The Use of Isotope Effects to Determine Enzyme Mechanisms

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When our laboratory started to carry out kinetic experiments on enzyme-catalyzed reactions we focused originally on initial velocity studies in which the concentrations of substrates, products, and inhibitors were varied. The notation and theory for these types of experiments were published as three papers in *Biochimica et Biophysica Acta* that have received many citations over the years (1–3). We used these methods to study various enzymes over the next decade or so. Although we used isotopes to measure isotopic exchanges in creatine kinase (4), galactokinase (5), shikimate dehydrogenase (6), alcohol dehydrogenase (7), and isocitrate dehydrogenase (8) and to measure rates in NDP kinase (9), we did not determine isotope effects.

However, in 1975 Dexter Northrop discovered how to exploit the Swain-Schaad relationship between deuterium and tritium isotope effects (10) to determine intrinsic isotope effects on the isotope-sensitive bond breaking step of an enzymatic reaction (11). The Swain-Schaad relationship says that the effect of tritium on a rate or equilibrium constant is the 1.442 power of the effect of deuterium substitution (this is derived from the relative masses of deuterium and tritium). Northrop assumed that there was no equilibrium isotope effect and thus that effects on $V/K$, the apparent first order rate constant at low substrate concentration and one of the independent kinetic constants, could be represented by Equation 1,

$$D(V/K)/H_{11005} (V/K)_H/(V/K)_D$$

where $D_k = k_H/k_D$, the intrinsic isotope effect on the bond breaking step, and $c$ is a commitment to catalysis. If the equilibrium isotope effect is unity and there is only one isotope-sensitive step, Equation 1 is valid regardless of how many steps precede or follow the isotope-sensitive one.

Northrop then subtracted one from each side of Equation 1 to get Equation 2.

$$D(V/K) - 1 = (V/K)_H/(V/K)_D - 1$$

The equation for the tritium isotope effect is the same except that the superscripts are T rather than D. Then if one takes the ratios of Equation 2 for deuterium and tritium, one gets Equation 3.

$$[V/K] - 1/[V/K]_T - 1 = (V/K)_T/(V/K) - 1$$

However, because the Swain-Schaad relationship makes $T_k = (D_k)^{1.442}$, one can substitute this value into Equation 3 to get an equation involving only experimental parameters and $D_k$.

$$[V/K] - 1/[V/K]_T - 1 = (V/K)_T/(V/K) - 1$$

Because this is a transcendental equation, one has to consult a table of values (12) or use a computer program to obtain a solution.
At the time Dexter discovered these relationships, Mike Schimerlik in my laboratory was studying malic enzyme. We wanted to determine whether there was an equilibrium isotope effect on the reaction, so Mike proceeded to determine $K_{eq}$ values with unlabeled and 2-deuterated malate. He used the most accurate way to determine $K_{eq}$, which is to make up reaction mixtures where the [products]/[reactants] ratio brackets $K_{eq}$ and then add enzyme. The $\Delta A$ that results as the reaction reaches equilibrium is plotted versus the [products]/[reactants] ratio, and the point where $\Delta A$ is zero is $K_{eq}$. This worked well with unlabeled malate, but when Mike used deuterated malate, the $\Delta A$ decreased greatly and then began to increase and returned to the starting point (Fig. 1). What he had forgotten was that he used unlabeled NADPH rather than deuterated nucleotide, which he didn’t have. He thus discovered the equilibrium perturbation method for determining isotope effects on reversible reactions (13).

The size of the perturbation is a function of the isotope effect although the relationship is only linear for small isotope effects.

\[
\text{fractional perturbation} = \frac{\text{isotope effect} - 1}{2.72} \quad \text{(Eq. 5)}
\]

For isotope effects above 1.2, the complete equation must be used (14). The fractional perturbation is the ratio of the perturbation size to the reciprocal of the sum of the concentrations of the perturbants (the molecules between which the label is exchanged). For malic enzyme, Mike was able to determine a deuterium isotope effect of 1.45 by equilibrium perturbation (1.47 on $V/K$ by direct comparison) and also a $^{13}$C isotope effect of 1.031, later confirmed by isotope ratio mass spectrometry (15).

Mike determined that there was a sizable equilibrium isotope effect on the malic enzyme reaction (later refined to be 1.18 (16)) and thus that the equation for the isotope effect had to be expanded to allow for this.

\[
D(V/K) = (Dk + c_f + c_r)K_{eq}(I + c_f + c_r) \quad \text{(Eq. 6)}
\]

The constants $c_f$ and $c_r$ are now commitments in forward and reverse directions. Each is the ratio of the rate constant for the bond breaking step to the net rate constant for release from the enzyme of the substrate whose $V/K$ is involved or the first product released. In an equilibrium perturbation experiment, the commitments are for the release of the perturbants.

The equation for the tritium isotope effect is the same except the leading superscripts are T rather than D. When one applies Northrop’s method to these equations, the third term in the numerator does not cancel out, and thus one gets only an approximate answer. However, if one divides the experimental $D(V/K)$ and $T(V/K)$ isotope effects by the respective equilibrium isotope effects (which gives the values for the reverse reaction) and carries out the Northrop analysis one obtains an approximate value for $Dk$ in the back reaction. Then the true $Dk$ in the forward reaction lies between the value determined in that direction and the one determined in the back reaction multiplied by the equilibrium isotope effect. This approach for malic enzyme gave limits of 5–8 for $Dk$ in the forward reaction and 4–6.5 in the reverse direction (17). The true value determined in 1985 by Chuck Grissom is 5.7 in the forward direction (18).
When we first started to work on isotope effects none of us knew very much about them, but Jack Shiner at Indiana University steered us in the right direction, and we discovered that the physical organic chemists knew quite a bit about isotope effects. By attending the Gordon Conferences on isotopes (I have attended every one since 1981) we got to know all of the major players in the field and learned what they knew as well as returning the favor by giving them information about isotope effects on enzymes. This Gordon Conference meets every 2 years in California in the winter (alternating with the ad hoc Enzyme Mechanism Conference, which is convenient) and will meet next in Ventura on February 15–20, 2004. Anyone interested in isotope effects on enzymatic reactions should attend; students and postdoctoral fellows are welcome.

As a result of our increasing interest in isotope effects, Marion O'Leary, Dexter Northrop, and I organized a Steenbock Symposium titled “Isotope Effects on Enzyme-catalyzed Reactions” here in Madison in 1976. This was very successful and the proceedings were published by University Park Press (19). This book includes computer programs for fitting isotope effect data and tables for use of Northrop’s method and for equilibrium perturbation analysis.

The year 1980 saw us publishing a number of measured equilibrium deuterium isotope effects (16) as well as kinetic isotope effects by John Blanchard on several enzymes (20, 21). The following year saw the development by Paul Cook in this laboratory of the theory for the variation of observed isotope effects with pH or the concentrations of other substrates (22–24). The forward commitment in Equation 6 represents the ratio of the rate constant for the isotope-sensitive step to the net rate constant for release from the enzyme of the varied substrate in a direct comparison experiment, the labeled substrate in an internal competition experiment, or the perturbant in an equilibrium perturbation one. In an ordered mechanism where the forward commitment is for the first substrate, the observed isotope effect on V/Ka will be unity at infinite concentration of the second substrate, increasing to 13(V/Kb) at very low levels of B. The value of 13(V/Kb) on the other hand is independent of the level of A. In a random mechanism, saturation with one substrate does not eliminate the V/K isotope effect for the other one, although the value may change. This sort of experiment is very useful for determining the kinetic mechanism. Cook used these methods to show that NAD and cyclohexanol added in that order with liver alcohol dehydrogenase, whereas NAD and 2-propanol added randomly to the yeast enzyme (22).

The effect of pH on observed isotope effects depends on whether the isotope-dependent and pH-dependent steps are the same. With a sticky substrate (one that reacts to give products faster than it dissociates), the isotope effect on V/K is reduced by an external forward commitment, but when one goes to a pH where the chemistry becomes rate-limiting, this external part of the forward commitment is eliminated (although any internal commitment remains), and so the V/K isotope effect increases (23). With liver alcohol dehydrogenase, however, the V/K isotope effect for cyclohexanol was 2.5 at low and neutral pH but decreased above a pK of 9.4 to unity. In the reverse direction, the value for cyclohexanone was 2.1 at lower pH values and decreased above the same pK to 0.85 (24). In this mechanism, proton removal from the alcohol to give a zinc-bound alkoxide precedes hydride transfer, and above the pK this proton is lost to the medium, thus committing the reaction to continue. In the reverse direction at high pH hydride transfer comes to equilibrium waiting for a proton to be added from the solvent.

In 1982 I was asked to write a review for Annual Reviews of Biochemistry on the use of isotope effects to elucidate enzyme mechanisms. I submitted the review, but it came back all marked up with many changes in wording and meaning, and after an unsatisfactory conversation with the redactor, I withdrew the paper. I then sent it to Critical Reviews in Biochemistry, where it was promptly accepted (25).

The next major advance in isotope effect theory was the use of multiple isotope effects by Jeff Hermes (15). When both isotope effects are on the same step, the effect of deuteration on 13C isotope effects allows one to determine intrinsic isotope effects or narrow limits on them. Thus, in Equation 6 where the superscripts are 13, deuteration decreases the rate of the isotope-sensitive step and thus decreases the commitments by the size of the intrinsic deuterium isotope effect in the forward or reverse direction. If one uses both primary and secondary deuterium substitution, one has five equations (13C isotope effect with unlabelled, primary, and secondary deuterated substrates plus primary and secondary deuterium isotope effects) in five unknowns (three intrinsic isotope effects and the two commitments). Jeff applied this tech-
nique to glucose-6-P dehydrogenase both in water and in D$_2$O (26). The intrinsic $^{13}$C isotope effect was 4% in both solvents, but the intrinsic primary deuterium isotope effect was 5.3 in water and 3.7 in D$_2$O. The $\alpha$-secondary deuterium isotope effect also decreased in D$_2$O. However, the surprise was that the sum of forward and reverse commitments increased from 1.24 in water to 2.5 in D$_2$O with most of the change being in the forward commitment. Thus the major effect of D$_2$O was on the conformation changes that precede the chemical step rather than on the chemistry itself. However, the decreased intrinsic deuterium isotope effects in D$_2$O reflect the coupled hydrogen motions in the transition state (proton from the 1-hydroxyl going to aspartate on the enzyme, hydride going from C-1 of glucose-6-P to C-4 of NADP, hydrogen at C-4 of NADP going from trigonal to tetrahedral; Fig. 2). This coupled motion effect, where the first deuterium substitution decreases the effect of further deuteration, shows that tunneling is involved in the hydrogen motions.

This coupled motion effect is very prominent in the formate dehydrogenase reaction (27). The deuterium isotope effect at C-4 of the ring of NAD was 1.23 despite the fact that the equilibrium isotope effect is 0.89. This shows that the motion of this hydrogen from trigonal to tetrahedral is coupled into the reaction coordinate so that there is little or no restoring force at the transition state. However, if deuterated formate is used, this secondary isotope effect decreases to 1.07, showing that the first deuterium substitution decreases the effect of the second deuterium. This is a nice system to study because there are no commitments (the $^{13}$C isotope effect in formate is independent of deuteration). When the nucleotide substrate was changed from NAD to thio-NAD and then to acetylpyridine-NAD, the transition state became earlier as the redox potential of the nucleotide became more positive. This led to larger primary deuterium isotope effects (2.17 to 2.60 to 3.32), smaller secondary deuterium isotope effects (1.23 to 1.18 to 1.06) as the coupling of the secondary motion into the reaction coordinate decreased, and smaller $^{13}$C isotope effects (4.2 to 3.8 to 3.6%) as the degree of C–C cleavage decreased. The secondary isotope effects decreased halfway to the equilibrium isotope effect with deuterated formate (1.07 to 1.03 to 0.95). Thus with multiple isotope effects one can really determine transition state structure.

When a deuterium and the $^{13}$C isotope effect are on different steps, deuteration makes the deuterium-sensitive step more rate-limiting and thus increases one of the commitments for the $^{13}$C-sensitive step so that the observed $^{13}$C isotope effect decreases. Further, the three measured isotope effects are not independent. In the direction where the deuterium-sensitive step comes first, the equation is,

$$[^{13}(V/K)_H - 1]/[^{13}(V/K)_D - 1] = \frac{V/K}{K_{eq}}$$

(Eq. 7)

although in the reverse direction where the $^{13}$C-sensitive step comes first,

$$[^{13}(V/K)_H - ^{13}K_{eq}]/[^{13}(V/K)_D - ^{13}K_{eq}] = \frac{V/K}{K_{eq}}$$

(Eq. 8)
These equations are really the same except that the first is expressed in terms of the parameters for the forward reaction, and the second one includes the parameters for the reverse reaction. When these equations were applied to data for malic enzyme, the data fitted Equation 7, but not Equation 8, showing that dehydrogenation precedes decarboxylation (15).

6-Phosphogluconate dehydrogenase was also shown to catalyze a stepwise reaction (28), but prephenate dehydrogenase provided some surprises (29). The isotope effects were measured with a substrate lacking the keto group in the side chain but having a V_max of 78% and V/K of 18% that of prephenate. The 13C isotope effect in the CO_2 product was 1.03% with deuterated substrate but only 0.33% with unlabeled substrate, whereas the deuterium isotope effect on hydride transfer was 2.34. Thus the reaction is concerted with intrinsic 13C and deuterium isotope effects of 1.0155 and 7.3 and a forward commitment of 3.7, assuming no reverse commitment for the irreversible reaction. The deuterium isotope effect is large, showing considerable C–H cleavage in the transition state, but the 1.55% 13C isotope effect shows that the reaction is asynchronous with little C–C cleavage in the transition state. The reason the reaction is concerted is presumably that the energy of aromatization is so great that the putative keto intermediate has no stability. In fact, if one removes one double bond from the ring of the prephenate analog with no ketone in the side chain the product of the reaction is a ketone and no decarboxylation takes place. The enzyme is thus a secondary alcohol dehydrogenase, and the decarboxylation takes place because of the instability of the keto product.

The multiple isotope method was also applied to 15N and deuterium isotope effects in studies on phenylalanine ammonia lyase (30), adenosine deaminase (31), and aspartate aminotransferase (32) to provide details of the mechanisms of these enzymes. The next development of the theory came with the discovery of intermediate partitioning by Chuck Grissom (18). With malic enzyme one can add oxaloacetate and NADPH and regenerate the putative intermediate on the enzyme. This will then partition both back to malate and NADP and forward to CO_2 and pyruvate. The partitioning ratio (pyruvate/malate \(= 0.47\)) is the forward commitment for the decarboxylation step and allowed determination of the intrinsic 13C isotope effect as 1.044. With the deuterium and tritium V/K isotope effects, the equations allow one to determine the intrinsic deuterium isotope effect as 5.7, the forward commitment to hydride transfer as 3.3, and the ratio of reverse hydride transfer to decarboxylation as 10 (18). Thus the tools are available to dissect the entire mechanism.

As the decade of the 90s dawned, we began to determine 18O and other isotope effects to study phosphoryl and acyl transfer. It is very difficult to extract the oxygen out of phosphate quantitatively and insert it into CO_2, so we adopted the use of the remote label method, which had been pioneered by Marion O’Leary (33). For example, to measure the secondary 18O isotope effect on the hydrolysis of glucose-6-P, one prepares two versions of this molecule. One has 13C at C-1 and three 18O’s in the phosphate group. The second has 12C at C-1 and no other labels. By 12C we mean carbon that has the 1% natural abundance of 13C removed (it is the by-product of making 13C). One then mixes 1% of the former with 99% of the latter to get a solution of glucose-6-P with the natural abundance of 12C at C-1 but with every 13C accompanied by three 18O’s. This remote labeled material is then used in a reaction, and the residual glucose-6-P and the glucose product (phosphorylated back to glucose-6-P by hexokinase) are then degraded by glucose-6-P and 6-P-glucuronate dehydrogenases to CO_2 and ribulose-5-P. Analysis of the CO_2 reveals the isotopic discrimination between the two species in the substrate mixture. One then uses glucose-6-P with no special labels in the same experiment, and this determines any 13C isotope effect at C-1. Division of the apparent isotope effect for the remote labeled substrate by the value from natural abundance substrate gives the desired 18O isotope effect.

The remote label method is very powerful and allows one to determine almost any isotope effect in any position of a molecule as long as there is a carbon that can be isolated as CO_2 or a nitrogen that can be isolated and converted to N_2. If there is only one nitrogen in a molecule this is simple as samples can be sealed in quartz tubes with CuO and heated to convert all organic matter to CO_2, H_2O, and N_2, which are readily separated for analysis of N_2 by the isotope ratio mass spectrometer. Convenient remote labels are nitro groups of p-nitrophenol or m-nitrobenzyl alcohol (inserted by nitration of triphenyl phosphate followed by hydrolysis or benzaldehyde followed by reduction, using either 15N- or 14N-labeled nitrate). Another useful remote label is the exocyclic amino group of adenine, which is readily inserted by the reaction...
of ammonia with chloropurine riboside and removed later for analysis by adenosine deaminase. This allows ATP, NAD, or other adenine-containing molecules to be remote-labeled.

Al Hengge and others in the laboratory carried out extensive measurements of 18O isotope effects on phosphoryl transfer using the remote label method for analysis. This showed that phosphate monoesters have dissociative transition states for their reactions, diesters have $S_n2$ reactions, and triesters have associative transition states although they do not form phosphorane intermediates unless geometry requires this (34).

Al Hengge and Rob Hess carried out a thorough study of the reactions of p-nitrophenyl acetate with various nucleophiles (35) and with several enzymes (36). In opposition to what most textbooks say, these reactions do not have a tetrahedral intermediate but are concerted. Only when the leaving group has a $pK_a$ of 16 or higher does a tetrahedral intermediate form and 18O exchange take place during hydrolysis. Al and Rob used five isotope effects to study these reactions. The nitro group was the remote label for measurement of the other isotope effects, and the 15N isotope effect itself told the degree of electron delocalization into the nitro group in the transition state. The other isotope effects were the primary 18O one in the phenolic oxygen, the secondary 18O in the carbonyl oxygen, the primary 13C in the carbonyl carbon, and the deuterium isotope effect from full deuteration of the methyl group. With all five isotope effects, one can really pin down transition state structures!

The major difference between the enzymatic and non-enzymatic cleavages of p-nitrophenyl acetate was in the $/H9252$-deuterium isotope effect in the methyl group. This isotope effect results from decreased hyperconjugation in the tetrahedral transition state and was 4–5% inverse for attack by oxygen nucleophiles (35). However, in the enzymatic reactions this value was 0–2% inverse (36). These data show that in the enzymatic reactions the enzymes polarize the carbonyl group and increase hyperconjugation when the substrate binds (Fig. 3). The fractionation factor then increases as the transition state is approached but not to the degree reached in the non-enzymatic reaction. Thus the isotope effect directly demonstrates the way the enzyme activates the carbonyl group for attack.

In 1992 we published a major study of aspartate transcarbamoylase. This was a collaborative effort by Marion O’Leary, Howie Schachman, and Laura Parmentier, who had been Marion’s graduate student before he left for Nebraska and was inherited by me. Laura developed what we called the “dribble-drip” method of measuring an isotope effect. To determine the 13C isotope effect in carbamoyl-P, she needed to start with 12 mM carbamoyl-P and convert half of it to product. The residual carbamoyl-P was then degraded with acid to liberate CO2 for analysis. This was no problem when she used high levels of aspartate, but because the isotope effect got larger at lower aspartate, she needed a way to determine the isotope effect at an aspartate level lower than that of carbamoyl-P. The answer was to add the aspartate at the same rate as it was consumed. In practice one adds the aspartate with a pump at a constant rate and uses the calculated level of enzyme. After half of the carbamoyl-P is consumed, the aspartate level is determined. If it has increased, the experiment is repeated with a higher enzyme level, but if it has decreased, the lower enzyme is used. The first reaction mixture is then discarded and the second one analyzed. This procedure allowed determination of the 13C isotope effect at aspartate levels from very low to over 100 mM.

Laura found that the 13C isotope effect with the holoenzyme varied from 1.022 at low aspartate to unity at infinite aspartate with the half-conversion point at 4.8 mM (37). Thus the kinetic mechanism is ordered with aspartate as the second substrate. With isolated catalytic
trimeric subunits, which no longer show allosteric kinetics, the $^{13}$C isotope effect varied from 1.024 at low aspartate to 1.004 at infinite aspartate (37). The reaction has thus become partly random. With the slow aspartate analog, cysteine sulfinate, the $^{13}$C isotope effect was 1.039 at any level of cysteine sulfinate and with both holoenzyme and catalytic subunits. The mechanism has now become completely random, and it appears that the chemistry is fully rate-limiting. The catalytic trimers of the H134A mutant also gave a $^{13}$C isotope effect of 1.04 that did not vary with aspartate level, showing a fully random mechanism with rate-limiting chemistry (38). These studies clearly show the power of isotope effects to determine the kinetic mechanism. Presumably the mechanism is ordered only when the complex of enzyme and substrates reacts to give products much faster than carbamoyl-P dissociates. The rate of dissociation of carbamoyl-P relative to forward reaction increases somewhat in the isolated catalytic trimers and much more with the slow alternate substrate or in the slow H134A mutant.

These experiments also gave information about the allosteric behavior of the enzyme. The holoenzyme of aspartate transcarbamoylase shows a highly sigmoid curve of rate versus aspartate concentration, which is made more so by the allosteric inhibitor CTP. ATP, on the other hand, activates by restoring Michaelis kinetics. Laura found that the curve of $^{13}$C isotope effect versus aspartate was identical when high levels of ATP or CTP sufficient to alter the initial velocity curve were present (37). Thus the Monod model of allosteric behavior is valid and there is only one active R form with the same properties regardless of the presence of ATP or CTP. The allosteric control is mediated by the ratio of R to inactive T forms with ATP binding selectively to R and CTP to T.

Our multiple isotope effect studies on malic enzyme with NADP and malate had indicated a stepwise mechanism, but with acetylpyridine-NADP, deuteration increased rather than decreased the $^{13}$C isotope effect (39). This suggested that the mechanism may have changed to a concerted one, or there was a large equilibrium isotope effect at C-4 on hydride transfer caused by hyperconjugation with the newly formed keto group. Bill Edens solved this puzzle by showing that the $^{13}$C isotope effect at C-3 (which would not be affected by hyperconjugation)
was also increased by deuteration of malate when acetylpyridine-NADP was the substrate (40). Thus the mechanism truly became concerted. We believe this results from the 2 orders of magnitude more favorable equilibrium constant for the oxidative decarboxylation to pyruvate. The shape of the free energy profile then changes to eliminate the small dip at the top for the oxaloacetate intermediate (Fig. 4). Interestingly, Jeff Urbauer showed that with erythro-fluoromalate and acetylpyridine-NADP the mechanism remained stepwise, because the fluorine substitution decreases the equilibrium constant for the reaction by an order of magnitude (41).

Lac Lee showed the power of isotope effects to deduce mechanism in his studies of L-ribulose-5-P 4-epimerase (42). He devised a way to degrade the xylulose-5-P product carbon by carbon and showed that there were over 2% 13C isotope effects at C-3 and C-4. The deuterium isotope effects at these carbons were 4 and 19% for a slow mutant, showing that C–H cleavage was not involved. The mechanism involves an aldol cleavage to the enediolate of dihydroxyacetone and glycolaldehyde-P, followed by rotation of the aldehyde group and condensation.

Our most recent work, a collaborative effort with Nigel Richards in Florida, is a study of the mechanism of oxalate decarboxylase using 13C and 18O isotope effects (43). This enzyme converts one end of oxalate to CO2 and the other end to formate. The enzyme contains Mn2+ and requires catalytic oxygen, which is not consumed during the reaction. Because both oxalate and formate as anhydrous salts are converted to CO2 by I2 in dimethyl sulfoxide without exchange of the oxygens, it is possible to determine both the 13C and 18O isotope effects going to each product. At pH 5.7 where the chemistry is rate-limiting, the 13C isotope effects were 1.9% going to formate and 0.8% going to CO2. The 18O isotope effects were 1.0% going to formate but 0.7% inverse going to CO2. The large isotope effects during formation of formate and the small 13C one for forming CO2 suggest that decarboxylation is not rate-limiting, and that in a prior step the C–O bond order is reduced in the end for oxalate going to formate. Application of the appropriate equations for such a model allowed calculation of the C–O bond order in the putative intermediate. This came out 1.16 from the 13C isotope effects and 1.14 from the 18O ones. We suspect that O2 and Mn2+ interact to give a Mn3+ species that coordinates the oxalate monoanion, which is the active form of the substrate. In the first step an electron is removed from the coordinated end of oxalate and a proton from the other end. The radical intermediate has ~70% positive charge in the coordinated end of oxalate (based on a C–O bond order of 1.15; Fig. 5), and this leads to decarboxylation that is four times faster than reversal of the first step. The remaining radical anion picks up an electron from Mn2+ and a proton to become formate. The observed isotope effects fit the equations for this mechanism very well.

At this point in time it is obvious that isotope effects are a very powerful tool for determining all aspects of enzyme mechanisms, starting with the kinetic mechanism and ending up with the chemical mechanism and the structure of the transition state. It has been fun seeing the theory and experiment develop together and to have been a part of the process. We are still determining isotope effects on enzymatic reactions and hope to continue doing so for some time in the future. I would like to thank all of the students, postdoctoral fellows, and other visitors who have actually done the work described above and other studies that I have not had space to discuss. They are the real heroes of the tale. Of course I thank the NIH, which has supported all of the work described here (Grant GM 18938).

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Reflections: Use of Isotope Effects

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