Use of Isotope Effects To Deduce the Chemical Mechanism of Fumarase†

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ABSTRACT: The pH variation of primary ^{18}O and primary and secondary deuterium isotope effects has been determined by the use of the equilibrium perturbation method for the reaction catalyzed by fumarase. The primary ^{18}O effect is 1.073 from the malate side at pH 5 (with an equilibrium ^{18}O effect of 1.033) but decreases rapidly to near unity above pH 7. The primary deuterium isotope effect is near unity at pH 5 and 9 but is strongly inverse at neutral pH (0.915 from the malate side at pH 7, compared to the equilibrium isotope effect of 0.98). Secondary isotope effects with dideuterated substrates from (Brant malate side were 1.31 at pH 5–6 but decreased to a value of 1.08 at pH 9.6 (the equilibrium isotope effect is 1.45). These data are interpreted to mean that the 3*R* proton of malate is transferred to a group (probably carboxyl) on the enzyme with a fractionation factor relative to water of at least 1.2, to give a carbanion intermediate with an aci-carboxylate structure which is tetrahedral at C-2 and trigonal at C-3. Carbon–oxygen bond cleavage accompanied by proton transfer from a group (probably imidazole) on the enzyme then gives water and fumarate. By quantitative analysis of the isotope effects, partition ratios for forward and reverse reaction of the E–malate, EH–carbanion, and EH–H_2O–fumarate intermediates are calculated as a function of pH. The commitments to catalysis of malate, fumarate, water, and the proton on the enzyme are small at pH 5, and carbon–oxygen bond breaking is totally rate limiting. At neutral and high pH, however, the commitment factors (except that for water) are large, so that no ^{18}O isotope effect is seen, and the other isotope effects are equilibrium ones, with the exact value seen depending on the ratio of forward and reverse commitments.

During the reversible trans addition of water to fumarate to form (2*S*)-malate catalyzed by fumarase (EC 4.2.1.2), a single nonexchangeable deuterium atom is incorporated from D_2O into the *pro-R* position at C-3 of malate (Alberty et al., 1957; Alberty & Bender, 1959):

$$\text{fumarate} + H_2O \rightleftharpoons (2S)\text{-malate} \qquad (1)$$

No primary kinetic isotope effect was observed by Fisher et al. (1955) when malate and (2*S*,3*R*)-malate-*3*-*d*[1] were compared at pH 8, although a primary equilibrium isotope effect of 1.07 has been reported (Thompson, 1960). Isotope-exchange studies at pH 7.3 (Hansen et al., 1969) have shown that in the enzymatic dehydration of malate a water molecule is the first product released, followed by fumarate and then the proton derived from the methylene carbon of malate. The pH variation of the kinetic parameters (Brant et al., 1963) suggested that the acidic and basic enzyme groups involved in catalysis are an imidazole and a carboxyl group.

In this report we present the pH variation of primary and secondary isotope effects for fumarase, from which the partition ratios for intermediates along the reaction path and a chemical mechanism have been determined. The initial step in malate dehydration appears to be the formation of a C_3 carbanion with an aci-acid structure in a step which is fast relative to subsequent C–O bond cleavage. Only at low pH where reactants are released from the enzyme much faster than the chemical reaction occurs, however, is C–O bond cleavage solely rate limiting. At neutral and high pH reactant release is slow relative to the chemical reaction, with fumarate release being more rate limiting at pH 7 and malate release being more rate limiting at pH 9.

Materials and Methods

Nomenclature. We will use the isotope effect nomenclature of Northrop (1975, 1977), in which a leading superscript D or 18 indicates the deuterium or ^{18}O isotope effect on the parameter given. Thus, $^{D}K_{eq}$, ^{D}V, and $^{D}(V/K_{malate})$ are deuterium isotope effects of K_{eq}, V, and V/K_{malate}. Isotope effects measured by equilibrium perturbation are expressed as ^{D}(Eq.P.) or ^{18}(Eq.P.), with the subscript indicating from which side the isotope effect is calculated, and not the reactant with the label, which determines only the direction of the perturbation. An equilibrium perturbation experiment determines ^{D}(Eq.P.) in both directions, with the ratio of the two values being $^{D}K_{eq}$ (Schimerlik et al., 1975).

Chemicals. Fumarase, aspartase, malic enzyme, malate dehydrogenase, yeast alcohol dehydrogenase, aldehyde dehydrogenase, and D_2O (99.8+ atom % D) were from Sigma Chemical Co. NAD, NADH, NADP, and glutamine–oxaloacetate transaminase were from Boehringer-Mannheim. Fumaric acid-*2,3*-d_2 (min 98 atom % ^2H) was from Merck and was recrystallized from water before use. $H_2{}^{18}O$ (99 atom % ^{18}O) was from Bio-Rad Laboratories. (2*S*)-[2-^{18}O]Malate (92 atom % ^{18}O) was synthesized for us by the Stable Isotopes Resource at the Los Alamos Scientific Laboratory. Resources).] [The S.I.R. is jointly supported by the U.S. Department of Energy and the National Institutes of Health (RR-00962-01, Division of Research Resources).] All other reagents were of the highest quality commercially available.

(3*R*)-Malate-*d* was prepared from fumarate and D_2O in the presence of fumarase, and malate-d_2 was similarly prepared in H_2O from fumarate-*2,3*-d_2. Malate-*2*-*d* was prepared by the reduction of oxaloacetate in the presence of malate dehydrogenase by A-side DPND [generated from perdeuterioethanol in the presence of yeast alcohol dehydrogenase and aldehyde dehydrogenase (Viola et al., 1979)].

(3*S*)-Malate-*d* was prepared in two steps from fumarate-*2,3*-d_2. In the first step, 100 units of aspartase was added to

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[1] Abbreviations used: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Taps, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Caps, (cyclohexylamino)propanesulfonic acid. We will use the following abbreviations for deuterated malates: (3*R*)-malate-*d*, (2*S*,3*R*)-[3-^{2}H]malate; (3*S*)-malate-*d*, (2*S*,3*S*)-[3-^{2}H]malate; malate-*2*-*d*, (2*S*)-[2-^{2}H]malate; malate-d_2, (2*S*,3*S*)-[2,3-$^2H_2$]malate.

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a 200-mL solution containing 50 mM fumarate-*2,3-d*$_2$, 500 mM $(NH_4)_2SO_4$, 10 mM $MgSO_4$, and 100 mM Pipes, pH 7.5. After equilibrium was reached, the reaction mixture was acidified to pH 2 with 85% perchloric acid. Precipitated protein and buffer were removed by filtration, the filtered solution was applied to a 2 × 30 cm column of Dowex 50-H^+, and the column was washed with water until no fumarate remained in the eluant. (2*S*,3*S*)-Aspartate-*2,3-d*$_2$ was eluted with 5% aqueous pyridine and was recrystallized from water. In the second step, 300 units of glutamate–oxaloacetate transaminase was added to a 400-mL solution at 4 °C containing 8 mM (2*S*,3*S*)-aspartate-*2,3-d*$_2$, 25 mM α-ketoglutarate, 500 μM NAD, 45 mM ethanol, 500 units of yeast alcohol dehydrogenase, 400 units of aldehyde dehydrogenase, and 10 000 units of malic dehydrogenase. A constant pH of 8 was maintained by the addition of 1 N KOH, and 100 mg of solid α-ketoglutaric acid was added after 4 h to ensure complete reaction. After an additional 6 h, the mixture was acidified to pH 2 with perchloric acid and flashed to 20 mL. One gram of acid-washed charcoal was added, and after being heated to 80 °C, the solution was filtered. The filtered solution was titrated to pH 7 with 6 N KOH and applied to a 2 × 40 cm column of Dowex 1-formate, which after being washed with 100 mL of water was developed with a 600-mL linear ammonium formate gradient (0–4 N). Fractions were analyzed for malate with malic enzyme in the presence of 1 mM NADP and 10 mM $MgSO_4$, and those containing malate were pooled, concentrated by rotary evaporation, and lyophilized. The proton NMR spectrum of this material showed a resonance corresponding to the 3*S* hydrogen, the intensity of which was 7% of the 3*R* peak. Racemization of the intermediate appears to occur slowly via the enol form at 4 °C before it is reduced to malate.

Chlorofumarate was synthesized by the procedure of Perkin (1888) and was recrystallized from water before use (mp 197–198 uncor). (2*S*,3*S*)-3-Chloromalate was prepared by enzymatic hydration in a 1-L solution containing 1 mM chlorofumarate, 500 units of fumarase, and 25 mM Pipes, pH 7. (2*S*,3*S*)-3-Chloromalate-*3-d* was prepared similarly from chlorofumarate by enzymatic hydration in D_2O. Chlorofumarate was separated from chloromalate in both of the above preparations on Dowex 1, as described above. The proton NMR spectra were consistent with the expected structures, and no detectable resonance corresponding to a C_{3R} proton was seen for (2*S*,3*S*)-3-chloromalate-*3-d*.

Initial Velocity Studies. Initial velocities were obtained with a Beckman DU monochromator equipped with a Gilford OD converter and a 10-mV strip chart recorder. Temperature was maintained at 25.0 ± 0.1 °C by using thermospacers and a circulating water bath. All studies were carried out by monitoring fumarate absorbance at wavelengths between 240 and 260 nm by using 1 or 10 cm path length cuvettes.

Fumarase solutions were prepared by centrifuging an aliquot of the ammonium sulfate suspension and dissolving the enzyme crystals in 50 mM Hepes, pH 7.5, containing 100 μM dithiothreitol and EDTA at 4 °C. All initial velocity studies and equilibrium perturbations were initiated by the addition of a small volume of cold enzyme solution to a temperature-equilibrated cuvette. The activity was stable over the measurement period at all pH values above 5, but at and below pH 5, a slow loss of activity took place which could not be reversed by increasing the pH.

The following buffers were used at 100 mM concentrations at the stated pH values to allow for overlap: acetate (4.5–5.5), Mes (5.0–6.5), Pipes (6.3–7.5), Hepes (7.0–8.5), Taps (8.0–9.0), Ches (9.0–10.0), and Caps (9.5–10.0). Only acetate showed any inhibition ($K_i \sim 360$ mM), while none of the buffers showed activation. Substrate activation was observed with malate between pH 6 and pH 9, and substrate concentrations used to determine V and V/K were chosen to avoid this activation.

Determination of Substrate Concentration. The concentrations of malate solutions were calibrated enzymatically in a system containing 1.5 units of malic enzyme, 100 μM malate, 2 mM NADP, 10 mM $MgSO_4$, and 100 mM Hepes, pH 8.5. Fumarate solutions were calibrated in a similar system also containing 4 units of fumarase and with fumarate replacing malate. An enzymatically calibrated solution of fumarate was used to determine $\epsilon^{fumarate}$ at wavelengths between 230 and 280 nm in both H_2O and D_2O.

Determination of Equilibrium Isotope Effects. The primary deuterium equilibrium isotope effect for malate was determined at pH 7.0 by allowing fumarase to hydrate fumarate in H_2O and 99% D_2O. After equilibrium was reached, fumarase was removed by ultrafiltration, and the equilibrium concentrations of fumarate and malate were determined enzymatically. The secondary equilibrium isotope effect for (3*S*)-malate-*d* was determined by adding fumarase to solutions of malate or (3*S*)-malate-*d*. After equilibrium was reached, fumarase was again removed by ultrafiltration, and the equilibrium concentrations of malate and fumarase were determined enzymatically. Chloromalate is not a substrate for malic enzyme, and the primary equilibrium isotope effect for this substrate was determined by allowing chlorofumarate to become enzymatically hydrated in H_2O and 99% D_2O. After correction for the change in $\epsilon_{240}^{chlorofumarate}$ in D_2O, $^DK_{eq}$ was calculated directly from the absorbance changes at 240 nm in H_2O and 99% D_2O.

To measure the ^{18}O equilibrium isotope effect, we made a stock solution containing 200 mM Pipes, pH 7.0, and concentrations of malate and fumarate that were close to their equilibrium concentrations. The addition of fumarase (1.5 units in 20 μL) to a 1-mL cuvette containing 0.5 mL of the stock solution and 0.5 mL of water caused a change in the absorbance at 240 nm. Additional substrate was added to the stock solution until the addition of enzyme caused no change in absorbance. An aliquot of the adjusted stock solution was then enzymatically calibrated to determine precisely the substrate concentrations. When enzyme was added to a cuvette containing 0.5 mL of the adjusted stock solution and 0.5 mL of $H_2{}^{18}O$, the absorbance decreased as the new equilibrium position was reached, and the ^{18}O equilibrium isotope effect was calculated from the initial and final concentrations in this system, correcting for the final concentration of $H_2{}^{18}O$ (48 atom % ^{18}O).

Equilibrium Perturbations. For equilibrium perturbations using $H_2{}^{18}O$ as the perturbing species, the concentration of malate in a stock solution was increased such that addition of enzyme to a cuvette containing 0.5 mL of the stock solution and 0.5 mL of water caused an increase in absorbance exactly equal to the decrease observed in 48% $H_2{}^{18}O$. An aliquot was removed, and the concentrations of substrates were determined by enzymatic analysis. Addition of enzyme to a cuvette containing 0.5 mL of $H_2{}^{18}O$ and 0.5 mL of the newly adjusted stock solution resulted in an equilibrium perturbation.

For equilibrium perturbations using (2*S*)-[2-^{18}O]malate, the concentration of malate was kept constant at 500 μM, and the concentration of fumarate was varied to maintain equilibrium at all pH values. For all other equilibrium perturbation experiments, the concentration of malate was ~3 mM, and the

Table I: pK Values Observed in pH Profiles,[a] 25 °C

		H_2O	D_2O[b]
		Fumarate Hydration	
V	pK_1	5.33 ± 0.14	5.80 ± 0.06
	pK_2	8.16 ± 0.06	8.13 ± 0.07
V/K	pK_1	5.81 ± 0.06	6.51 ± 0.13
	pK_2	7.67 ± 0.03	7.92 ± 0.13
		Malate Dehydration	
V	pK_1	6.28 ± 0.04	5.83 ± 0.13
	pK_2	9.98 ± 0.09	9.92 ± 0.27
V/K	pK_1	5.53 ± 0.06	5.86 ± 0.21
	pK_2	7.78 ± 0.04	8.07 ± 0.20

[a] The pK values are fits of the data to eq 6. See Materials and Methods for experimental details. [b] The pK values are in terms of pD, which was the pH meter reading + 0.4.

concentration of fumarate was varied to remain at equilibrium at all pH values.

Data Analysis. Reciprocal initial velocities were plotted against the reciprocal of substrate concentration and the data were fitted by the least-squares method assuming equal variances for the values of v or log Y (Wilkinson, 1961) by using a digital computer and the program of Cleland (1979). Individual saturation curves were fitted to eq 2. Initial velocities

$$v = VA/(K + A) \quad (2)$$

obtained by varying the concentration of hydrogen- and deuterium-containing substrates were fitted to eq 3–5, which

$$v = VA/[K + A(1 + F_iE_V)] \quad (3)$$

$$v = VA/[K(1 + F_iE_{V/K}) + A] \quad (4)$$

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (5)$$

assume isotope effects on V only, V/K only, or both V and V/K. The best fit was chosen on the basis of the lowest residual least square and the lowest standard errors of the fitted parameters. pH profiles in which the log of the parameter plotted decreased both above pK_2 with a slope of −1 and below pK_1 with a slope of 1 were fitted to eq 6. The secondary

$$\log Y = \log [C/(1 + [H]/K_1 + K_2/[H])] \quad (6)$$

isotope effects as a function of pH were fitted to eq 7, where

$$\log Y = \log [(a + bK_1/[H])/(1 + K_1/[H])] \quad (7)$$

a is the pH-independent value of Y at low pH and b is the pH-independent value of Y at high pH. In eq 2–5, V is the maximum velocity and K is the apparent Michaelis constant of A. In eq 3–5, F_i is the fraction of isotope and E_V and $E_{V/K}$ are the isotope effects minus 1 on V and V/K. In eq 6 K_1 and K_2 are the acid dissociation constants of groups that must be deprotonated and protonated, respectively, for activity, and in eq 7 K_1 is for the group whose protonation shifts Y from a to b. [H] is the hydrogen ion concentration, and in eq 6 C is the pH-independent value of Y.

Equilibrium perturbation isotope effects were calculated from reactant concentrations and perturbation sizes by two Fortran programs, one of which makes an exact solution to the equations when water is a perturbant and the other of which gives an exact solution when water is not a perturbant. Readers may obtain a listing of these programs from W. W. Cleland.

Results

pH Profiles. In H_2O the pH profiles for V and V/K in both directions decreased at both high pH and low pH, and the pK values from fits to eq 6 are given in Table I. The ratio of V/K values gave a pH-independent value of K_{eq} for eq 1 of 4.4 ± 0.1, after correcting for the pK values of malate and fumarate and assuming only the dianions to be substrates. The pH variation of the kinetic parameters was also measured in 99% D_2O (Table I). The equilibrium constant in D_2O calculated from the Haldane relationship remains pH independent with a value of 5.1 ± 0.1.

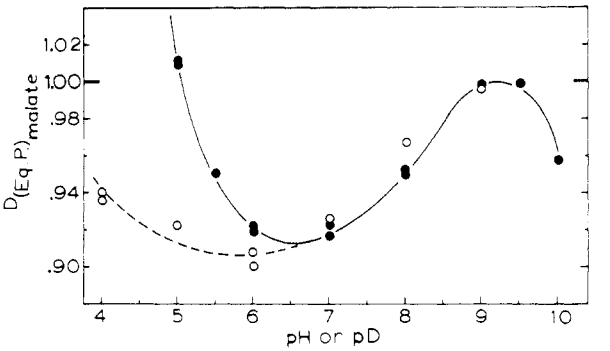


FIGURE 1: pH variation of the primary deuterium isotope effect from the malate side. Filled circles represent data where label was originally in (3R)-malate-d. Open circles represent data where label was originally in D_2O. pD = pH_{obsd} + 0.4.

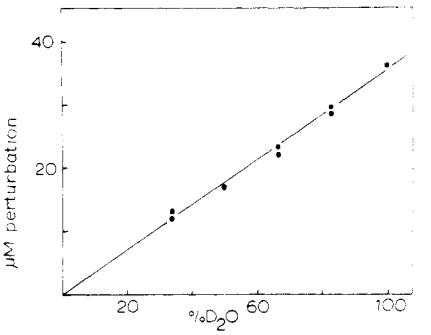


FIGURE 2: Dependence of equilibrium perturbation size on the concentration of labeled reactant (D_2O) at pH(D) 7. The concentration of malate was 3.0 mM, and fumarate was varied to remain at equilibrium. The calculated isotope effect remains unchanged after correcting for the change in $\epsilon_{240}^{fumarate}$ in D_2O.

Primary Isotope Effects. The apparent primary deuterium equilibrium isotope effect for fumarase measured by comparing K_{eq} in H_2O and D_2O was 0.939 ± 0.006. Because K_{eq} for eq

$$H_2O + D_2O \rightleftharpoons 2HOD \quad (8)$$

8 is 3.78 instead of 4.0 (Friedman & Shiner, 1966; Van Hook, 1972), all fractionation factors in D_2O are 0.945 times those in H_2O, and thus the true value of $^DK_{eq,malate}$ in H_2O is 0.98. The pH dependence of the primary kinetic deuterium isotope effect was determined with (3R)-malate-d by using the equilibrium perturbation technique and is shown in Figure 1. Isotope effects were calculated from the perturbation data by using a value of 0.98 for the equilibrium isotope effect in H_2O (see above).

Equilibrium perturbations were also carried out with unlabeled substrates in D_2O. The size of the perturbation is a linear function of the concentration of D_2O at pD 7, as can be seen in Figure 2. The pH profile for $^D(Eq.P.)_{malate}$, where the label was originally in D_2O, was essentially identical with that determined with (3R)-malate-d at pD >7, but at pD <7 the isotope effect in D_2O remained inverse and did not approach the normal value as quickly as in H_2O (Figure 1).

The measured primary equilibrium ^{18}O isotope effect ($^{18}K_{eq,malate}$) of 1.033 ± 0.003 was used in the calculation of ^{18}O isotope effects measured by equilibrium perturbation with [2-^{18}O]malate. $^{18}(Eq.P.)_{malate}$ increased from <1.007 at pH

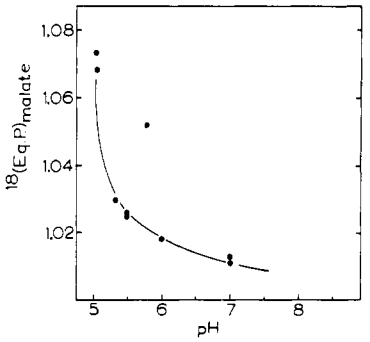


FIGURE 3: pH variation of the primary ^{18}O isotope effect. The concentration of [2-^{18}O]malate was 0.5 mM, and fumarate was varied to remain at equilibrium.

8 to 1.073 at pH 5.03 (Figure 3). ^{18}(Eq.P.)$_{malate}$ at pH 5 was 1.071 when $H_2{}^{18}O$ was used with unlabeled malate.

Secondary Isotope Effects. $^{D}K_{eq,(3S)\text{-malate-}d}$ was determined as 1.17 ± 0.02. Since scrambling of deuterium between C_2 and C_3 via monodeuteriofumarate occurs, the value should equal half of the equilibrium isotope effect in going from monodeuteriofumarate to (3*S*)-malate-*d* multiplied by 1 plus the equilibrium isotope effect in going from (3*S*)-malate-*d* to malate-*2*-*d* [that is, 1.11(1 + 1.18)/2 = 1.21] and is in reasonable agreement. The equilibrium isotope effect with malate-d_2 has been determined by Cook et al. (1980) to be 1.45. The secondary kinetic isotope effects for both fumarate-d_2 and malate-d_2 were measured as a function of pH by equilibrium perturbation (Figure 4A). ^{D}(Eq.P.)$_{malate\text{-}d_2}$ increased from a value of 1.08 at pH 9.6 to 1.31 at pH 5, and identical values were obtained with label in either fumarate or malate. A fit of the data to eq 7 gave a p*K* of 8.12 ± 0.03.

^{D}(Eq.P.)$_{(3S)\text{-malate-}d}$ increased from 1.03 at pH 10 to 1.13 at pH 5 (Figure 4B) with a p*K* of 8.12 ± 0.03 from a fit of the data to eq 7. Secondary isotope effects were also measured by direct comparison of the initial rates of dehydration of malate-*2*-*d* and unlabeled malate. Values of $^{D}(V/K_{malate\text{-}2\text{-}d})$ were 1.07 ± 0.07, 1.16 ± 0.06, 1.24 ± 0.08, and 1.27 ± 0.16 at pH values of 9.10, 7.75, 7.00, and 6.02. While the errors are sizable, the trend in values with pH is similar to that seen in Figure 4.

Alternate Substrates. Chlorofumarate is hydrated by fumarase to (2*S*,3*S*)-3-chloromalate (Tiepel et al., 1968) with a $V \sim 1\%$ that of fumarate and exhibited marked substrate inhibition at pH 7 (K_m = 120 μM; K_i = 1.86 mM), while (2*S*,3*S*)-3-chloromalate was dehydrated with a $V \sim 0.25\%$ that of malate. The apparent primary deuterium equilibrium isotope effect for chloromalate measured by comparing K_{eq} in H_2O and D_2O was 1.02 ± 0.01, but when corrected for the different fractionation factors of H_2O and D_2O (see above), the true value in water is 1.08. When (2*S*,3*S*)-3-chloromalate and (2*S*,3*S*)-3-chloromalate-*3*-*d* were compared as substrates under initial velocity conditions, ^{D}V was ~1.0, while $^{D}(V/K_{chloromalate})$ was 0.86 ± 0.06, 0.83 ± 0.06, and 0.78 ± 0.06 at pH 9, 7, and 5, respectively.

Discussion

pH Variation of Kinetic Parameters. In a reaction with one substrate and one product, the pH dependence of V/K and V reflects the ionizations of free enzyme or substrate groups and of enzyme–substrate complex groups, respectively, which are required in a specific protonation state for binding and catalysis. Since K_{eq} is pH independent, the V/K profiles are required by the Haldane relationship to show the same pH dependence. The p*K* values in the V/K profiles are the true

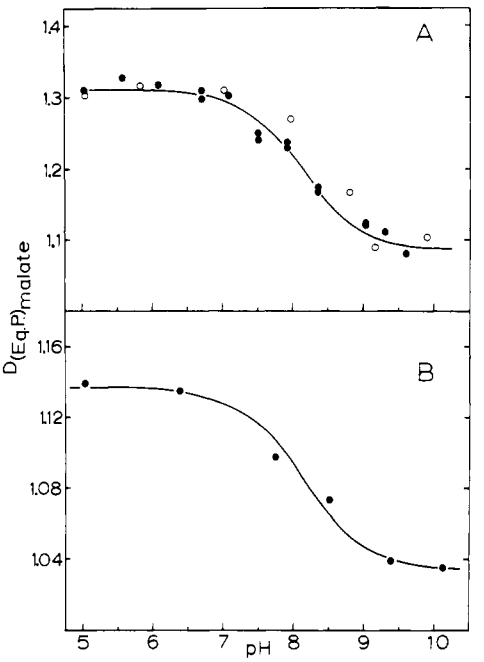


FIGURE 4: pH variation of secondary deuterium isotope effects. (A) Isotope effects calculated from data obtained with malate-d_2 (●) and fumarate-d_2 (○). The curve is a fit of the data to eq 7. (B) Isotope effects calculated from data obtained with (3*S*)-malate-*d*. The curve is a fit of the data to eq 7.

ones for free enzyme or free substrate, unless the substrates are sticky (that is, react to give products as fast or faster than they dissociate as unchanged substrates), in which case the p*K* values may be displaced outward on the pH profile (Cleland, 1978).[2] The groups observed in the V/K profiles for fumarase have been tentatively identified as a carboxyl and an imidazole group on the basis of their enthalpic behavior (Brant et al., 1963).

The p*K* values in the V profiles reflect the ionization of catalytic groups on the enzyme with substrate bound and may thus differ from p*K* values obtained from the V/K profiles. In the V profile using malate as the substrate, p*K* values of 6.3 and 10 are observed, which appear to be the carboxyl and imidazole p*K* values in the enzyme–malate complex (Brant et al., 1963). Both p*K* values appear to have been raised by malate binding, the carboxyl p*K* by 0.6 unit and the imidazole p*K* by greater than 2 units. In the direction of fumarate hydration, the V profile breaks above a p*K* of 8.2 and below a p*K* of 5.3, which again have been identified as the carboxyl and imidazole p*K* values of the enzyme–fumarate complex, respectively (Brant et al., 1963). The carboxyl p*K* has been raised by 2.5 units upon fumarate binding, while the imidazole p*K* is apparently lowered by 2.4 units.

In D_2O the p*K* values of the carboxyl and imidazole are raised 0.3–0.5 unit. Both V profiles in D_2O show a similar pH dependence to the V profiles in H_2O, and the identity of the groups responsible for this behavior is presumably the same.

Primary Isotope Effects. The primary ^{18}O equilibrium isotope effect of 1.033 reflects the preference of ^{18}O for the

[2] Both malate and fumarate are somewhat sticky except at pH 5 (see eq 22 and Table II), and thus the p*K* values in the V/K profiles should be displaced outward somewhat on the pH profile. In the pH profiles for competitive inhibitors observed by Wigler & Alberty (1960), the p*K* values (which should be seen in their correct position in these profiles) were in fact closer together as expected. This situation has been discussed in detail by Cleland (1978).

stiffer bond in the secondary alcohol, malate, over water. This experimental value is in quantitative agreement with the value calculated for the fractionation of ^{18}O between 2-propanol and water by Cleland (1980).

Sawyer & Kirsch (1973) have reported a value of 1.062 for rate-determining carbon–oxygen bond cleavage in the hydrazinolysis of methoxy-labeled methyl formate, while O'Leary & Marlier (1979) have reported a value of 1.041 for rate-determining carbon–oxygen bond scission in the hydrazinolysis of methoxy-labeled methyl benzoate. The $^{18}(Eq.P.)_{malate}$ value of 1.073 at pH 5.03 [and corresponding value for $^{18}(Eq.P.)_{H_2O}$ of 1.039 in the reverse reaction] indicates that carbon–oxygen bond breaking is completely or very nearly rate limiting at pH 5. At higher pH, however, carbon–oxygen bond breaking is no longer rate limiting.

The apparent value of $^{D}K_{eq,malate}$ of 0.94 agrees with the value of 0.93 reported earlier (Thompson, 1960), and the corrected value of 0.98 in H_2O reflects slightly stiffer bonding in the hydrogen-bonded solvent vs. the sp^3 methylene carbon of malate. When chlorine replaces hydrogen at C_3, however, deuterium enriches in malate, and the change in $^{D}K_{eq}$ value ($1.08/0.98 = 1.10$) is in good agreement with the expected stiffening effect of replacing hydrogen with chlorine on the carbon holding the hydrogen of interest (Hartshorn & Shiner, 1972).

The inverse values of $^{D}(Eq.P.)_{(3R)\text{-malate-}d}$ at neutral pH appear to result from rapid quasi-equilibrium formation of a malate C_{3R} carbanion, with transfer of the removed proton to a group on the enzyme with a fractionation factor higher than that of malate. Inverse primary isotope effects are seen only when the equilibrium isotope effect is inverse and the reaction is nearly at equilibrium; the kinetic isotope effect on the bond breaking step is always normal (see eq 17 below). Neither a concerted reaction, in which C–O bond cleavage would also have to be in equilibrium, in disagreement with the large normal ^{18}O kinetic isotope effect seen at pH 5, nor a mechanism with a carbonium ion intermediate, in which C–O cleavage would have to precede C–H cleavage and thus again be in equilibrium, is consistent with these data.[3]

The carboxyl group whose pK is seen in the V/K profiles and is raised by the binding of either substrate presumably lies at the bottom of the active site and is the group which removes the malate C_{3R} proton. Since $^{D}(Eq.P.)_{(3R)\text{-malate-}d}$ is more inverse than $^{D}K_{eq,malate}$, this carboxyl group when protonated has to have a larger fractionation factor than water. Although carboxylic acids in aqueous solution are reported to have the same fractionation factor as water (Schowen, 1977), the environment of the active site apparently causes stiffer vibrational frequencies of the protonated carboxyl group than would exist in aqueous solution. The fractionation factor for the acidic hydrogen of protonated formic acid in the gas phase is 1.045 relative to liquid water (V. J. Shiner, Jr., personal communication), and this is certainly a better model for the protonated carboxyl in the active site than is a carboxyl free to hydrogen bond in solution. The major vibrational stiffening, however, probably comes from restricted rotation of the hydrogen of the OH group around the C–O axis as the result of steric effects. Tentative calculations by Dr. Warren Buddenbaum (personal communication) suggest that an overall fractionation factor of 1.20 requires an increase in the torsional force constants for O–C–O–H and H–C–O–H rotation by a factor of 2.24.[4]

The above proposal requires that the proton derived from C_{3R} of malate be released only after the release of fumarate, in agreement with the isotopic exchange studies at equilibrium at pH 7.3 of Hansen et al. (1969). These authors found that at saturating concentrations of substrates, the ^{14}C exchange between malate and fumarate was 2.5 times faster than the ^{3}H exchange between malate and water, but at low substrate levels, the ^{3}H exchange was slightly faster than ^{14}C exchange. The C_{3R}-derived proton thus cannot exchange from enzyme–substrate complexes but is rapidly exchanged from the free enzyme. Since ^{18}O exchange between malate and water was faster than either ^{14}C or ^{3}H exchange at both high and low substrate concentrations, the products of malate dehydration are released in the following order: water, fumarate, and the C_{3R} proton derived from malate.

Chloromalate reacts much slower than malate, but this is not caused by rate limiting C–H bond breaking, since $^{D}(V/K_{chloromalate})$ appears to be inverse and essentially pH independent with an average value of 0.82. Equilibrium perturbations could not be performed with this substrate as a result of the limited solubility of fumarase and the large amount of enzyme required, and thus it was not possible to measure the ^{18}O isotope effect with chloromalate. As a consequence we cannot tell whether slow carbon–oxygen bond cleavage, a high degree of nonproductive binding, or slow isotope independent steps are responsible for the slow V with chloromalate, but in view of our conclusions concerning the cause of the inverse primary deuterium isotope effect with (3R)-malate-d (see above), it is tempting to postulate that substitution with chlorine at C-3 leads to a more stable carbanion intermediate so that carbon–oxygen bond breaking is completely rate limiting.[5] The inverse V/K effect of 0.82 then would reflect equilibrium transfer of hydrogen from the 3R position to the carboxyl group on the enzyme, and the fractionation factor of this protonated carboxyl group would have to be 1.20 relative to H_2O. These speculations are predicated on the inverse $^{D}(V/K)$ values being real. Since ^{D}V is 1 instead of 0.82, it is possible that the value of 0.82 is an artifact resulting from incorrect calibration of the concentrations of labeled and unlabeled chloromalate (such an error has no effect on ^{D}V). However, ^{D}V can be near unity and $^{D}(V/K)$ can have a value

[3] Nigh & Richards (1969) postulated a carbonium ion mechanism because water was added to fluorofumarate in the Markovnikov sense, while addition to other halofumarates yielded 3-halomalates. They also felt that a carbanion mechanism would give faster or at least equal reaction of difluorofumarate relative to fluorofumarate, while it was in fact nearly fivefold slower. However, the carbanion mechanism also predicts reverse hydration of fluorofumarate because of the differential effect of fluorine vs. the other halogens on the stability of carbanions developed on the carbons they are attached to (see footnote 5), and the relative rates of reaction with mono- and difluorofumarate could easily result from slightly different interactions with the enzyme.

[4] Formic acid in the gas phase is planar with the H of the OH group largely cis to the carboxyl oxygen. There is 4% trans form; the planarity results from the estimated 20% double-bond character in the single C—O bond caused by resonance with the form $^{-}O—CH=OH^{+}$ (Miyazawa & Pitzer, 1959). In aqueous solution, however, hydrogen bonding may diminish the preference for the planar cis form and lead to a broader distribution of torsion angles. The calculations mentioned above are for the cis form in the vapor phase, but in the active site of fumarase the H—O—C—O torsional angle may well have a different value. These calculations do indicate, however, that steric restraints on the motion of the proton can cause the observed increase in fractionation factor to a value of 1.2.

[5] Chlorine lowers the pK of protons on CH or CH_2 groups it is bonded to and thus stabilizes the resulting carbanions. The shift in pK is 1.6 units in ethyl nitroacetate or the corresponding amide (Adolph & Kamlet, 1966) and 2.8 units in p-nitrotoluene (Chatrousse et al., 1979). Conversely, fluorine has an opposite effect (Adolph & Kamlet, 1966), and thus it is not surprising that fluorofumarate is hydrated by adding the proton to the CH, rather than the CF, carbon, and the reaction is 3 times faster than that with fumarate (Clarke et al., 1968; Tiepel et al., 1968).

equal to $^{D}K_{eq}$ for proton transfer from malate to the carboxyl on the enzyme if $k_3 \gg k_4$, $k_5 \gg k_6$, or $k_7 \gg k_8[H_2O]$ in mechanism 9 (see below).

Secondary Isotope Effects. The secondary equilibrium isotope effect for fumarate-*2,3-d*$_2$ of 1.45 ± 0.02 (Cook et al., 1980) reflects the strong preference of deuterium to be in the stiffer sp^3 bonds of malate as opposed to the sp^2 bonds of fumarate. The equilibrium isotope effects at C_2 and C_3 are different, and the rules of Schimerlik et al. (1975) allow calculation of the equilibrium effects at both positions. C_3 undergoes a change from sp^2 to sp^3 hybridization for which the fractionation factor is 1.11 (Cleland, 1980), while the equilibrium effect at C_2 reflects the replacement of hydrogen with oxygen (for which the fractionation factor is 1.18) as well, so that the equilibrium isotope effect is 1.11 × 1.18 = 1.31.

The pH dependence of both $^{D}(Eq.P.)_{malate\text{-}d_2}$ and $^{D}(Eq.P.)_{(3S)\text{-}malate\text{-}d}$ shows a plateau at low pH and a lower plateau at high pH with identical apparent p*K* values of 8.12 for the transition. At pH 5, where carbanion formation is rapid compared to carbon–oxygen bond scission, we expect a full equilibrium isotope effect at C_3, and a kinetic one at C_2. As we will show below, C_3 of the carbanion is probably sp^2 rather than sp^3, so that the equilibrium isotope effect for its formation is 1.11. The kinetic effect at C_2 would then be 1.31/1.11 = 1.18, which in view of the equilibrium isotope effect of 1.31 at C_2, corresponds to a slightly late transition state for carbon–oxygen bond cleavage.

Quantitative Analysis of Isotope Effects. The following mechanism may be written for fumarase:

$$E \underset{k_2}{\overset{k_1A}{\rightleftharpoons}} E\text{–}A \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EH\text{–}X \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EH\text{–}P\text{–}H_2O \underset{k_8[H_2O]}{\overset{k_7}{\rightleftharpoons}} EH\text{–}P \underset{k_{10}P}{\overset{k_9}{\rightleftharpoons}} EH \overset{k_{11}}{\rightharpoonup} E \quad (9)$$

where A, P, H, and X represent malate, fumarate, the C_{3R}-derived proton, and the C_3 carbanion of malate, respectively. k_3 and k_4 are the steps sensitive to deuterium in the 3*R* position of malate, and k_5 and k_6 are the ^{18}O-sensitive steps. In this mechanism, the expression for $^{18}(Eq.P.)_{malate}$ is

$$^{18}(Eq.P.)_{malate} = \frac{^{18}k_5 + c_{malate} + c_{water}{}^{18}K_{eq}}{1 + c_{malate} + c_{water}} \quad (10)$$

where

$$c_{malate} = (k_5/k_4)(1 + k_3/k_2) \quad (11)$$

$$c_{water} = k_6/k_7 \quad (12)$$

and $^{18}k_5$ is the intrinsic isotope effect on carbon–oxygen bond scission.

At pH 5, both commitments are small since $^{18}(Eq.P.)_{malate}$ is large and, in comparison with reported ^{18}O isotope effects in chemical systems, probably close to the intrinsic isotope effect. As the pH is increased, $^{18}(Eq.P.)_{malate}$ is reduced due to an increase in c_{malate}. Since $^{18}K_{eq}$ is 1.033, c_{water} is always much less than c_{malate}, or values near 1.00 could not be seen for $^{18}(Eq.P.)_{malate}$. This is a very reasonable result, since water can certainly escape from the enzyme faster than it can react with fumarate. We will thus assume c_{water} to be negligible in further calculations.

Assuming[6] that $^{18}k_5$ is 1.080 and that c_{water} is near zero, values of c_{malate} as a function of pH can be calculated from eq 10 and the experimental values of $^{18}(Eq.P.)_{malate}$ in Figure 3. While no perturbations were observed at pH 8 or 9, we have adopted 1.006 at pH 8 and 1.005 at pH 9 as reasonable maximum values for $^{18}(Eq.P.)_{malate}$, and thus the resulting values of c_{malate} are minimum ones. Calculated values of c_{malate} are in Table II.

Table II: pH Variation of Commitment Factors and Rate Constants

	value at pH				
	5	6	7	8	9
c_{malate}	0.096	3.44	6.27	12.3	15
$c_{fumarate}$	0.21	7.6	12.5	10.4	3.5
c_{proton}	36	96	116	80	36
k_3/k_2	1.85	37	53	85	104
k_5/k_4	0.034	0.090	0.12	0.14	0.14
$k_1{}^a$	2.3	2.3	2.5	0.74	0.23
$k_{10}{}^a$	4.2	4.2	5.0	3.4	3.4

[a] These are relative rather than absolute values.

For secondary isotope effects with dideuterated substrates, where k_3, k_4, k_5, and k_6 in mechanism 9 are all isotope sensitive, the expression for the isotope effect is

$$^{D}(Eq.P.)_{malate\text{-}d_2} = \frac{^{D}k_5{}^{D}K_{eq2} + (k_5/k_4)(^{D}k_3 + k_3/k_2) + c_{fumarate}{}^{D}K_{eq3}}{1 + c_{malate} + c_{fumarate}} \quad (13)$$

where c_{malate} is given by eq 11, and

$$c_{fumarate} = (k_6/k_7)(1 + k_8[H_2O]/k_9) \quad (14)$$

In eq 13 $^{D}k_5$ and $^{D}k_3$ are the intrinsic secondary kinetic isotope effects on k_5 and k_3, $^{D}K_{eq2}$ is the equilibrium secondary isotope effect for conversion of E–A to EH–X, and $^{D}K_{eq3}$ is the overall secondary equilibrium isotope effect.

Since at pH 5 all commitments are low, we will assume that $^{D}k_5{}^{D}K_{eq2} = 1.31$, the observed kinetic isotope effect. $^{D}k_3$ is probably between 1.00 and 1.11 ($=^{D}K_{eq2}$), but since k_5/k_4 is never greater than 0.14, while k_3/k_2 is quite large except at pH 5 (see below and Table II), we set $^{D}k_3$ equal to 1 so that eq 13 can be rewritten as

$$^{D}(Eq.P.)_{malate\text{-}d_2} = \frac{1.31 + c_{malate} + 1.45c_{fumarate}}{1 + c_{malate} + c_{fumarate}} \quad (15)$$

The data in Figure 4A (using values from the fit to eq 7) can then be used with eq 15 to calculate the value of $c_{fumarate}$ in terms of c_{malate}. At pH 5 and 6, $c_{fumarate}$ is 2.21 times c_{malate}, while at pH 7–9 the relationship is

$$c_{fumarate} = ac_{malate} - b \quad (16)$$

where *a* is 2, 0.875, and 0.27 and *b* is 0.02, 0.4, and 0.6 at pH 7, 8, and 9, respectively. These ratios are quite well determined by the secondary isotope effects, while the absolute values of $c_{fumarate}$ shown in Table II depend on the accuracy of the c_{malate} values derived from the ^{18}O isotope effects.

The observed primary deuterium isotope effect will be given by eq 17:

$$^{D}(Eq.P.)_{(3R)\text{-}malate\text{-}d} = \frac{^{D}k_3 + c'_{malate} + c_{proton}{}^{D}K_{eq1}}{1 + c'_{malate} + c_{proton}} \quad (17)$$

where

$$c'_{malate} = k_3/k_2 \quad (18)$$

$$c_{proton} = (k_4/k_5)(1 + c_{fumarate}) \quad (19)$$

and $^{D}K_{eq1}$ represents the equilibrium isotope effect for transfer

[6] $^{18}k_5$ must be greater than the observed value of 1.073 at pH 5 and is probably less than 1.09. Choosing a larger value for $^{18}k_5$ alters the calculated values of c_{malate}, particularly at pH 5, but does not affect any of the conclusions described below.

of hydrogen between C-3 of malate and the protonated carboxyl of the enzyme in EH–X.[7] This value must be more inverse than 0.91, but the exact value is not known. For purposes of calculation we will use the value of 0.82 observed with deuterated chloromalate.[8] For $^{D}k_3$ we will use 7.5 as a reasonable value for the intrinsic isotope effect on C–H bond cleavage (using a different value alters the numerical values of the commitments but not the pattern seen in Table II).

From the experimental values in Figure 1 for H_2O solution,[9] it is now possible to obtain from eq 17 an expression for k_4/k_5 in terms of k_3/k_2. To do this, we substitute eq 11 into eq 16, eq 16 into eq 19, and eq 18 and 19 into eq 17. The resulting expression is

$$k_4/k_5 = c - d(k_3/k_2) \quad (20)$$

where the values at pH 5, 6, 7, 8, and 9 are 34, 64, 67, 50, and 36 for c and 2.2, 1.4, 1.1, 0.5, and 0.27 for d. Equation 20 can now be combined with eq 11 to give a solution for both k_3/k_2 and k_5/k_4, and these ratios are shown as a function of pH in Table II, along with values for c_{proton}.

Since we have earlier concluded that c_{water} $(=k_6/k_7)$ has a very low value, $c_{fumarate}$ is essentially equal to $k_6k_8[H_2O]/(k_7/k_9)$, and since k_5/k_4 is also small, c_{malate} is very little different from $k_3k_5/(k_2k_4)$. Thus

$$k_1/k_{10} = K_{eq}c_{fumarate}/c_{malate} \quad (21)$$

The value of k_1/k_{10}, which depends for its accuracy only on the analysis of the secondary isotope effects, is 0.55, 0.55, 0.50, 0.22, and 0.068 at pH 5, 6, 7, 8, and 9. Since V/K_{malate} for mechanism 9 can be written in terms of our approximations above as

$$V/K_{malate} = \frac{k_1c_{malate}}{1 + c_{malate} + c_{fumarate}} \quad (22)$$

we can determine from the pH dependence of V/K_{malate} shown in Table I the relative values of k_1 and, since k_1/k_{10} is known, of k_{10} as a function of pH, and these values are shown in Table II.

The values in Table II show a very interesting pH dependence. First, k_{10}, the bimolecular rate constant for combination of fumarate with the enzyme, appears essentially pH independent. This is a perfectly reasonable result, since neither the catalytic carboxyl nor imidazole form any bond to fumarate. On the other hand, k_1, the bimolecular rate constant for addition of malate to free enzyme, decreases at high pH to a new plateau value. A fit of the k_1 values in Table II to eq 7 gave a pK of 7.6 ± 0.1 and a decrease from the low pH value by a factor of 18 to a limiting value of 0.14 ± 0.03 at high pH. This rate constant, as expected, is not affected by the state of ionization of the catalytic carboxyl but is sensitive to the protonation state of the catalytic imidazole (pK 7.7), with which malate hydrogen bonds.

The partition ratio of the carbanion intermediate (k_5/k_4) might be expected to be pH independent if the state of protonation of the catalytic groups in the EH–X intermediate were frozen. However, it appears to be lower by a factor of 4 at pH 5 than at neutral and high pH. Since the catalytic imidazole is protonated already in EH–X, and the carboxyl is inaccessible to protonation (which would lower k_4 and thus raise, rather than lower, k_5/k_4), we presume that the observed change at low pH results from a conformation change in the enzyme resulting from protonation of a group or groups other than the catalytic ones.

The ratio k_3/k_2 is fairly constant at neutral and high pH, and its high value shows that malate is deprotonated more rapidly than it is released from the enzyme. At pH 5, however, the dramatic decrease in k_3/k_2 (a 20-fold change from pH 6) suggests both that k_3 is decreasing as the catalytic carboxyl becomes protonated[10] and that k_2 increases. Since the second pK of malate is 5.1, it is possible that protons are combining with bound malate and increasing its rate of dissociation.[11] Alternatively, the conformation change which alters k_5/k_4 at pH 5 may also increase k_2 or decrease k_3.

As discussed above, $c_{fumarate}$ is essentially equal to $k_6k_8[H_2O]/k_7k_9$. This commitment varies only slightly above pH 6 (see below) but drops by a factor of 36 from pH 6 to pH 5. Part of the change will result from a drop in k_6 as the catalytic imidazole becomes protonated. The ratio of k_8/k_7 should also drop at the same time, since H_2O hydrogen bonds to unprotonated imidazole, and protonation may thus tend to expel water from its site. There may also be an increase in k_9 as the result of proton-assisted dissociation,[11] but the effect is predicted to be only a factor of 1.25 at pH 5 because the second pK of fumarate is 4.4. The conformation change that affects k_5/k_4 may also affect this commitment.

The commitment which is critical for the variation in the observed primary deuterium isotope effect is c_{proton}. The 3.2-fold range shown in Table II seems small but is responsible for the range in observed isotope effects of 0.915–1.01.[12] Changes in k_3/k_2 are much less important, and in fact when the observed isotope effect is near 1.00, the value of k_3/k_2 makes no difference in eq 17. These changes in c_{proton} reflect largely changes in $c_{fumarate}$, except at low pH where the decrease in $c_{fumarate}$ is partly overcome by the increase in k_4/k_5. The

[7] The inclusion of $c_{fumarate}$ (as given by eq 14) in eq 19 is rigorously correct only if k_{11} is much greater than k_{10}[P] in mechanism 9. Since the levels of substrates used in our studies cause isotope exchange of tritium between malate and water to be only 1.8-fold slower than the ^{14}C exchange between malate and fumarate (Hansen et al., 1969), this approximation does not introduce a serious error.

[8] We have also made these calculations with other values for $^{D}K_{eq1}$, and while the numerical values of k_3/k_2, k_5/k_4, and c_{proton} change somewhat, the patterns seen in Table II do not.

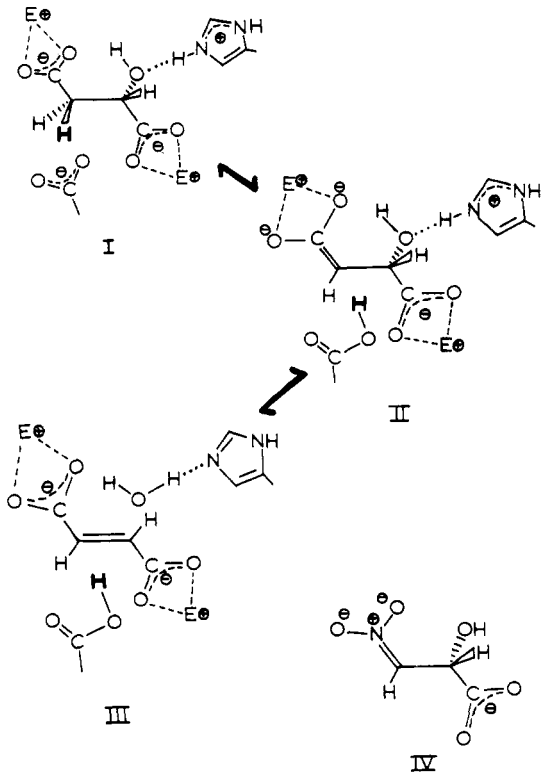
[9] It is apparent that the isotope effects calculated from equilibrium perturbation data in D_2O coincide with isotope effects calculated from equilibrium perturbation data obtained with the label originally in (3R)-malate-d at high pH but not at pD values below 7. Inspection of eq 17 reveals that this can only occur if c_{proton} remains larger at low pH in D_2O than in H_2O, since any decrease in c_{proton} or an increase in c_{malate} would serve to make the observed effect less inverse. The discrepancy between the data obtained in D_2O and with (3R)-malate-d may result from a more gradual drop in c_{proton} at low pH in D_2O than in H_2O as the result of a solvent isotope effect on carbon–oxygen bond cleavage (k_5). Since proton transfer from imidazole to the hydroxyl group accompanies C–O bond cleavage, such a solvent isotope effect is expected on k_5.

[10] While the state of protonation of the catalytic carboxyl can not be changed once substrates are bound (Hansen et al., 1969), the pH profiles of V_{malate} and $V_{fumarate}$ show clearly that both substrates will combine with enzyme with the carboxyl in either protonation state. This leads to an apparent pH effect on k_3 or, in the reverse direction, on k_6 or $k_6k_8[H_2O]/k_7$.

[11] A dicarboxylic acid will dissociate stepwise via an intermediate in which one carboxyl is bound and the other is free. Protonation of the free carboxyl will reduce the rate of recombination, which normally will greatly exceed the rate of complete dissociation, and thus increase the rate of overall dissociation. An effect of 2.3 is predicted in the present case at pH 5 if 5.1 is taken as the pK of the free carboxyl in the intermediate.

[12] At pH 5, c_{proton} is just small enough that proton removal to form the carbanion is not quite at equilibrium, and the inverse effect from this near-equilibrium state is just balanced by the large normal primary kinetic isotope effect. At pH 6–8, c_{proton} is larger, proton removal is closer to equilibrium, and thus an inverse isotope effect is seen. At pH 9, c_{proton} has decreased until the inverse equilibrium and normal kinetic isotope effects are again in balance.

Scheme I



decrease in $c_{fumarate}$ and c_{proton} at high pH probably reflects a generally looser binding of fumarate at high pH with k_9 increasing somewhat.

The preceding analysis shows the power of detailed isotope effect studies to deduce the internal structure of an enzymatic mechanism. In place of simple qualitative statements about where the "rate-limiting" steps lie, we can calculate internal partitioning ratios for each key intermediate and thus gain a clearer quantitative understanding of what is going on. In the present case, at pH 5 we can say from the very low k_5/k_4 value and the low values of c_{malate} and $c_{fumarate}$ that carbon–oxygen bond breaking is rate limiting. At neutral and high pH, however, the commitments to catalysis of all reactants other than water are high, and thus reactant release is rate limiting for both directions, and isotope effects, when seen, are largely equilibrium ones determined by the balance between forward and reverse commitment factors.[13]

Chemical Mechanism. A chemical mechanism consistent with the data presented is outlined in Scheme I. The aci-acid structure for the carbanion intermediate (II) is suggested by the very strong inhibition of fumarase by the corresponding aci form of the anion of 3-nitro-2-hydroxypropionic acid (IV) (Porter & Bright, 1980). It thus appears that fumarase catalyzes its reaction by stabilizing an intermediate which is trigonal at C_3 and tetrahedral at C_2. This mechanism appears to apply to enzymes such as aconitase and aspartase as well, which are also strongly inhibited by aci-nitro anion analogues of their substrates (Schloss et al., 1980; Porter & Bright, 1980). The mechanism by which carbanion formation is enhanced by the enzyme is not clear, however; theoretical calculations are needed in this area.

[13] At pH 7, for instance, the commitment for fumarate is higher than that of malate, but at pH 9 the reverse is true, and thus one sees the shift in secondary isotope effects seen in Figure 4. As noted in footnote 12, the size of the primary isotope effect depends on the balance between $^{D}k_3$, which is normal, and $c_{proton}{}^{D}K_{eq1}$, which is inverse.

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