[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

Studies of the Enzyme Fumarase. VI.[1] Study of the Incorporation of Deuterium into L-Malate during the Reaction in Deuterium Oxide[2,3]

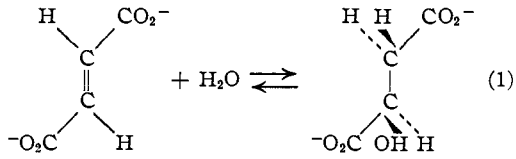
BY ROBERT A. ALBERTY, WILMER G. MILLER AND HARVEY F. FISHER

RECEIVED JANUARY 28, 1957

The incorporation of deuterium into L-malate during the dehydration to fumarate by fumarase in deuterium oxide has been determined. After various periods of time the reaction was stopped and the L-malate analyzed for non-exchangeable deuterium. Deuterium is incorporated into L-malate more slowly than fumarate is produced, and the rate is in quantitative agreement with that expected for the reversal of the over-all reaction. In order to explain the absence of exchange and of a deuterium rate effect it is necessary to introduce a further intermediate into the mechanism of the fumarase reaction.

Introduction

The addition of the proton to form the methylene group in L-malate in the fumarase reaction



(1)

has been shown to be stereospecific by carrying out the reaction in deuterium oxide.[4] The L-malate which is formed by the action of pig heart fumarase contains one atom of deuterium in the methylene group. Since the hydroxyl deuterium exchanges rapidly with an aqueous medium, and is always exchanged out before analysis for deuterium, the product will be referred to as monodeutero-L-malate. By use of nuclear magnetic resonance experiments at low temperatures it has been possible to show that the two protons in the monodeutero-L-malate are in the gauche position in solid L-malic acid.[5] When the monodeutero-L-malate was dehydrated enzymatically in ordinary water it was found that both the maximum initial velocity and the Michaelis constant are the same as for ordinary L-malate. If the breaking of the methylene carbon–hydrogen bond were rate determining, substitution of deuterium for the hydrogen would be expected to produce a decrease in rate of approximately two to sevenfold.[6,7] The absence of deuterium rate effect in the dehydration of monodeutero-L-malate suggests that there might be an exchange of this specific hydrogen atom with hydrogen atoms of the medium proceeding at a rate considerably faster than that of the dehydration reaction. Usually an exchange reaction is studied in the absence of an over-all reaction so that there is only a "virtual" reaction.[8] If in the present case we started with an equilibrium mixture of fumarate and L-malate in D_2O it would be difficult to detect a slow direct exchange of a methylene hydrogen of L-malate with the medium because the amount of exchange would be obtained from the experimental data by subtraction of the incorporation of deuterium due to reaction 1. Greater sensitivity in the detection of direct exchange is obtained by starting with L-malate in D_2O. A correction is made for the incorporation of deuterium into L-malate in the hydration of the fumarate produced by the use of equations recently derived for first-order reactions.[9]

Theory

A considerable simplification in the calculation of the deuterium incorporation into L-malate by the reverse reaction is made possible by carrying out the reaction under conditions where the approach to equilibrium is first order.

$$M \underset{k_2}{\overset{k_1}{\rightleftharpoons}} F \ (+H_2O) \qquad (2)$$

If only L-malate is initially present

$$\ln \frac{(M)_0 - (M)_{eq}}{(M) - (M)_{eq}} = \ln \frac{(F)_{eq}}{(F)_{eq} - (F)} = (k_1 + k_2)t = 0.693t/t_{1/2} \qquad (3)$$

where $(M)_0$ is the initial concentration of L-malate, $(M)_{eq}$ is the equilibrium concentration and (M) is the concentration at time t. The concentrations used throughout are total molar concentrations without regard to the extent to which the weak acids exist in various ionized forms. The second form of the logarithmic term comes from the fact that $(M)_0 = (M) + (F) = (M)_{eq} + (F)_{eq}$. The first-order rate constants k_1 and k_2, which for the enzymatic reaction are directly proportional to the enzyme concentration, may be calculated from a plot of $\log [(F)_{eq} - (F)]$ *versus* t, and the apparent equilibrium constant for reaction 2.

$$K_{app} = \frac{(F)_{eq}}{(M)_{eq}} = \frac{(F)_{eq}}{(M)_0 - (F)_{eq}} = \frac{k_1}{k_2} \qquad (4)$$

It is shown[10] readily that for mechanisms of the type

$$M + E \rightleftharpoons X_1 \rightleftharpoons X_2 \rightleftharpoons \cdots \rightleftharpoons F + E \qquad (5)$$

where E represents the enzymatic site and X_1, X_2, . . . are intermediates, the over-all reaction will be pseudo first order under conditions where the Michaelis constants for the forward and reverse re-

(1) The preceding article in this series is R. A. Alberty and W. H. Peirce, THIS JOURNAL, **79**, 1526 (1957).

(2) Presented at the 129th meeting of the American Chemical Society in Dallas, April 10, 1956.

(3) This research was supported by grants by the National Science Foundation and by the Research Committee of the Graduate School of the University of Wisconsin from funds supplied by the Rockefeller Foundation and by the Wisconsin Alumni Research Foundation.

(4) H. F. Fisher, C. Frieden, J. S. McKinley McKee and R. A. Alberty, THIS JOURNAL, **77**, 4436 (1955).

(5) T. C. Farrar, H. S Gutowsky, *et al.*, *ibid.*, **79**, 3978 (1957).

(6) F. H. Westheimer and N. Nicolaides *ibid.*, **71**, 25 (1949).

(7) K. B. Wiberg, *Chem. Revs.*, **55**, 713 (1955).

(8) H. A. C. McKay, *Nature*, **142**, 997 (1938).

(9) R. A. Alberty and W. G. Miller, *J. Chem. Phys.*, **26**, 1231 (1957).

(10) R. A. Alberty and B. E. Meyers, THIS JOURNAL, **79**, in press (1957).

actions are equal.[11] This statement applies under conditions where $(E)_0 << (M)_0$ and the time required to reach the steady state is negligible. Fortunately, conditions under which the Michaelis constants for the two substrates are equal can be achieved experimentally.[12,13] Carrying out the reaction under first-order conditions does not lead, of course, to any actual simplification of the mechanism but just to a simpler calculation of the fraction of L-malate present at any time that has been formed by the reverse reaction. The integrated rate equation[14] for mechanism 5 shows that the pseudo first-order rate constants k_1 and k_2 in equation 3 are proportional to enzyme concentration and are functions of the initial concentration of L-malate.

When the over-all reaction is carried out under pseudo first-order conditions in D_2O, the incorporation of deuterium through the reversal of the forward reaction may be represented by

$$\mathrm{M} \underset{k_2(\mathrm{T})}{\overset{k_1}{\rightleftharpoons}} \mathrm{F} \underset{k_1}{\overset{k_2(\mathrm{T}^*)}{\rightleftharpoons}} \mathrm{M}^* \qquad (6)$$

where M* is L-malate with deuterium in the specific methylene position, and (T) and (T*) are the mole fractions of the two isotopically different forms of the solvent. Thus (T) + (T*) = 1. The use of k_1 for the specific rate constant for the dehydration of both M and M* is justified by the absence of any isotope rate effect.[4] Since (T*) >> (T) in the experiments to be considered, the fact that F may react with H_2O and D_2O at somewhat different rates in the same solution would not introduce an appreciable error in these calculations.

The integrated rate equations for reaction 6 for the boundary condition (M) = $(M)_0$, (F) = 0, and (M*) = 0[15] at $t = 0$ are[9]

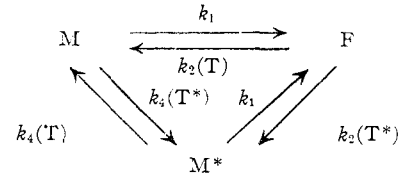
$$\frac{(\mathrm{M})}{(\mathrm{M})_0} = \frac{(\mathrm{T})}{(k_1 + k_2)} [k_2 + k_1 e^{-(k_1+k_2)t}] + (\mathrm{T}^*)e^{-k_1 t} \qquad (7)$$

$$\frac{(\mathrm{F})}{(\mathrm{M})_0} = \frac{k_1}{(k_1 + k_2)} [1 - e^{-(k_1+k_2)t}] \qquad (8)$$

$$\frac{(\mathrm{M}^*)}{(\mathrm{M})_0} = \frac{(\mathrm{T}^*)}{(k_1 + k_2)} [k_2 + k_1 e^{-(k_1+k_2)t}] - (\mathrm{T}^*)e^{-k_1 t} \qquad (9)$$

Once the values of k_1 and k_2 have been obtained from studies of the over-all reaction, the concentration of deutero-L-malate, M*, expected from mechanism 6 may be calculated for any time. There is an induction period in the formation of M* which arises initially as a function of t^2.

If deuterium is incorporated into the specific methylene position of L-malate by an additional process, for example by a rapid exchange of an intermediate with the medium, the first-order rate constant of this additional process is represented by the value of k_4 in



(10)

The integrated rate equations for this mechanism are[9]

$$\frac{(\mathrm{M})}{(\mathrm{M})_0} = \frac{(\mathrm{T})}{(k_1 + k_2)} [k_2 + k_1 e^{-(k_1+k_2)t}] + (\mathrm{T}^*)e^{-(k_1+k_4)t} \qquad (11)$$

$$\frac{(\mathrm{F})}{(\mathrm{M})_0} = \frac{k_1}{(k_1 + k_2)} [1 - e^{-(k_1+k_2)t}] \qquad (12)$$

$$\frac{(\mathrm{M}^*)}{(\mathrm{M})_0} = \frac{(\mathrm{T}^*)}{(k_1 + k_2)} [k_2 + k_1 e^{-(k_1+k_2)t}] - (\mathrm{T}^*)e^{-(k_1+k_4)t} \qquad (13)$$

It will be noted that the equation for $(F)/(M)_0$ is the same as for mechanism 6. According to mechanism 10, M* appears initially as a linear function of time.

Procedure

The reactions were carried out in deuterium oxide[16] solutions containing potassium succinate which acted as a buffer and provided a convenient dilution of the D_2O obtained by burning the dry sample. The potassium succinate, prepared from Merck succinic acid (m.p. 185–187°), contained less than 0.01 mole % fumarate as determined spectrophotometrically by treatment with fumarase. Succinate acts as a competitive inhibitor of fumarase and so it does combine with the enzymatic site, but investigation showed that fumarase does not catalyze the exchange of the hydrogen atoms of succinate with the medium under the conditions of the present experiments. The L-malic acid was obtained from the Pfanstiehl Chemical Company and was recrystallized from ethyl acetate.[17] Absorbancy measurements at 250 mμ showed that the L-malate preparation contained less than 0.03 mole % fumarate. If malic acid absorbs at all at this wave length, the percentage of fumarate impurity is less than 0.03%.

In view of the importance of the observation[4] that deutero-L-malate is dehydrated enzymatically at the same rate as ordinary L-malate when a rather large effect might be expected,[6,7] this rate experiment was repeated. Monodeutero-L-malate prepared enzymatically was purified by partition chromatography,[18] using a 35% butanol–65% chloroform mixture as the flowing phase and 0.5 *N* sulfuric acid supported on Hyflo Super-Cel as the stationary phase. The Michaelis constants in ordinary water obtained using 0.05 *M* phosphate buffer of *p*H 7.3 were 2.2 m*M* for ordinary L-malate and 2.7 m*M* for the deutero-L-malate. The ratio of the maximum initial velocities in H_2O for the normal to the deutero compound was 0.94, which is equal to unity within the experimental error in conformance with the earlier result.[4]

The solutions for the exchange experiments were prepared by dissolving L-malic acid and potassium succinate in D_2O, titrating to the desired *p*H with potassium hydroxide dissolved in D_2O, and finally diluting to the desired concentration. The *p*H values of the D_2O solutions determined with a Beckman Model G *p*H meter cannot be given to simple interpretation, but it was noted that they are very nearly equal to the *p*H values obtained after reconstituting the dried reaction mixtures in ordinary water at 25°.

(11) W. E. Lands and C. Niemann, THIS JOURNAL, **77**, 6508 (1955), have shown that an enzymatic reaction which goes to completion is pseudo first order if the competitive inhibition constant for the product is equal to the Michaelis constant for the substrate.

(12) R. A. Alberty, V. Massey, C. Frieden and A. R. Fuhlbrigge, *ibid.*, **76**, 2485 (1954).

(13) C. Frieden and R. A. Alberty, *J. Biol. Chem.*, **212**, 859 (1955).

(14) R. A. Alberty, *Advances in Enzymology*, **17**, 1 (1956).

(15) By dealing with atom per cent. excess it is possible to avoid the necessity of a small correction for the normal isotopic abundance of deuterium in the methylene position of malate.

(16) The D_2O(99.5%) was supplied by the Stuart Oxygen Company, Berkeley, Calif., and obtained on allocation from the Isotopes Division, U. S. Atomic Energy Commission.

(17) C. Frieden, R. G. Wolfe and R. A. Alberty, THIS JOURNAL, **79**, 1523 (1957).

(18) W. W. Cleland and M. L. Johnson, *J. Biol. Chem.*, **220**, 595 (1956).

Fumarase crystallized from pig heart muscle extract by the method of Frieden, Bock and Alberty[19] was used in these experiments. The reaction was started by the addition of enzyme which had been diluted to the desired concentration in 99.5% D_2O. The reaction mixture was placed in cuvettes in a Beckman DU spectrophotometer, and the absorbancy was recorded at one minute intervals at 300 mμ. The spectrophotometer was equipped with thermospacers held at 25 ± 0.3° by water circulated from a thermostat.

In order to stop the reaction at appropriate times 3-ml. aliquots of the reaction mixture were pipetted into 3 ml. of boiling ethyl alcohol. The samples were evaporated to dryness on a steam-bath and the ionizable deuterium was exchanged out by redissolving the samples three times in 3 ml. of water and each time evaporating to dryness. Finally the samples were dried at 35° in a vacuum oven. Blanks were run to determine the rate of non-enzymatic incorporation of deuterium into L-malate and succinate during the reaction period and during the evaporation on the steam-bath. These blanks were found to contain the normal isotopic abundance of deuterium.

Before combustion the samples containing appreciable deuterium were diluted with malic acid so that they contained less than 0.8 atom % deuterium. The samples were burned and the water converted to hydrogen by use of a zinc furnace at 410–414° as described by Graff and Rittenberg.[20] The H_2–HD samples were analyzed with a Consolidated mass spectrometer.[21] The normal abundance of deuterium in the L-malate used was found to be 0.012 atom per cent.

The fumarase concentration was determined at various times during the reaction by diluting 0.1 ml. of the reaction mixture to 4.1 ml. in ordinary water containing 0.05 *M* L-malate and 0.05 *M* phosphate buffer of *p*H 7.3. The turnover number for the enzyme under these conditions[17] is 10^5 min.$^{-1}$. The initial steady-state rate was determined using a DUR spectrophotometer.[12]

Experimental Data

A plot of the apparent first-order rate constant according to equation 3 is given in Fig. 1 for experiment B over a period of eight half-lives. The fact that the plot has a slope of 5×10^{-4} min.$^{-2}$ *M* rather than a zero slope, as would be expected for a first-order reaction, indicates that under these conditions the Michaelis constants for the forward and reverse reactions are not quite equal. The deviation from first-order behavior is small enough to be negligible. The rate constants calculated from equations 3 and 4 are $k_1 = 0.107$ min.$^{-1}$ *M* and $k_2 = 0.024$ min.$^{-1}$ *M* for this enzyme concentration which is 3.3×10^{-7} *M*. Therefore, the first-order constants for one molar enzyme are

$$k_1' = (0.024 \text{ min.}^{-1} M)/(3.3 \times 10^{-7} M)(60) = 1.2 \times 10^3 \text{ sec.}^{-1}$$

$$k_2' = (0.107 \text{ min.}^{-1} M)/(3.3 \times 10^{-7} M)(60) = 5.3 \times 10^3 \text{ sec.}^{-1}$$

Under conditions where the Michaelis constants for the forward and reverse reactions are equal, the integral equation[14] for mechanism 5 reduces to

$$\ln \frac{(F)_{eq}}{(F)_{eq} - (F)} = \frac{V_M + V_F}{K_M + (M)_0} t \qquad (14)$$

where V_M and V_F are maximum initial velocities for L-malate and fumarate as substrate and K_M is the Michaelis constant for L-malate. Since in the present experiments $(M)_0 >> K_M$, k_1' above would be interpreted as $V_M/(M)_0$ and k_2' as $V_F/(M)_0$. The experimental values of k_1' and k_2' are slightly smaller than would be expected from the values of V_F and V_M for phosphate and tris-(hydroxymethyl)-aminomethane buffers.

(19) C. Frieden, R. M. Bock and R. A. Alberty, THIS JOURNAL, **76**, 2482 (1954).

(20) J. Graff and D. Rittenberg, *Anal. Chem.*, **24**, 878 (1952).

(21) The deuterium analyses were made in the Biochemistry Department by courtesy of Professor Robert H. Burris and in the Department of Chemistry by courtesy of Professor Irving Shain.

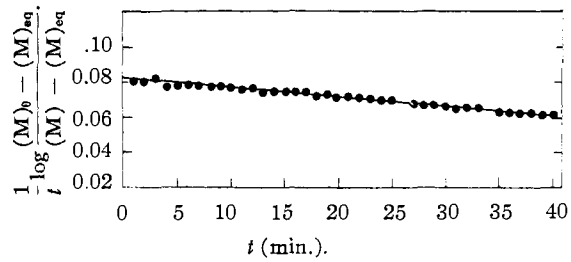


Fig. 1.—Plot of kinetic data for the fumarase reaction in D_2O at 25°. The reaction mixture contained 0.952 *M* potassium L-malate, 0.952 *M* potassium succinate and had a *p*H of 5.8 when reconstituted in H_2O.

The fractions of total substrate deuterated at various times are given for two experiments in Table I. For experiment A the *p*H of the reaction mixture reconstituted in ordinary water was 6.6 at 25° and for experiment B was 5.8. In experiment A the initial concentrations of potassium L-malate and potassium succinate were each 0.1 *M*. The enzyme concentration was such that the half time ($t_{1/2}$) for the over-all reaction was 21.8 min., and samples were taken for analysis after 3.7 and 40 min. In experiment B the initial concentrations of potassium L-malate and potassium succinate were each 0.0952 *M*. The half-time for the over-all reaction was 5.25 min., and the reaction was stopped in samples for analysis 2, 10, 25, 75, 226 and 291 minutes after addition of fumarase. The calculated values of the fractions of the substrate which are expected to be deutero-L-malate were obtained using mechanism 6 and equation 9, letting $k_1 = 1$, $k_2 = 4.55$, (T) = 0.03, and (T*) = 0.97. The time variable in equation 9 is a generalized time τ and is determined by correlation with the half-time of the fumarate to L-malate reaction. The error in measuring the equilibrium constant causes most of the uncertainty in the calculated values, since the values for k_1, k_2 and the relationship between the generalized time τ and the experimental half-times are dependent upon the equilibrium constant. The experimental data

TABLE I

INCORPORATION OF DEUTERIUM INTO L-MALATE DURING THE FUMARASE REACTION

$t/t_{1/2}$	τ	Molecules of deutero-malate / Total molecules of substrate: Exptl.	Eq. 9
		Expt. A	
0.18	0.022	0.002 ± 0.001	0.003 ± 0.002
1.5	0.186	0.046 ± 0.005	0.055 ± 0.007
		Expt. B	
0.38	0.047	0.0067 ± 0.0005	0.004 ± 0.002
1.91	0.236	.071 ± .004	.075 ± .005
4.76	0.590	.26 ± .01	.263 ± .008
14.3	1.77	.59 ± .01	.631 ± .007
43.0	5.34	.77 ± .02	.791 ± .005
55.5	6.88	.75 ± .02	.794 ± .005

plotted in Fig. 2 show that k_4 in mechanism 10 is too small to be detected.[22]

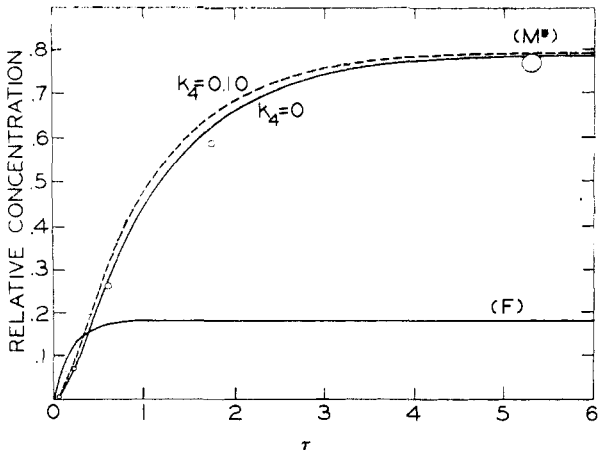


Fig. 2.—Relative concentrations of fumarate and monodeutero-L-malate as a function of generalized time. The lines are calculated using equations 8 and 9 letting $k_1 = 1$, $k_2 = 4.55$, (T) = 0.03 and (T*) = 0.97. The dashed line shows the relative concentration of monodeutero-L-malate expected if k_4 in mechanism 10 is 0.10 (calculated with equation 13).

The concentration of active enzyme is given in Table II as a function of time for experiment B.

TABLE II

CONCENTRATION OF ACTIVE ENZYME AS A FUNCTION OF TIME

Time (min.)	Molar concn. of active enzyme × 10^7	Time (min.)	Molar concn. of active enzyme × 10^7
18	3.7	131	3.3
48	2.9	279	3.3
81	3.3		

Av. = 3.3×10^{-7} M

The concentration of active enzyme as determined by the standard assay remained constant within the experimental accuracy during the entire reaction period.

The apparent equilibrium constant for the fumarase reaction defined by equation 4 has been determined earlier[23] as a function of ionic strength, pH and temperature. Since the values in heavy water are required in this research they have been determined at 99.5% D_2O. At 25°, pH values from 5.8 to 7.5 and ionic strengths up to 0.4, the equilibrium constant is approximately 5% lower than the corresponding values for ordinary water. In this connection it is interesting to note that the equilibrium constant for the hydrolysis of ethyl acetate is the same in D_2O and H_2O.[24]

Table III gives the molar absorbancy indices of dipotassium fumarate in D_2O at 25° and also the ratios of the absorbancy indices determined for D_2O and H_2O solutions.

(22) This conclusion is in contradiction with that reached in preliminary experiments[4] in which only very small extents of reaction were used.

(23) R. M. Bock and R. A. Alberty, THIS JOURNAL, **75**, 1921 (1953).

(24) Nachod, *Z. Elektrochem.*, **44**, 72 (1938); Kailan and Ebeneder, *Z. physik. Chem.*, **A180**, 157 (1937).

TABLE III

ABSORBANCY INDICES OF DIPOTASSIUM FUMARATE IN D_2O AT 25°

Wave length (mμ)	a, M^{-1} cm.$^{-1}$	a_{D_2O}/a_{H_2O}	Wave length (mμ)	a, M^{-1} cm.$^{-1}$	a_{D_2O}/a_{H_2O}
300	34	0.90	250	1325	0.95
290	101	.93	240	2210	.89
280	250	.96	230	3950	.89
270	475	.95	220	8350	.91
260	805	.95			

Discussion

The observed concentration of monodeutero-L-malate is in good agreement with that calculated for mechanism 6. Actually all that can be done experimentally is to set a maximum value for k_4 in mechanism 10. The dashed line in Fig. 2 for $k_4 = 0.10$ shows that if exchange accompanies the over-all reaction, the value of k_4 must be less than a tenth the value of k_1. Since no exchange has been detected the carbon–hydrogen bond must be formed in a step preceding the rate-determining step, or in the rate-determining step, in the hydration of fumarate. Since there is no isotope rate effect, the carbon–hydrogen bond must be formed in a step preceding the rate-determining step. Earlier kinetic calculations[1] had shown that the dissociation of fumarate from the enzyme is not the rate-determining step in the dehydration of L-malate. If this were not true it would be expected that an appreciable value of k_4 would have been obtained in the present experiments. Englard and Colowick[25] have obtained results showing that deuterium is incorporated into citrate under the action of aconitase *via* the process: citrate → aconitate ⇄ labeled citrate.

The significance of the absence of both a deuterium rate effect and exchange in the fumarase reaction may be discussed with reference to the possibilities indicated in Table IV.[26] These are the general types of simple mechanisms which might be followed by the fumarase reaction. The abbreviations HCCOH, C=C and ⁻CCOH represent the enzyme–substrate complexes and intermediates in the reaction from which the indicated ions, H^+ or OH^-, have been removed. The HCCOH intermediate dissociates to produce free enzyme and L-malate, while the C=C intermediate dissociates to produce free enzyme and fumarate. The fact that no deuterium rate effect and no exchange are observed would appear to eliminate a concerted mechanism[27] and indicates that the reaction follows a mechanism of the first type. Thus in order to account for these observations it has been necessary to introduce a further intermediate into the mechanism for the fumarase reaction. Thus the earlier representation[28] of the intermediates which were required to account for the effect of pH on the forward and reverse reactions may be extended as shown in Fig. 3. According to this mechanism the acid dissociation constants for EM obtained as described in the preceding article in this series would

(25) S. Englard and S. P. Colowick, *J. Biol. Chem.*, in press.

(26) The authors are indebted to Dr. John F. Brown for helpful discussion of these possibilities.

(27) C. G. Swain and J. F. Brown, THIS JOURNAL, **74**, 2538 (1952).

(28) R. A. Alberty, *J. Cellular and Comp. Physiol.*, **47**, 245 (1956).

TABLE IV

EXPECTED DEUTERIUM RATE EFFECTS AND EXCHANGE FOR VARIOUS FUMARASE MECHANISMS

			Isotope rate effect	Exchange
Hydroxyl removed first				
$HCCOH \rightleftharpoons OH^- + HCC^+ \rightleftharpoons H^+ + C{=}C$				
(1)	slow	fast	No	No
(2)	fast	slow	Yes	No
Hydrogen removed first				
$HCCOH \rightleftharpoons H^+ + {}^-CCOH \rightleftharpoons OH^- + C{=}C$				
(3)	slow	fast	Yes	No
(4)	fast	slow	No[a]	Yes
Concerted mechanism				
(5)	$HCOOH \rightleftharpoons H^+ + C{=}C + OH^-$		Yes[b]	No

[a] Isotope effects on equilibria would be expected to be too small to show up in measured rates. [b] A smaller isotope rate effect than the "normal" might be expected.

be those for the enzyme-L-malate complex, but those for EF would be a weighted average of the acid dissociation constants for the two intermediates shown on the left. The movement of the positive charge on RH^+ to the dicarboxylic acid in the new intermediate may be the cause of the acid strengthening of K_{aEF} over K_{aE} which is found experimentally and which stands in contrast with the acid weakening caused by the binding of L-malate.

The data of Table I are further evidence for the absence of even a small effect of the substitution of deuterium for hydrogen on the rate of dehydration of L-malate. If ordinary L-malate were dehydrated faster than the monodeutero-L-malate formed, the concentration of fumarate would go through a maximum which easily could be observed. If the ratio of first-order rate constants for the dehydration of ordinary malate and monodeutero-L-malate was 1.36, a maximum fumarate concentration 10% greater than the equilibrium fumarate concentration would be reached after six half lives for the over-all reaction.

The treatment of the data in terms of mechanisms 6 and 10 is required by the complicated nature of the actual mechanism and the fact that kinetic data can be obtained only in the steady state. The actual mechanism is of the type

$$\left.\begin{array}{l} M + E \rightleftharpoons EM \overset{\text{slow}}{\rightleftharpoons} \\ M + E \rightleftharpoons EM^* \overset{\text{slow}}{\rightleftharpoons} \end{array}\right\} EX \rightleftharpoons EF \rightleftharpoons E + F \qquad (15)$$

where the reactions in the unmarked equilibria are faster. According to this mechanism the values of k_3 and k_4 calculated earlier[1] from steady-state studies over a range of *p*H would be identified with the rate constants for EM $\rightleftarrows$ EX rather than EX $\rightleftarrows$ EF. It is necessary to postulate an intermediate EX in order to explain the absence of an isotope rate effect and of exchange. The branch in the mechanism is placed at EX because if EM could exchange with D_2O to give M*, there would be no induction period in the formation of M*. If the branch was placed at EF it would be necessary to introduce a further intermediate to keep the mechanism symmetrical with respect to M and M*. Speyer and Dickman[29] believe that the aconitase reaction follows mechanism 14 and have suggested that EX contains a "bound" carbonium ion.

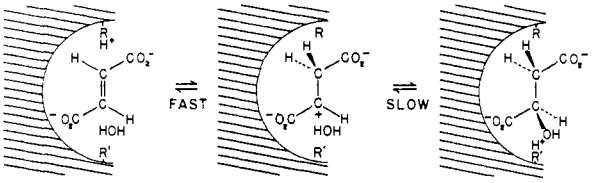


Fig. 3.—Structures of intermediates in the fumarase reaction suggested by the study of the effect of *p*H on the kinetics and the absence of a deuterium isotope rate effect or exchange.

On the basis of the mechanisms in Table IV it is predicted that L-malate containing O^{18} in the hydroxyl group would not show exchange during dehydration but that there should be an isotope rate effect. This prediction is being tested.

Acknowledgment.—The authors are indebted to Professor E. L. King for helpful discussions and to Drs. Raymond G. Wolfe, Jr., and Nehemia Shavit for preparations of crystalline fumarase.

MADISON, WISCONSIN

(29) J. F. Speyer and S. R. Dickman, *J. Biol. Chem.*, **220**, 193 (1956).