Secondary Isotope Effects in the Dehydration of Malic Acid by Fumarate Hydratase

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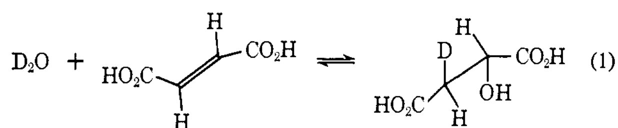
Abstract: The kinetic isotope effects for the dehydration by fumarate hydratase of 2-^{3}H-1,4-^{14}Cl-*l*-malic acid and 2,3-^{3}H-1,4-^{14}C-*l*-malic acid have been determined and support a carbonium ion mechanism for the enzymatic reaction. The isotope effect on the equilibrium between *l*-malate and fumarate has also been determined experimentally and agrees closely with that anticipated on the basis of a Bigeleisen treatment.

Fumarate hydratase catalyze the reversible hydration of fumaric acid to *l*-malic acid[1] and until recently these were the only known substrates for the enzyme although now fluorofumaric acids[2,3] and difluorofumaric acids[3] have been found to be hydrated by fumarate hydratase; the unnatural (−)-tartaric acid,[4,5] though a relatively poor substrate, is dehydrated by fumarate hydratase. The addition of water to fumaric acid occurs with a *trans* stereochemistry[6-9] and fumarate formed from this monodeuteriomalate contains no deuterium,[6] showing the absolute stereospecificity of the hydration reaction 1.



The kinetic isotope effect for that hydrogen removed during the hydration has been determined[6] by comparing the maximum initial velocity of unlabeled *l*-malic acid with the *erythro*-3-deuterio-*l*-malic acid (2-(*S*)-2-hydroxy-3-(*R*)-3-^{2}H-succinic acid); both the maximum initial velocity and the Michaelis constant were, within the limits of experimental error, unchanged by substitution of hydrogen by deuterium. The method of determining the catalytic constant for an enzymatic reaction by measuring the maximum initial velocity has been criticized[10] since the actual asymptotic value of the rate at high substrate concentrations is difficult to determine.

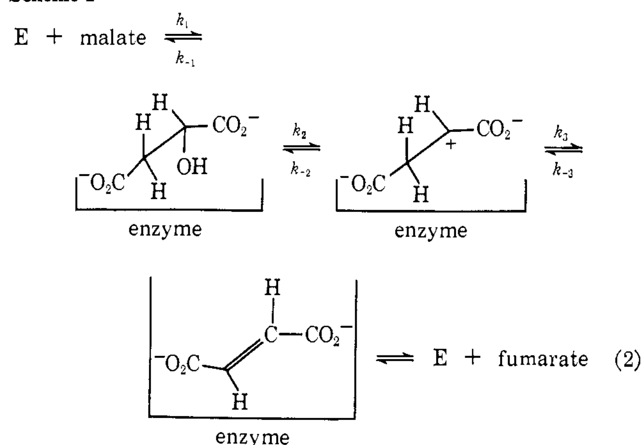
The rate of incorporation of deuterium into *l*-malate during its dehydration to fumarate in deuterium oxide was also determined[11] and found to be in quantitative agreement with that expected if the only path for deuterium incorporation into *l*-malate is by hydration of fumarate, *i.e.*, no exchange of the C-3 hydrogen of *l*-malate occurs without intermediate formation of fumarate.

For the enzymatic hydration of fumarate in water relative to the addition of deuterium oxide in deuterium oxide a value of $V_m^H/V_m^D = 2.1$ has been reported.[12] The apparent contradiction between this result and that of Alberty[6] on the dehydration of *l*-malate may arise from the variation in protein hydration and conformation, with resultant effects on catalytic activity for enzymes in water or deuterium oxide.

Though the suggestion had been made[13] that the rate-determining step could be dissociation of the fumarate–enzyme complex (which would explain the observed[6,11] absence of a primary isotope effect) Alberty[14] has presented kinetic arguments that the dissociation of the fumarate–enzyme complex is not the rate-limiting step; indeed, the combination of fumarate and enzyme are thought to be diffusion controlled with a minimum value for the second-order rate constant of 10^9 l. mol^{-1} sec^{-1}.

On the basis of these observations, a mechanism involving a carbonium ion intermediate was proposed[11] (Scheme I).

Scheme I



The purpose of the present work was to determine the kinetic isotope effects for tritium at C-2 and for that hy-

(1) For a general review see: R. Alberty, *Enzymes*, **5**, 531 (1961).
(2) D. D. Clarke, W. J. Nicklas, and J. Palumbo, *Arch. Biochem. Biophys.*, 123, 205 (1968).
(3) W. G. Nigh and J. H. Richards, *J. Am. Chem. Soc.*, in press.
(4) S. Nakamura and H. Ogata, *J. Biol. Chem.*, **243**, 528 (1968).
(5) S. Nakamura and H. Ogata, *ibid.*, **243**, 533 (1968).
(6) H. Fisher, C. Frieden, J. S. M. McKee, and R. A. Alberty, *J. Am. Chem. Soc.*, 77, 4436 (1955).
(7) O. Gawron and T. P. Fondy, *ibid.*, **81**, 6333 (1959).
(8) F. A. L. Anet, *ibid.*, 82, 994 (1960).
(9) R. A. Alberty and P. Bender, *ibid.*, **81**, 542 (1959).
(10) C. Walter, "Steady State Applications in Enzyme Kinetics," The Ronald Press Co., New York, N. Y., 1965, p 60.
(11) R. A. Alberty, W. G. Miller, and H. F. Fisher, *J. Am. Chem. Soc.*, 79, 3973 (1957).
(12) P. A. Sere, G. W. Kosicki, and R. Lumry, *Biochim. Biophys. Acta*, **50**, 184 (1963).
(13) M. Dixon and E. C. Webb, "Enzymes", Academic Press, New York, N. Y., 1964, p 293.
(14) R. A. Alberty and W. H. Pierce, *J. Am. Chem. Soc.*, **79**, 1526 (1957).

Table I

Substrate	k_H/k_T 20 sec	40 sec	60 sec	K_H/K_T
2-^{3}H-1,4-^{14}C-*l*-Malic acid	1.13 ± 0.02	1.12 ± 0.02	1.14 ± 0.02	1.23 ± 0.03
2,3-^{3}H-1,4-^{14}C-*l*-Malic acid	1.11 ± 0.02	1.12 ± 0.02	1.13 ± 0.02	1.24 ± 0.03
	60 sec	90 sec		
2-^{3}H-3-^{14}C-*l*-Malic Acid[a]	1.12 ± 0.02	1.11 ± 0.02		1.22 ± 0.03

[a] These values for this acid were determined by Mrs. B. Monroe.

drogen at C-3 which is not removed during the dehydration reaction. Moreover, the effect of tritium at C-2 and C-3 on the equilibrium between malate and fumarate was observed.

Results

2-^{3}H-*d,l*-Malic acid was synthesized by reduction of oxaloacetic acid with sodium borotritide. Resolution was carried out *via* the cinchonine salt which was crystallized six times from methanol before liberation of 2-^{3}H-*l*-malic acid. This was then mixed with 1,4-^{14}C-*l*-malic acid prepared by enzymatic hydration of 1,4-^{14}C-fumaric acid to give 2-^{3}H-1,4-^{14}C-*l*-malic acid.

2,3-^{3}H-1,4-^{14}C-*l*-Malic acid was prepared by enzymatic hydration of 2,3-^{3}H-1,4-^{14}C-fumaric acid which had been obtained by enzymatic dehydration of 2-^{3}H-1,4-^{14}C-*l*-malic acid.

For the observation of the kinetic isotope effects for dehydration, appropriately labeled malic acid was added to a solution of fumarate hydratase buffered to pH 7.3 with 10 m*M* sodium phosphate at 25°. Aliquots of reaction mixture were withdrawn at 20, 40, and 60 sec during which time 0.8, 1.6, and 2.3%, respectively, of the *l*-malate had been converted to fumarate. For equilibration experiments, four times as much enzyme relative to substrate was used as in the determination of kinetic isotope effects and the reaction was allowed to proceed for 24 hr.

The *l*-malic acid and fumaric acid samples were counted in Bray's solution using a Packard Tri-Carb scintillation counter Model 3324 for which channel settings and gain had been optimized for determining ^{3}H and ^{14}C activity in the same sample. The ratio of the ratios of ^{3}H/^{14}C for malate relative to fumarate so obtained represent k_H/k_T and are listed in Table I. The numbers for the equilibration experiments represent the ratio of ^{3}H/^{14}C activity in *l*-malic acid relative to the ^{3}H/^{14}C activity in fumaric acid.

$$M_H \rightleftharpoons F_H$$

$$K_H = (F_H)/(M_H)$$

$$M_T \rightleftharpoons F_T$$

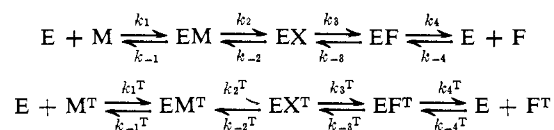
$$K_T = (F_T)/(M_T)$$

$$K_H/K_T = \frac{(F_H)/(M_H)}{(F_T)/(M_T)}$$

Discussion

Determination of Tritium Isotope Effect. The method of determining kinetic and equilibrium isotope effects in this work depends on the ability to estimate, accurately, ^{3}H/^{14}C ratios. Both ^{3}H and ^{14}C emit β particles though of different energies and this allows the accurate determination of the activity due to each in a single sample.[15] Tritium should cause a secondary isotope effect of about 10 to 30%.[16] On the other hand, carbon-14 will have little effect on the reaction. Data on secondary carbon isotope effects are rather scanty because the effect is small and difficult to measure. For example, the conversion of phenyl methyl ketone to a Schiff's base shows a secondary isotope effect of $k_{^{14}C}/k_{^{12}C} = 1.008$.[17] The secondary isotope effect in the decarboxylation of malonic acid is $k_{^{14}C}/k_{^{12}C} = 1.00 \pm 0.002$.[18] Even primary carbon isotope effects are relatively small; the Hofmann elimination of a number of trimethylalkylammonium hydroxides show $k_{^{14}C}/k_{^{12}C} = 1.03$ to 1.07.[16] As prepared in this work, the ^{14}C and ^{3}H are necessarily in different molecules and since the isotope effects due to ^{14}C in 1,4-^{14}C-*l*-malic or 1,4-^{14}C-fumaric acid will be small relative to those anticipated for tritium substitution, the rate of reaction of ^{14}C-containing substrates will be identical with that of ^{12}C substrates containing no tritium. Thus the rate of reaction of ^{14}C substrate will measure the rate of reaction of ^{1}H substrate and the change in ^{3}H/^{14}C ratio will be, therefore, a direct measure of the ^{3}H isotope effect obtained without the necessity of measuring accurately two separate kinetic constants. This method, therefore, avoids many of the criticisms of direct measurement of kinetic isotope effects of enzymatic reactions.[10]

When the doubly labeled substrate is added to a solution of enzyme, the following reactions occur simultaneously (assuming a mechanism with an intermediate–enzyme complex, EX).



where E = free enzyme, M^{14} = *l*-malic acid (^{1}H-^{12}C and ^{1}H-^{14}C), M^T = ^{3}H-*l*-malic acid, F^{14} = fumaric acid (^{1}H-^{12}C and ^{1}H-^{14}C), F^T = ^{3}H-fumaric acid; EM^{14}, EM^T, EF^{14}, EF^T are enzyme complexes with substrates; EX^{14} and EX^T are enzyme complexes with intermediate X.

A third set for ^{12}C substrates could be added, but as noted above, all constants for reactions of ^{12}C will be indistinguishable from the analogous reactions of ^{1}H-^{14}C substrates.

By use of the steady-state assumption for the change in concentration of enzyme–substrate complexes, eq 3, 4, and 5 can be derived for the disappearance of ^{3}H-*l*-malic acid

(15) C. G. Bell and F. N. Hayes, Ed., "Liquid Scintillation Counting," Pergamon Press, Inc., New York, N. Y., 1958.
(16) H. Simon and G. Mullhofer, *Pure Appl. Chem.*, 8, 379 (1964).
(17) V. F. Ragen, A. K. T. Somis, and C. J. Collins, *J. Am. Chem. Soc.*, 82, 5502 (1960).
(18) L. Melander, "Isotope Effects on Reaction Rates," The Ronald Press Co., New York, N. Y., 1960, p 136.

$$V^{T} = -\frac{d(M^{T})}{dt} = [k_f^{T}(M^{T}) - k_r^{T}(F^{T})](E) \quad (3)$$

where

$$k_f^{T} = \frac{k_1^{T}k_2^{T}k_3^{T}k_4^{T}}{k_{-1}^{T}k_{-2}^{T}k_{-3}^{T} + k_{-1}^{T}k_{-2}^{T}k_4^{T} + k_2^{T}k_3^{T}k_4^{T} + k_{-1}^{T}k_3^{T}k_4^{T}} \quad (4)$$

$$k_r^{T} = \frac{k_{-1}^{T}k_{-2}^{T}k_{-3}^{T}k_{-4}^{T}}{k_{-1}^{T}k_{-2}^{T}k_{-3}^{T} + k_{-1}^{T}k_{-2}^{T}k_4^{T} + k_2^{T}k_3^{T}k_4^{T} + k_{-1}^{T}k_3^{T}k_4^{T}} \quad (5)$$

Additional intermediates do not change the form of eq 3, 4, or 5.[19] Equation 3 can be integrated after substitution of (6) to give an equation containing a natural

$$(F^{T}) = (M_0^{T}) - (M^{T}) \quad (6)$$

logarithm term which can be expanded after substituting (7). This leads to (8)

$$1 - \frac{(M^{T})}{(M_0^{T})} = \frac{(F^{T})}{(M_0^{T})} \quad (7)$$

$$k_f^{T}\int_{t=0}^{t=t}(E)dt = \frac{(F^{T})}{(M_0^{T})}\sum_{m=0}^{\infty}\left[\frac{1}{m+1}\frac{(F^{T})}{(F_e^{T})}\right] \quad (8)$$

where (F_e^{T}) = equilibrium concentration of ^{3}H-fumaric acid. Equation 8 can be divided by the analogous one for non-^{3}H substrate to give (9)

$$\frac{k_f^{H}\int_{t=0}^{t=t}(E)dt}{k_f^{T}\int_{t=0}^{t=t}(E)dt} = \frac{(F^{14})(M_0^{T})}{(F^{T})(M_0^{14})}(1 + e) \quad (9)$$

where

$$e = \frac{\sum_{m=1}^{\infty}\left\{\frac{1}{m+1}\left[\frac{(F^{14})^m}{(F_e^{14})^m} - \frac{(F^{T})^m}{(F_e^{T})^m}\right]\right\}}{\sum_{m=1}^{\infty}\left\{\frac{1}{m+1}\frac{(F_T)^m}{(F_e^{T})^m}\right\}} \quad (10)$$

As the experiment is carefully arranged so that $(F^{14})/(F_e^{14})$ and $(F^{T})/(F_e^{T})$ are both less than 0.1[20] and $[(F^{14})/(F_e^{14})] - [(F^{T})/(F_e^{T})]$ is then very nearly zero, eq 10 reduces, under the conditions of this work, to (11) which was used to obtain the various values of k_H/k_T reported in Table I. In eq 11 $k_H = k_f^{H}$, $k_T = k_f^{T}$,

$$k_H/k_T = \frac{(F^{14})(M_0^{T})}{(F^{T})(M_0^{14})} \quad (11)$$

and $(F^{14}/(F^{T})$ = the inverse of the ^{3}H/^{14}C ratio for fumaric acid at time t and $(M_0^{T})/(M_0^{14})$ = the ^{3}H/^{14}C ratio of *l*-malic acid at time zero. (As a maximum of 2% of the original ^{3}H-^{14}C-*l*-malate is converted to ^{3}H-^{14}C-fumarate, the ^{3}H/^{14}C ratio for the ^{3}H-^{14}C-malate substrate is, within experimental limits, constant for the reaction over these small conversions of substrate to product.)

(19) Reference 10, pp 156–158.
(20) Krebs[21] has reported that the equilibrium between fumarate and *l*-malate is 18.45% fumarate. Thus at a conversion of 2% of the initial *l*-malate to fumarate, $F/F_e \simeq 0.02/0.18 = 0.1$.
(21) H. Krebs, *Biochem. J.*, **54**, 78 (1953).

Relation between ^{3}H and ^{2}H Effects. As most work on kinetic isotope effects in solvolyses, for example, has been done with ^{2}H, a conversion of the results of the present work to those for analogously deuterated substrates will be helpful. A treatment of this relationship has been presented[22] for primary deuterium and tritium isotope effects which is also directly applicable to secondary isotope effects if the same assumptions are made in both cases: (i) all differences except those in zero point vibrational energy are insignificant, (ii) the hydrogen isotope is attached to a heavy polyatomic molecule so that isotopic substitution affects the vibrations only of the bond which holds the hydrogen isotope, (iii) the bond can be treated as a harmonic oscillator, and (iv) all molecules pass over, and not through, the reaction potential barrier (no tunneling). In this way, the force constants for bonds to hydrogen, deuterium, or tritium are all assumed to be the same and, whatever vibrations are involved, these force constants will cancel when the ratio of the two partition functions is taken; the ratio of isotope effects is then related only to the masses of the hydrogen isotopes (12 and 13). When

$$\frac{k_H}{k_T} = \frac{k_H}{k_D}\left[\frac{(1/\sqrt{m_1}) - (1/\sqrt{m_3})}{(1/\sqrt{m_1}) - (1/\sqrt{m_3})}\right] = \left(\frac{k_H}{k_D}\right)^{1.442} \quad (12)$$

$$\frac{k_H}{k_D} = \left(\frac{k_H}{k_T}\right)^{0.694} \quad (13)$$

applied to the results of Table I, this gives the results in Table II for calculated deuterium isotope effects.

Table II

Substrate	k_H/k_T (calcd) 20 sec	40 sec	60 sec	k_H/k_D (calcd)
2-^{3}H-1,4-^{14}C-*l*-Malic acid	1.09	1.08	1.10	1.16
2,3-^{3}H-1,4-^{14}C-*l*-Malic acid	1.08	1.08	1.09	1.16

α-Kinetic Isotope Effect. The magnitude of the α-secondary kinetic isotope effect ($k_H/k_T = 1.13 \pm 0.02$; $k_H/k_D = 1.09 \pm 0.02$) is somewhat smaller for one α-tritium or α-deuterium than normally found for similar substitutions in solvolytic reactions involving carbonium ion intermediates for which values around k_H/k_D = 1.12–1.20 are more common.[23] The somewhat lower value in the enzymatic case could be explained by some neighboring group participation (for example, k_H/k_D = 1.11 per deuterium at 30° for the acetolysis and solvolysis of 1,1-dideuterio-2-anisylethyl tosylate)[24] or, more plausibly, by the intervention of some nucleophile. Such nucleophilic intervention would restrict the degree of out-of-plane vibration of the α-hydrogen or tritium and in so doing would reduce the magnitude of the isotope effect. When the degree of such nucleophilic participation is that encountered in an S_N2 reaction, the value of k_H/k_D is sharply reduced; for example, the reaction of isopropyl bromide with sodium ethoxide has k_H/k_D = 1.00.[25] The nucleophile in question may be supplied by a group on the enzyme or, alternatively,

(22) C. G. Swain, E. L. Stivers, J. F. Reuwer, Jr., and L. J. Schaad, *J. Am. Chem. Soc.*, **80**, 5885 (1958).
(23) See Table VI in M. J. Nugent, R. E. Carter, and J. H. Richards, *ibid.*, in press.
(24) W. H. Saunders and R. Glaser, *ibid.*, 82, 3586 (1960).
(25) V. J. Shiner, *ibid.*, 74, 5285 (1952).

Table III

Compound	Reaction	k_H/k_D	Ref
trans-2-^{2}H-Cyclopentyl tosylate	Acetolysis at 50°	1.16	30
cis-2-^{2}H-Cyclopentyl tosylate	Acetolysis at 50°	1.22	30
2,2,5,5-Tetra-^{2}H-cyclopentyl tosylate	Acetolysis at 50°	2.06	30
$CH_3CH_2C(Cl)(CH_3)CD_3$	Solvolysis in 80% aqueous alcohol at 25°	1.34	31
$(CH_3)_2CHCH_2C(Cl)(CH_3)CD_3$		1.34	31
$(CH_3)_3CCH_2C(Cl)(CH_3)CD_3$		1.34	31
$CH_3CD_2CCl(CH_3)_2$		1.40	31
$CH_3CH_2CD_2CCl(CH_3)_2$		1.37	31
$(CH_3)_2CHCH_2CD_2CCl(CH_3)_2$		1.44	31
$(CH_3)_3CCH_2CD_2CCl(CH_3)_2$		1.08	31
$HO_2CCHOHCHDCO_2H$	Enzymolysis	1.09	Calcd from k_H/k_T in present work

intramolecular neighboring group participation by one of the carboxylate groups of the substrate is possible; for example, a situation analogous to that which occurs in the conversion of α-bromopropionate to lactate[26,27] (participation by the C-1 carboxylate anion) or the formation of β-butyrolactone from β-bromobutyric acid[28] (participation by the C-4 carboxylate anion).

β-Kinetic Isotope Effect. The magnitude of the β-secondary kinetic isotope effect can be estimated from the observed isotope effect for 2,3-^{3}H-1,4-^{14}C-*l*-malic acid if one assumes that there is an equal probability of a carbon–tritium bond at C-2 and at C-3. The validity of this assumption rests on the relative energies of sp^3 C–^{1}H and sp^3 C–^{3}H bonds being the same whether the carbon in question carries a hydroxyl group (as it does for C-2) or a hydrogen (as it does for C-3) in addition to the other substituents which are virtually the same for these two carbons.

Such a possibility seems reasonable as substituents have only small effects on the stretching frequencies of secondary sp^3 C–H bonds.[29] On this basis, the isotope effect due to tritium at C-3 is $0.5(k_H/k_T)_{2-^3H} + 0.5(k_H/k_T)_{3-^3H} = 1.12 \pm 0.02$ where $(k_H/k_T)_{3-^3H} = 1.12 \pm 0.03$. (The statistical probability that a single molecule of malic acid will contain two atoms of tritium, at both C-2 and C-3, is negligibly small.) If the assumption that equal amounts of tritium are present at C-2 and C-3 is invalid, the appropriate expression would be $X(k_H/k_T)_{2-^3H} + (1 - X)(k_H/k_T)_{3-^3H} = (k_H/k_T)_{2,3-^3H}$ where X is the mole fraction of tritium at C-2. In any event, since the α- and β-kinetic isotope effects seem to have such similar magnitudes, corrections due to unequal distribution of tritium between C-2 and C-3 will probably not change the result for C-3 appreciably in the present case. Thus we shall consider that $(k_H/k_T)_{3-^3H} = 1.12 \pm 0.03$ and $(k_H/k_D)_{3-^2H} = 1.09 \pm 0.02$.

The kinetic isotope effect of substitution β to a carbonium ion is generally thought to be largely due to overlap (hyperconjugation) of the vacant p orbital on the carbonium ion center with the sp^3 C–H bond in question.[30,31] As such, the magnitude of the effect is significantly dependent on the dihedral angle between empty p orbital and the C–H bond. Some representative values are given in Table III. The absence of an appreciable isotope effect in the solvolysis of 3,3-dideuterio-2-chloro-2,4,4-trimethylpentane has been attributed[31] to the operation of severe crowding in the highly branched molecule which prevents effective overlap between the vacant 2p orbital on C-2 and the C–H or C–D bonds of C-3, *i.e.*, the steric crowding prevents the methylene group from adopting a transition-state conformation favorable to hyperconjugative interaction between the carbonium ion center at C-2 and the C–H or C–D bonds at C-3.

A similar explanation may account for the relatively small β-isotope effect observed during the enzymatic dehydration of *l*-malic acid. If the conformation of the substrate molecule at the active site of the enzyme is the eclipsed one shown in eq 1 with the –OH group and the hydrogen which is eliminated from C-3 being *trans* to each other, the dihedral angle between the empty p orbital on C-2 and the (*S*)-hydrogen at C-3 will be about 60° making overlap relatively inefficient and thereby reducing the effect of isotopic substitution of the 3-(*S*)-hydrogen. If this is indeed the correct explanation for the magnitude of the β-secondary isotope effect, significant limitations on acceptable conformational arrangements on the substrate molecule at the active site of the enzyme during reaction are imposed.

Another possibility is that distribution of the positive charge away from C-2, as by neighboring group partic-

(26) W. A. Cowdrey, E. D. Hughes, and C. K. Ingold, *J. Chem. Soc.*, 2, 1208 (1937).
(27) E. Grunwald and S. Winstein, *J. Am. Chem. Soc.*, **70**, 841 (1948).
(28) H. Johansson and S. M. Hagman, *Ber.*, **55**, 647 (1922).
(29) L. J. Bellamy, "The Infra-red Spectra of Complex Molecules," 2nd ed, John Wiley & Sons, New York, N. Y., 1958.
(30) A. Streitwieser, Jr., R. H. Jagow, R. C. Fahey, and S. Suzuki, *J. Am. Chem. Soc.*, **80**, 2326 (1958).
(31) V. J. Shiner, Jr., *Tetrahedron*, **5**, 243 (1959).

ipation, would reduce the β-isotope effect because of a lessened demand on the hydrogens at C-3 for hyperconjugation, a phenomenon which has recently been documented by studies of isotope effects of *para*-substituted 1-phenylethyl halides.[32] Thus the presence of some degree of neighboring group participation may account, in part, for the observed value of $(k_H/k_T)_{3-{}^3H}$ also.

Equilibrium Isotope Effect. The equilibration of *l*-malic acid and fumaric acid by fumarate hydratase allows the first direct experimental determination of the relative ground-state energies of a molecule which has C–^{1}H *vs.* C–^{3}H sp^3 bonds (*l*-malic acid) and one that has C–^{1}H *vs.* C–^{3}H sp^2 bonds (fumaric acid). In the case of *l*-malic acid the ground-state energies will, as discussed above, be averaged over the sp^3 C–H bonds at C-2 and at C-3.

The anticipated isotope effect can be calculated on the assumption that the change between zero-point energy between hydrogen and deuterium is the dominant effect.[30,33] (The calculation is done for deuterium because the stretching and bending frequencies for carbon–tritium bonds in the appropriate molecules have not been experimentally observed.) Use is made of the modified Bigeleisen equation (14)[34] which includes the approximation that $\nu_D = \nu_H/1.35$.[35]

$$k_H/k_D = \pi \exp\left(1 - \frac{1}{1.35}\right)\frac{h}{kT}(\nu_H - \nu_D)/2 \quad (14)$$

The frequencies which have been assigned to the in-phase and out-of-phase C–H stretching vibrations of a secondary C–H bond (in $-CH_2-$) are 2926 ± 10 and 2853 ± 10 cm^{-1}, respectively.[36] The deformation or bending modes of these hydrogens give rise to absorption at 1465 ± 20 cm^{-1}. If an olefinic C–H of a *trans* –CH=CH– is used as a model for fumaric acid and the frequency changes 2890–3025 cm^{-1} (C–H stretching), 1340–965 (C–H out-of-plane bending), and 1340–1300 cm^{-1} (C–H in-plane bending) are considered, one calculates an isotope effect for $K_H/k_D = 1.18$ which is in remarkably good agreement with the value $K_H/K_D = 1.16 \pm 0.03$ derived from the experimentally observed $K_H/K_T = 1.24 \pm 0.03$.

Mechanism

The various kinetic isotope effects can now be discussed in terms of a mechanism for the conversion of *l*-malate to fumarate catalyzed by fumarate hydratase. One additional experimental fact should be included: in the conversion of *l*-malate to fumarate the oxygen of the C-2 hydroxyl group of *l*-malate exchanges with oxygen of water faster by a factor of 2.5 than the carbon of *l*-malate is converted to fumarate.[37] Thus in the mechanism of Scheme I $k_{-2} = 2.5k_3$. In such a case, if one assumes that $k_{-1} > k_2$, that $(k_1/k_{-1})^H = (k_1/k_{-1}{}^T$,[38] and that steady-state conditions apply, the following rate expression 15 can be derived.

$$\frac{d(F^H)/dt}{d(F^T)/dt} = \frac{(M_0{}^H)}{(M_0{}^T)}\frac{k_3{}^H}{k_3{}^T}\frac{k_2{}^H}{k_2{}^T}\frac{(k_3{}^T + k_{-2}{}^T)}{(k_3{}^H + k_{-2}{}^H)} \quad (15)$$

When deuterium is substituted for hydrogen at C-3 in that position from which elimination occurs in step 3, no change in rate is observed,[6] and k_H/k_D is probably less than 1.36.[11] On this basis k_2 was considered to be the rate-determining step and a preliminary report appeared that, in agreement with this proposal, no exchange of oxygen in *l*-malate occurred except by dehydration to fumarate and rehydration.[1]

However, the recently observed oxygen exchange[37] suggests that k_3 be explicit in any rate law for the dehydration reaction. How then is one to reconcile the rate law of eq 15 with the absence of a kinetic isotope effect larger than 1.36 in step 3 during which hydrogen is eliminated? We suggest that the intermediate carbonium ion is of such high energy that little activation is required for proton elimination. Accordingly, the transition state for this elimination has a structure very similar to that of the carbonium ion[40] and the bonding of the hydrogen to C-3 is little changed in going from the carbonium ion like intermediate to the transition state for elimination of the hydrogen at C-3. Accordingly, this step (k_3) will manifest little, if any, kinetic isotope effect.

There is some precedence for this suggestion. The decomposition of 2-pentyl chlorosulfite extensively deuterated in the 1 and 3 positions yields 2-chloropentane and 2-pentene. The rate of disappearance of deuterated chlorosulfite is slower than undeuterated substrate by a factor of 1.4 in dioxane and 3.3 in isooctane; however, the relative yields of 2-chloropentane and 2-pentene are unaffected by the presence of deuterium. Moreover, no isotope fractionation occurs in the elimination process since the starting alcohol has 86% of the 1 and 3 positions occupied by deuterium and 85% of the same positions of the olefin are deuterated. Thus, in this reaction, which is considered to involve a solvated carbonium ion intermediate, no deuterium isotope effect for the elimination step is observed, nor is there any partitioning of product in favor of elimination of hydrogen.[41]

Similar results have been observed in the solvolysis of 3-methyl-2-butyl *p*-toluenesulfonate in which only a small isotope effect on product composition was observed.[42] For elimination from *t*-butyl cation and *t*-butyl-d_9 cation a partitioning in favor of hydrogen was observed and k_H/k_D was estimated to be 2.[43] In this case, the tertiary carbonium ion intermediate will be more stable and, therefore, more selective than the secondary carbonium ions involved in the first two cases

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(33) L. Melander "Isotope Effect on Reaction Rates," The Ronald Press, New York, N. Y., 1960, p 89.

(34) J. Bigeleisen, *J. Chem. Phys.*, 17, 675 (1949).

(35) G. Herzberg, "Molecular Spectra and Molecular Structure," Vol. II, D. Van Nostrand, Co., Inc., Princeton, N. J., 1950, p 227.

(36) Reference 26, pp 13, 34.

(37) P. D. Boyer, reported at the Western Regional Meeting of the National Academy of Sciences, Pasadena, Calif., Nov 13, 1968.

(38) As the principal bonding of substrate (malate or fumarate) to the enzyme is probably due primarily to ionic, hydrogen bonding, or van der Waals' interactions, the only effect of replacing the hydrogens at C-2 or C-3 by tritium on the equilibrium constant for dissociation of the enzyme–substrate complex should be caused by a change in "effective size." The magnitude of the isotope due to this effect can be judged to be negligibly small by comparison to the value $k_H/k_D = 1.030 \pm 0.003$ observed[39] for the reaction of 2-methylpyridine *vs.* 2-(α,α,α-tri-^{2}H)-methylpyridine with methyl iodide.

(39) H. C. Brown and G. J. McDonald, *J. Am. Chem. Soc.*, 88, 2514 (1966).

(40) G. S. Hammond, *ibid.*, 77, 334 (1955).

(41) C. E. Boozer and E. S. Lewis, *ibid.*, 76, 794 (1954).

(42) S. Winstein and J. Takahashi, *Tetrahedron*, 2, 316 (1958).

(43) G. J. Frisone and E. R. Thornton, *J. Am. Chem. Soc.*, 90, 1211 (1968).

cited and postulated as in intermediate during the enzymatic dehydration of *l*-malic acid.

The possibility of a mechansim involving carbanion intermediates was previously eliminated because no exchange of the 3-(*R*)-hydrogen at C-3 occurs without conversion of *l*-malate to fumarate.[11] Indeed, the rate of exchange of this hydrogen with solvent is slower by a factor of 2.5 than the rate of conversion of *l*-malate to fumarate.[37] Thus the possibility is very real that the hydrogen that is removed resides at a locus in the active site of the enzyme from which exchange with solvent is possible only after the substrate molecule has dissociated from the active site. Accordingly, the absence of exchange of the 3-(*R*)-hydrogen at C-3 does not of itself eliminate mechanisms that involve removal of this hydrogen as the first step in the dehydration and that involve, thereby, anionic intermediates. Mechanisms of this type can, however, be eliminated by results of studies of mono-[2] and difluorofumarates[2,3] as substrates and the observation[3] that the rate for hydration of difluorofumarate is slower by a factor of about 5 than the rate for hydration of fluorofumarate.[3]

In summary, the mechanism for the dehydration of *l*-malic acid involves the heterolytic cleavage of the carbon oxygen bond at C-2 of *l*-malic acid with the possibility that some nucleophilic group either on the enzyme or from the substrate molecule itself assists the departure of the hydroxyl group and partially stabilizes the resultant carbonium ion like intermediate. The intermediate so formed is unstable and can either reacquire a hydroxylic oxygen and revert to *l*-malate or lose a hydrogen from C-3 and form fumarate; this elimination step occurs with little if any isotope effect because of the high reactivity of the intermediate with carbonium ion character.

Experimental Section

2-^{3}H-*l*-Malic Acid. Sodium borohydride-^{3}H (19 mg, 0.5 mmol, 100 mCi, specific activity 200 mCi/mmol, New England Nuclear Corp. Lot No. 380–146) was mixed with sodium borohydride (220 mg, 5.6 mmol) in water (10 ml) at pH 8–10. The resulting solution was added to a cold solution of oxaloacetic acid (2.112 g, 16 mmol) in water (10 ml) neutralized to the phenolphthalein end point with sodium hydroxide solution. The cold solution was allowed to warm to room temperature over a period of 1 hr and then stirred at room temperature for 1 day. After neutralization with 1 *N* hydrochloric acid, calcium chloride was added to precipitate the calcium salt. The salt was converted to the acid by passage through an IR-120-H ion-exchange column, and, after lyophilization, the resulting acid was mixed with cinchonine (4.7 g, 16 mmol) and diluted with cold salt prepared from *l*-malic acid (3.3 g, 24.5 mmol) and cinchonine (7.26 g, 24.5 mmol) in methanol. The resulting salt was recrystallized six times from methanol. Addition of hydrochloric acid freed the 2-^{3}H-*l*-malic acid which was precipitated as the calcium salt; the salt was converted to the acid by passage over an IR-120-H column. After lyophilization the product was chromatographed over an AG-1-X2 (formate form) column and combined with 1,4-^{14}C-*l*-malic acid to give 2-^{3}H-1,4-^{14}C-*l*-malic acid with a ratio of ^{3}H/^{14}C = 4.85 ± 0.01.

Enzymolyses of 2-^{3}H-2,3-^{14}C-*l*-Malic Acid. 2-^{3}H-1,4-^{14}C-*l*-Malic acid (48.7 mg) was dissolved in 119 cc of 10 m*M* sodium phosphate buffer at pH 7.3, and the solution was adjusted to pH 7.3 with sodium hydroxide–10 m*M* sodium phosphate solution. The resulting solution was rapidly stirred at 25° while 1 ml of a solution of fumarate hydratase (the solution contained about 0.1 mg of enzyme/cc) in 10 m*M* sodium phosphate buffer at pH 7.3 was added. The rate of the reaction of an aliquot of the reaction mixture was followed by observing the change in absorption at 230 mμ and showed that during 1 min, 2.3% of the *l*-malate was converted to fumarate. Other samples (60 cc, 40 cc, and 20 cc) were withdrawn after 20, 40, and 60 sec, respectively, and added to equal volumes of boiling ethanol containing 10 mg of unlabeled fumaric acid. After removal of the ethanol and lyophilization, the solid acids were chromatographed on an AG-1-X2 column. The resulting samples of *l*-malic were recrystallized from ethyl acetate–ligroin; samples of fumaric acid were recrystallized from toluene–dioxane. Both were dried under vacuum over phosphorus pentoxide. The samples were then counted.

Counting. All samples were counted in 15 cc of fresh Bray's solution using a Packard Tri-Carb scintillation counter, Model 3324, for which channel settings and gain had been optimized for determining ^{3}H and ^{14}C activity in the same sample. In all cases, both with standards and blanks, cold malic or fumaric acid was added so that in any group of samples where ratios were to be compared, all counting vials had identical compositions.

2,3-^{3}H-1,4-^{14}C-*l*-Malic Acid. 2,3-^{3}H-1,4-^{14}C-Fumaric acid was obtained by the enzymatic dehydration of 2-^{3}H-1,4-^{14}C-*l*-malic acid. After purification, as described above, this labeled fumaric acid was then enzymatically rehydrated to give 2,3-^{3}H-^{14}C-*l*-malic acid.

Enzymolysis of 2,3-^{3}H-1,4-^{14}C-*l*-Malic Acid. This was carried out exactly under the same conditions used for 2-^{3}H-1,4-^{14}C-*l*-malic acid reported above.

Equilibration of 2-^{3}H-1,4-^{14}C-*l*-Malic Acid and of 2,3-^{3}H-1,4-^{14}C-*l*-Malic Acid with Fumarate Hydratase. In these cases, 4 ml of the solution of fumarate hydratase in 10 m*M* sodium phosphate buffer at pH 7.3 referred to previously was added to 120 ml of solution containing 48.7 mg of the appropriately labeled *l*-malic acid in 10 m*M* sodium phosphate buffer at pH 7.3. The solutions were allowed to stand at 25° for 24 hr and then added to boiling ethanol and the acids separated and counted as before.

Enzymolysis of 2-^{3}H-3-^{14}C-*l*-Malic Acid. The substrate was synthesized and purified as described for 2-^{3}H-1,4-^{14}C-*l*-malic acid. An enzymolysis was carried out essentially as in previous cases except that the enzyme solution had an activity such that 0.46% of the substrate was converted to fumarate during a period of 1 min. The ^{3}H/^{14}C activities are given for the initial malic acid and for samples of malic acid and fumaric acid recovered after quenching the reaction after 60 and 90 sec. Initial malic acid ^{3}H/^{14}C, 11.97; malic acid at t = 60 sec, 11.73; malic acid at t = 90 sec, 11.82; fumaric acid at t = 60 sec, 10.59; fumaric acid at t = 90 sec, 10.72.

Equilibration of 2-^{3}H-3-^{14}C-*l*-Malic Acid. 2-^{3}H-3-^{14}C-*l*-Malic acid (8.9 mg) was dissolved in 16 ml of 0.01 *M* phosphate buffer and the pH adjusted to 7.3. A 1-ml aliquot was removed and added to 10 mg of cold *l*-malic acid. To this solution was added 4 ml of fumarate hydratase solution, the pH readjusted to 7.3; the resulting solution was allowed to stand for 12 hr at 25°. The reaction was quenched and the acids were separated and purified as before. The activities of the purified acids were: *l*-malic acid ^{3}H/^{14}C, 12.39; fumaric acid ^{3}H/^{14}C, 10.16.

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