Malic Dehydrogenase. II. Kinetic Studies of the Reaction Mechanism*

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Detailed kinetic experiments have been carried out with pig heart malic dehydrogenase at *p*H 8.0 and 25° in Tris-acetate buffer (0.05 M with respect to acetate). Results of these experiments are in good agreement with theoretical predictions for a compulsory substrate binding order in both reaction directions. It was not possible to distinguish the presence of a ternary complex by kinetic methods. Several kinetic parameters, equilibrium constants, and dissociation constants have been evaluated. The rate-limiting step, in both reaction directions, appears to be the dissociation of coenzyme from the enzyme-coenzyme complex.

Malic dehydrogenase appears to have the lowest molecular weight of any known diphosphopyridine nucleotide–requiring enzyme. Therefore it is attractive for catalytic mechanism studies as the simplest available enzyme in the group. Several recent publications have described isolation procedures and other properties (Davies and Kun, 1957; Siegel and Englard, 1960) of the enzyme from beef heart or pig heart. Since detailed kinetic studies were lacking, the work reported here was undertaken.

Recent theoretical developments have facilitated kinetic studies of several diphosphopyridine nucleotide enzymes (Segal *et al.*, 1952; Alberty, 1953). Detailed kinetic studies of lactic dehydrogenase have been reported by Schwert (1958). Kinetic studies of alcohol dehydrogenase have been published by Theorell and co-workers (Theorell and Bonnichsen, 1951; Theorell and Chance, 1951). Glutamic dehydrogenase has been subjected to detailed kinetic study by Frieden (1959), and very recently Nordlie and Fromm (1959) published the results of kinetic studies of ribitol dehydrogenase. The addition of malic dehydrogenase to the growing list of diphosphopyridine nucleotide enzymes subjected to kinetic study may facilitate examination of the comparative aspects of enzyme mechanism among this group of enzymes.

EXPERIMENTAL PROCEDURE

Enzyme Isolation.—Malic dehydrogenase was prepared from acetone powder of pig heart by a modification of the previously published method (Wolfe and Neilands, 1956). This procedure was supplemented by hydroxylapatite chromatography (Tiselius *et al.*, 1958). This isolation procedure has proved reproducible in the hands of four different individuals in this laboratory. Criteria of purity of the enzyme have been presented in the first paper of this series (Wolfe and Neilands, 1956).

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Reagents.—Tris,[1] DPN (96% pure), and DPNH (90% pure) were obtained from the Sigma Chemical Company. Oxalacetic acid was obtained from the California Corporation for Biochemical Research. *l*-Malic acid, obtained from the Commercial Solvents Corporation, was recrystallized as described elsewhere (Frieden *et al.*, 1957).

Determination of the Equilibrium Constant.—Equilibrium concentrations of DPNH were determined by measuring the optical density at 340 mμ, assuming the molar extinction coefficient to be 6.22×10^6 (Horecker and Kornberg, 1948), DPNH to be 90% pure, and DPN to be 96% pure. A photomultiplier attachment was used as required for certain measurements at high optical density. Equilibrium concentrations of reactants other than DPNH were calculated from the observed change in DPNH concentration and the known initial concentrations of the reactants.

Assays.—All spectrophotometric assays were made with a Beckman DU spectrophotometer. The temperature was maintained at 25° by circulating water from a thermostat through thermospacers at both ends of the cell compartment. Protein concentrations were determined by optical density measurements at 280 mμ, the previously determined extinction coefficient (Wolfe and Neilands, 1956) being used.

Initial reaction rates were measured by observation of the rate of change in the optical density at 340 mμ. Measurement of the rate of DPN reduction is very difficult at lower *p*H values, with the available instruments, because of the unfavorable equilibrium. In order to study the reaction in both directions, *p*H 8.0 (Tris-acetate buffer 0.05 M with respect to acetate) was chosen as a suitable compromise value. The reaction was started by adding 10 μl of appropriately diluted enzyme to 20 ml of the reaction mixture contained in a cuvet having a 10-cm light path. The concentration of the enzyme was adjusted so that the initial reaction rate could be accurately ascertained. The sub-

[1] The abbreviations used in this paper are as follows: DPN for diphosphopyridine nucleotide, DPNH for reduced diphosphopyridine nucleotide, and Tris for tris(hydroxymethyl)aminomethane.

strates DPN, DPNH, and oxalacetate were observed to inhibit the enzyme at relatively high concentrations. The minimum concentration at which substrate inhibition was observed varied with each of the above substrates and with the concentration of the appropriate substrate partner. For example, the minimum inhibiting concentration of oxalacetate depended on the concentration of DPNH. Malate produced substrate activation (reaction rates in excess of that predicted by simple Michaelis-Menten theory) at concentrations which were dependent upon the concentration of DPN. Concentration ranges within which substrate inhibition or activation occurred were carefully avoided in the experimental evaluation of the kinetic parameters. Similar substrate inhibitions and activations of malic dehydrogenase have been reported (Davies and Kun, 1957; Pfleiderer and Hohnholz, 1959). Apparently different forms of malic dehydrogenase have been observed which differ in the degree of inhibition by oxalacetate (Englard *et al.*, 1960).

Theoretical Basis and Method of Kinetic Study

Various investigators have shown that it is possible to obtain evidence regarding possible reaction mechanisms for two-substrate enzymes by appropriate kinetic measurements (Segal *et al.*, 1952; Alberty, 1953; Theorell and Bonnichsen, 1951). The following rate law has been derived (Alberty, 1953),

$$\frac{1}{v} = \frac{1}{V_f} + \frac{K_A}{V_f(A)} + \frac{K_B}{V_f(B)} + \frac{K_{AB}}{V_f(A)(B)} \qquad (1)$$

where A and B are substrates in the forward direction for the general reaction $A + B \rightleftharpoons C + D$, and v is the prevailing initial velocity, V_f is the maximum initial velocity in the forward reaction, K_A and K_B are Michaelis constants, and K_{AB} is a complex Michaelis constant which has been defined elsewhere (Alberty, 1958). A similar equation may be written for the reverse reaction.

Dalziel (1957) has expressed the above rate law in slightly different form by the use of four "kinetic coefficients," as given in equation (2),

$$\frac{e}{v} = \phi_0 + \frac{\phi_1}{(A)} + \frac{\phi_2}{(B)} + \frac{\phi_{12}}{(A)(B)} \qquad (2)$$

where e is the concentration of enzyme active sites. The relationship of the kinetic coefficients to the more familiar experimental parameters is apparent by comparison of equation (2) with equation (1) above. In the tables, the kinetic coefficients for the reverse reaction are distinguished from those for the forward reaction by prime signs.

In the case of two-substrate enzymes the true Michaelis constants and maximum initial velocities must be obtained by extrapolation to conditions which are zero order with respect to both substrates. This extrapolation was accomplished as described by Dalziel (1957). A series of double reciprocal (reciprocal initial velocity versus reciprocal substrate concentration) "primary plots" were made as described by Lineweaver and Burk (1934). In such experiments the concentration of one substrate, the "fixed substrate," was adjusted to a constant initial value while the concentration of the second substrate was varied in the usual manner. In each of a series of such experiments the concentration of the "fixed substrate" was adjusted to an appropriate, though different, initial concentration. Data obtained in this way yield a series of "primary" double reciprocal plots (see Fig. 1), each of which represents a different concentration of the "fixed substrate." The role of the "fixed" and the variable substrates may be reversed in a second series of experiments (see Fig. 2). Similar experiments may be carried out for the reverse reaction. Two types of "secondary" plots were made from each series of "primary" plots; the first, a plot of the ordinate intercept ($1/(S) = 0$) versus the reciprocal of the "fixed substrate" concentration, and the second, a plot of the slope of the primary plot lines versus the reciprocal of the "fixed substrate" concentration. It is possible to evaluate all of the kinetic constants from these "secondary plots" (Dalziel, 1957). The kinetic coefficients for malic dehydrogenase were evaluated in this manner (see Fig. 6 and 8 for graphic representation).

Results

The apparent equilibrium constant at *p*H 8.0 in Tris-acetate buffer (0.05 M with respect to acetate) was determined by equilibrium concentration measurements to be 1.03×10^{-4}. Table I sum-

Table I
Equilibrium Concentration Data

Added Initially		Equilibrium Starting with DPN and Malate at *p*H 8.0 in 0.05 M Tris-acetate: Equilibrium Values				
DPN $\times 10^4$ M	Malate $\times 10^4$ M	DPNH $\times 10^6$ M	OAA $\times 10^6$ M	DPN $\times 10^4$ M	Malate $\times 10^4$ M	K_{eq}^{app}[a] (25°)
1.00	10.0	3.22	3.22	0.997	9.97	1.04×10^{-4}
1.00	5.00	2.25	2.25	0.998	4.98	1.01×10^{-4}
1.00	1.00	1.04	1.04	0.999	0.999	1.08×10^{-4}
4.00	1.00	1.93	1.93	3.98	0.998	0.925×10^{-4}
4.00	10.0	6.6	6.6	3.93	9.93	1.09×10^{-4}

Equilibrium Starting with DPNH and Oxalacetate (OAA) at *p*H 8.0 in 0.05 M Tris-Acetate						
DPNH $\times 10^5$	OAA $\times 10^5$	DPNH $\times 10^7$	OAA $\times 10^7$	DPN $\times 10^5$	Malate $\times 10^5$	K_{eq}^{app}[a] 25°)
2.0	2.0	2.0	2.0	1.98	1.98	1.02×10^{-4}
Av.						1.03×10^{-4}
						0.998×10^{-4}[b]

[a] Apparent over-all equilibrium. [b] Calculated from Burton and Wilson (1953).

TABLE II

EXPERIMENTAL VALUES OF VARIOUS KINETIC COEFFICIENTS AT *p*H 8.0 IN 0.05 M TRIS-ACETATE BUFFER

Forward direction (DPN reduction)	ϕ_0 (M.A.[a])$^{-1}$	ϕ_1 Moles × (M.A.[a])$^{-1}$	ϕ_2 Moles × (M.A.[a])$^{-1}$	ϕ_{12} Moles^2 × (M.A.[a])$^{-1}$
	1×10^{-4}	2×10^{-8}	8×10^{-8}	5.5×10^{-11}
Reverse direction (DPNH oxidation)	ϕ'_0 (M.A.[a])$^{-1}$	ϕ'_1 Moles × (M.A.[a])$^{-1}$	ϕ'_2 Moles × (M.A.[a])$^{-1}$	ϕ'_{12} Moles^2 × (M.A.[a])$^{-1}$
	2.9×10^{-5}	5.1×10^{-10}	11.4×10^{-10}	5.5×10^{-15}

[a] M.A., the molecular activity, is defined as moles of substrate decomposed per mole of enzyme per minute based on a molecular weight of 65,000 for the enzyme.

marizes the data. This value is in reasonable agreement with the measurements of Burton and Wilson (1953). The value of the apparent equilibrium constant calculated from kinetic parameters (1.00×10^{-4} assuming the validity of mechanisms B-1, B-2, or B-3 below) is in agreement with the value determined from the measurement of equilibrium concentrations (1.03×10^{-4}).

Kinetic data are presented in the form of "primary plots" in Figures 1 through 4. Figures 3 and 4 represent data for the reverse reaction (the oxidation of DPNH). Figures 5 through 8 represent "secondary plots" of the experimental data obtained for the forward and reverse reactions catalyzed by malic dehydrogenase. Each point on a "secondary plot" curve represents a slope or an intercept ($1/(S) = 0$) from a "primary plot." All reaction velocities are expressed in terms of molecular activity, based on a molecular weight of 65,000. Molecular activity, formerly known as the turnover number, is defined as the number of moles

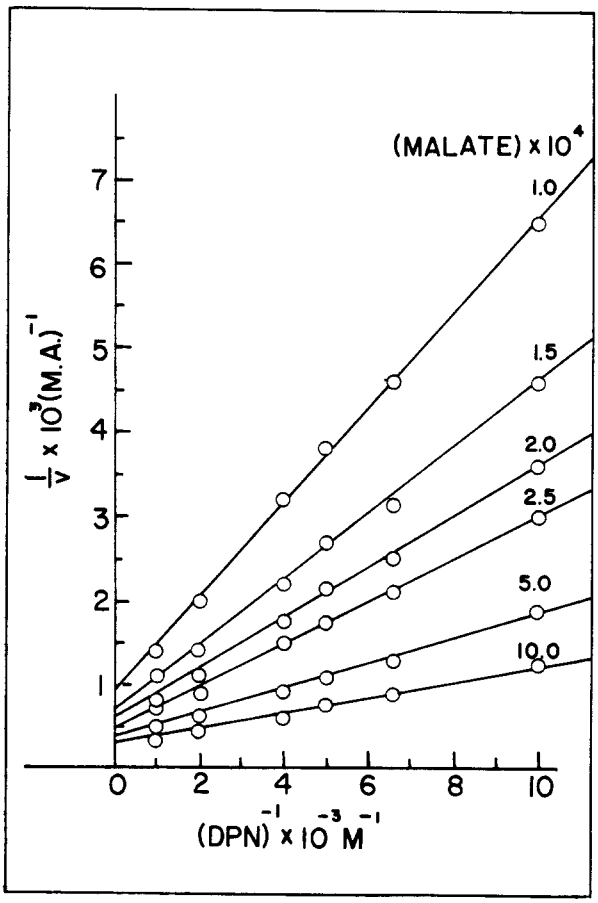

FIG. 1.—This figure represents a series of "primary plots" of reciprocal initial velocity versus reciprocal DPN concentration. The concentration of malate, the "fixed" substrate (see text), was adjusted to an appropriate though different concentration in each of the series of experiments. The initial molar concentration of malate used in each experiment is indicated on the right near the relevant curve. The molecular activity (M.A.) is defined as the number of moles of substrate decomposed per mole of enzyme per minute. All data were taken at 25° in Tris-acetate buffer, 0.05 M with respect to acetate.

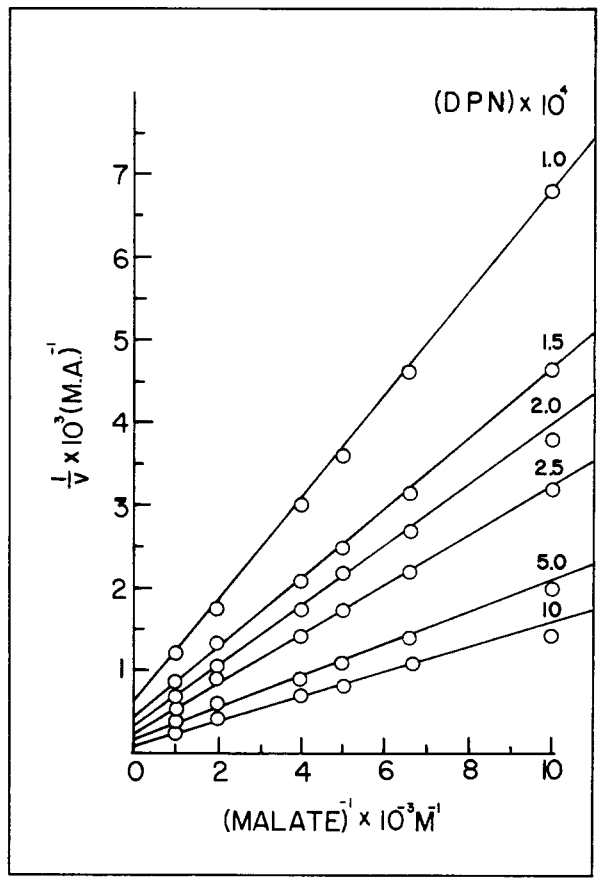

FIG. 2.—A series of "primary plots" of reciprocal initial velocity versus reciprocal malate concentration. The concentration of DPN, the "fixed" substrate, was adjusted to an appropriate though different concentration in each of the series of experiments. The initial molar concentration of DPN used in each experiment is indicated on the right near the relevant curve. The roles of the fixed and variable substrates have been reversed compared to the series of experiments given in Figure 1. All other conditions are identical to those given in Figure 1.

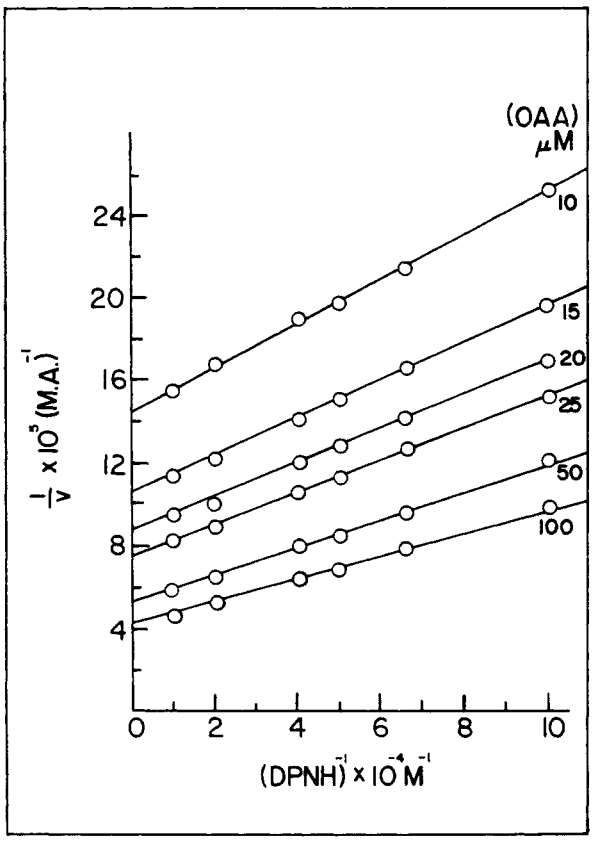

FIG. 3.—A series of "primary plots" of reciprocal initial velocity versus reciprocal DPNH concentration. The concentration of oxalacetate (OAA), the "fixed" substrate, was adjusted to an appropriate though different concentration in each of the series of experiments. The initial concentration of oxalacetate used in each experiment is indicated near the appropriate line. All other conditions are identical to those given in Figure 1.

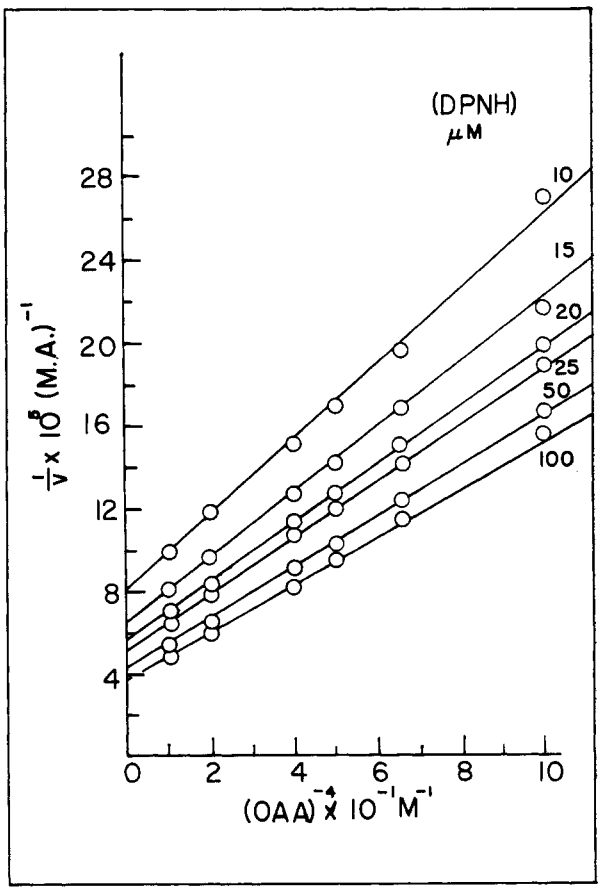

FIG. 4.—A series of "primary plots" of reciprocal initial velocity versus reciprocal oxalacetate concentration. The concentration of DPNH, the "fixed" substrate, was adjusted to an appropriate though different concentration in each of the series of experiments. The initial concentration of DPNH used in each experiment is indicated near the appropriate line. The role of the "fixed" and variable substrates have been reversed compared to the experiments illustrated in Figure 3. All other conditions are identical to those given in Figure 1.

of substrate decomposed per mole of enzyme per minute.

Table II lists the experimental values for the different kinetic coefficients. The following mechanisms can be excluded on the basis of theoretically required values of the various coefficients:

(1) No binary enzyme substrate complexes, but a single ternary complex is formed. This mechanism requires that ϕ_1, ϕ_2, ϕ'_1, and ϕ'_2 be zero.

(2a) A binary complex mechanism in which the enzyme is itself reversibly oxidized and reduced. This mechanism requires that values of ϕ_{12} and ϕ'_{12} be zero.

(2b) Reversible oxidation and reduction of the enzyme without the formation of an enzyme-substrate complex. This mechanism requires that the values of ϕ_0, ϕ'_0, ϕ_{12}, and ϕ'_{12} all be zero.

Two reaction mechanisms (and certain special cases), however, deserve further consideration:

(A-1) A random order of substrate binding by the enzyme involving four binary and two ternary complexes. All equilibria are rapidly adjusted except for the interconversion of the two kinetically important ternary complexes.

(A-2) The special case of (A-1) in which the binding of one substrate does not influence the affinity of the enzyme for the second substrate.

(B-1) A compulsory substrate binding order involving a single kinetically distinguishable ternary enzyme substrate complex.

(B-2) A special case of compulsory substrate binding order involving more than one kinetically significant ternary complex.

(B-3) A special case of compulsory order mechanism involving no kinetically discernible ternary complex, the mechanism proposed by Theorell and Chance (1951).

These mechanisms can be distinguished on the basis of two theoretical criteria, the relationships between kinetic coefficients, and the agreement of the apparent equilibrium constant expressed in terms of kinetic coefficients (Dalziel, 1957) with the thermodynamic equilibrium constant. Table III lists the special relationships between kinetic

TABLE III[a]

PREDICTED RELATIONSHIPS BETWEEN KINETIC COEFFICIENTS FOR VARIOUS MECHANISMS

Mechanism	Special Relations Between Coefficients[b]	K_{eq}^{app}[c] in Terms of the Kinetic Coefficients
(A-1)		$\frac{\phi'_{12}}{\phi_{12}}$
(A-2)	$\frac{\phi_1\phi_2}{\phi_{12}} = \phi_0$	$\frac{\phi'_{12}}{\phi_{12}} = \frac{\phi_0\phi'_1\phi'_2}{\phi'_0\phi_1\phi_2}$
(B-1) and (B-2)	$\frac{\phi_1\phi_2}{\phi_{12}} < \phi'_0$	$\frac{\phi'_{12}}{\phi_{12}}$
(B-3)	$\frac{\phi_1\phi_2}{\phi_{12}} = \phi'_0$	$\frac{\phi'_{12}}{\phi_{12}} = \frac{\phi'_0\phi'_1\phi'_2}{\phi_0\phi_1\phi_2}$

[a] Condensed from Dalziel (1957). [b] Relations for the reverse direction can be written by substituting primed for unprimed coefficients and removing primes from each coefficient so marked in this column. [c] Apparent over-all equilibrium.

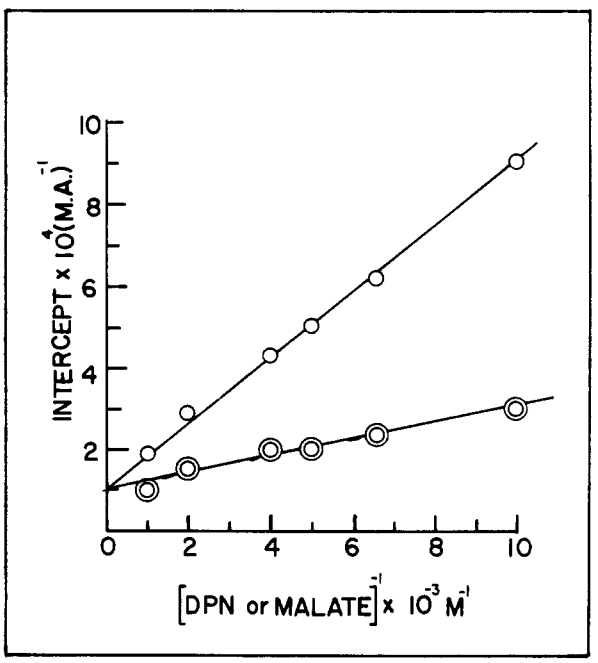

FIG. 5.—"Secondary plots" of ordinate intercepts ($1/(S) = 0$) from the series of "primary plots" given in Figures 1 and 2 as a function of the reciprocal "fixed" substrate concentration. Open circles represent data from experiments in which malate (Fig. 1) was the "fixed" concentration substrate. Concentric circles represent data from experiments in which DPN (Fig. 2) was the "fixed" concentration substrate. M.A. is the molecular activity.

coefficients as well as the apparent equilibrium constant in terms of kinetic coefficients for each of the above mechanisms. Table IV presents calculated experimental values for the various pertinent theoretical relationships listed in Table III. Mechanisms A-2, B-1, and B-2 can be excluded because the experimental data show no agreement with predicted relationships between kinetic coefficients. Mechanism B-3 is consistent with experimental data in each reaction direction by both of the distinguishing criteria.

Mechanisms A-1 and B-3 are both apparently compatible with the experimental data. It is desirable, however, to exclude one of these mechanisms in order to determine the nature of the reac-

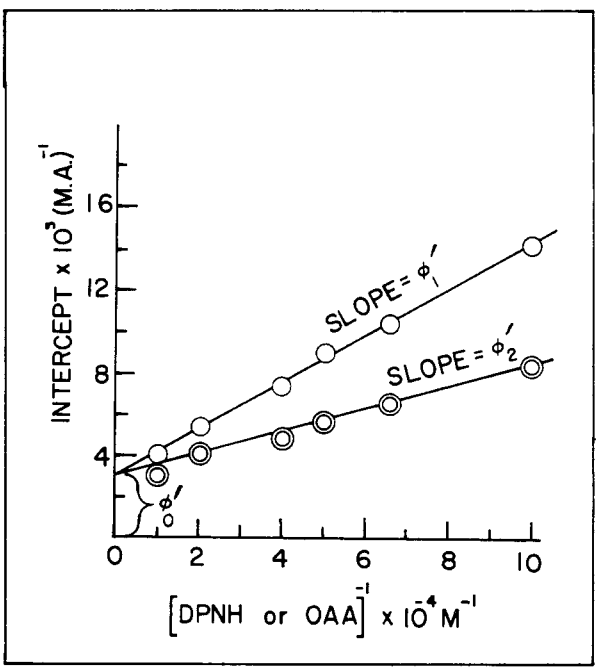

FIG. 6.—"Secondary plots" of ordinate intercepts ($1/(S) = 0$) from the series of "primary plots" given in Figures 3 and 4 as a function of the reciprocal "fixed" substrate concentration. Open circles represent data from experiments in which oxalacetate (Fig. 3) was maintained at a fixed initial concentration. Concentric circles are for data taken with DPNH at a fixed initial concentration (Fig. 4). M.A. is the molecular activity. This figure also indicates how the kinetic coefficients ϕ_0', ϕ_1', and ϕ_2' are evaluated. Unprimed kinetic coefficients are evaluated similarly from data for the opposite reaction direction.

TABLE IV

EXPERIMENTAL VALUES CALCULATED FOR VARIOUS PERTINENT DISTINGUISHING RELATIONSHIPS BETWEEN KINETIC COEFFICIENTS

Experimental Values of Pertinent Relations Listed in Table III, Column 2				Experimental Values of Apparent Over-all Equilibrium Expressions for Mechanisms Listed in Table III			K_{eq}^{app}[a]
ϕ_0	ϕ'_0	$\frac{\phi_1\phi_2}{\phi_{12}}$	$\frac{\phi'_1\phi'_2{}^{b}}{\phi'_{12}}$	$\frac{\phi'_{12}}{\phi_{12}}$	$\frac{\phi_0\phi'_1\phi'_2}{\phi'_0\phi_1\phi_2}$	$\frac{\phi'_0\phi'_1\phi'_2{}^{c}}{\phi_0\phi_1\phi_2}$	$\frac{(\text{OAA}^{d})(\text{DPNH})}{(\text{Malate})(\text{DPN})}$
1×10^{-4}	2.9×10^{-5}	2.9×10^{-5}	1.1×10^{-4}	1.0×10^{-4}	1.2×10^{-3}	1.0×10^{-4}	1.03×10^{-4}

[a] Apparent overall equilibrium, determined by measurements of equilibrium concentration in *p*H 8.0 Tris-acetate (0.05 M with respect to acetate). See Table I for experimental data. [b] Expressions for the reverse reaction (primed) included here were omitted from Table III. [c] Kinetic equilibrium expression for mechanism B-3, which is compatible with the experimental data. [d] Oxalacetic acid.

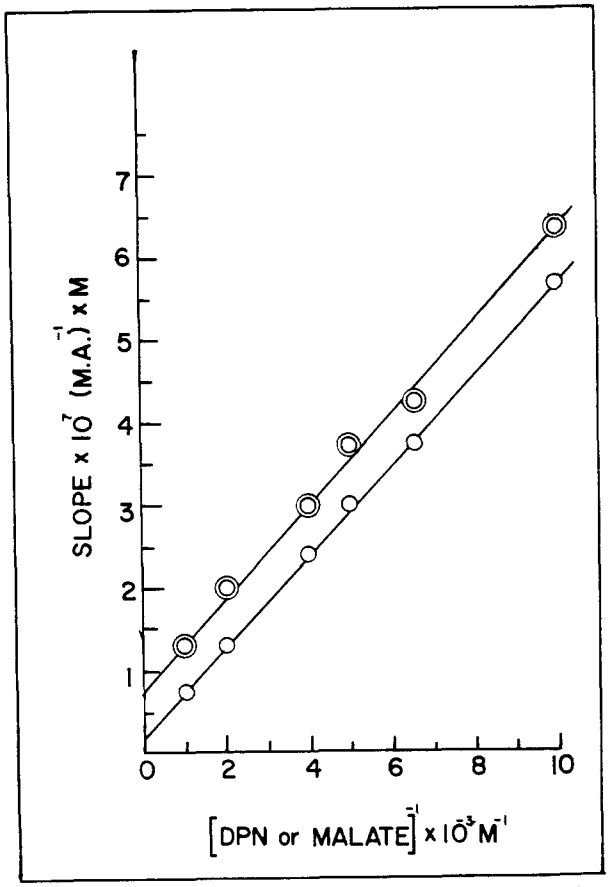

FIG. 7.—"Secondary plots" of the "primary plot" line slopes versus reciprocal "fixed" substrate concentration. Concentric circles represent data from experiments in which DPN (Fig. 2) was held at a fixed initial concentration. Open circles represent data from experiments in which malate (Fig. 1) was held at fixed initial concentration. M.A. is the molecular activity.

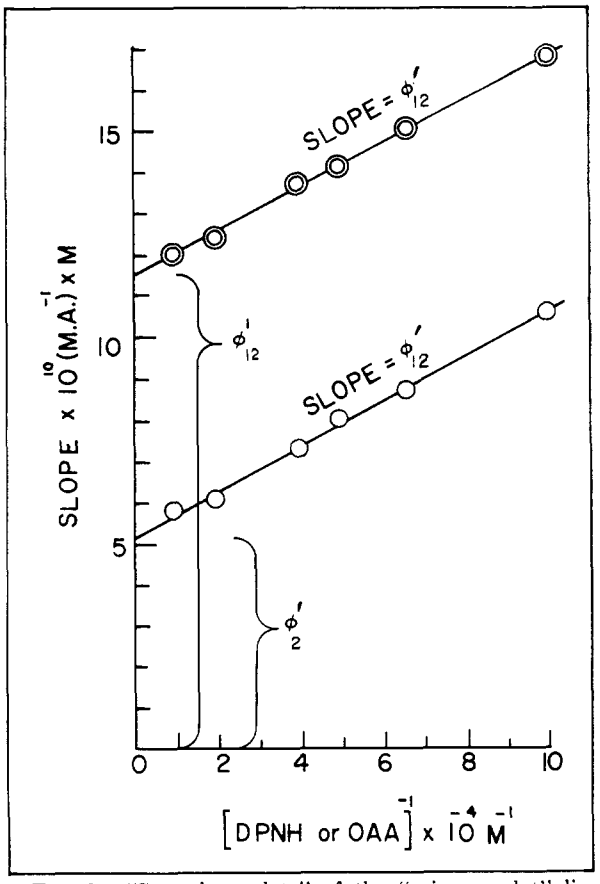

FIG. 8.—"Secondary plots" of the "primary plot" line slopes versus reciprocal "fixed" substrate concentration. Concentric circles represent data taken when the DPNH was maintained at fixed initial concentration (Fig. 4). Open circles are from data taken when oxalacetate was held at a fixed initial concentration (Fig. 3). M.A. is the molecular activity. This figure also indicates how the kinetic coefficients ϕ_1', ϕ_2', and ϕ_{12}' are evaluated. Unprimed kinetic coefficients are evaluated similarly from data for the opposite reaction direction.

tion intermediates and to evaluate appropriate rate constants. Mechanism B-3, proposed by Theorell and Chance (1951), is to be preferred for the following reasons:

(1) Two criteria support mechanism B-3, whereas one indeterminant criterion, the kinetic expression for the equilibrium constant, is available in support of mechanism A-1. The most general mechanism (A-1) would be expected to have properties in common with other mechanisms, which are, in a sense, special cases of the general mechanism. Moreover it is highly improbable that mechanism A-1, which has no predicted relationships between coefficients, would exhibit the observed experimental relationships in agreement with those predicted for mechanism B-3.

(2) Mechanism B-3 is also supported by agreement of the experimental data with the following unique relationships.

$$K_{eq}^{app} = \frac{\phi'_0 \cdot \phi'_1 \cdot \phi'_2}{\phi_0 \cdot \phi_1 \cdot \phi_2} \qquad \text{(i)}$$

and also,

$$\phi'_0 = \frac{\phi_1 \cdot \phi_2}{\phi_{12}}$$

$$\phi_0 = \frac{\phi'_1 \cdot \phi'_2}{\phi'_{12}} \qquad \text{(ii)}$$

The relationships between coefficients (ii) are a more sensitive test than the kinetic parameter expression for the equilibrium constant (i) because the former involve fewer experimental quantities.

Assuming that the mechanism proposed by Theorell and Chance (1951) is the actual one for malic dehydrogenase at 25°, in 0.05 M Tris-acetate buffer, *p*H 8.0, the equilibria involved can be expressed as follows:

$$\text{MDH} + \text{DPN} \underset{k_2 = 5.8 \times 10^2 \text{ sec.}^{-1}}{\overset{k_1 = 8.3 \times 10^5 \text{ M}^{-1} \text{ sec.}^{-1}}{\rightleftharpoons}} \text{MDH·DPN}$$

$$\text{MDH·DPN} + \text{malate} \underset{k_4 = 1.5 \times 10^7 \text{ M}^{-1} \text{ sec.}^{-1}}{\overset{k_3 = 2.0 \times 10^5 \text{ M}^{-1} \text{ sec.}^{-1}}{\rightleftharpoons}} \text{MDH·DPNH} + \text{oxalacetate} + \text{H}^+$$

$$\text{MDH·DPNH} \underset{k_6 = 3.3 \times 10^7\ \text{M}^{-1}\ \text{sec.}^{-1}}{\overset{k_5 = 1.7 \times 10^2\ \text{sec.}^{-1}}{\rightleftharpoons}} \text{MDH} + \text{DPNH}$$

Table V summarizes the experimental values of various kinetic parameters. The above mechanism is not intended to exclude explicitly the possibility of a ternary complex of the enzyme and its two substrates. It is not possible, by use of the techniques employed, to distinguish the presence of such intermediates if they are formed and removed by very rapid reactions.

TABLE V

KINETIC PARAMETERS AND CONSTANTS AT 25° IN *p*H 8.00, 0.05 M TRIS-ACETATE

Parameters and Constants	Forward Direction (Reduction of DPN)		Reverse Direction (Oxidation of DPNH)	
Michaelis constants (μM)	K_{DPN} 200	K_{Malate} 800	K_{DPNH} 18	K_{OAA}[a] 40
Maximum velocity (molecular activity[b])	V_f 1×10^4		V_r 3.5×10^4	
Dissociation constants (μM)	E·DPN 700		E·DPNH 5.1	
Kinetic constants (M^2)	$K_{DPN\cdot Malate}$ 5.5×10^{-7}		$K_{DPNH\cdot OAA}$[a] 1.9×10^{-10}	

[a] Oxalacetic acid. [b] Molecular activity is defined as moles of substrate decomposed per mole of enzyme per minute.

DISCUSSION

Since reversible oxidation of the enzyme itself (mechanism 2a) has apparently been eliminated as a possible reaction mechanism, it is difficult to imagine a direct stereospecific transfer of deuterium from substrate to coenzyme, as demonstrated by Graves and Vennesland (1956), without the occurrence of a ternary complex between both substrates and the enzyme. It might be possible to ascertain the presence of a ternary complex in the reaction sequence by the product inhibition method which was suggested by Alberty (1958). Fromm and Nelson (1961) applied the product inhibition technique in eliminating the random binding order mechanism for the enzyme ribitol dehydrogenase. The possibility of a ternary complex is supported by the recent observation of Theorell and Langan (1960) in which the addition of malate to the DPNH malic dehydrogenase complex resulted in a change in the properties of the fluorescence spectrum. Boyer (1960) has reported kinetic studies of rat liver malic dehydrogenase at *p*H 9.0 and ionic strength of 0.2. He finds that the liver enzyme also has properties consistent with the mechanism proposed by Theorell and Chance (1951).

A limited number of diphosphopyridine nucleotide enzymes have been subjected to kinetic study. Malic dehydrogenase, lactic dehydrogenase, and ribitol dehydrogenase (Theorell and Bonnichsen, 1951; Nordlie and Fromm, 1959) all appear to have a compulsory binding order mechanism in both reaction directions. Yeast and liver alcohol dehydrogenase appear to have compulsory binding order mechanisms (Dalziel, 1957). Glutamic dehydrogenase, a three-substrate enzyme, appears to have a compulsory binding order mechanism for the three substrates (Frieden, 1959). On the basis of kinetic studies of a limited number of enzymes it appears that the compulsory binding order mechanism may be a rather general one for enzymes reacting with diphosphopyridine nucleotide enzymes.

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