Malic Dehydrogenase. III. Kinetic Studies of the Reaction Mechanism by Product Inhibition*

DILIP N. RAVAL AND R. G. WOLFE

From the Chemistry Department, University of Oregon, Eugene

Received April 2, 1962

Kinetic studies of the reaction mechanism of pig heart malic dehydrogenase have been made by the method of product inhibition, at *p*H 8.0 and 25° in Tris acetate buffer (0.05 M with respect to acetate). Results of experiments in both reaction directions are in good agreement with a compulsory substrate binding order mechanism involving at least one ternary complex between the enzyme, coenzyme, and substrate. Coenzyme substrates bind first to the enzyme. The eight rate constants for the four reversible reactions involved in the ternary complex compulsory order mechanism are calculated. The dissociation of the ternary complex is found to be much faster than the dissociation of binary complexes.

Detailed initial rate kinetic studies of the reaction catalyzed by pig heart malic dehydrogenase at *p*H 8.0 and 25° were reported in paper II of this series (Raval and Wolfe, 1962). The data obtained were compatible with the two following mechanisms.[1] The first of these is a compulsory order mechanism without a ternary complex as follows:[2]

$$E + DPN \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot DPN$$

$$E\cdot DPN + M \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\cdot DPNH + OAA$$

$$E\cdot DPNH \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + DPNH$$

The second mechanism is a special case of the compulsory binding order mechanism in which a ternary complex is formed and decomposes so rapidly that it escapes detection by ordinary initial rate kinetic studies. This reaction mechanism is represented as:

$$E + DPN \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot DPN$$

$$E\cdot DPN + M \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EXY$$

$$EXY \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E\cdot DPNH + OAA$$

$$E\cdot DPNH \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + DPNH$$

(where $k_{-2} >> k_{-1}$ and $k_3 >> k_4$)

The mechanism lacking a ternary complex is often called the Theorell-Chance mechanism (1951), though it is apparent that Theorell and Chance also considered possible the special case of the ternary complex mechanism in their work on alcohol dehydrogenase.

The compatibility of the experimental data to the two mechanisms was tested by use of two criteria unique to these mechanisms. The first criterion, defined by Takenaka and Schwert (1956), expresses the expected relationships between kinetic parameters as follows:

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

The second unique criterion derived by Alberty (1953) relates the kinetic constants to the over-all thermodynamic equilibrium as follows:

$$\frac{V_f^3\cdot K_{OAA}\cdot K_{DPNH}}{V_r^3 K_{DPN}\cdot K_M} = K_{eq} \quad (2)$$

Alberty (1958) recently proposed the product inhibition method as a device for obtaining information useful in distinguishing certain possible mechanisms for two-substrate enzymes. The product inhibition method appears to be capable of discriminating between mechanisms lacking, or containing, a ternary complex, even though a possible ternary complex may have a very short half life.

Since the two criteria discussed above are

* This investigation was supported in part by a Public Health Service Grant H 3226 from the National Heart Institute.

[1] The random order mechanism could not be unequivocally excluded but it was considered highly improbable that data for the random order mechanism would satisfy the above two mechanisms.

[2] The abbreviations used in this paper include: E for enzyme, DPN, DPNH, M, and OAA for oxidized coenzyme, reduced coenzyme, malate, and oxalacetate respectively; V_f and V_r are maximum initial velocities for the forward and reverse reactions, K_X represents a Michaelis constant for the substrate represented in the subscript, $K_{X\cdot Y}$ are special kinetic constants as defined by Alberty (1953), and K_{eq} is the equilibrium constant. DPN is assumed to be the first substrate bound in the reaction.

indeterminate with regard to the presence of a ternary complex in the malic dehydrogenase mechanism, this matter was investigated by use of the product inhibition method.

Experimental Procedure

Materials.—The sources of the reagents and the isolation procedure for pig heart malic dehydrogenase have been described in previous papers (Raval and Wolfe, 1962; Wolfe and Neilands, 1956).

Method.—Initial reaction rates were estimated by measurements of the rate of change in the absorbancy at 340 mμ with a Beckman DU spectrophotometer. The temperature was maintained at 25° by water circulated 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 was used (Wolfe and Neilands, 1956).

All determinations were made at 25° in Tris acetate buffer (0.05 M with respect to acetate). The reaction was started by adding 10 ml of appropriately diluted enzyme to 20 ml of the reaction mixture contained in a cuvet having a 10-cm light path. When the product inhibition was being studied, the enzyme was added to the reaction mixture containing one product, the coenzyme, and the substrate. The concentration of the product was adjusted to produce very high (up to 80%) enzyme inhibition. The enzyme concentration was adjusted so that the initial reaction rate could be accurately ascertained.

Product inhibition by all four substrates—DPN, malate, DPNH, and oxalacetate—was investigated in detail. Measurements of reaction rates were made by varying the concentrations of both substrates independently at a fixed concentration of product inhibitor. All kinetic parameters were evaluated by extrapolation to conditions which were zero order with respect to both substrates.

Results

Four primary plots [1/v versus 1/(substrate)][3] of the product inhibition data are given in Figures 1–4. Four other primary plots of the data, which can be constructed from data included in Figures 1–4, have been omitted. Two representative secondary plots of the data are presented in Figures 5 and 6. Table I summarizes the values of the kinetic parameters obtained from studies in both reaction directions. It is apparent from Table I that the presence of DPNH in the reaction mixture containing malic dehydrogenase, DPN, and malate increases the apparent values of K_{DPN} and $K_{DPN \cdot M}$, but does not change either V_f or K_M. Similarly, in the reverse reaction product inhibition by DPN changes the apparent values of K_{DPNH} and $K_{DPNH \cdot OAA}$ only. Moreover, the inhibition constants for DPN and DPNH are found to be the same as the dissociation constants for E·DPN and E·DPNH complexes calculated from the data taken in the absence of product inhibitor.

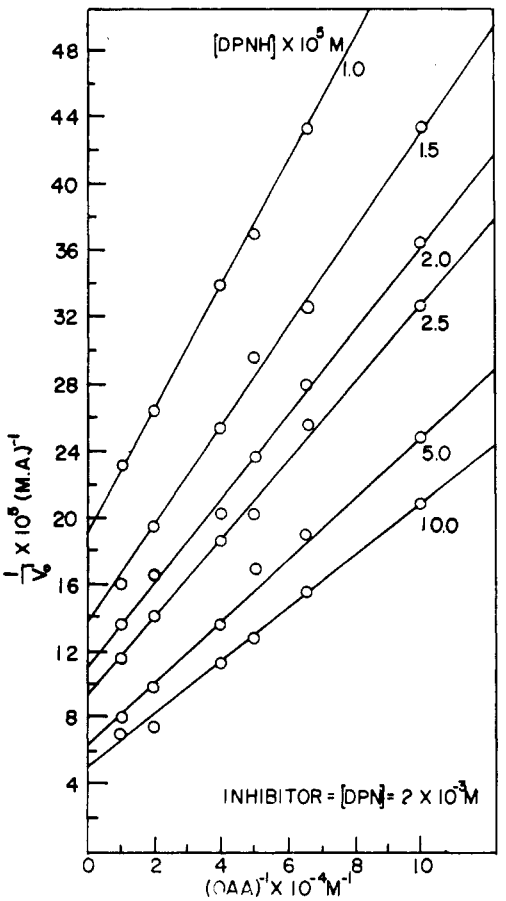

FIG. 1.—A double reciprocal plot of initial rate data taken in the presence of 2×10^{-3} M DPN as product inhibitor. Oxalacetate was the variable substrate. Concentrations of the fixed substrate (DPNH) are given under each line. Data were taken at 25° and *p*H 8.0 in Tris acetate buffer which was 0.05 M with respect to acetate.

$K_{I,DPN} = 920 \times 10^{-6}$ M

$K_{E \cdot DPN}$ (dissociation) $= 810 \times 10^{-6}$ M

$K_{I,DPNH} = 5.0 \times 10^{-6}$ M

$K_{E \cdot DPNH}$ (dissociation) $= 5.1 \times 10^{-6}$ M

These results are in agreement with the theoretical predictions involving the coenzyme as the first substrate bound by the enzyme (Alberty, 1958).

As suggested by Alberty (1958) the definitive product inhibition experiment necessary to test for the presence of a ternary complex is one in which oxalacetate (malate in the reverse reaction)

[3] $1/v_0$ is substituted for e/v_0 in the consideration of the data, since the enzyme is assumed to have only one active site and the activity is expressed as molecular activity.

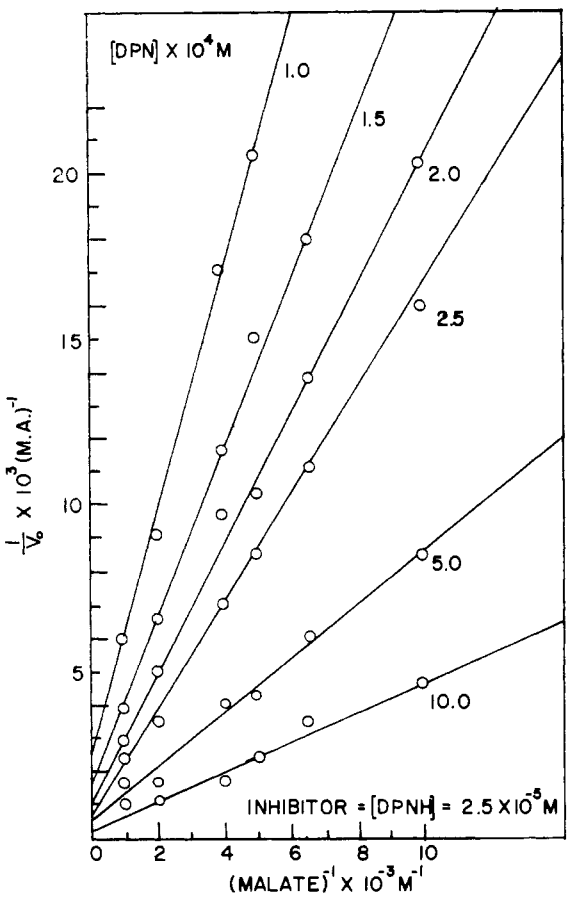

FIG. 2.—Double reciprocal plots of initial rate data taken in the presence of 2.5 × 10^{-5} M DPNH as product inhibitor. Malate was the variable substrate. Concentrations of the fixed substrate (DPN) are given under each line. Data were taken at 25° and *p*H 8.0 in Tris acetate buffer which was 0.05 M with respect to acetate.

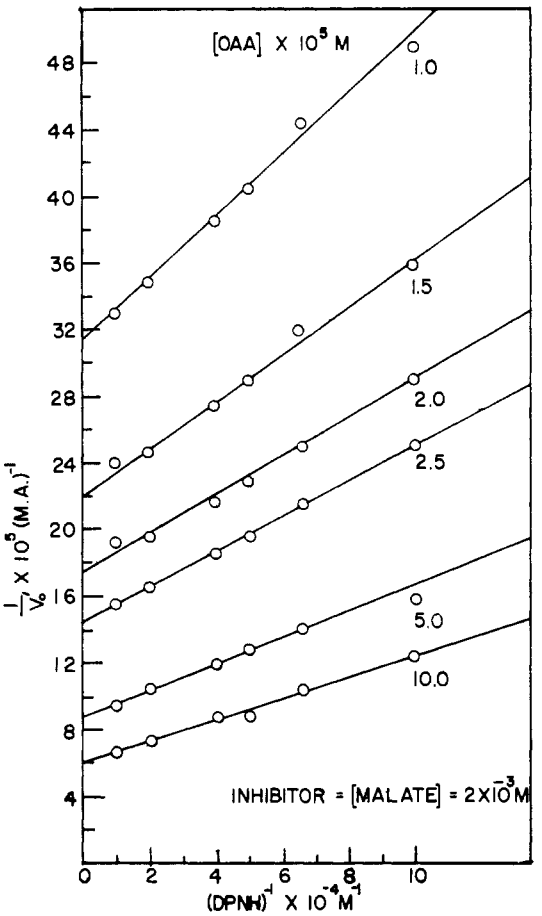

FIG. 3.—Double reciprocal plots of initial rate data taken in the presence of 2 × 10^{-3} M malate as product inhibitor. DPNH was the variable substrate. Concentrations of the fixed substrate (oxalacetate) are given under each line. Data were taken at 25° and *p*H 8.0 in Tris acetate buffer which was 0.05 M with respect to acetate.

is used as the product inhibitor. This statement is valid when oxalacetate is the first product dissociated from the enzyme in the compulsory binding order mechanism. The exact form of the rate law in the presence of a product inhibitor will depend upon the mechanism and also upon which product is present as the inhibitor. The steady state rate law expression for a ternary complex compulsory order mechanism in the presence of product oxalacetate (Alberty, 1958), assuming only a mass law effect, is:

$$\frac{e}{v_0} = \frac{1}{V_f}\left\{1 + \frac{k_{-3}}{k_3 + k_4}(OAA)\right\} + \frac{K_{DPN}}{V_f(DPN)} \cdots + \frac{K_M}{V_f(M)}\left\{1 + \frac{k_{-2}k_{-3}}{k_4(k_{-2} + k_3)}(OAA)\right\} + \frac{K_{DPN\cdot M}}{V_f(DPN)(M)}\left\{1 + \frac{k_{-2}k_{-3}}{k_4(k_{-2} + k_3)}(OAA)\right\} \quad (3)$$

The rate law expression in the case of a compulsory order mechanism lacking a ternary complex in the presence of oxalacetate is:

$$\frac{e}{v_0} = \frac{1}{V_f} + \frac{K_{DPN}}{V_f(DPN)} + \frac{K_M}{V_f(M)}\left[1 + \frac{k_{-2}}{k_3}(OAA)\right] + \frac{K_{DPN\cdot M}}{V_f(DPN)(M)}\left[1 + \frac{k_{-2}}{k_3}(OAA)\right] \quad (4)$$

Similar expressions could be written for the reverse reaction since the mechanism is symmetrical. The terms in the above equations may be evaluated as described by Dalziel (1957) or Raval and Wolfe (1962). Since the first term in the equation for the mechanism involving a ternary complex (equation 3) carries an inhibition factor it follows that the maximum initial velocity should be altered. Such an effect would not be expected for the mechanism lacking a ternary complex (equation 4). It is apparent from the data in Table I or the secondary plot in Figure 5 that a decrease in the apparent maximum initial velocity is observed experimentally. The data

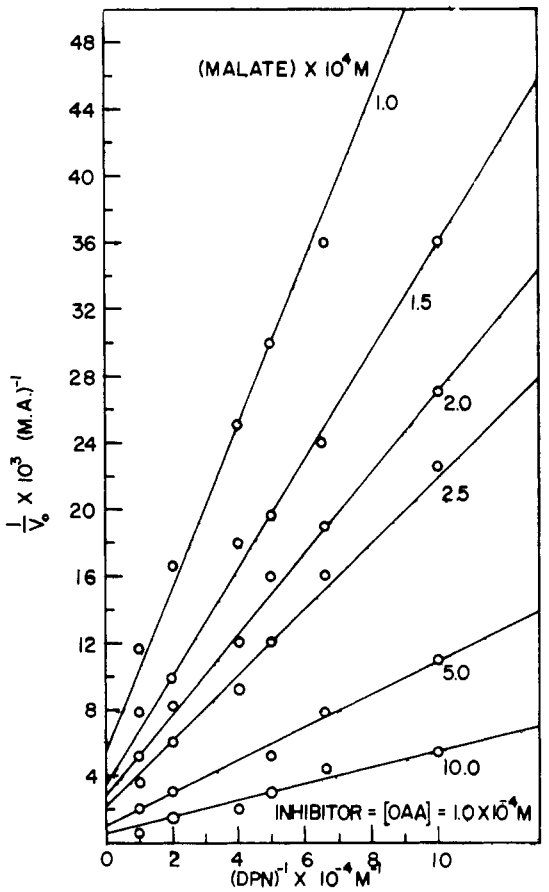

FIG. 4.—Double reciprocal plots of initial rate data taken in the presence of 1.0×10^{-4} M oxalacetate as product inhibitor. DPN was the variable substrate. Concentrations of the fixed substrate (malate) are given under each line. Data were taken in the presence of Tris acetate buffer which was 0.05 M with respect to acetate at *p*H 8.0 and at 25°.

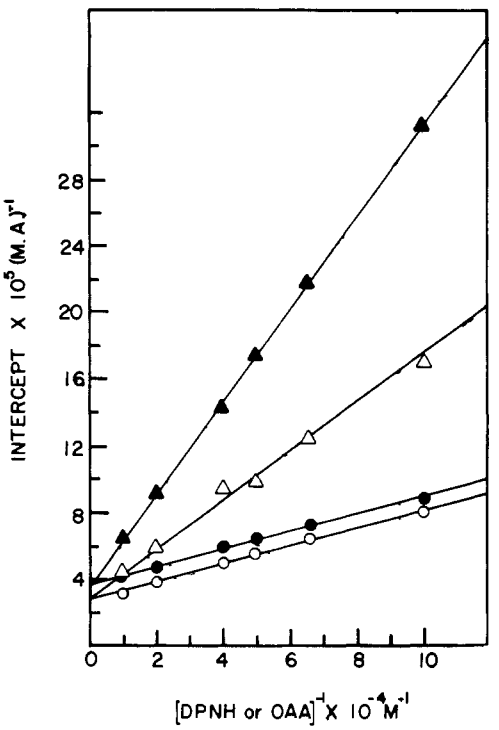

FIG. 5.—Secondary plots of primary plot intercepts (see Figure 3) versus the reciprocal of fixed substrate concentration. Circles, whether open or filled, represent data in which DPNH was present as the fixed substrate. Triangles, whether open or filled, represent data taken with oxalacetate as the fixed substrate. Open circles or triangles represent data taken in the absence of product inhibitor. Filled circles or triangles represent data taken in the presence of malate (2×10^{-3} M) as product inhibitor. All data were taken at 25° in *p*H 8.0 Tris acetate buffer.

therefore support a compulsory order mechanism with a kinetically insignificant ternary complex. If more than one ternary complex is present in the reaction mechanism this fact can not be established by known available methods.

The data also argue against the presence of a random order mechanism. It should be pointed out, however, that limitations in the sensitivity of the analytical methods make it unlikely that a subordinate reaction pathway accounting for approximately 1% or less of the reaction could be detected.

Table II shows that there is good agreement between the thermodynamic equilibrium constant and its equivalent calculated from kinetic parameters or kinetic constants obtained experimentally for a mechanism involving a kinetically insignificant ternary complex.

The reaction mechanism for malic dehydrogenase involving a compulsory substrate binding order (coenzyme binding first) and a kinetically insignificant ternary complex EXY, can now readily be written as follows:

$$\text{MDH} + \text{DPN} \underset{k_{-1} = 5.7 \times 10^{2}\ \text{Sec.}^{-1}}{\overset{k_1 = 5.5 \times 10^{5}\ \text{M}^{-1}\ \text{Sec.}^{-1}}{\rightleftharpoons}} \text{MDH·DPN}$$

$$\text{MDH·DPN} + \text{Malate} \underset{k_{-2} = 3.3 \times 10^{4}\ \text{Sec.}^{-1}}{\overset{k_2 = 2.0 \times 10^{3}\ \text{M}^{-1}\ \text{Sec.}^{-1}}{\rightleftharpoons}} \text{EXY}$$

$$\text{EXY} \underset{k_{-3} = 1.7 \times 10^{7}\ \text{M}^{-1}\ \text{Sec.}^{-1}}{\overset{k_3 = 8.3 \times 10^{3}\ \text{Sec.}^{-1}}{\rightleftharpoons}} \text{MDH·DPNH} + \text{Oxalacetate} + \text{H}^{+}$$

$$\text{MDH·DPNH} \underset{k_{-4} = 3.3 \times 10^{7}\ \text{M}^{-1}\ \text{Sec.}^{-1}}{\overset{k_4 = 1.7 \times 10^{2}\ \text{Sec.}^{-1}}{\rightleftharpoons}} \text{MDH} + \text{DPNH}$$

The rate constants were calculated from product inhibition data, assuming only one ternary complex was involved.

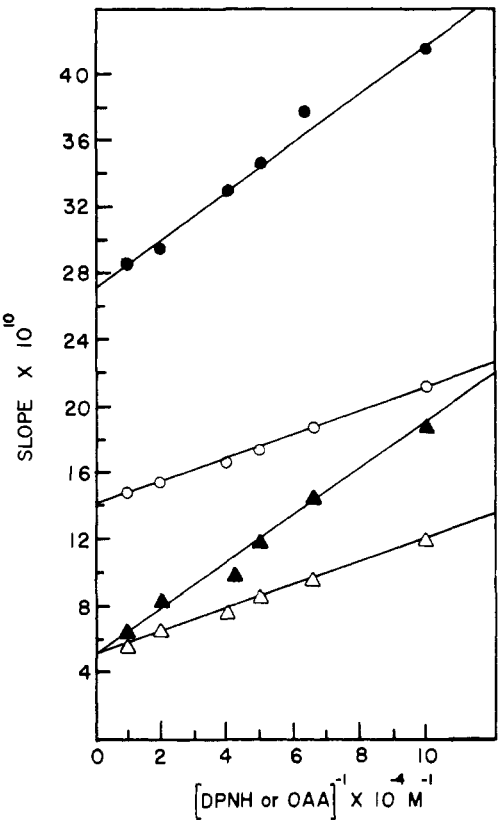

FIG. 6.—Secondary plots of primary plot slopes (see Figure 3) *versus* the reciprocal of the fixed substrate concentration. Circles, whether open or filled, represent data taken with DPNH as the fixed substrate. Triangles, whether open or filled, represent data taken with oxalacetate as the fixed substrate. Open circles or open triangles represent data taken in the absence of product inhibitor. Closed circles or triangles represent data taken in the presence of malate (2×10^{-3} M) as product inhibitor. All data were taken at 25° in pH 8.0 Tris acetate buffer.

TABLE I

EXPERIMENTAL VALUES OF KINETIC CONSTANTS IN VARIOUS REACTION MIXTURES

(These were evaluated from "secondary plots" and in in some cases "apparent" rather than true values are given)

Reaction Mixture	V_f (M.A.[a]) $\times 10^{-3}$	K_{DPN} M $\times 10^4$	K_M M $\times 10^4$	$K_{DPN \cdot M}$ $M^2 \times 10^7$
Forward Direction				
MDH + DPN + Malate	10.0	3.0	6.0	6.3
MDH + DPN + Malate + DPNH (2.5×10^{-5} M)	10.0	18.0[c]	6.0	37.0[c]
MDH + DPN + Malate + Oxalacetate (1.0×10^{-4} M)	8.3[b]	3.1	40.5[c]	40.0[c]
Reverse Direction				
Reaction Mixture	V_r (M.A.[a]) $\times 10^{-3}$	K_{DPNH} M $\times 10^5$	K_{OAA} M $\times 10^6$	$K_{DPNH \cdot OAA}$ $M^2 \times 10^{10}$
MDH + DPNH + OAA	33.0	1.6	4.75	2.4
MDH + DPNH + OAA + DPN (2×10^{-3} M)	33.0	5.35[c]	4.8	7.85[c]
MDH + DPNH + OAA + Malate (2×10^{-3} M)	29.0[b]	1.6	9.0[c]	4.7[c]

[a] Molecular activity—formerly termed the turnover number. [b] Apparent maximum initial velocities. [c] Apparent values of Michaelis constants. Apparent values are true values multiplied by an inhibition factor.

DISCUSSION

When one of the reaction products is present initially in the reaction mixture, the initial rate of the reaction is reduced. This reduction in the initial rate could possibly be due to two main influences. First, the product could compete with either one of the substrates to form an inactive binary complex (between enzyme and product inhibitor) or an inactive ternary complex (for example one between enzyme, coenzyme, and the wrong substrate). The formation of such an inactive complex would reduce the concentration of free enzyme or active enzyme-substrate complex. Second, the presence of product could, by the law of mass action, shift the equilibrium of the reaction step in which it is formed.

Fromm and Nelson (1962) and Zewe and Fromm (1962) have found product inhibition data for ribitol dehydrogenase and muscle lactic dehydrogenase, respectively, to be consistent with the formation of inactive binary and ternary complexes as well as the mass law effect. The formation of an inactive ternary complex (between enzyme, coenzyme, and the wrong substrate) was observed by them only when relatively high concentrations of the product inhibitor were employed.

When malate or oxalacetate was used as the product inhibitor for malic dehydrogenase the data were consistent with a mass law effect only. When DPN or DPNH was used as a product inhibitor an inactive binary complex between the enzyme and the product-coenzyme was observed. The fact that relatively low concentrations of product inhibitor were used may account for the inability to observe the presence of an inactive ternary complex. The latter speculative statement is based on the following two observations: (1) Theorell and Langan (1960) have reported spectrofluorometric evidence for the occurrence of E·DPNH·malate as a ternary complex at relatively high concentrations of malate. (2) Unpublished studies of substrate inhibition of malic dehydrogenase by oxalacetate in this laboratory

TABLE II

SUMMARY OF EXPERIMENTAL VALUES OF THE OVER-ALL EQUILIBRIUM CONSTANT CALCULATED FROM KINETIC AND EQUILIBRIUM CONSTANTS, AND EXPERIMENTAL VALUES OF PERTINENT RELATIONSHIPS BETWEEN KINETIC CONSTANTS, ASSUMING THE COMPULSORY BINDING ORDER MECHANISM WITH A KINETICALLY INSIGNIFICANT TERNARY COMPLEX

Expected Relations (Experimental values in parentheses)	Equilibrium Constant		
	$\frac{k_1 \cdot k_2 \cdot k_3 \cdot k_4}{k_{-1} \cdot k_{-2} \cdot k_{-3} \cdot k_{-4}}$	$\frac{V_f^3 \cdot K_{DPNH} \cdot K_{OAA}}{V_r^3 \cdot K_{DPN} \cdot K_M}$	$\frac{(DPNH)\ (OAA)\ (H)}{(DPN)\ (Malate)}$
$\frac{K_{DPN} \cdot K_M}{K_{DPN \cdot M} \cdot V_f} = 1/V_r$ (2.9×10^{-5}) (3.0×10^{-5})	1.14×10^{-12}	1.18×10^{-12}	1.04×10^{-12}
$\frac{K_{DPNH} \cdot K_{OAA}}{K_{DPNH \cdot OAA} \cdot V_r} = 1/V_f$ (0.96×10^{-4}) (1.0×10^{-4})			

are consistent with the formation of an inactive E·DPN·OAA complex at very high oxalacetate concentrations.

Ternary complex compulsory binding order mechanisms have been reported for liver alcohol dehydrogenase by Baker (1962), for rabbit muscle lactic dehydrogenase by Zewe and Fromm (1962), for beef heart muscle lactic dehydrogenase by Takenaka and Schwert (1956), and for ribitol dehydrogenase by Fromm and Nelson (1962). It appears that the ternary complex compulsory order mechanism may be a rather general one for the diphosphopyridine nucleotide dehydrogenases.

REFERENCES

Alberty, R. A. (1953), *J. Am. Chem. Soc. 75*, 1928.

Alberty, R. A. (1958), *J. Am. Chem. Soc. 80*, 1777.

Baker, R. H., Jr. (1962), *Biochemistry 1*, 41.

Dalziel, K. (1957), *Acta Chem. Scand. 11*, 1706.

Fromm, H. J., and Nelson, D. R. (1962), *J. Biol. Chem. 237*, 215.

Raval, D. N., and Wolfe, R. G. (1962), *Biochemistry 1*, 263.

Takenaka, Y., and Schwert, G. W. (1956), *J. Biol. Chem. 223*, 157.

Theorell, H., and Chance, B. (1951), *Acta Chem. Scand. 5*, 1127.

Theorell, H., and Langan, T. H. (1960), *Acta Chem. Scand. 14*, 933.

Wolfe, R. G., and Neilands, J. B. (1956), *J. Biol. Chem. 221*, 61.

Zewe, V., and Fromm, H. J. (1962), *J. Biol. Chem. 237*, 1668.