mechanisms are operating, namely, that a ternary complex is formed.

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The authors wish to acknowledge the able technical assistance of Miss Frances Oltman in this investigation. We are also deeply indebted to Dr. J. Reed of the Red Star Yeast Co., Milwaukee, Wis., who provided without charge the great quantities of cake yeast needed during this study. Appreciation is extended to Dr. R. K. Morton, who kindly supplied the authors with a preprint of "Symposium on Haematin Enzymes" prior to actual publication.

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Studies on the Mechanism of Enzyme-Catalyzed Oxidation-Reduction Reactions. VII.* *p*H Effects on the Kinetics of the Reaction Catalyzed by Yeast L(+)-Lactate Dehydrogenase

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The effects of *p*H upon the kinetics of binding of L(+)-lactate, D(−)-lactate, and pyruvate by crystalline yeast L(+)-lactate dehydrogenase have been studied by means of initial velocity measurements. These studies implicate a group on the free enzyme with a pK_a' of 6.5–7.0, which is acceptor independent and appears to be concerned with binding the groups attached to the α-carbon of the substrate and the product. The dissociation constants for the complexes of the free enzyme with L-lactate, caprylate, D-lactate, and pyruvate are 7.2×10^{-4} M, 4.2×10^{-3} M, 7.2×10^{-3} M, and 1.5×10^{-2} M, respectively, all at *p*H 7.5 and 20°.

From studies of the *p*H dependence of an enzyme-catalyzed reaction inferences may be made concerning the dissociation constants (pK_a values) of group(s) on the free enzyme involved in the binding of the substrate and/or product. Dixon (1953a) has presented a method for evaluating such data which has been used in the present investigations.

In the companion paper (Hinkson and Mahler, 1963) we have shown that the oxidation of lactate by ferricyanide (at concentrations ≥2 mM) and cytochrome *c* with YLDH[1] obeys the rate laws of equations (1) and (2), respectively,

$$E_t/v_0 = \phi_0 + \phi_1/S + \phi_2/A + \phi_{12}/S{\cdot}A + P(\phi_0' + \phi_1'/S + \phi_2'/A + \phi_{12}'/S{\cdot}A) \quad (1)$$

$$E_t/v_0 = \phi_0 + \phi_1/S + \phi_2/A + \phi_1'/S{\cdot}A \quad (2)$$

where the ϕ_n's are experimentally determined parameters, interpretable in terms of mechanism-dependent specific rate constants, and E_t, S, A, and P are the stoichiometric concentrations of enzyme, lactate, acceptor, and pyruvate, all in moles/liter. It appeared appropriate to determine the effect of *p*H on v_0 and

* For paper VI of this series see Hinkson and Mahler (1963).

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[1] The following abbreviations will be used in this paper: YLDH = crystalline yeast L(+)-lactate dehydrogenase (cytochrome b_2); $p\phi_n$ = negative $\log_{10}$ of the kinetic parameter ϕ_n; FMN = riboflavin-5'-phosphate; OD = optical density.

TABLE I
EFFECTS OF pH UPON THE FOUR KINETIC PARAMETERS

Conditions	ϕ_0 (M sec.)	ϕ_1 (M sec.)	ϕ_2 (M sec.)	ϕ_{12} (M² sec.)
		Ferricyanide as Acceptor		
pH 5.5,[a] no NaCl added	1.5×10^{-2}	4.6×10^{-5}	6.6×10^{-7}	7.2×10^{-11}
pH 7.5, no NaCl added	6.3×10^{-3}	4.5×10^{-6}	4.8×10^{-7}	1.5×10^{-10}
pH 5.5,[b] 0.13 M NaCl	6.6×10^{-3}	8.4×10^{-5}	3.5×10^{-6}	3.7×10^{-9}
pH 7.5, 0.13 M NaCl	1.2×10^{-2}	7.2×10^{-6}	3.4×10^{-6}	3.0×10^{-9}
		Cytochrome *c* as Acceptor[c]		
pH 5.5, no NaCl added	2.8×10^{-2}	3.8×10^{-5}	1.3×10^{-7}	—
pH 7.5, no NaCl added	4.8×10^{-3}	5.2×10^{-6}	5.5×10^{-7}	—
pH 5.5, 0.13 M NaCl	3.1×10^{-2}	8.4×10^{-5}	6.0×10^{-7}	—
pH 7.5, 0.13 M NaCl	3.9×10^{-3}	9.0×10^{-6}	7.2×10^{-7}	—

[a] The values of the parameters reported for these experiments without NaCl in the reaction mixture are an average of three determinations. Ferricyanide was varied from 0.102 mM to 1.02 mM. L(+)-Lactate was varied from 0.33 mM to 1.57 mM. The enzyme concentration for all experiments was 4.6×10^{-8} M. [b] There is a greater error involved in the values for these parameters obtained when NaCl was present due to experimental difficulties unforeseen at the time these experiments were performed. The comparison of the values at different pH values is, however, considered to be legitimate. [c] Oxidized cytochrome *c* was varied from 4.4 mM to 44.0 mM. L(+)-Lactate was varied from 0.33 mM to 1.57 mM. Enzyme was 6.3×10^{-9} M for these experiments.

thus on the various kinetic parameters ϕ_n. This paper presents the results of the studies, which implicate a group on the enzyme with a pK_a' in the range of 6.5–7.0 in the binding of L-lactate. The pH-dependence of the inhibition of the reaction produced by D-lactate and pyruvate has also been investigated, and values of the dissociation constants of the complexes between these compounds and YLDH have been determined and compared with that of the enzyme-substrate complex.

EXPERIMENTAL PROCEDURES

Crystalline YLDH was prepared from air-dried Red Star baker's yeast by the method of Appleby and Morton (1959a). K_i, the dissociation constants of various competitive enzyme-inhibitor complexes, was determined by the method of Dixon (1953b). Four concentrations of inhibitor and three concentrations of L(+)-lactate were used to determine K_i. Thus K_i in this investigation represents an intersection of three lines in the Dixon plot. From equation (1), assuming that P acts as the sole inhibitor, $K_i = (\phi_{1A} + \phi_{12})/(\phi_1'_A + \phi_{12}')$. K_i, the constant evaluated in the Dixon plot for pyruvate or any inhibitor analogous to pyruvate, is then equal to this product. If $\phi_{12} \simeq \phi_{12}' \ll \phi_1$ and ϕ_1', $K_i = \phi_1/\phi_1'$ and becomes independent of A, the acceptor concentration. Evaluation of the ϕ_n's was accomplished as previously described (Hinkson and Mahler, 1963). Tenth molar sodium pyrophosphate–acetic acid buffers were used throughout these studies.

RESULTS

To ascertain that the putative effects of pH were due only to this variable and not to the differences in the ionic strength of the various buffers used, preliminary studies were conducted. Effects of the buffer used on the different ϕ_n values were studied at two different pH values in the presence or absence of added 0.13 M NaCl and with two different acceptors, ferricyanide and cytochrome *c*. As shown in Table I, when ferricyanide serves as the acceptor only the values of ϕ_1 are simultaneously dependent upon pH and independent of added electrolyte. With cytochrome *c* as the acceptor both ϕ_0 and ϕ_1 exhibit these properties. A plot of $p\phi_0$ *vs.* pH (cytochrome *c* as acceptor) did not result in a curve which permitted any simple and unambiguous interpretation in terms of one (or more) pK_a's (unpublished observations). Accordingly, we have investigated in detail only the effects of pH upon ϕ_1 for both acceptors.

Shown in Figures 1 and 2 are Dixon plots describing the effects of pH on the negative log of ϕ_1 when ferricyanide or cytochrome *c* was used as the final electron acceptor, respectively. As seen from Figure 1 the straight lines intersect at a point corresponding to pH 6.7 when ferricyanide is used as the acceptor. The corresponding intersection for cytochrome *c* (Fig. 2) occurs in a pH range between 6.5 and 7.0.

During the course of these investigations D(−)-lactate was found to compete for the same site on the crystalline enzyme as L(+)-lactate (see Fig. 3). Previous workers had disagreed as to the ability of D(−)-lactate to inhibit the enzyme in crude prepara-

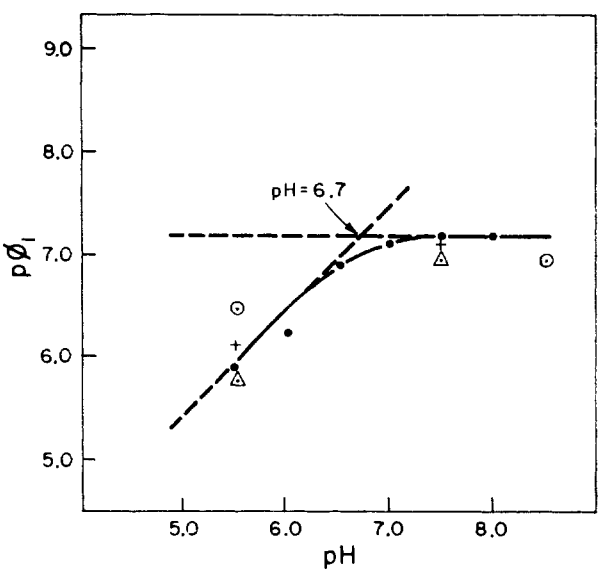


FIG. 1.—Effects of pH on $p\phi_1$; ferricyanide as acceptor. The solid circles represent data obtained during a single experiment done in one day. The points marked with an O and a + were determined in second and third experiments, respectively, done on different days. The points marked with triangles were determined in the presence of 0.13 M NaCl. Potassium ferricyanide was varied from 1.1×10^{-4} M to 1.1×10^{-3} M. L(+)-Lactate was varied from 0.31 mM to 1.56 mM. Room temperature (*ca.* 20°) was used. The dashed lines have slopes of zero and one, respectively. The points were corrected for loss of activity during the experiment and for pH instability of the enzyme where necessary.

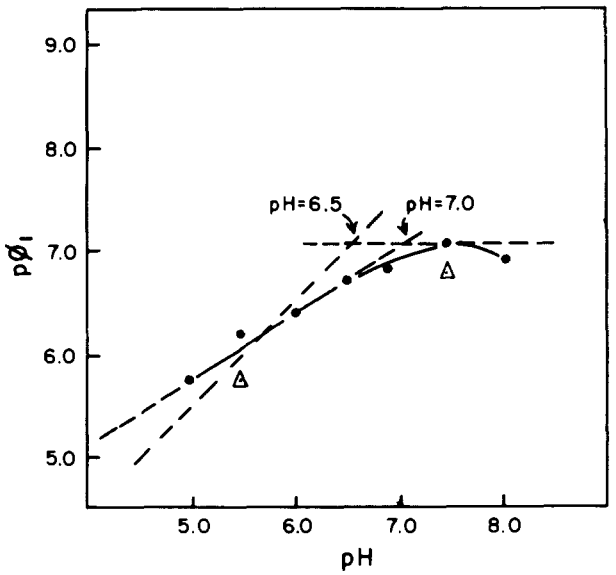


FIG. 2.—Effects of *p*H upon $p\phi_1$; cytochrome *c* as acceptor. The solid circles represent data obtained during a single experiment done in one day. The points marked with a triangle were determined in the presence of 0.13 M NaCl. Oxidized cytochrome *c* was varied from 3.5 × 10^{-5} M to 3.5 × 10^{-6} M. L(+)-Lactate was varied from 0.31 mM to 1.56 mM. Room temperature (*ca.* 20°) was employed. The points were corrected for loss of activity during the course of the experiment and for the *p*H instability of the enzyme where necessary. The two positively sloped lines represent two cases of subjective bias in constructing the plot.

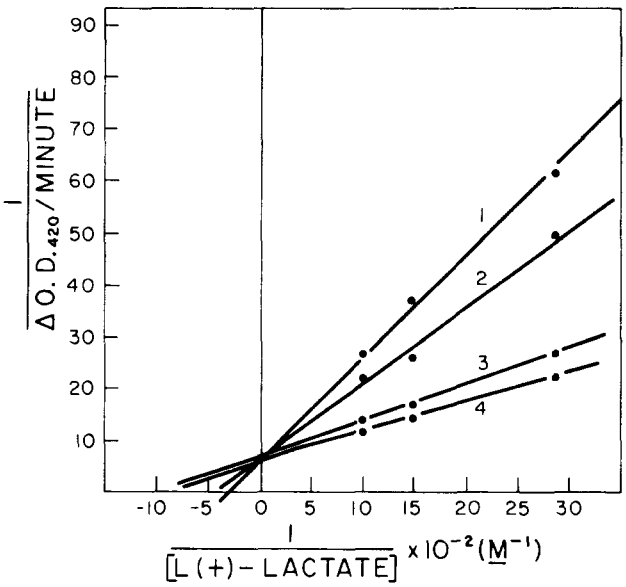


FIG. 3.—Lineweaver-Burk plots when D(−)-lactate was used as the inhibitor, *p*H 7.0. Curve 1, 2 × 10^{-2} M D(−)-lactate; curve 2, 1.3 × 10^{-2} M D(−)-lactate; curve 3, 6.7 × 10^{-3} M D(−)-lactate; curve 4, no D(−)-lactate. Potassium ferricyanide (0.67 mM) served as the acceptor. All experiments done at room temperature, *ca.* 20°.

tions (Boeri *et al.*, 1955; Dikstein, 1959), but to the authors' knowledge the inhibition of the enzyme-catalyzed reaction by D(−)-lactate when crystalline enzyme was used had not as yet been investigated. Figure 4*A* indicates the interesting fact that no definite effect of *p*H upon the binding of D(−)-lactate as measured by changes in K_i occurred within the *p*H range examined.

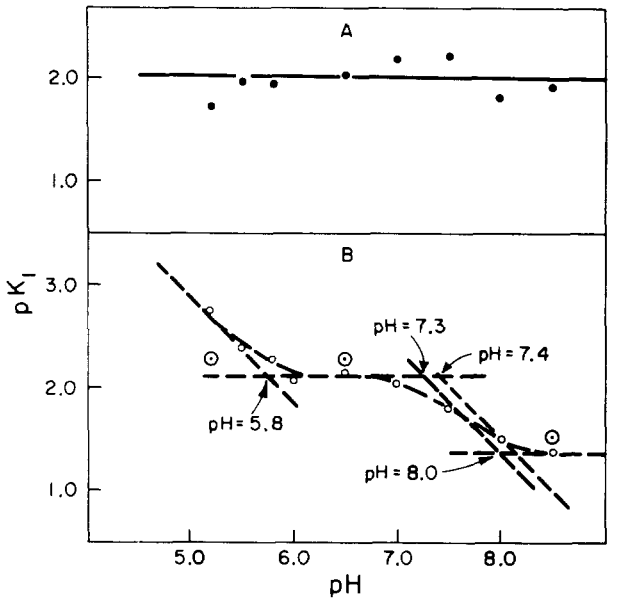


FIG. 4.—Effect of *p*H upon K_i when D(−)-lactate and pyruvate were used as inhibitors. *A*, sodium D(−)-lactate varied from 0 to 2 + 10^{-2} M; *B*, sodium pyruvate varied from 0 to 2 × 10^{-2} M; ○, same as *A* but determined in 0.2 M NaCl. The ○ at *p*H 8.5 is a maximal value and actually may be less than shown. 0.67 mM potassium ferricyanide was used as the acceptor. 0.1 M acetate-pyrophosphate buffers were used. All dashed lines are drawn with slopes of zero or minus one. Room temperature was used, *ca.* 20°.

Since pyruvate, the product of the dehydrogenation reaction, is also an inhibitor of the reaction (Hinkson and Mahler, 1963; Bernheim, 1928), the effects of *p*H upon the binding of this inhibitor were also investigated. Without the presence of NaCl in the reaction mixture there are three breaks in a Dixon plot, at *p*H ≃ 6, 7.3, and 8.0. Since the break at *p*H ≃ 6 disappears if the reaction is studied in the presence of 0.2 M NaCl, it undoubtedly represents the effects of a change in ionic strength rather than the effects of *p*H.

In Table II the apparent dissociation constants, all determined kinetically, for L(+)-lactate, D(−)-lactate, pyruvate, and caprylate (also found to be a competitive inhibitor to L(+)-lactate) are compared at the different *p*H values for which data have been collected. It is seen that at *p*H 8.0 the dissociation constant for pyruvate is 42 times the dissociation constant for L(+)-lactate. It will also be noted that in a range where the respective dissociation constants are relatively *p*H-independent, both D(−)-lactate and pyruvate exhibit essentially the same dissociation constants. Presumably, the dissociation constant for caprylate in this range is also near this value.

DISCUSSION

When ferricyanide is used as the acceptor $\phi_1 = 1/2k_1$, where k_1 is the rate constant in reaction (3): $E + S \xrightarrow{k_1} ES$ (Hinkson and Mahler, 1963). Of the two components in this reaction, enzyme (*E*) and lactate (*S*), free lactate, with a pK_a of 3.86 (Lange, 1952), cannot be responsible for the pK_a' of 6.7 as determined in Figure 1. The experimentally obtained pK_a' can then be assigned to the free enzyme.

With cytochrome *c* as acceptor it has previously been shown that in the mechanism which may describe the sequence of reactions in this instance $k_2 \simeq k_3$ (Hinkson and Mahler, 1963). With this assumption ϕ_1 is

TABLE II
COMPARISON OF DISSOCIATION CONSTANTS[a] OF ENZYME WITH VARIOUS COMPOUNDS
(BOUND AT ESSENTIALLY THE SAME SITE)

Compound	Dissociation Constant		
	At pH 7.5	At pH 8.0	In pH-independent region (range shown)
Caprylate	4.2×10^{-3}[a]	—	—
D(−)-Lactate	7.2×10^{-3}[a]	—	6.10×10^{-3} (pH 5.5–8.5)
Pyruvate	1.4×10^{-2}[a] 1.6×10^{-2}[b]	3×10^{-2}[a]	7×10^{-3} (pH 6–7)
L(+)-Lactate	7.2×10^{-4}[c]	7.2×10^{-4}[c]	7.2×10^{-4}[c] (pH 7–8)

[a] Determined by the method of Dixon (1953a). [b] Calculated from $\phi_1/\phi_1'P = k_{12}/k_{11}$; see equation (3) of Hinkson and Mahler (1963). [c] Calculated from $\phi_{12}/\phi_2 \simeq k_2/k_1$ from equation (3) of Hinkson and Mahler (1963); ferricyanide used as acceptor.

approximately equal to $1/k_1$ where k_1 has the significance indicated in the previous paragraph. Thus, it is quite likely that the pK_a' of 6.5–7.0 as determined in Figure 2 also corresponds to the free enzyme, which must be deprotonated in order to bind L-lactate. As would be expected *a priori* this pK_a' is therefore independent of the acceptor employed.

There is no evidence of any pK_a' for the enzyme in the region of pH = 7.0 for the binding of D(−)-lactate (see Fig. 4*A*). Yet D(−)-lactate, a competitive inhibitor to L(+)-lactate, must be bound at essentially the same site as the latter. The only difference between the structures of the two enantiomers is the configuration of the groups attached to the α-carbon. In all other respects the two molecules are identical. It is therefore suggested that the group on the free enzyme responsible for the $pK_a' \simeq 6.7$–7.0 is implicated by virtue of interaction with one (or more) of the groups attached to the α-carbon of L(+)-lactate and most probably specifically with the α-hydroxy group. D(−)-lactate then is a competitive inhibitor by virtue of its binding to the enzyme through its carboxyl and/or its methyl group. The fact that caprylate (which lacks the α-hydroxyl group and has the methyl group replaced with a hexyl group) is also a competitive inhibitor for L(+)-lactate with approximately the same value for its dissociation constant as D(−)-lactate strongly suggests that it is mainly the carboxyl group which is responsible for the binding of these inhibitors. L(+)-lactate is probably bound more strongly through its carboxyl group than by virtue of the *R*—OH-enzyme interaction just described, since the decrease in the dissociation constant ascribable to this second effect is only one order of magnitude (*cf.* the K_1' for L-lactate and caprylate in Table II): *i.e.*, ~1.0 kcal for *R*—OH··· binding, ~4.2 kcal for the binding of *R*—OH plus *R*—COO^-.

Again, by applying Dixon's rules to the data of Figure 4*B* we observe that there exists a group on the enzyme (or on an enzyme-acceptor complex,[2] Hinkson and Mahler, 1963) with a pK_a' in the range of 7.3–7.4, and perhaps a group in an enzyme-pyruvate complex responsible for a pK_a' of around 8. It is possible though not very likely that, owing to experimental error, the two groups with experimentally determined pK_a' values of 6.7 ± 0.2 and 7.3 concerned in the binding of L-lactate and pyruvate, respectively, are actually identical and reside on the free enzyme. A more likely alternative is that the pK_a' measured in the second case is predominantly that of the same group but on the *EZ* complex, where it may well be acid weakened; for if, as previously suggested by us, this complex is one in which the enzyme has become wholly or partially reduced, it is quite likely that the electron density in the immediate vicinity of the substrate binding site has been slightly increased. Therefore we conclude that it is highly probable that the same group (with a pK_a' of 6.7 when free) on the enzyme is involved in the binding both of the substrate and of the product and is capable of interacting with either an α-hydroxy or an α-keto group. A corollary of this statement is that the substrate remains stationary with respect to the substrate binding area on the enzyme surface in the course of the dehydrogenation.

[2] A probable sequence of events is

$$E \xrightarrow{S} ES \xrightarrow{A} EXY \xrightarrow{P} EZ \xrightarrow{2A_{red}} E.$$

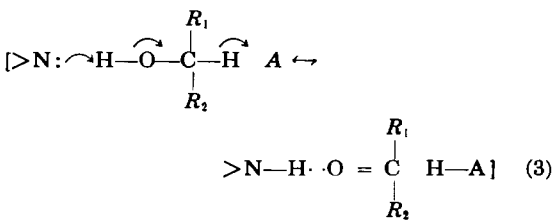
At the "pH optimum" for the reaction with ferricyanide, pH 8.0 (Appleby and Morton, 1959b), the dissociation constant for pyruvate is 42 times that for L(+)-lactate. This observation may help to account for the fact that the apparent pH optimum for reaction is at 8.0 instead of near pH 7.0, the pK_a of the substrate binding site, since at the pH optimum the dissociation of the product is greatly facilitated while the binding of the substrate remains unaffected.

It would be premature to propose a definite assignment of this kinetically determined pK_a' to a particular group on the free enzyme (see, for example, the excellent studies of Bruice and his group on this point [Bruice and Schmir, 1959; Bruice and Bruno, 1962]). But as pointed out by others (Koshland, 1960), a pK_a in the region of 6.5 is most probably due to an imidazole group, although Erlanger (1960) has shown that acetylated arginine would also have a pK_a' in this region and it is known that pepsin has a carboxyl group with a pK_a in the region of 6.5 (Edelhoch, 1958).

Most workers interested in YLDH *assume* that L(+)-lactate binds directly to the enzyme-bound FMN and then transfers electrons directly to this moiety (Morton *et al.*, 1961; Dikstein, 1959; Boeri and Tosi, 1956). Of the various pK_a' values for FMN in this range (10.4 and 6.7 for the oxidized and reduced forms of the *iso*alloxazine ring, respectively [Lowe and Clark, 1956] and 6.6 for the phosphoryl group [Cerletti, 1959]) only those of the reduced *iso*alloxazine and of the phosphoryl groups are close enough to the pK_a measured in these experiments to merit serious consideration. Further reduction of a reduced *iso*alloxazine by a reduced substrate is unlikely. At the pH optimum, pH 8, the negatively charged phosphoryl group would be expected to repel the negatively charged lactate ion instead of attracting it. The only other possibility for FMN binding is that a semiquinoid form of FMN is involved in binding the lactate, since the semiquinone of riboflavin has a pK_a of 6.5 (Michaelis and Schwarzenbach, 1936). This latter possibility is also consistent with the present observations.

Boeri and Tosi (1956) have previously proposed that

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)

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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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