Use of Multiple Isotope Effects To Determine Enzyme Mechanisms and Intrinsic Isotope Effects. Malic Enzyme and Glucose-6-phosphate Dehydrogenase†

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ABSTRACT: By determining the ^{13}C isotope effect on V/K with both a deuterated and an unlabeled substrate, and the deuterium isotope effect on V/K, it is possible to tell whether the ^{13}C-sensitive and deuterium-sensitive steps are the same or not and, if they are different, to determine which comes first in the mechanism. If the two isotope-sensitive steps are the same, (1) deuteration increases the size of the observed ^{13}C isotope effect, (2) narrow limits can be calculated for the intrinsic deuterium and ^{13}C isotope effects, and (3) wider limits can be placed on the size of the commitments in the system. If an accurate determination of the tritium isotope effect on V/K is available, or if an α-secondary deuterium isotope effect which originates from the same step as the primary deuterium one and the corresponding ^{13}C isotope effect with α-secondary deuterated substrate can be measured, an exact solution for the intrinsic isotope effects and the commitments is possible. With glucose-6-phosphate dehydrogenase, $^{13}K_{eq}$ for label at C-1 of glucose is 0.9920, and ^{13}C isotope effects are 1.0165, 1.0316, and 1.0176 with glucose 6-phosphate, glucose-*1-d* 6-phosphate, and glucose 6-phosphate (with TPN-*4-d*), respectively. The primary deuterium isotope effect on V/K of 2.97 and the α-secondary deuterium isotope effect of 1.00 allow calculation of intrinsic isotope effects and commitments in this system: $^{D}k = 5.27$, $^{13}k = 1.0408$, $^{\alpha\text{-}D}k = 1.054$, $c_f = 0.75$, and $c_r = 0.49$. When deuterium- and ^{13}C-sensitive steps are different, deuteration decreases the size of the observed ^{13}C isotope effect. The data fit the equation $[^{13}(V/K)_H - 1]/[^{13}(V/K)_D - 1] = {}^{D}(V/K)/{}^{D}K_{eq}$ when the deuterium-sensitive step comes first but fit the equation $[^{13}(V/K)_H - {}^{13}K_{eq}]/[^{13}(V/K)_D - {}^{13}K_{eq}] = {}^{D}(V/K)$ when the ^{13}C-sensitive step comes first. With malic enzyme [using triphosphopyridine nucleotide (TPN)], ^{13}C isotope effects of 1.031 and 1.025 with malate or malate-*2-d*, plus a deuterium isotope effect of 1.47, show that the deuterium-sensitive step comes first and that reverse hydride transfer is 6–12 times faster than decarboxylation. With 3-acetylpyridine-TPN, however, a deuterium isotope effect of 2.18 and a ^{13}C one of 1.0037 show that if the intrinsic isotope effects are similar to those with TPN, reverse hydride transfer is slowed relative to decarboxylation by a factor of 25, presumably as the result of the more positive redox potential of 3-acetylpyridine-TPN.

The multistep nature of enzyme-catalyzed reactions usually decreases the magnitudes of observed isotope effects from the intrinsic isotope effects on the bond-breaking steps to somewhat lower values. Though this less than full expression can be a hindrance when trying to correlate isotope effects with transition-state structure, it can be quite useful in determining the relative rates of various steps and the sequence of these steps in a reaction mechanism. Often an isotopic substitution can be made which will affect the rate of only one particular step in a mechanism (typically via a primary deuterium isotope effect). By thus selectively changing the rate of this one step in the reaction mechanism and observing another isotope effect (either a deuterium or a heavy atom isotope effect) which is also expressed on a particular step of a mechanism, one can tell which isotope-sensitive step comes first in the mechanism or whether both isotope effects are on the same step. The concerted vs. stepwise controversy long associated with various enzymatic mechanisms can thus be unambiguously settled in many cases by application of the techniques which we will describe. We will show that the malic enzyme catalyzed oxidative decarboxylation of malate by triphosphopyridine nucleotide (TPN)[1] is a stepwise reaction with hydride transfer (deuterium sensitive) preceding decarboxylation (^{13}C sensitive). Conversely, ^{13}C and deuterium isotope effects from substitution at C-1 of glucose 6-phosphate are on the same step in the glucose-6-P dehydrogenase reaction, and by also measuring the α-secondary deuterium isotope effect at C-4 of TPN, and the effect of this substitution on the ^{13}C isotope effect, we have solved the resulting five equations simultaneously for the intrinsic primary ^{13}C, primary deuterium, and α-secondary deuterium isotope effects, as well as the forward and reverse commitments.

Materials and Methods

Chemicals. L-Malate-*2-d* was synthesized by the procedure of Viola et al. (1979), using ethanol-d_6 (Merck, 99 atom % D), DPN, and oxalacetate with liver alcohol dehydrogenase, yeast aldehyde dehydrogenase, and malate dehydrogenase. Glucose-*1-d* 6-phosphate was prepared by hexokinase-catalyzed phosphorylation of glucose-*1-d*. The reaction mixture contained 30 mM glucose-*1-d* (Stohler, 98 atom % D), 50 mM ATP, 50 mM Mg^{2+}, and 500 units of yeast hexokinase in 50 mL total volume. The reaction was allowed to proceed for several hours with the pH maintained at 8.0 by addition of

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[1] Abbreviations: TPN, triphosphopyridine nucleotide; 3-AcTPN, 3-acetylpyridine-TPN; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; DTT, dithiothreitol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DPN, diphosphopyridine nucleotide; HPLC, high-pressure liquid chromatography.

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2 N KOH, and the extent of reaction was monitored by assay with glucose-6-phosphate dehydrogenase. An Amicon PM 10 ultrafiltration was used to remove hexokinase, and repeated activated charcoal treatments removed ATP and ADP. The solution was then run through a 2.4 × 10 cm Chelex-100 column (Na^+ form) to remove Mg^{2+} ions. Glucose-*1-d* 6-phosphate was precipitated from solution by rotary evaporation and ethanol addition.

TPN deuterated at the 4 position of the nicotinamide ring (TPN-*4-d*) was synthesized by introduction of deuterium with an A-side enzyme (malic enzyme) and reoxidation with a B-side one (glutamate dehydrogenase). A 2-fold excess of L-malate-*2-d* (1.0 mmol) was incubated with 0.5 mmol of TPN, 1.0 mmol of α-ketoglutarate, 1.0 mmol of NH_4Cl, 2 mM $Mg(OAc)_2$, and 15 units of malic enzyme in 40 mL at pH 8.0 (unbuffered). After the absorbance at 340 nm reached a maximum, 100 units of glutamate dehydrogenase was added. When the A_{340nm} was near zero, the solution was filtered through an Amicon PM30 ultrafilter and lyophilized. One-fifth of this material was purified on a 1.6 × 17 cm column of AG-MP-1 (Bio-Rad) in the chloride form with 0.2 M LiCl, pH 8.2, as the eluant, followed by use of a 1.2 × 50 cm column of Bio-Gel P-2 to desalt the nucleotide, giving material which was 93% deuterated by 270-MHz ^1H NMR. Final purification was by reverse-phase HPLC (Waters semiprep C_{18} column, flow rate 2.5 mL/min, elution with 20 mM LiCl, pH 6.3, 2 mg/injection). After this material was desalted on a Bio-Gel P-2 column, the 259:231-nm absorbance ratio was 2.15.

All enzymes (except malate dehydrogenase obtained from Boehringer-Mannheim) and TPN were obtained from Sigma. The malic enzyme used was from chicken liver. The glucose-6-phosphate dehydrogenase was from *Leuconostoc mesenteroides*. 3-Acetylpyridine-TPN was obtained from either P-L Biochemicals or Sigma. All other chemicals were reagent grade and available commercially.

Nomenclature. The nomenclature used is that of Northrop (1977), in which isotope effects on kinetic or thermodynamic parameters are defined by leading superscripts. Thus, T, 13, 15, 18, D, or α-D refers to tritium, ^{13}C, ^{15}N, ^{18}O, primary deuterium, or α-secondary deuterium isotope effects, respectively. Where necessary, following subscripts are used. For example, $^{13}(V/K)_{malate\text{-}2\text{-}d}$ is the ^{13}C isotope effect on V/K with malate-*2-d* as substrate. For a further discussion of nomenclature, see Cook & Cleland (1981a).

Initial Velocity Studies. Initial velocity studies were performed at 25 °C by monitoring absorbance changes at 340 nm with a Cary 118 spectrophotometer. Deuterium isotope effects on initial velocities were obtained at saturating nucleotide concentration by comparing deuterated and nondeuterated substrates. V/K isotope effects determined by comparing the slopes of reciprocal plots are not sensitive to the presence of inhibitors in the deuterated or unlabeled substrates, but are only as precise as the relative concentrations of the substrates are known (see the methods of calibration below). The V isotope effects do not depend on substrate calibration but are sensitive to the presence of impurities in the substrates (since we were primarily interested in V/K isotope effects, special efforts to assure purity were not employed).

Conditions for the malic enzyme deuterium isotope effect determinations were 1 mM TPN ($K_{TPN} \approx 4$ μM) or 1 mM 3-AcTPN ($K_{3\text{-}AcTPN} \approx 3$ μM), 5 mM $MgSO_4$, and 70 μg/mL bovine serum albumin in 100 mM hepes, pH 8.0, at 25 °C. For determination of the glucose-6-phosphate dehydrogenase deuterium isotope effects, 400 μM TPN (saturating), 100 mM $Mg(OAc)_2$, and 0.2 mM DTT in 100 mM Hepes, pH 8.0, at 25 °C were used. pH values were measured with a Radiometer 26 pH meter equipped with a combined microelectrode standardized to ±0.01 pH unit. Standard buffers were from Beckman.

Determination of Substrate Concentrations. L-Malate-*2-(h,d)* concentrations were determined enzymatically by using chicken liver malic enzyme and 2 mM 3-acetylpyridine-TPN in pH 8.0 K-Hepes buffer. Assays also contained 5 mM $Mg(OAc)_2$, 70 μg/mL bovine serum albumin, and 100 μM dithiothreitol. TPN and 3-AcTPN concentrations were determined in the same system with 20 mM malate. Glucose-*1-(h,d)* 6-phosphate concentrations were determined enzymatically with glucose-6-phosphate dehydrogenase and 2 mM TPN in pH 8.0 K-Hepes buffer containing 100 mM $Mg(OAc)_2$ and 0.2 mM dithiothreitol.

^{13}C Kinetic Isotope Effects. The ^{13}C isotope effects on the malic enzyme reaction were determined by the method discussed by O'Leary (1980), using the natural abundance of ^{13}C in the substrate as the label. The assay solutions consisted of a TPN (or 3-AcTPN) recycling system using yeast glutathione reductase (or glutamate dehydrogenase in the case of 3-AcTPN) and a pyruvate-consuming reaction catalyzed by lactate dehydrogenase. The conversion of pyruvate to lactate circumvents problems which arise from the reversibility of the malic enzyme reaction (lower ^{13}C isotope effects result if the lactate dehydrogenase couple is not utilized) and also dead-end inhibition by formation of E–pyruvate–TPN. Reaction mixtures contained 20 mM L-malate-*2-(h,d)*, 400 μM TPN or 3-AcTPN, 2 mM DPNH, 5 mM $Mg(OAc)_2$, 100 μM DTT, 100 mM Hepes, and either 40 mM oxidized glutathione (for determinations with TPN as nucleotide) or 25 mM α-ketoglutarate and 25 mM NH_4Cl (when 3-AcTPN was used as nucleotide). Aliquots (20.0 mL) of the above solutions were degassed overnight by using CO_2-free N_2 and titrated to pH 8.0 with saturated KOH. In determinations with TPN, malic enzyme, lactate dehydrogenase, and glutathione reductase were added in the units ratio of 1:2:2, respectively, with enough malic enzyme to give 10% reaction in 2 h at 25 °C. With 3-AcTPN determinations, glutamate dehydrogenase was substituted for glutathione reductase, and the units ratio was changed to 1:5:5. Reactions were quenched with 1.5 mL of concentrated H_2SO_4 after appropriate times, and the CO_2 was isolated on a high vacuum line. Complete conversion samples were obtained in the same manner with 3–10 times greater enzyme concentrations and overnight incubations. Malate assays indicated that greater than 99.7% reaction had occurred in all cases.

In glucose-6-phosphate dehydrogenase determinations, carbon 1 of the product, 6-phosphogluconic acid lactone, was converted into CO_2 by use of the TPN-dependent 6-phosphogluconate dehydrogenase reaction. So that problems with the possibly rate-limiting (and probably ^{13}C sensitive) hydrolysis of 6-phosphogluconic acid lactone could be avoided, the CO_2-producing reaction (6-phosphogluconate dehydrogenase) was initiated only after the glucose-6-phosphate dehydrogenase reaction had been terminated at some low conversion. For the complete conversions, however, simultaneous incubation of glucose 6-phosphate with 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase was used. α-Ketoglutarate and NH_4Cl with glutamate dehydrogenase were used to recycle TPNH. Reaction mixtures contained 20 mM D-glucose 6-phosphate, 1 mM TPN or TPN-*4-d*, 2.0 mM $Mg(OAc)_2$, 200 μM DTT, 25 mM α-ketoglutarate, and 50 mM NH_4Cl in 100 mM

Hepes, pH 8.0. To 15.0 mL of this solution were added 1.0 unit of glucose-6-P dehydrogenase and 20 units of glutamate dehydrogenase, and incubation at 25 °C was allowed to proceed for 50 min. (Note that for determinations with TPN-*4-d*, the deuterium label stays in the nucleotide through the recycling since both glucose-6-P dehydrogenase and glutamate dehydrogenase are B-side enzymes.) Five minutes of vortexing with 1 mL of CCl_4 left no detectable glucose-6-P dehydrogenase activity. The aqueous phase was then degassed with CO_2-free N_2 overnight. An additional 15 μmol of TPN, 300 μmol of α-ketoglutarate (α-KG), and 600 μmol of NH_4Cl were added, the pH was readjusted to 8.0, and the solution was degassed for an additional 30 min. To the sealed reaction flask were then added 100 units of glutamate dehydrogenase and 10 units of 6-phosphogluconate dehydrogenase. The incubation was allowed to proceed overnight, and the following day CO_2 was isolated on a high vacuum line. For complete-conversion samples, 5.0 mL of the initial reaction solution described above was diluted to 15.0 mL with 100 mM Hepes buffer, and an additional 300 μmol of α-KG was added. After being degassed overnight, 600 μmol of NH_4Cl was added, the pH adjusted to 8.0, and the solution degassed for an additional 30 min. To the sealed reaction vessel were added 100 units of glutamate dehydrogenase, 10 units of 6-phosphogluconate dehydrogenase, and 200 units of glucose-6-P dehydrogenase. Incubation was allowed to proceed overnight, and CO_2 was isolated as described by O'Leary (1980).

The procedure for determination of $^{13}(V/K)_{\text{glucose-}1\text{-}d\text{-}6\text{-P}}$ was identical with that described above for $^{13}(V/K)_{\text{glucose-6-P}}$ except that glucose-*1*-*d*-6-P generated from glucose-*1*-*d* with hexokinase was used. The glucose-*1*-*d*-6-P was generated in situ, but care was taken to allow the hexokinase reaction to reach completion [500 units of hexokinase, 21 mM ATP, 23 mM $Mg(OAc)_2$, and 20 mM glucose-*1*-*d* preincubated for 30 min] before glucose-6-P dehydrogenase and glutamate dehydrogenase were added. The complete-conversion sample was treated identically with that for $^{13}(V/K)_{\text{glucose-6-P}}$ except that 7 mM ATP, 7.7 mM $Mg(OAc)_2$, and 5 mM glucose-*1*-*d* were added before degassing and 200 units of hexokinase was added 30 min before the other enzyme additions (this preincubation prevented glucose-6-P dehydrogenase from reacting with unphosphorylated glucose, which is a poor substrate, and producing gluconolactone, which is not a substrate for 6-phosphogluconate dehydrogenase).

^{13}C Equilibrium Isotope Effect for the Glucose-6-P Dehydrogenase Reaction. A 15.0-mL sample of 34 mM glucose-6-P, 3.58 mM DPN (V_{max} higher than that for TPN), 2 mM $Mg(OAc)_2$, and 0.2 mM DTT in 100 mM Mes, pH 5.9, were incubated at 25 °C with 350 units of glucose-6-phosphate dehydrogenase for 10 min (chemical equilibrium achieved in less than 1 min as followed by UV at 340 nm). It was important to achieve isotopic equilibrium as quickly as possible since hydrolysis of the product lactone competes with the back-reaction. At pH 5.9, insignificant hydrolysis was observed in 10 min. The solution was then filtered through an Amicon PM 10 filter at 30 psi N_2 (3.5 min required). The filtered solution showed no enzyme activity. To this solution were added 500 μmol of α-ketoglutarate and 20 μmol of TPN, and the pH was adjusted to 7.5. After being degassed overnight, 600 μmol of NH_4Cl and the same enzymes used for the low-conversion reaction in determination of $^{13}(V/K)_{\text{glucose-6-P}}$ were added to convert 6-phosphogluconate to CO_2. After reaction overnight, the CO_2 was isolated as usual.

Instrumentation. For both the malic enzyme and the glucose-6-phosphate dehydrogenase experiments, the ratios of $^{13}C/^{12}C$ in the CO_2 from both low- and complete-conversion samples were measured with a Nuclide Associates RMS 6-60 isotope-ratio mass spectrometer equipped with a dual inlet system. The low- and complete-conversion samples from a particular experiment were always analyzed on the same day to minimize any day to day variations in the mass spectrometer.

Data Analysis. In determinations of deuterium isotope effects, reciprocal initial velocities were plotted against reciprocal substrate concentrations, and the data were fitted to eq 1 by the least-squares method by using the computer

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (1)$$

program of Cleland (1979). F_i is the fraction of deuterium label in the substrate, and E_V and $E_{V/K}$ are the isotope effects minus one for V and V/K, respectively. ^{13}C kinetic isotope effects were determined by use of eq 2 where R_f is the $^{13}C/^{12}C$

$$^{13}(V/K) = \ln(1 - f)/\ln[1 - f(R_f/R_0)] \quad (2)$$

ratio at a fraction of reaction f and R_0 is the $^{13}C/^{12}C$ ratio at $f = 1.0$.

For calculation of $^{13}K_{eq}$ for the glucose-6-P and 6-phosphogluconolactone equilibrium, eq 3 was used where x_1

$$^{13}K_{eq} = \frac{1 + x_1(1 - y_1/y_2)}{y_1/y_2} \quad (3)$$

= [gluconolactone-6-P]/[glucose-6-P] at equilibrium, y_1 = $^{13}C/^{12}C$ ratio in gluconolactone at equilibrium, and y_2 = initial $^{13}C/^{13}C$ ratio in glucose-6-P before equilibration.

Theory

In any enzyme reaction which shows an isotope effect on a single step in the mechanism, the following equation will always apply in the forward direction (Cook & Cleland, 1981a):

$$^{D}(V/K) = \frac{^{D}k + c_f + c_r{}^{D}K_{eq}}{1 + c_f + c_r} \quad (4)$$

In this equation, ^{D}k is the isotope effect on the forward rate constant for the isotope-sensitive step (i.e., the intrinsic isotope effect), and $^{D}K_{eq}$ is the equilibrium isotope effect in the forward direction. The constants c_f and c_r are the forward and reverse commitments, respectively. A commitment for a given reactant is the ratio of the rate constant for the unlabeled molecule for the isotope-sensitive step to the net rate constant for release of the given reactant into solution from the intermediate which faces the isotope-sensitive step.[2] Equation 4 is for a deuterium isotope effect, but identical expressions hold for other isotope effects; for example, the superscript D is changed to 13 for a ^{13}C isotope effect. If these isotope effects are on different steps, c_f and c_r will be different in the two equations, but if the isotope effects are on the same step, it is possible for c_f and c_r to be the same in the two equations as long as these commitments are calculated for the same molecules. c_f is calculated for the variable substrate (this is not necessarily the labeled one) when V/K values with labeled and unlabeled substrates are compared but is calculated for the labeled substrate in an internal competition experiment with tritium or natural-abundance ^{13}C labels, or for an equilibrium perturbation experiment (Schimerlik et al., 1975). Except in the

[2] The determination of "net rate constants" is described by Cleland (1975). They represent the net rate at which an overall sequence of steps occurs. See Cook & Cleland (1981a) for further discussion.

equilibrium perturbation method where c_r is for the perturbant, c_r is calculated for the first irreversible step, which is usually release of the first product.[3] For comparison of different isotope effects on the same step, one should design the experiments so that the commitments are for the same molecules in the different experiments. For example, with glucose-6-P dehydrogenase, where for the ^{13}C isotope effects determined in internal competition experiments the forward commitment is for glucose-6-P (the labeled molecule), the primary and α-secondary deuterium isotope effects were determined in direct comparison experiments by varying glucose-6-P or glucose-*1-d*-6-P concentration in the former case, and by varying glucose-6-P concentration at identical saturating levels of TPN or TPN-*4-d* in the latter case, so that all forward commitments for all of the isotope effects were for glucose-6-P.

When C–H bond cleavage occurs in a mechanism so that there is a primary deuterium isotope effect, and an additional isotope effect (typically a ^{13}C or other heavy atom isotope effect) can be conveniently measured, one can distingush among three possibilities by measurement of $^{D}(V/K)$ and the ^{13}C isotope effects with deuterated and unlabeled substrates [$^{13}(V/K)_D$ and $^{13}(V/K)_H$]: (1) The deuterium-sensitive step precedes the ^{13}C-sensitive step; (2) the ^{13}C-sensitive step precedes the deuterium-sensitive step; or (3) the deuterium- and ^{13}C-sensitive steps are the same.

The two equilibrium isotope effects ($^{D}K_{eq}$ and $^{13}K_{eq}$) must also be known, but these can be easily measured (Cook et al., 1980) or in many cases estimated (Cleland, 1980).

Deuterium-Sensitive Step Precedes ^{13}C-Sensitive Step. The equations which apply in such a case are the following:

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA' \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EX \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EPQ' \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EPQ \xrightarrow{k_{11}} EQ \xrightarrow{k_{13}} E \quad (5)$$

$$w = {}^{T}(V/K) = \frac{({}^{D}k)^{1.442} + a + ({}^{D}K_{eq})^{1.442}(1/c)(1 + b)}{1 + a + (1/c)(1 + b)} \quad (6)$$

$$x = {}^{D}(V/K) = \frac{{}^{D}k + a + {}^{D}K_{eq}(1/c)(1 + b)}{1 + a + (1/c)(1 + b)} \quad (7)$$

$$y = {}^{13}(V/K)_H = \frac{{}^{13}k + c(1 + a) + {}^{13}K_{eq}b}{1 + c(1 + a) + b} \quad (8)$$

$$z = {}^{13}(V/K)_D = \frac{{}^{13}k + (c/{}^{D}K_{eq})({}^{D}k + a) + {}^{13}K_{eq}b}{1 + (c/{}^{D}K_{eq})({}^{D}k + a) + b} \quad (9)$$

where a is the forward commitment for the deuterium-sensitive step [$(k_5/k_4)(1 + k_3/k_2)$ in mechanism 5], b is the reverse commitment for the ^{13}C-sensitive step [$(k_8/k_9)(1 + k_{10}/k_{11})$ in mechanism 5], and c is the ratio of rate constants for the forward reaction through the ^{13}C-sensitive step to the reverse reaction through the deuterium-sensitive step (k_7/k_6 in mechanism 5). The interconversion of EA′ and EX is the D-sensitive step, and the interconversion of EX and EPQ′ is the ^{13}C-sensitive step. In eq 9, the commitment a is divided by ^{D}k, while $1/c$ is divided by $^{D}k/^{D}K_{eq}$; these factors are the intrinsic isotope effects in the forward and reverse directions for the deuterium-sensitive step. With a deuterated substrate, the deuterium-sensitive step is slowed down, while other steps are not, and thus the commitments are decreased by the same factor as the deuterium-sensitive step.

[3] See Cook & Cleland (1981a) for the mechanism dependence of V/K isotope effects.

In this system, z is less than y. Further

$$\frac{y - 1}{z - 1} = x/{}^{D}K_{eq} \quad (10)$$

and thus there are only three independent equations above. Equation 10 holds even when one or more isotope-insensitive steps lie between the deuterium- and ^{13}C-sensitive steps.

^{13}C-Sensitive Step Precedes Deuterium-Sensitive Step. In this case, the pertinent equations are the following:

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA' \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EX \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EPQ' \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EPQ \xrightarrow{k_{11}} EQ \xrightarrow{k_{13}} E \quad (11)$$

$$w = \frac{({}^{D}k)^{1.442} + c(1 + a) + ({}^{D}K_{eq})^{1.442}b}{1 + c(1 + a) + b} \quad (12)$$

$$x = \frac{{}^{D}k + c(1 + a) + {}^{D}K_{eq}b}{1 + c(1 + a) + b} \quad (13)$$

$$y = \frac{{}^{13}k + a + {}^{13}K_{eq}(1/c)(1 + b)}{1 + a + (1/c)(1 + b)} \quad (14)$$

$$z = \frac{{}^{13}k + a + {}^{13}K_{eq}(1/c)({}^{D}k + b{}^{D}K_{eq})}{1 + a + (1/c)({}^{D}k + b{}^{D}K_{eq})} \quad (15)$$

where a is the forward commitment for the ^{13}C-sensitive step [$(k_5/k_4)(1 + k_3/k_2)$ in mechanism 11], b is the reverse commitment for the deuterium-sensitive step [$(k_8/k_9)(1 + k_{10}/k_{11})$ in mechanism 11], and c is the ratio of rate constants for the forward reaction through the deuterium-sensitive step to the reverse through the ^{13}C-sensitive step (k_7/k_6 in mechanism 11). The interconversion of EA′ and EX is the ^{13}C-sensitive step, and the interconversion of EX and EPQ′ is the D-sensitive step. z is again smaller than y, but now

$$\frac{y - {}^{13}K_{eq}}{z - {}^{13}K_{eq}} = x \quad (16)$$

Equation 16 holds even when one or more isotope-sensitive steps lie between the deuterium- and ^{13}C-sensitive steps. Equations 10 and 16 are different as long as

$${}^{D}K_{eq} \neq \frac{(y - {}^{13}K_{eq})(z - 1)}{(y - 1)(z - {}^{13}K_{eq})} \quad (17)$$

and thus when z is less than y and the mechanism is a two-step one, we have a method of determining which isotope-sensitive step comes first. At the same time, these equations prevent a general solution for the intrinsic isotope effects and commitments, since only three of the four equations given in each case above are independent, and there are five unknowns.

However, if the ^{13}C isotope effect is measured on the *same* step in which C–H bond-breaking occurs (for example, if the effect is measured at C-2 rather than C-4 of malate with malic enzyme), solution for ^{D}k and the commitments in this system is possible by the method in eq 29–41 below. When we then reconsider the ^{13}C isotope effect at C-4 (that is, on the step which is not deuterium sensitive), knowing ^{D}k and the commitment for hydride transfer, we can calculate limits on ^{13}k for the step which is not the deuterium-sensitive one. Thus, when the deuterium-sensitive step precedes the ^{13}C-sensitive one, a knowledge of ^{D}k and $(1/c)(1 + b)$, which is the reverse commitment in mechanism 5, provides the following two independent equations:

$$d = (1 + b)/c \quad (18)$$

$$y = \frac{^{13}k + c(1 + a) + {}^{13}K_{eq}b}{1 + c(1 + a) + b} \quad (19)$$

which combine to give

$$^{13}k = y + (y - 1)(1 + a)/d + b[(y - 1)(1 + a)/d + y - {}^{13}K_{eq}] \quad (20)$$

The value of ^{13}k must be at least $y + (y - 1)(1 + a)/d$ (the value when $b = 0$) but can have any reasonable value larger than this, corresponding to increasing values of b and c.

Fortunately, b is probably zero for decarboxylations (that is, the reverse commitment for CO_2 is very small), and thus exact calculation of ^{13}k is possible if ^{D}k is known. When ^{D}k is not exactly known, deuterium, tritium, and ^{13}C isotope effects can be combined to give limits on ^{D}k, ^{13}k, a, and c. This is done by assuming different values of ^{D}k and calculating the following values (a computer program is available to do this):

$$g = 1/(ac) = \frac{[(^{D}k)^{1.442} - w](x - 1) - (^{D}k - x)(w - 1)}{(^{D}k - x)[w - (^{D}K_{eq})^{1.442}] - [(^{D}k)^{1.442} - w](x - {}^{D}K_{eq})} \quad (21)$$

$$a = \frac{^{D}k - x}{x - 1 + (x - {}^{D}K_{eq})g} \quad (22)$$

$$c = 1/(ag) \quad (23)$$

$$^{13}k = y + (y - 1)(1 + a)c \quad (24)$$

By discarding negative values of a or c, and values of ^{13}k larger than is reasonable for a ^{13}C effect, one obtains limits on the various parameters. With the data of Schimerlik et al. (1977) for malic enzyme, for example ($w = 2.02$, $x = 1.47$, $y = 1.031$), c becomes negative below a value of 4.3 for ^{D}k (^{13}k reaches a singularity at $\pm\infty$), 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 1.04–1.06 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 (see Table III).

Deuterium and ^{13}C-Sensitive Steps Are the Same. The equations which apply in this case are

$$w = {}^{T}(V/K) = \frac{(^{D}k)^{1.442} + c_f + c_r(^{D}K_{eq})^{1.442}}{1 + c_f + c_r} \quad (25)$$

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

$$y = {}^{13}(V/K)_H = \frac{^{13}k + c_f + c_r{}^{13}K_{eq}}{1 + c_f + c_r} \quad (27)$$

$$z = {}^{13}(V/K)_D = \frac{^{13}k + c_f/{}^{D}k + c_r{}^{13}K_{eq}{}^{D}K_{eq}/{}^{D}k}{1 + c_f/{}^{D}k + c_r{}^{D}K_{eq}/{}^{D}k} \quad (28)$$

where c_f and c_r are the forward and reverse commitments as defined for eq 4. Note that in eq 28 c_f is reduced by ^{D}k and c_r by $^{D}k/^{D}K_{eq}$ (there are the intrinsic deuterium isotope effects in the forward and reverse directions) because the isotope-sensitive steps are slowed down while other steps are not, and c_f and c_r both include the rate constant for the isotope-sensitive step as a factor. Thus, z will now be greater than y, and such an observation proves that both isotope effects are on the same step (z was less than y for the two-step mechanisms discussed above).

Equations 26–28 can be used to obtain narrow limits on ^{D}k, ^{13}k, c_f, and c_r, and in favorable cases, eq 25 can be combined with the other three to give a complete solution for all of the unknowns, since eq 25–28 are independent. (Equations 26–28 can also be combined with measurements of the α-secondary deuterium isotope effect on the same step, as well as the ^{13}C isotope effect in the presence of α-secondary deuterium substitution, and this procedure will be described below.)

If we consider only eq 26–28, the following rules apply: (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 or 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 sizable 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

$$^{D}k = \frac{(z - d)x}{y - d} \quad (29)$$

where $d = (c_f + c_r{}^{13}K_{eq})/(c_f + c_r)$ and

$$^{13}k = \frac{xz - yc}{x - c} \quad (30)$$

where $c = (c_f + c_r{}^{D}K_{eq})/(c_f + c_r)$. Thus, for any assumed ratio of c_r and c_f given by $r = c_r/c_f$, we can calculate d and c as

$$d = \frac{1 + r^{13}K_{eq}}{1 + r} \quad (31)$$

$$c = \frac{1 + r^{D}K_{eq}}{1 + r} \quad (32)$$

and calculate ^{D}k and ^{13}k from the equations above.

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

$$^{D}k = \frac{b + \sqrt{b^2 - 4x(yp)(zp)}}{2(yp)} \quad (33)$$

$$^{13}k = y + (^{D}k - x)(yp)/(xp) \quad (34)$$

where

$$xp = x(1 + r) - (1 + r^{D}K_{eq}) \quad (35)$$

$$yp = y(1 + r) - (1 + r^{13}K_{eq}) \quad (36)$$

$$zp = z(1 + r^{D}K_{eq}) - (1 + r^{D}K_{eq}{}^{13}K_{eq}) \quad (37)$$

$$b = x(yp) + (z - y)(xp) + (zp) \quad (38)$$

The value of ^{D}k is then used to calculate c_f and c_r:

$$c_f = (^{D}k - x)/(xp) \quad (39)$$

$$c_r = rc_f \quad (40)$$

If $c_f = 0$, however

$$c_r = (^{D}k - x)/(x - {}^{D}K_{eq}) \quad (41)$$

The analysis so far gives limits on ^{D}k and ^{13}k and possible ranges of c_f and c_r. A computer program has been written which makes these calculations for c_r/c_f ratios varying from zero to infinity, and obviously c_r/c_f ratios which give a negative value for one of the intrinsic isotope effects, or a value which is ridiculous (over 1.08 for ^{13}k, for example), can be discarded. In favorable cases, the values of $^{T}(V/K)$ calculated from eq 25 for each set of possible c_f, c_r, ^{D}k, and ^{13}k values can then be compared with the experimental value of $^{T}(V/K)$, and the c_r/c_f ratio adjusted until the calculated and experimental values agree (the computer program does this). The problem in practice is that unless the commitments are such that the observed $^{D}(V/K)$ value is in the range of 1.5–2, the solution becomes very ill conditioned (Albery & Knowles, 1977). This problem is the same as that met in the original method of Northrop (1975) in which $^{D}(V/K)$ and $^{T}(V/K)$ are compared to give limits on ^{D}k (Schimerlik et al., 1977) or an exact solution when $c_r = 0$. We are, therefore, attempting to develop methods for measuring $^{D}(V/K)$ and $^{T}(V/K)$ simultaneously by isotope ratio mass spectrometry, and if this is successful, the precision should be sufficient to allow more exact solution of the full set of eq 25–28.

An alternative to the use of the tritium isotope effect for determination of intrinsic isotope effects and commitments involves the use of secondary deuterium isotope effects. The analysis of ^{D}k, ^{13}k, c_f, and c_r for a reaction mechanism in which the deuterium-sensitive and ^{13}C-sensitive steps are the same relies on the fact that c_f and c_r are identical for both isotope effects. We know that the $^{D}(V/K)$ and $^{13}(V/K)$ isotope effects measured for glucose-6-P dehydrogenase involve the same step since the bond which connects both atoms is broken. Another isotope effect which we can be reasonably sure corresponds to the same transition state is the α-secondary deuterium isotope effect caused by deuteration at C-4 of the nucleotide (Cook et al., 1981). Measurement of $^{\alpha\text{-}D}(V/K)_{\text{glucose-6-P}}$ and $^{13}(V/K)_{\text{glucose-6-P }(\alpha\text{-D})}$ (the ^{13}C isotope effect with α-secondary deuterated nucleotide) allows one to write two more equations analogous to eq 26 and 28, while introducing only one more unknown ($^{\alpha\text{-}D}k$, the intrinsic α-secondary deuterium isotope effect).

$$^{\alpha\text{-}D}(V/K) = \frac{^{\alpha\text{-}D}k + c_f + c_r{}^{\alpha\text{-}D}K_{eq}}{1 + c_f + c_r} \quad (42)$$

$$^{13}(V/K)_{\alpha\text{-}D} = \frac{^{13}k + c_f/{}^{\alpha\text{-}D}k + c_r{}^{13}K_{eq}{}^{\alpha\text{-}D}K_{eq}/{}^{\alpha\text{-}D}k}{1 + c_f/{}^{\alpha\text{-}D}k + c_r{}^{\alpha\text{-}D}K_{eq}/{}^{\alpha\text{-}D}k} \quad (43)$$

Together with eq 26–8, we now have five equations in five unknowns (^{D}k, ^{13}k, $^{\alpha\text{-}D}k$, c_f, and c_r), and an exact solution is possible without assuming the Swain relationship. The solution is achieved by successive approximation by assuming a ratio of $r = c_r/c_f$ and applying eq 29–41 to give values of ^{D}k, ^{13}k, c_f, and c_r. These values of c_f and c_r are substituted into eq 42, and it is solved for $^{\alpha\text{-}D}k$, and eq 43 is then used to compute a value for $^{13}(V/K)_{\alpha\text{-}D}$. This value is compared with the experimental one for this isotope effect, and the partial derivative of this parameter with respect to r is used to calculate a new estimate of r and the process repeated until experimental and computed values of $^{13}(V/K)_{\alpha\text{-}D}$ agree. A computer program has been written to do this.

Table I: ^{13}C Isotope Effects at pH 8.0, 25 °C, for Malic Enzyme

nucleotide	substrate	% reaction[a]	isotope ratios[b] (×10⁵) low conversion	100% conversion	$^{13}(V/K)$
TPN	malate-*2-h*	20.6	1138.5	1167.1	1.0307
		17.2	1138.6	1167.4	1.0302
		14.0	1138.2	1167.0	1.0295
		13.9	1138.0	1167.0	1.0297
		16.4	1138.3	1167.3	1.0303
		15.8	1138.1	1167.0	1.0306
	$^{13}(V/K)_{\text{malate}} = 1.0302 \pm 0.0005$				
TPN[c]	malate-*2-h*	46.7	1142.4	1167.8	1.0334
		42.6	1141.8	1167.4	1.0325
		19.4	1136.6	1167.2	1.0326
		18.9	1136.6	1167.0	1.0323
		15.2	1136.4	1167.1	1.0317
		13.7	1136.2	1167.5	1.0320
	$^{13}(V/K)_{\text{malate,Mn}^{2+}} = 1.0324 \pm 0.0006$				
TPN	malate-*2-d*	6.4	1195.0	1223.8	1.0250
	$^{13}(V/K)_{\text{malate-2-d}} = 1.0250 \pm 0.0007$				
3-AcTPN	malate-*2-h*	3.5	1188.0	1191.7	1.0032
		3.5	1186.9	1191.7	1.0041
	$^{13}(V/K)_{\text{malate}} = 1.0037 \pm 0.0006$				

[a] Value for the low-conversion sample determined by recovery of CO_2 from acidified reaction mixtures. [b] Ratios were adjusted for ^{17}O contribution to *m/e* 45 by subtracting 74 from decade settings for *m/e* 45/44 which were corrected to tank standard of 1260 (O'Leary, 1980). [c] In the presence of 0.5 mM Mn^{2+} in place of 5 mM Mg^{2+}. The isotope effect in the presence of 5 mM Mg^{2+} is four standard deviations from the isotope effect in the presence of 0.5 mM Mn^{2+} and thus appears significantly different.

Results

Deuterium Isotope Effects. For malic enzyme, L-malate-*2-(h,d)* was varied at saturating TPN or 3-AcTPN concentrations, and isotope effects were observed on both V and V/K_{malate}. With TPN, these effects were $^{D}(V/K)_{\text{malate}} = 1.47 \pm 0.03$[4] and $^{D}V = 1.05 \pm 0.05$, while with 3-AcTPN $^{D}(V/K)_{\text{malate}} = 2.18 \pm 0.33$ and $^{D}V = 2.18 \pm 0.32$.

For glucose-6-phosphate dehydrogenase, glucose-*1*-(*h,d*)-6-P was varied at saturating TPN concentrations, giving $^{D}(V/K)_{\text{glucose-6-P}} = 2.97 \pm 0.14$ and $^{D}V = 1.80 \pm 0.04$. The α-secondary isotope effects, determined by varying glucose-6-P concentration at equal saturating levels (49 μM) of TPN or TPN-*4-d*, were $^{\alpha\text{-}D}(V/K)_{\text{glucose-6-P}} = 1.000 \pm 0.015$ and $^{\alpha\text{-}D}V = 1.04 \pm 0.02$.

^{13}C Kinetic and Equilibrium Isotope Effects. The $^{13}(V/K)_{\text{malate-2-(h,d)}}$ and $^{13}(V/K)_{\text{glucose-1-(h,d)-6-P}}$ isotope effects were determined from comparison of $^{13}C/^{12}C$ ratios in CO_2 from partial reactions and $^{13}C/^{12}C$ ratios in complete conversion reactions. These ratios are tabulated, together with the ratios needed for determination of $^{13}K_{eq}$ for the reaction glucose-6-P $\rightleftharpoons$ 6-phosphogluconate, in Table I and II. The isotope effects calculated by use of either eq 2 [$^{13}(V/K)$ kinetic isotope effects] or eq 3 ($^{13}K_{eq}$ equilibrium isotope effect) are given below each set of experimental data. The very small size of the ^{13}C isotope effect with 3-AcTPN as the nucleotide makes impractical the determination of $^{13}(V/K)_{\text{malate-2-d}}$. By use of eq 9, this isotope effect can be predicted to be 1.0022, and the difference between this value and 1.0037 is little more than the standard error associated with these types of measurements. For this

[4] Determined by Dr. P. F. Cook in this laboratory.

Table II: ^{13}C Isotope Effects at pH 8.0, 25 °C, for Glucose-6-phosphate Dehydrogenase

nucleotide	substrate	% reaction[a]	isotope ratios[b] (×10⁵) low conversion	100% conversion	$^{13}(V/K)$
TPN	glucose-*1-h*-6-P	9.3	1200.1	1218.4	1.0160
		10.5	1199.8	1219.0	1.0170
	$^{13}(V/K)_{\text{glucose-1-h-6-P}}$ = 1.0165 ± 0.0007				
TPN	glucose-*1-d*-6-P	26.7	1187.0	1218.5	1.0311
		20.0	1184.7	1218.6	1.0320
	$^{13}(V/K)_{\text{glucose-1-d-6-P}}$ = 1.0316 ± 0.0006				
TPN-*4-d*	glucose-*1-h*-6-P	20.9	1196.2	1216.1	1.0177
		17.4	1197.1	1215.1	1.0175
	$^{13}(V/K)_{\text{glucose-6-P (α-D)}}$ = 1.0176 ± 0.00014				
DPN[c]	glucose-*1-h*-6-P	4.5	1229.0	1219.1	0.992[c]
		4.5	1228.7	1218.3	0.991[c]
	$^{13}K_{\text{eq,glucose-6-P⇌gluconolactone-6-P}}$ = 0.992 ± 0.001				

[a] Value for the low-conversion sample determined by recovery of CO_2 from acidified reaction mixtures. [b] Ratios were adjusted for ^{17}O contribution to *m/e* 45 by subtracting 74 from decade settings for *m/e* 45/44 which were corrected to tank standard of 1260 (O'Leary, 1980). [c] Determination of equilibrium isotope effect. The low-conversion samples were at chemical and isotopic equilibrium.

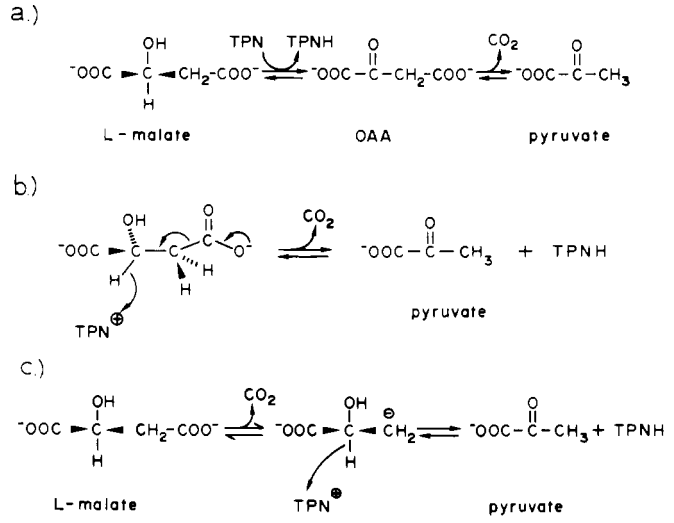

FIGURE 1: Mechanisms for malic enzyme. (a) Stepwise mechanism with C–H cleavage preceding C–C cleavage. (b) Concerted C–H and C–C cleavages. (c) Stepwise mechanism with C–C cleavage preceding C–H cleavage.

reason, this effect was not determined.

Discussion

Mechanism of Malic Enzyme. For the decarboxylation catalyzed by malic enyzme, it has long been assumed that formation of oxalactate, via hydride transfer to TPN, preceded the C–C bond breakage. [For pH and isotope effect studies bearing on the chemical mechanism, see Schimerlik et al. (1977) and Schimerlik & Cleland (1977).] Oxalacetate would thus be a distinct enzyme-bound intermediate which partitions between hydride transfer and decarboxylation steps (Figure 1a). This mechanism has firm footings in solution chemistry since it is well-known that β-keto acids readily decarboxylate. Is solution chemistry relevant to enzyme-bound substrates, however? One could imagine a concerted mechanism which, if the proper orientation between TPN and malate were promoted by the enzyme, might provide a quite facile oxidative

Table III: Comparison of Forward Commitments and Internal Partition Ratios Calculated for Malic Enzyme with TPN or 3-Acetylpyridine-TPN as Substrates

assumed isotope effects[a]		calcd values[b] TPN		3-AcTPN	
$^{D}k_s$	$^{13}k_7$	k_6/k_7	c_{malate}	k_6/k_7	c_{malate}
5.2	1.063	5.46	4.56	0.23	2.36
5.5	1.051	7.59	3.89	0.30	2.56
5.8	1.044	9.89	3.11	0.38	2.75
6.1	1.039	12.4	2.22	0.45	2.94

[a] These limits are based on the experimental isotope effects of Schimerlik et al. (1977) [$^{T}(V/K)$ = 2.02, $^{D}(V/K)$ = 1.47, $^{13}(V/K)$ = 1.031] and the assumption that the reverse commitment for CO_2 is zero (see Theory for the method of calculating possible ^{D}k and ^{13}k values). These data were obtained with the pigeon liver enzyme, but the agreement of the $^{D}(V/K)$, ^{D}V, and $^{13}(V/K)$ isotope effects with those obtained with chicken liver enzyme suggests that the $^{T}(V/K)$ effect is also the same for pigeon liver and chieken liver malic enzymes. The intrinsic isotope effects are assumed to be the same for TPN and 3-AcTPN. [b] The rate constants refer to mechanism 5. $c_{\text{malate}} = (k_5/k_4)(1 + k_3/k_2)$. k_6/k_7 = (rate of reverse hydride transfer)/(rate of decarboxylation).

decarboxylation (Figure 1b). The last alternative, the most unlikely, is that the decarboxylation of malate provides the driving force for hydride transfer and that C–C bond breakage precedes the C–H bond cleavage (Figure 1c).

The results in Table I distinguish between these three possibilities. Since $^{13}(V/K)_{\text{malate-2-d}}$ is less than $^{13}(V/K)_{\text{malate}}$, a concerted mechanism (Figure 1b) is immediately ruled out. Equations 10 and 16 are utilized to distinguish between the mechanisms in parts a and c of Figures 1. The equilibrium isotope effects for the reaction are $^{13}K_{eq}$ = 0.999[5] and $^{D}K_{eq}$ = 1.18 (Cook et al., 1980). Substituting these values and the data from Table I into eq 10 and 16 gives the following comparisons. From eq 10:

$$\left[\frac{^{13}(V/K)_H - 1}{^{13}(V/K)_D - 1} = 1.21 \pm 0.05\right] = [^{D}(V/K)/^{D}K_{eq} = 1.25 \pm 0.03] \quad (44)$$

From eq 16:

$$\left[\frac{^{13}(V/K)_H - ^{13}K_{eq}}{^{13}(V/K)_D - ^{13}K_{eq}} = 1.20 \pm 0.05\right] \neq [^{D}(V/K) = 1.47 \pm 0.03] \quad (45)$$

Since eq 10 is fitted by the data and eq 16 is not, a two-step mechanism with hydride transfer preceding decarboxylation has been verified.[6]

Comparison of Free-Energy Profiles for TPN and 3-AcTPN. Having established that the deuterium-sensitive step precedes decarboxylation, we decided to try to increase the rather modest $^{D}(V/K)_{\text{malate}}$ of 1.47 by selectively slowing down hydride transfer. With the alternate nucleotide, 3-acetyl-

[5] The ^{13}C fractionation factor of the center carboxyl of isocitrate relative to dissolved CO_2 has been measured by O'Leary & Yapp (1978) as 1.0027 [note that this value is incorrectly quoted as the reciprocal on p 108 of Cleland (1980)]. Since substitution of H for C on a carbon attached to the isotopic one appears to decrease the ^{13}C fractionation factor by 1.004 [footnote r, p 110, of Cleland (1980)], this gives 0.999 for C-4 of malate, and 0.995 for the carboxyl of acetate relative to dissolved CO_2.

[6] Chicken liver malic enzyme catalyzes the decarboxylation of oxalactate, while the bacterial enzyme does not. This has been taken as evidence, by some, that the chicken liver enzyme proceeds by a stepwise mechanism, while the bacterial enzyme uses a concerted process. This hypothesis would be readily testable by using the technique described.

Table IV: Intrinsic Isotope Effects and Commitments for Glucose-6-phosphate Dehydrogenase[a]

assumed c_r/c_f	c_f	c_r	^Dk	$^{\alpha D}k$	^{13}k	calcd $^{13}(V/K)_{\alpha D}$	calcd $^T(V/K)$
0	1.380	0	5.688	1.000	1.0393	1.0165	5.734
0.3	0.992	0.298	5.427	1.033	1.0402	1.0171	5.625
1.0	0.602	0.602	5.173	1.066	1.0412	1.0179	5.516
3.0	0.285	0.854	4.973	1.094	1.0421	1.0186	5.429
∞	0	1.083	4.800	1.119	1.0430	1.0192	5.352

[a] Experimental parameters: $^{13}(V/K)_H = 1.0165 \pm 0.0007$, $^{13}(V/K)_D = 1.0316 \pm 0.0006$, $^{13}(V/K)_{\alpha D} = 1.0176 \pm 0.00014$, $^{13}K_{eq} = 0.992 \pm 0.001$, $^D(V/K) = 2.97 \pm 0.14$, and $^{\alpha D}(V/K) = 1.000 \pm 0.015$ from the present work; $^DK_{eq} = 1.28 \pm 0.02$ and $^{\alpha D}K_{eq} = 0.887 \pm 0.005$ from Cook et al. (1980). The values tabulated were calculated with eq 33–43. See the text for the set of values resulting from comparison of calculated and experimental $^{13}(V/K)_{\alpha D}$ values.

pyridine-TPN, the deuterium isotope effect increased to 2.18 while the ^{13}C isotope effect decreased dramatically from 1.0302 to 1.0037. We can illustrate what rate changes are causing these variations when the nucleotide is changed from TPN to 3-AcTPN by an analysis based on mechanism 5. If we assume that CO_2 does not have a reverse commitment to the decarboxylation step (that is, that b is zero in eq 8 and 9), the analysis presented in eq 21–24 under Theory gives the limiting values for $^Dk_5$ and $^{13}k_7$ in mechanism 5 shown in the left two columns of Table III, and the corresponding k_6/k_7 and c_{malate} values with TPN as substrate shown in the third and fourth columns. We have assumed that $^Dk_5$ and $^{13}k_7$ are the same with TPN and 3-AcTPN[7] and used eq 7 and 8 with $b = 0$ and the observed isotope effects with 3-AcTPN to solve for k_6/k_7 and c_{malate} in this case (last two columns in Table III). While c_{malate} is not much different with 3-AcTPN, the major difference lies in the relative rate of reverse hydride transfer to decarboxylation, which is decreased by a factor of 24–27 with 3-AcTPN, presumably because of its more positive redox potential. A free-energy profile illustrating these differences between TPN and 3-AcTPN is shown in Figure 2.

An additional difference between TPN and 3-AcTPN is the size of the V_{max} isotope effect. With TPN, this effect is not significantly different from 1.0, and TPNH release is believed to be quite slow ($k_{13} \ll k_5$ in mechanism 5; Schimerlik et al., 1977). With 3-AcTPN, on the other hand, this release rate is greatly increased, since $^DV = ^D(V/K) = 2.18$. The V_{max} for 3-AcTPN is nearly 3-fold greater than that for TPN, and the rate-limiting steps involve the reaction sequence between substrate addition and CO_2 release, rather than 3-AcTPNH release.

Determination of Intrinsic Isotope Effects for Glucose-6-P Dehydrogenase. Isotope effects are a powerful tool for studying enzyme mechanisms and transition-state structure (Cleland, 1982), but any detailed analysis requires that one knows the intrinsic isotope effects on the isotope-sensitive steps. Until now, the only method for doing this has been to compare $^D(V/K)$ and $^T(V/K)$ according to the method of Northrop (1975). When $c_r = 0$ in eq 25 and 26, eq 46 results:

$$\frac{^D(V/K) - 1}{^T(V/K) - 1} = \frac{^Dk - 1}{(^Dk)^{1.442} - 1} \quad (46)$$

This method has given ^Dk values of 6.3 with liver alcohol dehydrogenase and cyclohexanol (Cook & Cleland, 1981c) and 5.7 with the yeast enzyme and 2-propanol (Cook & Cleland, 1981b). When c_r is not zero, eq 46 gives a limiting value of ^Dk. If $^D(V/K)$ is then divided by $^DK_{eq}$ and $^T(V/K)$ by $^TK_{eq}$ [$=(^DK_{eq})^{1.442}$] and these values (now representing the reverse reaction) are used in eq 46, one obtains the other limiting value of ^Dk. This approach was used by Schimerlik et al. (1977) to give limits of 5–8 on ^Dk for malic enzyme.

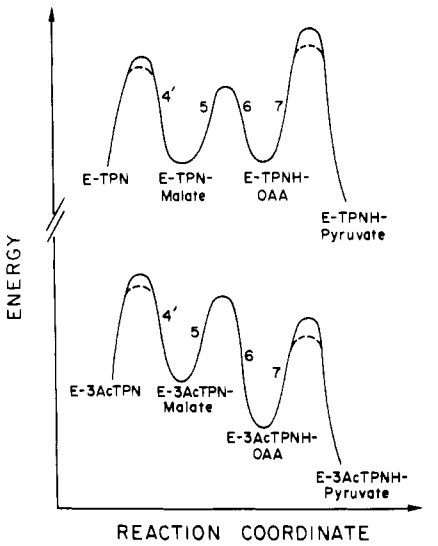

FIGURE 2: Comparison of free-energy profiles for TPN and 3-acetylpyridine-TPN as substrates for malic enzyme when malate is subsaturating. The numbers next to activation barriers correspond to the rate constants in mechanism 5 (the reverse commitment for the decarboxylation step is assumed to be very small). $k_{4'}$ is the net rate constant for release of malate from the ternary complex [$k_{4'} = k_4/(1 + k_3/k_2)$]. The profiles are drawn so that $k_5/k_{4'} = 2.2$–4.6 and $k_6/k_7 = 6$–12 for TPN, while $k_5/k_{4'} = 2.3$–3 and $k_6/k_7 = 0.2$–0.4 for 3-AcTPN (the dotted lines in the profiles define these limits; see calculated values in Table III). The equilibrium constant for the hydride transfer step with TPN was assumed to be unity. With 3-AcTPN, the equality of V and V/K isotope effects implies that reduced nucleotide release is not slow and that $k_{4'} = k_7$. This relationship was used to determine the equilibrium constant for the hydride transfer step in the 3-AcTPN case. For both profiles, the relative energies of the activated complexes are correct regardless of assumptions made concerning the internal equilibrium constants.

Northrop's method has the drawback, aside from the fact that an exact solution is obtained only when $c_r = 0$, that the solutions to eq 46 are ill conditioned if the commitment is either too large or too small (Albery & Knowles, 1977). The method described in the present work of using ^{13}C isotope effects with deuterated and unlabeled substrates in combination with the deuterium isotope effect to solve for limits on the ^Dk and ^{13}k values does not suffer from this problem, and the limits obtained are narrow. This approach was previously used with formate dehydrogenase by Blanchard & Cleland (1980) to show that all commitments were zero, since the ^{13}C isotope effect was the same with deuterated and unlabeled formate.

The data in Table IV for glucose-6-P dehydrogenase illustrate the general case where finite commitments exist. As

[7] This assumption may not be valid for $^Dk_5$, since the intrinsic isotope effect observed for formate dehydrogenase varies with the nucleotide substrate (Blanchard & Cleland, 1980), but is probably valid for $^{13}k_7$, since it is the same molecule (oxalacetate) which is being decarboxylated in either case.

Table V: Error Analysis for Solution for Intrinsic Isotope Effects and Commitments in the Glucose-6-P Dehydrogenase Reaction[a]

exptl parameters	solution parameters				
	$^{D}k-1$	$^{13}k-1$	$^{\alpha\text{-}D}k-1$	c_f	c_r
$^{13}K_{eq}-1$	−0.08	0	−0.37	−0.08	−0.25
$^{D}K_{eq}-1$	0.01	0.05	0	0.11	0
$^{\alpha\text{-}D}K_{eq}-1$	0.08	−0.05	0	0.67	−0.82
$^{13}(V/K)_H-1$	0.21	−0.74	−14.4	8.8	−14.4
$^{13}(V/K)_D-1$	1.31	1.2	0.37	3.7	0.29
$^{13}(V/K)_{\alpha\text{-}D}-1$	−1.3	0.59	14	−12	14
$^{D}(V/K)-1$	0.78	−0.25	0	−0.67	−0.08
$^{\alpha\text{-}D}(V/K)$[b]	1.69	−0.98	22.2	15	−18

[a] The values shown are the percent change in the solution parameter for a 1% change in the experimental parameters. [b] Since the value of $^{\alpha\text{-}D}(V/K)$ was near unity, 1 was not subtracted from its value. The percent change is in the isotope effect itself.

the ratio of c_r/c_f changes from zero to infinity, the limits on the intrinsic ^{13}C isotope effect are 1.039–1.043, while ^{D}k varies from 4.8 to 5.7. Because the commitments are low, the range of calculated $^{T}(V/K)$ values is only from 5.35 to 5.73, and thus very careful measurements would be needed to achieve an exact solution in this case. Simulation has shown, however, that with somewhat larger commitments, the overall solution is not so ill conditioned, and exact answers could be obtained with a tritium isotope effect determined with the usual precision.

By contrast, the calculated $^{13}(V/K)_{\alpha\text{-}D}$ values in Table IV show a variation which is considerably larger than the error associated with this type of measurement, and thus a comparison with the experimental value of 1.0176 can be used to give an exact solution for all parameters in the system, using the computer program we have written for this purpose. To evaluate the errors in the parameters resulting from this solution, we made small changes in the experimental input data and observed the resulting effects on each of the solution values. Table V shows the percent change in the final values with a 1% change in each experimental value. Using the values in Table V and the percent errors of the experimental data, and assuming that all of the experimental errors are independent since they came from different experiments, we assign the following standard errors to the derived values:[8] $^{D}k = 5.27 \pm 0.29$, $^{13}k = 1.0408 \pm 0.0018$, $^{\alpha\text{-}D}k = 1.054 \pm 0.035$, $c_f = 0.75 \pm 0.26$, and $c_r = 0.49 \pm 0.27$.

This method should find broad application, and its use will yield for the first time exact values of intrinsic isotope effects and commitments. In further studies with glucose-6-P dehydrogenase, for example, we have found that in D_2O the commitments are increased, but that ^{13}k is probably unchanged, and ^{D}k somewhat decreased from the values seen in water. We will defer a discussion of the size of the observed intrinsic isotope effects for this enzymatic reaction until these and other studies are complete.

[8] The percent error in each solution parameter was calculated as the square root of the sum of the squares of the percent errors contributed by each experimental parameter. The percent error contributed by each experimental parameter was obtained by multiplying the appropriate value in Table V by the percent error in the experimental parameter.

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