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Relationships of pH to Exchange Rates and Deuterium Isotope Effects in the Fumarase Reaction[1]

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The rates of the $^{3}H_{malate} \rightleftarrows {}^{3}H_{HOH}$, $^{14}C_{fumarate} \rightleftarrows {}^{14}C_{malate}$ and $^{18}O_{malate} \rightleftarrows {}^{18}O_{HOH}$ exchanges were measured in the fumarase reaction at equilibrium and high substrate concentration over the pH range 5.0–9.6. Exchange rates were near maximal at pH 7.3, with a ratio of 0.38:1.0:1.8 for the ^{3}H, ^{14}C, and ^{18}O exchanges, respectively. All exchanges approached zero at the pH extremes. The ratio of exchange rates approached equality as pH was increased, indicative of change in relative rate-limiting steps. As pH was decreased, the ratio of the ^{18}O:^{14}C exchange reached a maximum of 2.3 at pH 6.7 and remained above 2 down to pH 5.0, indicative that C–O bond breaking remains the most rapid step even at low pH.

Replacement of H by D in the C–H bond broken when HOH is eliminated had no effect on the rate of fumarate formation at pH 7.3 and 9.6, but a small effect ($k_H/k_D = 1.3$) was revealed at pH 5. The deuterium substitution had only a very small effect on the rate of ^{18}O exchange at pH 7.3 ($k_H/k_D = 1.15$). The findings add to the probability of an intermediate with considerable carbonium ion character in fumarase catalysis.

Previous studies from this laboratory reported the relative equilibrium exchange rates catalyzed by fumarase (*1*). At neutral pH and high substrate concentration the ^{18}O-malate $\rightleftarrows$ HOH exchange was faster but the ^{3}H-malate $\rightleftarrows$ HOH exchange slower than the interconversion of the carbon skeleton as measured with ^{14}C. These results and the lack of a primary isotope effect on the exchange rates (*1*) as well as in the initial reaction (*1*, *2*) indicated that a slow dissociation of an enzyme-bound proton was a principal rate-limiting step in fumarate formation. Such dissociation must involve changes not showing a primary isotope effect, such as an enzyme conformation change.

The rapid ^{18}O exchange and other data, summarized recently by Hill and Teipel (*3*), are consistent with a carbonium ion intermediate, but alternative possibilities for the mechanism must still be considered. The slow dissociation of an enzyme-bound proton considered by itself, for example, still leaves open the question of whether the cleavage of the C–H bond occurs prior to, concomitant with, or after cleavage of the C–O bond of malate. It appeared that additional light could be shed on this and related questions by assessment of pH effects on the exchanges; with pH change the relative contributions

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of the various reaction steps to rate limitation may be modified as revealed by changes in exchange rates. Pertinent questions, for example, are whether at high pH, where carbonium ion formation by OH^- release might be considerably slowed, the ^2H or ^3H exchange exceeds the ^{18}O exchange, and whether changes in contribution of various steps to rate limitation result in changes in exchange rates as pH is decreased or increased.

In addition to measurements of effect of pH on exchange rates, the present report includes additional tests for possible primary isotope rate effects. Even though a slow proton release from enzyme occurs, rupture of the C–H bond prior to C–O cleavage in a slow step or concomitant with C–O cleavage would necessitate a primary isotope effect on the ^{18}O exchange using (3*R*)-^{2}H-2-^{18}O-malate. This paper gives results demonstrating only a quite small deuterium primary isotope effect on the ^{18}O-malate $\rightleftarrows$ HOH exchange rate at neutral pH, and a small deuterium isotope rate effect on fumarate formation at low pH. The various findings suggest changes in prominent rate-limiting steps at various pH values and add to the probability of formation of an intermediate with considerable carbonium ion structure in the catalysis.

EXPERIMENTAL PROCEDURE

Materials and procedure were similar to those utilized previously (*1*) with additions or modifications as presented herein. 2 (S)-malate (L-malate) was used in all experiments.

Preparation of (3R)-^{2}H-2-^{18}O-malate. A 1 *M* solution of Na fumarate in water containing 92.4 mole % deuterium and 4.5 at. % ^{18}O was equilibrated with fumarase. Enzyme activity was quenched by heating at 100°C for 5 min. The water was removed by lyophilization and 0.5 *M* HCl added to the residue. The fumarate was removed by ether extraction and barium malate isolation as previously described (*1*).

(3R)-^{2}H-malate. 99.8 mole % ^2H and 2-^{18}O-malate without excess deuterium were prepared in a similar manner.

Determination of equilibrium constants. Equilibrium constants at different pH values were calculated from the data of Bock and Alberty (*4*) or determined using both 2 m*M* fumarate or 2 m*M* malate as substrates. NaCl was added to maintain ionic strength at a value equal to the ionic strength of 0.1 *M* sodium malate as used in the equilibrium exchange experiments.

Equilibrium exchange rate assays. Exchange rates for ^{14}C, ^3H, and ^{18}O were determined in 0.1 *M* Tris–acetate buffer, pH 5.0–9.1 or glycine–NaOH pH 9.6, with 0.1 *M* sodium (3*R*)-^{3}H-malate, 0.019–0.023 *M* ^{14}C sodium fumarate (depending on pH), and $H_2{}^{18}O$ present. The temperature was 25°C. Fumarase concentration ranged from 19 μg/ml (pH 7.3) to 190 μg/ml (pH 5.0), and incubation times of 0.75–30 min at pH 7.3 and 4–148 min at pH 5.0. Rates were calculated from values from at least six determinations at different time intervals, using a simple computer program (*1*, *5*).

After removal of 0.2 ml zero time samples into 0.5 ml of 0.5 *M* HCl, fumarase was added in a small volume and 0.2-ml samples were withdrawn at various times into 0.5 ml of 0.5 *M* HCl. Samples taken at approximately 20 half times were used as "infinity" samples. Water was removed by distillation from the frozen state and a portion of the distillate counted for tritium. Separations and assays for ^{14}C and ^{18}O in the malate were made as described previously (*1*).

Rates of exchange closely followed first-order kinetics and indicated no deviations due to loss of enzyme activity during incubation time. A marked stabilizing effect of substrates on enzyme activity was noted. In absence of substrate fumarase (33 μg/ml)

in 0.1 *M* Tris–acetate buffer at pH 5.0 showed a first-order loss of activity with $t_{1/2}$ of 72 min. Presence of 0.1 *M* malate and 0.023 *M* fumarate prevented any loss of activity during incubations at this pH.

Malate analysis. Malate was determined by the formation of DPNH in the malate dehydrogenase reaction at pH 9.5 in the presence of hydrazine to pull the reaction towards the formation of oxalacetate (*6*).

Fumarase concentration. The concentration of fumarase was determined using an extinction coefficient of 0.53 ml mg^{-1} cm^{-1} at 280 nm (*7*). The enzyme had a specific activity of 2.8×10^5 when assayed according to Frieden *et al.* (*7*).

RESULTS

pH dependence of ^3H, ^{14}C, and ^{18}O exchanges. The observed pH dependence of ^{14}C, ^3H, and ^{18}O exchange rates is shown in Fig. 1. All measurements were with excess substrate present. Alberty *et al.* (*8*) showed that the K_m for malate increases as the pH

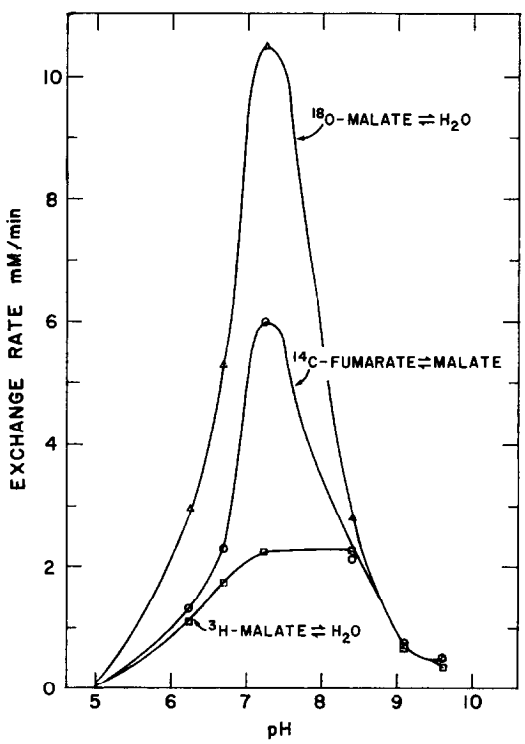


FIG. 1. pH dependence of the equilibrium ^{14}C, ^3H, and ^{18}O exchange rates in the fumarase reaction.

increases whereas the K_m for fumarate remains approximately the same. Calculations based on their equations for the pH dependence of the K_m of malate and fumarate indicate that under the least favorable conditions used in our studies (pH 9.6), the concentration of malate was still about an order of magnitude higher than the K_m.

One important point shown in Fig. 1 is that at low and high pH ranges all exchange rates tend to approach zero. However, the ratios of the exchange rates as pH is changed

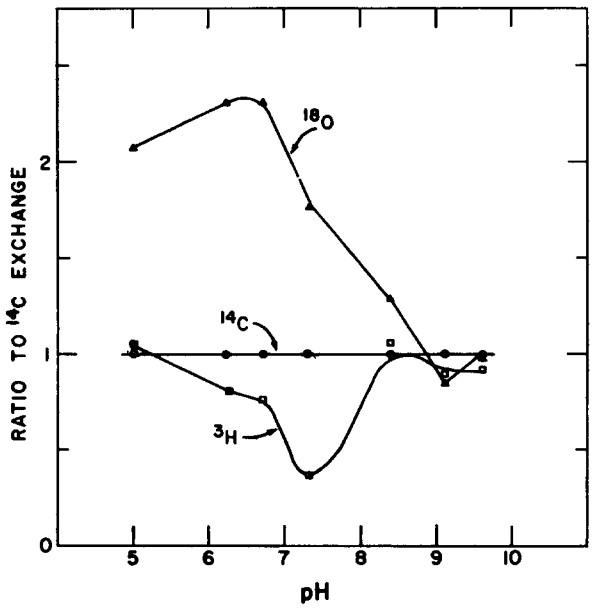


FIG. 2. pH dependence of equilibrium exchange rates relative to ^{14}C exchange. Data were taken from the experiments reported in Fig. 1.

do not remain constant. A plot of these ratios, relative to the ^{14}C exchange rate, is given in Fig. 2. At high pH, all the exchanges approach equality within experimental error as they decrease. At low pH, the ^{3}H exchange becomes about equal to the ^{14}C exchange but the ^{18}O exchange remains faster than ^{14}C exchange, maintaining a ratio only slightly less than the value at pH 7.3.

A primary deuterium isotope effect on initial rate. The effect of substitution of the 3*R* hydrogen of malate with deuterium on the initial rate of fumarate formation at four different pH values is shown in Table 1. A small but definitive isotope effect (k_H/k_D=

TABLE 1

DEMONSTRATION OF A PRIMARY DEUTERIUM ISOTOPE EFFECT ON INITIAL RATE AT LOW pH[a]

pH	k_H/k_D
4.6	1.31
5.0	1.31
7.3	1.00
9.6	0.97

[a] Reaction mixtures contained 0.1 *M* Tris–acetate or 0.1 *M* glycine–NaOH (pH 9.6), 0.1 *M* sodium 3*R*-(^{1}H)-malate or 3*R*-(^{2}H)-malate, 99.88 mole % ^{2}H, in a 1-ml cuvette. Fumarase was added and the change in absorbancy at 250 nm recorded.

1.31) was observed at pH 4.6 and 5.0 but none at pH 7.3 or 9.6 within experimental error. The absence of a primary isotope effect near neutral pH is in harmony with earlier results on initial rates (*1*, *2*) and on rate of ^3H and ^2H release from malate at equilibrium (*1*).

A small primary deuterium isotope effect on equilibrium ^{18}O-malate $\rightleftarrows$ H_2O exchange rate. Because the ^{18}O-malate $\rightleftarrows$ H_2O exchange is the fastest rate at equilibrium and reflects a partial reaction that occurs prior to the slow dissociation of the enzyme-bound proton (*1*), the observation of a definitive deuterium isotope effect on the ^{18}O exchange rate would indicate that the cleavage of the C–H (or C–D) bond and transfer of the hydrogen to an enzyme-bound form occurs concomitantly with or prior to C–O bond cleavage. On the other hand, lack of a significant isotope effect would imply that C–O cleavage occurred without appreciable C–H cleavage.

Data in Table 2 show the relative rates of ^{18}O-malate $\rightleftarrows$ H_2O exchange at equilibrium using (3*R*)-^2H-malate in one reaction mixture and nonisotopic malate in the other.

TABLE 2

PRIMARY DEUTERIUM ISOTOPE EFFECT ON ^{18}O-MALATE $\rightleftarrows$ H_2O EXCHANGE AT EQUILIBRIUM[a]

Exchange	Rate, m*M*/min	
	H	D
^{14}C-fumarate $\rightleftarrows$ malate	3.78 ± 0.06 (5)[b]	3.62 ± 0.12 (5)
^{18}O-malate $\rightleftarrows$ H_2O	6.91 ± 0.26 (5)	5.75 ± 0.29 (5)
Ratio $k^{18}O/k^{14}C$	1.83	1.59
Ratio H/D	1.15	

[a] Reaction mixtures contained 0.1 *M* Tris-acetate pH 7.3, 0.1 *M* sodium 3*R*-(^2H), 2-(^{18}O)-malate (92.4 mole % ^2H, 4.5 atom % ^{18}O) or 0.1 *M* sodium 3*R*-(^1H), 2(^{18}O)-malate (4.8 atom % ^{18}O) and 0.022 *M* sodium ^{14}C-fumarate in 2 ml at 25°C. After removal of zero time samples into 0.5 *M* HCl, 25 μg fumarase was added, 0.050-ml samples removed for ^{14}C-malate determination, and 0.2-ml samples removed for ^{18}O determination in malate. Samples were taken over the range 10–70% of isotopic equilibrium.

[b] Values ± the average deviation are given; the number in parentheses refers to the number of separate determinations.

^{18}O loss from malate was measured rather than ^{18}O incorporation into malate from $H_2{}^{18}O$. The former approach has the advantage that the reverse reaction is negligible since ^{18}O is diluted from 0.1 *M* in malate to 55.6 *M* water. The rate of ^{14}C exchange was also determined in each reaction mixture. This serves as a control to correct for any differences in exchange rate due to differences in the enzyme activity in the two reaction mixtures. The differences found in ^{14}C exchange were, however, very small and within experimental error. A significant difference of 15% was, however, found in the ^{18}O-malate $\rightleftarrows$ H_2O exchange rates between the deuterated and nondeuterated malates.

DISCUSSION

It is instructive to consider the possible effects of pH on the ^3H, ^{14}C, and ^{18}O exchange reactions based on a schematic representation of the fumarase active site as shown in

Fig. 3. Such a representation is based on various experimental data as summarized by Teipel and Hill (*3*). For discussion, the initial assumption is made that the only effects of pH are on the protonation of the catalytic groups, and not on enzyme structure. As depicted in Fig. 3, an aprotic base (B) on the enzyme facilitates the cleavage of the (3*R*)-C–H bond and a protonated base (B′H⊕) cleavage of the C–O bond. If a concerted elimination of the –H and –OH occurs, the participation of both catalytic groups will be required for $^{3}H_{malate} \rightleftarrows H_{HOH}$ or $^{18}O_{malate} \rightleftarrows O_{HOH}$ exchanges. As the pH is lowered, the catalytically active form of B′, B′H⊕, will increase to a maximum concentration, whereas the active form of B will decrease as B is protonated. Consequently, the cleavage of the C–H bond will become sufficiently slow until it becomes rate limiting for the entire reaction. Hydrogen and oxygen exchange will become equal to the carbon exchange as all exchange rates approach zero.

By analogous considerations, for a concerted reaction as pH is increased, C–O bond cleavage will become rate limiting, oxygen exchange will approach equality with carbon and hydrogen exchange rates, and all exchange rates will approach zero.

For a mechanism in which C–O bond cleavage may occur independently of C–H bond cleavage, tending toward a carbonium ion intermediate, oxygen exchange would increase to a constant level as the pH is lowered while hydrogen exchange and carbon

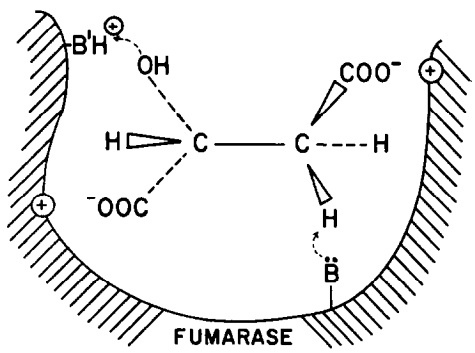


FIG. 3. A representation of the active site of fumarase with bound malate.

exchange would decrease in rate and become equal. As mentioned earlier, this pattern is based on the assumption that pH affects only the state of protonation of the groups B′ and B.

The data in Figs. 1 and 2 do not fit either of the simple possibilities presented above completely. The tendency toward a bell-shaped pH profile observed for the ^{3}H and ^{14}C exchange rates may be explained by a predominantly concerted cleavage of both C–O and C–H bonds of malate. However, the approach to equality of all exchanges at high pH is also consistent with a carbonium mechanism with the C–O cleavage becoming rate limiting because of a lack of –BH⊕ to protonate a departing OH^{-} group. In addition, an alternative explanation to the concerted scheme is clearly necessary to account for the observations that the ratio of ^{18}O to ^{14}C exchanges, which reaches a maximum value at neutrality, does not change appreciably as the pH is lowered even though the rates of exchange decrease sharply and the ^{14}C exchange becomes equal to the ^{3}H exchange. These results are consistent with a mechanism in which C–O bond breaking still occurs faster than C–H bond breaking with continued participation of a carbonium ion intermediate at the low pH. The sharp decrease in exchange rates at low pH may be

due to a decrease in the concentration of active enzyme as a result of secondary structural changes. At about pH 9 all equilibrium exchanges become equal within experimental error. The unprotonated base B′, however, is at optimal concentration for removal of the 3*R* proton. The fact that hydrogen exchange is not faster than ^{18}O exchange under these conditions suggests that hydroxyl cleavage must precede or occur at the same time as C–H bond cleavage.

The experiments on the primary isotope effect on initial rate and ^{18}O exchange at equilibrium favor a mechanism with participation of a carbonium ion intermediate as mentioned earlier. The slow hydrogen exchange at equilibrium and lack of a primary deuterium isotope effect at pH 7.3 indicate that slow release of an enzyme-bound proton, derived from the 3*R* position of malate, can be a slow step in the overall reaction. A change in the relative contribution of rate-limiting steps at pH 4.6–5.0 is indicated by the appearance of a small deuterium isotope effect on initial rate at this pH. This suggests that the C–H bond breaking step is now contributing measurably to the rate of the reaction. An isotope effect of 2–7 is normally expected for reactions in which C–H bond breaking is rate limiting in an α,β elimination (*9*). The fact that a value of only 1.3 was found may indicate that the rate of other steps such as proton and substrate dissociation are still the principal steps limiting the velocity. It is important to note again that ^{18}O exchange still proceeds at a faster rate than ^{3}H exchange under conditions where C–H bond breaking may be partially rate limiting. This distinctly favors a carbonium ion intermediate. At high pH the lack of a deuterium isotope effect is consistent with C–O bond breaking as the rate-limiting step.

A primary deuterium isotope effect might be observable if C–H bond breaking were concerted with C–O bond breaking at alkaline pH, where the latter step may determine the rate. The fact that no such effect is observed gives further argument against a concerted reaction.

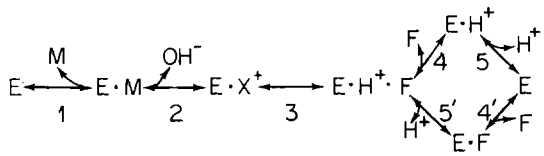
The finding of only a very small primary deuterium isotope effect on equilibrium ^{18}O exchange ($k_H/k_D = 1.15$) at neutral pH also favors a carbonium ion intermediate in the reaction. This experiment permits a firmer interpretation than isotope effects on the overall reaction. Even though the dissociation of the enzyme–proton complex is slow at pH 7.3, it is possible that the C–H bond is cleaved and reformed several times, concomitant with C–O bond breaking, before dissociation of fumarate. In this case a pronounced deuterium isotope effect could be observed if C–H bond cleavage were a necessary concomitant step with C–O bond cleavage. A limitation to this interpretation needs mention. The observed deuterium isotope effect could be small if the rate of equilibration of an enzyme-bound hydroxyl with bulk water were slow relative to cleavage of the C–O bond.

The value of 1.15 observed for k_H/k_D near pH 7.3 is of the order of magnitude of a β deuterium secondary isotope effect (*9*), and this, rather than any contribution of C–H bond breaking to the C–O cleavage, could explain the small rate effect. For a slow removal of hydrogen relative to $-OH^-$, hyperconjugation effects might stabilize the carbonium ion formed from nondeuterated malate relative to deuterated malate by this small amount. The magnitude of the deuterium effect is close to that observed by Schmidt et al. (*10*) for a secondary isotope effect of deuterium on fumarate formation.

Although participation of a carbonium ion in the fumarase reaction has been suggested by a number of studies (*1, 2, 8, 10, 11*), alternate explanations have not been eliminated. For example, Rose (*12*) has suggested that the secondary isotope effect noted (*10*) might result from a quasi-equilibrium among enzyme forms prior to the slow dissociation of the enzyme–proton complex, rather than carbonium ion formation.

Results of the present studies appear to add to the probability of formation of a carbonium ion intermediate.

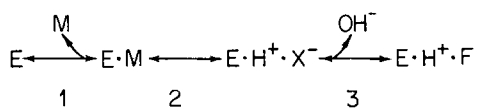
Our data give evidence that formation of a carbonium ion by C–H (or C–D) bond breaking does not occur concomitant with C–O bond breaking. Cleavage of the C–O prior to the C–H bond would give an enzyme-bound carbonium ion as an additional step in the scheme presented previously for the fumarase reaction (*1*). The reaction sequence may thus be depicted as follows:



(1)

When starting with malate at zero fumarate concentration, rapid reversal of steps 1 and 2 would give the observed oxygen exchange into unreacted malate and would be compatible with data of the present paper. Steps up through 3 would be in quasi-equilibrium to account for the lack of a primary isotope effect. Dissociation of the proton or of fumarate would be principal rate-limiting steps. Reaction by the lower pathway, where step 5′ precedes step 4′, would necessitate fumarate dissociation by step 4′ to be considerably more rapid than reversal of step 5′ to account for lack of incorporation of solvent protons into unreacted malate (*12*). Dissociation of fumarate via step 4′ could be more rapid than by step 4. In reaction of $E \cdot H^+ \cdot F$ by the upper pathway, step 4 would be irreversible at low fumarate concentration, and no proton incorporation into unreacted malate would occur by this pathway irrespective of the rate of proton association and dissociation in step 5. At high fumarate concentration, reversal of step 4 could lead to carbon exchange without proton exchange of malate, as observed (*1*).

Although a carbonium ion intermediate offers a rational explanation for present data, the probable participation of a carbanion structure in related nonenzymic eliminations (*13*, *14*) and in the elimination of H_2O catalyzed by enolase (*15*) suggests that, unless conclusively ruled out, possibilities of a carbanion participation in the fumarase reaction should remain in consideration. The demonstration from equilibrium exchange data (*1*) that the proton released from malate can be retained on the enzyme even with interchange of carbon skeleton allows one mode of carbanion participation. The proton retention means that a relatively rapid and quasi-equilibrium C–H bond breaking step could actually precede OH^- loss. The first three steps of sequence (1) given above might thus be as follows:



(2)

As before, the $E \cdot H^+ \cdot F$ form would be in quasi-equilibrium with free malate to account for ^{18}O exchange data. Interchange through steps 1 and 2 would need to be even more rapid to account for lack of a primary deuterium isotope effect. The secondary isotope data of Schmidt et al. could reflect decreased quasi-equilibrium accumulation of the tritiated form in $E \cdot H^+ \cdot X^-$. This might depend in part on the geometry of the carbanion form, X^-.

There are limitations, however, in accepting sequence (2). The $E \cdot H^+ \cdot X^-$ form would need to show no interchange of the bound H^+ with solvent to account for the lack of proton exchange into unreacted malate (*12*). Another point is that an inverse equilibrium isotope effect might be expected from preferential accumulation of the deuterium released by C–D cleavage to become bound to N (imidazole?) or O (carboxyl?) at the active site. Such an inverse effect was noted, for example, by Cram *et al.* (*16*) in exchange of a C–H proton involving carbanion formation. The lack of a primary deuterium isotope effect in the fumarase reaction could reflect a balance between slowing due to C–D vs. C–H bond breaking and acceleration due to the quasi-equilibrium mentioned above. Such equality and cancellation of effects are unlikely.

As a final comment, the pH data given in this paper are somewhat difficult to reconcile with a carbanion mechanism. At low pH the C–H bond breakage should become more rate limiting and oxygen exchange thus would not exceed carbon exchange. At high pH, hydrogen exchange more rapid than oxygen exchange would seem likely. Neither of these effects was observed.

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