Kinetic Studies with Liver Alcohol Dehydrogenase*

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ABSTRACT: Dissociation constants for enzyme–nucleotide complexes have been determined by product inhibition studies with seven different aldehydes and eight different alcohols as the nonvaried substrates. The nature of the alcohol or aldehyde does not affect the enzyme–nucleotide dissociation constants, which is consistent with an Ordered mechanism. Alternate product studies were carried out using *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde as substrates and several alcohols and aldehydes as alternate products. Intercept-hyperbolic activation and slope-linear inhibition were produced by acetaldehyde and benzaldehyde with *p*-hydroxybenzyl alcohol as the variable substrate, while ethyl, phenethyl, and cycloheptyl alcohols produced linear competitive inhibitions *vs.* *p*-hydroxybenzaldehyde. These data are consistent with an Ordered mechanism which includes abortive ternary complexes. Methanol, which has been shown not to be a substrate for horse liver alcohol dehydrogenase, acts as a partial inhibitor of either ethanol oxidation or acetaldehyde reduction. Although the initial velocity data for liver alcohol dehydrogenase are not inconsistent with a Random mechanism where addition of nucleotides is diffusion controlled and the rates of dissociation of alcohol and aldehyde from the ternary complexes greatly exceed the rates of release of nucleotides from and the rates of interconversion of the ternary complexes, the available product inhibition data require that the contribution of the alternate pathway be quite small.

Liver alcohol dehydrogenase (LADH)[1] catalyzes the following reaction.

$$DPN^+ + \text{alcohol} \rightleftharpoons \text{aldehyde} + DPNH + H^+ \quad (1)$$

This enzyme exhibits a rather broad specificity; many primary and secondary alcohols and many aldehydes and ketones can be utilized as substrates (Sund and Theorell, 1963). The kinetic mechanism has been known for some time to be sequential (Theorell and Chance, 1951) (that is, both substrates must combine with the enzyme before either product can be released), but until recently it was not clear whether central ternary complexes were kinetically important, or whether the addition of substrates was ordered or random. Dalziel (1963) concluded from careful initial velocity experiments that central ternary complexes were not kinetically important, but, by using the more direct and sensitive method of product inhibition, Wratten and Cleland (1963) were able to demonstrate the presence of these complexes and postulated the following Ordered mechanism[2] for LADH (eq 2). These data also suggested the formation of dead-end E–DPNH–ethanol and E–DPN^+–acetaldehyde complexes similar to those shown to exist for other dehydrogenases (Fromm and Nelson, 1962; Zewe and Fromm, 1962).

(2)

However, it has been reported that the dissociation constants of enzyme–nucleotide complexes, as determined by initial velocity studies, varied with the alcohol or aldehyde used as the second substrate (Baker, 1960). These data are inconsistent with mechanism 2, which requires that these dissociation constants be independent of the second substrate. In order to check on this point, these dissociation constants have been determined more precisely from certain product inhibition experiments. Data are reported here from experiments

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[1] Abbreviations used: LADH, liver alcohol dehydrogenase; DPN^+, diphosphopyridine nucleotide; DPNH, reduced DPN^+; E, enzyme.

[2] The nomenclature used to describe kinetic mechanisms is that of Cleland (1963a).

carried out with seven different aldehydes and eight different alcohols as the second substrate.

The broad substrate specificity of this enzyme makes it ideally suited for alternate product inhibition studies which can provide evidence concerning the order of addition of reactants to the enzyme and the importance of ternary central and ternary dead-end complexes in the kinetic mechanism. Alternate product studies have been carried out using *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde as substrates.

It has been reported (Merritt and Tomkins, 1959) that despite its broad substrate specificity horse LADH will not oxidize methanol. This has been confirmed, but it has been found that at high concentrations methanol acts as a partial inhibitor and causes nonlinear reciprocal plots when either ethanol or acetaldehyde is the variable substrate.

Methods and Materials

Chemicals. Horse LADH (C. F. Boehringer) was obtained as a crystalline suspension in potassium phosphate buffer containing 10% ethanol. This suspension was diluted, dialyzed against 0.02 M phosphate, pH 7.0, to remove ethanol, and centrifuged to remove any insoluble material. The resulting solutions contained about 1 mg/ml of protein and 5×10^{-3} M ethanol and were stable for as long as 6 weeks at 0°. The specific activity was equal to that obtained by Bonnichsen and Brink (1956). For an experiment this stock solution was diluted with 0.1% bovine serum albumin; the diluted enzyme was stable for at least 2 hr. Enzyme concentrations were calculated as site equivalents per liter, assuming a molecular weight of 84,000 and two active sites per molecule.

Experiments to determine dissociation constants of enzyme–nucleotide complexes and the experiments with methanol were carried out in 0.1 M sodium glycylglycinate, pH 8.6. All other experiments were carried out in 0.05 M potassium phosphate, pH 7.0.

DPN^+ (Sigma) and the disodium salt of DPNH (Sigma) were kept desiccated at −20°. DPNH solutions were freshly prepared for each experiment. The DPN^+ used in experiments designed to determine the dissociation constants of enzyme–nucleotide complexes was first chromatographed on DEAE-cellulose–bicarbonate according to the method of Cha (1963).

p-Hydroxybenzaldehyde (Eastman Organic Chemicals) and *p*-hydroxybenzyl alcohol (Aldrich Chemicals Co.) were sublimed at 67° under vacuum (0.01 mm) before use. All other alcohols and aldehydes used were of reagent grade. The alcohols were redistilled and kept at 4°; fresh solutions were prepared on the day of an experiment. Aldehydes were redistilled on the day they were to be used in an experiment.

Apparatus. Normally the reaction was followed by measuring the appearance or disappearance of DPNH at 340 mμ using a Beckman DU monochromator with a deuterium lamp, a Gilford Model 200 optical density converter, and a 10-mv recorder equipped with an adjustable zero and a multispeed chart drive. For alternate product experiments, however, the reaction was followed by measuring the appearance or disappearance of the alcohol or aldehyde substrate at 281 mμ (an isosbestic point for DPN^+–DPNH). The electronic circuits of the optical density converter allowed optical densities up to 3.0 to be blanked out, so that the full-scale range on the recorder could be set to as low as 0.05 optical density even in the presence of large initial concentrations of DPNH. To keep the slopes of the recorded lines close to 45° the full scale of the recorder was varied from 0.05–0.2 optical density and chart speeds were varied from 0.5–6 in./min. Silica cuvets (1-cm light path) were filled with all reaction components except enzyme in a volume of 2.9 ml and preincubated in thermostated boxes at 25°. The reaction cell compartment was also kept at 25° by the use of thermospacers. The reaction was initiated by the addition of 0.1 ml of diluted enzyme solution with an "adder mixer" (Boyer and Segal, 1954). The recorder curves obtained were extrapolated to the time of enzyme addition and the tangents to the curves at this time were taken as initial velocities.

Data Processing. Reciprocal velocities were first plotted graphically against the reciprocals of substrate concentrations. When these plots were linear, the data were first fitted to eq 3 using a least-squares method and assuming equal variance for the velocities (Wilkinson, 1961). All least-square fits reported here were performed by a digital computer using the Fortran programs of Cleland (1963c). These programs provide values for the constants in a fitted equation, the standard errors of their estimates, and weighting factors for further analysis. Slopes (K/V) and intercepts ($1/V$) obtained from eq 3 were then plotted graphically against either the inhibitor concentration (for inhibition experiments) or the reciprocal of the nonvaried substrate concentration (for initial velocity experiments) to determine the form of the over-all rate equation. Final values for kinetic constants were obtained by fitting all data points used in the first analysis to this over-all equation. Data conforming to a sequential initial velocity pattern were fitted to eq 4, data conforming to competitive inhibition were fitted to eq 5, data conforming to linear uncompetitive inhibition were fitted to eq 6, data conforming to linear noncompetitive inhibition were fitted to eq 7, and data conforming to slope-linear, intercept-hyperbolic noncompetitive inhibition were fitted to eq 8.

$$v = \frac{VA}{K + A} \tag{3}$$

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \tag{4}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \tag{5}$$

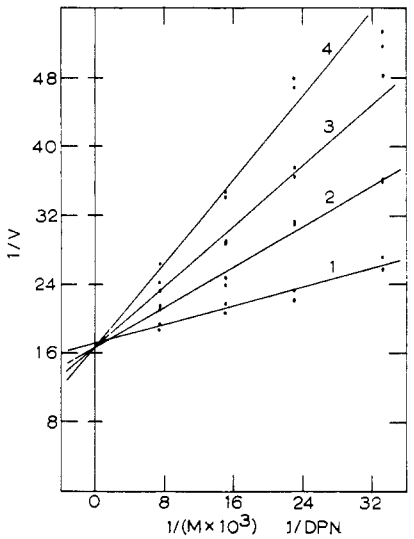


FIGURE 1: Reciprocal plot with DPN^+ as the variable substrate and DPNH as the inhibitor. 1-Pentanol, 2.56×10^{-3} M; DPNH: (1) 0, (2) 3.0×10^{-6} M, (3) 6.0×10^{-6} M, (4) 9.0×10^{-6} M; enzyme, 3×10^{-8} N; 0.1 M sodium glycylglycinate, pH 8.6.

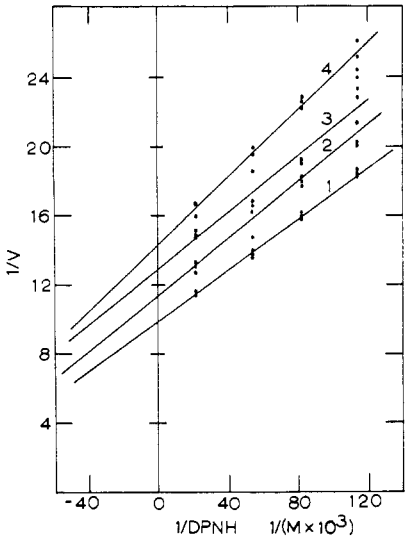


FIGURE 2: Reciprocal plot with DPNH as the variable substrate and butyraldehyde as the changing fixed substrate. Butyraldehyde: (1) 7.07×10^{-4} M, (2) 2.35×10^{-4} M, (3) 1.41×10^{-4} M, (4) 1.01×10^{-4} M; enzyme, 6×10^{-8} N; 0.1 M sodium glycylglycinate, pH 8.6.

$$v = \frac{VA}{K + (1 + I/K_{ii})A} \tag{6}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + (1 + I/K_{ii})A} \tag{7}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + \left(\frac{1 + I/K_{i\ num}}{1 + I/K_{i\ denom}}\right)A} \tag{8}$$

If the original double reciprocal plot were not linear, the data were fitted either to eq 9 when the plot appeared to be a parabola or to eq 10 when it appeared to be a 2/1 function.[3] In doubtful cases, fits to all realistic equations were tried and the one giving the lowest residual least square was taken as the most likely equation.

$$v = \frac{VA^2}{a + bA + A^2} \tag{9}$$

$$v = \frac{V(A^2 + cA)}{a + bA + A^2} \tag{10}$$

The points drawn in figures showing double reciprocal plots are the experimentally determined values. The lines drawn through these points are calculated from fits of these data to eq 3 unless otherwise stated in the figure caption. The slopes and $1/v$ intercepts of these lines were then used as the points for the figures showing replots of slopes and/or intercepts. The lines shown in these replot figures, however, were calculated from a fit of the experimental data to the appropriate over-all rate equation.

Results

Dissociation Constants of Enzyme–Nucleotide Complexes. In product inhibition experiments precautions were taken to avoid concentrations of alcohols or aldehydes giving substrate activation or inhibition. Experiments to detect substrate inhibition or activation were carried out at either 10^{-4} M DPN^+ or 3.8×10^{-4} M DPNH with the following results: cyclohexanol and cycloheptanol activated at concentrations above 2×10^{-3} M; benzyl alcohol, phenylacetaldehyde, 1-butanol, 1-pentanol, and valeraldehyde inhibited at concentrations above 5×10^{-4} M; benzaldehyde, butyraldehyde, ethanol, and acetaldehyde inhibited at concentrations above 6×10^{-3} M; cyclohexanone and phenethyl alcohol produced neither activation nor inhibition at concentrations up to 1×10^{-2} M; 1-propanol and propionaldehyde produced neither activation nor inhibition at concentrations up to 5×10^{-3} M.

With any of eight alcohols as the nonvaried substrate and DPN^+ as the variable substrate, DPNH gave competitive inhibition. A typical experiment with 1-pentanol is shown in Figure 1. Initial velocity experiments were also carried out with DPNH and either acetaldehyde or butyraldehyde as substrates. In both cases intersecting double reciprocal plots were obtained,

[3] A 2/1 function, as described by the following equation, $y = (a + bx + cx^2)/(1 + dx)$, is linear at high values of x but curves near the y axis (Cleland, 1963b). The curve is actually a hyperbola.

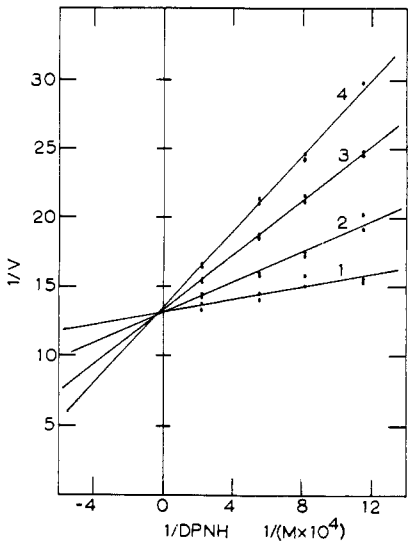


FIGURE 3: Reciprocal plot with DPNH as the variable substrate and DPN^+ as the inhibitor. Cyclohexanone, 9.95×10^{-3} M; DPN^+: (1) 0, (2) 5.28×10^{-5} M, (3) 10.55×10^{-5} M, (4) 15.86×10^{-5} M; enzyme, 1×10^{-8} N; 0.1 M sodium glycylglycinate, pH 8.6.

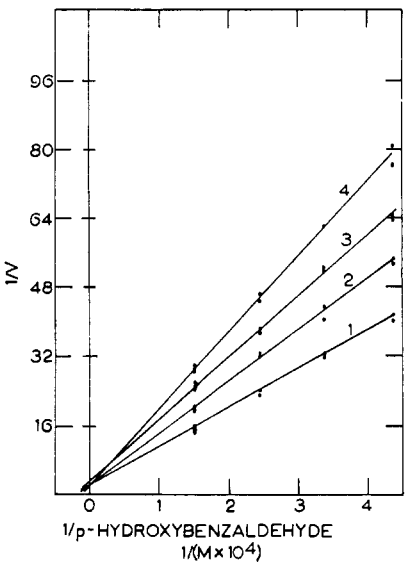


FIGURE 4: Reciprocal plot with *p*-hydroxybenzaldehyde as the variable substrate and phenethyl alcohol as the inhibitor. DPNH, 1.21×10^{-4} M; phenethyl alcohol: (1) 0, (2) 2.0×10^{-3} M, (3) 4.0×10^{-3} M, (4) 6.0×10^{-3} M; enzyme, 8×10^{-8} N; 50 mM potassium phosphate, pH 7.0.

as shown in Figure 2. Dissociation constants for the enzyme–DPNH complex obtained from these product inhibition and initial velocity experiments are given in Table I.

TABLE I: Dissociation Constants for DPNH from Liver Alcohol Dehydrogenase at pH 8.6.

Aldehyde or Alcohol Chain	This Work: Product Inhibition (M × 10^6)	This Work: Initial Velocity (M × 10^6)	Baker (1960) Initial Velocity (M × 10^6)
C_2	2.1 ± 0.1	4.6 ± 1.3	0.87 ± 0.08
C_3	2.7 ± 0.2		1.3 ± 0.1
C_4	2.2 ± 0.1	3.1 ± 1.1	1.3 ± 0.1
C_5	2.7 ± 0.2		3.2 ± 0.1
Cyclo-C_6	3.0 ± 0.1		0.80 ± 0.04
Cyclo-C_7	2.7 ± 0.1		
Benzyl	2.1 ± 0.1		0.47
Phenyl-C_2	2.5 ± 0.1		0.31

With DPNH as the variable substrate, DPN^+ gave competitive inhibition with any of seven aldehydes as the nonvaried substrate. A typical experiment with cyclohexanone is shown in Figure 3. Initial velocity experiments were carried out in this case with DPN^+ and either ethanol or butanol as substrates, and again double reciprocal plots were intersecting. Dissociation constants for the enzyme–DPN^+ complex obtained from these experiments are given in Table II.

TABLE II: Dissociation Constants for DPN from Liver Alcohol Dehydrogenase at pH 8.6.

Aldehyde or Alcohol Chain	This Work: Product Inhibition (M × 10^5)	This Work: Initial Velocity (M × 10^5)	Baker (1960) Initial Velocity (M × 10^5)
C_2	4.0 ± 0.2	4.6 ± 0.5	2.4 ± 0.2
C_3	4.2 ± 0.3		23 ± 7
C_4	5.5 ± 0.2	2.1 ± 0.4	19 ± 3
C_5	4.9 ± 0.2		
Cyclo-C_6	2.9 ± 0.2		18
Benzyl	4.6 ± 0.2		0.73
Phenyl-C_2	1.8 ± 0.2		4.4

Alternate Product Studies. Attempts were made to use vitamin A alcohol as the variable substrate with several aldehydes as alternate product inhibitors by following the oxidation of vitamin A alcohol by DPN^+ at 391 mμ, where DPNH does not absorb. The Michaelis constant for vitamin A alcohol was found to be approximately 8×10^{-6} M determined at a DPN^+ concentration of 1.88×10^{-4} M; however, these experiments were abandoned because the results were not reproducible.

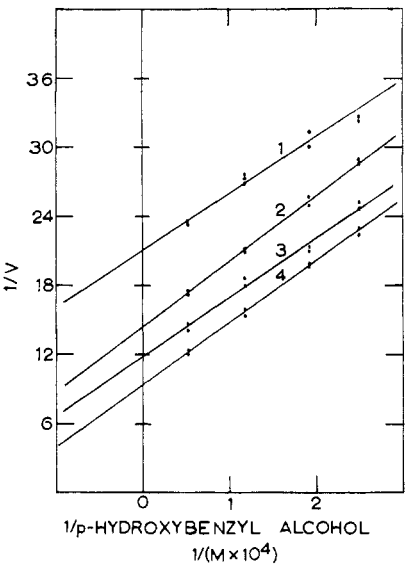


FIGURE 5: Reciprocal plot with *p*-hydroxybenzyl alcohol as the variable substrate and acetaldehyde as the activator. DPN, 5.68×10^{-4} M; acetaldehyde: (1) 0, (2) 0.645×10^{-5} M, (3) 1.94×10^{-5} M, (4) 7.1×10^{-5} M; enzyme, 6×10^{-8} N; 50 mM potassium phosphate, pH 7.0.

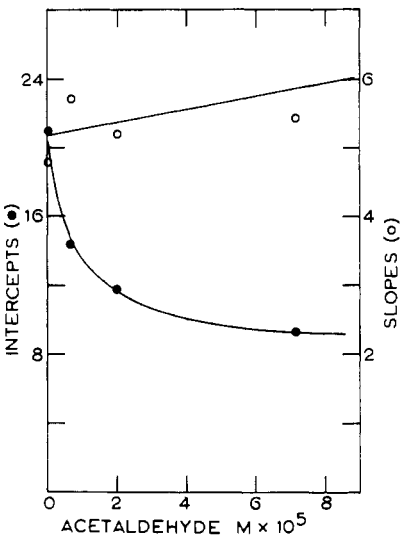


FIGURE 6: Secondary plot from Figure 5 of the slopes and intercepts *vs.* the acetaldehyde concentration. Slope units: (min)(M $\times 10^4$)/OD; intercept units: min/OD.

At 281 mμ, an isosbestic point for DPN^+ and DPNH, there is a millimolar extinction difference of 10 between *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde. The appearance or disappearance of *p*-hydroxybenzaldehyde could therefore be followed at 281 mμ in the presence of alternate products. With *p*-hydroxybenzaldehyde as the variable substrate, ethanol, phenethyl alcohol, and cycloheptanol gave competitive inhibition, as shown in Figure 4. K_{is} values obtained from fits to eq 5 were $3.84 \pm 0.09 \times 10^{-2}$ M for ethanol (weighted average of three experiments), $5.85 \pm 0.14 \times 10^{-3}$ M for phenethyl alcohol, and $20.7 \pm 0.6 \times 10^{-3}$ M for cycloheptanol. With *p*-hydroxybenzyl alcohol as the variable substrate, acetaldehyde and benzaldehyde gave linear inhibition of the slopes (S-linear) and hyperbolic activation of the intercepts (I-hyperbolic) when the data were plotted in double reciprocal form (Figures 5 and 6). Weighted averages for kinetic constants obtained from fitting the S-linear I-hyperbolic data to eq 8 are as follows: acetaldehyde as the alternate product (2 experiments), $K_{is} = 70.7 \pm 23.4 \times 10^{-5}$ M, $K_{i\,num} = 2.07 \pm 0.20 \times 10^{-5}$ M, $K_{i\,denom} = 0.733 \pm 0.058 \times 10^{-5}$ M; benzaldehyde as the alternate product (3 experiments), $K_{is} = 36.8 \pm 5.3 \times 10^{-5}$ M, $K_{i\,num} = 1.49 \pm 0.17 \times 10^{-5}$ M, $K_{i\,denom} = 0.583 \pm 0.054 \times 10^{-5}$ M.

Relative Maximum Velocities with Different Alcohols and Aldehydes. Maximum velocities were obtained by varying the concentrations of both substrates at a constant ratio. If $A = xB$ is substituted into eq 4 the resulting equation has the form of eq 9 with positive constants. In double reciprocal form this equation becomes a parabola, the $1/v$ intercept of which determines $1/V$. However, the concentrations of substrates used often produced little curvature, and when a significant fit of the data to eq 9 was not obtained, the data were fitted to eq 3. The maximum velocity for *p*-hydroxybenzyl alcohol was obtained by varying its concentration at a high constant level of DPN^+ and fitting the data to eq 3. Relative maximum velocities were obtained by determining maximum velocities for several compounds in a single experiment with the same enzyme solution. The values obtained for three alcohol–aldehyde pairs are given in Table III.

Inhibition Studies with Methanol. To determine if LADH would oxidize methanol, an aliquot of LADH was added to a solution containing 4.8×10^{-4} M DPN^+, 0.1 M sodium glycylglycinate, pH 8.6, and methanol.

TABLE III: Relative Maximum Velocities for Liver Alcohol Dehydrogenase with Several Substrates at pH 7.0.

Substrates	Relative Maximum Velocities[a]
DPNH, acetaldehyde	92.9 ± 10.3
DPNH, *p*-hydroxybenzaldehyde	1.50 ± 0.37
DPNH, benzaldehyde	60.9 ± 6.0
DPN^+, ethanol	2.07 ± 0.29
DPN^+, *p*-hydroxybenzyl alcohol	0.738 ± 0.114
DPN^+, benzyl alcohol	1

[a] Values are expressed relative to the maximum velocity of benzyl alcohol and DPN^+.

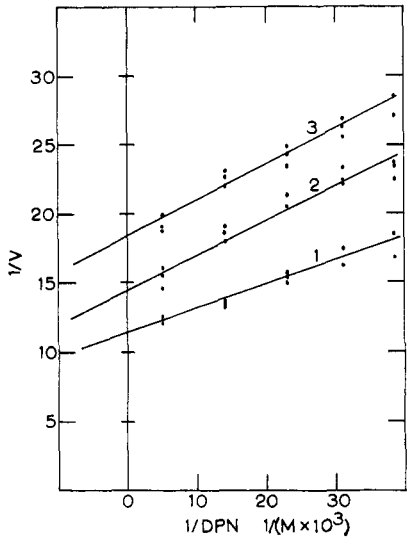


FIGURE 7: Reciprocal plot with DPN^+ as the variable substrate and methanol as the inhibitor. Ethanol, 3.08×10^{-3} M; methanol: (1) 0, (2) 0.08 M, (3) 0.16 M; enzyme, 5×10^{-8} N; 50 mM potassium phosphate, pH 7.0.

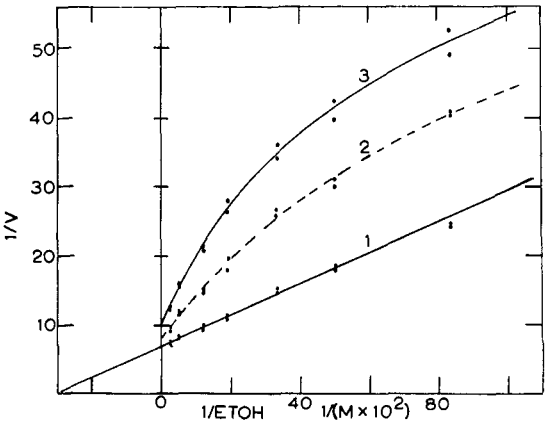
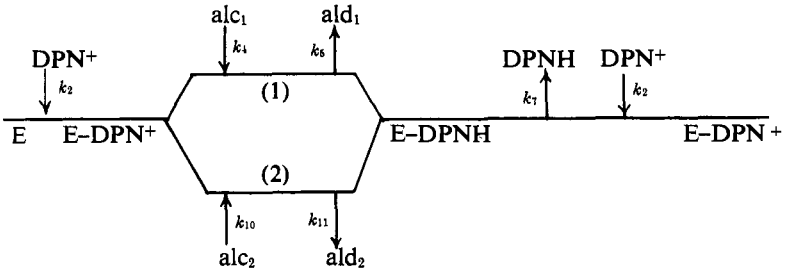


FIGURE 8: Reciprocal plot with ethanol as the variable substrate and methanol as the inhibitor. DPN^+, 4×10^{-4} M; ethanol, $0.12\text{–}4.0 \times 10^{-3}$ M; methanol: (1) 0, (2) 0.11 M, (3) 0.22 M; enzyme, 7×10^{-8} N; 50 mM potassium phosphate, pH 7.0. Data from line 1 were fitted to eq 3; data from lines 2 and 3 were fitted to eq 10, but since a significant fit could not be obtained for the data from line 2 the dotted line drawn on the figure does not represent a computer fit of these data.

Methanol concentrations between 10^{-5} and 10^{-1} M did not produce any measureable increase in the ΔOD at 340 mμ over the normal blank due to enzyme-bound ethanol. Methanol (0.1 M), however, inhibited the oxidation of ethanol by DPN^+ and LADH. With DPN^+ as the variable substrate, methanol gave the inhibition pattern shown in Figure 7. Fits of these data to either eq 6 (uncompetitive inhibition) or to eq 7 (noncompetitive inhibition) gave equal residual least squares. When ethanol was the variable substrate, methanol produced the pattern shown in Figure 8. A significant fit to eq 10 was obtained for the data in line 3 but not for the data in line 2. With ethanol varied at concentrations higher than those in Figure 8, methanol produced a noncompetitive inhibition (Figure 9). Methanol produced the pattern shown in Figure 10 with acetaldehyde as the variable substrate. Significant fits to eq 10 were obtained for the data from both lines 2 and 3.

Discussion

Dissociation Constants of Enzyme–Nucleotide Complexes. Examination of Tables I and II reveals that the nature of the second substrate does not significantly influence the dissociation constant of enzyme–nucleotide complexes except in the case of phenylacetaldehyde. These data suggest that the order of substrate addition and product release given in mechanism 2 holds, regardless of the alcohol or aldehyde used as second substrate. The differences observed by Baker (1960) most likely resulted from experimental error, since these values were obtained from initial velocity data by determining the intersection point of the lines on a double reciprocal plot such as that shown in Figure 2. If the dissociation constant and the Michaelis constant of the nucleotide are not of the same order of magnitude, this extrapolation becomes highly inaccurate. The relative accuracy of values determined by inhibition studies as opposed to initial velocity experiments can be seen from Table I. Although the values obtained by Baker appear significantly different, the standard errors given are undoubtedly too small, since unweighted fits were made to eq 4 (the proper weighting factors to use, assuming equal variance for experimental velocities, are the fourth powers of the experimental velocities).



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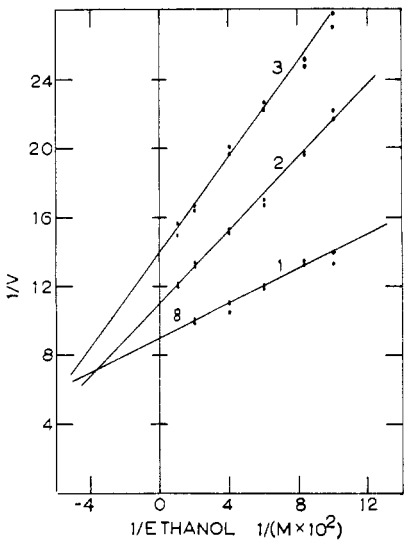


FIGURE 9: Reciprocal plot with ethanol as the variable substrate and methanol as the inhibitor. Ethanol, 1–10 × 10^{-3} M; DPN^+, 4 × 10^{-4} M; methanol: (1) 0, (2) 0.11 M, (3) 0.22 M; enzyme, 7 × 10^{-8} N; 50 mM potassium phosphate, pH 7.0. The open circles were not used in the computer analysis of line 1.

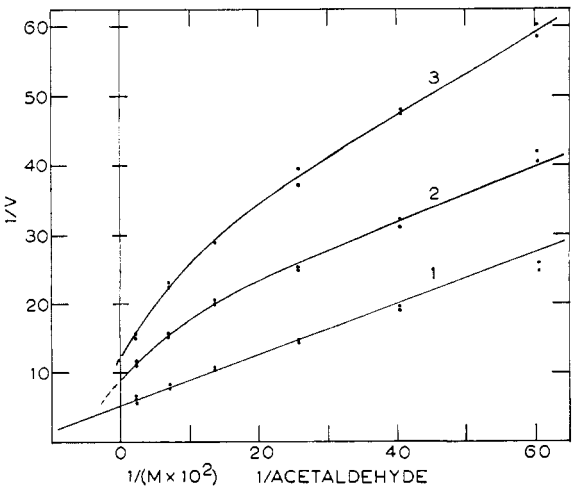
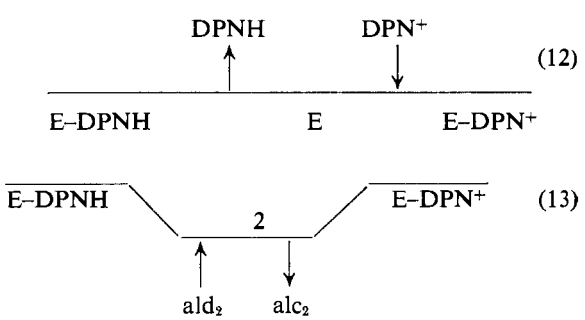


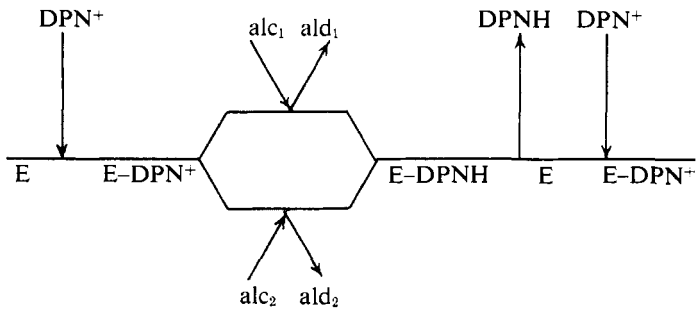
FIGURE 10: Reciprocal plot with acetaldehyde as the variable substrate and methanol as inhibitor. DPNH, 1.22 × 10^{-4} M; methanol: (1) 0, (2) 0.08 M, (3) 0.16 M; enzyme, 2 × 10^{-8} N; 50 mM potassium phosphate, pH 7.0. Data from line 1 were fitted to eq 3; data from lines 2 and 3 were fitted to eq 10.

Alternate Product Studies. Cleland (1963b) has shown that when alternate reaction sequences are established by an alternate product, nonlinear inhibitions may be observed. When mechanism 2 is expanded to allow for the presence of alternate substrates and products we have the situation shown in mechanism 11 where k_2, k_4, k_5, k_7, k_{10}, and k_{11} represent the unimolecular rate constants for release of reactants from the enzyme surface. If DPN^+ and alc_1 are present, the addition of ald_2 produces two reaction pathways by which E–DPNH can be reconverted into E–DPN^+, as shown in mechanisms 12 and 13. When alc_1 is the substrate whose concentration is varied, ald_2 gives linear noncompetitive inhibition (eq 7) if the appearance of DPNH is followed, since only reaction *via* pathway 12 is being followed. This type of inhibition would also be produced by the normal product, ald_1. If the formation of ald_1 is followed, however, the effects of ald_2 are those described by eq 14 which represents hyperbolic uncompetitive activation or inhibition. Addition of ald_2 diverts the reaction from pathway 12 to pathway 13 in a hyperbolic manner. The ratio of $K_{i\,num}$ to $K_{i\,denom}$ determines whether an activation, inhibition, or an unaffected rate is observed. If central ternary complexes are not kinetically important in the mechanism (that is, the steady-state concentration of (1) and (2) in mechanism 11 are nearly zero), the Ordered mechanism becomes a Theorell–Chance mechanism (mechanism 15) and eq 14 becomes eq 16, which represents hyperbolic uncompetitive activation with the unusual characteristic that, as the activator concentration is raised to infinity, the intercept is lowered to zero (that is, the velocity of the reaction when saturated with both alc_1 and ald_2 becomes infinite). Experiments of this type are thus sensitive indicators of the importance of the central ternary complexes in the mechanism.



$$\frac{1}{v} = \frac{K}{V}\left(\frac{1}{alc_1}\right) + \left(\frac{1}{V}\right)\frac{(1 + ald_2/K_{i\,num})}{(1 + ald_2/K_{i\,denom})} \qquad (14)$$

Equations 14 and 16 assume that ald_2 combines only with E–DPNH, and predict no effect of ald_2 on the slopes of reciprocal plots. If ald_2 also combines with E–DPN^+ in dead-end fashion to form an E–DPN^+–ald_2 complex, however, the first terms in eq 14 and 16 are multiplied by the factor $(1 + ald_2/K_i)$ where K_i is the dissociation constant of ald_2 from the dead-end complex. These equations then predict a linear slope effect, and the presence of such an effect serves as a clear indication of the formation of a dead-end ternary complex, the existence of which can be demonstrated in normal product inhibition experiments only by observation of a parabolic slope or intercept replot. The value of K_i can thus be accurately and unambiguously determined.



(15)

$$\frac{1}{v} = \frac{K}{V}\left(\frac{1}{\text{alc}_1}\right) + \frac{1}{V(1 + \text{ald}_2/K_{i_1})} \qquad (16)$$

The first attempts to carry out alternate product experiments were made using vitamin A alcohol as variable substrate, and recording optical density changes at 391 mμ. Although vitamin A alcohol is a good substrate, it was not possible to prepare an aqueous dispersion which was sufficiently stable to give identical rates over a 2-hr period, even when stringent precautions were taken to exclude light and oxygen. As a result these experiments were abandoned.

The spectra of *p*-hydroxybenzyl alcohol and *p*-hydroxybenzaldehyde enable the appearance or disappearance of the aldehyde to be measured at 281 mμ, an isosbestic point of DPN+ and DPNH. The hyperbolic activation of the intercepts produced by acetaldehyde and benzaldehyde with *p*-hydroxybenzyl alcohol as substrate indicates that in these cases the ratio $K_{i\,denom}/K_{i\,num}$, eq 8, is less than one. In terms of (11) this means that the alternate pathway is faster than the normal pathway, or $[(1/k_5) + (1/k_{10})] < [(1/k_5) + (1/k_7)]$. The fact that the intercepts do not decrease to zero eliminates the Theorell–Chance mechanism from consideration, and demonstrates that the central ternary complexes are kinetically important.

In the cases where ethyl, phenethyl, and cycloheptyl alcohols were used as inhibitors with *p*-hydroxybenzaldehyde as substrate, the results were inconclusive. The low substrate concentrations used in these experiments did not permit accurate values to be obtained for the intercepts. Experiments carried out at higher substrate concentrations would give more precise values, but the large extinction of *p*-hydroxybenzaldehyde at 281 mμ prevents this.

The linear slope effects produced by alcohols and aldehydes as alternate products demonstrate the formation of dead-end enzyme–DPNH–alcohol and enzyme–DPN+–aldehyde complexes, and the K_{is} values obtained from fits to either eq 5 or 8 are the dissociation constants for the alcohols and aldehydes from these dead-end ternary complexes. Such dead-end complexes have been proposed previously on the basis of product inhibition studies (Wratten and Cleland, 1963) which showed that ethanol gave S-parabolic I-linear noncompetitive inhibition against acetaldehyde, and *vice versa*. The dissociation constants for ethanol and acetaldehyde, calculated from normal product inhibition data were 3.47 ± 0.60 × 10^{-2} M from enzyme–DPNH–ethanol and 4.57 ± 1.15 × 10^{-5} M from enzyme–DPN+–acetaldehyde. The values obtained from the alternate product data presented here were 3.84 ± 0.09 × 10^{-2} M and 70.7 ± 23.4 × 10^{-5} M. The agreement of the values for the enzyme–DPNH–ethanol dissociation constant is excellent and provides further support for the existence of this complex. Comparison of the values for the enzyme–DPN+–acetaldehyde complex is not so conclusive because of the large standard errors of the estimates which do not permit realistic statistical calculations.

Relative Maximum Velocities with Different Alcohols and Aldehydes. Equations 17 through 21 relate the relative maximum velocities of Table III, the $K_{i\,num}$ and $K_{i\,denom}$ values obtained from the effect of ald_2 with alc_1 as substrate, and the unimolecular rate constants

$$\frac{1}{k_5} + \frac{1}{k_7} = \frac{1}{V_{max\ alc_1}} \qquad (17)$$

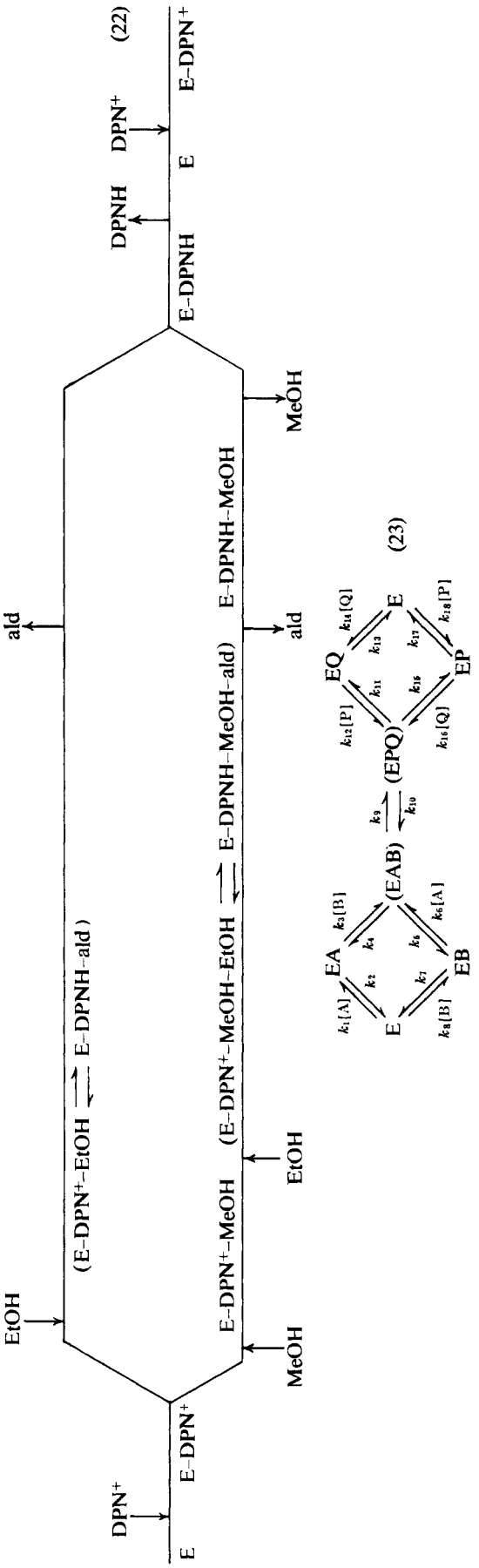
$$\frac{1}{k_2} + \frac{1}{k_4} = \frac{1}{V_{max\ ald_1}} \qquad (18)$$

$$\frac{1}{k_7} + \frac{1}{k_{11}} = \frac{1}{V_{max\ alc_2}} \qquad (19)$$

$$\frac{1}{k_2} + \frac{1}{k_{10}} = \frac{1}{V_{max\ ald_2}} \qquad (20)$$

$$\left(\frac{1}{k_5} + \frac{1}{k_7}\right) = \left(\frac{K_{i\,num}}{K_{i\,denom}}\right)\left(\frac{1}{k_5} + \frac{1}{k_{10}}\right) \qquad (21)$$

shown in mechanism 11. With alc_2 and ald_2 representing benzyl alcohol and benzaldehyde and ald_1 and alc_1 representing *p*-hydroxybenzaldehyde and *p*-hydroxybenzyl alcohol, the relative values obtained from these equations were (these values are the true values divided by the turnover number for oxidation of *p*-hydroxybenzyl alcohol by DPN+) k_2 and k_{10}, >60.9 ± 6.0; k_{11}, ≧9; k_7, 1.2 ± 0.3; k_5, 2.0 ± 0.4; and k_4, 1.5 ± 0.4. Similarly, with ald_2 and alc_2 representing acetaldehyde and ethanol and ald_1 and alc_1 as above, the following relative values were obtained: k_2 and k_{10}, >92.0 ± 10.3; for k_{11} the standard errors of the constants involved in the calculation of this value were such that it could not be distinguished from infinity; k_7, 1.2 ± 0.3;



k_5, 2.1 ± 0.3; and k_4, 1.5 ± 0.4. It appears that in the direction of alcohol oxidation, the release of DPNH is largely rate limiting but that in the other direction the release of alcohol may also influence the rate, or in the case of *p*-hydroxybenzyl alcohol be the rate-limiting step.

Inhibition Studies with Methanol. If the inhibition of methanol with DPN^+ as the variable substrate (Figure 7) were uncompetitive, it could be explained adequately by postulating dead-end combination of methanol with the enzyme–DPN^+ complex, while noncompetitive inhibition would indicate a more complex mechanism of inhibition. Equally significant fits were, however, obtained to both eq 6 and 7. The patterns observed with either ethanol or acetaldehyde as the variable substrate (Figures 8 and 10) indicate more than a simple dead-end combination of methanol with the enzyme–DPN^+ complex. The inhibited lines in Figures 8 and 10 in the presence of methanol are best described by a 2/1 function (eq 10). These data are consistent with mechanism 22 which represents a partial inhibition where reaction of the enzyme forms which include bound inhibitor (E–DPN^+–MeOH, E–DPN^+–MeOH–EtOH, E–DPNH–MeOH–ald, and E–DPNH–MeOH) is slower than that of the normal enzyme forms (E–DPN^+, E–DPN^+–EtOH, E–DPNH–ald, and E–DPNH). This situation is different from that caused by dead-end inhibitors which combine with the enzyme to form complexes incapable of further reaction. The rate equation for mechanism 22 predicts S-hyperbolic I-2/1 noncompetitive inhibition with DPN^+ or DPNH varied at several fixed levels of methanol, while with ethanol or acetaldehyde varied the effect of methanol is to change the double reciprocal plots from straight lines to 2/1 functions. Since, as suggested earlier, ethanol may combine with both E–DPN^+ and E–DPNH it is likely that methanol will also combine with both complexes. The open circles of Figure 9 probably represent substrate inhibition caused by ethanol combining with the E–DPNH complex. The fact that this inhibition is not seen when methanol is present (lines 2 and 3) suggests that methanol competes with ethanol for the E–DPNH complex. Mechanism 22 thus appears to describe the effect of methanol on LADH adequately.

The data presented here as well as the product inhibition data presented earlier (Wratten and Cleland, 1963) have been shown to be consistent with an Ordered mechanism for LADH. The isotopic exchange data of Silverstein and Boyer (1964), however, suggest a Random mechanism with a small percentage of the reaction flux going through a pathway with the nucleotides as the second substrates to add to the enzyme and the first products to be released. Such a mechanism can be diagrammed as shown in (23) where A and Q are the nucleotides and B and P the other reactants. Such a Random mechanism normally leads to curved reciprocal plots which are 2/1 functions (eq 10) when any substrate is varied at nonsaturating levels of the other one. These plots with LADH have always been straight lines. Cleland and Wratten (1965) have recently shown, how-

ever, that when certain relationships exist between the rate constants, the reciprocal plots in the absence of products for such a Random mechanism will become straight lines. One such case, which occurs when k_9 and k_{10} are the sole rate-limiting steps, is ruled out both by the exchange data of Silverstein and Boyer (1964) and by the product inhibition data of Wratten and Cleland (1963). The most general case, which requires $k_2 = k_7$ when A and B are substrates, is also quite unrealistic for LADH. A third case, however, requires only that k_4 be considerably larger than k_5 and k_9 and that $k_1 = k_6$ when A and B are substrates. Since the addition of the nucleotide may very well be diffusion limited, the latter requirement is realistic, while the former is consistent with the isotopic exchange data. This case is of special interest in that the kinetic constants in the initial velocity rate equation for this case (eq 4) have the same definitions in terms of rate constants as they would have if the addition of substrates were completely ordered and EB did not form; that is, the rate constants for the lower branch in mechanism 23 are not involved, even though this branch carries considerable reaction flux at high B levels.

When one carries out the same type of analysis of the equations for product inhibition in mechanism 23, however, the requirements for linearity of the reciprocal plots are more stringent. In general, these plots will never become completely linear, although the curvature will become small as the importance of the alternate reaction branch decreases. From the above discussion it is clear that, although the initial velocity data for LADH are not inconsistent with a Random mechanism where addition of nucleotides is diffusion controlled and the rates of dissociation of alcohol and aldehyde from the ternary complexes greatly exceed the rates of release of nucleotides from and the rates of interconversion of the ternary complexes, the product inhibition data require that the contribution of the alternate pathway be quite small.

References

Baker, R. H., Jr. (1960), Ph.D. dissertation, Indiana University.

Bonnichsen, R. K., and Brink, N. G. (1956), *Methods Enzymol. 1*, 495.

Boyer, P. D., and Segal, H. L. (1954), *in* The Mechanism of Enzyme Action, McElroy, W. D., and Glass, B., eds., Baltimore, Johns Hopkins Press, p. 523.

Cha, S. (1963), Ph.D. dissertation, University of Wisconsin.

Cleland, W. W. (1963a), *Biochim. Biophys. Acta 67*, 104.

Cleland, W. W. (1963b), *Biochim. Biophys. Acta 67*, 173.

Cleland, W. W. (1963c), *Nature 198*, 463.

Cleland, W. W., and Wratten, C. C. (1965), Symposium on the Mechanism of Action of the Pyridine Nucleotide Dependent Dehydrogenases, The University of Kentucky Press (in press).

Dalziel, K. (1963), *J. Biol. Chem. 238*, 2850.

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

Merritt, D. A., and Tomkins, G. M. (1959), *J. Biol. Chem. 234*, 2778.

Silverstein, E., and Boyer, P. D. (1964), *J. Biol. Chem. 239*, 3908.

Sund, H., and Theorell, H. (1963), *Enzymes 7*, 25.

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

Wilkinson, G. N. (1961), *Biochem. J. 80*, 324.

Wratten, C. C., and Cleland, W. W. (1963), *Biochemistry 2*, 935.

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