude of the true isotope effect. The second and third are steady-state considerations which have not been adequately described, but which must be evaluated prior to an appeal to possible thermodynamic causes when evaluating experimental data, since these considerations determine the difference between true and apparent values.

The first area of steady-state consideration concerns the degree to which an isotope effect on a single rate constant is expressed on the kinetic terms k_a, k_b, and k_c, due to pre- and post-equilibration steps. In most instances, k_a will not express an isotope effect if a significant effect is present on V/K. The kinetic relationships which suppress the isotope effect on k_b are described by eq 22–24. Similar equations can be derived for k_c, since the principles responsible for the suppression are identical for k_b and k_c.

The second area of steady-state consideration concerns the degree to which an isotope effect appearing in k_b or k_c will be expressed in V and V/K. The maximal velocity is a function of the apparent first-order rate constants for the second and third components of an enzymatic reaction, i.e., k_b and k_c in eq 15, and approaches the rate of the slower component when a large disparity exists between the rates of the two components. In this situation, an isotope effect on the slower component will be fully expressed on V, whereas an effect on the faster component will not. This relationship is described by eq 18 and is presented graphically in Figure 2. The extreme right and left portions of Figure 2 reveal that the disparity between the apparent first-order rate constants must approach a factor of 100 in order to either fully express or depress the isotope effect. Between these extremes, the faster component does play a significant role, and the maximal velocity cannot be considered equal to the rate of the slower component.

The other side of this question is what magnitude of a deuterium isotope effect on the maximal velocity is necessary in order to conclude that cleavage of a carbon–hydrogen bond is the rate-determining step. Values of two or more are commonly accepted (Richards, 1970, Collins and Bowman, 1970). However, Figure 2 shows that given an isotope effect on k_b of 15, an apparent value of two is observed for V_H/V_D when the rate of k_b exceeds k_c by a factor of 13, and is therefore hardly "rate determining". Considering possible thermodynamic and steady-state suppression of the isotope effect on k_b, the lower curve of Figure 2 shows that even at a suppressed level of k_{bH}/k_{bD} = 3, a value of two is observed for V_H/V_D when k_b and k_c are equivalent. Furthermore, if this suppression is due to steady-state factors within k_b, i.e., k_H/k_D is greater than 5, then the carbon–hydrogen bond cleavage step cannot be the slowest step contributing to k_b. Consequently, the current practice of accepting deuterium isotope effect values as low as two for evidence of a rate-determining step in enzyme catalysis is unfounded. Unless additional kinetic evidence is available which characterizes the steady-state contributions to k_b, or the true isotope effect is determined, a maximum effect of k_{bH}/k_{bD} = 15 must remain in the realm of possible values. This places the lower limit for apparent isotope

[4] For example, using the data of Bright and Gibson (1967)

$$f_v = \frac{(V_H/V_D) - 1}{(k_H/k_D) - 1} = \frac{(330\ \text{sec}^{-1}/67\ \text{sec}^{-1}) - 1}{(1000\ \text{sec}^{-1}/67\ \text{sec}^{-1} - 1} = 0.28$$

Thus, the carbon–hydrogen bond cleavage is said to be 28% partially rate limiting, and the observed maximal velocity is 28% less than the expected velocity if the bond cleavage were not partially rate limiting.

[5] Equations 32 and 33 yield only minimal values for k_H and k_2/k_1 in the presence of an equilibration step preceding catalysis. For example, in Scheme II with an isotope effect on k_5, $V/f_v = k_{5H}/(1 + k_4/k_3)$, $V/K/f_{v/k} = k_{5H}k_1k_3/k_2k_4$, and the apparent dissociation constant is $k_2/k_1(1 + k_3/k_4)$. When $k_3 > k_4$, the apparent value of k_5 approaches the true value; conversely, when $k_4 > k_3$, the apparent dissociation constant approaches the true value. Events following catalysis, however, do not influence the calculation.

effects on the maximal velocity, attributable to rate determination from carbon–hydrogen bond cleavage, at a value of eight. The upper curve of Figure 2 shows that at $V_H/V_D = 8$ and $k_{bH}/k_{bD} = 15$, k_b is equal to k_c, and the two components contribute equally to the maximal velocity. To the extent that unknown thermodynamic and steady-state considerations have suppressed k_{bH}/k_{bD}, k_b will actually be less than k_c. Hence, a value of eight for an isotope effect on the maximal velocity means that no other step of the reaction can be slower than C–H bond cleavage, but may be equal in the absence of suppression. Under these conditions bond cleavage may be considered a major "rate-limiting step" of the reaction, a wording more precise than the designation "rate-determining step," since the maximal velocity may be as low as one-half the rate of the designated rate-limiting step.

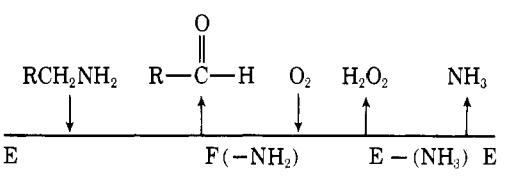
The expression of an isotope effect on V/K is subject to restraints similar to those discussed for V, and is dependent upon the ratio of k_b to k_a as shown in eq 19 and 21. These equations are similar in form to eq 18, and produces a graphical profile similar to Figure 2. Consequently, when the ratio of k_b to k_a is significantly greater than 0.01, an isotope effect appearing in k_b will not be fully expressed in V/K.

The dependence of V/K on k_a determines the meaning and subsequent interpretation of isotope effects on V/K. The kinetic term k_a represents that fraction of enzyme–substrate complex which is not available to undergo catalysis. It is determined by the extent to which the preequilibrium step illustrated in Scheme II favors ES, plus the rate that ES dissociates to free enzyme and substrate. The magnitude of k_a relative to k_b determines the degree to which the binding substrate to enzyme results in a "commitment to catalysis". Therefore, the expression of an isotope effect on V/K may provide a measure of the commitment to catalysis, but does not by itself yield information concerning the rate-limiting steps of an enzymatic reaction.[6] The absence of an isotope effect on V/K may be due either to the suppression of an effect on k_b (see above), meaning carbon–hydrogen bond cleavage is not the slowest step contributing to k_b, or to a large commitment to catalysis. On the other hand, a large isotope effect on V/K indicates both a low commitment to catalysis plus rate limitation of k_b by carbon–hydrogen bond cleavage, but does not reveal whether k_b is fast or slow relative to the remaining portion of the enzymatic reaction represented by k_c. Only in the special case of a freely reversible reaction producing a single product[2] will an isotope effect on V/K by itself yield conclusive information concerning the contribution of a carbon–hydrogen bond cleavage step to the overall rate of an enzymatic reaction, and then only if the observed effect has a value of eight or more.

Comparison of V and V/K Isotope Effects. The major difficulty encountered in evaluating isotope effects on the kinetic parameters V and V/K derives from their dependence upon a pair of the components contributing to the enzymatic reaction. Unless some information can be obtained concerning one of the pair of components, a definitive conclusion as to which one is slower may not be possible. Unfortunately, the effect of isotopic substitution on only one of the components cannot be determined directly from steady-state data, even if the true isotope effect is known. Nevertheless, advantage can be taken of the fact that V and V/K show a common dependence on the second component, k_b, and it is this portion of an enzymatic reaction that frequently contains the rate constants for catalytic steps. Comparing V and V/K isotope effects, the larger effect represents a minimum value for k_{bH}/k_{bD}. This minimum value plus either an assumed maximal value of 15 or the true isotope effect if determined can then be substituted into either eq 18 or 21, to obtain a range of values for the ratio of a pair of components. When $(V/K)_H/(V/K)_D$ exceeds V_H/V_D, use of eq 18 allows a calculation of the limits of the ratio of k_b to k_c, to give an estimation of the relative contribution of the two reaction components following substrate binding. In a sequential mechanism, this ratio may reveal whether or not product release is rate limiting; for a ping-pong mechanism, the ratio represents a comparison of the rates of the two half-reactions. In the converse situation, when V_H/V_D exceeds $(V/K)_H/(V/K)_D$, use of eq 21 allows a calculation of the limits of the ratio of k_b to k_a, and an estimation of the commitment to catalysis. Although this information is less useful, the predominance of low values for V/K isotope effects calculated from the literature data indicates that the commitment to catalysis is generally rather high, and that the rate of dissociation of substrates from enzymes is not as rapid as is often assumed (Seltzer et al., 1959; O'Leary, 1971). The following two examples will serve to illustrate the utility of comparing isotope effects on V and V/K when interpreting data.

Bardsley et al. (1973) have examined the kinetics of diamine oxidase using *p*-dimethylaminomethylbenzylamine and *p*-dimethylaminomethyl-α,α-bis(deuteriobenzyl)amine as substrates. Steady-state kinetics using the hydrogen substrate support a ping-pong reaction mechanism illustrated in Scheme III. Subsequent initial velocity measurements

SCHEME III



employing the deuterium substituted substrate (RCD_2NH_2) under a variety of experimental conditions and substrate concentrations gave a range of v_H/v_D values from 1.14 to 5.5. These results plus the presence of the isotope effect at low O_2 concentrations were interpreted as proving that the rate-limiting process in the catalytic sequence occurs prior to the reactive step involving oxygen, namely the first half of the ping-pong mechanism. But calculation of V/K effects from their data reveals equal or greater values than present on V, the mean values being 3.5 and 2.0, respectively. Substitution of $(V/K)_H/(V/K)_D$ values for k_{bH}/k_{bD} in eq 18 together with observed values for V_H/V_D yields a mean value of 1.5 for k_b/k_c. Assuming an upper limit of 15 for k_{bH}/k_{bD}, a mean value of 13 is obtained. Therefore, the rate of the first half of the ping-pong mechanism must exceed the second by a factor between 1.5 and 13, and cannot be the slowest step as suggested.

Bright and Gibson (1967) have investigated the kinetics of glucose oxidase in a very thorough study employing initial velocity, stopped flow, and isotopic substitution experi-

[6] Primary isotope effects on V/K are not usually reported nor interpreted to implicate a rate-limiting step. However, enrichment experiments have been undertaken for this purpose (O'Leary, 1971; Seltzer et al., 1959). The dependence of enrichment experiments on V/K is described in footnote 1.

SCHEME IV

$$E_{ox} + G \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E_{ox}\text{-}G \xrightarrow{k_3} E_{red}\text{-}P_1 \xrightarrow{k_5} E_{red} + P_1$$

$$E_{red} + O_2 \xrightarrow{k_7} E_{ox}\text{-}P_2 \xrightarrow{k_9} E_{ox} + P_2$$

ments. Comparison of the three experimental approaches provides a unique opportunity to test the present method of analysis. Bright and Gibson proposed Scheme IV for glucose oxidase. At 3°, 1-D-glucose produced isotope effects of 1.98 and 4.92 on V/K and V, respectively.[7] The smaller effect on V/K than on V suggests a large commitment to catalysis causing a suppression of the isotope effect on V/K. Using values of 4.92 and 15 as the limits for k_{3H}/k_{3D} in eq 7 (this equation may be used instead of eq 18 since no preequilibrium step was detected by stopped-flow measurements) the ratio of k_3/k_2 is found to be between 3 and 13.3. Stopped-flow data show in fact, a ratio of 11.1 and a large isotope effect on k_3 of 14.9. Clearly, the suppression of the isotope effect on catalysis from a value of 14.9 to a value of 1.98 on V/K is the result of a relatively slow rate of release of substrate from the enzyme. In addition, the isotope effect on V also appears suppressed, suggesting slower steps following k_3. In fact, stopped-flow measurements reveal values of 784 and 570 sec^{-1} for k_5 and k_9, respectively, as compared to 1000 sec^{-1} for k_3. Although an isotope effect of almost five is observed on the maximal velocity of glucose oxidase, the effect is clearly not sufficient to conclude that the isotopically sensitive step is solely rate limiting.

Presence of Isotope Effects. The presence of an isotope effect on either V or V/K, or on both, yields some diagnostic value as to where in a reaction mechanism an isotopic substitution exerts a specific effect. Examination of eq 2 and 4 or 12 and 14 shows that V and V/K are each rather simple functions of only two of the three components of an enzymatic reaction, but not the same pair of components. Isotopic substitution which alters the first component (i.e., binding of substrate defined by k_1 and k_2) will be expressed in V/K but not V. Substitution which alters the second component (i.e., enzyme complex interconversion up to and including the first irreversible step, governed by k_3 or k_b, normally representing catalysis) will be expressed in both V and V/K. That which affects the third component (i.e., events following the first irreversible step, governed by k_5 or k_c, normally representing product release) will be expressed in V but not V/K.

The utility of these diagnostic features lies in the study of enzymes for which there is little information available concerning the sequence of events leading to the overall reaction. For example, the involvement of molecular oxygen in many oxidase enzymes introduces an irreversible catalytic step into the reaction mechanisn. Determining whether the introduction of deuterium into the transferrable position of a substrate produces an isotope effect on both V and V/K or on V only, will establish whether O_2 precedes or follows the proton abstraction step. It must be stressed, however, that conclusions based upon an absence of an isotope effect on either V or V/K must be confirmed by other evidence since certain relationships between rate constants will result in complete suppression of an isotope effect on V and/or V/K.

In contrast to V and V/K, the Michaelis constant is a complex function of all three components as shown in eq 3 and 13, and thus has little diagnostic utility for interpretation of isotope effects. Values for K_H/K_D are frequently tabulated and discussed (Simon and Palm, 1966; Jencks, 1969) but are often the subject of ambiguous interpretation, particularly when values of less than one are obtained (which are neither abnormal nor infrequent). Since all three components do contribute to K, interpreting K_H/K_D in terms of only one component, particularly substrate binding, is therefore hazardous. Even in the extreme case where K is considered to have the significance of a dissociation constant (i.e., the first catalytic step is much slower than the rate of substrate dissociation) close examination of eq 13 reveals that K may remain sensitive to isotopic effects occurring after substrate binding.

The observation of a kinetic isotope effect on a measured rate of an enzymic reaction (v_H/v_D) has little meaning regarding the mechanism of catalysis. This is due to the shift in steady-state distribution of enzyme forms as a function of substrate and cofactor concentration. At different concentrations of substrate, the isotope effect might well be abolished, as has been observed in studies of alcohol dehydrogenase (Bush et al., 1973). On the other hand, a single set of conditions might fail to express the effect, indicating erroneously that no isotope effect is present. Accurate interpretation is therefore dependent upon obtaining limiting values for V_H/V_D and $(V/K)_H/(V/K)_D$.

This discussion addresses the expression of isotope effects as a function of rate dependence on a labeled substrate only. In enzymatic reactions involving two or more substrates, the concentrations of additional substrates contribute to k_a, k_b, or k_c, and in turn influence the expression of isotope effects on V and V/K. The diagnostic utility of determining the expression of isotope effects as a function of nonlabeled substrates (or products) has yet to be fully examined, but two conditions are worthy of mention. First, in an ordered reaction mechanism, saturation with the second substrate abolishes the isotope effect on V/K for the first substrate, thus providing another means of identifying ordered mechanisms. Second, no isotope effect should be present on V/K of the unlabeled substrate in a ping-pong mechanism, nor will the concentration of the unlabeled substrate influence the expression of an isotope effect on V/K for the labeled substrate (Bright and Gibson, 1967). Hence, the observation of Bardsley et al. (1973) that low O_2 concentrations did not abolish the deuterium isotope effect of diamine oxidase is entirely consistent with the predicted behavior of enzymes displaying a ping-pong mechanism.

[7] A low temperature was apparently necessary for accurate determinations of rate constants by stopped-flow measurements. Steady-state assays at 25° gave values for $(V/K)_H/(V/K)_D$ and V_H/V_D of 3.84 and 7.83, respectively.

References

Bardsley, W. G., Crabbe, M. J. C., and Schindler, J. S. (1973), *Biochem. J. 131*, 451.

Bigeleisen, J., and Wolfsberg, M. (1958), *Adv. Chem. Phys. 1*, 15.

Bright, H. J., and Gibson, Q. H. (1967), *J. Biol. Chem. 242*, 994.

Bush, K., Shiner, V. J., and Mahler, H. R. (1973), *Biochemistry 12*, 4802.

Collins, J. C., and Bowman, N. S. (1970), *ACS Monogr. 167*, 319.

Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N.Y., McGraw-Hill, Chapter 4.

Lewis, E. S., and Robinson, J. K. (1968), *J. Am. Chem.*

Soc. 90, 4337.
Melander, L. (1960), Isotope Effects on Reaction Rates, New York, N.Y., Roland Press.
O'Leary, M. H. (1971), *Biochim. Biophys. Acta 235,* 14.
Richards, J. H. (1970), *Enzymes, 2nd Ed. 2,* 321.
Seltzer, S., Hamilton, G. A., and Westheimer, F. H. (1959), *J. Am. Chem. Soc. 81,* 4018.
Simon, H., and Palm, D. (1966), *Angew. Chem., Int. Ed. Engl. 5,* 920.
Swain, C. G., Stivers, E. C., Reuwer, Jr., J. F., and Schaad, L. J. (1958), *J. Am. Chem. Soc. 80,* 5885.
Westheimer, F. H. (1961), *Chem. Rev. 61,* 265.

Proton Nuclear Magnetic Resonance Investigations of Fraying in Double-Stranded d-ApTpGpCpApT in H_2O Solution†

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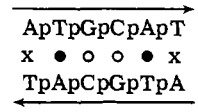
ABSTRACT: The chemical shifts and line widths of the Watson–Crick ring NH resonances of the self-complementary duplex of d-ApTpGpCpApT have been monitored at low ionic strength and in the presence of Mg ions at neutral pH in aqueous solution to determine the thermodynamic parameters associated with *fraying* (D. J. Patel (1974), *Biochemistry 13,* 2396) at the terminal and internal base pairs as a function of temperature and pH. From studies in H_2O–MeOH (3:2), the fraying process persists down to ~−20° for the internal TA base pair and down to and probably beyond −30° for the terminal AT base pair. The observed average chemical shift at each of these base pairs as a function of temperature suggests rapid exchange on the nuclear magnetic resonance (NMR) time scale between helix and coil (chemical shift separation of 3.2 ppm) and have been utilized to determine the dissociation constant at the terminal and internal base pairs. Comparison of the reaction enthalpies elucidated from the chemical shift parameters with those reported from optical studies suggests that the symmetry related internal TA base pairs break in a coupled manner at low ionic strength, with the coupling removed in the presence of Mg ions and high salt. By contrast, the symmetry related terminal AT base pairs break independently of each other in the absence and presence of Mg ions and high salt. The terminal base pair exhibits a T_m of 10–15° lower than that of the internal base pair in the hexanucleotide, with divalent Mg ions and high salt stabilizing the double helix as reflected in the T_m values of these base pairs. The observed line width changes as a function of temperature provide an estimate of the exchange rate of the proton from the coil form with water. The exchange reaction from the coil state is base catalyzed with rate constants in the diffusion limit.

The availability of short double-stranded nucleic acids of defined sequence has resulted in the evaluation of the thermodynamic and kinetic parameters associated with their helix–coil transition (Bloomfield et al., 1974). These results followed from optical changes monitored as a function of temperature in equilibrium studies and temperature jump experiments. They were first undertaken on self-complementary riboadenylic acid–uridylic acid block copolymers (Martin et al., 1971; Craig et al., 1971; Pörschke, 1971; Pörschke and Eigen, 1971) and later extended to GC containing ribonucleic acid sequences $(Ap)_nCpG(pU)_n$, where n = 2,3,4 (Uhlenbeck et al., 1971) and $(Ap)_nGpC(pU)_n$, where n = 2,3,4 (Pörschke et al., 1973; Ravetch et al., 1975). In parallel investigations, thermodynamic parameters have been determined for the helix–coil interconversion in self-complementary duplexes of $(dA\text{-}dT)_n$ (Scheffler et al., 1968, 1970; Scheffler and Sturtevant, 1969) and $(dG\text{-}dC)_n$ (Pohl, 1974).

It has been demonstrated that the Watson–Crick hydrogen-bonded ring imino protons (guanine N_1H and thymine N_3H) resonate between 11 and 15 ppm downfield of sodium 2,2-dimethyl-2-silapentane-5-sulfonate in the high-resolution nuclear magnetic resonance (NMR) spectrum of double-stranded nucleic acids in H_2O solution (Kearns et al., 1971). The observed chemical shifts of these resonances reflect the ring current contributions from stacked nearest neighbor base pairs to the intrinsic chemical shifts of the ring imino protons (Shulman et al., 1973; Patel and Tonelli, 1974).

Self-complementary single strands of the hexanucleotide d-ApTpGpCpApT form a double helix which exhibits twofold symmetry so that the six base pairs are pairwise equivalent.



This study reports on the helix–coil transition of double-stranded d-ApTpGpCpApT in H_2O solution as monitored by the chemical shift and line-width changes of the guanine N_1H and thymine N_3H resonances as a function of temperature and pH. It will be demonstrated that the thermodynamic parameters associated with the fraying of the termi-

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