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Studies on the Mechanism of Enzyme-Catalyzed Oxidation Reduction Reactions. IV. A Proposed Mechanism for the Over-all Reaction Catalyzed by Liver Alcohol Dehydrogenase*

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The kinetic data for liver alcohol dehydrogenase acting on acetaldehyde-ethanol obtained in the preceding paper are analyzed in terms of a variety of possible modifications of the mechanisms proposed by Theorell and Chance (1951) and ourselves. It is concluded that the most likely mechanism is one entailing the formation of both unreactive and reactive complexes between both enzyme and DPN and enzyme and DPNH. The kinetic and equilibrium dissociation constants obtained on the basis of the proposed mechanism are shown to be in good agreement with values determined independently for $K_{eq.}^{over\text{-}all}$ by two techniques by ourselves and by direct measurement by Bäcklin; with values for the dissociation constant of the enzyme-coenzyme complexes obtained by direct measurement by Theorell and Winer; and with values for the kinetic constants obtained by Theorell *et al.* The isotope effects for the binding constant for DPNH and for the rate constant of the hydrogen transfer step proper have been determined by comparing DPNH with α-DPND in the reactions catalyzed by both liver and yeast alcohol dehydrogenase, and found to be of the order of 1.3 for the equilibrium constant and 2–3 for the rate constant. A complete kinetic analysis is also presented for the reaction in the presence of the inhibitor *o*-phenanthroline. It is concluded, in agreement with Vallee and co-workers, that both DPNH and DPN bind at a site also capable of interacting with the inhibitor and therefore probably at the enzyme-bound Zn^{++}. Ethanol binds at a site not identical with this, but related to it or closely adjacent, while acetaldehyde seemingly does *not* bind at the same site (or in the same manner) as ethanol.

In the preceding paper (Baker, 1962), the mechanism of the liver alcohol dehydrogenase system has been investigated by a study of its kinetics. It became apparent that the most satisfactory description involved some type of compulsory binding mechanism, but that none of the examples in this group (homeomorphs) described by Dalziel (1957) could account for some of the characteristics of the reaction, notably the fact that it did not obey the "Dalziel criteria." Therefore some modification or perturbation has to be introduced. In general, with mechanisms which do not postulate alternate pathways (forks) between reactants and products, only two classes of perturbations exist which can alter the Dalziel relationships in the observed direction.[1] The first is the formation of isomeric forms of the binary complexes between enzyme and the leading substrate (Peller and Alberty, 1959). As an example, this particular modification of the Theorell-Chance mechanism (1951), as the simplest example of a compulsory binding type, is shown in Equation (1)

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EX$$

$$EY \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EA' \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E + A' \quad (1)$$

A and A′ correspond to DPNH and DPN in the present case.

$$EX + B \underset{k_6}{\overset{k_5}{\rightleftharpoons}} B' + EY$$

$$E/v_0 = \varphi_0 + \varphi_1/(A) + \varphi_2/(B) + \varphi_{12}/(A)(B) \quad (1a)$$

where (A) and (B) are the initial concentrations of the two substrates and

$$\varphi_0 = \frac{k_3(k_7 + k_8 + k_9) + k_7k_9}{k_3k_7k_9}, \quad \varphi_1 = \frac{k_2 + k_3}{k_1k_3},$$

$$\varphi_2 = \frac{k_4 + k_3}{k_3k_5}, \quad \varphi_{12} = \frac{k_2 + k_4}{k_1k_3k_5} \quad (1b)$$

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[1] This becomes readily apparent if the schematic method of King and Altman (1956) is applied.

$$\varphi_0' = \frac{k_8(k_2 + k_3 + k_4) + k_2k_4}{k_2k_4k_6},\ \varphi_1' = \frac{k_8 + k_9}{k_8k_{10}},$$

$$\varphi_2' = \frac{k_7 + k_8}{k_6k_8},\ \varphi_{12}' = \frac{k_7k_9}{k_6k_8k_{10}}$$

Now the ratio $\varphi_1\varphi_2/\varphi_{12}$ may be equal to, smaller than, or greater than φ_0, depending solely on the relative magnitudes of k_2 and k_8; thus the Dalziel criterion $\varphi_1\varphi_2/\varphi_{12} \lessgtr \varphi_0'$ may or may not be fulfilled.

The second type of perturbation of a compulsory, reversible mechanism which can lead to inverted Dalziel relationships postulates the rapid and reversible formation of unreactive binary complexes, *i.e.*, entities which cannot give rise to product [equation (2)].

$$E + X \underset{}{\overset{K_1}{\rightleftharpoons}} [EX]\ \text{(inactive)} \qquad (2)$$

$$\text{(inactive) } [EX'] \overset{K_2}{\rightleftharpoons} E + X'$$

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \qquad EA' \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + A'$$

$$EA + B \underset{k_4}{\overset{k_3}{\rightleftharpoons}} B' + EA'$$

K_1 and K_2 are association constants; A and A′ correspond to DPNH and DPN, respectively, in the present case.

$$E/v_0 = \varphi_0 + \varphi_1/(A) + \varphi_2/(B) + \varphi_{12}/(A)(B)$$

If X and Y are the "leading" substrates A and A′, respectively, then

$$\varphi_0 = \frac{k_1 + k_5K_1}{k_1k_5},\ \varphi_1 = 1/k_1,$$

$$\varphi_2 = \frac{k_1 + k_2K_1}{k_1k_3},\ \varphi_{12} = k_2/k_1k_3 \quad (2a)$$

$$\varphi_0' = \frac{k_6 + k_2K_2}{k_2k_6},\ \varphi_1' = 1/k_6,$$

$$\varphi_2' = \frac{k_6 + k_5K_2}{k_4k_6},\ \varphi_{12}' = k_5/k_4k_6$$

On the other hand, if X and Y are B and B′, respectively, then

$$\varphi_0 = 1/k_5,\ \varphi_1 = \frac{k_3 + K_1(k_2 + k_3B)}{k_1k_3},$$

$$\varphi_2 = 1/k_3,\ \varphi_{12} = k_2/k_1k_3 \quad (2b)$$

$$\varphi_0' = 1/k_2,\ \varphi_1' = \frac{k_4 + K_2(k_5 + k_4B')}{k_4k_6},$$

$$\varphi_2' = 1/k_4,\ \varphi_{12}' = k_5/k_4k_6$$

In the latter case the concentration terms B and B′ appear in the coefficients φ_1 and φ_1'; therefore there is some possibility that the reciprocal plot of initial velocity against reciprocal substrate concentration (equation 1b, above, discussed in Baker and Mahler, 1962) would no longer be linear. If $k_3B \ll k_2$ and $k_4B \ll k_5$, however, this difficulty is circumvented and the plots remain linear.

It can be shown quite rigorously that for any two-substrate mechanism, provided the path of the reaction is unbranched[1] (Wong and Hanes, 1962 and private communication), the two perturbations just described are the *only* ones which can lead to the conditions $\varphi_1\varphi_2/\varphi_{12} > \varphi_0'$ and $\varphi_1'\varphi_2'/\varphi_{12}' > \varphi_0$ (*i.e.*, an "inverted" Dalziel relationship). Inclusion of any number of ternary complexes in the pathway between the coenzyme and the reactants, whether on the direct path or not, will still lead to the reverse inequality (the ordinary Dalziel criteria). For instance, the suggestion recently advanced by Theorell (1961) (in order to account for some observations on inhibition by fatty acids and amides)—that in the liver alcohol dehydrogenase system there may occur the formation of ternary, unreactive complexes between both reactants and the enzyme—can be shown to lead to a rate law similar to equation (1a) with φ coefficients so constituted as to yield the normal and not the inverted Dalziel relationship.

Results

Selection of Mechanism.—In order to determine more precisely the applicability of these considerations to our data we therefore wish to reexamine the mechanism proposed in the previous paper (Baker, 1962). In that paper a compulsory binding mechanism involving one or more ternary complexes was suggested, largely on the basis of the kinetic isotope effect and reversible continuous rate criteria (the latter obtained in experiments with a different pH and buffer system). This introduction of one or more additional steps between the two binary complexes of equations (1) or (2) leads to a secondary perturbation and φ values more complicated than those shown. For the sake of focusing our attention on the primary perturbations here under consideration, we shall for the moment turn our attention to the modified Theorell-Chance mechanisms of equations (1) and (2); this constitutes an over-simplification and will give results which at best can be considered as first approximations only.

In the mechanism involving isomeric, reactive forms of the binary complexes (equation 1) we find that we must postulate at least two such forms each for E-DPNH and E-DPN—and probably three of the latter kind—in order to account for the Dalziel relationship actually observed. Physically this is possible, but as a consequence of these manipulations it can be shown that now φ_2/φ_{12} and φ_2'/φ_{12}' are no longer equal to the ratios k_2/k_1 and k_9/k_{10}, respectively, *i.e.*, the dissociation constants for the complexes between the leading substrate (DPN and DPNH) with the enzyme. Yet comparison of our kinetic data with Theorell's values for these dissociation constants determined directly (discussed below in Table III) suggests that they are the same. For this reason and because this mechanism does not permit the evaluation of individual rate constants—and therefore cannot be examined critically—we shall not consider it further.

In the second type of mechanism [that of equation (2)], let us first examine the formation of unreactive complexes with the substrates [equation (2b)]; this mechanism is inconsistent with the kinetic isotope effects reported in the previous paper (Baker, 1962). We find an isotope effect in φ_0, but the postulated mechanism shows that φ_0 depends on k_5 only, a rate constant which cannot exhibit an isotope effect. This conclusion is verified by the fact that there is *no* isotope effect

TABLE I

ESTIMATE OF THE CONSTANTS: MECHANISM (2a)

(All in 0.1 M glycylglycine-Na^+, pH 8.58, t = 27.0°)

Constants	Magnitude and Range (±)	Isotope Effect, H/D
$k_1(H)$	3.50×10^6 (3.5%) sec.$^{-1}$ M^{-1}	1.62 ± 0.19
$k_1(D)$	2.16×10^6 (8.5%) sec.$^{-1}$ M^{-1}	
$k_2(H)$	3.43 (20%) sec.$^{-1}$	2.12 ± 0.76
$k_2(D)$	1.62 (17%) sec.$^{-1}$	
$k_2/k_1(H) = K_{diss}^{E\cdot DPNH}$	9.80×10^{-7} (24%) M	1.31 ± 0.64
$k_2/k_1(D) = K_{diss}^{E\cdot DPND}$	7.50×10^{-7} (25%) M	
$k_3(H)$	8.14×10^4 (11%) sec.$^{-1}$ M^{-1}	3.11 ± 0.55
$k_3(D)$	2.62×10^4 (6.2%) sec.$^{-1}$ M^{-1}	
$k_4(H)$	1.76×10^4 (6.9%) sec.$^{-1}$ M^{-1}	2.28 ± 0.27
$k_4(D)$	7.71×10^3 (5.1%) sec.$^{-1}$ M^{-1}	
$k_4/k_3(H)$	0.216 (18%)	0.735 ± 0.23
$k_4/k_3(D)$	0.294 (11%)	
k_5	17.6 (15%) sec.$^{-1}$	...
k_6	2.33×10^5 (4.7%) sec.$^{-1}$ M^{-1}	...
$k_5/k_6 = K_{diss}^{E\cdot DPN}$	7.55×10^{-5} (20%) M	...
$K_1(H)$	8.52×10^4 (max.) or 4.26×10^4 (100%)	
$K_1(D)$	1.17×10^5 (45%) M	
$K_1^{-1}(H) = K_{diss}^{[E\cdot DPNH]}$	2.34×10^{-5} M	2.7
$K_1^{-1}(D) = K_{diss}^{[E\cdot DPND]}$	8.55×10^{-6} M	
K_2	2.93×10^4 (24%) M	...
$K_2^{-1} = K_{diss}^{[E\cdot DPN]}$	3.41×10^{-5} M	

observed in the ratio $\varphi_{12}'/\varphi_2' = k_5/k_6$; since k_6 also should not exhibit an isotope effect *a priori*, this implies an absence of isotope effect in k_5. The only alternative is the unlikely possibility that the isotope effects for both k_5 and k_6 are identical. Finally, we experimentally find no isotope effect in φ_1', yet the mechanism would predict the occurrence of such an effect. These considerations permit us to discard the mechanism of equation (2b).

The final mechanism to be considered involves the formation of unreactive binary complexes between the enzyme and the leading substrates (or coenzyme): equation (2a). This mechanism predicts isotope effects for all of the φ_p values except for φ_1' and the ratio φ_{12}'/φ_2'. The implication is that no isotope effect is to be expected in the binding of DPN to the enzyme to yield either a reactive or an unreactive complex—a proposition which is obvious, since the DPN used did not contain any deuterium. This mechanism predicts an interesting and critical identity, equation (3). Our data, summarized below [relation (4)], indicate that this relation is obeyed to a first approximation.

$$\varphi_0 + \varphi_0' \equiv \varphi_1\varphi_2/\varphi_{12} + \varphi_1'\varphi_2'/\varphi_{12}' \qquad (3)$$

Nature of Atom Transferred	$\varphi_0 + \varphi_0'$	$\varphi_1\varphi_2/\varphi_{12} + \varphi_1'\varphi_2'/\varphi_{12}'$	
Hydrogen	0.435 ± 0.017	0.523 ± 0.053	(4)
Deuterium	0.594 ± 0.057	0.823 ± 0.114	

Calculation of Rate and Equilibrium Constants.—In order to evaluate the individual rate and equilibrium constants we find that each set of experiments (hydrogen or deuterium-containing reactants) can be described in terms of eight unknowns: six rate and two equilibrium constants. As a result of the identity of the factors in equation (3), however, the number of independent equations is reduced to seven. If we use both sets of results we find that ten rate constants and three equilibrium constants have to be evaluated, since the rates k_5 and k_6 and the constant K_2 are assumed to be unaffected by isotopic substitution in the reactants. This assumption would appear to be correct, since no isotope effect is observed for φ_1' or for the ratio φ_{12}'/φ_2'. These relationships, however, serve to reduce the number of independent algebraic equations available to us to twelve. Since a total of thirteen unknowns is to be evaluated, no complete algebraic solution is possible.

An approximate method of solution which yields values for the thirteen constants is available, however. As an example,[2] the equation for φ_0^H is given below (equation 5). Since $k_1^H = 1/\varphi_1^H$ we can obtain limits for the two constants k_5^H and K_1^H, *viz.*

$$\varphi_0^H = 1/k_5 + K_1^H/k_1^H = 1/k_5 + K_1^H\varphi_1^H \qquad (5)$$

a maximum value for K_1^H, if $1/k_5$ is assumed to be zero, and a minimum value for k_5^H, if K_1^H is assumed to be equal to zero. The numerical values obtained in this manner, using our earlier data (Baker, 1962) are shown in equation (6).

$$k_5^H \text{ (minimum)} \simeq 1/\varphi_0^H = 13.9;$$
$$K_1^H \text{ (maximum)} \simeq \varphi_0^H/\varphi_1^H = 2.58 \times 10^5 \qquad (6)$$

If this technique is applied to the twelve independent equations, one obtains corresponding limits for all constants and can estimate "highest values" for their minima and "lowest values" for the corresponding maxima, all of which are self-consistent. This leads to certain restrictions in the allowed values of the thirteen constants which are then used for a new set of estimates. By reiteration of this process twice more the constants (with their range) tabulated in Table I are obtained. It is evident that a self-consistent set of constants can be calculated in this manner from the data for ethanol–DPNH, ethanol–α-D-DPND and deuteroethanol–DPNH. The values for the dissociation constants of the unreactive complexes between the enzyme and the coenzyme are virtually identical for DPN and DPNH, and therefore probably involve the identical binding site on the enzyme.

[2] φ_p, k_p^H, or K_p^H are constants for hydrogen containing reactants, φ_p^D, k_p^D or K_p^D, the corresponding ones with deuterium in the *reactive* position substituted for hydrogen.

TABLE II
ESTIMATE OF THE CONSTANTS FOR THE REACTION WITH β-D-DPND
Constant assumed to be unaffected

Values Computed for	k_2	k_3	k_5	K_1
k_2	3.43 (20%)	7.75 (19%)	9.80 (42%)	7.46 (20%)
k_3	3.54×10^4 (28%)	8.14×10^4 (11%)	9.90×10^4 (26%)	7.75×10^4 (12%)
k_5	4.06 (35%)	13.2 (50%)	17.6 (15%)	11.4 (30%)
K_1	-5.10×10^5 (55%)	8.50×10^4 (100%)	1.50×10^5 (53%)	4.26×10^4 (100%)

The complex so formed appears to be more easily dissociated than the reactive complex of DPNH, but less easily dissociated than the corresponding one for DPN.

Turning to the data collected for β-D-DPND, we find that there is no isotope effect in φ_1; this implies a corresponding absence of an isotope effect in k_1. Four additional constants (k_2, k_3, k_5, and K_1) remain to be computed, but only three independent relations are available. One additional relationship is provided by assuming at least one of these remaining constants to be unaffected by isotopic substitution. The results of the calculations making this assumption for each of the four parameters in turn is shown in Table II. The values which seem to have the greatest internal consistency are those calculated on the basis that k_3, k_5, or K_1 are essentially invariant under isotopic substitution. If we assume *all* of them to possess this property the most likely values become:

$k_2^{\beta\text{-D}} = 7.61$ (±18%), $k_3^{\beta\text{-D}} = 7.90 \times 10^4$ (±6.3%), $k_5^{\beta\text{-D}} = 11.4$ (±30%), and $K_1^{\beta\text{-D}} = 7.76 \times 10^4$ (±9.8%).

Comparison with Other Results.—It is now possible to compare the values of the various parameters determined in this investigation for the hydrogen-containing reactants with those obtained by Theorell and his collaborators under similar, but not identical, conditions (Table III).

Most of the results appear to be in reasonable agreement. The two least satisfactory ones are those for K_{eq} and K_{diss}^{DPN}. Considering the complexities involved in estimating equilibrium constants from kinetic data, the observed agreement within 20% of the values determined by direct measurement is probably reasonably satisfactory. This in itself constitutes good presumptive evidence that the mechanisms are linear with respect to all reactants, *i.e.*, that they are a modification of one of those discussed by Dalziel (1958). The fact that the kinetically determined dissociation constant for DPN, $K_{diss}^{DPN^+}$, appears to be some three times that determined by Theorell and Winer (1959) is of some interest. Actually their value corresponds more closely to ours for K_2, the dissociation constant for the *unreactive* complex between enzyme and DPN. Now the determination by Theorell and Winer was a static binding study by means of fluorometric techniques. Frieden (1961) has recently presented evidence that in some analogous cases the interactions measured by this technique frequently are those which "do not represent binding to the site of the enzyme which determines the kinetic parameters of the reaction" and are "unrelated to the enzymic activity." This conclusion is identical with our own, arrived at by purely kinetic means, and suggests the plausibility of the mechanism shown in equation (2a).

Isotope Effects.—Some comment appears germane concerning the magnitude of the isotope effects and a comparison of those obtained in this investigation with the ones previously observed by us (Mahler and Douglas, 1957; Shiner *et al.*, 1960) on the related reaction catalyzed by yeast alcohol dehydrogenase. The pertinent results are summarized in Table IV.

The magnitude of the isotope effects on the binding constants for α-D-DPND at the kinetically active site are quite comparable, and so, considering the differences in the conditions used, are those of the rate constants for the rate-limiting step. The latter are lower than might be anticipated for a pure, primary isotope effect (Wiberg, 1955; Melander, 1960; Streitwieser, 1960), while the former appear abnormally high for a secondary isotope effect (Shiner *et al.*, 1960). A possible interpretation, to be explored in more detail in a future communication, is that in the reaction catalyzed by *either* enzyme there occurs, as a consequence of the binding step, some kind of

TABLE III
COMPARISON OF KINETIC AND EQUILIBRIUM CONSTANTS

Constant	This Investigation (0.1 M glycylglycine-Na^+, pH 8.58, 27.0°)	Previous Investigations	Reference
$K_{eq}^a \times 10^{11} = \varphi_{12}H^+/\varphi_{12}'$	0.738 ± 0.07 M	1.06 ± 0.03[b]	Bäcklin (1958)
continuous rate	0.911 ± 0.04 M		
direct determination	1.14 ± 0.08 M		
$k_1 \times 10^{-6}$	3.50 ± 0.12 M^{-1} sec.$^{-1}$	2.9[c]	Theorell *et al.* (1955)
k_2	3.43 ± 0.70 sec.$^{-1}$	1.8[c]	Theorell *et al.* (1955)
$K_{diss}^{E\cdot DPNH} = k_2/k_1 \times 10^7$	9.80 ± 2.4 M	8.8[d]	Theorell and Winer (1959)
$k_3 \times 10^{-4}$	8.14 ± 0.93 M^{-1} sec.$^{-1}$	4.0[c]	Theorell *et al.* (1955)
$k_4 \times 10^{-4}$	1.76 ± 0.12 M^{-1} sec.$^{-1}$	0.6[c]	Theorell *et al.* (1955)
$k_5 \times 10^{-1}$	1.76 ± 2.6 sec.$^{-1}$	1.0[c]	Theorell *et al.* (1955)
$k_6 \times 10^{-5}$	2.33 ± 0.11 M^{-1} sec.$^{-1}$	2.3[c]	Theorell *et al.* (1955)
$K_{diss}^{E\cdot DPN^+} = k_5/k_6 \times 10^5$	7.55 ± 1.5 M	2.2[d]	Theorell and Winer (1959)
$K_2 \times 10^5$	3.41 ± 0.5 M		

[a] Equilibrium constant for over-all reaction. [b] Calculated for 27.0° from Fig. 2 of Bäcklin (1958). [c] Extrapolated to the pH used in this investigation from Fig. 6–10 of Theorell *et al.* (1955) all at 23.5°. [d] In phosphate buffer, ionic strength = 0.1, 23.5°.

TABLE IV

MAGNITUDE OF IMPORTANT ISOTOPE EFFECTS[a] IN STEPS CATALYZED BY YEAST AND LIVER ALCOHOL DEHYDROGENASE

Type of Constant	Step	Yeast Alcohol Dehydrogenase 0.01 M Phosphate, pH 7.45, 24.0° α-DPND	Liver Alcohol Dehydrogenase 0.10 M Glycylglycine, pH 8.58, 27.0° α-DPND	β-DPND
Equilibrium	Binding of DPNH	$\bar{K}_1 = 1.43 \pm 0.34$ Av.[b] $= 1.39 \pm 0.28$ $\bar{K}_4 = 1.34 \pm 0.21$	$\bar{K} = 1.31 \pm 0.64$	$\bar{K} = 0.50 \pm 0.1$
Rate of H transfer	Forward	$\bar{k} = 1.75 \pm 0.5$	$\bar{k}_3 = 3.11 \pm 0.55$	$\bar{k}_3 = 1.0 \pm 0.1$
	Reverse		$\bar{k}_4 = 2.28 \pm 0.27$	

[a] $\bar{k}$, $\bar{K}$ indicate k_p^H/k_p^D and K_p^H/K_p^D respectively. [b] Mean of $\bar{K}_1$ and $\bar{K}_4$

direct interaction between the hydrogen to be transferred and the enzyme. In line with this assumption is the fact that β-DPND shows a *reverse* isotope effect for $\bar{K}$ (due probably entirely to a reverse effect on $\bar{k}_2$: Table II). The relatively small magnitude of the isotope effect for the H-transfer step proper indicates a high conservation of zero-point energy in the appropriate transition state—perhaps not unexpected for an enzymic reaction and one which probably involves coordinated electronic displacements in the transition state (Shiner *et al.*, 1960).

Inhibition of Liver Alcohol Dehydrogenase by o-Phenanthroline.—In the hands of Vallee and his collaborators (1959), *o*-phenanthroline has proved to be an exceedingly useful reagent in studies on the importance of enzyme-bound Zn in liver alcohol dehydrogenase. We hoped that a more comprehensive kinetic analysis of *o*-phenanthroline inhibition along the lines indicated in our earlier paper (Baker and Mahler, 1962) might be of help in a further confirmation and/or elaboration of the mechanisms presented here. The results of our studies with this inhibitor are summarized in Table V. A comparison of our data and conclusions with those of the earlier investigators indicates qualitative agreement. The only discrepancy is our assignment of a "competitive-uncompetitive" type to the inhibition observed when acetaldehyde and *o*-phenanthroline are varied. Vallee interpreted his data in terms of pure uncompetitive inhibition—a difference in degree only. We confirm his conclusion that the substitution of both DPN and DPNH by *o*-phenanthroline is strictly competitive and that at least one of the binding sites is identical for both coenzymes and can be identified as the enzyme-bound Zn^{++}. The dissociation constant for the enzyme–Zn–*o*-phenanthroline complex determined kinetically equals $\sim 7.5 \times 10^{-5}$ M (0.10 M glycylglycine, pH 8.58, 27.0°). This is in good agreement with the value of 3.3×10^{-5} M (in 0.10 M Tris buffer, pH 7.5 at 23°) obtained by Vallee and Coombs (1959) by spectrophotometric titration. Our conclusions are also similar to theirs with regard to the nature of the binding of alcohol and acetaldehyde; the inhibition data suggest that *neither* substrate is bound directly to the Zn^{++} site on the enzyme. To us, the partially competitive nature of the ethanol binding by *o*-phenanthroline would suggest, however, that the alcohol binding site interacts with or is closely adjacent to that responsible for coenzyme (and *o*-phenanthroline) binding. The aldehyde, finally, seemingly is *not* bound at the same site (or in the same manner) as is ethanol.

As far as consistency of the data obtained in the presence of *o*-phenanthroline with the mechanism postulated earlier is concerned, the following conclusions may be drawn: equation (2a) of this paper, together with equations (7) and (9) of Baker and Mahler (1962), suggests that the values for φ_0 and φ_0' determined in the presence of an inhibitor should be identical to those observed in its absence: a comparison of the appropriate

TABLE V

INHIBITION OF LIVER ALCOHOL DEHYDROGENASE BY *o*-PHENANTHROLINE

Reaction Mixture	Component Varied: Acetaldehyde or DPNH		Ethanol or DPNH	
Buffer	0.10 M glycylglycine, pH 8.58			
o-Phenanthroline	0.0 to 1.26×10^{-4} M			
Substrate	$5.85 \times 10^{-5} \rightarrow 4.54 \times 10^{-4}$ M		$1.10 \times 10^{-3} \rightarrow 8.5 \times 10^{-3}$ M	
Coenzyme	$3.35 \times 10^{-5} \rightarrow 2.59 \times 10^{-5}$ M		$2.67 \times 10^{-5} \rightarrow 2.1 \times 10^{-4}$ M	
Enzyme	8.71×10^{-8} site equivalents/liter		2.27×10^{-7} site equivalents/liter	
Parameter[a]	Acetaldehyde[b]	DPNH[b]	Ethanol[b]	DPN[b]
φ_0 or $\varphi_0' \times 10^2$ sec./site	6.92 (2.5)	5.98 (3.0)	19.7 (0.55)	16.7 (1.2)
φ_1 or $\varphi_1' \times 10^7$ M sec./site	27.6 (4.3)	3.92 (4.4)	944 (2.0)	50.1 (1.6)
φ_2 or $\varphi_2' \times 10^{-1}$ M sec./site	176. (1.3)	3.58 (83)	68.7 (1.9)	3.67 (45)
φ_{12} or $\varphi_{12}' \times 10^2$ M² sec./site	5.84 (2.8)	0.734 (0.33)	44.6 (5.8)	5.41 (1.2)
$K_S' \times 10^5$ M	40.0 ± 2.7	6.56 ± 0.48	4.79 ± 0.23	30.1 ± 1.4
$K_I' \times 10^5$ M	4.75 ± 0.34	5.35 ± 0.41	21.1 ± 1.7	9.25 ± 0.26
$K_I'' \times 10^5$ M	3.92 ± 0.15	$\gg K_I'$	28.6 ± 0.7	$\gg K_I'$
Inhibition type	Noncompetitive-uncompetitive transition	Competitive	Competitive-uncompetitive transition	Competitive

[a] See equation (9) of Baker and Mahler: $\varphi_0 = 1/k_3$; $\varphi_1 = K_S'/k_3$; $\varphi_2 = 1/k_3K_I''$; $\varphi_{12} = K_S'/k_3K_I'$. [b] Standard error in % in parentheses.

values in Table V with those of Table IV of the preceding paper (Baker, 1962) shows that this is the case. Equations (7) and (9) also permit us an independent estimate of the dissociation constants for DPNH and DPN. This is simplified by the fact that k_3 of equation (7) $= 1/\varphi_0 \ll k_1$ or k_6, and therefore the K values for DPNH and DPN $\simeq$ their respective dissociation constants. The values for these constants in Table V are to be compared to those tabulated earlier (as $K_{diss}^{E.DPNH}$ and $K_{diss}^{E.DPN}$) in Table II. Again the qualitative agreement is satisfactory. Especially in the case of DPN we conclude that inhibition by *o*-phenanthroline by virtue of interaction of the reagent with the site responsible for the formation of the active E·DPNH and E·DPN complex of equation 2a is consistent with this mechanism.

Discussion[3]

The studies described in the previous and discussed in the present paper can best be interpreted in terms of the mechanism summarized in scheme I (Fig. 1). Here we indicate that under the particular conditions tested and to a first approximation the two ternary complexes shown, although known to be formed from a variety of different lines of evidence, are not highly significant kinetically (Theorell, 1956, 1958, 1961). Primary perturbations are, however, introduced by the binding of the coenzyme[4] at secondary sites on the enzyme and by the binding of *o*-phenanthroline, which involves a binding site (probably Zn^{++}) also important for the binding of both DPN and DPNH. In the course of the latter process there occurs a strong interaction between the enzyme and the hydrogen to be transferred subsequently. In the scheme only one of the two equivalent enzymatically active sites is actually indicated, and the binding of acetaldehyde and alcohol is shown to occur in a qualitatively different manner.

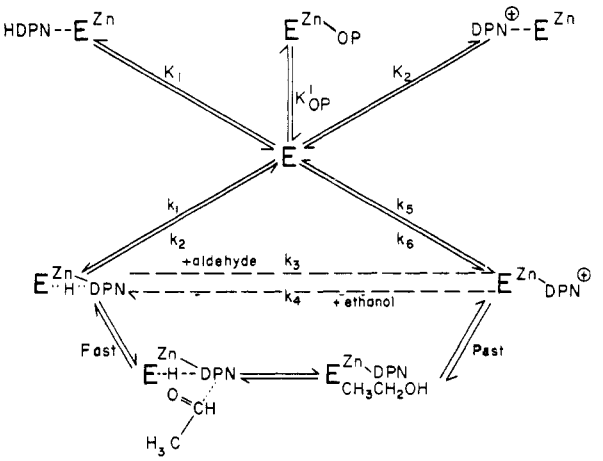

Fig. 1.—Scheme I: The mechanism of the reaction catalyzed by liver alcohol dehydrogenase. Not all bonds required to link reactants to their binding sites on the enzyme are shown.

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[3] Note Added in Proof: In a recent article Theorell and McKee (1961) cite a series of φ values, determined fluorometrically in 0.1 μ phosphate, pH 7, at 23.5°, which are in good qualitative agreement with those obtained in this investigation (Baker 1960, 1962). Their new values for the dissociation constant $K_{diss.}^{E\text{-}DPNH}$ determined by direct measurement again agree closely with the value of this constant obtained from kinetic parameters both by them and by ourselves (Table III). The new estimates of $K_{diss.}^{E\text{-}DPN}$ are, however, significantly higher than those published previously. Theorell and McKee's kinetic and equilibrium measurements now agree very well at pH 7 and permit them to assert, with considerable justification, that the Theorell-Chance mechanism provides an adequate description of the reaction under these conditions. By the same token the discrepancy between the two types of measurement (their direct *vs.* our kinetic determination) indicates either that the composition of the buffer (*i.e.*, glycylglycine *vs.* phosphate) has a very pronounced influence on the magnitude of $K_{diss.}^{E\text{-}DPN}$ or that there is a very considerable departure from Theorell-Chance or even Dalziel kinetics under our conditions, especially in the reaction of ethanol with DPN.

[4] The magnitude of the binding constants is such as to make this perturbation of much greater relative significance when the enzyme interacts with the oxidized as compared to the reduced coenzyme.