Malic Dehydrogenase. IV. *p*H Dependence of the Kinetic Parameters*

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The *p*H dependence of the kinetic parameters of the reaction catalyzed by pig heart malic dehydrogenase has been investigated. The kinetic behavior of the system is explained through a model in which groups on the enzyme surface which have *pK* values of approximately 7 and 10 are involved in the catalytic function, and the proton generated during the oxidation of malate is taken up by the enzyme and not by the solvent. Calculated values of the individual rate constants for the reaction between various charged forms of the enzyme and the coenzyme are given. A compulsory substrate binding order mechanism involving one or more ternary complexes is compatible with the experimental data at every *p*H value studied. The dissociation of the coenzyme-product from the enzyme-coenzyme complex appears to be the rate limiting reaction in both forward and reverse reactions.

Detailed kinetic studies of pig heart malic dehydrogenase involving initial rate measurements at *p*H 8.0 and 25° have been reported in paper II (Raval and Wolfe, 1962a) of this series. Product inhibition studies have been reported in paper III (Raval and Wolfe, 1962b) of this series. The most plausible mechanism, the compulsory binding order mechanism given below, is supported by three types of evidence:

1. The over-all thermodynamic equilibrium constant is in agreement with that obtained with use of kinetic constants on the basis of the compulsory binding order mechanism.
2. Experimental values are in good agreement with theoretically predicted relationships between kinetic constants, assuming the compulsory binding order mechanism with a rapidly dissociating ternary complex.
3. Product inhibition studies indicate the presence of a ternary complex.

The compulsory binding order mechanism, which may contain one or more ternary complexes, can be represented as follows:[1]

$$E + DPN \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot DPN$$

$$E\cdot DPN + M \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EXY$$

$$EXY \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E\cdot DPNH + OAA + H^+$$

$$E\cdot DPNH \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + DPNH$$

Previous kinetic studies have not considered the role of the proton generated as a product or absorbed as a reactant in the above reaction. Moreover, Dalziel (1957) and Theorell (1958) have reported an apparent change in the reaction mechanism with *p*H in the case of yeast alcohol dehydrogenase. Variation in the values of kinetic parameters with *p*H has been interpreted in terms of ionizable groups on the enzyme by Alberty (1953). The above considerations have led to the *p*H dependence studies reported here.

METHODS

The enzyme isolation and assay methods used were identical to those described previously (Raval and Wolfe, 1962a) except that a Cary model 11 recording spectrophotometer was used at acid *p*H values. Tris acetate buffer, 0.05 M with respect to acetate, was used in all experiments. The ionic strength was therefore constant at 0.05 except for a small decrease in the extreme acid range because of the association of protons to form acetic acid. The *p*H of all solutions was adjusted with Tris (Sigma 121) so that the concentration of uncharged Tris increased at the higher *p*H values. The stability of the reagents was studied at each *p*H, and experimental conditions were carefully controlled to avoid decomposition.

Substrate inhibition or activation (rates less than or in excess of that predicted by simple Michaelis-Menten theory) occurred at concentrations which were a function of *p*H. It was found possible to avoid concentration ranges where such effects were observed though velocity measurements were made over substrate concentration ranges which bracketed the Michaelis constant value in each experiment.

The temperature was maintained constant at 25° by circulating water from a thermostated

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[1] The following abbreviations are used throughout this paper: DPN = oxidized coenzyme, DPNH = reduced coenzyme, M = malate, OAA = oxalacetate, E = free enzyme, EXY = the ternary complex, and Tris = tris(hydroxymethyl)aminomethane.

water bath through thermospacers in the spectrophotometer.

All experimental parameters reported in this paper were obtained by extrapolation to conditions which were zero order with respect to both substrates. The reader is referred to previous work (Dalziel, 1957, or Raval and Wolfe, 1962a) for the details of extrapolation methods and methods of evaluating the experimental parameters.

Results

In the following discussion the reaction in the direction of malate oxidation will be referred to as the forward and that in the direction of oxalacetate reduction as the reverse reaction. Table I summarizes the values of various kinetic parameters at a series of *p*H values in the range between *p*H 5.5 and 10.25.

Table I

Kinetic Parameters for Malic Dehydrogenase at 25° as a Function of *p*H in Tris Acetate Buffer, Ionic Strength 0.05

*p*H	K_{DPN} × 10^4 M	K_M × 10^4 M	$K_{DPN \cdot M}$ × 10^6 M^2	V_f × 10^{-4} M.A.[a]
5.5	8.0	120.0	40.0	0.3
6.0	3.0	55.0	5.0	0.3
7.0	2.2	12.0	1.0	0.5
7.5	1.4	8.0	0.6	0.6
8.0	2.0	8.0	0.6	1.0
8.5	1.3	7.0	0.4	1.0
9.0	2.5	10.0	0.7	1.6
9.5	4.8	20.0	1.5	2.5
10.0	17.0	65.0	4.5	5.0
10.25	22.0	82.0	6.0	6.3

*p*H	K_{DPNH} × 10^5 M	K_{OAA} × 10^5 M	$K_{DPNH \cdot OAA}$ × 10^{10} M^2	V_r × 10^{-4} M.A.[a]
5.5	0.5	0.2	0.06	0.4
6.0	1.2	0.8	0.2	0.8
7.0	1.5	2.0	0.5	2.0
7.5	1.8	3.0	0.9	3.0
8.0	1.8	4.0	1.9	3.5
8.5	2.1	6.0	3.4	4.0
9.0	2.0	10.0	7.6	3.8
9.5	2.2	25.0	32.0	3.6
10.0	3.5	40.0	210.0	3.0
10.25	3.0	55.0	400.0	2.7

[a] M.A. is the molecular activity of the enzyme.

Table II presents the over-all equilibrium constant as calculated from kinetic parameters at each *p*H studied. The thermodynamic equilibrium constant is also included in this table to facilitate comparison with kinetic values. It is apparent that there is reasonably good agreement between thermodynamic and kinetically determined values of the over-all equilibrium constant except at very acidic *p*H values. There is also very good agreement, except at acidic *p*H values, between the expected relationships between various kinetic parameters and their experimental values for a mechanism involving a kinetically insignificant ternary complex (Table IV). It appears, therefore, that the compulsory binding order mechanism involving a kinetically insignificant ternary complex (one with a very short half life) applies over the broad *p*H range included in this study, and also that the ternary complex becomes kinetically significant at acidic *p*H. The existence of a ternary complex has been suggested previously (Raval and Wolfe, 1962a), and experimental evidence of its presence was given subsequently (Raval and Wolfe, 1962b).

Table II

Thermodynamic and Kinetic Over-all Equilibrium Constants for the Reaction Catalyzed by Malic Dehydrogenase at Various *p*H Values

*p*H	app.[a] K_{eq}	$V_f \cdot K_{DPNH \cdot OAA}$[b] $V_r \cdot K_{DPN \cdot M}$	$V_f^3 \cdot K_{DPNH} \cdot K_{OAA}$[c] $V_r^3 \cdot K_{DPN} \cdot K_M$
5.5	3.1×10^{-7}	1.0×10^{-7}	6.6×10^{-7}
6.0	1×10^{-6}	1.5×10^{-6}	3.0×10^{-6}
7.0	1×10^{-5}	1.2×10^{-5}	1.8×10^{-5}
7.5	3.1×10^{-5}	3×10^{-5}	3.5×10^{-5}
8.0	1×10^{-4}	1.0×10^{-4}	1.0×10^{-4}
8.5	3.1×10^{-4}	2.0×10^{-4}	2.3×10^{-4}
9.0	1×10^{-3}	1.0×10^{-3}	0.8×10^{-3}
9.5	3.1×10^{-3}	4.0×10^{-3}	2.4×10^{-3}
10.0	1×10^{-2}	1.0×10^{-2}	0.6×10^{-2}
10.25	1.7×10^{-2}	1.7×10^{-2}	1.2×10^{-2}

[a] Thermodynamic equilibrium constant (Raval and Wolfe, 1962a). [b] The over-all equilibrium constant in terms of kinetic constants assuming a compulsory substrate binding order mechanism involving one or more kinetically significant ternary complexes (Alberty, 1953). [c] The over-all equilibrium constant in terms of kinetic constants assuming a compulsory substrate binding order mechanism involving a kinetically insignificant ternary complex.

Figure 1 represents the variation of the pre-

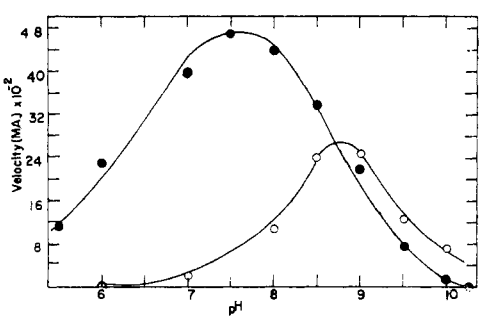

Fig. 1.—A plot of molecular activity (M.A.) as a function of *p*H for both reaction directions. The open circles represent experimental points obtained with a reaction mixture containing 5×10^{-4} M malate, 2×10^{-5} M DPN, and 2.4×10^{-8} g/ml of malic dehydrogenase. Solid circles represent experimental points obtained with 1×10^{-5} M oxalacetate, 1×10^{-5} M DPNH, and 2.4×10^{-8} g/ml of malic dehydrogenase. The continuous curves represent calculated values from equations (1) and (2) using the values of kinetic parameters given in Table I.

vailing initial velocity with *p*H in both reaction directions. The *p*H optimum for the forward reaction is approximately 8.9, whereas the *p*H optimum for the reverse reaction is approximately 7.5. The solid lines represent theoretical curves calculated from the following rate law equations by use of the values of the kinetic parameters given in Table I:

$$V_f/v_0^* = 1 + K_{DPN}/(DPN) + K_M/(M) + K_{DPN.M}/(DPN)(M) \quad (1)$$

and

$$V_r/v_0 = 1 + K_{DPNH}/(DPNH) + K_{OAA}/(OAA) + K_{DPNH.OAA}/(DPNH)(OAA) \quad (2)$$

Figure 1 indicates the *p*H optima as well as the agreement between the experimental data and the general rate law expressions.

Although the steady state derivations of equations (1) and (2) give complex definitions of kinetic parameters, simple relationships exist between some of these parameters (Takenaka and Schwert, 1956) as follows:

$$V_f/K_{DPN} = k_1;\ K_{DPN.M}/K_M = k_2/k_1 = K_{E.DPN};\ \text{and } V_fK_{DPN.M}/K_{DPN}K_M = k_2$$

$$V_r/K_{DPNH} = k_8;\ K_{DPNH.OAA}/K_{OAA} = k_7/k_8 = K_{E.DPNH};\ \text{and } V_rK_{DPNH.OAA}/K_{DPNH}K_{OAA} = k_7$$

Table III lists the values of the coenzyme dissociation constants and the related rate constants. In order to reduce the effects of random errors, smooth curves were drawn through the points describing the variation of each kinetic parameter with *p*H and values taken from these curves were used to calculate the rate constants given in Table III.

Discussion

As shown in Table III the values of $K_{E.DPNH}$ are essentially constant from *p*H 5.5 to 8.5. The average value of $K_{E.DPNH}$ between *p*H 5.5 and 8.5 is 4×10^{-6} M. Above *p*H 8.5 the values of $K_{E.DPNH}$ rise very sharply as the *p*H increases, attaining a value of 8.0×10^{-5} at *p*H 10.25. If one assumes that the variation of $K_{E.DPNH}$ with *p*H is determined by the dissociation of a proton from a single group which is essentially unprotonated at *p*H 10.25, then the value of $K_{E.DPNH}$ at 8.0 (two *p*H units removed) should not be more than 1% of its value at *p*H 10.25. The fact that the experimental value is greater than expected for $K_{E.DPNH}$ at *p*H 8.0 suggests that more than one dissociating group is involved in binding DPNH.

The curve describing the rate of combination of DPNH and malic dehydrogenase, k_8, with *p*H is given in Figure 2. The shape of this

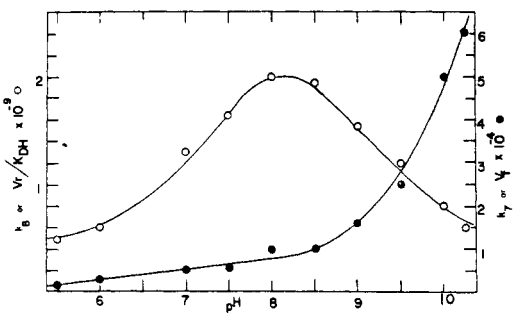

Fig. 2.—Plots describing (1) the rate of combination of DPNH and malic dehydrogenase, k_8 or V_r/K_{DPNH} (open circles) and (2) the rate of dissociation of the enzyme-DPNH complex, k_7 (solid circles). The continuous curves are theoretical curves calculated as described in the text. The circles represent experimental points.

curve suggests that there are three charged forms of the enzyme, $^+E^+$, $^0E^+$, $^0E^0$, which have kinetic significance in the *p*H range studied. Assuming that DPNH binds to all charged forms of the enzyme, the scheme given in Figure 3 was calculated as described by Winer (1957). The main features of this scheme are as follows:

(1) The various equilibrium constants shown in Figure 3 are not independent. A typical relationship required between them, thermodynamically, is shown in equation (3).

Table III

Coenzyme Dissociation Constants and Related Rate Constants

*p*H	$k_1 \times 10^{-7}$ (M)(min.$^{-1}$)	$k_2 \times 10^{-4}$ (min.$^{-1}$)	$K_{E.DPN} \times 10^4$ (M)	$k_7 \times 10^{-4}$ (min.$^{-1}$)	$k_8 \times 10^{-9}$ (M)(min.$^{-1}$)	$K_{E.DPNH} \times 10^6$ (M)
5.5	0.4	1.25	31.0	0.3	0.8	2.8
6.0	1.2	0.9	7.5	0.3	0.6	5.0
7.0	2.5	2.0	8.0	0.5	1.4	3.5
7.5	4.3	3.2	7.5	0.5	1.7	3.1
8.0	5.0	3.5	7.0	1.0	2.0	5.0
8.5	6.3	4.4	7.0	0.9	1.9	4.8
9.0	6.0	4.2	7.3	1.5	1.7	9.5
9.5	5.0	3.8	7.6	2.4	1.3	18.6
10.0	3.5	2.8	8.0	4.8	1.0	48.0
10.25	3.2	2.5	7.8	6.0	0.8	80.0
			Mean 7.6			

$$\frac{k_7'}{k_8'} \cdot K_{a_1} \cdot K_{a_2} = \frac{k_7''}{k_8''} \cdot K_{a'_1} \cdot K_{a_2} = \frac{k_7'''}{k_8'''} \cdot K_{a'_1} \cdot K_{a'_2} \quad (3)$$

The numerical values of the three equivalent expressions are 8.7×10^{-22}, 4×10^{-22}, and 3×10^{-22} respectively. Variation by a factor of two does not seem excessive considering the probable experimental error.

(2) The rate-limiting step in the forward direction of the over-all reaction is the rate of the dissociation of E·DPNH complex. When the more strongly acidic group dissociates, the rate of dissociation of E·DPNH complex is increased by a factor of 5. When the second functional group dissociates, the rate of dissociation of E·DPNH is increased by an additional factor of 70. The increase in V_f parallels the rate of increase in the dissociation of E·DPNH. The theoretical curve through the solid circles in Figure 2, describing the variation of V_f with pH, was calculated by using the above values of k_7', k_7'', and k_7''' and the relative concentration of the appropriate form of E·DPNH complex.

(3) Since V_r/K_{DPNH} is defined as k_8, it would be expected that the sum of the products of the fractional concentration of each form of the enzyme at any pH multiplied by its appropriate rate constant k_8', k_8'', or k_8''' should yield the observed value of V_r/K_{DPNH} at that pH. The theoretical curve through the open circles in Figure 2 was calculated using the dissociation constant and the rate constants shown in Figure 3.

(4) The mechanism described in Figure 3 indicates that two ionizable groups with pK values of 6.7 and 9.2 are involved in the reaction of DPNH with the enzyme. Since the pK values of the titrable groups of the coenzyme and the substrate are outside the pH range covered in the present study it must be assumed that these groups lie on the enzyme surface.

The dissociation constant of MDH·DPN complex is essentially constant at all the pH values studied except pH 5.5. Calculation of the over-all equilibrium constant at pH 5.5 using the kinetic constants yields a value lacking agreement with the thermodynamic equilibrium constant. If the value of $K_{DPN\cdot M}$ at pH 5.5 is assumed to be 1×10^{-5} instead of the observed value of 4×10^{-5}, it yields not only the correct value for the over-all equilibrium constant but also a value of $K_{E\cdot DPN}$ which is in agreement

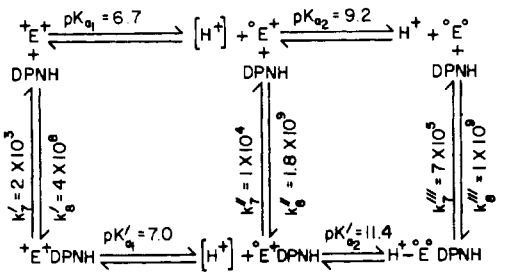

FIG. 3.—The mechanism of binding of DPNH to malic dehydrogenase. Calculated values of the rate and dissociation constants are given in the text.

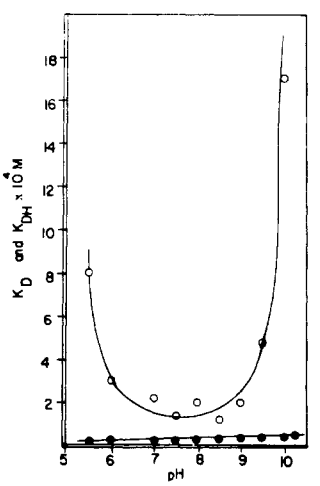

FIG. 4.—Plots describing the variation of K_{DPN} (K_D, open circles) and K_{DPNH} (K_{DH}, solid circles) with pH.

TABLE IV

EXPERIMENTAL VALUES FOR THE EXPECTED RELATIONSHIPS BETWEEN VARIOUS KINETIC PARAMETERS FOR A COMPULSORY BINDING ORDER MECHANISM INVOLVING A KINETICALLY INSIGNIFICANT TERNARY COMPLEX

Expected Relationships[a]

$$\frac{K_{DPN}\cdot K_M}{K_{DPN\cdot M}\cdot V_f} = \frac{1}{V_r} \text{ and } \frac{K_{DPNH}\cdot K_{OAA}}{K_{DPNH\cdot OAA}\, V_r} = \frac{1}{V_f}$$

pH	$\frac{K_{DPN}\cdot K_M}{K_{DPN\cdot M}\cdot V_f}$ × 10⁵	$\frac{1}{V_r}$ × 10⁵	$\frac{K_{DPNH}\cdot K_{OAA}}{K_{DPNH\cdot OAA}\cdot V_r}$ × 10⁵	$\frac{1}{V_f}$ × 10⁵
5.5	8.0	25.0	42.0	33.0
6.0	11.0	12.0	60.0	33.0
7.0	5.3	5.0	30.0	20.0
7.5	3.1	3.3	20.0	17.0
8.0	2.7	2.9	11.0	10.0
8.5	2.3	2.5	13.0	10.0
9.0	2.2	2.6	8.0	6.3
9.5	2.5	2.8	4.8	4.0
10.0	4.9	3.3	2.2	2.0
10.25	4.7	3.7	1.55	1.6

[a] Dalziel (1957).

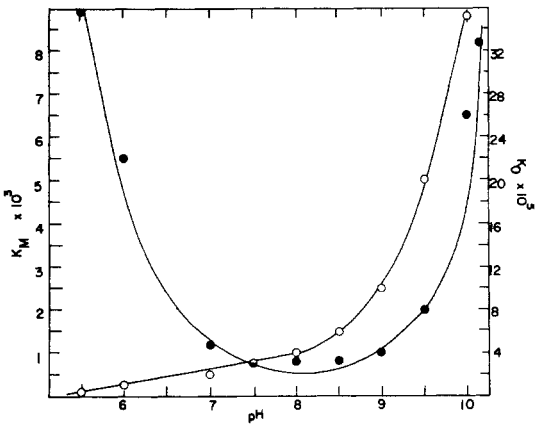

FIG. 5.—Plots describing the variation of K_M (solid circles) and K_{OAA} (K_O, open circles) with *p*H.

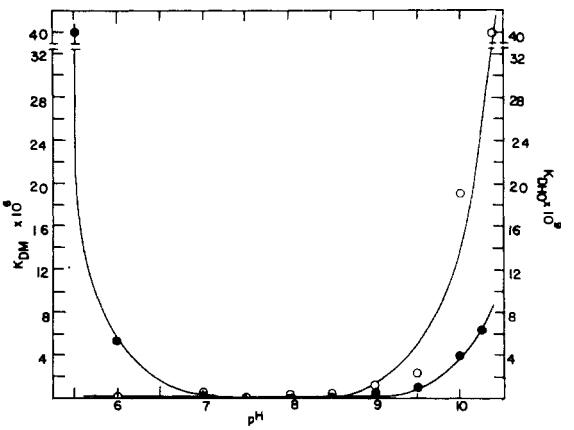

FIG. 6.—Plots describing the variation of $K_{DPN\cdot M}$ (K_{DM}, solid circles) or $K_{DPNH\cdot OAA}$ (K_{DHO}, open circles) with *p*H.

with the values of $K_{E\cdot DPN}$ observed at the higher *p*H values. It appears possible, therefore, that the value of $K_{DPN\cdot M}$ at *p*H 5.5 is erroneous.

Malic dehydrogenase shows a striking resemblance to lactic dehydrogenase in that the values of K_{DPN}, K_M, and $K_{DPN\cdot M}$ vary in the same way with *p*H (Fig. 4, 5, and 6). Moreover, $K_{E\cdot DPN}$ for both enzymes is essentially constant with *p*H. The mechanism proposed for lactic dehydrogenase by Winer and Schwert (1958) therefore applies equally well to malic dehydrogenase as depicted in Figure 7. Winer and Schwert (1958) have derived the steady state rate law expression for such a model. The values of k_1, k_2, k_9, k_{10}, k_{11}, and k_{12} calculated according to the method described by Schwert (1958) are as follows:

$$k_1 = 6 \times 10^7\ \text{M}^{-1}\ \text{min.}^{-1};\quad k_2 = 4.6 \times 10^4\ \text{min.}^{-1}$$

$$k_9 = 1 \times 10^{11}\ \text{M}^{-1}\ \text{min.}^{-1};\quad k_{10} = 4.1 \times 10^3\ \text{min.}^{-1}$$

$$k_{11} = 8 \times 10^{13}\ \text{M}^{-1}\ \text{min.}^{-1};\quad k_{12} = 8 \times 10^3\ \text{min.}^{-1}$$

By definition (Winer and Schwert, 1958),

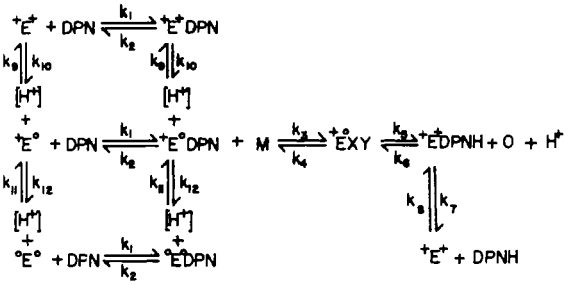

FIG. 7.—The mechanism of binding of DPN and malate (M) to malic dehydrogenase.

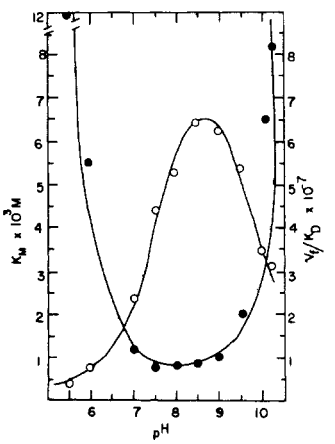

FIG. 8.—Plots showing the variation of (1) the rate of DPN binding to malic dehydrogenase, V_f/K_D (open circles) and (2) K_M with *p*H (solid circles). Continuous curves are theoretical curves calculated as described in the text.

$$V_f/K_{DPN} = \frac{k_1k_{10}k_{11}H^+(k_2 + k_9H^+ + k_{10} + k_{12})}{(k_2 + k_{10})(k_9k_{11}H^{+2} + k_{10}k_{11}H^+ + k_{10}k_{12})} \quad (4)$$

The theoretical curve through the open circles in Figure 8, describing the variation of V_f/K_{DPN} with *p*H, is calculated using equation (4) and the values of rate constants given above.

The mechanism proposed in Figure 7 assumes that a proton released by malate is accepted by the unprotonated group on the surface of MDH. If this assumption is correct then the rate of reactions (3), (4), and (5) should be independent of the hydrogen ion concentration. This point can be tested as follows:

K_M, Michaelis constant for malate, is defined as:

$$K_M = \frac{k_7(k_4 + k_5)(k_9k_{11}H^{+2} + k_{10}k_{11}H^+ + k_{10}k_{12})}{k_3k_{11}H^+(k_5k_7 + k_5k_{10} + k_7k_{10})} \quad (5)$$

k_7, the rate of dissociation of E·DPNH complex is found to be equal to V_f; it follows, therefore, that k_5k_{10} is much larger than $k_5k_7 + k_7k_{10}$ at any given *p*H. Thus equation (5) becomes

$$K_M = \left(\frac{k_7(k_9k_{11}H^{+2} + k_{10}k_{11}H^+ + k_{10}k_{12})}{k_{10}k_{11}H^+}\right)\left(\frac{k_4 + k_5}{k_3k_5}\right) \quad (6)$$

If k_3, k_4, and k_5 are true constants and do not vary with hydrogen ion concentration, then the ratios of observed values of K_M to the first bracketed term in equation (6) should be constant and equal to $(k_4 + k_5)/k_3k_5$. Table V gives the values

TABLE V

THE pH DEPENDENCE OF RATE CONSTANTS INVOLVED IN THE HYDROGEN ION TRANSFER REACTION

pH	$\frac{K_M/k_7(k_9k_{11}H^{+2} + k_{10}\cdot k_{11}H^+ + k_{10}\cdot k_{12})}{(k_{10}\cdot k_{11}H^+)} \times 10^8$
5.5	5.8
6.0	7.0
7.0	6.9
7.5	7.15
8.0	6.4
8.5	7.0
9.0	6.3
9.5	6.0
10.0	6.5
10.25	5.0
Mean	$6.0 \pm 1 \times 10^{-8}$

of this quotient at all pH values studied. The mean value is $6.0 \pm 1 \times 10^{-8}$. The product of this value and of the values for the first bracket in equation (6) were used to calculate the curve shown in Figure 8.

The above treatment suggests that the proton given off by malate is taken up on the enzyme surface. Combination of the substrate malate with the enzyme-coenzyme complex seems to be favored when a participating group with a pK of approximately 7.3 is unprotonated and a second participating group with a pK of approximately 10.0 is protonated. It seems plausible that the group having a pK of about 7.3 could function by accepting the proton removed from malate in the reaction. This same group, possibly an imidazole, might act as a proton donor in the conversion of oxalacetate to malate in the reverse reaction. The same mechanism has been suggested for lactic dehydrogenase by Winer and Schwert (1958).

Hydrogen ion may act simultaneously as a reactant and by influencing the ionization of groups on the enzyme. Consideration of both effects simultaneously leads to extreme complexity. Therefore, the data presented here have been interpreted in terms of the latter effect only.

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