# α-Secondary Tritium Kinetic Isotope Effects Indicate Hydrogen Tunneling and Coupled Motion Occur in the Oxidation of L-Malate by NAD-Malic Enzyme†

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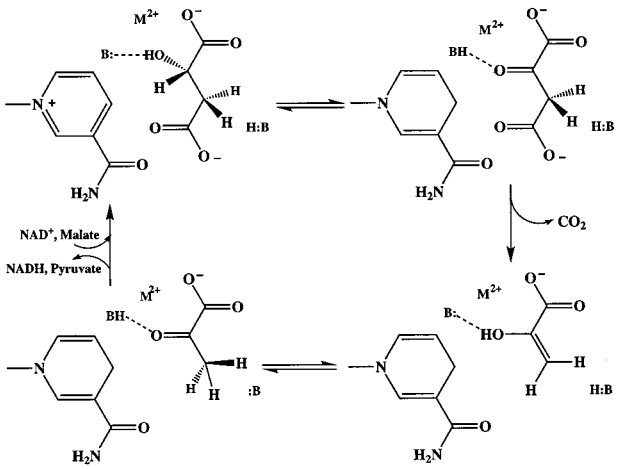
*Received October 13, 1998; Revised Manuscript Received December 28, 1998*

ABSTRACT: The NAD-malic enzyme from *Ascaris suum* catalyzes the divalent metal ion-dependent oxidative decarboxylation of L-malate to give pyruvate and $CO_2$, with $NAD^+$ as the oxidant. α-Secondary tritium kinetic isotope effects were measured with $NAD^+$ or $APAD^+$ and L-malate-2-H(D) and several different divalent metal ions. The α-secondary tritium kinetic isotope effects are slightly higher than 1 with $NAD^+$ and L-malate as substrates, much larger than the expected inverse isotope effect for a hybridization change from $sp^2$ to $sp^3$. The α-secondary tritium kinetic isotope effects are reduced to values near 1 with L-malate-2-D as the substrate, regardless of the metal ion that is used. Data suggest the presence of quantum mechanical tunneling and coupled motion in the malic enzyme reaction when $NAD^+$ and malate are used as substrates. Isotope effects were also measured using the D/T method with $NAD^+$ and $Mn^{2+}$ as the substrate pair. A Swain−Schaad exponent of 2.2 (less than the value of 3.26 expected for strictly semiclassical behavior) is estimated, suggesting the presence of other slow steps along the reaction pathway. With $APAD^+$ and $Mn^{2+}$ as the substrate pair, inverse α-secondary tritium kinetic isotope effects are observed, and a Swain−Schaad exponent of 3.3 is estimated, consistent with rate-limiting hydride transfer and no quantum mechanical tunneling or coupled motion. Data are discussed in terms of the malic enzyme mechanism and the theory developed by Huskey for D/T isotope effects as an indicator of tunneling [Huskey, W. P. (1991) *J. Phys. Org. Chem. 4*, 361−366].

Malic enzyme catalyzes the oxidative decarboxylation of L-malate to pyruvate and $CO_2$ using NAD(P)[1] as the oxidant. As such, it is a member of a class of pyridine nucleotide-linked dehydrogenases that catalyze the oxidative decarboxylation of β-hydroxy acids. Other members of the class include isocitrate dehydrogenase, isopropylmalate dehydrogenase, 6-phosphogluconate dehydrogenase, and tartrate dehydrogenase.

A general base, general acid chemical mechanism with Lewis acid catalysis by the divalent metal ion has been proposed for the *Ascaris suum* malic enzyme, on the basis of the pH dependence of kinetic parameters for the malate oxidative decarboxylation (*1*) and oxalacetate decarboxylation (*2*) reactions. In the proposed reaction (Scheme 1), malate is bound such that the hydroxyl is positioned near the divalent metal ion. L-Malate is first oxidized to oxalacetate, assisted by an enzyme general base which accepts a proton from the β-hydroxyl of malate. Decarboxylation of the oxalacetate intermediate is effected by the divalent metal ion acting as a Lewis acid. Enolpyruvate is then tautomerized to pyruvate with a general acid donating a proton to the 3-carbon to give pyruvate.

Scheme 1: Proposed Mechanism for the NAD-Malic Enzyme




The sequence of chemical steps during the course of the oxidative decarboxylation of malate has been studied extensively using the technique of multiple isotope effects (*3*). Studies of the NADP-malic enzyme (*4*) suggested a two-step oxidation−decarboxylation with NADP as the reactant. Isotope effect data obtained for the *A. suum* NAD-malic enzyme by Karsten and Cook (*5*), and confirmed in the recent studies of Edens et al. (*6*) with the chicken liver NADP-malic enzyme, indicate a change from a stepwise mechanism for oxidative decarboxylation of L-malate with NAD(P) as

† This work was supported by a grant from the National Institutes of Health (GM 36799) and a PI Research Enhancement Award from the University of Oklahoma to P.F.C.

[1] Abbreviations: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; MDH, malate dehydrogenase; NAD, nicotinamide adenine dinucleotide (the positive charge is omitted for convenience); NADP, nicotinamide adenine dinucleotide 2′-phosphate; APAD, 3-acetylpyridine adenine dinucleotide; NADH, reduced NAD; NADD, NADH-4-D.

10.1021/bi982439y CCC: $18.00 
Published on Web 03/19/1999

the dinucleotide substrate to a concerted mechanism with APAD(P).

The role of the metal ion in the transition state for decarboxylation was probed via $^{13}C$ isotope effects in the enzymatic oxidative decarboxylation of malate using NADP as the dinucleotide substrate and in the nonenzymatic metal-catalyzed decarboxylation of oxalacetate (*7*, *8*). An identity in the intrinsic $^{13}C$ isotope effects for these two systems suggests that the enzyme serves to properly position the divalent metal ion and the substrate malate. The role of the divalent metal ion in the hydride transfer step was recently investigated by obtaining multiple isotope effects with different metal ions and dinucleotide substrates (*9*). Data suggest that the positions of the metal and dinucleotide substrate do not change whatever the identity of the two, and that malate (as suggested above) is bound such that the hydroxyl is in the vicinity of the metal ion. As the ionic radius of the divalent metal ion increases, the distance between the hydride to be transferred and the 4-position of the nicotinamide ring decreases; that is, the reaction coordinate is compressed. Compression of the reaction coordinate results in an increase in the extent of hydrogen tunneling as demonstrated by an increase in the estimated intrinsic deuterium isotope effect from a value near 3 for the smallest metal ion ($Mn^{2+}$) to near 30 for the largest ($Cd^{2+}$).

To further probe the possible role of hydrogen tunneling in the malic enzyme reaction, α-secondary tritium kinetic isotope effects and D/T kinetic isotope effects were measured for the NAD-malic enzyme. Data suggest that hydrogen tunneling does occur and that there is a coupling of the motion of the hydride that is transferred to the motion of the α-secondary hydrogen in the transition for the oxidation of L-malate.

## MATERIALS AND METHODS

*Chemicals and Enzymes*. Pyruvate carboxylase from bovine liver was purchased from Sigma. Ethanol-$d_6$ (99.9 at. % D) was from CIL. Sodium [1-$^{14}C$]pyruvate (11.1 mCi/mmol), $NaB^3H_4$ (1000 mCi/mmol), glucose-1-T, and [$^{14}C$]-ribose NAD were purchased from NEN Dupont. The NAD-malic enzyme was prepared as described previously (*25*).

α-*Secondary Tritium Isotope Effects.* Preparation of B-side NADT was carried out according to the methods of Viola et al. (*10*) using glucose-1-T. Reduction of oxalacetate using malate dehydrogenase (A-side) gave NAD-4-T (143 000 cpm/$\mu$mol), which was mixed with [$^{14}C$]ribose NAD (81 000 cpm/$\mu$mol). For experiments with APAD, the single label method was used and APAD-4-T (156 000 cpm/$\mu$mol) was prepared in a manner similar to that used for NAD-4-T. Full and partial conversion reactions were carried out in a 1 mL volume, and the mixtures contained 100 mM Hepes (pH 8), 25 mM malate, 1.5 mM [$^3H$,$^{14}C$]NAD, and either 5 mM $MnSO_4$ or 25 mM $MgSO_4$. The reaction was terminated by vortexing the reaction mixture with 100 $\mu$L of $CCl_4$. The mixture was immediately applied to either a DEAE-Sephadex A25 column (1 cm × 13 cm) or a DEAE Spherilose column (1 cm × 15 cm) using an ISCO liquid chromatography system and eluted with a linear 25 to 600 mM $(NH_4)_2CO_3$ gradient at pH 8. Aliquots of fractions containing labeled NADH(T) were counted on either a Beckman or a Packard Tri-Carb 2100TR liquid scintillation counter. The α-secondary tritium kinetic isotope effect was calculated using the following expression

$$^{T}(V/K) = \log(1 - f)/\log(1 - fR_f/R_o) \qquad (1)$$

where $f$ is the fractional conversion (between 10 and 15%), $R_f$ is the ratio of $^3H$ and $^{14}C$ in the product NADT at fractional conversion $f$, and $R_o$ is the ratio of $^3H$ and $^{14}C$ in the product after complete conversion of NAD-4-T to product. Experiments were repeated using L-malate-2-D (*10*) to obtain the multiple primary deuterium/secondary tritium isotope effect. With APAD, experiments were carried out in an identical manner. The reaction was followed by monitoring the appearance of APADH(T) at 363 nm ($\epsilon_{363}$ = 9100 $M^{-1}$ $cm^{-1}$). Data were analyzed using eq 1, but $R_f$ and $R_o$ are the specific radioactivities of APADH(T) and APAD-4-T, respectively.

*Synthesis of Labeled Malates*. The most difficult and time-consuming aspect of applying the D/T method (*11*) to an enzyme reaction can be the synthesis of stereospecifically singly and doubly labeled compounds. There are three molecules that must be prepared: [1-$^{14}C$]-L-malate, [1-$^{14}C$,2-$^2H$]-L-malate, and [2-$^3H$]-L-malate. The [1-$^{14}C$]malate-labeled malic acid was prepared by carboxylation of [1-$^{14}C$]pyruvate with pyruvate carboxylase to form [1-$^{14}C$]oxalacetate, which was then reduced with NADD (NADH) by malate dehydrogenase. The NADH or A-side NADD was prepared in situ using ethanol or ethanol-$d_6$ with alcohol and aldehyde dehydrogenases at pH 7.6. Both labeled and unlabeled nucleotides were prepared using the same method to ensure that if minor contaminants were present, they would be in both reaction mixtures. To a solution of pyruvate (0.91 $\mu$mol, SA = 11.1 mCi/mmol) at pH 7.6 were added 50 mM Tris, 70 mM $MgCl_2$, 0.25 mM acetyl CoA, 2.5 mM ATP, 50 mM $KHCO_3$, 1.2 mM NADD (NADH), 50 units of malate dehydrogenase, and 0.25 unit of pyruvate carboxylase to initiate the reaction. The formation of malate was monitored spectrophotometrically at 340 nm. The reaction was quenched when the absorbance was at a minimum by the addition of 100 $\mu$L of $CCl_4$, followed by vortexing to denature the enzymes. The solution was added to 0.2 g of activated charcoal, heated for 10 min in boiling water, and filtered to remove nucleotides. The labeled malate was purified using an anion exchange resin (Dowex 1, formate form). Malate was eluted using a 0 to 4 N formic acid gradient (200 mL). Fractions containing malate were pooled, lyophilized, and dissolved in $H_2O$. The specific activities of the [1-$^{14}C$,2-$^2H$]-malate and [1-$^{14}C$]malate were 6.6 and 7.4 mCi/mmol, respectively. Analytical HPLC results for the labeled malates were identical. [2-$^3H$]-L-Malate was prepared according to the method of Karsten and Cook (*5*) with a final specific activity of 0.97 mCi/mmol.

*Measurement of Primary Kinetic D/T Isotope Effects.* Isotope effects were measured using the competitive technique as used for measurement of the α-secondary tritium kinetic isotope effects above. Reactions were performed in a 0.3 mL volume, and the reaction mixture contained 2 mM [$^3H$,$^{14}C$]malate, 10 mM NAD, 5 mM $MnSO_4$, 1 mM DTT, and 100 units of lactate dehydrogenase in 100 mM Hepes (pH 7.1). The temperature was maintained at 25 °C. Aliquots (20 $\mu$L) were removed prior to injection of malic enzyme for the calibration of zero time points, and the reaction was

Table 1: α-Secondary Tritium Isotope Effects for the *A. suum* NAD-Malic Enzyme[a]

| | $Mg^{2+}$ and NAD | $Mn^{2+}$ and NAD | $Mn^{2+}$ and APAD | $Cd^{2+}$ and NAD |
|---|---|---|---|---|
| | | L-malate-2-H | | |
| | 1.016 | | | |
| | 1.043 | | | |
| | 1.030 | | | |
| | 1.057 | 1.053 | | |
| | 1.026 | 1.012 | | |
| | 1.027 | 1.046 | | |
| | 1.015 | 1.023 | 0.861 | |
| | 1.009 | 1.039 | 0.867 | 1.007 |
| | 1.009 | 1.005 | 0.893 | 1.005 |
| $^{T}(V/K_{malate})_{H}$ | 1.025 ± 0.016 | 1.028 ± 0.018 | 0.874 ± 0.018 | 1.006 ± 0.001 |
| $^{D}(V/K_{malate})_{H}$[b] | 1.018 ± 0.017 | 1.019 ± 0.018 | 0.920 ± 0.018 | 1.004 ± 0.002 |
| | | L-malate-2-D | | |
| | 1.015 | | | |
| | 0.990 | | | |
| | 0.995 | 1.001 | | |
| | 1.007 | 1.002 | ND | 0.976 |
| $^{T}(V/K_{malate})_{D}$ | 1.002 ± 0.01 | 1.0015 ± 0.0005 | | 0.976 |
| $^{D}(V/K_{malate})_{D}$[a] | 1.001 ± 0.01 | 1.001 ± 0.0005 | | 0.984 |

[a] Each of the values represents a result from a separate experiment. [b] $^{D}(V/K)$ is calculated from $^{T}(V/K)$ using the relationship of Swain et al. (*13*).

initiated by the addition of malic enzyme. Aliquots (20 μL) were removed at appropriate time points (representing 15−80% fractional conversion), the reactions quenched with 4 μL of 0.4 M EDTA, and the mixtures stored at −20 °C until they were analyzed by HPLC. The remaining malate and lactate, produced from the pyruvate product of the malic enzyme reaction, were separated using an ionic exclusion organic acid column (ORH-801, Alltech) by HPLC, isocratically eluting with 0.005 N $H_2SO_4$ at a flow rate of 0.5 mL/min. Malate and lactate were well separated with retention times of 9.6 and 12.2 min, respectively. Samples were counted, and the isotope effect was obtained using eq 1.

## RESULTS

*α-Secondary Tritium Isotope Effects.* α-Secondary tritium kinetic isotope effects with the label at the 4-position of the nicotinamide ring of NAD were measured using the NAD-malic enzyme from *A. suum*. Data were obtained using several divalent metal ion activators and either NAD or APAD as the dinucleotide substrate. Experiments were carried out with protium-labeled malate, and with L-malate-2-D, to determine the multiple primary deuterium/secondary tritium isotope effect. The data are summarized in Table 1.

As a control, the α-secondary tritium kinetic isotope effect for yeast alcohol dehydrogenase was measured using 2-propanol as the substrate. The reported value is 1.08 for $^{\alpha-D}(V/K)$ (*12*). The tritium effects measured in these studies are 1.075, 1.089, and 1.064, giving an average of 1.076 for $^{\alpha-T}(V/K_{2-proponal})$. Using the Swain−Schaad relationship (*13*), a value of 1.052 is obtained for $^{\alpha-D}(V/K_{2-proponal})$, similar to the reported value of 1.08.

*Primary Kinetic D/T Isotope Effects.* The isotope effects, $^{T}(V/K)$ and $^{T}(V/K)_{D}$, have been measured with $Mn^{2+}$ as the divalent metal ion and $NAD^+$ as the oxidant as a function of fractional conversion of reaction (Figure 1). With $NAD^+$ as the oxidant, duplicate experiments gave $^{T}(V/K)_{H}$ values of 3.56 ± 0.05 and 3.60 ± 0.08 (a standard error of 2.3%) with an average value of 3.58 ± 0.05, and $^{T}(V/K)_{D}$ values of 1.73 ± 0.04 and 1.70 ± 0.04 (an error of 1.5%) with an average value of 1.72 ± 0.03. Using the measured value of

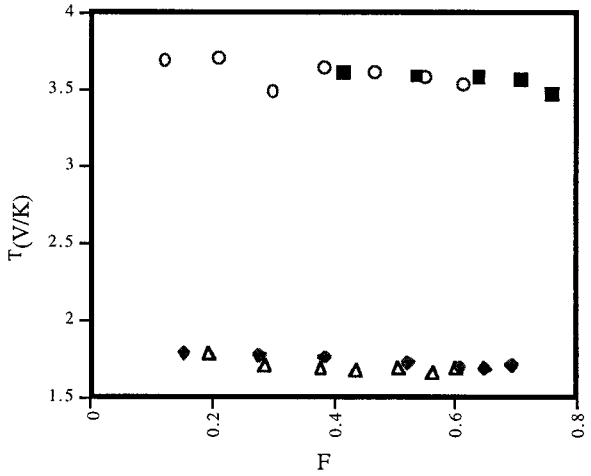



FIGURE 1: Isotope effects, $^{T}(V/K)$ and $^{T}(V/K)_{D}$, obtained with $Mn^{2+}$ as the divalent metal ion and NAD as the oxidant, have been obtained as a function of the fractional conversion of reaction. With $NAD^+$ as the oxidant, duplicate experiments yielded $^{T}(V/K)_{H}$ values of 3.56 ± 0.05 (■) and 3.60 ± 0.08 (○) (a standard error of 2.3%), and $^{T}(V/K)_{D}$ values of 1.73 ± 0.04 (◆) and 1.70 ± 0.04 (△) (an error of 1.5%).

1.72 ± 0.03, a value of 5.81 ± 0.31 was calculated assuming strictly semiclassical behavior, a value much greater than the experimentally measured value of 3.58 ± 0.05.[2] The experimentally obtained exponents that satisfy the relationship $[^{T}(V/K)_{D}]^{exp} = {}^{T}(V/K)_{H-calc}$ were estimated to be 2.32 and 2.41 for the two experiments carried out, with an average value of 2.35. A repeat of the above experiment in duplicate with $Mn^{2+}$ and APAD (Figure 2) gave $^{T}(V/K)_{H}$ values of 4.82 ± 0.07 and 4.85 ± 0.05 (a standard error of 1.2%) with an average value of 4.84 ± 0.04, and $^{T}(V/K)_{D}$ values of 1.65 ± 0.04 and 1.61 ± 0.06 (a standard error of 3%) with an average value of 1.63 ± 0.04. The value calculated for $^{T}(V/K)_{H}$ assuming semiclassical behavior was 4.92 ± 0.35, in excellent agreement with the observed values. Estimates

[2] The calculation of $^{T}(V/K)_{H-calc}$ is based on the Swain−Schaad relationship (*13*): $[^{T}(V/K)_{D}]^{3.26} = {}^{T}(V/K)_{H-calc}$; errors are calculated from the following expression (*26*): $\delta^{T}(V/K)_{H-obs} = \{3.26[^{T}(V/K)_{D-obs}]^{2.26}\}\delta^{T}(V/K)_{D-obs}$.

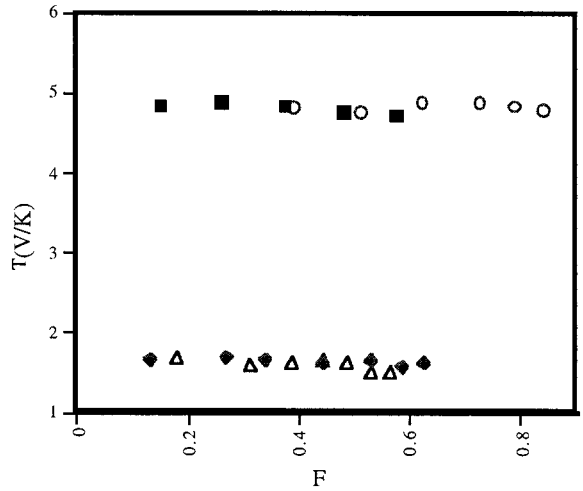



FIGURE 2: Repeating the experiment whose results are depicted in Figure 1 in duplicate with APAD as the oxidant yielded $^{T}(V/K)_{H}$ values of 4.82 ± 0.07 (■) and 4.85 ± 0.05 (○), and $^{T}(V/K)_{D}$ values of 1.65 ± 0.04 (◆) and 1.61 ± 0.06 (△).

of the exponents that satisfy the relationship $[^{T}(V/K)_{D}]^{exp} = {}^{T}(V/K)_{H-calc}$ are 3.14 and 3.32 for the two experiments that were carried out, with an average value of 3.23. The latter average value is within error identical of the theoretical exponent of 3.26 expected for semiclassical behavior (see below).

## DISCUSSION

*Interpretation of* α*-Secondary Tritium Kinetic Isotope Effects.* Kinetic secondary isotope effects largely reflect differences in bending modes between ground and transition states (*14*, *15*). The frame of reference for secondary kinetic isotope effects is the equilibrium isotope effects which reflect differences in the bending modes between reactant and product ground states (*15*, *16*). In the case of a rate-limiting chemical step, a secondary kinetic isotope effect of 1 thus suggests the transition state resembles the reactant; that is, little or no change in hydridization has occurred at the carbon of interest in the transition state. On the other hand, a secondary kinetic isotope effect equal to the secondary equilibrium isotope effect suggests the transition state resembles the product; that is, the hydridization at the carbon of interest is complete in the transition state. The extent of the hybridization change in the transition state measured with the kinetic isotope effect is thought to increase linearly from a value of 1 to a value equal to the equilibrium effect.

There are several reports in the literature of secondary kinetic isotope effects outside the limits of 1 and the measured secondary equilibrium isotope effect (*12*, *17*−*19*). Isotope effects, in all but the last case, were measured for reduction of aldehydes or ketones by alcohol dehydrogenase. The last example is the oxidation of formate by formate dehydrogenase. The large effects have been attributed to a coupling of the motion of the secondary hydrogen to that of the hydride (primary hydrogen) that is transferred. Indeed, Huskey and Schowen (*20*), in modeling these "anomalous" secondary kinetic isotope effects, were led to the conclusion that to generate the large values and the values observed for the primary isotope effect in these systems, a significant amount of hydrogen tunneling of the primary hydrogen is accompanied by a coupling of the motions of the primary and secondary hydrogens. The large secondary kinetic isotope effect can be thought to arise from a propagation of the tunneling of the primary position to the secondary position (*11*, *21*).

A consequence of the propagation of tunneling leading to the large secondary kinetic isotope effects is that the effects will be mass-dependent (*20*). In fact, it has been shown experimentally in the above-mentioned dehydrogenase systems that deuteration of the primary position results in a significant decrease in the measured secondary kinetic isotope effect.

The data obtained for the NAD-malic enzyme reaction agree very well with the predictions made above. In the direction of malate oxidative decarboxylation, the 4-position of the nicotinamide ring of NAD (labeled position) changes its hybridization state from $sp^2$ to $sp^3$. The secondary equilibrium isotope effect measured experimentally for this change is 0.89 (*22*), an inverse effect. The measured secondary tritium kinetic isotope effects (Table 1) using NAD as the dinucleotide substrate are normal (>1), larger than the expected inverse effect. Repeating these effects with L-malate-2-D, increasing the mass at the primary position, results in a decrease in the measured secondary kinetic tritium isotope effect to a value very close to 1, with some of the measurements giving slightly inverse effects. Thus, it would appear that both tunneling and coupled motion occur in the hydride transfer step of the NAD-malic enzyme reaction. Because of the errors in the isotope effects, it is not possible to analyze the data quantitatively. A quantitative analysis will have to await more accurate isotope effect determination, as will be discussed below.

With APAD and $Mn^{2+}$ as reactants, however, very different results were observed. Inverse isotope effects were measured as expected for semiclassical behavior (*14*). Previous results with these dinucleotides (*5*) indicate a concerted oxidative decarboxylation of malate with rate-limiting chemistry. The estimated intrinsic primary deuterium isotope effect is 2.7, which suggests, given strictly semiclassical behavior (*15*), a significant amount of bond cleavage in the transition state for oxidative decarboxylation. The authors interpreted results in terms of an early, not late transition state, for oxidative decarboxylation. A calculated secondary deuterium kinetic isotope effect of 0.92 was obtained for APAD and $Mn^{2+}$, between 1 and 0.89, consistent with a change in hybridization between $sp^2$ and $sp^3$.

*Interpretation of D/T Kinetic Isotope Effects*. Use of the Swain−Schaad relationship to test for a breakdown in the Rule of the Geometric Mean have generally made use of protium as a frame of reference for rates obtained with deuterated and tritiated molecules (*13*). The relationship in this case is $[^{D}(V/K)]^{1.44} = {}^{T}(V/K)$, and changes in the value of the exponent (1.44) are used as an indicator of hydrogen tunneling (exponent of >1.44, since hydrogen tunnels more than deuterium and much more than tritium) or kinetic complexity, that is, the existence of other rate-limiting steps along the reaction pathway (exponent of <1.44). Using tritium as the reference point in these experiments (*23*) generates a new Swain−Schaad exponent in the case where the kinetic complexity or hydrogen tunneling does not exist. Thus, $[^{T}(V/K)_{D}]^{3.26} = {}^{T}(V/K)$; kinetic complexity will tend to decrease the experimentally determined exponent, while tunneling tends to increase its value for the reasons explained

above. However, whether the predicted breakdown in the Rule of the Geometric Mean is observed will depend on the isotope effect that is measured and the position that is labeled, that is, whether the primary isotope effect is measured with protium or deuterium in the secondary position, or vice versa (*24*). Huskey (*24*) has suggested that evidence for tunneling will be obtained using the D/T method, only if reaction coordinate coupled motion also exists. Tunneling alone will give essentially semiclassical behavior, while coupling the primary and secondary position motions leads to a breakdown in the Rule of the Geometric Mean, and the large exponents observed by Klinman (*11*, *21*). Theory further indicates that only when the secondary isotope effect is measured with protium or deuterium in the primary position will the breakdown in the Rule of the Geometric Mean be observed since the smaller isotope effects at the secondary position are more sensitive to tunneling and coupled motion than are the larger primary isotope effects (*21*, *24*).

To test the above prediction, isotope effects determined with the D/T method were carried out by measuring the primary isotope effect with protium in the secondary position. The results agree with the theory developed by Huskey (*24*). The data obtained with NAD and $Mn^{2+}$ give an exponent of 2.2, suggesting the presence of other slow steps along the malic enzyme reaction pathway. Previously published data for the NAD-malic enzyme, obtained using the technique of multiple isotope effects, indicated the existence of rate limitation by steps prior to and following the oxidation step; that is, both $c_f$ and $c_r$ are finite (*5*). However, secondary kinetic tritium isotope effects clearly indicate the presence of tunneling and coupled motion using this dinucleotide−metal ion pair, yet no indication of either is given using D/T kinetic isotope effects with the primary position labeled, as suggested by Huskey.

In the case of $Mn^{2+}$ and APAD, a concerted oxidative decarboxylation of malate is proposed with an intrinsic primary deuterium isotope effect of 2.7 (*5*). With APAD and Mn, commitment factors are very small to zero; that is, intrinsic deuterium and $^{13}C$ kinetic isotope effects are observed. From the D/T data that were collected, an exponent of about 3.3 is calculated, suggesting strictly semiclassical behavior, in agreement with the inverse secondary tritium kinetic isotope effects that are observed (see above). It is interesting to note that although hydrogen tunneling is implicated in the cases where NAD is the substrate it is not with APAD as the substrate. Results may have implications concerning the length of the reaction coordinate or the geometry of hydride transfer in stepwise versus concerted oxidative decarboxylation reactions catalyzed by malic enzyme.

To further test the theory developed by Huskey, future experiments making use of the D/T method will be carried out as described above, with the exception that the secondary position will be labeled, so that (4-T-Nic)AD (or 4-T-AcPyr)-AD will be compared to either (4-H-Nic)([$^{14}C$]AD) or (4-H-AcPyr)([$^{14}C$]AD) and (4-D-Nic)([$^{14}C$]AD) or (4-D-AcPyr)-([$^{14}C$]AD), and the comparisons will be carried out with the primary position (C2 of malate) labeled with protium or deuterium. In this way, we should be able to corroborate results obtained from secondary tritium kinetic isotope effects concerning whether coupled motion and tunneling effects can be separated.

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BI982439Y