Kinetic Studies of Liver Alcohol Dehydrogenase and pH Effects with Coenzyme Preparations of High Purity

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Liver alcohol dehydrogenase has been the subject of repeated and detailed kinetic study. Because of its abundance, ready crystallization (1, 2) and stability, the availability of pure, stable substrates, and the marked changes of absorption and fluorescence spectrum which accompany its combination with the reduced coenzyme (3), it has been to a considerable extent the immediate stimulus for the development of both experimental techniques, notably fluorescence methods (4-6), and theoretical foundations for initial rate and equilibrium studies of coenzyme-substrate reactions (6-12).

One reason for continued attention to this enzyme is that as the detail and precision of kinetic and equilibrium studies increased, discrepancies with the earlier work emerged (9, 13, 14), as regards both the absolute values of the kinetic parameters and conformity of the data to the theoretical requirements of the mechanism proposed by Theorell and Chance (7, 9). Some of the discrepancies were recently resolved by the finding that reduced nicotinamide adenine dinucleotide (NADH) preparations contain variable amounts of a competitive inhibitor (15, 16), and this is no doubt the main reason why the maximal rate of reduction of aldehyde at pH 6 and 7 was found to be much greater in later work, with purer NADH preparations (2, 6, 13, 17), than in earlier work (18). However, in experiments with NADH of high purity (6) the chief discrepancy from the requirements of the Theorell-Chance mechanism persisted, and was clearly outside the random error of the measurements: at pH 6.0, and to a lesser extent at pH 7.1, the maximal rate of aldehyde reduction by NADH exceeded the apparent rate of the last step, the dissociation of enzyme from its NADH compound, as calculated from the initial rate parameters for the reverse reaction on the assumption of the Theorell-Chance mechanism (9). This finding, first reported by Dalziel and Theorell (9, 13), was particularly puzzling because, as these authors pointed out, it could not be explained by the inclusion of rate-limiting ternary complexes in the Theorell-Chance mechanism; furthermore, in other respects kinetic data with two pairs of substrates satisfied criteria characteristic of the Theorell-Chance mechanism (6).

A simple explanation of this anomaly emerged from theoretical and experimental studies of the kinetic effects of inhibitors present as impurities in coenzymes (12, 21). It seemed that a very small proportion of inactive nucleotide in NAD+1, able to compete with the latter, might well cause large errors in initial rate parameters, of a different kind to those caused by inhibitor in NADH preparations, but precisely of the kind required to cause the observed discrepancies from the Theorell-Chance mechanism. The high purity of NAD+ preparations commercially available in recent years, therefore, was not reassuring, and the hypothesis was substantiated by the separation of such an impurity from several preparations and demonstration of its kinetic effects with liver alcohol dehydrogenase (22). It was pointed out that kinetic data for other enzymes may be subject to similar errors.

In this paper initial rate data for the ethanol-NAD+ reaction at pH 6.0 and 7.1 obtained with purified NAD+ are correlated with data for the reverse reaction previously obtained with NADH of high purity (6). New data for the reaction in both directions at pH 8.0 and 9.0 with purified coenzymes are reported. The mechanism of the reaction is considered in the light of these and other results.

EXPERIMENTAL PROCEDURE

Crystalline alcohol dehydrogenase was prepared from horse liver and assayed as described previously (2). The molecular weight is 84,000, and the concentration of active centers, E, is twice the molar concentration (29). NADH was freshly prepared as described by Dalziel (16).

NAD+ was purified by ion-exchange chromatography as previously described (22). The phosphate content of the eluates, 2 mm NAD+ in 0.06 M NaH2PO4, was not sufficient to significantly affect the pH or ionic strength of reaction mixtures at pH <7.5. For experiments at higher pH, the NAD+ solutions were titrated with NaOH immediately before use.

For experiments at pH 5.35 to 8.0, sodium phosphate buffers of ionic strength 0.1 were used. For pH 0.0 and 0.9, the buffers were 0.033 M Na2HPO4 (ionic strength 0.1) containing 0.0036 M glycine-NaOH buffer pH 9 and 0.0021 M glycine-NaOH buffer pH 9.9, respectively.

Initial rate measurements were made with an Aminco fluorometer at 23.5°C. Detailed descriptions have already been given (6, 9) of the apparatus, technique, and method of estimation of the kinetic coefficients in the initial rate shown in Equation 1.

\[ E/v_0 = \phi_0 + \phi_1/S_1 + \phi_2/S_2 + \phi_12/S_1S_2 \] (1)

Unprimed symbols \( \phi_0 \) etc. will be used to denote kinetic coefficients for the reduction of acetaldehyde \( (S_2) \) by NADH \( (S_1) \), and primed symbols \( \phi'_0 \) etc. for those for oxidation of ethanol \( (S'_2) \) by NAD+ \( (S'_1) \).

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1 Despite recent statements and misquotations to the contrary (19, 20).
RESULTS

Initial rate data for the ethanol-NAD\(^+\) reaction at pH 7.1 are shown in the primary plots of Figs. 1 and 2. The intercepts and slopes obtained are plotted in Figs. 3A and B, and the slopes and intercepts of these secondary plots yield values for the four kinetic coefficients in Equation 1. Similar experiments were made at pH 6.0. The values obtained for the kinetic coefficients are compared in Table I with earlier estimates from experiments with commercial NAD\(^+\) preparations (6). Except for 1'\(0\), the two sets of data agree within the experimental error. The earlier values for 1'\(0\), 1'\(1\), and 1'\(12\) were averages of three independent and more detailed experiments (6) and are retained as the most reliable estimates.

New estimates of 1'\(0\), the reciprocal of the maximal specific rate of the acetaldehyde-NADH reaction, were also obtained at pH 6.0 and pH 7.1, and agreed with one another, and with earlier estimates (6) to within 10%.

Complete initial rate studies of both the ethanol-NAD\(^+\) reaction and the acetaldehyde-NADH reaction were made at pH 8.0 and pH 9.0. The results of one experiment at pH 9.0 are shown in Figs. 4 to 9. Three such experiments on the ethanol-NAD\(^+\) reaction gave kinetic coefficients which agreed to within 10%. Less detailed measurements were made at pH 5.35 and pH 9.9.

DISCUSSION

The removal of a small proportion of foreign nucleotide from commercial NAD\(^+\) preparations of high purity results in much lower values for 1'\(2\) (and for the Michaelis constant for ethanol, 1'\(20\)/1'\(0\)), by a factor of 0.35 at pH 6.0 and 0.6 at pH 7.1. The other three kinetic coefficients for ethanol oxidation (and the Michaelis constant for NAD\(^+\), 1'\(20\)) are unchanged (Table I). These findings are in accordance with previous experiments, and with theoretical predictions (12, 22) of the effects of an impurity which competes with NAD\(^+\) in a compulsory order mechanism:

\[
E + S_1 \xrightarrow{k_{11}} ES_1 \quad (K_{11} = k_{11}/k_{-1})
\]

\[
ES_1 + S_2 \xrightarrow{k_{12}} ES_S_1 \xrightarrow{k_{-12}} ES_1 + S_2 \quad (1)
\]

\[
ES_1 \xrightarrow{k_{11}'} E + S_1 \quad (K_{11}' = k_{11}'/k_{-1}')
\]

The best estimates of the kinetic coefficients for pH 6.0 to 0.0 from this and earlier work (6) are given in Table II.

<table>
<thead>
<tr>
<th>NAD(^+) preparation</th>
<th>pH</th>
<th>1'(0)</th>
<th>1'(1)</th>
<th>1'(12)</th>
<th>1'(2)</th>
<th>1'(20)</th>
</tr>
</thead>
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<tr>
<td>Purified(^*)</td>
<td>6.0</td>
<td>0.08</td>
<td>2.7</td>
<td>280</td>
<td>67,000</td>
<td></td>
</tr>
<tr>
<td>Commercial(^\dagger)</td>
<td>6.0</td>
<td>0.62</td>
<td>2.5</td>
<td>800</td>
<td>70,000</td>
<td></td>
</tr>
<tr>
<td>Purified(^*)</td>
<td>7.1</td>
<td>0.37</td>
<td>1.1</td>
<td>66</td>
<td>6,600</td>
<td></td>
</tr>
<tr>
<td>Commercial(^\dagger)</td>
<td>7.1</td>
<td>0.37</td>
<td>1.1</td>
<td>110</td>
<td>7,200</td>
<td></td>
</tr>
</tbody>
</table>

\(^*\) This work.
\(^\dagger\) Reference (6).

The best estimates of the kinetic coefficients for pH 6.0 to 0.0 from this and earlier work (6) are given in Table II.
In earlier kinetic studies at pH 6.0 and pH 7.1 (6) with NADH preparations of high purity, but with commercial NAD+ preparations, it was shown that within the experimental errors, $\phi_1/\phi_0 = K_1$, the dissociation constant of the enzyme-NADH complex, and $\phi'_1/\phi'_0 = K'_1$, the dissociation constant of the NAD+ complex. Further, it was found that the values of $\phi_0$ and $\phi_1$ in the NADH reaction, and $\phi'_0$ and $\phi'_1$ in the NAD+ reaction, were the same with two different substrates. These results indicated that not only could $\phi_1$ be identified with $1/k_1$, and $\phi'_1$ with $1/k'_1$, as required by Mechanism I, but also that $\phi_0 = 1/k_{-1}$ and $\phi'_0 = 1/k'_{-1}$, that is, that the maximal specific rate in each direction is equal to the rate of the last step, the dissociation of the product coenzyme from its complex. This is the essential feature of the Theorell-Chance mechanism, the limiting case of Mechanism I in which the ternary complexes are not kinetically significant under maximum rate conditions (6, 9).
Mechanism I also requires that \( \varphi_2/\varphi_2 = K_1 \) and that \( \varphi_1/\varphi_2^* \) be less than unity if ternary complexes are kinetically significant under maximal rate conditions, or equal to unity in the limiting case of the Theorell-Chance mechanism (9). These relations were also satisfied by the earlier data (6). However, the complementary relations \( \varphi_1/\varphi_2 = K_1 \) and \( \varphi_1/\varphi_2^*/\varphi_2^* \) \( \leq 1 \) were not, suggesting that neither the Theorell-Chance mechanism nor the general form of Mechanism I were satisfactory. The present results show that these discrepancies are due to impurities in the NAD\(^+\) preparations. The data of Table III show that with the lower values of \( \varphi_2^* \) obtained with purified NAD\(^+\), these deviations from the requirements of the Theorell-Chance mechanism largely disappear. None of the other tests of mechanism are affected, since they do not involve \( \varphi_2^* \), and this mechanism, therefore, may be regarded as satisfactory at pH 6.0 and pH 7.1.

Theorell and McKee (17) reported kinetic parameters for liver alcohol dehydrogenase at pH 7.0 obtained with commercial coenzyme preparations which also satisfied all the requirements of the Theorell-Chance mechanism. It seems likely that this was partly due to fortuitous cancellation of errors. Their values\(^2\) of 0.016 sec for \( \varphi_a \) and 115 \( \mu \)M sec for \( \varphi_2^* \) are both twice the present values, but agree very well with those found by Dalziel (6) with similar commercial coenzyme preparations, and indicate the presence of inhibitors.\(^3\) However, their values of 2.3 \( \mu \)M for \( K_1 \), and 5.0 \( \mu \)M for \( K_2 \) (\( = \varphi_1/\varphi_2^* \)) for NAD\(^+\), are also twice the values found in the present and earlier work (6). These discrepancies cannot be attributed to the effects of an inhibitor, but are perhaps within the experimental error of their measurements, since the lowest NAD\(^+\) concentration used was 25 \( \mu \)M, and their values for \( K_1 \) and \( K_2 \), therefore, were estimated from rates not less than 80% of the maximal rates (reference (17), Figs. 5 and 12).

The conformity of kinetic data at pH 8.0 and 9.0 to the requirements of the Theorell-Chance mechanism is tested in Table IV, by comparisons between various ratios of the kinetic coefficients from Table II with independent values for the dissociation constants for coenzyme complexes at the same temperature and in the same buffer (24) and with the equilibrium constant (25) for the over-all reaction, \( K = [NADH] [CH\(_2\)CHO][H\(^+\)]/[NAD\(^+\)][CH\(_2\)OH] \). At pH 9.0, all the criteria of the mechanism are fulfilled within close limits. At pH 8.0 also, there is satisfactory agreement between \( \varphi_2/\varphi_2^* \) and \( K_1 \), and \( \varphi_1/\varphi_2^* \) and \( K_1 \), consistent with the essential feature of the Theorell-Chance mechanism, that the maximal specific rate may be identified with the rate of dissociation of the product coenzyme complex. This also requires that the functions in columns 10 and 11 of Table IV be equal to unity; although larger than at other pH values, the discrepancies at pH 8.0 are not outside the combined experimental errors of the four parameters involved. The difference between \( \varphi_1 \) \( [H\(^+\)]/\varphi_2 \) and \( K_1 \) is also not clearly significant, because \( \varphi_1 \) at this pH value, as at pH 6.0 and 7.1 (6), is small and can be estimated only approximately even with the sensitive fluorometric method. The most serious discrepancy is between \( \varphi_1 \), \( \varphi_2^* \), and \( K_1 \) at pH 8.0. This seems to be outside the random error of measurements of \( \varphi_1 \) and \( \varphi_2^* \), but also is not conclusive in view of the possible error in the direct estimation of \( K_1 \) (5, 24).

It may be concluded that initial rate data for liver alcohol dehydrogenase at pH 6 to 9 with pure coenzymes are satisfactorily described by the compulsory order Mechanism 1, in which the intramolecular reactions of the ternary complexes and the dissociation of substrate product are rapid, and the maximal rate is determined by the rate of dissociation of the product coenzyme, as originally suggested by Theorell and Chance (7).

On this basis, the reciprocals of \( \varphi_2^* \), \( \varphi_2^*/\varphi_2^* \), and \( \varphi_1 \) may be

\begin{table}[ht]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{pH} & \multicolumn{3}{|c|}{\textbf{NAD\(^+\) + CH\(_2\)CHO}} & \multicolumn{3}{|c|}{\textbf{NAD\(^+\) + CH\(_2\)OH}} \\
\hline
 & \( \varphi_1 \) & \( \varphi_2 \) & \( \varphi_2^* \) & \( \varphi_1 \) & \( \varphi_2 \) & \( \varphi_2^* \) \\
\hline
6.0 & 0.008 & 0.115 & 3.7 & 1.5 & 0.62 & 2.5 & 280 & 70,000 \\
7.1 & 0.008 & 0.100 & 3.3 & 1.4 & 0.07 & 1.1 & 56 & 7,200 \\
8.0 & 0.021 & 0.132 & 3.9 & 2.4 & 0.31 & 0.9 & 50 & 1,300 \\
9.0 & 0.132 & 0.22 & 13.9 & 11.4 & 0.26 & 1.2 & 103 & 940 \\
\hline
\end{tabular}
\caption{Kinetic coefficients for liver alcohol dehydrogenase at 28.5\(^\circ\)C with coenzymes of high purity}
\end{table}

\begin{table}[ht]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
\textbf{NAD\(^+\) preparation} & \textbf{pH} & \( \varphi_1/\varphi_2^* \) & \( K_1 \) & \( \varphi_1/\varphi_2^* \) & \( \varphi_2^*/\varphi_2^* \) \\
\hline
\textbf{Purified} & 6.0 & 250 & 256 & 1.2 & \\
& 6.0 & 88 & 3.6 & \\
\textbf{Commercial} & 7.1 & 110 & 160 & 1.3 & \\
& 7.1 & 65 & 2.2 & \\
\hline
\end{tabular}
\caption{Tests of conformity of kinetic coefficients for ethanol oxidation by NAD\(^+\) with requirements of compulsory order mechanism}
\end{table}
identified with the specific rates of formation and dissociation of 2,854 1.3 × 10⁻¹⁰ from the present kinetic data.

Values of K₁ and K₁ are those of Theorell and McKee (24), and K₁ is taken from Backlin (25). Values for kinetic coefficients are from Table II. The relations by which the mechanism is tested were derived by Dalsgard (9).
FIG. 10. Variation with pH of log $K_{2}$, the equilibrium constant of the reaction of enzyme-coenzyme complexes with substrates, calculated from kinetic parameters ($\bullet$, $K_{2} = \varphi \left( \frac{H^{+}}{\varphi} \right)$) and from the dissociation constants of enzyme-coenzyme complexes (24) and the over-all equilibrium constant ($\circ$, $K_{2} = K_{2}K_{2}'/K_{1}$).

FIG. 11. Variation with pH of the dissociation constants of $E\cdot NAD^{+}$ ($K_{r}^{+}$) and $E\cdot NADH$ ($K_{r}$). The filled symbols represent estimates from kinetic parameters ($K_{r} = \varphi / \varphi$ and $K_{r}^{+} = \varphi / \varphi$) and the open symbols are the equilibrium data of Theorell and McKee (24) or Theorell and Winer (5).

FIG. 12. Variation with pH of the specific rates of formation, $k_{1}$, and dissociation, $k_{-1}$, of the enzyme compound with NAD$. The curves are theoretical acidic dissociation curves for $pK$ values of (a) 6.4, (b) 9.6, and (c) 8.1.

FIG. 13. Variation with pH of the specific rates of formation, $k_{1}$, and dissociation, $k_{-1}$, of the enzyme compound with NADH. The curve (a) is a theoretical acidic dissociation curve for $pK = 8.7$.

could be either a thiol group or a water molecule coordinated to zinc (24). The shift of $pK$ could again be an electrostatic effect of NAD$, and is responsible for the marked decrease of $K_{r}$ from pH 7 to pH 10.

The dissociation of $E\cdot NADH$ is promoted by ionization of two groups in the enzyme. It appears that ionization of a group with $pK$ 8.5 to 9.0 in the free enzyme and $pK > 9.5$ in the complex both inhibits formation of the latter and increases its rate of dissociation (Fig. 13), and the difference of $pK$ is responsible for the large increase of $K_{r}$ from pH 8 to pH 10. These results are consistent with other evidence that a thiol group is involved in the binding of NADH to the enzyme, either by addition to the pyridine ring (24, 26) or by hydrogen bonding (27). In either case the acidity of the group would be decreased in $E\cdot NADH$, and a proton would be released during oxidation to $E\cdot NAD^{+}$ at pH >7.

The liberation of protons from the enzyme during oxidation of $E\cdot NADH$ and the uptake of protons during the reduction of $E\cdot NAD^{+}$ may provide the means of proton transfer to and from the substrate in the intramolecular reaction of the ternary complex. The involvement of the proton in the over-all reaction would be represented by

$$EH\cdot NADH + CH_{2}CHO \rightarrow E^{+}NAD^{+} + CH_{2}OH$$

$$E^{+}NAD^{+} + H^{+} \rightarrow EH + NAD^{+}$$

Such a role has been suggested for a histidine group in lactate dehydrogenase (28). The presence of two groups, with $pK$ values of 6.5 to 7.0 and >9.5 in $E\cdot NADH$, and 6.0 and ~8.0 in $E\cdot NAD^{+}$, would perhaps provide a more efficient mechanism for the reaction in both directions at pH ~7 to 8.

Consideration of More Complex Mechanisms—Two more complicated reaction schemes have been proposed for liver alcohol dehydrogenase, involving enzyme-substrate complexes (17) and isomeric or inactive enzyme-coenzyme complexes (11). A random order mechanism with kinetically significant enzyme-substrate complexes, in addition to enzyme-coenzyme complexes, does not in the general case (29) yield a steady state initial rate equation of the simple form of Equation 1. In the special case that the complexes are in rapid equilibrium (30) which does con-
form to Equation 1, the intramolecular reaction of the ternary complex determines the maximal rate, and, therefore, the fact that the relations \( \varphi_1^o/\varphi_1' > 1 \) and \( \varphi_1'/\varphi_1 = K' \) are satisfied, with two pairs of substrates (6), makes this case unlikely. These particular relations may be satisfied by a random order mechanism if other assumptions are made (6, 17), but strict simplification of the complex initial rate equation to the form of Equation 1 has not been demonstrated. The suggestion (6) that this might explain the discrepancies of earlier initial rate data from the requirements of a compulsory order mechanism now has no experimental justification. Nevertheless, the possibility that binary enzyme-substrate complexes are kinetically significant cannot be dismissed. Theorell and McKee (17, 31) achieved a consistent interpretation of initial rate data with and without inhibitors on the basis of a random order mechanism. They assumed that the dissociation constant of the enzyme substrate complex may be identified with the ratio of initial rate parameters \( \varphi_1/\varphi_1' \), by analogy with the relation \( \varphi_1/\varphi_2 = K_1 \) derived for the enzyme-coenzyme complex in a compulsory order mechanism. Closely similar values for substrate dissociation constants were calculated from inhibition data, on the assumption that with excess substrate the enzyme would be largely in the form of the substrate complex. Agreement between these two quantities does not, however, appear to establish their identity as dissociation constants of enzyme-substrate complexes, or the kinetic significance of the latter, and it seems possible that an equally consistent interpretation might be achieved on the basis of a compulsory order mechanism, in which inhibitors compete with substrates for the enzyme-coenzyme complex rather than the free enzyme. It has been shown that the kinetics of ortho-phenanthroline inhibition are consistent with a simple compulsory order mechanism (34). Direct estimates of dissociation constants of enzyme-substrate complexes are lacking.

Mahler, Baker, and Shiner (11) showed that the assumption of isomeric or inactive complexes in the Theorell-Chance mechanism permits values for the ratio \( \varphi_1'/\varphi_1 \) greater than unity. For liver alcohol dehydrogenase they suggested the Theorell-Chance case of the compulsory order mechanism I with the addition of reactions \( E + S_1 = EX \) and \( E + S_1' = EX' \) by which unreactive enzyme-coenzyme complexes are formed. Since the formation of the reactive \( ES_2 \) and inactive \( EX' \) is competing reactions, the initial rate equation for this mechanism (11) is identical in form to that for a competitive inhibitor present as an impurity in the coenzyme (12), viz.

\[
\frac{E}{v_1} = \frac{\varphi_1'}{\varphi_1} \left( 1 + \frac{\varphi_1'}{\varphi_1} \varphi_1' \right) + \frac{\varphi_1'}{S_1} \left( 1 + \frac{\varphi_1'}{\varphi_1} \varphi_1' \right) + \frac{\varphi_1'}{S_1} \frac{\varphi_1'}{S_2} + \frac{\varphi_1'}{S_1} \frac{\varphi_1'}{S_2}
\]

where the kinetic coefficients are those for Mechanism 1, \( K_1 = K' \), the dissociation constant of the inactive coenzyme compound, and \( r \), the molar ratio of inhibitor to coenzyme, is unity. Thus, the demonstration of the occurrence and effects of inhibiting impurities in coenzymes renders equivocal the existing evidence for the occurrence of inactive or isomeric coenzyme complexes in the reaction of this and other (22) pyridine nucleotide-linked dehydrogenases, insofar as this is based on initial rate data or discrepancies between kinetic and equilibrium values for dissociation constants of coenzyme complexes (35).

Further work with purified coenzymes and other dehydrogenases may of course reveal significant discrepancies from the Theorell-Chance mechanism. The steady state initial rate equation for both take the form of Equation 1. The kinetic coefficients for the forward reactions in terms of the rate constants in the mechanisms are given in Table VI, and certain ratios of kinetic coefficients are also evaluated. The corresponding expressions for the kinetic coefficients for the reverse reactions, and the complementary ratios, are obtained simply by substituting primed for unprimed rate constants and vice versa. From these data the following conclusion may be drawn.

(a) Either \( \varphi_1/\varphi_2 = k_1/k_2 \) or \( \varphi_1'/\varphi_2' = k_1'/k_2' \) may be greater than unity, but not both, depending simply upon the relative values of \( k_1K_2 \) and \( k_2K_1' \) for Mechanism A and of \( k_1K_1' \) and \( k_2K_2' \) for Mechanism B. If \( \varphi_1'/\varphi_1 = \varphi_2'/\varphi_2 = \varphi_1/\varphi_2 > 1 \), more than one form of complex from the reaction of enzyme and \( S_1 \) would be indicated, but no conclusion could be drawn regarding the presence of isomers or inactive forms of \( ES^1 \).

(b) For neither mechanism is \( \varphi_1/\varphi_2 = k_1/k_2 \), the dissociation constant of the species \( ES \). However, in both cases it can be shown from Table VI that \( \varphi_1/\varphi_2 = k_{app} = (E)(S_2)/ES_2 + (EX) \), where these are equilibrium concentrations. Direct equilibrium measurements with coenzyme and enzyme would also yield \( K_{app} \) provided the method of analysis is such that the total bound coenzyme and free coenzyme are distinguished. Thus comparison of the kinetic value of \( \varphi_1/\varphi_2 \) with direct estimates of the “dissociation constant” of the enzyme-coenzyme compound (11) will not distinguish these two mechanisms, nor does agreement between these quantities preclude the existence of either isomeric or inactive compounds. It may be recalled that such a conclusion is predicted for Mechanism I with or without rate-limiting ternary complexes and for an equilibrium random order mechanism (9).

(c) It follows directly from (a) and (b) that agreement between \( \varphi_1/\varphi_2 \) and direct measurement of the “dissociation constant” of the enzyme-coenzyme compound is not predicted for either mechanism. Such agreement, like the equality \( \varphi_1/\varphi_2 = \varphi_1'/\varphi_2' = 1 \), is characteristic of the Theorell-Chance case of the simple compulsory order Mechanism 1. The present data at pH 8.0 (Table IV) do satisfy the relations \( \varphi_1/\varphi_2 = K_1 \) and \( \varphi_1'/\varphi_2' = K_1' \).

(d) The identity \( \varphi_1 + \varphi_1' = \varphi_2 + \varphi_2' = \varphi_1 + \varphi_2 + \varphi_1' + \varphi_2' \), deduced by Mahler, Baker, and Shiner (11) for Mechanism A, is predicted also for Mechanism B and for the Theorell-Chance mechanism. It appears, therefore, that while values of either \( \varphi_1/\varphi_2 = \varphi_1'/\varphi_2' \) or \( \varphi_1'/\varphi_1 = \varphi_2'/\varphi_2 \) significantly greater than unity, obtained with pure coenzymes, would justify the assumption of more than one form of enzyme-coenzyme compound, initial rate data will not permit

Some of these conclusions have also been reached for a generalized form of Mechanism B with unspecified numbers of isomeric coenzyme complexes and ternary complexes by Blochfield, Peller, and Alberty (36) and also by Cleland (37). For Mechanism B, \( k_1 \) and \( k_1' \) correspond to \( k_2 \) and \( k_2' \) in reference (11), where \( k_2 \) and \( k_2' \) are specified in this context.
Kinetic coefficients in initial rate equation $E/v_0 = \phi_1 + \phi_2/S_1 + \phi_3/S_2 + \phi_4/S_3S_4$ for Theorell-Chance mechanism with (A) inactive coenzyme complexes and (B) isomeric coenzyme complexes (11)

<table>
<thead>
<tr>
<th>Theorell-Chance mechanism</th>
<th>$\phi_1$</th>
<th>$\phi_2$</th>
<th>$\phi_3$</th>
<th>$\phi_4$</th>
<th>$\phi_1/\phi_3$</th>
<th>$\phi_2/\phi_4$</th>
</tr>
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<tr>
<td>Original</td>
<td>1</td>
<td>$k_{-1}$</td>
<td>$k_1$</td>
<td>$k_2$</td>
<td>$k_{-1}$</td>
<td>$k_1$</td>
</tr>
<tr>
<td>A. Inactive coenzyme complexes</td>
<td>$k_{-1} + kK_z$</td>
<td>$k_1 + kK_z$</td>
<td>$k_1$</td>
<td>$k_1 + kK_z$</td>
<td>$k_1 + k_{-1}/k'K_{-1}$</td>
<td></td>
</tr>
<tr>
<td>B. Isomeric coenzyme complexes</td>
<td>$k'<em>{-1}(1 + k'</em>{-2}/k_2) + k'_{-2} + k'$</td>
<td>$k_{-1} + k_2$</td>
<td>$k_2 + k_3$</td>
<td>$k_{-1}$</td>
<td>$k_1$ + k_2</td>
<td>$k_1 + k_{-1}/k'K'$</td>
</tr>
</tbody>
</table>

* Mechanism A is: $E + S_1 \rightleftharpoons EX, K_z = (E)(S_1)/(EX)$

$$E + S_1 \xrightarrow{k_{-1}} ES,$$
$$ES_1 + S_1 \xrightarrow{k_{-2}} ES_1 + S'$$
$$ES'_{1} \xrightarrow{k'_{-1}} E + S'$$
$$EX' \xrightarrow{k'_{-2}} E + S'$$

† Mechanism B is: $E + S_1 \xrightarrow{k_{-1}} ES, \xrightarrow{k_{-2}} EX$

$$EX + S_2 \xrightarrow{k_{-3}} EX' + S'$$
$$EX' \xrightarrow{k'_{-3}} ES'$

† There appear to be misprints in the expressions for $\phi_1$ and $\phi_3$ in reference (11).

SUMMARY

1. Initial rate data for liver alcohol dehydrogenase at pH 5.35 to 9.9 with purified coenzymes are presented. The data at pH 6.0 to 9.0 are shown to be consistent, within the experimental error, with the limiting case of a compulsory order mechanism, proposed by Theorell and Chance, in which the maximal rate in each direction is equal to the rate of dissociation of the enzyme-coenzyme product complex. Divergences of earlier data from the requirements of this simple mechanism were due to the effects of a competitive inhibitor in commercial nicotinamide adenine dinucleotide preparations.

2. On the basis of this mechanism, and agreement between kinetic and equilibrium values for the dissociation constants of enzyme-coenzyme complexes, experimentally determined parameters in the initial rate equation are identified with the specific rates of formation and dissociation of the enzyme-coenzyme product complex. Divergences of earlier data from the requirements of this simple mechanism were due to the effects of a competitive inhibitor in commercial nicotinamide adenine dinucleotide preparations.

3. Variations of initial rate parameters and equilibrium constants with pH are tentatively ascribed to the presence of two coenzyme-linked acidic groups in the enzyme, with lower pH values in the nicotinamide adenine dinucleotide complex than in the reduced nicotinamide adenine dinucleotide complex. It is suggested that these groups may serve in proton transfer to and from the substrates.

4. More complex mechanisms which have been proposed for liver alcohol dehydrogenase are discussed. It is concluded that there is no definite evidence for the occurrence of kinetically significant binary enzyme-substrate complexes, or of more than one form of each enzyme-coenzyme complex. Mechanisms of the latter type involving inactive or isomeric coenzyme complexes are considered theoretically and shown to be indistinguishable from one another by initial rate studies.

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REFERENCES

Kinetic Studies of Liver Alcohol Dehydrogenase and pH Effects with Coenzyme Preparations of High Purity
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