Mechanistic Deductions from Isotope Effects in Multireactant Enzyme Mechanisms†

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ABSTRACT: In an enzymatic mechanism with two or more substrates, comparison of the isotope effects on the maximum velocity and on the apparent V/K values when each substrate concentration is varied allows one to deduce the kinetic mechanism and obtain quantitative information on the relative rates at which substrates dissociate from the enzyme, as opposed to undergoing reaction to give products. Theory is also presented for using the effects of other reactants on the apparent isotope effects determined by the equilibrium perturbation method to determine the same information. With liver alcohol dehydrogenase, DPN is not released at an appreciable rate from the E–DPN–cyclohexanol complex, while cyclohexanol is released much more rapidly than it reacts to give products, so that the mechanism appears ordered. With DPNH and cyclohexanone, however, the reaction is random since DPNH can be released from the ternary complex at a finite rate. With yeast alcohol dehydrogenase, acetone, when present, prevents DPNH release from the enzyme so that the mechanism appears ordered, but 2-propanol and DPN are released at equal rates from E–DPN–2-propanol so that the reaction is random in this direction.

The first step in determining the mechanism of action of an enzyme is to ascertain the kinetic mechanism, that is, the order of addition of substrates and release of products. This is most commonly done by looking at the patterns obtained when substrate concentrations are varied at different levels of other substrates or in the presence and absence of products or dead-end inhibitors. Such studies are often not conclusive and must be supplemented by other techniques such as isotope exchange at equilibrium (Boyer, 1959) and isotope partitioning (Rose et al., 1974). In this paper, we present two very sensitive techniques for determining kinetic mechanisms which involve isotope effects. In the first, initial velocities in the absence of products are obtained by using both labeled and unlabeled substrates. From the magnitude of the isotope effects on V and on V/K for the various substrates, information can be obtained on the order of addition of reactants, as well as on the location and degree of rate limitation of the isotope-sensitive steps. The second technique makes use of the equilibrium perturbation method of Schimerlik et al. (1975) to determine the effect of reactants other than those involved in the perturbation on the rate of release of the perturbant molecules from the enzyme, and thus to distinguish ordered from random binding. These techniques are illustrated by data for both horse liver and yeast alcohol dehydrogenases.

Materials and Methods

Chemicals. 2-Propanol-*2-d* and cyclohexanol-*1-d* were prepared by reduction of the corresponding ketones with $NaBD_4$ and recovered by distillation. A-side DPND was prepared by the procedure of Viola et al. (1979). All other reagents and enzymes were obtained from commercially available sources.

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots were linear. All data were fitted to appropriate equations by the FORTRAN programs of Cleland (1979). Data obtained by varying the concentration levels of two substrates were fitted to eq 1. When the levels of hydrogen- and deuterium-containing alcohols were varied at different DPN concentrations, or when DPNH and DPND were varied at different levels of ketone concentration, data were fitted to eq 2–5, which assume isotope effects on V, V/K_a, V/K_b, and K_{ia} (eq 2); V, V/K_a, and V/K_b (eq 3); V and V/K_b (eq 4); or only on V/K_b (eq 5). In addition, eq 3 was also used in the log form [that is, log v = log (VAB/denominator)]. In eq 2–5, F_i is the fraction of deuterium label in the substrate while E_V, E_{V/K_b}, E_{V/K_a}, and $E_{K_{ia}}$ are the isotope effects minus one for the respective parameters. Data for the variation with ketone concentration of the isotope effect obtained by equilibrium perturbation were fitted to eq 25 where P is ketone concentration, y is the observed isotope effect, a is the value of y at $P = 0$, and $K_{I\,num}$ and $K_{I\,denom}$ are inhibition constants for the numerator and denominator. This equation was also fitted in a slightly modified form (see eq 26). Isotope effects were extracted from equilibrium perturbation data by a FORTRAN program which gives an exact solution to the equations of Schimerlik et al. (1975).

$$v = VAB/[K_{ia}K_b + K_bA + K_aB + AB] \quad (1)$$

$$v = VAB/\{[K_{ia}K_b(1 + F_iE_{K_{ia}}) + K_bA] \times (1 + F_iE_{V/K_b}) + K_aB(1 + F_iE_{V/K_a}) + AB(1 + F_iE_V)\} \quad (2)$$

$$v = VAB/[(K_{ia}K_b + K_bA) \times (1 + F_iE_{V/K_b}) + K_aB(1 + F_iE_{V/K_a}) + AB(1 + F_iE_V)] \quad (3)$$

$$v = VAB/[(K_{ia}K_b + K_bA) \times (1 + F_iE_{V/K_b}) + K_aB + AB(1 + F_iE_V)] \quad (4)$$

$$v = VAB/[(K_{ia}K_b + K_bA)(1 + F_iE_{V/K_b}) + K_aB + AB] \quad (5)$$

Nomenclature. In this and the three following papers in this issue, we shall use the nomenclature of Northrop (1977) in which isotope effects on a kinetic or thermodynamic parameter are indicated by a leading superscript. Thus, ${}^{D}V$ is the isotope effect on the maximum velocity (V_H/V_D), and ${}^{D}(V/K_a)$ is the deuterium isotope effect on V/K_a. ${}^{D}K_{eq}$ is the equilibrium isotope effect ($K_{eq\,H}/K_{eq\,D}$). Where necessary,

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following subscripts will be used to identify in which direction ${}^{D}V$ and ${}^{D}K_{eq}$ are calculated. Thus, ${}^{D}V_{cyclohexanol}$ is the deuterium isotope effect on V with cyclohexanol as substrate. For isotope effects determined by the equilibrium perturbation method of Schimerlik et al. (1975), ^{D}(Eq.P.) will be used with the following subscript identifying the perturbant in the desired direction. T is used in place of D as the superscript for tritium isotope effects on V/K; since tritium is always used as a trace label, ${}^{T}V$ and ^{T}(Eq.P.) values are not obtainable (they could, of course, be determined by the use of nearly carrier-free tritiated compounds). The use of trace labels such as tritium or natural-abundance ^{13}C gives only V/K effects while the equilibrium perturbation method requires a high level of isotopic substitution to see any effect. When reciprocal plots with deuterated or unlabeled substrates are compared, the ratio of intercepts gives ${}^{D}V$, and the ratio of slopes gives ${}^{D}(V/K)$. For heavier atom isotope effects, only the mass number is used. Thus, ${}^{15}(V/K_a)$ represents an ^{15}N isotope effect on V/K_a, and ${}^{18}(\text{Eq.P.})_a$ represents an ^{18}O effect from the side with A as a substrate obtained by equilibrium perturbation.

If there is an isotope effect on the rate constants for only one step in an enzymatic reaction, the following equations will always apply in the forward direction when one varies the concentration level of one substrate (Schimerlik et al., 1977):[1]

$$ {}^{D}(V/K) = \frac{{}^{D}k + c_f + c_r({}^{D}K_{eq})}{1 + c_f + c_r} \tag{6} $$

$$ {}^{D}V = \frac{{}^{D}k + c_{Vf} + c_r({}^{D}K_{eq})}{1 + c_{Vf} + c_r} \tag{7} $$

In these equations, ${}^{D}k$ is the isotope effect on the forward rate constant for the isotope-sensitive step, and ${}^{D}K_{eq}$ is the equilibrium isotope effect in the forward direction. The constants c_f and c_r were originally called "commitments to catalysis" by Northrop (1977), but we will simply call them commitments. The subscripts f and r in eq 6 and 7 indicate commitments in forward and reverse directions, but in actual cases we will use the name of the reactant whose commitment it is. A commitment for a given reactant is the ratio of the rate constant for the unlabeled molecule for the isotope-sensitive step to the net rate constant for release of the reactant into solution from the complex which undergoes the isotope-sensitive step.[2] In equations for ${}^{D}(V/K)$, c_f is always for the varied substrate, regardless of which substrate is labeled, and c_r is for the first product released.

Commitments have both an internal and an external part, and this is particularly important for the forward commitment when a V/K isotope effect is being determined. The internal part is that which remains when the rate constant for release of the varied substrate is made infinite. The external part is the term containing the partition ratio with the rate constant for release of the varied substrate in the denominator. For example, for mechanism 8, where k_7 and k_8 are isotope sensitive:

$$ \text{E} \underset{k_2}{\overset{k_1\text{A}}{\rightleftharpoons}} \text{EA} \underset{k_4}{\overset{k_3\text{B}}{\rightleftharpoons}} \text{EAB} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \text{EAB}' \underset{k_8}{\overset{k_7}{\rightleftharpoons}} \text{EPQ}' \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} \text{EPQ} \underset{k_{12}\text{P}}{\overset{k_{11}}{\rightleftharpoons}} \text{EQ} \underset{k_{14}\text{Q}}{\overset{k_{13}}{\rightleftharpoons}} \text{E} \tag{8} $$

c_f in eq 6 when B is varied is given by

$$ c_f = (k_7/k_6)[1 + (k_5/k_4)] \tag{9} $$

The internal part is k_7/k_6, and the external part is k_5k_7/k_4k_6. When a varied substrate is sticky (that is, reacts to give products as fast or faster than it dissociates from the enzyme), its commitment has a finite external part; conversely, when a substrate is not sticky and its binding is in rapid equilibrium, its commitment is solely internal. These distinctions will become particularly important in the following two papers (Cook & Cleland, 1981a,b) in which we will develop the theory of the pH variation of isotope effects.

In eq 7 for ${}^{D}V$, c_f is replaced by c_{Vf}. This constant is not a commitment but is essentially the sum of the ratios of the forward rate constant for the isotope-sensitive step to each net forward rate constant for the unimolecular steps in the mechanism.[3]

If the numerator of eq 6 is divided by ${}^{D}K_{eq}$, the equation now yields the V/K isotope effect in the reverse reaction, since ${}^{D}k/{}^{D}K_{eq}$ is the isotope effect on the reverse rate constant for the isotope-sensitive step. For ${}^{D}V$ in the reverse direction, however, one must not only divide the numerator of eq 7 by ${}^{D}K_{eq}$ but also replace c_{Vf} in the numerator and denominator with c_f, and c_r by c_{Vr}, which is defined in similar fashion to c_{Vf}. The actual values of c_f, c_r, c_{Vf}, and c_{Vr} in these equations depend on the kinetic mechanism, and it is the purpose of the present paper to show how the variation of these values with the concentration levels of the substrates can be used to deduce the kinetic mechanism.

The equilibrium perturbation method of Schimerlik et al. (1975) involves adding enzyme to a reaction mixture at equilibrium which contains a labeled reactant on only one side of the reaction. For example, with alcohol dehydrogenase, one might use a deuterated alcohol and unlabeled DPNH, or vice versa. The molecules between which the label is transferred are called the perturbants (alcohol and DPNH in this case). Because of the isotope effect, the initial rate of reaction of the labeled perturbant will be less than that of the unlabeled one,[4] and the reaction will thus be perturbed from the equilibrium position. However, as isotopic mixing between the perturbants proceeds, the reaction will eventually reach both chemical and isotopic equilibrium. The size of the perturbation depends (nearly linearly for small isotope effects) on the size of the isotope effect, and the method is extremely sensitive, being

[1] When more than one step is isotope sensitive, which will usually happen only with secondary isotope effects, the equations are more complex. See Cook et al. (1981) and Blanchard & Cleland (1980) for a further discussion of such cases. With primary isotope effects, normally only the bond-breaking step shows an appreciable isotope effect, and eq 6 and 7 apply.

[2] The term "net rate constant" is used here as defined by Cleland (1975). It represents a collection of the actual rate constants for a series of steps which gives the net rate at which the overall sequence of steps occurs. Thus, for an EA complex about to undergo a bond-breaking step, c_f is $k_{\text{bond breaking}}/k_{off}$, where k_{off} is the net rate constant for release of A from EA. Cleland (1975) describes the calculation of net rate constants for any series of steps.

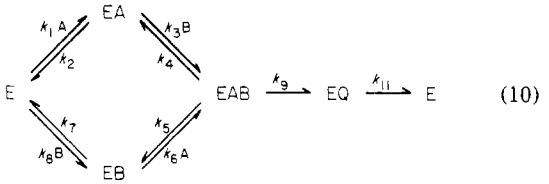
[3] When some step other than the isotope-sensitive one becomes rate limiting for V, it is c_{Vf} that becomes large and reduces the size of ${}^{D}V$ toward 1.0. To be more accurate about the definition of c_{Vf}, it consists of the sum of the ratios between k^* and each net rate constant in the forward direction, with the step prior to the isotope-dependent step being considered an irreversible step in calculating net rate constants by the method of Cleland (1975). The constant k^* is the forward rate constant for the isotope-sensitive step divided by the ratio at equilibrium between the sum of the concentrations of all reversibly connected complexes which precede the isotope-sensitive step and the concentration of the complex which actually undergoes this step. For mechanism 8, for instance, $k^* = k_7/[1 + (k_6/k_5)]$, and c_{Vf} is thus the sum of the ratios of k^* and k_5, $k_9k_{11}/(k_{10} + k_{11})$, k_{11}, and k_{13}. See Schimerlik et al. (1977) for the definition of c_{Vf} for a more complicated mechanism.

[4] This applies for normal isotope effects; for inverse ones, the labeled perturbant reacts faster, of course.

capable of determining isotope effects less than 1.01. Comparison of the perturbation size with the levels of the perturbants and other reactants allows calculation of $^{D}(\text{Eq.P.})_f$ and $^{D}(\text{Eq.P.})_r$, the ratio of which is $^{D}K_{eq}$ in the forward direction.[5] The equation for ^{D}(Eq.P.) is identical with eq 6, except that c_f and c_r are for the two perturbants, rather than for the varied substrate and first product released. In this paper, we shall discuss the way in which the commitments for the two perturbants vary with the levels of the nonperturbant molecules and show how this variation determines the kinetic mechanism.

Theory

Mechanism Dependence of Deuterium and Tritium Isotope Effects. For purpose of determining the order of addition of reactants in a bireactant mechanism, we can employ eq 10,



(10)

in which only k_9 is isotope sensitive. For simplicity, we have lumped together in k_9 all of the steps that the initially formed EAB complex undergoes, up to and including the release of the first product. The constant k_{11} then includes release of the second product and any other steps (such as isomerization of EQ) which limit V, but not V/K for either substrate. In reality, EAB undergoes a number of discrete steps, such as conformation changes prior to the bond-breaking step, the bond-breaking step itself, conformation changes following the bond-breaking step, and, finally, product release. Of these, only the bond-breaking step will usually show an isotope effect, and, thus, unless the bond-breaking step is slow with respect to all other steps included in k_9, the apparent isotope effect on k_9 will be less than that on the bond-breaking step itself.[6]

Since the internal partition ratios in the part of the mechanism represented by k_9 will not depend on the levels of the substrates, however, the apparent isotope effect on k_9 will also not vary with substrate levels, and, thus, eq 10 is adequate for our purposes. We will consider in the following two papers in this series (Cook & Cleland, 1981a,b) the further breakdown of k_9 into its discrete steps.

In reaction 10, the isotope effect on V will be given by

$$^{D}V = \frac{^{D}k_9 + k_9/k_{11}}{1 + k_9/k_{11}} \qquad (11)$$

where k_9/k_{11} corresponds to c_{VT} in eq 7. (Because of our simplification, there is no c_r for eq 10.) Equation 11 is true regardless of the order or randomness of substrate addition.

The isotope effect on V/K_a and V/K_b will, however, depend on the kinetic mechanism and will have the form of eq 6, except that there are no c_r terms. The commitment c_f will be the ratio of k_9 to the net rate constant for release of the varied substrate. When A is varied, the net rate constant for its release from EAB is $k_5 + k_2k_4/(k_2 + k_3B)$, while when B is varied, the net rate constant for its release is $k_4 + k_5k_7/(k_7 + k_6A)$. We thus have when A is varied

$$c_f = k_9/[k_5 + k_2k_4/(k_2 + k_3B)] \qquad (12)$$

and when B is varied

$$c_f = k_9/[k_4 + k_5k_7/(k_7 + k_6A)] \qquad (13)$$

We will interpret the variation of the V/K isotope effects with kinetic mechanism in terms of these two equations, and we will consider six possible mechanisms.

(1) Equilibrium ordered (k_5, k_6, k_7, and k_8 not present; $k_2 \gg V/E_t$). When A is varied, $k_5 = 0$, and k_2 will be greater than k_3B at any finite level of B, so that $c_f = k_9/k_4$, and the isotope effect does not vary with the level of B. When B is varied, $k_5 = 0$, so that c_f is also k_9/k_4 at all levels of A. The V/K isotope effect is thus the same regardless of which substrate is varied, and regardless of the level of the second substrate. The full $^{D}k_9$ is seen only if B is not sticky, however, so that $k_4 \gg k_9$. This mechanism gives distinctive initial velocity patterns which cross on the vertical axis when B is varied, but cross in the second quadrant with the slope replot going through the origin when A is varied.

(2) Equilibrium ordered with a dead-end EB complex. The isotope effects are the same as they are without the EB complex, but its presence causes the initial velocity pattern to become a normal intersecting one. This mechanism cannot be distinguished from the fifth case below.

(3) Steady-state ordered (k_5, k_6, k_7, and k_8 not present; k_2 less than or not much greater than V/E_t). When B is varied, c_f is k_9/k_4 (since $k_5 = 0$), regardless of the level of A. The $^{D}(V/K_b)$ isotope effect is thus seen when B is varied at any A level. When A is varied, k_5 is zero, but k_2 will not always be larger than k_3B, so eq 12 reduces to $c_f = (k_9/k_4)(1 + k_3B/k_2)$. At infinite B, c_f is also infinite, so $^{D}(V/K_a)$ will be 1.0 (the limiting V/K value is determined at saturating levels of the other substrate). As the level of B goes to zero, however, c_f becomes k_9/k_4, and the apparent V/K isotope effect becomes equal to $^{D}(V/K_b)$. The level of B giving a value of $[^{D}(V/K_b) + 1]/2$ is $K_{ia}K_b/K_a$.

(4) Rapid equilibrium random (k_2, k_4, k_5, and $k_7 \gg V/E_t$). Because all substrates are released from the enzyme much faster than V/E_t, all commitments are zero, and all V/K isotope effects are equal to $^{D}k_9$ regardless of the level of the nonvaried substrate.

(5) Random, with substrates in rapid equilibrium for the binary complexes, but sticky from the ternary ones (k_2 and $k_7 \gg V/E_t$; k_4 and k_5 less than or not much larger than V/E_t). When A is varied, k_2 will exceed k_3B at any finite level of B, so $c_f = k_9/(k_4 + k_5)$ regardless of the level of B. When B is varied, k_7 will exceed k_6A at any finite level of A, so $c_f = k_9/(k_4 + k_5)$, regardless of the level of A. The full value of $^{D}k_9$ is seen only if either A or B is not sticky from the ternary complex (that is, either k_5 or k_4 greatly exceeds k_9). As noted above, this mechanism cannot be distinguished from an equilibrium-ordered one with a dead-end EB complex, since in both mechanisms the V/K isotope effects do not depend on the substrate varied, or the level of the nonvaried one.

(6) Steady-state random (k_2, k_4, k_5, and k_7 less than or not much larger than V/E_t). When A is varied, c_f varies from

[5] These calculations were originally made graphically (Schimerlik et al., 1975) or with a short computer program and a table (Cleland, 1977) but can now be done by a more powerful computer program which makes an exact solution by successive approximation. It does not matter which of the perturbant molecules is initially labeled; this determines only the direction of the perturbation, not its size. To make these calculations in the usual manner, we must know $^{D}K_{eq}$ [a compilation of values useful for biochemists is in Cleland (1980)], but we will show in the fourth of the four papers in this issue (Cook et al., 1981) how the equilibrium perturbation method can be used to determine the equilibrium isotope effect as well as the kinetic isotope effects in both directions by varying the ratio of the perturbant molecules in the experiment. This procedure is especially useful when the equilibrium isotope effect is small and difficult to determine by direct comparison of equilibrium constants for labeled and unlabeled molecules, as is usually the case with heavy atoms such as ^{15}N.

[6] $^{D}k_9$ will be given by an equation analogous to eq 6 with only the internal part of c_f, but all of c_r, being included.

k_9/k_5 at infinite B to $k_9/(k_4 + k_5)$ at near zero B, so the apparent V/K isotope effect will stay the same as B is lowered if A is not sticky ($k_5 \gg k_9$) or if B is very sticky ($k_4 \ll k_9$). If A is sticky and B is not, however, the isotope effect increases as B is lowered and reaches $^Dk_9$ at low B. When B is varied, c_f varies from k_9/k_4 at infinite A to $k_9/(k_4 + k_5)$ at near zero A, and the isotope effect does not change as A is lowered if B is not sticky, or if A is very sticky. The isotope effect increases to $^Dk_9$ at low A, however, if B is sticky and A is not. Thus, the isotope effect for the stickiest substrate shows the greatest dependence on the level of the other substrate. In theory, a steady-state random mechanism should show nonlinear reciprocal plots, but in practice the curvature is often too small to detect, and the dependence of the V/K isotope effects on which substrate is varied, and the level of the fixed one, can prove a valuable tool for identifying such mechanisms.

It is clear from the above examples that the expected V/K isotope effects in bireactant mechanisms are mechanism dependent and can be used to distinguish between various mechanisms. For deuterium isotope effects, it does not matter which substrate is deuterated (as long as k_9 is the only isotope-dependent step), but which substrate is varied and the level of the nonvaried substrate may affect the magnitude of the isotope effect. For tritium isotope effects, the level of the tritiated substrate used does not matter, and the equations and analysis used above for deuterium isotope effects can be used by replacing "varied substrate" with "tritiated substrate", and "level of nonvaried substrate" with "level of nontritiated substrate". $^Tk_9$ also replaces $^Dk_9$, of course. Finally, it should again be emphasized that $^{D(T)}k_9$ will usually not be an intrinsic isotope effect, but will be reduced by internal commitments resulting from non-isotope-dependent steps before and/or after the bond-breaking step.

Isotope Effects in Terreactant Mechanisms. In a terreactant mechanism that is fully random, the denominator of the equation for c_f (analogous to eq 12 or 13) has three terms, each corresponding to the release of one of the three substrates from the EABC complex (if a substrate cannot be released from EABC, then the corresponding term is of course missing.) If all substrates are at low levels, the denominator is just the sum of the three rate constants for substrate release from EABC, but as substrate concentrations are raised each term will decrease to zero whenever saturation with another prevents substrate release by that path (or will change from one value to another if the presence of the other substrate only alters the rate of, and does not totally prevent, substrate release). The isotope effects thus give two types of information: (1) the stickiness of each of the substrates from the EABC complex and (2) any obligate order of addition among the substrates.

Determination of Kinetic Mechanism by Use of Equilibrium Perturbation. Schimerlik et al. (1975) showed that ^{D}(Eq.P.) varied with the concentration of a reactant other than the two perturbant molecules when this reactant slowed down or prevented release of a perturbant molecule from the enzyme. In the ordered mechanism:

$$\mathrm{EA} \underset{k_4}{\overset{k_3\mathrm{B}}{\rightleftharpoons}} \mathrm{EAB} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{EPQ} \underset{k_8\mathrm{P}}{\overset{k_7}{\rightleftharpoons}} \mathrm{EQ} \underset{k_{10}\mathrm{Q}}{\overset{k_9}{\rightleftharpoons}} \mathrm{E} \qquad (14)$$

where B and Q are the molecules between which label is exchanged during the perturbation experiment, and only k_5 and k_6 are isotope dependent. The isotope effect obtained by equilibrium perturbation is given by an equation analogous to 6, except that it involves $^Dk_5$ and commitment factors for the perturbant molecules. Thus, c_b and c_q replace c_f and c_r, where c_b is k_5/k_4 and

$$c_q = (k_6/k_7)/(1 + k_8P/k_9) \qquad (15)$$

As P goes to zero,[7] $c_q = k_6/k_7$ while as P goes to infinity c_q becomes very large, and the isotope effect from the B side becomes equal to $^DK_{eq}$, and that from the Q side is 1.0.

If, however, Q is released from the ternary complex at a finite rate as in mechanism 16, the equation for the isotope

$$\mathrm{EA} \underset{k_4}{\overset{k_3\mathrm{B}}{\rightleftharpoons}} \mathrm{EAB} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{EPQ} \underset{k_8\mathrm{P}}{\overset{k_7}{\rightleftharpoons}} \mathrm{EQ} \underset{k_{10}\mathrm{Q}}{\overset{k_9}{\rightleftharpoons}} \mathrm{E} \qquad (16)$$
$$\mathrm{EPQ} \underset{k_{12}\mathrm{Q}}{\overset{k_{11}}{\rightleftharpoons}} \mathrm{EP}$$

effect still has the same general form as eq 6, and c_b is the same (k_5/k_4), but the reverse commitment factor becomes

$$c_q = \frac{k_6}{k_{11} + [k_7k_9/(k_8P + k_9)]} \qquad (17)$$

Now as P goes to zero, $c_q = k_6/(k_7 + k_{11})$ while as P goes to infinity $c_q = k_6/k_{11}$, and the isotope effect from the B side will decrease to a constant value greater than $^DK_{eq}$, with the degree of change dependent on the ratio of k_7 to k_{11}, and the relative sizes of k_5/k_4 and k_6/k_{11}. This pattern clearly establishes the random nature of the mechanism, as well as determining the stickiness of Q from the ternary complex (k_{11}/k_6) when c_b is small or is known.

The extension of this theory to terreactant mechanisms is a logical one. In mechanism eq 18 where B and R are the perturbant molecules and k_5 and k_6 are the only isotope-dependent steps, c_b is k_5/k_4 and

$$\mathrm{EA} \underset{k_4}{\overset{k_3\mathrm{B}}{\rightleftharpoons}} \mathrm{EAB} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \mathrm{EPQR} \underset{k_8\mathrm{P}}{\overset{k_7}{\rightleftharpoons}} \mathrm{EQR} \underset{k_{10}\mathrm{Q}}{\overset{k_9}{\rightleftharpoons}} \mathrm{ER} \underset{k_{12}\mathrm{R}}{\overset{k_{11}}{\rightleftharpoons}} \mathrm{E} \qquad (18)$$

$$c_r = k_6/k_7\,[1 + (k_8P/k_9)(1 + k_{10}Q/k_{11})] \qquad (19)$$

Thus, as either P or Q is increased, c_r becomes very large and the isotope effect in the forward direction given by the equation analogous to eq 6 becomes $^DK_{eq}$. As P and Q go to zero, c_r goes to k_6/k_7 (the expression for c_p).

The other extreme in Bi-Ter reactions is complete randomness for release of P, Q, and R, as illustrated in mechanism 20, where B and R are the perturbants and k_5 and k_6 are the only isotope-dependent steps:

$$\begin{array}{ccccccccc} & & & & \mathrm{EPQ} & & & & \\ & & & \overset{k_{11}}{\nearrow} & & & & & \\ \mathrm{EA} & \underset{k_4}{\overset{k_3\mathrm{B}}{\rightleftharpoons}} & \mathrm{EAB} & \underset{k_6}{\overset{k_5}{\rightleftharpoons}} & \mathrm{EPQR} & \underset{k_8\mathrm{P}}{\overset{k_7}{\rightleftharpoons}} & \mathrm{EQR} & \xrightarrow{k_9} & \mathrm{EQ} \\ & & & & \underset{k_{16}\mathrm{Q}}{\swarrow} & \underset{k_{14}\mathrm{Q}}{\overset{k_{13}}{\rightleftharpoons}} & & & \\ & & & & \mathrm{EPR} & \underset{k_{18}\mathrm{P}}{\overset{k_{17}}{\rightleftharpoons}} & \mathrm{ER} & \xrightarrow{k_{19}} & \mathrm{E} \\ & & & & & \searrow^{k_{21}} & & & \\ & & & & & & \mathrm{EP} & & \end{array} \qquad (20)$$

In eq 20, R can be released from two ternary complexes (EPR and EQR) in addition to the quaternary and binary complexes. For determination of whether R is released from either of the ternary complexes and the quaternary complex and also what the flux is through these pathways, the terreactant mechanism is reduced to two bireactant mechanisms. If P is maintained

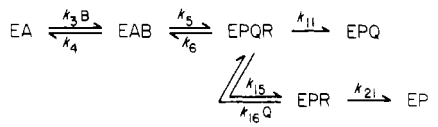
[7] As P goes to zero, the commitment of Q becomes that of P. This is very useful where Q is a nucleotide or other sticky molecule, and k_9 is smaller than k_6. If P is not sticky (that is, $k_7 \gg k_6$), use of low P in an equilibrium perturbation experiment allows one to eliminate the commitment for the nucleotide and see the maximum possible isotope effect.

Table I: Deuterium Isotope Effects for Yeast Alcohol Dehydrogenase[a]

$^{D}(V/K_{2\text{-propanol}})$	$^{D}V_{2\text{-propanol}}$	$^{D}(V/K_{DPN})$	$^{D}(K_{i\,DPN})$	eq fitted[b]	σ[c]
3.06 ± 0.37	0.59 ± 0.45	2.38 ± 0.34	0.60 ± 0.17	2	0.38
2.22 ± 0.04	2.11 ± 0.38	1.91 ± 0.05		3L[d]	0.02[d]
2.40 ± 0.10	1.31 ± 0.28	1.87 ± 0.17		3	0.40
1.35 ± 0.60	556 ± ∞			4	499

[a] 2-Propanol-2-(*h,d*) concentration was varied at several levels of DPN at pH 8 and 25 °C, and all data were fitted to the equations indicated. [b] The fit to eq 5, which assumes an isotope effect on $V/K_{2\text{-propanol}}$ only, did not converge. [c] σ is equal to (sum of squares of residuals)/(degrees of freedom) where degrees of freedom are defined as (number of points) minus (number of parameters). [d] Equation 3 was used in the log form. σ now represents the average idfference between the natural logarithms of calculated and experimental velocities, so that 0.02 means an average 2% error. This value of σ cannot be compared with the others, which are in velocity units.

at a very high level, and Q is varied, eq 20 reduces to mechanism 21:

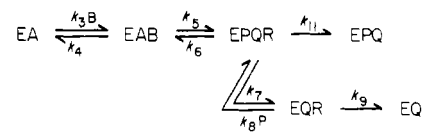


(21)

In this case, c_b remains k_5/k_4, and

$$c_r = \frac{k_6}{k_{11} + [k_{15}k_{21}/(k_{16}Q + k_{21})]} \quad (22)$$

Thus, as Q goes to zero, c_r becomes $k_6/(k_{11} + k_{15})$ while at infinite Q, c_r becomes k_6/k_{11}. Similarly, if Q is maintained at a very high level, and P is varied, mechanism 20 reduces to mechanism 23:



(23)

where c_b is k_5/k_4, and

$$c_r = \frac{k_6}{k_{11} + [k_7k_9/(k_8P + k_9)]} \quad (24)$$

As P goes to zero, c_r becomes $k_6/(k_{11} + k_7)$ while as P becomes infinite $c_r = k_6/k_{11}$.

The extension of this method to the left side of a Bi-Ter reaction, or either side of a Ter-Ter reaction, is straightforward. Thus, the variation of the observed isotope effect obtained by equilibrium perturbation with the concentrations of reactants other than the perturbant molecules allows one to tell from which complexes the perturbants are released and their rate of release from each of the complexes relative to the bond-breaking step.

Results

Yeast Alcohol Dehydrogenase. In the direction of 2-propanol oxidation, the kinetic parameters at pH 8 in 100 mM Tris-HCl were the following: $V_{2\text{-propanol}}/E_t = 44\ s^{-1}$, $K_{DPN} = 1.12 \pm 0.22$ mM, $K_{i\,DPN} = 0.75 \pm 0.12$ mM, $K_{2\text{-propanol}} = 38.5 \pm 7.6$ mM, $K_{i\,2\text{-propanol}} = 25.8 \pm 4.1$ mM. When 2-propanol-2-(*h,d*) concentration was varied at different levels of DPN, the data in Table I were obtained. Isotope effects are clearly present on both V/K values and are about equal. The standard errors of all three parameters, $^{D}(V/K_{2\text{-propanol}})$, $^{D}V_{2\text{-propanol}}$, and $^{D}(V/K_{DPN})$, were improved by fitting the data to eq 3 in the log form. Thus, a significant isotope effect on V is most likely also present. It is unlikely that any effect is really present on $K_{i\,DPN}$ since the fit to eq 2 gives large standard errors and an unrealistically large inverse effect on V and $K_{i\,DPN}$, without a significantly lower value of σ.

Isotope effects determined by using the equilibrium perturbation method at pH 8.2 for yeast alcohol dehydrogenase as a function of acetone concentration are shown in Figure 1. Since data could not be obtained above 170 mM acetone, the limiting value at infinite acetone concentration is not well defined. A fit to eq 25 which describes a hyperbola, where

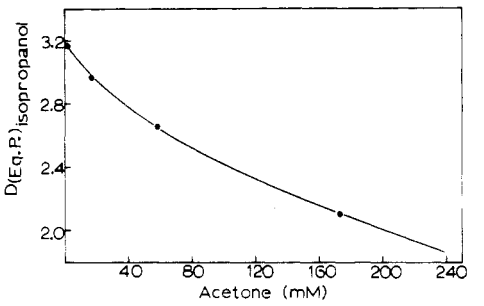


FIGURE 1: Dependence of $^{D}(Eq.P.)_{2\text{-propanol}}$ on the concentration of acetone. The solid line represents a fit to eq 26. Conditions were the following: Tris-HCl, pH 8.2, 100 mM; acetone, 2–170 mM; 2-propanol-2-*d*, 4–25 mM; DPN, 0.2–0.3 mM; DPNH, 0.2 mM; yeast alcohol dehydrogenase, 35 units.

$$y = \frac{a(1 + P/K_{I\,num})}{(1 + P/K_{I\,denom})} \quad (25)$$

y is $^{D}(Eq.P.)_{2\text{-propanol}}$, a is the value of $^{D}(Eq.P.)_{2\text{-propanol}}$ at zero acetone concentration, P is acetone concentration, and $K_{I\,num}$ and $K_{I\,denom}$ are constants, gave 0.7 ± 0.3 for the limiting effect at infinite acetone concentration $[a(K_{I\,denom})/(K_{I\,num})]$. Since the isotope effect should not go below $^{D}K_{eq}$, which is 1.18 (Cook et al., 1980), the data were fitted to eq 26 (a modified

$$y = \frac{a + 1.18(P/K_I)}{1 + P/K_I} \quad (26)$$

form of eq 25 in which y becomes 1.18 at infinite P), giving $K_I = 176 \pm 7$ mM [the value of P that gives $y = (a + 1.18)/2$] and $a = 3.16 \pm 0.02$.

Liver Alcohol Dehydrogenase. Kinetic parameters were obtained at pH 8 in 100 mM Tris-HCl for both oxidation of cyclohexanol and reduction of cyclohexanone: $V_{cyclohexanol}/E_t = 3.0 \pm 0.1\ s^{-1}$; $V_{cyclohexanone}/E_t = 2.2 \pm 0.1\ s^{-1}$; $K_{DPN} = 30.0 \pm 1.3\ \mu M$; $K_{i\,DPN} = 0.012 \pm 0.001$ mM; $K_{cyclohexanol} = 0.56 \pm 0.03$ mM; $K_{DPNH} = 3.1 \pm 0.3\ \mu M$; $K_{i\,DPNH} = 1.6 \pm 0.6\ \mu M$; $K_{cyclohexanone} = 4.0 \pm 0.5$ mM. These values are in reasonable agreement with those obtained by Ainslie & Cleland (1972) at pH 8.6.

At pH 8, when cyclohexanol-1-(*h,d*) concentration was varied at different levels of DPN, or when cyclohexanone concentration was varied at different levels of DPNH and A-side DPND, the data shown in Table II were obtained. In the direction of alcohol oxidation, a significant isotope effect of 3.0–3.2 is obtained on $V/K_{cyclohexanol}$ and a small effect may be present on V, but, clearly, there are no significant isotope effects on V/K_{DPN}, or $K_{i\,DPN}$. In the direction of ketone reduction, significant isotope effects of 1.4, 2.2, and 1.2 are obtained for V, $V/K_{cyclohexanone}$, and V/K_{DPNH}, respectively.

Table II: Deuterium Isotope Effects for Horse Liver Alcohol Dehydrogenase[a]

Cyclohexanol Oxidation					
$^{D}(V/K_{cyclohexanol})$	$^{D}V_{cyclohexanol}$	$^{D}(V/K_{DPN})$	$^{D}(K_{i\,DPN})$	eq fitted	σ[c]
3.14 ± 0.30	1.08 ± 0.09	0.90 ± 0.18	0.43 ± 0.66	2	0.285
3.04 ± 0.23	1.10 ± 0.09	0.85 ± 0.20		3[b]	0.283
3.04 ± 0.24	1.06 ± 0.08			4	0.282
3.19 ± 0.16				5	0.280
Cyclohexanone Reduction					
$^{D}(V/K_{cyclohexanone})$	$^{D}V_{cyclohexanone}$	$^{D}(V/K_{DPNH})$		eq fitted[d]	σ[c]
2.25 ± 0.04	1.46 ± 0.04	1.16 ± 0.05		3L[e]	0.012[e]
2.22 ± 0.08	1.41 ± 0.07	1.25 ± 0.11		3	0.701
2.01 ± 0.08	1.48 ± 0.06			4	0.759
2.63 ± 0.12				5	1.381

[a] Cyclohexanol-*1*-(*h*,*d*) concentration was varied at several levels of DPN, or cyclohexanone concentration was varied at several levels of DPNH or A-side DPND at pH 8 and 25 °C, and the full set of data was fitted to the equation indicated. [b] Isotope effects and their standard errors were virtually identical by using eq 3 in either log or normal forms. [c] σ is equal to (sum of squares of residuals)/(degrees of freedom) where degrees of freedom are defined as (number of points) minus (number of parameters). [d] The fit to eq 2, which assumes isotope effects on $V/K_{cyclohexanone}$, $V_{cyclohexanone}$, V/K_{DPNH}, and $K_{i\,DPNH}$, did not converge. [e] Equation 3 was used in the log form. σ now represents the average difference between the natural logarithms of calculated and experimental velocities, so that 0.012 means an average 1.2% error. This value of σ cannot be compared with the others, which are in velocity units.

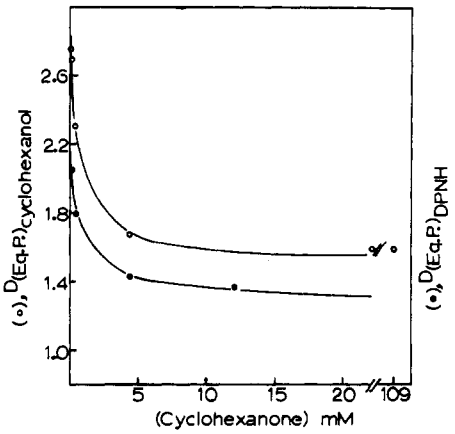


FIGURE 2: Dependence of ^{D}(Eq.P.) on the concentration of cyclohexanone. (○) Label present in cyclohexanol; cyclohexanone concentration varied from 0.011 to 109 mM, and cyclohexanol concentration varied from 0.165 to 61.9 mM. Other conditions were as follows: DPN, 0.13 mM; DPNH, 0.16 mM; phosphate, pH 8.0, 100 mM; liver alcohol dehydrogenase, 0.2 unit. (●) Label present in A-side DPND; cyclohexanone concentration varied from 0.044 to 12 mM, and cyclohexanol concentration varied from 0.57 to 61.9 mM. Other conditions were as follows: DPN, 0.13 mM; DPND, 0.166 mM; Tris-HCl, pH 8, 100 mM; liver alcohol dehydrogenase, 0.25 unit. Solid lines are fits to eq 25.

Isotope effects obtained by equilibrium perturbation at pH 8 by using cyclohexanol-*1*-*d* or A-side DPND as a function of cyclohexanone concentration are shown in Figure 2. The observed isotope effect does not decrease to a value of $^{D}K_{eq}$ (1.18) in the direction of alcohol oxidation or a value of 1 in the direction of ketone reduction, indicating a finite rate of release of DPNH from the ternary E–DPNH–cyclohexanone complex. A fit of the data obtained with cyclohexanol-*1*-*d* to eq 25 gave values of 2.86 ± 0.04 for $^{D}(Eq.P.)_{cyclohexanol}$ at zero cyclohexanone concentration, 1.54 ± 0.03 for $^{D}(Eq.P.)_{cyclohexanol}$ at infinite cyclohexanone concentration, and 0.52 ± 0.09 mM for $K_{cyclohexanone}$ (= $K_{I\,denom}$). A fit of the data obtained with A-side DPND to eq 25 gave values of 2.08 ± 0.01 for $^{D}(Eq.P.)_{DPND}$ at zero cyclohexanone concentration, 1.31 ± 0.01 for $^{D}(Eq.P.)_{DPND}$ at infinite cyclohexanone concentration, and 0.67 ± 0.04 mM for $K_{cyclohexanone}$.

For determination of whether there was any difference in the mechanism for liver alcohol dehydrogenase when ethanol was used as the alcohol substrate instead of cyclohexanol, isotope effects were obtained by equilibrium perturbation as a function of acetaldehyde concentration with ethanol as the alcohol substrate with label in DPND. $^{D}(Eq.P.)_{DPND}$ was 2.86 ± 0.16 at zero acetaldehyde concentration and 1.15 ± 0.20 at infinite acetaldehyde concentration, and $K_{acetaldehyde}$ was 22 ± 11 μM.

Discussion

Yeast Alcohol Dehydrogenase. Since the isotope effects obtained by the equilibrium perturbation method decrease with increasing acetone concentration to a value apparently equal to $^{D}K_{eq}$, an ordered release of acetone and DPNH is indicated, with DPNH being released much more slowly from the ternary E–DPNH–acetone complex than 2-propanol is produced and released as a product.

In the direction of 2-propanol oxidation, $^{D}V_{2\text{-propanol}} \cong {}^{D}(V/K_{2\text{-propanol}}) \cong {}^{D}(V/K_{DPN}) = 2.1$, so that as seen under Theory these data may correspond to a rapid equilibrium addition of DPN and 2-propanol. However, there is a small increase in the isotope effect on both $V/K_{2\text{-propanol}}$ and $V_{2\text{-propanol}}$ (Cook & Cleland, 1981a) as the pH is decreased below the $V/K_{2\text{-propanol}}$ pK, so that there is a small amount of stickiness associated with 2-propanol [the external part of the commitment is eliminated at pH values where V/K is below its pK; see Cook & Cleland (1981a)]. Thus, 2-propanol and DPN are about equally sticky, and DPNH release is slow enough to be partially rate limiting, so that in terms of mechanism 10, k_4, k_5, and k_{11} are roughly equal and just slightly larger than k_9.

Liver Alcohol Dehydrogenase. In the direction of alcohol oxidation, a significant isotope effect (about 3.0) is obtained on only $V/K_{cyclohexanol}$. Thus, since no significant $^{D}(V/K_{DPN})$ is observed, DPN is not released from the ternary E–DPN–cyclohexanol complex at a rate that is appreciable relative to the rate of production and release of cyclohexanone. By direct comparison of initial velocities at saturating levels of DPN, an isotope effect of about 10% was also consistently obtained on $V_{cyclohexanol}$ so that the $^{D}V_{cyclohexanol}$ of 1.06–1.10 shown in Table II is real, even though the standard error is large. Since it will be shown by Cook & Cleland (1981b) that neither cyclohexanol nor cyclohexanone is sticky, the observed isotope effect on $V_{cyclohexanol}$ is lower than the intrinsic deuterium isotope effect (6.3; Cook & Cleland, 1981b) as a result of slower release of reduced nucleotide than hydride transfer from alcohol to DPN at pH 8. Since cyclohexanol is not sticky, $^{D}(V/K_{cyclohexanol})$ is equal to $^{D}k_9$ in eq 11, so that values of 3.0 for $^{D}k_9$ and 1.10 for ^{D}V in eq 11 allow us to calculate that the

rate of hydride transfer from alcohol to DPN is 19 times the rate of DPNH release at pH 8.

In the direction of ketone reduction, a ${}^{D}(V/K_{DPNH})$ value of about 1.2 is observed, indicating a finite rate of release of DPNH from E–DPNH–cyclohexanone relative to the rate of cyclohexanol formation and release. Since cyclohexanone is not sticky, ${}^{D}k_9$ is 2.2, and we can use eq 27 to estimate that

$$ {}^{D}(V/K) = \frac{{}^{D}k_9 + c_f}{1 + c_f} \qquad (27) $$

hydride thansfer from DPNH to cyclohexanone is 5 times the rate of release of DPNH from the ternary E–DPNH–cyclohexanone complex. Since a value of ${}^{D}V_{cyclohexanone}$ less than ${}^{D}(V/K_{cyclohexanone})$ is observed, the partly rate-limiting release of DPN is most likely responsible for the observed isotope effect of 1.4 being lower than the V/K isotope effect. [A ${}^{D}V_{cyclohexanone}$ value of 1.15 has been obtained repeatedly by varying cyclohexanone concentration at saturating DPNH(D) (Cook & Cleland, 1981b), so that this value of 1.15 is probably more accurate than the values of 1.4 in Table II.] From eq 11, we can thus estimate that hydide transfer from DPNH to cyclohexanone is from 2 (corresponding to ${}^{D}V = 1.4$) to 7 (if ${}^{D}V$ is 1.15) times faster than DPN release.

The equilibrium perturbation data for liver alcohol dehydrogenase can be described by mechanism 16 where A, B, P, and Q are DPN, cyclohexanol, cyclohexanone, and DPNH. Since cyclohexanol has a very low commitment, the equation analogous to 6 reduces to

$$ {}^{D}(\text{Eq.P.})_{cyclohexanol} = \frac{{}^{D}k_5 + {}^{D}K_{eq\ cyclohexanol}(c_{DPNH})}{1 + c_{DPNH}} \qquad (28) $$

where c_{DPNH} is given by eq 17. At zero cyclohexanone concentration, ${}^{D}(\text{Eq.P.})_{cyclohexanol}$ (2.86) is in good agreement with the value of ${}^{D}(V/K_{cyclohexanol})$ (3.0), as predicted; that is, at zero concentration of the molecule which can slow down or prevent release of a perturbant, the observed isotope effect becomes equal to the V/K effect for the labeled reactant. At high cyclohexanone concentration, substitution of a value of 2.86 for ${}^{D}k_5$ (the maximum observed isotope effect on k_5, which is smaller than the intrinsic effect due to internal commitments), 1.18 for ${}^{D}K_{eq\ cyclohexanol}$, and 1.54 for ${}^{D}(\text{Eq.P.})_{cyclohexanol}$, a value of 3.6 is calculated for $c_{DPNH} = k_6/k_{11}$. Thus, at high cyclohexanone concentration, release of DPNH from the ternary complex occurs at 28% the rate of hydride transfer from DPNH to cyclohexanone. The value of 3.6 for k_6/k_{11} is in good agreement with the value of 5 calculated for this same ratio above by using the direct comparison of initial velocities. At the cyclohexanone concentration that produces half the change in the isotope effect, ${}^{D}(\text{Eq.P.})_{cyclohexanol} = 2.21$, while ${}^{D}k_5$ and ${}^{D}K_{eq\ cyclohexanol}$ have the same values given above, so that $c_{DPNH} = 0.65$. Solving eq 17 for cyclohexanone concentration gives eq 29. The value of P which produces half

$$ P = (k_7/k_8)[k_9/(1.54k_6 - k_{11}) - k_9/k_7] \qquad (29) $$

the change in the isotope effect is 0.53 mM, while k_7/k_8 ($K_{i\ cyclohexanone}$) = 49 mM (Ainslie & Cleland, 1972) and $k_6 = 3.6k_{11}$. Substituting into eq 29:

$$ 0.011 = k_9/4.4k_{11} - k_9/k_7 \qquad (30) $$

Since ${}^{D}V_{cyclohexanol}$ is small (1.15) due to the slow release of DPNH from the binary complex relative to hydride transfer, and cyclohexanone has a very low commitment, $k_7 \gg k_9$, and k_9/k_{11} has a lower limit of 0.048. An upper limit can be calculated by using $k_7 \cong 20k_9$ [from the isotope exchange data of Ainslie & Cleland (1972)], giving a value of 0.27 for k_9/k_{11}. Thus, the off rate for DPNH from the ternary complex is approximately 3.7–21 times the off rate of DPNH from the binary complex. Cyclohexanone thus acts in a manner similar to cyclohexanol at high concentrations to produce an increase in the rate of dissociation for DPNH (the cyclohexanol effect results in substrate activation of about 5-fold on the intercepts of reciprocal plots when DPN concentration is varied; Dalziel & Dickinson, 1966; Wratten & Cleland, 1963; Ainslie & Cleland, 1972).

The calculations presented about show the power of isotope effect studies to give quantitative, as well as qualitative, information on the mechanism of an enzyme-catalyzed reaction. In the next two papers of this series, we will show how the pH variation of isotope effects can be used to give even more information concerning the mechanism.

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