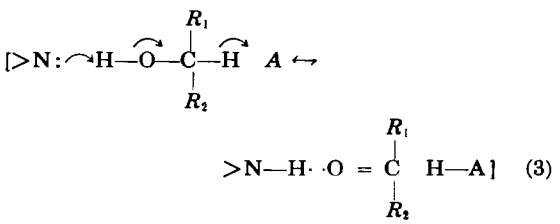
a pK_a' of 5.65, which they obtained from a *p*H-activity curve, was associated with the reduced FMN bound to the enzyme. Since these measurements were performed at substrate concentrations close to saturation, any assignment to groups in the *free* enzyme is highly speculative (see Alberty, 1956, and Krupka and Laidler, 1960, for a more complete discussion of this point.) A pK_a' of 5.68 has been obtained by us from a *p*H-activity curve at near saturating substrate levels and a pK_a' of 6.3 from a *p*H-activity curve at non-saturating substrate levels. The pK_a' determined under the latter conditions more nearly approximated the true pK_a' of the free enzyme, as shown by the results reported here (in agreement also with the theoretical discussion given by Krupka and Laidler, 1960). In any event, bonding of a base on the enzyme to the hydroxyl proton of a carbinol substrate, followed by the formation of the conjugate acid simultaneously with the dehydrogenation proper (equation 3), may well be a characteristic feature of most dehydrogenase mechanisms.



(3)

ACKNOWLEDGMENTS

We are happy to express our thanks to the Red Star Yeast Co. (especially to Dr. J. Reed of that company), Milwaukee, Wis., who supplied the vast quantities of yeast needed for the preparation of the enzyme used in these studies. Miss Frances Oltman provided capable technical assistance for most of the experimental work reported here.

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Malic Dehydrogenase. V. Kinetic Studies of Substrate Inhibition by Oxalacetate*

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Detailed initial rate kinetic studies of substrate inhibition by oxalacetate, at *p*H 8.0 in 0.05 M Tris acetate buffer, are reported. Substrate inhibition by oxalacetate occurs at concentrations above approximately 2.5×10^{-4} M when the DPNH concentration is 1×10^{-4} M or less. The experimental data are consistent with a reaction mechanism involving competitive inhibition by the formation of an inactive E·oxalacetate complex, and independent uncompetitive inhibition by the formation of an E·DPN·oxalacetate complex. A previously unpublished method of evaluating kinetic parameters is presented.

Initial rate studies of a considerable number of enzymes have shown that deviations from the predictions of the Michaelis-Menten theory are surprisingly common at relatively high substrate concentrations. Two fundamentally different types of deviation are observed. In the first type of deviation the reaction rate is apparently inhibited at high substrate concentration. The second, less commonly observed deviation is that in which the reaction is apparently accelerated at relatively high substrate concentrations. These deviations have come to be known as "substrate inhibition" and "substrate activation" respectively.

Substrate activation or inhibition is observed in various types of enzymes, including hydrolases, transferases, and oxidoreductases. Moreover, the number of substrates participating in the reaction seems to have no obvious relationship to the occurrence of this anomalous behavior. For example, Wolf and Niemann (1959) have observed substrate activation of the enzyme α-chymotrypsin by the substrate methylace-

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turate, Frieden (1959) has observed substrate activation of glutamic dehydrogenase by high concentrations of glutamate, Wold and Ballou (1957) have reported substrate inhibition of the enzyme enolase by glyceric acid 2-phosphate, and Bodansky (1961) has reported substrate inhibition of phosphoglucomutase. Other examples too numerous to mention are present in the literature.

Previous studies of malic dehydrogenase by Wolfe (1955), Davies and Kun (1957), Pfleiderer and Hohnholz (1959), and Siegel and Englard (1960) have shown that high substrate concentrations produce activation or inhibition. More recently Raval and Wolfe (1962a) have shown that substrate inhibition of malic dehydrogenase is produced by DPN, DPNH, and oxalacetate. Substrate activation of malic dehydrogenase by high concentrations of malate was also reported.

The relatively common and apparently indiscriminate occurrence of substrate activation and inhibition among a variety of enzymes suggests that this behavior may in some way be related to the poorly understood properties of enzymes as catalysts. Consequently, detailed studies of substrate inhibition and activation of malic dehydrogenase were undertaken. The results of part of this study, substrate inhibition by oxalacetate, are presented in this communication.

Experimental Procedure

Tris 121[1] and DPNH were obtained from the Sigma Chemical Company. Oxalacetic acid was obtained from the California Corporation for Biochemical Research, and reagent-grade sodium sulfate was obtained from Baker and Adamson.

The enzyme isolation and assay methods used were identical to those described previously (Raval and Wolfe, 1962a). Since it was desirable to study the influence of oxalacetate at high concentrations, the DPNH concentrations employed were restricted to a range in which they produced no substrate inhibition. Oxalacetate concentration was varied over a wide range so as to cover (*i*) the concentration range in which initial velocity was in agreement with the predictions of the Michaelis-Menten theory, and (*ii*) the concentration range which produced substrate inhibition by oxalacetate of up to 90%.

Initial rate kinetic studies of monovalent (Tris acetate) and divalent (sodium sulfate) anions were carried out to study possible nonspecific ionic strength effects. The maximum ionic strength used in this ionic strength study was greater than that produced by the highest oxalacetate concentration as outlined above.

All kinetic measurements were carried out at *p*H 8.0 in Tris acetate buffer which was 0.05 M with respect to acetate (ionic strength was 0.05). The temperature was held constant at 25° in all experiments. Triplicate assays were made at each substrate concentration.

An IBM 1620 computer was used in the statistical analysis of all data by the method of Wilkinson (1961). A least squares fit to a hyperbola program was used in evaluating the apparent values of the kinetic parameters at fixed concentrations of the second substrate. Employing data taken at oxalacetate concentrations less than 2.5×10^{-4} M, the output from the hyperbola program, with weighting factors, was used in a least squares fit to a straight line program to evaluate the zero order kinetic constants. Weighted output results from the hyperbola program were evaluated for least squares fit to a bell-shaped curve.

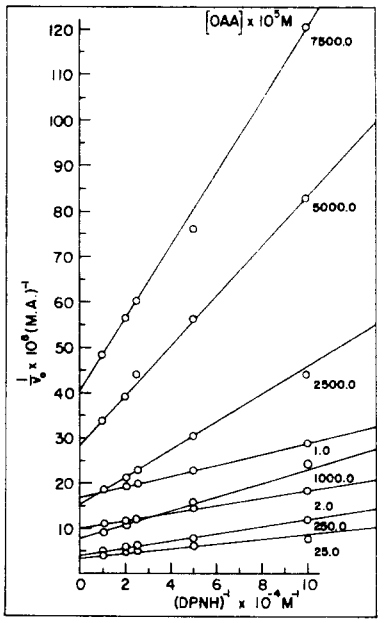

FIG. 1.—Plots of reciprocal initial velocity versus reciprocal DPNH concentration. The concentration of the fixed substrate, oxalacetate (OAA), is given under each line. The figure shows the range of concentrations employed in this study, but curves at OAA concentrations of 4.0, 5.0, 10.0, 50.0, 100.0, 500.0, and 750 (all $\times 10^{-5}$ M) have been omitted in order to simplify the presentation. Experimental points represent the average of three measurements.

Results

Figure 1 shows that double reciprocal plots [$1/v_0$ versus $1/(\text{DPNH})$][2] are linear for all concentrations of oxalacetate in the range from 1×10^{-6} to 7.5×10^{-2} M. This indicates that DPNH did not produce substrate inhibition at the concentrations used. Figure 2 shows that the double reciprocal plots of $1/v_0$ versus $1/(\text{OAA})$ is a complex, nonlinear function when studied over a very large substrate concentration range. Malic dehydrogenase apparently obeys the simple Michaelis-Menten theory at oxalacetate concentrations less than 2.5×10^{-4} M under our experimental conditions. Substrate inhibition apparently occurs at concentrations in excess of 2.5×10^{-4} M. Since both the slope and the intercepts of these plots are observed to be increased with increasing oxalacetate concentration in the range producing substrate inhibition (see Fig. 1), the inhibition is of a mixed type.[3]

Malic dehydrogenase apparently has a compulsory order substrate binding mechanism in which the presence of a ternary complex is not distinguishable by

[1] The following abbreviations are used throughout this paper: DPNH = reduced coenzyme, OAA = oxalacetate, EXY = active ternary complex, DPN = oxidized coenzyme, M = malate, v_0 = initial prevailing velocity, and Tris = tris(hydroxymethyl)aminomethane (Tris 121 is primary standard-grade reagent).

[2] The expression $1/v_0$ is substituted for e/v_0 in treating the data, since the activity is expressed as molecular activity and one active site per molecule of enzyme is assumed.

[3] If in the double reciprocal plots $1/v_0$ *vs.* $1/(S)$: (1) the ordinate intercept ($1/v_0 = 0$) is unchanged by the presence of inhibitor, the inhibition is of the competitive type; (2) the ordinate intercept is changed but the slope unaltered by the presence of inhibitor, the inhibition is uncompetitive; (3) the ordinate intercept and the slope are both altered by the presence of inhibitor, the inhibition is said to be of the mixed competitive and uncompetitive type.

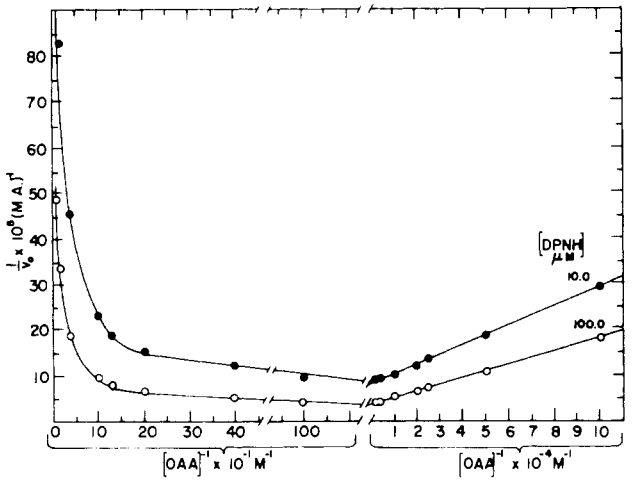

FIG. 2.—Plots of reciprocal initial velocity versus reciprocal oxalacetate (OAA) concentration. The concentration of DPNH, the fixed substrate, is given above each line. Solid circles represent data taken at the lowest concentration of the fixed substrate (1×10^{-5} M DPNH) used in this study. Open circles represent data taken at the maximum concentration (1×10^{-4} M) of DPNH used in this study. Data obtained at the intermediate concentrations of DPNH are not included in the interest of simplicity. The three highest OAA concentrations were corrected for ionic strength effects. Experimental points represent the average of three velocity measurements.

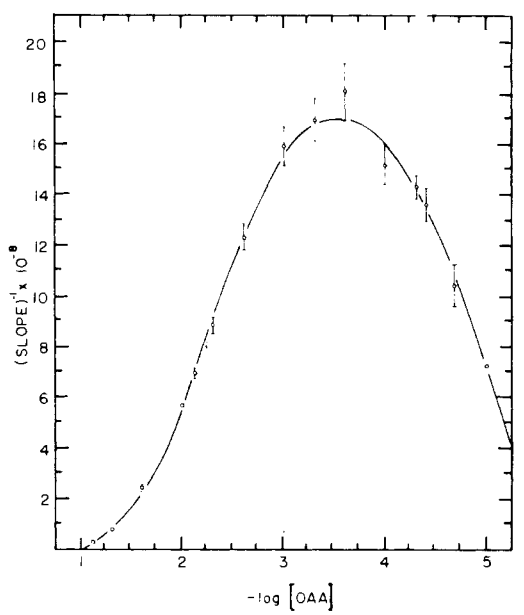

FIG. 3.—A plot of the reciprocal of the slope of each line in Figure 1 against the −log (OAA), the fixed concentration substrate. The vertical bars represent the standard error which was obtained by analysis of least squares fit to a hyperbola. Standard errors of 0.15 ordinate units or less have been omitted. The smooth curve is a least squares fit curve calculated from the data with the application of weighting factors.

ordinary initial rate kinetic studies (Raval and Wolfe 1962a,b). Assuming that at high concentrations (greater than 2.5×10^{-4} M) oxalacetate forms an inactive ternary complex E·DPN·OAA and also an inactive binary complex E·OAA, the mechanism for substrate inhibition by oxalacetate can be written as follows:

$$\mathrm{E} + \mathrm{DPNH} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E{\cdot}DPNH} \qquad (a)$$

$$\mathrm{E} + \mathrm{OAA} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E{\cdot}OAA}\ (K'_I) \qquad (b)$$

$$\mathrm{E{\cdot}DPNH} + \mathrm{OAA} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{EXY} \qquad (c)$$

$$\mathrm{EXY} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \mathrm{E{\cdot}DPN} + \mathrm{M} \qquad (d)$$

$$\mathrm{E{\cdot}DPN} + \mathrm{OAA} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \mathrm{E{\cdot}DPN{\cdot}OAA}\ (K''_I) \qquad (e)$$

$$\mathrm{E{\cdot}DPN} \underset{k_{-6}}{\overset{k_6}{\rightleftharpoons}} \mathrm{E} + \mathrm{DPN} \qquad (f)$$

where K'_I and K''_I are inhibition constants.[4] The steady state treatment of this mechanism by the method of King and Altman (1955) gives the following rate equation in terms of kinetic parameters.[5]

$$e/v_0 = \left\{\phi_0 + \frac{(\mathrm{OAA})}{K''_I k_6} + \frac{\phi_2}{(\mathrm{OAA})}\right\} + \left\{\left(1 + \frac{(\mathrm{OAA})}{K'_I}\right)\left(\phi_1 + \frac{\phi_{12}}{(\mathrm{OAA})}\right)\right\} \frac{1}{(\mathrm{DPNH})} \qquad (1)$$

With low concentrations of oxalacetate such that

$$\phi_0 >> (\mathrm{OAA})/K''_I k_6 \qquad (2)$$

$$1 >> (\mathrm{OAA})/K'_I \qquad (3)$$

the initial rate data will conform to simple Michaelis-Menten kinetic behavior. As the concentration of oxalacetate is increased the curve representing a double reciprocal plot [$1/v_0$ *versus* $1/(\mathrm{OAA})$] passes through a minimum value, increasing at higher oxalacetate concentrations (smaller $1/(\mathrm{OAA})$ values) because of substrate inhibition.

The kinetic constants, ϕ_0, ϕ_1, ϕ_2, and ϕ_{12} can be evaluated as described by Dalziel (1957) if the data obtained at low concentrations are used so that equations (2) and (3) apply (no substrate inhibition is apparent). Since this method fixes the upper limit of the substrate concentration that can be used, an alternate method is proposed as follows. If the expression for the slope term of equation (1) is rearranged as:

$$\mathrm{Slope} = \phi_1 + \phi_{12}/K'_I + \phi_1(\mathrm{OAA})/K'_I + \phi_{12}/(\mathrm{OAA}) \qquad (4)$$

it is apparent that a plot of the reciprocal of the slope against −log (OAA) over a wide concentration yields a bell-shaped curve (see Fig. 3) from which ϕ_1, ϕ_{12}, and K'_I may be determined. The equations used in computing the values of these parameters are derived in the appendix. The possibility of applying this method of evaluating inhibition constants was suggested by Alberty as cited by Dalziel (1957). Similar treatment of the intercept data obtained from Figure 1 will give a bell-shaped curve [1/intercept versus −log (OAA)] as depicted in Figure 4. The values of ϕ_0, ϕ_2, and $K''_I k_6$ can be calculated as discussed in the appendix. Since k_6 is known (Raval and Wolfe, 1962b),[6] K''_I can be calculated. It is therefore possible to

[4] The data are also compatible with a perhaps less plausible alternate to reaction (*e*) above. This is:

$$\mathrm{EXY} + \mathrm{OAA} \rightleftharpoons \mathrm{EXY{\cdot}OAA}$$

These two possibilities cannot be distinguished with the available kinetic data.

[5] $\phi_0 = 1/V$, $\phi_1 = K_{\mathrm{DPNH}}/V$, $\phi_2 = K_{\mathrm{OAA}}/V$, $\phi_{12} = K_{\mathrm{DPNH{\cdot}OAA}}/(\mathrm{DPNH})(\mathrm{OAA})$.

[6] Since different symbols were used, the constant k_6 in this publication is the same rate constant as k_4 in the previous paper (Raval and Wolfe, 1962a).

evaluate all of the kinetic parameters and the inhibition constants by this method. Table I lists the values of these experimental parameters and compares them with values obtained at low OAA concentrations when obtained by linear extrapolation. It is apparent that the two inhibition constants have nearly identical expermental values.

TABLE I

EXPERIMENTAL VALUES OF VARIOUS KINETIC COEFFICIENTS AND THE INHIBITION CONSTANTS AT *p*H 8.0, AND 25°, IN 0.05 M TRIS ACETATE BUFFER

Coefficients and Constants	Obtained from Substrate Inhibition Studies[a]	Obtained by Extrapolation[b]
ϕ_0	3.0×10^{-5}	$2.9 \pm 0.04 \times 10^{-5}$
ϕ_1	4.8×10^{-10}	$5.3 \pm 0.13 \times 10^{-10}$
ϕ_2	1.3×10^{-9}	$1.4 \pm 0.03 \times 10^{-9}$
ϕ_{12}	1.0×10^{-14}	$6.8 \pm 0.3 \times 10^{-15}$
K'_I	5.0×10^{-3} M	—
K''_I	5.7×10^{-3} M	—

[a] Values given were calculated from least squares fit to bell-shaped curve data using slope equation (2) (in the appendix) and the corresponding intercept equation. [b] Values given are those obtained by extrapolation to zero-order conditions with respect to both substrates. Apparent values were evaluated by least squares fit to a hyperbola at each of a series of "fixed substrate concentrations." Zero-order values were obtained by least squares fit to a straight line analysis of V^{-1} versus (fixed concentration substrate)$^{-1}$ using apparent values and weighting factors [(S. E.)$^{-2}$] from the fit of a hyperbola treatment of the data.

DISCUSSION

The competitive part of the inhibition might occur through oxalacetate binding reversibly at the DPNH binding site or through enzyme configurational alteration when combined at some locus adjacent to the DPNH binding site.

The uncompetitive part of the inhibition could occur when oxalacetate combines with certain reaction intermediates. There are three possible ways that oxalacetate might inhibit uncompetitively with respect to DPNH; (1) by forming an inactive ternary complex, E·DPNH·OAA, (2) by forming an inactive quaternary complex, EXY·OAA, and (3) by forming an inactive ternary complex, E·DPN·OAA. Since at lower concentrations OAA combines with the E·DPNH complex to give an active and important intermediate it is hard to conceive of the formation of an inactive E·DPNH·OAA complex. The formation of EXY·OAA or E·DPN·OAA complexes is theoretically possible in the case of malic dehydrogenase. Spectrophotometric studies support the existence of inactive complexes between enzyme, coenzyme, and the wrong substrate in the case of malic dehydrogenase (Theorell and Langen, 1960), and lactic dehydrogenase (Fromm, 1961). The E·DPN·OAA complex seems more plausible because product inhibition data from ribitol dehydrogenase (Fromm and Nelson, 1962) and lactic dehydrogenase (Zewe and Fromm, 1962) are consistent with the formation of ternary complexes of enzyme, coenzyme, and wrong substrate. Moreover, the nearly identical values of K''_I and K'_I argue for a similar binding mechanism in both the competitive and the uncompetitive inhibition reactions. If the proposed mechanism is correct, the presence of DPN apparently has little effect on oxalacetate binding by the enzyme.

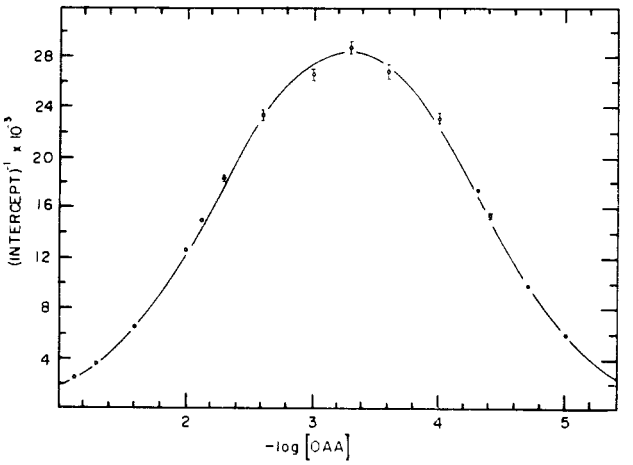

FIG. 4.—A plot of the reciprocal of the ordinate intercept ($1/(S) = 0$) of each experimental double reciprocal plot against −log (OAA), the fixed concentration substrate. The vertical bars represent the standard error obtained by least squares fit to a hyperbola analysis of the data. Bars were omitted if standard error values were less than 0.2 ordinate unit. The smooth curve is a calculated least squares fit to the data.

If one assumes that a single ternary complex is present in the compulsory substrate binding order mechanism it is possible to calculate the value of the dissociation constant for oxalacetate (from E·DPNH·OAA) using initial rate data. The value of this constant is 100 times smaller than the inhibition constant values for oxalacetate which are reported in this paper. If the assumed mechanism is correct the dissociation constants for the E·DPNH·OAA complex is quite different from that for the E·DPN·OAA complex. This would seem to imply an altered or possibly a different binding site for oxalacetate in these two complexes. If, however, more than one ternary complex is present in the reaction mechanism it may not be possible to calculate the true dissociation constant for oxalacetate in the E·DPNH·OAA complex and this comparison of dissociation and inhibition constants is not possible.

The E·OAA complex is proposed as an intermediate in the case of the random substrate binding order mechanism. The observation that this complex is apparently inhibitory might be interpreted as an argument against the presence of the random order mechanism with one very subordinate pathway. Some caution seems desirable in interpreting the data in this way since it appears possible that oxalacetate may be bound by the enzyme in at least two different ways (*vide supra*).

APPENDIX

$$\text{Slope} = \phi_1 + \frac{\phi_{12}}{K'_I} + \frac{\phi_1}{K_I'}[\text{OAA}] + \frac{\phi_{12}}{[\text{OAA}]} \qquad (1)$$

$$\therefore 1/\text{Slope} = \frac{1/\left(\phi_1 + \frac{\phi_{12}}{K'_I}\right)}{1 + \frac{\phi_1}{(\phi_1 K'_I + \phi_{12})}\cdot[\text{OAA}] + \frac{\phi_{12}}{\left(\phi_1 + \frac{\phi_{12}}{K'_I}\right)}\cdot\frac{1}{[\text{OAA}]}} \qquad (2)$$

For large values of (OAA) [low -log (OAA)] the term $\phi_1/\phi_1 K'_I + \phi_{12}$ will dominate the denominator, and, as (OAA) gets sufficiently large, 1 slope approaches zero.

For very small values of (OAA) [high -log (OAA)], the term

$$\phi_{12}/\phi_1 + \frac{\phi_{12}}{K'_I}$$

will dominate the denominator, and for sufficiently small (OAA) values 1/slope will again approach zero. There must, therefore, be a maximum value of 1/slope in the range of (OAA) somewhere. Since (OAA) is confined to the denominator, when the derivative of the denominator with respect to (OAA) becomes zero, *i.e.*, when

$$\phi_1/(\phi_1 K'_I + \phi_{12}) - \phi_{12}/\left(\phi_1 + \frac{\phi_{12}}{K'_I}\right)\frac{1}{(\text{OAA})^2} = 0$$

1/slope has the maximum value.

Thus, the plot of 1/slope versus −log (OAA) will be a bell-shaped curve with a *maximum* when

$$(\text{OAA})^2_{max} = \phi_{12}K'_I/\phi_1 \qquad (3)$$

Substituting the above equation in equation (1), the maximum value of 1/slope is

$$1/\text{slope}_{max} = \left(\sqrt{\phi_1} + \sqrt{\phi_{12}/K'_I}\right)^{-2} \qquad (4)$$

If $(\text{OAA})_1$ is the oxalacetate concentration at which

$$(1/\text{slope})/(1/\text{slope}_{max}) = 1/2$$

on the left hand side of the bell-shaped curve and $(\text{OAA})_2$ is the concentration of oxalacetate at the corresponding point on the right hand side of the curve then

$$(\text{OAA})_1 = \frac{\phi_1 K'_I + \phi_{12}}{\phi_1} \qquad (5)$$

and

$$(\text{OAA})_2 = \frac{\phi_{12}}{\phi_1 + \frac{\phi_{12}}{K'_I}} \qquad (6)$$

Since the values of $(\text{OAA})_{max}$, $1/\text{slope}_{max}$, $(\text{OAA})_1$, and $(\text{OAA})_2$ can be read directly from the curve of 1/slope versus −log (OAA) (Fig. 3), ϕ_1, ϕ_{12}, and K'_I can be calculated by using equations (3), (4), (5), and (6). A correction in the values of $(\text{OAA})_1$ and $(\text{OAA})_2$ obtained from the bell-shaped curve is necessary if the negative logs of their values differ by less than three units. Correct values given in the Table were obtained by computer calculation.

A similar treatment for the intercept can be derived readily.

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The Use of Deuterated DPNH Mixtures as an Aid in Establishing Dehydrogenase Mechanism*

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The use of a mixture of the stereoisomers of DPND as a coenzyme in dehydrogenase reactions may provide information permitting a distinction between reaction mechanisms. In the case of an equilibrium mechanism, there is a linear relationship between the reciprocal of the initial velocity (v) and the reciprocal of either the substrate (S) or coenzyme (C) concentration. For steady-state mechanisms, however, $1/v$ varies linearly with $1/C$, but not with $1/S$. Data are presented for two enzymes, yeast alcohol dehydrogenase and rabbit muscle lactate dehydrogenase, which obey different mechanisms.

The kinetic equations for an enzyme acting on a mixture of two substrates have been derived for unimolecular reactions. Whether a steady-state (Reiner, 1959) or a rapid-equilibrium (Thorn, 1949) mechanism is postulated, the following expression holds:

$$v = \frac{V_1 S_1}{S_1 + K_1(1 + S_2/K_2)} + \frac{V_2 S_2}{S_2 + K_2(1 + S_1/K_1)} \qquad (1)$$

where S_1 and S_2 are the concentrations of the two substrates, K_1 and K_2 are the Michaelis constants, and V_1 and V_2 are the maximum velocities obtainable with each substrate individually.

When the concentration of S_1 is any fraction a of

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