# USE OF ISOTOPE EFFECTS TO ELUCIDATE ENZYME MECHANISMS

Author: **W. Wallace Cleland**
Department of Biochemistry
University of Wisconsin
Madison, Wisconsin

Referee: Richard L. Schowen
Department of Chemistry
University of Kansas
Lawrence, Kansas



## I. INTRODUCTION

Kinetic studies are one of the best ways to study enzyme mechanisms, since one is looking at the reaction while it occurs. X-ray structures of enzymes are very useful to have, and chemical modification studies may identify groups in the active site, but the kinetics are the ultimate arbiter. Kinetic analysis takes place in three stages. First, one determines the kinetic mechanism; that is, the order of combination of enzyme and substrates and of dissociation of products. Second, one determines the relative rates of the various steps in the kinetic mechanism. Third, one attempts to describe the chemical mechanism of the reaction in the terms of the physical organic chemist. One thus is concerned with acid-base catalysis, geometry changes, electron movements during the reaction, and transition state structure. For a general overview of this process of studying an enzyme mechanism, see References 1 and 2.

One of the newest but most powerful types of kinetic study of enzyme mechanisms involves the use of isotope effects. Chemists have been using isotope effects for years to study chemical reactions,[3-6] but enzymologists have made much less use of isotope effect studies, both because the theory for the experiments was not fully developed and because suitably precise methods for measuring heavy atom or secondary deuterium isotope effects were not available. The last several years, however, have seen a tremendous development in both theory and experimental techniques, and we can now say that the field is wide open for exploitation. This review is thus timely and will summarize the new theory and techniques and the uses to which they have been put to date in the study of enzyme mechanisms. The reader may also find the recent reviews of Klinman[6a] and Northrop [6b] useful.

## II. NATURE OF ISOTOPE EFFECTS

### A. Equilibrium Isotope Effects

Equilibrium isotope effects are changes in equilibrium constants as the result of isotopic substitution. They result from differences in the bonding of the substituted atom in reactant and product and in particular from differences in vibrational modes and frequencies. The force constants for these vibrations can be determined from infrared spectra, so when a force field can be accurately determined, one can calculate equilibrium isotope effects.[7] They can also be measured experimentally.[8] Such effects are determined almost entirely by the immediate structure surrounding the isotopic atom, and more remote structural differences make little contribution. It is thus practical to construct a table of fractionation factors which lists equilibrium isotope effects relative to a standard compound.[9]

In the table in Reference 9, the reference compound for deuterium and $^{18}O$ isotope effects is liquid water, while $CO_2$ in aqueous solution is used for $^{13}C$ and $NH_3$ in aqueous solution for $^{15}N$. Other reference compounds can be used; Hartshorn and Shiner[7] used acetylene as the reference for deuterium and $^{13}C$ fractionation factors. In comparing two structures in such a table, the heavy isotope (deuterium, for example) becomes enriched in the compound with the highest fractionation factor and the light isotope in the one with the lower value. The equilibrium isotope effect for going from one structure to the other ($K_{eq\ H}/K_{eq\ D}$) is thus the fractionation factor of the reactant divided by that of the product. We will designate isotope effects with a leading superscript corresponding to the isotope (D, T, 13, 15, 18 meaning deuterium, tritium, $^{13}C$, $^{15}N$, or $^{18}O$; this notation will be used for kinetic as well as equilibrium isotope effects), and thus $K_{eq\ H}/K_{eq\ D}$ will be written $^{D}K_{eq}$, and $^{15}K_{eq}$ would be an $^{15}N$ equilibrium isotope effect.

Tritium isotope effects are the 1.442 power of deuterium ones,[10] and this relationship holds for equilibrium as well as for intrinsic kinetic isotope effects (it will not necessarily hold for observed isotope effects; see Section IV below). Thus:

$$^{T}K_{eq} = (^{D}K_{eq})^{1.442} \quad (1)$$

$^{14}C$ isotope effects are calculated from $^{13}C$ ones by the expression:[3]

$$^{14}K_{eq} - 1 = 1.9(^{13}K_{eq} - 1) \quad (2)$$

Again, the same equation holds for intrinsic kinetic isotope effects.

Equilibrium isotope effects can either be primary (such as for deuterium transfer from ethanol to DPN in a dehydrogenase reaction) or secondary (for reduction of deuterated acetaldehyde to ethanol), but the same fractionation factors are used to calculate the value, and it makes no difference whether the isotopic atom is in the same or a different molecule after the reaction. Thus the equilibrium isotope effect for the first example is the ratio of fractionation factors for the $-CH_2OH$ group of ethanol and the $-CH_2-$ group at C-4 of DPNH, while that for the second example is the ratio of fractionation factors for the equilibrium mixture of $-CHO$ and $-CH(OH)_2$ and that for $-CH_2OH$.

As noted above, equilibrium isotope effects can be calculated from infrared frequencies, but this works well only for spectra of small molecules in the gas phase, and the resulting values apply only in the gas phase. Fractionation factors for $^{13}C$, and for deuterium attached to carbon, will not be appreciably different in the gas phase and in water. For $^{15}N$ and especially $^{18}O$ isotope effects, however, there will be hydrogen bonding to the isotopic atom in water, and not in the gas phase, and thus vapor pressure isotope effects must be applied to values calculated in the gas phase. For example, $^{18}O$ enriches in liquid water compared to the vapor by 1.0091,[11] and similar effects are expected for alcohols, carbonyls, and carboxylates (with alcohols probably showing somewhat smaller and ionized carboxyls somewhat higher vapor pressure isotope effects; experimental values are badly needed here!). The value for methanol vapor relative to liquid methanol is 1.006,[11a] but the value relative to an aqueous solution of methanol is not known.

It thus becomes important to determine equilibrium isotope effects experimentally, and the majority of values in the table in Reference 9 are based on experimental as well as calculated values. For equilibrium deuterium isotope effects, it is usually sufficient simply to compare equilibrium constants with deuterated and unlabeled substrates, and this can be done with 1% accuracy in most cases.[8] For equilibrium $^{18}O$ isotope effects when water is one of the reactants (the hydration of fumarate to malate catalyzed by fumarase, for example[12]) one can make up a reaction mixture in water that is at equilibrium, and add to two aliquots either an equal volume of $H_2O$ or $H_2[^{18}O]$, followed by a

catalytic amount of enzyme. The difference in final optical density (from fumarate, in the example given) is then used to calculate the equilibrium $^{18}O$ isotope effect (one must multiply the [isotope effect — 1] by 2, of course, since one is comparing equilibrium constants in unlabeled and in 50% [$^{18}O$]-labeled water). The same approach can be used with $D_2O$ when hydrogen is transferred from a reactant to water (again, fumarase is an example), but now corrections must be made for the altered extinction coefficients of the colored molecule in $D_2O$, $H_2O$, or mixtures of the two[12] and for the different fractionation factors of deuterium in $H_2O$ and in $D_2O$. This latter effect results from the fact that $K_{eq}$ for the reaction $H_2O + D_2O \rightleftharpoons 2HDO$ is not 4.00, but about 3.78,[13,14] so that deuterium fractionation factors measured in $D_2O$ must be divided by 3.78/4.00 = 0.945 to correct them to $H_2O$. Thus the fumarase equilibrium constant is 7% different in $D_2O$ and in $H_2O$, but the true fractionation factor of the $-CH_2-$ group in malate is 0.98 relative to water (0.93 relative to $D_2O$).

Equilibrium isotope effects for heavy atoms such as $^{13}C$ or $^{15}N$ may be determined in favorable cases by isotope ratio mass spectrometry. For example, the equilibrium isotope effect in the isocitrate dehydrogenase reaction:

$$\text{TPN} + \text{Isocitrate} \rightleftharpoons CO_2 + \alpha\text{-Ketoglutarate} + \text{TPNH} \qquad (3)$$

has been measured by letting the reaction reach equilibrium in a closed system and then determining the $^{13}C$ content of the $CO_2$ in the gas phase and of the isocitrate (by complete decarboxylation enzymatically to $CO_2$).[15] A similar approach has been used to measure the equilibrium $^{15}N$ effect for conversion of glutamate to ammonia in the glutamate dehydrogenase reaction (1.026),[18] since it is possible to isolate ammonia and measure its mass ratio accurately after conversion to $N_2$. In this case, the mass ratio of ammonia remaining after 85% had been converted to glutamate, isotopic equilibrium had been reached, and NaOH added to inactivate the enzyme and allow recovery of the ammonia was compared with that of the starting material, and the ratio was corrected for the incomplete conversion to glutamate.

As a final resort, it is possible to measure equilibrium isotope effects directly in favorable cases by the equilibrium perturbation method. This is normally a method for determining a kinetic isotope effect (see Section III.C.), and to use it one needs to know the equilibrium isotope effect, since the kinetic isotope effects in forward and reverse directions are calculated from the size of the perturbation by making their ratio equal to the equilibrium isotope effect. However, the size of the perturbation seen depends on the ratio of the concentrations of the two reactants between which label is exchanged during the perturbation, and by changing this ratio (by altering the concentrations of the other reactants) one can alter the apparent isotope effect calculated from the perturbation size from that in the forward direction to that in the reverse direction. This method has so far only been used to determine an equilibrium $^{15}N$ isotope effect of 1.038 for conversion of DPN to DPNH, and the equations and procedures for the analysis are given in that work.[16]

### B. Kinetic Isotope Effects

Kinetic isotope effects result from altered rates caused by isotopic substitution. They represent equilibrium isotope effects in going from the reactant to the transition state for the reaction, but since the vibrational frequencies in the transition state can only be guessed at, calculations of kinetic isotope effects are much less reliable than those for equilibrium isotope effects. It should be noted that the ratio of kinetic isotope effects in forward and reverse directions on a chemical reaction is the equilibrium isotope effect, since one is comparing fractionation factors of the same transition state with those of reactant or product. For an enzymatic reaction, however, things may be more complex,

and the actual equations which apply will be discussed in Section IV.

Kinetic isotope effects can be either primary or secondary. Primary isotope effects involve bond making or breaking to the isotopic atom and are almost always normal (that is, slower reaction for the heavier atom), since the force constants for some vibrational frequencies in the transition state are greatly decreased or even negative for motion along the reaction coordinate which constitutes the reaction. An exception to this rule is the inverse $^{37}Cl$ isotope effect on reactions of unsolvated $Cl^-$ acting as a nucleophile;[17] the chloride is more tightly bonded in the transition state since it is not bonded at all as the free ion. This will be discussed further in Section IX, which concerns the size of isotope effects and transition state structure.

Secondary isotope effects involve changes in bonding of the isotopic atom, but no actual bond making or breaking during the reaction. While primary isotope effects are usually much larger than the equilibrium isotope effect, so that normal values are seen in both forward and reverse reactions, it is common for secondary isotope effects to be closer to unity than the equilibrium isotope effect, and thus normal in one direction and inverse in the other. Thus if $^{D}K_{eq} = 0.80$ in the forward direction, $^{D}k$ (that is $k_H/k_D$) in this direction might be 0.9, and $^{D}k$ in the reverse direction 1.125, compared to a $^{D}K_{eq}$ value of 1.25 in the reverse reaction. This situation will occur whenever the fractionation factor of the transition state is intermediate between that of reactant and product.

If the fractionation factor for the transition state is higher than that of either reactant or product, the secondary isotope effect may be inverse in both directions (there may be no equilibrium isotope effect, in fact). Conversely, if the isotopic atom is more loosely bound bonded in the transition state than in either reactant or product, a normal isotope effect is seen in both directions. This was the case with [$^{15}N$]-DPN and liver alcohol dehydrogenase, for example ($^{D}k$ was 1.06 from the DPN side and 1.02 from the DPNH side[16]).

Secondary deuterium isotope effects are called $\alpha$ when deuterium substitution is on a carbon undergoing bond cleavage during the reaction. Examples are the hydrogen at C-4 of DPN during conversion to DPNH or at C-1 of an aldehyde during reduction to an alcohol. The effects are called $\beta$ when the deuterium is on a carbon next to the one undergoing bond cleavage. $\beta$-Secondary deuterium isotope effects result from hyperconjugation in aldehydes, ketones, or carbonium ions and its absence in the corresponding molecules with tetrahedral carbon.

## III. METHODS OF MEASURING ISOTOPE EFFECTS ON ENZYME-CATALYZED REACTIONS

### A. Direct Comparison

In this technique, one runs reciprocal plots ($1/v$ vs. $1/S$) in a system with labeled or unlabeled substrates and determines the isotope effects on V and V/K ($^{D}V$ and $^{D}(V/K)$) from the ratios of vertical intercepts or slopes of the lines for labeled and unlabeled substrates. Any substrate can be varied; it does not need to be the labeled one, but one obtains $^{D}(V/K)$ for the varied substrate rather than for the labeled one. Computer programs are available to analyze such data and give standard errors of the estimates.[6] In these experiments it is necessary to have a high degree of isotopic substitution and isotope effects of a reasonable size, and thus this method is used almost exclusively to measure deuterium isotope effects. It has, however, been used to measure the isotope effect with [$^{15}N$]-DPN on formate dehydrogenase,[18] which is 1.07, and whenever the experiment can be so arranged that the isotope effect is the same on V and V/K, values can be determined to 1 or 2% accuracy. By the use of automated digital readout with a sensitive spectrophotometer, Rosenberg and Kirsch[18a] have refined the direct comparison of rates

with $^{18}$O-labeled and unlabeled substrates under saturating conditions to determine $^{18}V$ values with a precision of 0.1% and a reproducibility of 0.5%.

The precision in measuring $^{D}(V/K)$ depends on how well the concentrations of labeled and unlabeled substrates are known, since V/K is the first-order rate constant for reaction at low substrate levels. Thus, solutions of labeled and unlabeled substrates should be calibrated by accurate enzymatic assay; with care this can be done to 1% accuracy.[8] The purity of the labeled and unlabeled substrates does not affect V/K when they are the variable substrates, but will cause problems if another substrate is the varied one and a competitive inhibitor is present in different levels in unlabeled and labeled substrates whose concentrations are held high and constant during the experiment.[19] (The problem can be overcome by keeping the fixed levels of these substrates *below* saturation; the inhibitors thus have no effect.)

Accurate determination of $^{D}V$ does not depend on knowing the concentrations of the labeled and unlabeled substrates, but their purity is critical. Since a competitive inhibitor present in the variable substrate affects V and not V/K,[20] it is essential that no competitive inhibitor be present in the labeled and unlabeled substrates. Grimshaw and Cleland[19] have outlined the problems one can meet with impurities in direct comparison experiments, and their paper should be thoroughly studied by anyone who does such experiments.

The advantages of the direct comparison method are that it is the only way to determine $^{D}V$ and it is an easy method to use, since deuterated substrates are not hard to prepare, and the data analysis is straightforward. The disadvantages are the purity and accurate concentration calibrations required for reactants and a usual limit on sensitivity of perhaps 1.05 when isotope effects are not the same on V and V/K.

**B. Internal Competition**

In this method the heavy isotope is normally used as a trace label and the change in isotopic ratio between substrate and product is determined. This is the method used for tritium isotope effects and with the isotope ratio mass spectrometer for following natural abundance D, $^{13}$C, $^{15}$N, or $^{18}$O isotope effects. One normally measures isotope ratios in product at low percent conversion and compares this with the isotope ratio in product at 100% conversion according to the equation:

$$^{T,\,13,\,etc}(V/K) = \frac{\log(1 - f)}{\log(1 - fR/R_o)} \qquad (4)$$

where $R_o$ and R are isotope ratios in initial substrate (or product at f = 1.0) and product at fractional conversion f. Double labeling is sometimes used in tritium experiments, with a $^{14}$C label in a part of the molecule not affected by the reaction serving as a marker for the nontritiated molecules. $^{3}H/^{14}C$ ratios in substrate (or product at f = 1.0) are thus compared with ratios in product at low f. A more sophisticated triple label technique was first used by Dahlquist et al.[20a] to determine a secondary deuterium isotope effect with lysozyme. They used a mixture of ([$^{14}$C]-phenyl)-$\beta$-D-glucoside-1-d and ([$^{3}$H]-phenyl)-$\beta$-D-glucoside as a substrate and determined the discrimination caused by the deuterium from the tritium to $^{14}$C ratio in the liberated phenol.

The precision of this method with the isotope ratio mass spectrometer is very good, with isotope effects less than 1.001 easily determined. The problem is that if one does not generate during the reaction a molecule easily measured in the spectrometer ($CO_2$, $H_2$, $N_2$), is is necessary to convert the product chemically or enzymatically into such a molecule with *no further isotopic fractionation or exchange.* In favorable cases, however, one may be able to follow the mass ratio of a product such as ethanol[20b] or

2,4-dinitrophenol[20c] directly in a normal mass spectrometer. Such an approach requires enriched substrates (preferably close to 50% labeled, so the two peaks being observed are nearly the same size), but has been used to measure $^{18}O$ isotope effects with errors reported to be 0.04 to 0.07% when ethanol was determined[20b] and 0.6% when 2,4-dinitrophenol was the product.[20c]

Another approach which preserves the sensitivity of the isotope ratio mass spectrometer involves the use of remote labels as sensors for isotopes in other positions. This technique, which was developed by O'Leary and Marlier,[21] is potentially a very powerful one. For example, the $^{18}O$ isotope effect with formate dehydrogenase has been determined [18] by using formate prepared by mixing 1.5% $H[^{13}C][^{18}O]_2^-$ with 98.5% $[^{12}C]$-formate depleted in $^{13}C$. This formate now contains close to the normal natural abundance of $^{13}C$ (thus minimizing the errors from any contamination with atmospheric $CO_2$ in the final analysis), but every $^{13}C$ is accompanied by two $^{18}O$ atoms ($^{18}O$ in formate does not exchange with water at neutral pH, but does do so at acid pH, so that $^{18}O$ is easily introduced by exchange with $H_2[^{18}O]$). The observed kinetic isotope effect is thus the product of the $^{13}C$ and $^{18}O$ effects. Since the $CO_2$ product is equilibrated with water before being subjected to isotopic ratio analysis, the $^{18}O$ washes out, and only the $^{13}C$ mass ratio is actually measured. The $^{13,18,18}(V/K)$ value determined in this way is divided by the $^{13}(V/K)$ value determined with normal formate containing natural abundance $^{13}C$ and the square root of the remainder gives the desired $^{18}(V/K)$ value.

A similar remote-label method should allow measurement of the $^{18}O$ isotope effect on hexokinase, which has so far not been detected by the equilibrium perturbation method.[22] If 1-$[^{13}C]$-glucose has $^{18}O$ introduced into the 6 position, one can mix 1.1% of this 1-$[^{13}C]$, 6-$[^{18}O]$-glucose with 98.9% of 1-$[^{12}C]$-glucose prepared by reaction of $^{13}C$-depleted KCN with arabinose to obtain a double-labeled glucose suitable for use in the hexokinase reaction at any pH value. The glucose-6-P produced at low extent of reaction and that produced by complete phosphorylation of the substrate can then separately be converted by glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase to ribulose-5-P with liberation of $CO_2$ from C-1. Since there should be no kinetic $^{13}C$ isotope effect at C-1 from the hexokinase reaction (this would be checked, however), the observed isotope effect should be that caused by $^{18}O$ substitution.

To determine isotope effects with ATP, DPN, or another adenine-containing compound, the remote label can be in the amino group of adenine. Thus mixing 0.4% DPN labeled with $^{15}N$ in the adenine amino group and $^{13}C$ at C-4 of the nicotinamide ring with 99.6% DPN containing depleted $^{14}N$ in the adenine amino group would give a molecule with near natural abundance $^{15}N$ in the amino group, but with every $^{15}N$ accompanied by a $^{13}C$ in the nicotinamide ring. For determination of the $^{13}C$ isotope effect in a dehydrogenase reaction the DPNH produced would be degraded to adenosine, which would be treated with adenosine deaminase to give ammonia, which is readily converted to $N_2$ for mass ratio analysis. This approach would also allow determination of isotope effects resulting from $^{18}O$ substitution in the $\beta,\gamma$-bridge, $\beta$ or $\gamma$ nonbridge, or any other position in ATP.

The precision of the internal competition method with tritium in our experience is not as good as that obtained by direct comparison experiments with deuterium labeling. The reason for this is not clear, but it probably results from the difficulty in accurately counting tritium. To obtain accurate results one should add an internal standard to every vial after the initial counting and count again, correcting each result for recovery of the internal standard.

For heavy atom and secondary tritium isotope effects, it is possible to determine the isotope effect in an internal competition experiment from the enrichment of the heavier isotope in the substrate as the reaction proceeds. If $R_f$ is now the specific activity or mass

ratio in the substrate, rather than in the product at extent of reaction f, the equation for the isotope effect is

$$^{T}(V/K) = \frac{1}{1 + \frac{\log(R_f/R_o)}{\log(1-f)}} \qquad (4a)$$

The value of $R_f/R_o$ is 1.0 at very low f and for normal isotope effects increases gradually at first (the initial slope is $1 - 1/^{T}[V/K]$) and then more rapidly, reaching infinity at $f = 1.0$. For small isotope effects the value of f at which $R_f/R_o$ reaches $^{T}(V/K)$ is $[1 - 1/^{T}(V/K)]$, or 0.63 for $^{T}(V/K)$ values near 1.0 and 0.70 for a value of 1.5. The value of $R_f/R_o - 1$ seen at $f = 0.3$ is about 28% of that seen at $f = 0.7$, and the value at $f = 0.5$ is twice that. Thus one must let the reaction go long enough to observe a value of $R_f/R_o$ sufficiently greater than unity to be determined accurately, but because $R_f/R_o$ is changing more rapidly with f as f increases, errors in f become more critical as the reaction proceeds. A value of f between 0.6 and 0.7 is probably best for tritium isotope effects, while values from 0.3 to 0.5 are practical when the isotope ratio mass spectrometer is used to determine heavy atom isotope effects, and the isotope effect is large enough to determine very precisely (above 1.03, for example). With tritium isotope effects, one can also sample the reaction at several f values and fit the resulting $R_f/R_o$ and f values to Equation 4a directly to determine $^{T}(V/K)$. Because of the difficulty in accurate determination of specific activity, the $R_f/R_o$ ratio will probably have considerably more error than the f values, so that f can be considered free of error in the statistical analysis. The opposite situation will probably apply with most heavy atom isotope effects, however, since $R_f/R_o$ can be determined in the isotope ratio mass spectrometer more accurately than f can be measured.

Following substrate-specific activity in an internal competition experiment is useful only for small isotope effects (less than 2) and thus cannot be used for large primary tritium isotope effects. The reason is that the discrimination is so large in such cases that essentially all of the tritium label remains in the substrate except at very high f values, so that one cannot tell the value of the isotope effect, except that it is large. For example, at $f = 0.5$ where half of the substrate has reacted, the specific activity of the substrate is 1.87 times the initial value when $^{T}(V/K)$ is 10, 1.92 times greater when $^{T}(V/K)$ is 18, and twice the initial value when the isotope effect is infinite. Even at $f = 0.9$, the corresponding ratios are 7.94, 8.80, and 10 for isotope effects of 10, 18, and $\infty$.

The major problem with use of the internal competition method is reversal of the reaction. Unless conditions are chosen so that the reaction goes to completion, reversal of the reaction lowers the isotopic discrimination and gives incorrect results. The method is thus best suited for irreversible reactions, but it can be used for reversible ones if the products other than the one being measured are removed by coupling enzymes. It can also be used for reversible systems in which there is only one substrate and one product by following the specific activity of either product or substrate as a function of fractional approach to equilibrium, $f_{eq}$ (which in terms of the substrate concentration A is given by: $(1 - A/A_o)(1 + K_{eq})/K_{eq}$, where $A_o$ is the initial substrate concentration). When tritium label in product is followed, the equation for $^{T}(V/K)$ from the substrate side is

$$^{T}(V/K) = \left(\frac{K_{eq} + {}^{T}K_{eq}}{K_{eq} + 1}\right)\frac{\log(1 - f_{eq})}{\log(1 - f_{eq}R_f/R_{eq})} \qquad (4b)$$

where $R_f$ and $R_{eq}$ are specific activities of the product at fractional approach to equilibrium, $f_{eq}$, and at final equilibrium, respectively. The $^{T}(V/K)$ value in the reverse direction

is that given by Equation 4b divided by ${}^{T}K_{eq}$. $R_{eq}$ can be calculated from the initial specific activity of the substrate, $R_o$, by the equation

$$R_{eq} = \frac{R_o(1 + K_{eq})}{({}^{T}K_{eq} + K_{eq})} \tag{4c}$$

When label in substrate is followed, the log term in the denominator of Equation 4b becomes

$$\log\left[\frac{R_f}{R_{eq}}\left(\frac{K_{eq} + 1}{K_{eq}} - f_{eq}\right) - \frac{1}{K_{eq}}\right] \tag{4d}$$

where $R_f$ is now the specific activity of substrate at $f_{eq}$, but $R_{eq}$ is still the specific activity of the product at equilibrium given by Equation 4c (the specific activity of substrate at equilibrium is ${}^{T}K_{eq}R_{eq}$). Equations 4b and 4c as modified with 4d change into Equations 4 and 4a, respectively, as $K_{eq}$ becomes infinite.

### C. The Equilibrium Perturbation Method[9,23]

In contrast to the internal competition method, this new technique for measuring isotope effects is carried out at equilibrium and thus works only with reactions that are freely reversible. The $^{13}C$ isotope effect at C-4 of malate with malic enzyme has been determined with both the equilibrium perturbation and isotope ratio mass spectrometer methods, however, with identical results.[23,24] In the equilibrium perturbation method, reaction mixtures are set up at equilibrium, except that one substrate contains the heavy atom and the corresponding product the light one (these two reactants are called the perturbants). Enzyme is then added, and the level of a colored reactant is followed until isotopic mixing is complete. Because of the isotope effect, the initial rate of reaction of the labeled perturbant is slower (for a normal isotope effect; faster, of course for an inverse one) than that of the unlabeled perturbant, and the reaction appears to be perturbed towards the labeled perturbant. As isotopic mixing occurs, however, the reaction returns to the original equilibrium position. The size of the perturbation relative to the concentration of the limiting perturbant varies with the isotope effect (nearly linearly for small isotope effects) according to the equation

$$\frac{A_{max} - A_o}{A_o'} = \alpha^{-1/(\alpha-1)} - \alpha^{-\alpha/(\alpha-1)} \tag{5}$$

where $\alpha$ is an apparent isotope effect related to those in forward, ${}^{D}(Eq.P.)_f$, and reverse directions, ${}^{D}(Eq.P.)_r$, by the equations

$$\alpha = {}^{D}(Eq.P.)_r(1 + K)/(1 + K/{}^{D}K_{eq}) \tag{6}$$

$${}^{D}(Eq.P.)_f = {}^{D}(Eq.P.)_r{}^{D}K_{eq} \tag{7}$$

In these equations $A_{max}$ and $A_o$ are the concentrations of the colored reactant being followed at the maximum of the perturbation and at the start (actually, the average of the starting and final values if they are not exactly the same), and $A_o'$ is the reciprocal of the sum of the reciprocal concentrations of the perturbants, with a correction added for low levels of any reactants that are not perturbants. The parameter K is the ratio at equilibrium of the levels of unlabeled perturbants in the system (this is not identical with the actual starting levels of perturbants because of the equilibrium isotope effect; in practice K is calculated from the equilibrium isotope effect and the concentrations used).

The method is quite sensitive, being capable of determining isotope effects as small as 1.003 in favorable cases. Exact calibration of concentrations is not required, since one adjusts concentrations empirically until the perturbation returns to the starting point, and it is primarily the perturbant present in the lowest concentration (or other reactant, if present at less than half the level of the most dilute perturbant) that limits the size of the perturbation. Inhibitors present in the reactants affect only the time course of the perturbation and not its size. The reactants must be stable for sufficient time to observe the perturbation, however; the breakdown of DPNH below pH 8 makes it difficult to study dehydrogenases by this method at low pH.

Two sources of artifact must be carefully controlled. First, the equilibrium constant for many reactions is quite temperature sensitive, and thus good temperature control is essential. For example, picking up a cuvette with the fingers to add enzyme will impart a pulse of heat (when the reaction is run at any temperature less than 35°) which takes several seconds to penetrate the glass and be transmitted to the solution. The result is a perturbation looking exactly like one caused by an isotope effect. This problem is easily overcome by using an adder-mixer and a small volume of enzyme.

Temperature problems are easily avoided, and when no perturbation is observed with unlabeled reactants, or with labeled ones on both sides of the reaction, but one is observed with a labeled perturbant on only one side of the reaction (and has opposite sign but equal amplitude when the label is on the other side of the reaction), one is on solid ground. The second type of artifact is more insidious, however, since it may not affect the control and labeled reaction mixtures equally. This artifact results from the presence of a small amount of a more rapidly or more slowly reacting alternate substrate as a contaminant in one of the reactants or in the enzyme solution. Since this contaminant will react to reach chemical equilibrium at a different rate than the major reactants present, a perturbation from equilibrium is observed when the initial concentrations of the major reactants are chosen so that they are the same distance from equilibrium initially as the contaminant, so that the final optical density returns to the initial value.

If the contaminant is in the enzyme or an unlabeled reactant, it will give a perturbation in an unlabeled system and thus be detected, but if it is only in the labeled reactant, and not in the corresponding unlabeled molecule used for the control, it will cause spurious results. It is for this reason that labeled perturbants should be used on both sides of the reactions as a second control; failure to observe a perturbation in such a system is excellent proof of the absence of alternate substrates in the labeled reactants. Observation of a perturbation of equal size but opposite direction when the label is put in the perturbant on the other side of the reaction also rules out these problems.

The equilibrium perturbation method has so far been used largely in the author's laboratory, but its simplicity and sensitivity will hopefully lead to its use by other workers in the near future.

## IV. THE EQUATION FOR THE OBSERVED ISOTOPE EFFECT

### A. Only One-Step Isotope Sensitive

We will consider first the case where only one step in the enzymatic mechanism shows an isotope effect. Primary isotope effects in dehydrogenase reactions are examples, with only the hydride transfer step showing an isotope effect. In mechanism 8,

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA^* \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EPQ^* \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EPQ \qquad (8)$$

$$\xrightarrow{k_9} EQ \xrightarrow{k_{11}} E$$

where only $k_5$ and $k_6$ are isotope sensitive, V/K and V may be expressed in the following way, in which $k_5$ is a factor of the numerator, and the denominator contains one term in $k_5$ and one in $k_6$:

$$V/K = \frac{k_1 k_3 k_5 E_t / k_2 k_4}{1 + c_f + c_r} \tag{9}$$

$$V = \frac{k_3 k_5 E_t / (k_3 + k_4)}{1 + c_{Vf} + c_r} \tag{10}$$

$$c_f = (k_5 / k_4)(1 + k_3 / k_2) \tag{11}$$

$$c_r = (k_6 / k_7)(1 + k_8 / k_9) \tag{12}$$

$$c_{Vf} = \left(\frac{k_5}{1 + k_4 / k_3}\right)[1/k_3 + (1/k_7)(1 + k_8/k_9) + 1/k_9 + 1/k_{11}] \tag{13}$$

When $(V/K)_H$ is divided by $(V/K)_D$, the observed isotope effect is given by

$$^D(V/K) = \frac{^Dk_5 + c_f + c_r\,{}^DK_{eq}}{1 + c_f + c_r} \tag{14}$$

where

$$^DK_{eq} = {}^Dk_5 / {}^Dk_6 = \frac{k_{5H} k_{6D}}{k_{5D} k_{6H}} \tag{15}$$

Likewise:

$$^DV = \frac{^Dk_5 + c_{Vf} + c_r\,{}^DK_{eq}}{1 + c_{Vf} + c_r} \tag{16}$$

Equations 14 and 16 are general and apply to any mechanism with only one isotope-sensitive stop. The definitions of $c_f$, $c_r$, and $c_{Vf}$ will depend on the particular mechanism, however. Equation 14 also describes isotope effects measured by the internal competition and equilibrium perturbation methods, although $c_f$ and $c_r$ may have different definitions in these types of experiments (see below). Equation 16 applies only to the direct comparison method, however, since only this method allows determination of $^DV$.

The constants $c_f$ and $c_r$ in Equation 14 are called "commitments to catalysis"[25] or simply commitments. A commitment is the ratio of the rate constant for the isotope-sensitive step to the net rate constant for release from the enzyme of the reactant whose commitment is being calculated. Thus, $c_f$ in mechanism 8 is the ratio of $k_5$ and $k_4 k_2 / (k_2 + k_3)$, and $c_r$ is the ratio of $k_6$ and $k_7 k_9 / (k_8 + k_9)$. Net rate constants are readily calculated by the method of Cleland.[26] In the direct comparison method of measuring isotope effects, $c_f$ is computed for the variable substrate, regardless of which substrate contains the label. In the internal competition or equilibrium perturbation methods, on the other hand, $c_f$ is computed for the labeled substrate or for the perturbant molecule, respectively.

A forward commitment has an external and an internal part. The internal part consists of those terms not including any rate constants for substrate release ($k_5 / k_4$ in mechanism 8) and does not vary with the levels of the other substrates or, except in certain cases, with pH. The external portion of the commitment consists of the terms containing rate

constants for substrate release ($k_3k_5/k_2k_4$ in mechanism 8) and may vary with the levels of nonvaried substrates in a direct comparison experiment, unlabeled substrates in an internal competition experiment, or nonperturbant substrates in an equilibrium perturbation experiment. This dependence is the basis for distinguishing different kinetic mechanisms (see Section VI.). The external portion of the commitment usually also becomes very small at pH values where V/K is decreasing by a factor of 10 per pH unit, since the chemical reaction is slowing down, while the rate constant for substrate release is not. A finite external commitment is seen only for partly sticky substrates (a sticky substrate is one which reacts to give products as fast or faster than it dissociates from the enzyme). Slow, nonsticky substrates will have only internal forward commitments. Reverse commitments also can have external and internal parts, and the elimination of the external part by pH changes can affect pH profiles of V/K and $^D(V/K)$ (see Section VII.A.).

The constant $c_r$ in the equilibrium perturbation method is computed for the product which is the perturbant molecule, and its variation with the level of the other reactants for the reverse reaction also can be used to distinguish between kinetic mechanisms. In contrast, $c_r$ for the direct comparison and internal competition methods is computed for the first irreversible step. This step is usually the release of the first product (as in mechanism 8), but may be an earlier irreversible step, or a later step if the first product is present at a finite level, so that its release is a reversible step. Thus in mechanism 8, $c_r$ would be $(k_6/k_7)[1+(k_8/k_9)(1+k_{10}P/k_{11})]$ if P were present at a finite level, but Q was absent.

A classic example of the interplay between commitments in determining the observed isotope effect is isocitrate dehydrogenase. In direct comparison experiments at neutral pH, $^D(V/K) = 1.0$ because $c_{isocitrate}$ (the forward commitment) is very large and $c_{CO_2}$ (the reverse commitment) is small. In an equilibrium perturbation experiment with high levels of $CO_2$ and $\alpha$-ketoglutarate present, however, $c_{TPNH}$ (the reverse commitment in this case) is even larger than $c_{isocitrate}$, and $^DK_{eq}$ is seen as the observed isotope effect from the isocitrate side.[28]

The symmetry inherent in Equation 14 is obvious if it is divided by $^DK_{eq}$ to give $^D(V/K)$ or $^D$(Eq.P.) in the reverse direction (the ratio of the $^D(V/K)$ or $^D$(Eq.P) values in forward and reverse reactions must equal $^DK_{eq}$). The numerator now becomes $^Dk_6$ (which is $^Dk_5/{}^DK_{eq}) + c_r + c_f/{}^DK_{eq}$, and $c_r$ has become the forward and $c_f$ the reverse commitment (we should also replace $^DK_{eq}$ by its reciprocal for consideration of reaction in this direction).

Equation 16 does not show this symmetry, however, and one does not obtain the correct equation for $^DV$ in the reverse direction by dividing Equation 16 by $^DK_{eq}$. The desired equation is rather analogous to Equation 16 with the numerator being $^Dk_6 + c_{Vr} + c_f/{}^DK_{eq}$, where $c_f$ and $^DK_{eq}$ are the same constants used in Equations 14 and 16 ($c_f$ is actually a reverse commitment in this equation, and we would use the reciprocal of $^DK_{eq}$, which is the equilibrium isotope effect in the reverse reaction), and $c_{Vr}$ is a term similar to $c_{Vf}$ in the forward reaction. $c_{Vf}$ and $c_{Vr}$ are not commitments, but can be called the V ratios. Each consists of the sum of the ratios of the rate constant for the isotope-sensitive step to each other net rate constant in the given direction, multipled by a precatalytic proportion factor. For this calculation, formation of the enzyme complex which undergoes the isotope-sensitive step is considered an irreversible step. Thus for mechanism 8 $c_{Vf}$ is the sum of the ratios of $k_5$ and $k_3$, $k_7k_9/(k_8+k_9)$, $k_9$, and $k_{11}$, multiplied by the precatalytic proportion factor $k_3/(k_3+k_4)$. This precatalytic proportion factor is that portion (at equilibrium) of the enzyme complexes preceding the isotope-sensitive step and reversibly connected to each other which is actually available to undergo the isotope-sensitive step. Thus, in mechanism 8, this factor represents $[EA^*]/-([EA]+[EA^*])$ at equilibrium.

The V ratio may include rate constants which do not occur in $c_f$ or $c_r$, such as $k_{11}$ in mechanism 8. If $k_{11}$ is smaller than $k_5$, it becomes rate limiting for V, $c_{Vf}$ is as a result large, and $^DV$ becomes 1.0. This situation is common for dehydrogenases where the release for the nucleotide product is often rate limiting for V.

### B. More Than One-Step Isotope Sensitive

While many primary isotope effects correspond to single isotope-sensitive steps, it is not uncommon, especially for secondary isotope effects, for more than one step to be affected. Thus the $^{15}N$-sensitive steps for liver alcohol dehydrogenase with DPN labeled at N-1 come before and after hydride transfer[16] while there should be at the 3 position of malate in the malic enzyme reaction a $\beta$-secondary isotope effect in the dehydrogenation step and an $\alpha$-secondary effect on the subsequent decarboxylation step. In mechanism 17:

$$E_1 \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} E_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_3 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_4 \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E_5 \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}}$$

$$E_6 \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E_7 \xrightarrow{k_{13}} E_8 \qquad (17)$$

where $k_5$ to $k_{10}$ are all isotope sensitive, but $k_1$ to $k_4$ and $k_{11}$ to $k_{13}$ are not, the isotope effect is

$$^D(V/K) = \frac{^DK_{eq\,5}\,^Dk_7 + (k_7/k_6)[^Dk_5 + (k_5/k_4)(1+k_3/k_2)] + {}^DK_{eq}(k_8/k_9)[^Dk_{10} + (k_{10}/k_{11})(1 + k_{12}/k_{13})]}{1 + (k_7/k_6)[1 + (k_5/k_4)(1 + k_3/k_2)] + (k_8/k_9)[1 + (k_{10}/k_{11})(1 + k_{12}/k_{13})]} \qquad (18)$$

where

$$^DK_{eq\,5} = {}^Dk_5/{}^Dk_6 \qquad (19)$$

and

$$^DK_{eq} = {}^Dk_5\,{}^Dk_7\,{}^Dk_9/({}^Dk_6\,{}^Dk_8\,{}^Dk_{10}) \qquad (20)$$

The same equation will apply for $^D$(Eq.P) if the other perturbant adds to $E_8$ to give $E_7$. Equation 18 is written for primary consideration of $^Dk_7$, but if numerator and denominator are multiplied by $k_6/k_7$, one obtains a form where the focus is on $^Dk_5$, and if one multiplies by $k_9/k_{10}$, the form focuses on $^Dk_9$. Equations similar to this one have been used in connection with secondary deuterium isotope effects on fumarase[12] and $^{15}N$ isotope effects on liver alcohol dehydrogenase.[16]

## V. DETERMINATION OF INTRINSIC ISOTOPE EFFECTS

### A. Northrop's Method

Before one can really apply Equations 14 or 16 to many problems, one must determine the intrinsic isotope effect on the isotope-sensitive step. Several methods for doing this, or for setting limits on the values, have been developed, but the first and so far the only one used is that of Northrop.[29] If 1.0 is subtracted from both sides of Equation 14 and of the corresponding equation for the tritium isotope effect, one obtains

$$^D(V/K) - 1 = \frac{^Dk - 1 + c_r(^DK_{eq} - 1)}{1 + c_f + c_r} \qquad (21)$$

$$^{T}(V/K) - 1 = \frac{(^{D}k)^{1.44} - 1 + c_r(^{T}K_{eq} - 1)}{1 + c_f + c_r} \quad (22)$$

Taking the ratio of the two equations gives

$$\frac{^{D}(V/K) - 1}{^{T}(V/K) - 1} = \frac{^{D}k - 1 + c_r(^{D}K_{eq} - 1)}{(^{D}k)^{1.44} - 1 + c_r(^{T}K_{eq} - 1)} \quad (23)$$

If either $c_r = 0$ or $^{D}K_{eq} = 1$, this equation reduces to

$$\frac{^{D}(V/K) - 1}{^{T}(V/K) - 1} = \frac{^{D}k - 1}{(^{D}k)^{1.44} - 1} \quad (24)$$

and the equation can be solved for $^{D}k$.

With alcohol dehydrogenases, $c_r$ *is* very small, and Cook and Cleland have used Northrop's method to measure $^{D}k$ values of 5.7 for isopropanol and the yeast enzyme[28] and 6.3 for cyclohexanol and the liver enzyme.[30] The solutions to Equation 24 are most precise when the sum of the commitments is between 0.4 and 1.5 times $^{D}k$, and as it gets smaller than $0.2\ ^{D}k$, the solution is so ill conditioned that it is not possible to determine $^{D}k$, except to say that it is larger than or equal to the observed $^{D}(V/K)$ value.[31]

When $c_r$ is finite and $^{D}K_{eq} \neq 1$, solution of Equation 24 gives only an apparent solution of $^{D}k_{forward}$. One then divides $^{D}(V/K)$ by $^{D}K_{eq}$ and $^{T}(V/K)$ by $^{T}K_{eq}$ ($=^{D}K_{eq}^{1.44}$) and solves Equation 24 with these values, which now represent isotope effects in the reverse direction. This solution yields an apparent value of $^{D}k_{reverse}$, and the true value of $^{D}k_{forward}$ then lies between its apparent value and (apparent $^{D}k_{reverse}$)/$^{D}K_{eq}$, and the true value of $^{D}k_{reverse}$ lies between its apparent value and (apparent $^{D}k_{forward}$)$^{D}K_{eq}$. The intrinsic isotope effects for malic enzyme were found by this approach to lie between 5 to 8 in the forward direction and 4 to 6.5 in the reverse direction.[32]

### B. Comparison of $^{13}C$ Isotope Effects with Deuterated and Unlabeled Substrates

As noted above, Northrop's method gives an exact solution only when $c_r = 0$, or $^{D}K_{eq} = 1$, and even then the solution is well conditioned only when the commitments are not too large or too small. A method which overcomes these problems and is capable in fact of giving a total solution to the parameters of the system involves comparing the $^{13}C$ isotope effect (or any other convenient heavy atom or $\beta$-secondary deuterium isotope effect resulting from the same step as C-H bond breaking) with a deuterated and unlabeled substrate. The equations for these isotope effects are

$$^{13}(V/K)_H = \frac{^{13}k + c_f + c_r\ ^{13}K_{eq}}{1 + c_f + c_r} \quad (25)$$

$$^{13}(V/K)_D = \frac{^{13}k + c_f/^{D}k + c_r{}^{13}K_{eq}{}^{D}K_{eq}/^{D}k}{1 + c_f/^{D}k + c_r{}^{D}K_{eq}/^{D}k} \quad (26)$$

Note that with a deuterated substrate $c_f$ is reduced by $^{D}k$ and $c_r$ by $^{D}k/^{D}K_{eq}$ (the intrinsic isotope effect in the reverse direction) because the isotope-sensitive steps are slowed down while other steps are not, and $c_f$ and $c_r$ both indlcude the rate constant for the isotope-sensitive step as a factor. These two equations and Equation 14 for the deuterium isotope effect form a set of three equations in four unknowns, but by alternately assuming $c_f$ or $c_r$ to be zero and solving the three equations for the remaining commitment, $^{D}k$, and $^{13}k$, one obtains limits for the various parameters. The rules which apply to these solutions are[27]

1. If either equilibrium isotope effect is 1.0, one gets an exact solution for the other intrinsic isotope effect, regardless of $c_f$ and $c_r$ values. An exact solution also is obtained if either $c_f$ and $c_r$ is zero.
2. If either equilibrium isotope effect is normal, one gets too high an intrinsic value for the other isotope effect when $c_f$ is assumed to be zero and too low a value when $c_r$ is assumed to be zero.
3. If either equilibrium isotope effect is inverse, one gets too low an intrinsic value for the other isotope effect when $c_f$ is assumed to be zero and too high a value when $c_r$ is assumed to be zero.
4. The range for the calculated intrinsic isotope effect depends on the size of the equilibrium isotope effect relative to the intrinsic isotope effect for the other isotope. Thus the range for $^{13}k$ will normally be narrow since ($^{D}K_{eq} - 1$) is usually far less than ($^{D}k - 1$). Conversely, the range for $^{D}k$ will be wide when ($^{13}K_{eq} - 1$) is a sizeable portion of ($^{13}k - 1$). In all cases, however, the ranges for $^{D}k$ and $^{13}k$ are smaller when $c_f$ and $c_r$ are small and larger when $c_f$ and $c_r$ are larger.
5. The value of $^{T}(V/K)$ calculated from the limiting values of $^{D}k$ and either $c_f$ (when $c_r$ is assumed to be zero) or $c_r$ (when $c_f$ is assumed to be zero) is too high when $^{D}k$ is too high and too low when $^{D}k$ is too low. An experimental value of $^{T}(V/K)$ can thus be used to achieve an exact solution by adjusting $c_f$ and $c_r$ until the calculated value of $^{T}(V/K)$ matches the experimental one.

If either equilibrium isotope effect is 1.0, or either $c_f$ or $c_r$ is zero, the equations for the solution are[27]

$$x = {}^{D}(V/K) \qquad y = {}^{13}(V/K)_H \qquad z = {}^{13}(V/K)_D \tag{27}$$

For any assumed ratio of $c_r$ and $c_f$ given by: $r = c_r / c_f$

$$d = \frac{1 + r^{13}K_{eq}}{1 + r} \qquad c = \frac{1 + r^{D}K_{eq}}{1 + r} \tag{28}$$

$$^{D}k = \frac{(z - d)\,x}{y - d} \qquad ^{13}k = \frac{(xz - yc)}{x - c} \tag{29}$$

$$(xp) = x(1 + r) - (1 + r^{D}K_{eq}) \tag{30}$$

$$c_f = (^{D}k - x)/(xp) \qquad c_r = r\,c_f \tag{31}$$

If $c_f = 0$, however,

$$c_r = (^{D}k - x)/(x - {}^{D}K_{eq}) \tag{32}$$

For the general case in which $c_f$ and $c_r$ are both finite and $^{D}K_{eq}$ and $^{13}K_{eq}$ are both different from unity, however, the above equations give only approximate solutions for $^{D}k$ and $^{13}k$. The exact solution is given by:

$$(yp) = y(1 + r) - (1 + r^{13}K_{eq}) \tag{33}$$

$$(zp) = z(1 + r^{D}K_{eq}) - (1 + r^{D}K_{eq}\,{}^{13}K_{eq}) \tag{34}$$

$$b = x(yp) + (z - y)\,(xp) + (zp) \tag{35}$$

$$^{D}k = \frac{b + \sqrt{b^2 - 4x(yp)\,(zp)}}{2(yp)} \tag{36}$$

$$^{13}k = y + (^{D}k - x)(yp)/(xp) \quad (37)$$

The value of $^{D}k$ is then used to calculate $c_f$ and $c_r$ by Equations 31 and 32. The value of $^{T}(V/K)$ calculated from

$$^{T}(V/K) = \frac{(^{D}k)^{1.44} + c_f + c_r(^{D}K_{eq})^{1.44}}{1 + c_f + c_r} \quad (38)$$

is then compared with the experimental value, and a new choice of r is tested. The process is continued until the calculated and experimental values agree. A computer program has been written to make these calculations.

This method has only been used so far in the formate dehydrogenase reaction to show that since the $^{13}C$ isotope effect was the same with deuterated and unlabeled formate, $c_f$ and $c_r$ were both zero in this system.[33] (It has also been used with D and $^{37}Cl$ isotope effects to demonstrate concerted elimination in a nonenzymatic reaction.)[34] It should find wide application, however, and experiments are underway in this laboratory to apply it to a number of enzymatic reactions. Unlike Northrop's method, this technique works better the smaller $c_f$ and $c_r$ are (as far as determining $^{D}k$ and $^{13}k$, that is) and should in particular give very precise estimates of $^{13}k$ values. Even without the use of $^{T}(V/K)$ values, the method will in many cases produce narrow limits on $c_f$ and $c_r$, in addition to $^{D}k$ and $^{13}k$, since all values of $c_r/c_f$ which produce negative values of $^{D}k$ or $^{13}k$ or totally unrealistic values (such as $>1.08$ for $^{13}k$) can be eliminated. When $c_f$ and $c_r$ are of suitable size, all parameters can be determined with some measure of confidence by the use of $^{T}(V/K)$ values.

Care must be taken to be sure that the $^{13}C$ and deuterium isotope effects result from the same step (see Section VIII.A. for the situation when they do not; in that case $^{13}(V/K)_D$ is smaller, rather than larger, than $^{13}(V/K)_H$). The $^{13}C$ isotope effects will have to be determined precisely, and thus the first applications of the method will probably be to cases where the $^{13}C$ effect can be measured with the isotope ratio mass spectrometer. It should be possible to use the equilibrium perturbation method to determine the $^{13}C$ isotope effects, however, although special precautions will be needed when both perturbants are deuterated to be sure that the extent of deuteration is precisely that required to be at isotopic equilibrium. Cleland has described how this can be done, but it is not yet known how easy it will be in practice to carry out such experiments.[35]

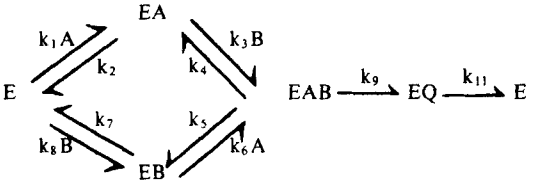
As noted earlier, it is possible to use a $\beta$-secondary deuterium isotope effect in place of a heavy atom isotope effect to help determine the parameters of the system (one cannot use $\alpha$-secondary deuterium isotope effects, since their size is dependent on the mass of the primary atom as the result of the coupled motion of the primary and secondary hydrogen atoms; see Section IX.C.). To date this has only been done for lactate dehydrogenase,[16] where comparison of Equation 14 for the primary and secondary deuterium isotope effects allowed estimation of the commitments for lactate (0.4 to 1.4) and pyruvate (12 to 13). The primary intrinsic isotope effect was assumed to be 6 (it could be determined by Northrop's method or by the $^{13}C$-D method described above), and the secondary intrinsic isotope effect was assumed to lie between 1.0 and $^{D}K_{eq}$. Since the relationship of an intrinsic $\beta$-secondary deuterium isotope effect to its equilibrium isotope effect is likely to differ from that for a $^{13}C$ isotope effect, one has an extra equation to help determine commitments, as was done in the lactate dehydrogenase case above, or if the commitments are known, to determine the intrinsic secondary isotope effect. The more isotope effects on the same step which are looked at (for all of which $c_f$ and $c_r$ are the same), the greater the redundancy in the equations and the better able one will be to determine all of the parameters of the system.

## VI. USE OF ISOTOPE EFFECTS TO DETERMINE KINETIC MECHANISM

The kinetic mechanism of an enzymatic reaction with two or more substrates or products consists of a description of the order of combination of substrates and release of products and thus of the stoichiometry of the enzyme-reactant complexes at each step of the reaction. Each kinetic mechanism corresponds to a rate equation containing different terms in the denominator, and thus initial velocity and product inhibition patterns are commonly used to distinguish between various kinetic mechanisms. Patterns of inhibition by dead end inhibitors and of isotopic exchange between reactants can also assist in determining the kinetic mechanism. A very sensitive method for distinguishing kinetic mechanisms, however, depends on the variation of forward and reverse commitments for isotope effects with the level of a nonvaried or nonlabeled reactant.[36,37]

### A. Direct Comparison Experiments with Deuterated Substrates

We will consider first mechanism 39, in which two substrates, A and B, add to the enzyme in sequential fashion to give a ternary complex which undergoes a chemical reaction prior to release of the products P and Q.




(39)

For simplicity, we have lumped together in $k_9$ all of the steps which the initial EAB complex undergoes up to and including release of the first product P. The rate constant $k_{11}$ includes all nonisotope-sensitive steps after release of the first product. In reality, EAB undergoes nonisotope-sensitive conformation changes prior to and following the isotope-sensitive bond-breaking step, in addition to the step for release of P, and thus $^{D}k_9$ is not the intrinsic isotope effect, but is given by an equation similar to Equation 14 including all of $c_r$ and the internal portion of $c_f$. In analyzing mechanism 39 we will be concerned only with the external part of $c_f$, since that is the only part which changes with substrate concentration, and thus mechanism 39 is adequate for our needs.

In mechanism 39, the equation for the deuterium isotope effect on V/K when A is varied at a fixed level of B is given by

$$\text{app } ^{D}(V/K) = \frac{^{D}k_9 + c_{f\text{-}ex}}{1 + c_{f\text{-}ex}} \tag{40}$$

where $c_{f\text{-}ex}$ (the external forward commitment) is given by:

$$c_{f\text{-}ex} = k_9/[k_5 + k_4k_2/(k_2 + k_3B)] \tag{41}$$

In the fully random mechanism it can readily be seen that as B goes to infinity, $c_{f\text{-}ex} = k_9/k_5$, and, since $V/K_a$ is the limiting V/K value reached at infinite B, app $^{D}(V/K) = ^{D}(V/K_a)$. On the other hand, as B goes to zero, $c_{f\text{-}ex} = k_9/(k_5 + k_4)$, and app $^{D}(V/K)$ will be greater than $^{D}(V/K_a)$ as long as $k_4$ is not much smaller than $k_5$. When B is varied at a fixed level of A, Equation 40 still holds, but

$$c_{f\text{-}ex} = k_9/[k_4 + k_5k_7/(k_7 + k_6A)] \quad (42)$$

As A goes to infinity, $c_{f\text{-}ex} = k_9/k_4$, and app $^D(V/K) = {}^D(V/K_b)$. As A goes to zero, $c_{f\text{-}ex} = k_9/(k_4 + k_5)$, which is of course the same value seen above as B approaches zero, since the variable substrate is extrapolated to zero to determine V/K, and thus this value of $c_{f\text{-}ex}$ corresponds to very low levels of both A and B. It is clear that under these conditions $c_{f\text{-}ex}$ must be at least a factor of 2 less than it is for calculation of the smaller of $^D(V/K_a)$ or $^D(V/K_b)$, and thus at least one app $^D(V/K)$ value will be sensitive to the level of the nonvaried substrate unless both $k_4$ and $k_5$ are much larger than $k_9$. Such variation shows clearly that one does not have a rapid equilibrium random mechanism, and the observation of isotope effects on both $V/K_a$ and $V/K_b$ shows that both $k_4$ and $k_5$ are of finite size relative to $k_9$, and thus that the mechanism is truly random.

There are random mechanisms in which the isotope effects are the same regardless of what substrate is varied. First, if $k_4$ and $k_5$ are both much larger than $k_9$, we have the classic rapid equilibrium random mechanism, and all app $^D(V/K)$ values equal $^Dk_9$. If $k_4$ and $k_5$ are not both large, however, but $k_2$ and $k_7$ are much larger than $k_9$, $k_3B$ in Equation 41 will not exceed $k_2$ at the highest levels of B it is practical to use, and likewise $k_6A$ will not exceed $k_7$ at the highest A levels one can use. Thus $c_{f\text{-}ex} = k_9/(k_4 + k_5)$ regardless of which substrate is varied, and the isotope effect given by Equation 40 is constant, although smaller than $^Dk_9$ as long as neither $k_4$ nor $k_5$ is much larger than $k_9$. This mechanism can usually be distinguished from one in which $k_4$ and $k_5$ are much larger than $k_9$ by the pH variation of $^D(V/K)$ values (see Section VII.A.); $^D(V/K)$ will usually increase to $^Dk_9$ in the pH range where V/K is decreasing by a factor of 10 per pH unit.

In an ordered mechanism, $k_8$ and $k_5$ are zero, and EB does not form. As a result, from Equation 42 $c_{f\text{-}ex} = k_9/k_4$ at all times when B is varied, and Equation 40 gives $^D(V/K_b)$. When A is varied, however, Equation 41 gives

$$c_{f\text{-}ex} = (k_9/k_4)(1 + k_3B/k_2) \quad (43)$$

and $c_{f\text{-}ex}$ varies from $k_9/k_4$ at low B, where app $^D(V/K) = {}^D(V/K_b)$, to infinity at infinite B, where app $^D(V/K) = {}^D(V/K_a) = 1.0$. The value of B giving an isotope effect which is the average of $^D(V/K_b)$ and 1.0 is $k_2(k_4 + k_9)/k_3k_9$, which is $K_{ia}K_b/K_a$ in Cleland's nomenclature.[38]

If $k_2$ is much larger than $k_9$ in an ordered mechanism, one has an equilibrium ordered mechanism, and the Michaelis constant for A is zero, the $K_aB$ term is missing in the denominator of the rate equation, and the initial velocity pattern is different from that of a normal intersecting one (it crosses on the vertical axis when B is varied and has a slope replot vs. 1/B which goes through the origin when A is varied). In such a mechanism, $k_2$ exceeds $k_3B$ at any level of B it is practical to use, and thus $c_{f\text{-}ex} = k_9/k_4$ at all times, and one sees $^D(V/K_b)$ as the V/K isotope effect regardless of which substrate is varied or the level of the nonvaried substrate. This pattern of constant isotope effects is the same as that seen with a rapid equilibrium random mechanism, but the initial velocity pattern is distinctive. A more insidious mechanism, however, is an equilibrium ordered one in which a dead end EB complex forms. This mechanism gives a normal intersecting initial velocity pattern, but the same constant isotope effects, and cannot be distinguished from an equilibrium random mechanism. The distinction is actually not possible by definition, since as long as E, EA, EB, and EAB are all at equilibrium, it does not matter whether or not the step between EB and EAB has a finite rate.

As a result of the above analysis, it should be obvious that regardless of which substrate is labeled, one would normally vary the second substrate to add in the ordered case, or the

one with the highest rate constant for release from the ternary complex in a random mechanism, in order to enhance the size of the observed V/K isotope effects and make it as easy as possible to study the chemical mechanism.

**B. Internal Competition Experiments with Tritium or Heavy Atom Isotopes**

This method of measuring isotope effects involves competition between labeled and unlabeled substrates, and thus the commitment is figured for the substrate which carries the label. The equations in Section VI.A. thus apply if one exchanges "labeled substrate" for "varied substrate" and "unlabeled substrate" for nonvaried substrate". The level of the labeled substrate has no effect on the observed isotope effect, but the level of the nonlabeled substrate affects the commitment in the same way as the nonvaried substrate does in a direct comparison study, and this variation can be used to distinguish random from ordered binding. Thus when tritiated DPNH is used with a dehydrogenase, the level of the molecule being reduced (pyruvate, acetaldehyde, etc.) will control the size of the observed isotope effect, and if one wants to see the maximum possible isotope effect, it is necessary to keep the pyruvate or acetaldehyde at levels will below their $K_{ia}K_b/K_a$ values. Conversely, high levels of these molecules in the experiment will eliminate the isotope effect if the mechanism is ordered. A similar problem arises in remote label experiments such as measurement of the $^{18}O$ isotope effect on the hexokinase reaction by use of glucose in which every $^{13}C$ at C-1 is accompanied by $^{18}O$ at C-6, and one determines the carbon mass ratio at C-1 of glucose-6-P as a measure of the $^{18}O$ isotope effect. Since glucose is released from the ternary E-MgATP-glucose complex very slowly, one must run this experiment at levels of MgATP of $10\,\mu M$ ($0.1\,K_m$) or lower in order not to have an external commitment for glucose. By recycling MgADP with pyruvate kinase and phosphoenolpyruvate, the MgATP level will stay constant and allow a high level of glucose to be converted irreversibly (an essential requirement for accurate measurement of the isotope effect) to glucose-6-P.

**C. Equilibrium Perturbation Experiments**

The same equations in Section VI.A. apply to isotope effects measured by the equilibrium perturbation method, except that both $c_f$ and $c_r$ have external portions, and thus both $c_{f-ex}$ and $c_{r-ex}$ may vary with the levels of the nonperturbant molecules when the presence of these on the enzyme alters the rate of dissociation of a perturbant. Thus in a dehydrogenase reaction where the perturbants are an alcohol and the reduced nucleotide, $c_{f-ex}$ for the alcohol is usually not sensitive to the level of DPN or TPN, since the alcohol dissociates more rapidly from the ternary complex than the nucleotide. Since the ketone or aldehyde product is normally released much more rapidly than DPNH or TPNH, however, $c_{r-ex}$ is sensitive to the level of the ketone or aldehyde, and by using a low level of this reactant one reduces $c_{r-ex}$ to that for the aldehyde or ketone, even when the rate constant for release of the nucleotide from its binary complex is very small (that is, the rate constant analogous to $k_2$ in Equation 41, small though it is, is larger than the rate corresponding to $k_3B$ when the product analogous to B is at low enough concentration). This trick for reducing the commitments of tightly bound reactants to those of the more rapidly dissociating ones was used by Cook et al.[16] to eliminate the external commitments of DPN and DPNH in order to determine the $^{15}N$ isotope effects with [$^{15}N$]-DPN.

The extension of these principles to V/K or equilibrium perturbation isotope effects in three substrate cases is clearly feasible, and Cook and Cleland[37] have discussed some of the possibilities. We will defer to the next section a discussion of V isotope effects, since these are primarily of use in determining which steps are rate limiting.

# VII. USE OF ISOTOPE EFFECTS TO DETERMINE RATE-LIMITING STEPS

Once the intrinsic isotope effect has been determined by one of the methods in Section V, the size of the observed isotope effects on V/K or V can be used to determine the relative rates of the various steps in the kinetic mechanism. Basically this process involves estimating the values of $c_f$, $c_r$, and $c_{vf}$ in Equations 14 and 16 and, in particular, dissecting the external from the internal portion of $c_f$ or $c_r$. We will consider several cases below.

## A. Stickiness and the External Commitment Factor

A sticky substrate is one which reacts to give products as fast or faster than it dissociates from the enzyme. In contrast, a nonsticky substrate dissociates faster than it reacts, so that its binding step comes to equilibrium, with considerable simplification in rate equations (especially those for pH profiles[2]). The quantitative measure of stickiness is the ratio of the net rate constant for reaction of the first collision complex of enzyme and substrate through the first irreversible step to the rate constant for dissociation of the substrate from the collision complex. This stickiness ratio can be expressed in terms of commitments as:

$$S_r = c_{f-ex} / (1 + c_{f-in} + c_r) \tag{44}$$

where $c_{f-ex}$ and $c_{f-in}$ are the external and internal parts of $c_f$, and $c_f$ and $c_r$ are the constants in Equation 14. It is clear that a substrate without an external forward commitment cannot be sticky, but a substrate with a finite external commitment will be sticky unless $c_r$ is much larger than $c_{f-ex}$. In this case a slow step after the isotope-sensitive one brings all previous steps, including the binding one, to equilibrium, despite the finite external commitment. Stickiness can be measured directly by the isotope partition method of Rose et al.[39]

The stickiness factor is an important parameter, since the apparent pK values in V/K profiles are displaced to the outside of the profile (that is, to lower pH on the acid side and to higher pH on the basic side of the profile) when the substrate is sticky and it is possible for the enzyme-substrate complex to be incorrectly protonated.[2] They are also displaced by the stickiness of the first product, however, and in fact the displacement in the pH profile is $\log(1 + S_r')$, where $S_r'$ is given by

$$S_r' = \frac{c_{f-ex} + c_{r-ex}}{1 + c_{f-in} + c_{r-in}} \tag{45}$$

This expression is symmetrical with respect to forward and reverse reactions (unlike Equation 44), and $S_r'$ is greater than the sum of $S_r$ values in forward and reverse reactions evaluated from Equation 44, except when there is no external commitment in one direction, in which case Equation 45 and Equation 44 for the sticky substrate are identical. $S_r'$ will thus be the same in forward and reverse reactions for the last substrates to add to the enzyme as long as $K_{eq}$ and the binding of the other substrates are pH independent. This is the case for fumarase, for example, but not for alcohol dehydrogenase, where $K_{eq}$ varies by a factor of 10 per pH unit, and the binding of DPN and DPNH varies with pH.

While the displacement in pKs caused by $S_r'$ values less than 0.1 is hardly noticeable, a value of $S_r'$ equal to unity causes a 0.3 pH unit displacement, which is not hard to dem-

onstrate by comparison of the pKs in V/K and $pK_i$ profiles (the correct values are seen for pKs in the $pK_i$ profile of a competitive inhibitor, since one extrapolates to the equilibrium situation of no variable substrate in determining a $K_i$ value[2]), or in V/K profiles for the sticky substrate and a much slower nonsticky one. An $S_f'$ value of 9 gives a full pH unit displacement, which does not even require statistical analysis to see. Such pH displacements have been seen for creatine-P with creatine kinase[40] and for L-alanine (but not for the much slower substrate, L-serine) with alanine dehydrogenase.[41]

Since incorrect protonation of catalytic groups on the enzyme prevents the catalytic reaction, but normally does not prevent dissociation of the substrate, the external commitment and along with it the stickiness of a substrate disappear as one goes past a pK and V/K begins to decrease a factor of 10 per pH unit. The result is an increase in the V/K isotope effect from its value at the pH optimum to a larger value closer to the intrinsic value as the pK is passed. If $^D(V/K)$ increases in this fashion as the pH is decreased, we can calculate a modified stickiness ratio as

$$S_f'' = \frac{[^D(V/K)_{\text{low pH}} - {}^D(V/K)_{\text{pH opt}}]}{[^D(V/K)_{\text{pH opt}} - 1]} = \quad (46)$$

$$\frac{[^Dk_5 - 1 + (^DK_{eq} - 1)c_{r-in}](c_{f-ex} + c_{r-ex}) - c_{r-ex}(^DK_{eq} - 1)(1 + c_{f-in} + c_{r-in})}{[^Dk_5 - 1 + (^DK_{eq} - 1)c_r](1 + c_{f-in} + c_{r-in})}$$

Unless $^DK_{eq} = 1.0$, or $c_{r-ex} = 0$, in which cases Equation 46 simplifies to Equation 45 or 44, respectively, $S_f''$ will not equal $S_f'$, but for normal isotope effects where $^Dk_5 > {}^DK_{eq}$ will be less than $S_f'$ in the direction where $^DK_{eq} > 1.0$ and will be greater than $S_f'$ when $^DK_{eq} < 1.0$.

This method has been used to show that L-alanine is sticky with alanine dehydrogenase ($^D(V/K_{alanine})$ goes from 1.4 to 2.0 as the pH is decreased from 11 to below 7) while L-serine is not[41] (the V/K isotope effect is pH independent at 2.0). With isocitrate dehydrogenase, $^D(V/K_{isocitrate})$ is 1.00 at neutral pH where isocitrate is very sticky, but increases to about 1.09 at low and high pH.[28] $^{13}(V/K_{isocitrate})$ is unity at neutral pH also,[42] but increases to 1.03 at pH 4.[42a] The pK of the acid-base catalytic group is seen at about 5.7 in the $pK_i$ profile for oxalylyglycine,[42a] but is displaced to low pH by at least a pH unit in the $V/K_{isocitrate}$ profile[28] (the third pK of isocitrate is seen in the V/K profile, but because only the trianion of isocitrate can bind this pK is not displaced, and it is the pK of the catalytic group, protonation of which does not prevent isocitrate binding, which is displaced by the stickiness of the substrate). Since the pH value at which $^D(V/K)$ and $^{13}(V/K)$ become the average of their values at low and neutral pH is also displaced to low pH by the stickiness of isocitrate, the limiting values of these isotope effects at low pH must be larger than those actually observed.

In contrast to isocitrate dehydrogenase, the closely related malic enzyme shows a pH-independent $^D(V/K_{malate})$ value of 1.5 so that malate clearly is not sticky.[32] An analysis of the $^{13}C$, tritium, and deuterium isotope effects (see Section VIII.A.) suggests that reverse hydride transfer is 6 to 12 times as fast as decarboxylation. With alcohol dehydrogenases, cyclohexanol is not sticky with the liver enzyme,[30] while isopropanol is somewhat sticky with the yeast enzyme[28] ($^D(V/K_{isopropanol})$ increases from 2.7 at pH 8.2 to 4.0 at pH 6.0).

This method is a very sensitive one for detecting the stickiness of substrates and is certainly one of the easiest to use, since it only requires comparison of isotope effects above and below a pK in the V/K profile, and the isotope effects are easily determined to the necessary degree of precision. It will certainly be the method of choice for enzymes

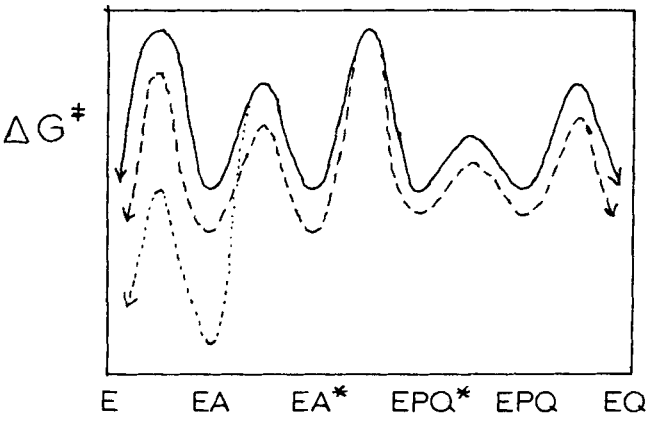



FIGURE 1. Free energy diagram for V/K in mechanism 8. The solid line is for unlabeled substrate and the dashed one for deuterated substrate. The dotted line corresponds to altered pH so that $k_3$ is decreased by a factor of 1000. (If $K_{eq}$ is not pH dependent, a decrease in $k_3$ with pH would have to be accompanied by a decrease in $k_8$, or in some other rate constant in the reverse direction, but if $K_{eq}$ is pH dependent, this might not be the case.) We assume $^{D}k_5 = 6$, $^{D}k_6 = 3$, and $^{D}K_{eq} = 2$, so that the left side of the deuterated profile is lowered from the unlabeled one more than the right side.

where a suitable isotope effect can be measured. However, it should be kept in mind that what is being measured is $S_r''$, which, unless the external reverse commitment is small or not pH dependent, will be somewhat larger than the true stickiness of the substrate and, in fact, could result solely from stickiness of the product.

### B. Determination of Rate-Limiting Steps for V/K from $c_f$ and $c_r$

When $c_f$ and $c_r$ can be separately determined, one can construct a full free energy diagram similar to Figure 1 and describe exactly where the rate limitation lies for V/K. In such a diagram the highest transition state is most rate limiting, and one sees a large isotope effect only when this corresponds to the isotope-sensitive step. The relative heights of the two transition states next to each intermediate enzyme complex determine the partition ratio for this intermediate. Thus for EA* in Figure 1, the fact that the barrier between EA* and EA is lower than that between EA* and EPQ* means that $k_4 > k_5$ in mechanism 8 and $c_{f-in}$ is small (0.1 here). Since the barrier between E and EA is higher than that between EA and EA*, $k_2 < k_3$, and since the E-EA barrier has the same height as the EA*-EPQ* one, $c_{f-ex}$ will be unity and the substrate somewhat sticky. The low EPQ*-EPQ barrier shows that $k_7 > k_6$, while the higher EPQ-EQ transition state corresponds to $k_8 > k_9$. However, since the barrier is lower than the EA*-EPQ* one, $c_r$ will be small (0.11 here).

We can thus see that in Figure 1 the heights of the barriers just before and after the one corresponding to bond-breaking relative to the latter determine the size of the internal portions of $c_f$ and $c_r$, while the heights of the outer barriers relative to the central one determine the external portions of $c_f$ and $c_r$. When the pH is changed so that the catalytic groups on the enzymes are largely in the wrong protonation state, the effect (shown by the dotted line in Figure 1) is to decrease the level of EA in the diagram without altering the barrier height between E and EA (if $k_2$ remains unchanged) and thus to make $c_{f-ex}$ very small and the substrate nonsticky. (This assumes that the EA complex can be

incorrectly protonated for catalysis, but that EA* cannot. If neither can be, the profile is not altered by pH, while if both can be incorrectly protonated, the level of the EA* complex is also decreased.)

The determination of rate-limiting steps from equilibrium perturbation isotope effects is similar to that described above and in Figure 1, and the free energy diagram includes that portion of the mechanism lying between the points of addition of the two perturbant molecules. The rate limitation applies to the flow of material from one perturbant to the other.

### C. Determination of Rate-Limiting Steps for V

The maximum velocity may be limited by steps that also limit V/K or by other steps that follow the first irreversible one or precede the addition of the variable substrate. The analysis of isotope effects on V can thus be more complex than analysis of those on V/K, but at the same time can yield information on portions of the mechanism which do not affect V/K. The classical free energy diagram in Figure 1 must be interpreted differently for analysis of the rate-limiting steps for V and thus of $^{D}V$ values, since the rate-limiting step involves reaction of the complex which occurs in highest concentration at saturating substrate. This complex will be the one which sees the highest total barrier in the forward direction to reach an irreversible step (such as product release), regardless of whether the barrier is for the immediate reaction of this complex or for a subsequent step. Thus in Figure 1, EA and EA* see equal barriers (the transition state between EA* and EPQ*) and forward reaction of these complexes will thus be equally rate limiting. Since this barrier is isotope sensitive, a large $^{D}V$ is expected if these steps are solely rate limiting. EPQ* and EPQ also see equal barriers (that between EPQ and EQ), but because this barrier is lower than that seen by EA and EA*, the reaction of these two complexes is not very rate limiting. One other step must also be considered in mechanism 8: the breakdown of EQ, which is not shown in Figure 1 because it comes after the release of P (the first irreversible step) and thus does not affect V/K. If the transition state for EQ breakdown is as high as that for reaction of EA and EA*, these steps will be equally rate limiting (thus lowering $^{D}V$ somewhat), while if it is higher, EQ breakdown would be totally rate determining for V and $^{D}V$ will be unity.

Even when the only important steps lie in the region of Figure 1 between EA and EPQ, isotope effects on V and V/K are not always the same.[6b] For example, if the level of the EPQ* complex is lowered drastically in Figure 1 without affecting the position of the transition states (that is, $k_6$ and $k_7$ are both reduced in mechanism 8 without changing their ratio or the values of the other rate constants), there is no effect on $c_r$ or $^{D}(V/K)$. Because the EPQ* complex now sees the highest barrier in the system (the transition state between EPQ and EQ), however, reaction of EPQ* becomes rate limiting, and $^{D}V$ becomes very small. Conversely, if the level of the EA complex in Figure 1 is raised drastically, along with the height of the transition state between EA and EA* (that is, $k_4$ is decreased in mechanism 8 without changing the other rate constants), $c_f$ will become large and $^{D}(V/K)$ will become small. The EA* complex now sees the highest barrier at substrate saturation, since the highest barrier for the EA complex is the EA-EA* transition state, and thus $^{D}V$ is still very large.

Another graph which is useful for analyzing $^{D}V$ effects is shown in Figure 2, in which pk', that is, log(1/k'), is plotted for each enzyme form in the mechanism. The parameter k' is the net rate constant (as defined by Cleland[26]) in the forward direction for reaction of the given enzyme form at saturation with the variable substrate. Each k' value is determined for both labeled and unlabeled reactants, and the difference between the values for the enzyme form with the highest position on the graph determines the isotope effect. (More precisely, $V_H$ or $V_D$ is the reciprocal of the sum of reciprocals of the k' values for H or D on the graph, and $^{D}V$ is $V_H/V_D$.) In the present case, breakdown of

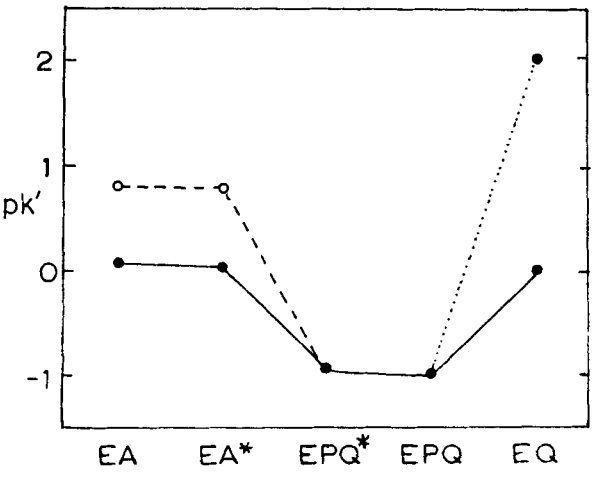



FIGURE 2. Rate limitation diagram for V for mechanism 8 with the same rate constants used in Figure 1 (1, 10, 10, 1, 1, 100, 100, 10 for $k_2$ to $k_9$, respectively, and $^Dk_5 = 6$, $^Dk_6 = 3$). In addition $k_{11}$ has been set equal either to 1.0 (solid line) or 0.01 (dotted line). The dashed line and open circles show the values for the deuterated substrate. pk' is the net rate constant in the forward direction at saturating substrate for reaction of each complex shown.

EQ is almost one third rate limiting for the unlabeled substrate, but is not rate limiting for the deuterated molecule, and $^DV = 3.89$, which is close to the $^D(V/K)$ value of 3.31. A lower rate constant for EQ breakdown (as shown by the dotted line in Figure 2) would make the process rate limiting for the deuterated molecule as well and lower the $^DV$ value to 1.1 with no change in $^D(V/K)$.

The effect of incorrect protonation of EA, and the corresponding reduction in the apparent value of $k_3$, is to raise the values of pk' for EA for both unlabeled and deuterated substrates until these values become totally rate limiting. $^DV$ is then 5.22, the same value obtained for $^D(V/K)$ when $c_{f-ex}$ is eliminated by the decreased value of $k_3$ as shown by the dotted line in Figure 1.

If, however, $k_{11}$ (or any other rate constant which limits V, but not V/K) is also pH dependent as well as $k_3$, $^DV$ will not become equal to $^D(V/K)$ when the EA complex is largely incorrectly protonated. Rather, the pk' values for both EA and EQ (or whatever complex is undergoing the other pH-dependent step limiting V) will be elevated together in Figure 2, and $^DV$ will become constant at some value different from and closer to unity than $^D(V/K)$, since another step which does not limit V/K is still partly rate limiting for V. Alanine dehydrogenase shows such a pattern.[41] Isomerization of E-DPN to E-DPN* (the form which can productively bind alanine and undergo reaction and which thus corresponds to E in mechanism 8) is largely rate limiting for V. The rate of this isomerization is pH dependent, decreasing a factor of 10 per pH unit at low pH, just as the rate of the dehydrogenation reaction does, so that their ratio remains constant. As a result $^DV$ is 1.35 at low pH, while $^D(V/K_{alanine})$ is 1.97.

Liver alcohol dehydrogenase also shows this phenomenon.[30] $^D(V/K_{cyclohexanol})$ is 2.5 at pH values below 8, while $^DV$ rises from 1.15 at pH 8 to 1.75 at pH 4.85. It is not known in this case what step partly limits V at low pH. The phenomenon is not general for all dehydrogenases, since lactate dehydrogenase,[19] isocitrate dehydrogenase,[28] malic enzyme,[28] and yeast alcohol dehydrogenase with isopropanol as substrate[28] all give equal isotope effects at low pH on V and V/K, as is predicted by mechanism 8 if $k_3$ is the only

pH-dependent step. In this simple model, $c_{V_f}$ becomes equal to $c_{f\text{-}in}$ at low pH (and thus $^{D}(V/K) = {}^{D}V$) because $k_3$ becomes so much smaller than all of the other rate constants in Equation 13.

At neutral pH, however, $c_{V_f}$ need not equal $c_f$ even when $k_{11}$ is not at all rate limiting, as in the cases discussed above in which $^{D}V$ and $^{D}(V/K)$ are drastically different.[6b] Clear cases where $^{D}V$ and $^{D}(V/K)$ are different for reasons other than the stickiness of the substrate or the partial rate limitation of $k_{11}$ are still rare, but the primary deuterium isotope effects with the methylene-bridged analog of 2-P-glycerate as a substrate for enolase are an apparent example.[43] $^{D}(V/K)_{Mg}$ is 7 to 8, but $^{D}V$ is only 1.3. It appears that the carbanion resulting from C-H bond cleavage is specifically stabilized so that its breakdown is partly rate limiting for V (this corresponds to the case described above in which $k_6$ and $k_7$ are both decreased in mechanism 8). In the reverse reaction, of course, the slow reaction of the carbanion is its protonation, and the $D_2O$ solvent isotope effect on V is large (4.1), as predicted by this model.

### D. Examples of Studies on Rate-Limiting Steps

An interesting isotope effect study involved the shift in rate-limiting steps seen with arginine decarboxylase when the solvent was switched from water to 16 mol % ethylene glycol.[44] With the slow substrate homoarginine (V 1% that of arginine), $^{13}(V/K)$ was 1.061, suggesting that decarboxylation was almost totally rate limiting. In the presence of ethylene glycol V increased by a factor of 3, and $^{13}(V/K)$ decreased to 1.044. Since it is thought that $^{13}k$ is not different in the two cases, it appears that a commitment of 0.4 has been caused by the ethylene glycol. This presumably reflects an increase in the decarboxylation rate (postulated to be the result of a medium effect) and a decrease in the rate of Schiff base formation between substrate and amino acid.

With the fast natural substrate arginine, $^{13}(V/K)$ decreased from 1.028 in water to 1.003 in 16 mol % ethylene glycol, while V decreased to 81% of the value in water. If $^{13}k$ is 1.06 for arginine as well as for homoarginine (the structures are identical in the vicinity of the carbon lost as $CO_2$), the commitments are 1.14 and 19. Again the ethylene glycol causes in increase in the decarboxylation rate and a decrease in the rate of Schiff base formation, but because the latter was partly rate limiting to start with, the result is a drop in V and a drastic increase in commitment. For both sets of data if one assumes that Schiff base formation has an equilibrium constant of unity (it is a transimination reaction between the amino acid and the Schiff base of pyridoxal-P with a lysine on the enzyme), the ethylene glycol increases decarboxylation by a factor of 5 to 7 and decreases Schiff base formation by a factor of 3. Further, it appears that the slow reaction with homoarginine is not the result of nonproductive binding of the substrate before Schiff base formation or of slow Schiff base formation, but rather of a slow rate constant for decarboxylation. Presumably the geometry of the Schiff base is not quite right for the decarboxylation process with the substrate which is one carbon too large for the active site.

Rosenberg and Kirsch[45,46] recently carried out $^{18}O$ isotope effect studies on a number of glycosidases. In each case $^{18}O$ was in the bridge between the sugar and the leaving group, and the bond cleavage is between the oxygen and the sugar. These are thus primary isotope effects and might be expected to show a reaction coordinate motion effect, as does C—O bond cleavage in the fumarase reaction.[12] Because the masses of the separating fragments are much larger for the glycosidase than with fumarase, however, these authors assumed only an equilibrium component to the isotope effects and compared the values to $^{18}K_{eq}$ for conversion of a p-nitrophenyl glycoside to p-nitrophenolate (1.0425; a value calculated from vibrational frequencies).

With β-galactosidase with the very unstable substrate 2,4-dinitrophenyl-β-D-galactoside,[45] $^{18}V$ was 1.002 because the second step in the ping pong reaction is rate limiting

with this substrate (the phenol is liberated during formation of galactosyl enzyme, which then is hydrolyzed). $^{18}(V/K)$ was 1.030, however, showing that C—O bond cleavage is partly rate limiting (or at least involved) for V/K. Because the $\alpha$-secondary deuterium isotope effect on V is 1.25 in this system[47] (and is 1.34 when methanol replaces water as the acceptor [48]), it was felt that C-1 of the sugar is very weakly bonded in the transition state and is nearly an oxycarbonium ion (for formation of which $^{D}K_{eq}$ for the $\alpha$-secondary hydrogen would be 1.33). Unfortunately nothing is known of the commitments in this system, so a more exact analysis is not possible.

With the more stable substrate p-nitrophenyl-$\beta$-D-galactoside, $^{18}V$ was 1.022 and $^{18}(V/K)$ was 1.014. It is not clear whether stickiness of the substrate, experimental error, or some other reason causes $^{D}V$ to exceed $^{D}(V/K)$, but since the $\alpha$-secondary deuterium isotope effect was only 1.04 with this substrate,[47] it was felt that the reaction proceeds via an $S_N2$ displacement of phenol by the catalytic group to give galactosyl-enzyme. Clearly, formation of galactosyl-enzyme is slower than its hydrolysis with this substrate, but since the commitments are unknown, further conclusions about transition state geometry are not really possible. Very similar data were seen for hydrolysis of p-nitrophenyl glucoside by $\beta$-glucosidase, with $^{18}(V/K) = 1.038$ and $^{\alpha\text{-}D}(V/K) = 1.015$, and similar conclusions were reached.[46]

With lysozyme and a p-nitrophenyl derivative of a disaccharide, $^{18}(V/K)$ was 1.047, and $^{\alpha\text{-}D}(V/K)$ is reported as 1.11 for a similar substrate.[46] This enzyme has long been thought to involve an oxycarbonium ion as either an intermediate or transition state,[20a] and it is clear that C-1 of the sugar does develop considerable $sp^2$ character during the reaction. Since the commitments are not known, however, it is not possible to deduce whether (1) oxycarbonium ion formation (stabilized by asp-52) is in rapid equilibrium prior to rate-limiting release of p-nitrophenol, (2) we are looking at a transition state for formation of an oxycarbonium ion, or (3) we have an $S_N2$ reaction with very low bond order to both the incoming and leaving oxygens in the transition state.

$^{18}O$ isotope effects, especially in conjunction with deuterium, $^{13}C$, and solvent isotope effects (see Section IX.D.), should certainly prove to be a very useful tool for studying enzymes like these, but ways have to be found to determine the commitments and to evaluate the contribution of reaction coordinate motion to the intrinsic $^{18}O$ isotope effects before full interpretation is possible.

## VIII. USE OF ISOTOPE EFFECTS TO DETERMINE CHEMICAL MECHANISM

The third stage of kinetic analysis of enzyme mechanisms is determination of the chemical mechanism.[2] pH studies can identify the groups on the enzyme which act as acid-base catalysts, or which have to be in a given protonation state for binding. A careful study of isotope effects and their pH dependence, however, can often provide additional evidence on the chemical mechanism and the nature of the intermediates in the reaction. We will give several examples of what has been or could be determined by such studies.

### A. Establishing the Order of Deuterium- and $^{13}C$-Sensitive Steps

There are a number of enzymatic reactions in which deuterium- and $^{13}C$-sensitive steps are not the same. For example, with malic enzyme, the primary deuterium isotope effect at C-2 of malate corresponds to the hydride transfer step, while the $^{13}C$ isotope effect at C-4 corresponds to the subsequent decarboxylation step. One can, of course, determine the primary $^{13}C$ isotope effect at C-2, which does correspond to the hydride transfer step; by doing this with deuterated and unlabeled malate, one can determine $^{D}k$ and $^{13}k$ for the hydride transfer step; as well as $c_f$ and $c_r$ as described in Section V.B.

By using deuterated and unlabeled malate to determine $^{13}(V/K)$ for C-4, however, one can establish whether the deuterium- or $^{13}C$-sensitive step comes first in the mechanism or whether the reaction is concerted, so that only one step is sensitive to both isotopes. While there is little question that the malic enzyme reaction involves hydride transfer prior to decarboxylation, the mechanism is not always so clear, and particularly when a concerted reaction is a possibility this method should prove very useful in establishing the order of events. There must be, of course, large enough deuterium and $^{13}C$ (or other heavy atom or $\beta$-secondary deuterium) isotope effects to measure accurately.

The equations which apply in this case when the deuterium-sensitive step comes first are[32a]

$$x = {}^{D}(V/K) = \frac{{}^{D}k + a + {}^{D}K_{eq}(1/c)(1 + b)}{1 + a + (1/c)(1 + b)} \tag{47}$$

$$y = {}^{13}(V/K)_{H} = \frac{{}^{13}k + c(1 + a) + {}^{13}K_{eq}\, b}{1 + c(1 + a) + b} \tag{48}$$

$$z = {}^{13}(V/K)_{D} = \frac{{}^{13}k + (c/{}^{D}K_{eq})({}^{D}k + a) + {}^{13}K_{eq}\, b}{1 + (c/{}^{D}K_{eq})({}^{D}k + a) + b} \tag{49}$$

where a is the forward commitment for the deuterium-sensitive step, b is the reverse commitment for the $^{13}C$-sensitive step, and c is the ratio of rate constants for forward reaction through the $^{13}C$-sensitive step to reverse reaction through the deuterium-sensitive step. In this system z is closer to unity than y, while the reverse is true when the deuterium- and $^{13}C$-sensitive steps are the same. Further,

$$\frac{y - 1}{z - 1} = x/{}^{D}K_{eq} \tag{50}$$

and thus there are only two independent equations above. Equation 50 holds even when one or more isotope-insensitive steps lie between the deuterium- and $^{13}C$-sensitive steps. If the deuterium-sensitive step follows the $^{13}C$-sensitive one, the equations are

$$x = \frac{{}^{D}k + c(1 + a) + {}^{D}K_{eq}\, b}{1 + c(1 + a) + b} \tag{51}$$

$$y = \frac{{}^{13}k + a + {}^{13}K_{eq}(1/c)(1 + b)}{1 + a + (1/c)(1 + b)} \tag{52}$$

$$z = \frac{{}^{13}k + a + {}^{13}K_{eq}(1/c)({}^{D}k + b\,{}^{D}K_{eq})}{1 + a + (1/c)({}^{D}k + b\,{}^{D}K_{eq})} \tag{53}$$

where a is the forward commitment for the $^{13}C$-sensitive step, b is the reverse commitment for the deuterium-sensitive step, and c is the ratio of rate constants for forward reaction through the deuterium-sensitive step and reverse reaction through the $^{13}C$-sensitive step. z is again closer to unity than y, but now:

$$\frac{y - {}^{13}K_{eq}}{z - {}^{13}K_{eq}} = x \tag{54}$$

Equations 50 and 54, both of which relate x, y, and z, will be different as long as

$$^{D}K_{eq} \neq \frac{(y - {}^{13}K_{eq})(z - 1)}{(y - 1)(z - {}^{13}K_{eq})} \quad (55)$$

and thus we have a way of telling the order of the deuterium- and $^{13}$C-sensitive steps. This method has been applied to malic enzyme,[18,32] where x = 1.47, y = 1.031, z = 1.0250, $^{13}K_{eq}$ = 0.999, and $^{D}K_{eq}$ = 1.18. With these values, the two sides of Equation 50 are 1.21 and 1.25, while the two sides of Equation 54 are 1.20 and 1.47. Clearly, Equation 50 holds while Equation 54 does not, and the deuterium-sensitive step precedes the $^{13}$C-sensitive one, as has always been assumed.

The fact that Equations 47 to 49 and 51 to 53 are not independent means that we cannot solve for $^{D}k$ and $^{13}k$ in the general case as one does when the isotope effects are on the same step. Even when the equation for $^{T}(V/K)$ is included, one has only three independent equations and five unknowns. Further analysis is possible in several special cases, however, First, if $^{D}k$, $^{13}k$, and the commitments are determined for the deuterium-sensitive step, the unknowns are reduced to b, c, and $^{13}k$ for a $^{13}$C-sensitive step which follows the deuterium-sensitive one and to a, c, and $^{13}k$ for one which comes first, and limits can be calculated for three parameters. Thus when the deuterium-sensitive step precedes the $^{13}$C-sensitive one, a knowledge of $^{D}k$, a, and the reverse commitment (1 + b) c provides two independent equations:

$$d = (1 + b)/c \quad (56)$$

$$y = \frac{^{13}k + c(1 + a) + {}^{13}K_{eq}\, b}{1 + c(1 + a) + b} \quad (57)$$

which combine to give

$$^{13}k = y + (y - 1)(1 + a)/d + b[(y - 1)(1 + a)/d + y - {}^{13}K_{eq}] \quad (58)$$

The value of $^{13}k$ must be at least the value when b = 0, but can have any reasonable value larger than this, corresponding to increasing values of b and c.

For most decarboxylations, b is probably zero (that is, $CO_2$ has no commitment), and thus exact calculation of $^{13}k$ is possible if $^{D}k$ is known. When $^{D}k$ has not been determined, deuterium, tritium, and $^{13}$C isotope effects can be combined to give limits on $^{D}k$, $^{13}k$, a, and c if b is assumed to be zero. This is done by assuming different values of $^{D}k$ and calculating the following values:

$$w = {}^{T}(V/K) \quad (59)$$

$$r = 1/ac = \frac{[(^{D}k)^{1.442} - w][x - 1) - (^{D}k - x)(w - 1)}{(^{D}k - x)[w - (^{D}K_{eq})^{1.442}] - [(^{D}k)^{1.442} - w](x - {}^{D}K_{eq})} \quad (60)$$

$$a = \frac{(^{D}k - x)}{x - 1 + (x - {}^{D}K_{eq})\, r} \quad (61)$$

$$c = 1/ar \quad (62)$$

$$^{13}k = y + (y - 1)(1 + a)c \quad (63)$$

By discarding negative values of a or c and values of $^{13}k$ larger than are reasonable for a $^{13}$C isotope effect, one obtains limits on the various parameters. With data for malic

enzyme,[18,32] for example (w = 2.02, x = 1.47, y = 1.031, $^{D}K_{eq}$ = 1.18), c becomes negative below a value of 4.3 for $^{D}k$ (this value for $^{D}k$ gives a singularity at $\pm\infty$ for $^{13}k$), while a becomes negative above a value of 6.7 for $^{D}k$ ($^{13}k$ is 1.033 at this point). If the range of 1.04 to 1.06 is taken as reasonable for $^{13}k$, $^{D}k$ could vary from 5.3 to 6.0, a from 2.5 to 4.4, and 1/c from 6 to 12.

### B. The pH Variation of Isotope Effects and the Chemical Mechanism of Liver Alcohol Dehydrogenase

As noted in Section VII.A., a simple model such as mechanism 8 in which the EA complex can be incorrectly protonated for reaction so that $k_3$ is pH dependent predicts that $^{D}V$ and $^{D}(V/K)$ will become equal when EA is largely incorrectly protonated and that the isotope effects will be the same size or larger than they are at the pH optimum. With liver alcohol dehydrogenase with DPN and cyclohexanol-1-d, or DPND and cyclohexanone as substrates,[30] however, where $V/K_{cyclohexanol}$ decreases below a pK of 7.1, while $V/K_{cyclohexanone}$ decreases above a pK of 8.8, $^{D}(V/K_{cyclohexanol})$ and $^{D}(V/K_{cyclohexanone})$ decrease from values of 2.5 and 2.1 at low pH to 1.0 and 0.85 at high pH with a pK of 9.4. This decrease in the V/K isotope effects as V/K for cyclohexanone is decreasing shows that the hydride transfer step precedes the pH-dependent step in the direction of ketone reduction and that the isotope-sensitive hydride transfer step is not pH dependent, although since the pK for the isotope effects is 0.6 pH unit higher than that for $V/K_{cyclohexanone}$, the hydride transfer step may go as much as fourfold slower when the acid-base catalytic group is not protonated. It thus appears that the ketone is reduced to an alkoxide intermediate by hydride transfer and that the pH-dependent step which follows is the protonation of the alkoxide to permit its release as a free alcohol.

In the direction of alcohol oxidation, the alkoxide intermediate must form before dehydrogenation by proton transfer to a base on the enzyme thought to be His-51. This histidine has its back side exposed to solvent[49] so that a proton can be lost to the solution from the EH-DPN-alkoxide intermediate at high pH, with the result that the reprotonation of the alkoxide cannot occur and $c_{f-in}$ for cyclohexanol becomes very large. Since there do not appear to be external commitments for cyclohexanol or cyclohexanone, the value of $c_{f-in}$ at low and neutral pH is 2.5 from comparison of the intrinsic isotope effect of 6.3 and the observed $^{D}(V/K_{cyclohexanol})$ value of 2.5. In the reverse reaction at high pH, alkoxide formation comes to equilibrium, and the inverse $^{D}K_{eq}$ value of 0.85 is seen above pH 11. A similar pH variation in the isotope effects has been seen in single turnover experiments for the rate of disappearance of the highly colored complex of E-DPNH with *trans*-4-(N,N-dimethylamino)-cinnamaldehyde[50] and also in steady state studies with the yeast enzyme with isopropanol and acetone as substrates.[28]

There has been considerable controversy over whether the substrates for the alcohol dehydrogenase reaction form an inner or outer sphere complex with the catalytic zinc. An interesting application of isotope effects to the problem comes from solvent isotope effect studies with p-methoxybenzaldehyde and the yeast enzyme.[51] pH profiles for V in $H_2O$ and $D_2O$ were fitted to the appropriate rate equations, and the extrapolated ratios at high pH for alcohol oxidation and at low pH for aldehyde reduction were compared. A significant isotope effect was not seen for alcohol oxidation, which for this slow substrate is limited solely by the rate of the hydride transfer step which involves no proton transfers. An isotope effect of 0.5 to 0.6 seen in the direction of reduction is thought to represent the effect of releasing a water molecule during formation of an inner sphere complex of aldehyde with zinc. The fractionation factor of each proton in water bonded to zinc would have to be 0.7 to 0.8 relative to bulk water, which seems reasonable in view of the value of 0.69 per proton in $H_3O^+$.[52]

When the pK values for the drop in V at low pH for alcohol oxidation and at high pH

for aldehyde reduction were compared in $D_2O$ and $H_2O$, the former was elevated by 0.21 pH unit and the latter by 0.02 pH unit, although the pKs of groups such as carboxyl, amino, and imidazole are elevated by 0.48 pH unit in $D_2O$. The pKs seen in the V profiles are not those of the acid-base catalytic group itself, however, but are displayed by factors which are functions of the rate constants in the mechanism. In the simplified mechanism,

$$\begin{array}{ccccc} \text{E-DPN-alcohol} & \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} & \text{EH-DPN-alkoxide} & \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} & \text{EH-DPNH-aldehyde} \\ \uparrow\downarrow K_1 & & \uparrow\downarrow K_2 & & \uparrow\downarrow K_3 \\ \text{EH-DPN-alcohol} & & \text{E-DPN-alkoxide} & \underset{k_{20}}{\overset{k_{19}}{\rightleftharpoons}} & \text{E-DPNH-aldehyde} \end{array} \qquad (64)$$

where $k_{11}, k_{12} \gg k_{13}, k_{14}, k_{19}, k_{20}$, because hydride transfer is so slow, the apparent pK for V for aldehyde reduction is[30]

$$pK_{app} = pK_2 + \log \frac{(k_{12}/k_{14})}{(1 + k_{19}/k_{20})} \qquad (65)$$

Since: (1) the ratio of $k_{19}$ and $k_{20}$ should not be solvent dependent, (2) the value of $k_{14}$, which is solely rate limiting at low pH for aldehyde reduction, is twice as high in $D_2O$ as in $H_2O$, and (3) $pK_2$ should be elevated 0.48 pH units in $D_2O$, the observed rise in $pK_{app}$ of 0.02 pH units requires that $k_{12}$ be slower in $D_2O$ by a factor of 1.45. Since $k_{12}$ involves proton transfer to the alkoxide to give an alcohol, a normal isotope effect is expected, although its small size suggests that the conformation change accompanying the proton transfer is more rate limiting than the proton transfer itself.

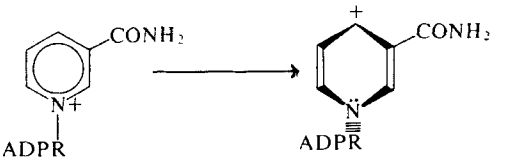
For alcohol oxidation, the apparent pK in the V profile is given by[30]

$$pK_{app} = pK_1 + \log \frac{(k_{12}/k_{13})}{(k_{11}/k_{19})} \qquad (66)$$

and since the ratio of $k_{13}$ to $k_{19}$ should not be affected by $D_2O$, we know the effects on $k_{12}$ and $pK_{app}$, and $pK_1$ should be increased by 0.48 pH unit, $k_{11}$ will show an inverse isotope effect of 0.78. This inverse effect presumably results from replacing water on zinc with the alcohol prior to the formation of the alkoxide and equals the fractionation factor relative to bulk water of the single net proton displaced ($H_2O$ has two protons, while ROH has only one; thus one fewer proton is attached to zinc-bonded oxygen when ROH replaces $H_2O$ on zinc). These data thus suggest that inner sphere complexes of substrate and zinc form prior to any chemical reaction in both directions of the reaction.

Still further details concerning the alcohol dehydrogenase mechanism come from isotope effects with DPN labeled with $^{15}N$ at N-1 of the nicotinamide ring.[16] By running equilibrium perturbations at low levels of cyclohexanol and cyclohexanone, it was possible to reduce the commitments in the system solely to the partition ratio for the alkoxide of hydride transfer to protonation, which is 2.5. Perturbations with ratios of DPNH/DPN varying from 0.12 to 2.0 showed that apparent $^{D}$(Eq.P.) varied from 1.018 at a very low ratio to 1.062 at a very high one, with $^{15}K_{eq} = 1.044$ from the DPN side. This

is so far the only case where the variation in perturbation size with the ratio of perturbants has been used to determine both the equilibrium isotope effect and the kinetic ones in both directions. The large normal $^{15}N$ isotope effects in both directions show that the reaction proceeds through an intermediate or transition state in which the bond order at N-1 is less than it is in either DPN (bond order 4) or DPNH (bond order around 3.4 as the result of resonance with the side chain). These data suggest that the bond order becomes 3 in the key intermediate and thus that the enzyme bends the absorbed nicotinamide ring to produce a trigonal nitrogen at N-1 and to promote the development of positive charge at C-4:




(67)

A quantitative analysis of the $^{15}N$ data in terms of Equation 14, assuming that hydride transfer is the $^{15}N$-sensitive step, gives

$$1.062 = \frac{^{15}k + 2.5}{1 + 2.5} \tag{68}$$

from which $^{15}k$ would have to have the unbelievably large value of 1.22. If, however, alkoxide formation (or some prior step) is $^{15}N$-sensitive and hydride transfer is not

$$1.062 = \frac{^{15}K_{eq\ 3} + 2.5\ ^{15}k_3}{1 + 2.5} \tag{69}$$

where $^{15}k_3$ and $^{15}K_{eq\ 3}$ are kinetic and equilibrium isotope effects on the $^{15}N$-sensitive step prior to hydride transfer. While an exact solution is not possible, it seems most likely that $^{15}K_{eq\ 3}$ is 1.07 to 1.08 and $^{15}k_3$ is 1.06 to 1.055, so that the transition state is rather late, as expected for such a deformation process.

N-1 of DPN presumably remains trigonal during hydride transfer and becomes planar only in the fast steps which accompany release of cyclohexanone. Note that in this mechanism a carbonium ion forms at C-4 of DPN in the same step that the alcohol becomes an alkoxide, so that less energy is needed for both processes. The net effect is to transfer the positive charge of N-1 to the acid-base catalytic group, while leaving the DPN and alcohol in chemically activated forms which readily react to give DPNH and the aldehyde or ketone.

### C. Isotope Effects on Fumarase[12]

The chemical mechanism of fumarase has been uncertain for many years, despite the fact that the elegant kinetic studies of Alberty and co-workers identified the acid-base catalytic groups as a carboxyl and a histidine.[53] A carbonium ion mechanism was proposed on the basis of the rates and products formed for reactions of mono- and difluorofumarates relative to other halo-fumarates,[54] but it is now known that these data are also consistent with the carbanion intermediate which is clearly established by isotope

effect studies[12] and by the very strong inhibition by the aci form of the dianion of 3-nitrolactate.[55]

The isotope effects on fumarase are small and very pH dependent and most were determined by equilibrium perturbation.[12] The primary $^{18}O$ isotope effect is 1.072 from the malate side (1.038 from the fumarate side) at pH 5, but decreases rapidly with pH until it is less than 1.01 above pH 7. Since $^{18}K_{eq} = 1.033$, it is clear that the effect of rising pH is to increase the commitment for malate, but not that for water (the product containing $^{18}O$), which presumably is very small at all pH values (any finite commitment for water would result in some portion of $^{18}K_{eq}$ being observed in $^{18}(Eq.P.)_{malate}$). The very large $^{18}O$ isotope effects in both directions at pH 5 show that C—O bond cleavage is totally rate limiting at this pH.

The primary deuterium isotope effect is near unity at pH 5, but from pH 5.5 to 8 is inverse, reaching a minimum value at pH 6.5 of about 0.92 from the malate side. This result requires that: (1) at neutral pH proton transfer from the 3R position of malate to the enzyme must come nearly to equilibrium, (2) this proton must not be exchangeable with the solvent when it is on the enzyme, and (3) the fractionation factor of the proton on the acid-base catalytic group must be greater than that of water by a factor which is probably at least 1.2 (the acid-base catalyst is thought to be a carboxyl group whose fractionation factor is raised relative to water by restricted rotation of the OH group,[12] and it is known that this proton is released only after fumarate leaves the enzyme[56]). These results are inconsistent with either a concerted reaction, in which C—O bond cleavage would also have to come to equilibrium, or with a carbonium ion mechanism, in which C—O bond cleavage would have to precede C—H bond cleavage, and thus again be at equilibrium, in contrast to the large observed $^{18}O$ isotope effects at low pH. They do support a carbanion mechanism, however, and all other data are consistent with such an intermediate.

The secondary isotope effects with dideuterofumarate, or the dideuteromalate made from it enzymatically, are seen largely from the malate side at low pH but largely from the fumarate side at high pH (1.31 from the malate side at pH 5 to 7, decreasing to 1.07 at high pH with a pK of 8.1, compared to $^{D}K_{eq} = 1.45$). At low pH where C—O bond cleavage is rate limiting, we expect an equilibrium isotope effect at C-3 (1.11 if the carbanion is in the aci form which is $sp^2$ at C-3; this matches closely the observed value with malate-3S-d of 1.13 at pH 5) and a kinetic one at C-2 ($1.31/1.11 = 1.18$, corresponding to a slightly late transition state, since $^{D}K_{eq} = 1.31$ for deuterium at C-2).

By making reasonable assumptions it was possible to deduce partition ratios as a function of pH for E-malate and E-carbanion and commitments for malate, fumarate, and the 3R proton.[12] The partition ratios were largely independent of pH except at low pH where malate became no longer sticky and where the ratio of C—O bond cleavage to protonation for the carbanion intermediate decreased by a factor of 4. The commitments for the reactants were high (up to 12 for malate and fumarate, except at pH 5 where both were very low, and from 36 to over 100 for the 3R proton), and thus except at pH 5 it is the balance between the forward and reverse commitments, rather than the intrinsic isotope effects, which determines the size of the observed isotope effects. In such a situation, Equation 14 becomes

$$^{D}(V/K) = \frac{^{D}K_{eq} + c_f/c_r}{1 + c_f/c_r} \qquad (70)$$

and the ratio of $c_f$ to $c_r$ determines how much of the equilibrium isotope effect is seen in the forward and reverse directions.

### D. The Mechanism of Ethanolamine Deaminase

Ethanolamine deaminase and diol dehydrase are enzymes catalyzing the following reactions:

$$HO-CH_2-CH_2-NH_3^+ \longrightarrow CH_3-CHO + NH_4^+ \quad (71)$$

$$HO-CH_2-CH_2-OH \longrightarrow CH_3-CHO + H_2O \quad (72)$$

in which hydrogen is transferred from one carbon to the other, and in turn either a $NH_3^+$ or OH group migrates in the other direction.[57,58] The resulting carbinolamine or gem-diol then breaks down to produce acetaldehyde.

These enzymes have a cobalamine coenzyme which in its resting state has the C-5′ of a 5′-deoxyadenosine group attached to the cobalt (III) in the corrin nucleus. The bond from the cobalt to C-5 can be broken, and denaturation of ethanolamine deaminase while it is operating on ethanolamine gives up to 4% of the coenzyme as free 5′-deoxyadenosine.[59] With the alternate substrate propanolamine, however, up to 90% of the coenzyme could be isolated at 5′-deoxyadenosine, depending on the method of denaturation (heat or trichloracetic acid gave high values; ethanol gave only 13%). The cleavage of the C—Co bond is apparently reversible on the enzyme (although externally added 5′-deoxyadenosine does not exchange with bound coenzyme), since a reaction mixture incubated first with propanolamine under conditions giving 80% 5′-deoxyadenosine on denaturation, and then with ethanolamine, gave only 11% as the free nucleoside.[59] Further, tritium from the propanolamine found its way into the 5′ position of the coenzyme (both that liberated as free nucleoside during denaturation, and that remaining bound to cobalt) and then upon addition of ethanolamine was found in acetaldehyde.

It thus appears that cleavage of the C—Co bond in the coenzyme yields a cobalamine containing Co (II) and a 5′-deoxyadenosine radical, which in turn extracts a hydrogen atom from the substrate to give 5′-deoxyadenosine and a substrate radical. Rearrangement of the substrate radical by migration of the amino group gives a product radical which removes a hydrogen atom from the 5′-deoxyadenosine to give product and allow regeneration of the coenzyme containing the C—Co bond.

When deuterated ethanolamine was used as a substrate, $^DV$ was found to be 7.4, a value which is quite reasonable for a rate-limiting hydrogen atom transfer ($^D(V/K)$ was similar in size).[60] Tritiated substrate gave puzzling results, however. First, tritium accumulated in the 5′ position of the coenzyme during reaction, and $^T(V/K)$ appeared to be 4.7 for the process. Second, under conditions where enzyme was in excess over coenzyme, comparison of the rate of washout of tritium from tritiated coenzyme into product with the rate of turnover gave an isotope effect of 160 when unlabeled substrate was used and 22 with deuterated substrate (these are the actual discriminations for the methyl hydrogens of 5′-deoxyadenosine after corrections have been made for the fact that each tritium is competing with two H or D atoms).[60] Although these results (and similar ones obtained for diol dehydrase[61]) have been published for over 10 years, a possible interpretation for these unreasonably large isotope effects has only recently been deduced. The value of 160 represents $k_H/k_T$, while the value of 22 is $k_D/k_T$ (the coenzyme rapidly becomes deuterated by deuterium transfer from the substrate during the first few turnovers, so that the discrimination is between D and T). The ratio of these values, $(k_H/k_T)/(k_D/k_T) = k_H/k_D$, is 7.3, an entirely reasonable value for a deuterium isotope effect, and in excellent agreement with $^DV$. What then causes the very large observed discrimination against tritium? The key assumption made in the calculations is that *one atom of hydrogen is washed out of the coenzyme during each turnover*. If we assume that

hydrogen is transferred from coenzyme to product only once in 9.1 turnovers, however, the $k_H/k_T$ and $k_D/k_T$ values become 17.6 and 2.4, in agreement with the expected relationships:

$$k_H/k_T = (k_H/k_D)^{1.44} \quad (73)$$

$$k_D/k_T = (k_H/k_D)^{0.44} \quad (74)$$

It is thus clear that the role of the coenzyme is to provide a source of radicals for beginning the reaction, but that the radical chain can then be propagated by some other group on the enzyme which is left as a radical when the product departs. With saturating substrate (the conditions of the washout experiments), the substrate combines with the enzyme and is converted to a radical before the other radical present has time to abstract a hydrogen atom from 5′-deoxyadenosine and permit the C—Co bond in the coenzyme to be regenerated. It would be interesting to carry out the experiments with a subsaturating level of substrate (added at a fixed rate so that many turnovers can be carried out); one would anticipate that the increased delay in each reaction cycle would lead to reformation of the coenzyme and isotope effects on tritium washout from the coenzyme closer to the expected values. The nature of the group which forms radicals on the enzyme is completely unknown, but hopefully this interpretation of the isotope effects will stimulate further study of the situation.

We should point out that there appears to be no commitment for reaction of product radicals with 5′-deoxyadenosine, since the observed isotope effect of 7.3 for $^Dk$ is the value expected for an intrinsic isotope effect in this case. Likewise there appears to be little commitment for reaction of the unknown radical which participates in eight of nine turnovers with the substrate, since $^DV$ has the same value. Reaction of substrate with the 5′-deoxyadenosine radical to transfer tritium into the coenzyme, however, shows a much lower isotope effect (corresponding to 2.9 for $^D(V/K)$, and a commitment of 2.3 if $^Dk$ is 7.3), showing that reformation of the C—Co bond is slower than rearrangement of the substrate radical to the product radical. Having a lower isotope effect for transfer of tritium into coenzyme than for its removal leads to a specific activity for each of the 5′ hydrogens of coenzyme in the steady state which is higher than that of substrate by the ratio of the isotope effects. For ethanolamine deaminase the expected ratio would be just over 3, but for diol dehydrase,[61] this ratio was 8 to 10. Tritium isotope effects much larger than are reasonable have also been seen for tritium transfer from coenzyme to product with diol dehydrase,[61] and presumably for this enzyme also the radical chain is propagated for a number of turnovers without regeneration of the C—Co bond of the coenzyme.

### E. The Use of An Isotope Effect on Internal Partitioning to Deduce Chemical Mechanism

An interesting use of isotope effects to distinguish chemical mechanisms is the recent study of the reaction of fluoromethylglyoxal in the presence of glutathione and glyoxylase-I.[61a] This enzyme normally transfers a hydrogen from carbon 1 of the thiohemiacetal of glutathione and methylglyoxal to carbon 2 without exchange with the solvent to give a lactyl thioester. Mechanisms have been proposed involving either a hydride shift or proton removal by a base on the enzyme to give an endiol intermediate. The fluoro substrate produces both S-fluorolactylglutathione as expected and also S-pyruvylglutathione as the result of fluoride elimination. When fluoromethylglyoxal-1-d was used, the proportion of fluoride released increased, with an isotope effect on the partition ratio of 1.4 to 1.8, depending on the source of the enzyme. These results support

the endiol mechanism, since fluoride elimination from the enediol would not show an isotope effect, while protonation of the enediol by a deuteronated base would be slower than with a protonated one. In the hydride shift mechanism HF elimination would presumably occur from the enzyme-bound thioester after the hydride shift and should be slower with a deuterated molecule, so that a lower proportion of the product would undergo fluoride elimination. The isotope effect on partition of the enediol intermediate is not large, suggesting that there may be a high commitment for protonation of the enediol intermediate when fluorine is in the in-plane position (that is, protonation is faster than rotation of the fluoromethyl group and elimination of fluoride). This is not surprising, and the variable partition ratios with enzymes from different sources (fluoride elimination occurs once in 3, 4, or 12 turnovers with enzymes from yeast, mouse, and rat) suggests that the enzyme tends to hold the fluorine in in-plane positions to a variable degree.

### F. The Use of Equilibrium Isotope Effects on Aldehyde Hydration to Deduce Chemical Mechanisms

Deuterium substitution on an aldehydic carbon increases the equilibrium constant for hydration of the aldehyde by a factor of 1.37.[61b] This large equilibrium isotope effect makes it possible to tell whether the free aldehyde or the hydrate is the active form of a substrate or inhibitor. For the aldehyde-induced ATPase reactions catalyzed by phosphofructokinase[61c], fructokinase[61d] the size of the deuterium isotope effect on V/K for the aldehydes (2,5-anhydromannose-6-P and 2,5-anhydromannose showed that the aldehyde and not the hydrate was the activator. Failure to see an isotope effect on V also showed that the hydrate did not bind as a competitive inhibitor of the aldehyde. Conversely, the hydrate of acetaldehyde was the active inhibitor vs. acetate with acetate kinase, although no ATPase activity was induced.[61d] Since $^{18}O$ transfer from the aldehyde to phosphate occurs during the ATPase reactions,[61d] it appears that MgATP phosphorylates the aldehyde to give an oxycarbonium ion stabilized by the acid-base catalyst which normally accepts the proton from the substrate and must be ionized for the ATPase reaction as well as for the kinase one. Attack of water on the original carbonyl carbon of the oxycarbonium ion gives the aldehyde and $P_i$. The failure of the hydrate to bind in place of the free aldehyde shows the geometric restraints of the kinase active sites (no room for the second oxygen), while inhibition of acetate kinase by acetaldehyde hydrate is expected from the fact that the active site must have room for both oxygens of acetate.

The deuterium isotope effect on the $K_i$ value (25 $\mu M$) of benzamidoacetaldehyde, a good competitive inhibitor of papain, was used by Lewis and Wolfenden[61e] to show that a thiohemiacetal formed in the active site. Since this aldehyde had an equilibrium constant for hydration of 11.8,[61b] the estimated $^{D}K_i$ values were 0.75, 0.93, and 1.02 if (1) the free aldehyde bound as such, (2) the free aldehyde reacted after binding to form a thiohemiacetal, or (3) the hydrate bound as such as the inhibitor. The experimental $^{D}K_i$ value of $0.90 \pm 0.05$ supports formation of a thiohemiacetal between the aldehyde and the active site cysteine.

## IX. USE OF ISOTOPE EFFECTS TO STUDY TRANSITION STATE STRUCTURE

Isotope effects are often used to determine transition state structure in chemical reactions where the rate-limiting step is the reaction itself. For enzymatic reactions, however, one has to be able to determine the intrinsic isotope effect on the bond breaking step in order to be able to reach useful conclusions, and this requires that one know the

commitments in the system and hopefully keep them as small as possible. Only recently has this been done to any extent, and thus many isotope effects that have been determined in the past for enzymatic reactions are not useful for study of transition state structure. We will discuss some of those cases where useful conclusions have resulted.

### A. Primary Isotope Effects

There does not appear to be general agreement on the interpretation of the size of primary isotope effects, except for certain broad principles.[3] When an atom or group such as $H^+$, $H^\cdot$, $H^-$, $-CH_3$, or $-CH_2-$ is transferred from one molecule to another, the deuterium or $^{13}C$ isotope effects on the atom being transferred are largest when the transition state is symmetrical in the sense that the force constants of the bonds being made and broken are equal.[62,63] If these two atoms do not form equally strong bonds, however, this maximum isotope effect may not correspond to equal bond order to the two atoms. For example, the maximum calculated $^{14}C$ isotope effects for chloride displacement from R-$CH_2$ by oxygen, chloride, or sulfur nucleophiles[63] occurs at transition states with bond order to the attacking nucleophile of 0.37, 0.5, and 0.57. Thus, the force constant of a C—O bond of order 0.37 is the same as that of a C—Cl bond of order 0.63, while a C—Cl bond of order 0.43 and a C—S bond of order 0.57 have the same force constant. For earlier or later transition states, the force constants for bonding of the atom being transferred to either the substrate or product are nearly those in substrate or product, and thus only reaction coordinate motion (that is, the loss of a vibrational mode corresponding to motion along the reaction coordinate) results in an appreciable isotope effect.

An example of variation in primary isotope effects with transition state structure has been seen with formate dehydrogenase.[18,33,64] The four nucleotides DPN, pyridinealdehyde-DPN, thio-DPN, and acetylpyridine-DPN gave primary deuterium isotope effects on $V/K_{formate}$ of 2.2, 2.8, 2.6, and 3.3, which are believed to represent a series from late to nearly symmetrical transition states, since the primary $^{13}C$ isotope effects decrease over the same series from 1.042 to 1.036 (the $^{13}C$ isotope effect, which must result mainly from reaction coordinate motion, is larger for the later transition state where C—H bond breaking is more advanced; the increasing bond order of the C—O bonds apparently does not compensate for this effect). While none of the nucleotides gives an early transition state with formate dehydrogenase, the nonenzymatic oxidation of formate in dimethylsulfoxide by $I_2$, which shows $^{D}k = 2.2$ and $^{13}k = 1.0155$, does correspond to an early transition state. In $H_2O$ this reaction is much slower, gives $^{D}k = 3.8$ and $^{13}k = 1.036$, and clearly has a later transition state.[18,65] It is not yet clear what parameters control the transition state structure for the formate dehydrogenase reaction. The redox potentials of all of the nucleotides are considerably more positive than the value of −0.42 v for formate (DPN, −0.32 v; thio-DPN, −0.285 v; pyridinealdehyde-DPN, −0.262 v; acetylpyridine-DPN, −0.258 v), but pyridinealdehyde-DPN is out of line if difference in redox potential controls transition state structure. This nucleotide shows an anomolous $^{18}O$ isotope effect and a very low V, however, and the transition state may be different from those of the other nucleotides (see below). The early transition state for $I_2$ oxidation of formate in dimethylsulfoxide is expected since $I_2$ is a more powerful oxidant than the nucleotides.

Analysis of the formate dehydrogenase results was simplified by the fact that there are no commitments for formate in this reaction, as demonstrated by identical $^{13}C$ isotope effects with formate and formate-d.[33] With alcohol dehydrogenases, the transition states with isopropanol and DPN for the yeast enzyme ($^{D}k = 5.7$)[28] and cyclohexanol and DPN for the liver enzyme ($^{D}k = 6.3$)[30] are apparently more symmetrical than for formate dehydrogenase. The variation in intrinsic isotope effect with nucleotide structure and

redox potential has not yet been determined for these enzymes. Klinman has determined what she believes to be intrinsic isotope effects for a series of para substituted benzyl alcohols and aldehydes which are slow substrates for yeast alcohol dehydrogenase.[66,67] $^{D}V$ values for alcohol oxidation ranged from about 3.5 to 5, and careful determination of $\alpha$-secondary isotope effects with 4-deuterated nucleotides might allow one to tell which of these transition states are early or late.

While large and thus possibly intrinsic isotope effects have been reported in a number of other cases, in no other case do we have sufficient information to know for sure the degree of symmetry of the transition state. This will require a study of secondary isotope effects along the lines of what has been done in the formate dehydrogenase case, as described below. What we can say, however, is that hydrogen atom transfers appear to show deuterium isotope effects in the 7 to 8 range (as with the ethanolamine deaminase data discussed above), and proton transfer reactions show effects in the 7 to 10 range (examples are the elimination of HCl from chlorolactate by enolase[68] where $^{D}k = 7$ to 8.5, and the oxidation via a carbanion intermediate of lactate or chlorolactate by L-$\alpha$-hydroxy acid oxidase[69] where $^{D}k$ is 8.4 or 10, respectively). Both of these types of reactions give slightly larger deuterium isotope effects than those seen for hydride transfer (2.2 to 6.3).

The formate dehydrogenase reaction is the only one where we know that the $^{13}C$ isotope effect we are observing is an intrinsic one (since it does not change with formate-d).[33] While $^{13}C$ isotope effects have been measured for a number of decarboxylases, in most cases commitments of finite size are clearly present because the observed isotope effects are relatively small. However, with arginine decarboxylase the decarboxylation of the slow substrate homoarginine shows $^{13}(V/K) = 1.061$, which is probably an intrinsic value.[44] The $^{13}V$ value for transfer of $[^{13}C]H_3$ from S-adenosyl methionine to 3,4-dihydroxyacetophenone catalyzed by catechol O-methyltransferase[70] is reported as $1.09 \pm 0.05$. While this value is not very well determined, its size does suggest that it may be an intrinsic value. There clearly is room for much more work on the variation of primary heavy atom isotope effects with transition state structure, and use of the remote label method (see Section III. B.) should make such isotope effects more readily obtainable.

### B. Secondary Isotope Effects

With the possible exception of $\alpha$-secondary deuterium isotope effects for reactions where hydrogen is the primary atom transferred (see below), secondary isotope effects are a powerful tool for directly determining transition state structure. This is because (except again for those $\alpha$-secondary cases where the motion of the $\alpha$-secondary hydrogen and the primary hydrogen is coupled) there is no reaction coordinate motion component to the isotope effect (that is, all vibrational modes of the isotopic atom have positive force constants and real frequencies in the transition state). Thus one normally expects to see a value between unity and the equilibrium isotope effect, although when the transition state structure is more loosely bonded than in either substrate or product, one will see a value more normal than $^{D}K_{eq}$ (as in the $^{15}N$ isotope effects in the alcohol dehydrogenase reaction[16]), and when the transition state structure is more stiffly bonded, one will see inverse effects. An example of the latter is the methyl transfer from S-adenosyl methionine catalyzed by catechol O-methyltransferase.[70] $(^{D}V)^3$ from comparison of $-CH_3$ and $-CD_3$ transfer was $0.83 \pm 0.05$, corresponding to a fractionation factor of 0.94 per D relative to the starting methyl group. This value was taken as evidence of a tight or compreessed $S_N2$-like transition state in which the vibrations of the hydrogens are restricted. In view of the size of this and the primary $^{13}C$ isotope effect mentioned above, there probably are not commitments in this system, but this has not been proven.

$\beta$-Secondary deuterium isotope effects result almost entirely from hyperconjugation

which loosens the bonding of hydrogen on carbon next to a carbonyl group or carbonium ion center. In the alcohol dehydrogenase-catalyzed reductions by DPNH of acetone with the yeast enzyme, or cyclohexanone with the liver one, these isotope effects were equal to $^{D}K_{eq}$, suggesting that there was no hyperconjugation in the transition state[16] (that is, that the fractionation factor of the transition state was the same as that of the product alcohol). However, a bond order of 1.5 for the C—O bond, and of 0.5 for the primary C—H bond, would give a transition state with no charge on the carbonyl carbon and thus no hyperconjugation. The same conclusion concerning the charge on this carbon in the transition state was reached by Klinman by the use of structure-reactivity relationships.[67]

One must be cautious in interpreting observed secondary isotope effects in systems where the commitments are unknown, however. With lactate dehydrogenase,[16] for example, pyruvate-$d_3$ gave an isotope effect of 0.984, but this value is completely determined according to Equation 70 by the balance between $c_f$ and $c_r$ and conveys no information concerning transition state structure. Unfortunately, many secondary isotope effects reported for enzymatic reactions suffer from this flaw.

A very interesting set of $^{18}O$ isotope effects were measured for the formate dehydrogenase reaction.[18] Since $^{18}K_{eq}$ is inverse (estimated to be 0.964), we expected to see only inverse secondary isotope effects. The value for pyridinealdehyde-DPN was inverse (0.996), but the values for the other nucleotides were all normal, although the trend was in the expected direction (DPN, 1.0038; thio-DPN, 1.0051; acetylpyridine-DPN, 1.0062). The value for $I_2$ oxidation of formate in dimethylsulfoxide was 0.9945. These data suggest that the normal values result from the equilibrium isotope effect of dehydrating formate ion as it enters the active site. Such dehydration is clearly necessary to give rapid reaction (the $I_2$ oxidation is nearly 7 orders of magnitude faster in dimethylsulfoxide than in water[65]), and the size of this equilibrium isotope effect should be for the partly negatively charged oxygens of formate at least as large as the vapor pressure isotope effect[11] for water of 1.0091 (that is, $^{18}O$ is enriched in liquid water relative to the vapor phase by this factor). Multiplication of this equilibrium isotope effect for dehydration by the kinetic isotope effect for the reaction thus gives the normal values which are, however, smaller for the later transition states.

The one value which is out of line is the one for pyridinealdehyde-DPN, where $^{18}(V/K)$ is inverse, although not as much so as expected for the rather late transition state indicated by the deuterium and $^{13}C$ primary isotope effects. The more inverse $^{18}O$ value and the low maximum velocity for this nucleotide (less than 0.2% that for DPN) suggest that with this nucleotide formate is not completely dehydrated when absorbed in the active site, but that at least one water molecule remains hydrogen-bonded to it. Either this nucleotide does not fully induce the conformation change which makes the formate binding site anhydrous (formate is being absorbed as the counterion for the positive charge on N-1 of the nucleotide) or absorption of the aldehyde as the hydrate later leads to release of water in the active site.

### C. Coupled Motion in $\alpha$-Secondary Deuterium Isotope Effects

It has now become apparent that $\alpha$-secondary deuterium isotope effects where the primary atom being transferred is hydrogen are considerably more normal than expected on the basis of the expected transition state structure. Values near unity from the aldehyde side have been seen for benzaldehyde-1-d at pH 8 with liver and yeast alcohol dehydrogenases,[16,51] although at higher pH where hydride transfer comes to equilibrium, the value decreases to $^{D}K_{eq}$. Similarly, at pH 9 $^{D}(V/K)$ for oxidation of benzyl alcohol and the p-methoxy derivative were equal to $^{D}K_{eq}$. These data if taken at face value call for a transition state resembling the aldehyde, although the $\beta$-secondary isotope effects with ketones say the transition state resembles the alcohol.[16]

Further, when DPN-4-d was used the intrinsic isotope effects from the DPN side

(calculated from the $^{D}(V/K)$ values by using the commitments determined for these systems) were 1.22 with isopropanol and yeast alcohol dehydrogenase and 1.34 for cyclohexanol and the liver enzyme.[16] The values in the reverse direction are still larger (they are divided by $^{D}K_{eq}$, which is 0.89), and thus it is clear that the $\alpha$-secondary hydrogen in the transition state is much more loosely bonded than in either DPN or DPNH. Similar results were seen with formate dehydrogenase, where $^{D}(V/K)_{formate}$) when DPN-4-d was used was 1.21.

The explanation for what is happening here became clearer when it was found[64] that with formate dehydrogenase and formate-d the $\alpha$-secondary isotope effect with DPN-4-d was reduced to 1.06. With acetylpyridine-DPN-4-d, the values were 1.07 and 0.95 with formate and formate-d, while with glucose-6-P dehydrogenase and DPN-4-d, the values were 1.068 with glucose and 0.97 with glucose-1-d.[18]

Thus deuteration of the primary position causes a considerable decrease towards $^{D}K_{eq}$. These data show that the motions of the primary and $\alpha$-secondary hydrogens are coupled in the transition state, so that bending of the $\alpha$-secondary hydrogen (54° between DPN and DPNH) becomes part of the reaction coordinate motion. The force constant for the out-of-plane bending of the $\alpha$-secondary hydrogen thus is greatly decreased (or perhaps even becomes negative) in the transition state, and the observed isotope effects reflect the loss or near loss of this vibrational mode. The large effect of deuterating the primary hydrogen proves that the motion of the two atoms is coupled, and the decrease in the observed secondary isotope effect shows that the motion of the heavier deuterium atom is less affected by being coupled to the motion of the secondary hydrogen than is the case with the lighter hydrogen atom in the primary position. It is clear that for primary atoms heavier than hydrogen (that is, for O, N, S) reaction coordinate motion will not contribute appeciably to the $\alpha$-secondary isotope effect, and the bonding of the hydrogen in the transition state will be intermediate between that of substrate and product.

We should note that the size of the isotope effect reflects the degree of coupling and thus tells something about whether the transition state is early or late. With formate dehydrogenase,[64] for example, acetylpyridine-DPN-4-d with its nearly symmetrical transition state gives a relatively low value of 1.07, while DPN with a later transition state, which should lead to tighter coupling of the motions of primary and secondary hydrogen atoms, gives the larger value of 1.21. Pyridinealdehyde-DPN-4-d gives 0.99 and 0.94 with formate and formate-d, suggesting that the transition state is even earlier than with acetylpyridine-DPN. The $^{13}C$ isotope effects, however, are 1.041 and 1.036, suggesting that the C—H bond of formate is more stretched in the transition state for the pyridinealdehyde-DPN. We believe these data mean that the entire reaction coordinate is longer for the very slow pyridinealdehyde-DPN, so that C—H distances in the transition state are longer both to formate and the nucleotide than with the other faster substrates. In support of this view, the activation energy for the maximum velocity of the pyridinealdehyde-DPN reaction is 23 kcal/mol, while that for DPN is 16 kcal/mol. These experiments show the tremendous value for defining transition state structure of knowing isotope effects sensitive to distances from the atom in flight to the atoms between which it is being transferred.

It is not clear whether the degree of coupling we see in $\alpha$-secondary deuterium isotope effects is solely a function of the distance from the $\alpha$-carbon to the primary hydrogen or varies with the nature of the substrates and enzyme. What is needed are experiments with other dehydrogenases in which transition state structure is modified by using different nucleotides, and $\alpha$-secondary (as well as primary) deuterium and primary $^{13}C$ isotope effects are measured for both the 4-position of the nucleotide and the 1-position of the alcohol. It would be of interest also in a system where the motion of three hydrogen atoms is coupled to determine the effect of deuteration of one $\alpha$-secondary position on the isotope effect for the other $\alpha$-secondary position.

### D. Solvent Isotope Effects in $D_2O$-$H_2O$ Mixtures[71]

When a reaction is run in $D_2O$, all exchangeable hydrogens are replaced with deuterium, and whenever reaction involves bonding changes of any water molecule or exchangeable hydrogen, there will be a solvent isotope effect. Such effects are primary when the hydrogen is transferred during the reaction and reaction coordinate motion is involved and secondary when the hydrogen is not tranferred, but does change its fractionation factor in the transition state relative to that of the starting reactants. See the table in Reference 9 for the fractionation factors of hydrogen in exchangeable positions or in $H_3O^+$ and $OH^-$.

The largest primary solvent isotope effects will be seen when hydrogen is in flight between water or an exchangeable position and a carbon, as in the reverse reaction of C—H bond cleavage with transfer to a base on the enzyme. The effects when hydrogen is transferred between two atoms such as oxygen or nitrogen tend to be somewhat smaller (2 to 4 for $k_{H_2O}/k_{D_2O}$, which we will write as $^{D20}k$), and will depend on the structure of the transition state. Since the hydrogen is in a covalent bond to one atom and a hydrogen bond to the other both before and after reaction and the interatomic distances are thus short, the bonding in the transition state is not that much weaker than it is in the reactant or product structures, and $^{D20}k$ is thus not as large as intrinsic isotope effects for transfer between carbon. The length of the reaction coordinate should affect $^{D20}k$, with compressed transition states showing smaller isotope effects than looser, extended ones where the force constants will be less.

When one determines the solvent isotope effects for chemical reactions, one always has the question of how many solvent molecules are involved in the transition state structure. For enzymatic reactions, however, the reactants are usually desolvated when adsorbed in the active site, and thus the reactants are no longer surrounded by solvent molecules. As a result only any water molecules actually adsorbed in the active site and exchangeable hydrogens on acid-base catalytic groups will be involved. Compensating for this degree of simplification, however, is the complication that there may be solvent isotope effects of a nonspecific nature on the enzyme structure and the rates of conformation changes not involving the chemical reaction. The tetramerization of formyltetrahydrofolate synthetase shows large solvent isotope effects on both rates and equilibrium constants, for example.[72] One must always keep this possibility in mind, but fortunately in many cases such complications seem not to be involved.

We have given an example above in Section VIII. B. of the application of simple solvent isotope effects in the liver alcohol dehydrogenase reaction. A more sophisticated analysis involves what is called a proton inventory.[71] In this method one varies the solvent from pure $H_2O$ to pure $D_2O$ and determines rates as a function of n, the mole fraction of deuterium. Either the rates or the ratios of the observed rates to that in $H_2O$ are plotted vs. n. The equation for this curve is

$$k_n = k_{H_2O} \prod_i (1 - n + n\phi_i^T)/\prod_i (1 - n + n\phi_i^R) \qquad (75)$$

where $\phi_i^T$ and $\phi_i^R$ are fractionation factors relative to $H_2O$ of corresponding hydrogens in either the transition state or starting reactants. As the product signs indicate, there are as many terms as there are exchangeable hydrogens which alter their bonding between the reactant and the transition state.

The curve expressed by Equation 75 can be linear, concave up, or concave down. Let us consider normal solvent isotope effects first (that is, cases where $\Pi\phi_i^T$ is less than $\Pi\phi_i^R$). A linear curve is seen whenever only one hydrogen is involved and $\phi^R$ is unity (when $\phi^T$ and $\phi^R$ are the same there is of course no isotope effect and we can ignore such hydrogens). It is also possible for a linear curve to result from fortuitous cancellation of terms, but this is

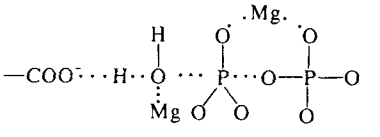
not very likely. Whenever $\phi^R$ is inverse but greater than $\phi^T$ for a single hydrogen, the curve is concave downwards, but the slope is always negative. An example of this would be a hydrogen transfer from an SH group to some nucleophile where the hydrogen was in flight in the transition state. A curve concave down can also result from two hydrogens with initial $\phi^R$ values of unity where $\phi^T$ is less than 1.0 for one and above 1.0 for the other. Unlike the curve for one hydrogen where $\phi^R$ is inverse, this plot will have a hump (that is an initial positive slope) if the sum of $\phi^R$ and $\phi^T$ exceeds 2.0.

The most common shape other than linear is for the curve to be concave upwards. Such a curve almost always means that more than one exchangeable hydrogen has changed its fractionation factor from values near unity in the reactants to lower values in the transition state. It is very difficult, without making assumptions about $\phi^T$ values, however, to determine whether 2, 3, or many hydrogens are involved. What one *can* do is to determine what combinations of $\phi^T$ values for 2, 3, or any other number of hydrogens will fit the data.

When the solvent isotope effect is inverse, the slope of the proton inventory curve is interpreted similarly. A linear plot suggests a single hydrogen with a $\phi^T$ value above unity and $\phi^R$ equal to 1.0. A curve concave down can result from $\phi^T$ being greater than $\phi^R$ and both being more than 1.0, or from two hydrogens, each with $\phi^R = 1.0$, but where $\phi^T$ is less than 1.0 for one and more than 1.0 by a greater factor for the other. Curves concave upward suggest more than one hydrogen with all $\phi^T$ values above unity and $\phi^R$ values of 1.0.

An interesting application of the proton inventory technique is a study of different substrates for trypsin.[73] The very specific substrate N-benzoyl-phenylalanyl-valyl-arginine-p-nitroanilide shows $^{D20}V = 4.3$ and a concave upward curve that suggests the involvment of two protons in the transition state, each with a kinetic isotope effect of 2.1. With N-benzoyl-arginine-ethyl ester, $^{D20}V = 3.0$ and the proton inventory curve was linear. These results suggest that the charge relay operates (in the sense that both protons are in flight at the same time) only with specific substrates.

With pyrophosphatase,[74] the proton inventory curve was concave downwards, and $^{D20}k$ was 1.9. In view of the mechanism indicated by pH studies,[75] it appears that the transition state will be




The proton inventory results are accommodated by giving the hydrogens on the water $\phi^R$ values of 0.82 as the result of coordination to $Mg^{2+}$ and assigning a value of 0.34 as $\phi^T$ for the proton transferred to the carboxyl and a value of 1.0 for $\phi^T$ for the other hydrogen (if the secondary hydrogen does not increase its fractionation factor in the transition state, the $\phi^R$ value for the primary proton must be decreased to 0.67, which is unrealistically low). As this example shows, proton inventory experiments are certainly much easier to interpret once one has run sufficient other kinetic studies to know as much as possible about the kinetic and chemical mechanisms of the reaction. They are a very useful kinetic tool, however, and can be used to confirm or rule out possible transition state structures. Certainly any transition state postulated must be consistent with the proton inventory results.

## X. CONCLUDING REMARKS

It can be seen from the scope of this review that the application of isotope effects in the

study of enzyme-catalyzed reactions has undergone tremendous development in only the last few years. The theory has in fact gotten ahead of experiment, but the experimental tools are there, and there are virgin fields to munch on in all directions. The next decade should witness an explosive expansion of our knowledge of enzyme mechanisms, and isotope effect studies will certainly constitute a major part of this effort.

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