Product Inhibition Studies on Yeast and Liver Alcohol Dehydrogenases*

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Received April 1, 1963

Product inhibition studies are reported which show clearly that in physiological concentration ranges the kinetic mechanism of both yeast and liver alcohol dehydrogenases is Ordered Bi Bi (the two substrates add to the enzyme in obligatory order to give a ternary complex or complexes which break down with release of products in obligatory order), with the nucleotide adding to free enzyme before the other substrate. These data, together with other data in the literature, show that all other reasonable mechanisms can be ruled out. There are probably two or more enzyme-DPN complexes present in both mechanisms, but it is impossible to tell if there is isomerization of the enzyme-DPNH complexes. Either the liver enzyme can form dead end enzyme-DPN-aldehyde and enzyme-DPNH-alcohol complexes, or alcohols and/or aldehydes are adsorbed at sites other than the active site and alter the catalytic properties of the enzyme.

Yeast and liver alcohol dehydrogenases catalyze the reaction:

$$DPN^+ + \text{ethanol} \rightleftharpoons \text{acetaldehyde} + DPNH + H^+ \quad (1)$$

While both enzymes catalyze the same reaction, they show considerable differences in their physical properties, substrate specificities, and kinetic behavior. They presumably also perform different functions, the yeast enzyme serving to reduce acetaldehyde to ethanol in an anaerobic fermentation, and the liver enzyme presumably serving to detoxify alcohols by oxidation to aldehydes, which can then be further oxidized to acids.

Kinetic studies designed to determine the mechanism by which each of these enzymes catalyzes the oxidation of ethanol have been carried out by many workers, and while there has been general agreement that the mechanisms are sequential (that is, both substrates add before either product can be released),[1] there has been considerable disagreement over whether the substrates must add in compulsory order, and over the number of intermediate enzyme-reactant complexes in the mechanism. Thus for the liver enzyme, Theorell and various co-workers (Theorell and Bonnichsen, 1951; Theorell and Chance, 1951; Theorell *et al.*, 1955; Dalziel and Theorell, 1957; Theorell, 1958; Theorell and McKinley-McKee, 1961a,b) have concluded that the order of addition of substrates is not compulsory, but that the rate equation is approximately that of a Theorell-Chance mechanism (Theorell and Chance, 1951), in which the only kinetically important intermediate complexes are ones between the enzyme and DPN or DPNH. Mahler *et al.* (1962), however, concluded that the mechanism probably was not random but ordered, and that ternary complexes of enzyme, nucleotide, and alcohol or aldehyde were present. For the yeast enzyme, Nygaard and Theorell (1955) and Mahler and Douglas (1957) both suggested mechanisms in which the interconversion of two ternary complexes was the rate-determining step, and the addition of substrates was rapid enough to be at equilibrium.

* Supported in part by grants (G-14388 and GB-449) from the National Science Foundation.

[1] The nomenclature used to describe kinetic mechanisms and types of inhibition is that of Cleland (1963a).

[2] The Fortran programs used in this work have been described by Cleland (1963c); copies of the programs themselves may be obtained from the authors. These programs will work on an IBM 1620 or any larger computer which accepts programs written in Fortran.

These previous attempts to distinguish between possible mechanisms have involved only initial velocity data obtained in the absence of products. The various kinetic constants obtained have been tested for consistency with predicted Haldane relationships (equations relating kinetic constants to the equilibrium constant [Alberty, 1953]), and with other relationships between the constants themselves (Dalziel, 1958). A more direct approach involves determining the product inhibition patterns (Alberty, 1958; Fromm and Nelson, 1962; Cleland, 1963b). In this paper are reported the results of key product inhibition studies which show clearly that both yeast and liver alcohol dehydrogenases have an ordered mechanism in which binary enzyme-nucleotide complexes as well as ternary complexes of enzyme with both substrates are present.

METHODS AND MATERIALS

Chemicals.—DPN and the disodium salt of DPNH were obtained from the Sigma Chemical Company. Solutions of DPNH were freshly prepared for each experiment. Reagent grades of ethanol and acetaldehyde were redistilled and solutions were freshly prepared on the day of an experiment. Crystalline yeast alcohol dehydrogenase was a gift from Dr. Stephen Kuby. Solutions of this enzyme (1 mg/ml in 0.001 M Versene and 0.005 M phosphate, *p*H 7) lost no activity when stored frozen for several months. Liver alcohol dehydrogenase (C. F. Boehringer) was obtained as a crystalline suspension in phosphate buffer containing 10% ethanol. This suspension was diluted, dialyzed to remove ethanol, and centrifuged to remove any insoluble material. Such stock solutions in 0.02 M phosphate, *p*H 7, 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 experiments, the stock solutions of both enzymes were diluted with 0.1% bovine serum albumin, *p*H 7; the diluted enzymes were stable at 0° for at least 40 minutes. Enzyme concentrations were calculated as site equivalents per liter, assuming molecular weights of 150,000 and 84,000 and active sites per molecule of 4 and 2 for the yeast and liver enzymes, respectively.

Apparatus.—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. The electronic circuits of the optical density converter allowed optical

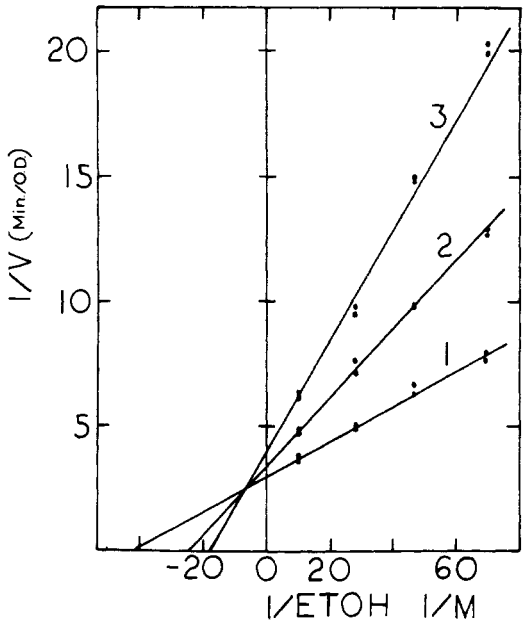


FIG. 1.—Product inhibition of yeast alcohol dehydrogenase by acetaldehyde with ethanol as variable substrate. DPN, 5.8×10^{-4} M. Enzyme, 4.5×10^{-8} N. Acetaldehyde: (1) 0, (2) 1.25×10^{-4} M, (3) 2.5×10^{-4} M.

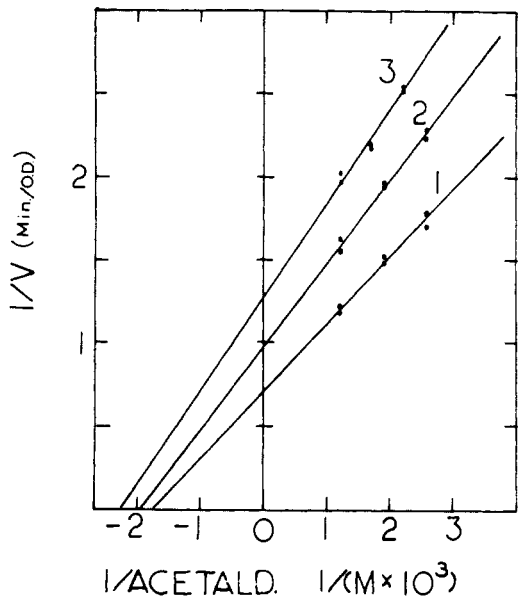


FIG. 2.—Product inhibition of yeast alcohol dehydrogenase by ethanol with acetaldehyde as variable substrate. DPNH, 2.08×10^{-4} M. Enzyme, 2×10^{-8} N. Ethanol: (1) 0, (2) 2.5×10^{-2} M, (3) 5×10^{-2} M.

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 between 0.05 and 0.2 optical density, and chart speeds were varied from 0.6 to 36 inches per minute.

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

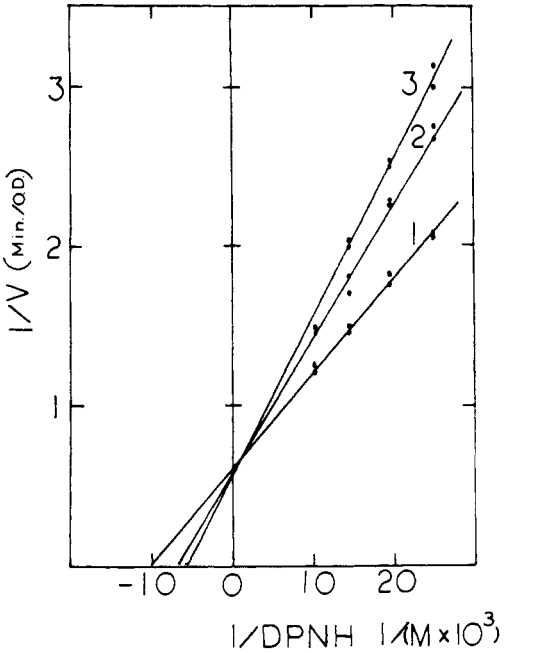


FIG. 3.—Product inhibition of yeast alcohol dehydrogenase by DPN with DPNH as variable substrate. Acetaldehyde, 6×10^{-3} M. Enzyme, 2×10^{-8} N. DPN: (1) 0, (2) 2.06×10^{-4} M, (3) 4.12×10^{-4} M.

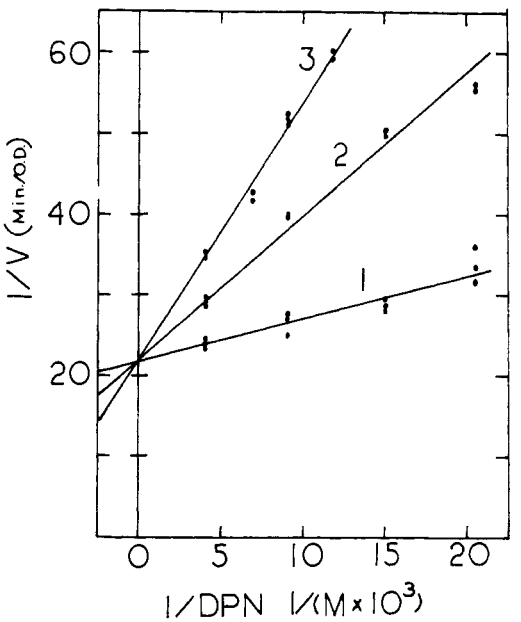


FIG. 4.—Product inhibition of liver alcohol dehydrogenase by DPNH with DPN as variable substrate. Ethanol, 7.44×10^{-3} M. Enzyme, 1×10^{-7} N. DPNH: (1) 0, (2) 3.3×10^{-6} M, (3) 6.6×10^{-6} M.

to the time of addition and the tangents to the curves at this time taken as initial velocities.

Data Processing.—Reciprocal velocities were plotted graphically against the reciprocals of substrate concentrations and any points which deviated greatly from a linear relationship were discarded. The remaining data were fitted to equation (2) using a least squares

$$v = \frac{VS}{K + S} \qquad (2)$$

method and assuming equal variance for the velocities (Wilkinson, 1961). All calculations were performed by a digital computer using a Fortran program which provides values of K, V, K/V, $1/V$, the standard errors

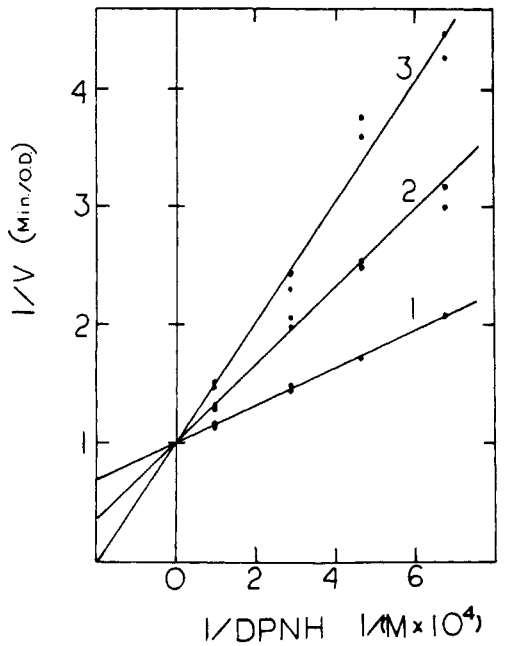


FIG. 5.—Product inhibition of liver alcohol dehydrogenase by DPN with DPNH as variable substrate. Acetaldehyde, 2×10^{-2} M. Enzyme, 5×10^{-8} N. DPN: (1) 0, (2) 1.2×10^{-4} M, (3) 2.4×10^{-4} M.

of their estimates, and weighting factors (reciprocals of squares of standard errors) for further analysis.[2] Slopes (K/V) and intercepts ($1/V$) were then plotted graphically against inhibitor concentration to determine the type of inhibition (linear, parabolic, etc.). Preliminary values of the inhibition constants and estimates of their accuracy were obtained by making weighted least square fits to these replots (assuming a line or parabola) using the weighting factors supplied by the fits to equation (2). In cases where the intercepts did not vary greatly with inhibitor concentration, t tests were applied to determine if they were significantly different. Final values for inhibition and other kinetic constants and the standard errors of their estimates were obtained by fitting all data points used in the first analysis to an over-all equation describing the observed type of inhibition. Data for linear competitive inhibition were fitted to equation (3), data for linear noncompetitive inhibition to equation (4), and data for S-parabolic I-linear noncompetitive inhibition to equation (5). All fits assumed equal variances for the observed velocities.

$$v = \frac{VS}{K(1 + I/K_i) + S} \quad (3)$$

$$v = \frac{VS}{K(1 + I/K_{is}) + S(1 + I/K_{ii})} \quad (4)$$

$$v = \frac{VS}{K(1 + aI + bI^2) + S(1 + I/K_{ii})} \quad (5)$$

RESULTS

Yeast Alcohol Dehydrogenase.—With ethanol as the variable substrate, acetaldehyde gave linear noncompetitive inhibition (Fig. 1). Although the slopes are increased more than the intercepts by the inhibitor, the intercepts are significantly different (P = 0.13% for the closest pair). The comparable experiment for the reverse reaction, with acetaldehyde as variable substrate and ethanol as inhibitor, also gave linear noncompetitive inhibition (Fig. 2). Replots of slopes and intercepts versus inhibitor concentration were linear for both of these experiments.

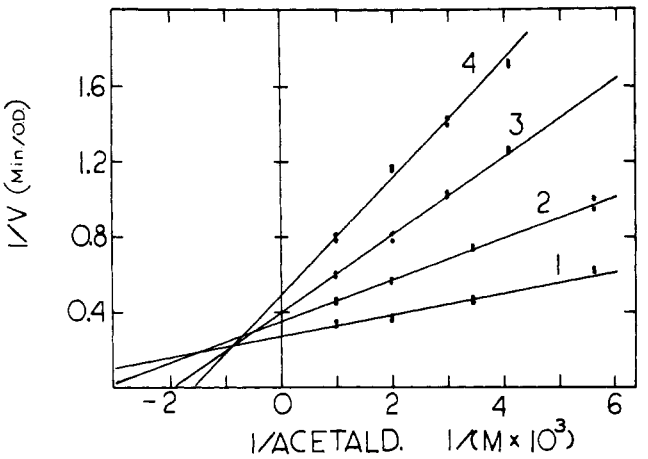


FIG. 6.—Product inhibition of liver alcohol dehydrogenase by ethanol with acetaldehyde as variable substrate. DPNH, 1.32×10^{-4} M. Enzyme, 5×10^{-7} N. Ethanol: (1) 0, (2) 6×10^{-3} M, (3) 1.2×10^{-2} M, (4) 1.8×10^{-2} M.

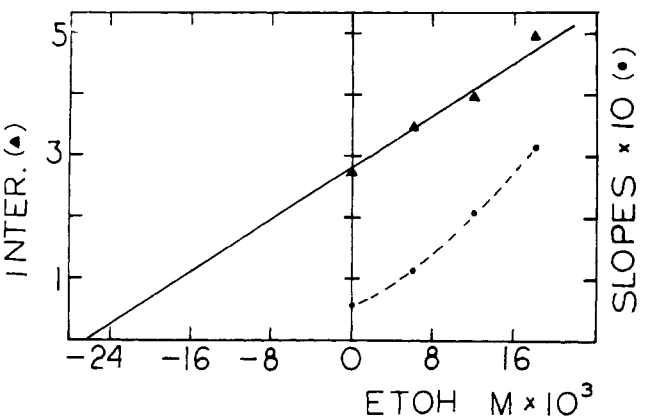


FIG. 7.—Replots of slopes and intercepts from Figure 6 versus ethanol concentration.

With DPNH as variable substrate, DPN gave linear competitive inhibition (Fig. 3). The intercepts were not significantly different (P = 56% for the pair differing the most), and the replot of slopes versus DPN concentration was linear.

Liver Alcohol Dehydrogenase.—With DPN as variable substrate, DPNH gave linear competitive inhibition (Fig. 4). The intercepts were not significantly different (P = 66% for the pair differing most) and the replot of slopes versus DPNH concentration was linear. By the same criteria DPN gave linear competitive inhibition when DPNH was the variable substrate (P = 20% for the intercept pair differing most) (see Fig. 5). The same pattern and a nearly identical inhibition constant were also obtained when acetaldehyde was present at lower concentration (5×10^{-3} M).

With acetaldehyde as the variable substrate, ethanol gave noncompetitive inhibition (Fig. 6). The intercepts were significantly different (P = 0.2% for the closest pair) and a replot of intercepts versus ethanol concentration was linear (Fig. 7). The slope was curved, however (Fig. 7). This curvature in the slope replot was observed in five separate experiments, and always seemed to be of similar form. The curvature was greatest near the vertical axis, with the points at high ethanol concentration nearly in a straight line. If the curve were a parabola the curvature should increase at higher ethanol levels. The "2/1" function shown in equation (6) can have the observed properties, but

$$\text{slope} = \text{slope}_0\left(\frac{1 + aI + bI^2}{1 + cI}\right) \quad (6)$$

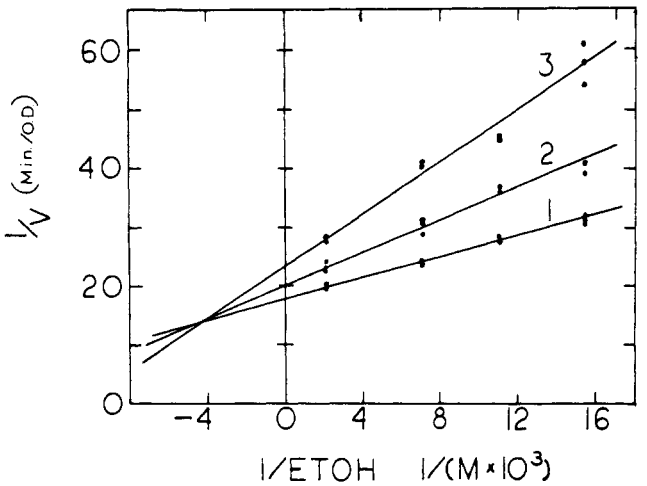


FIG. 8.—Product inhibition of liver alcohol dehydrogenase by acetaldehyde with ethanol as variable substrate. DPN, 8.8×10^{-4} M. Enzyme, 1×10^{-7} N. Acetaldehyde: (1) 0, (2) 1.3×10^{-5} M, (3) 2.6×10^{-5} M.

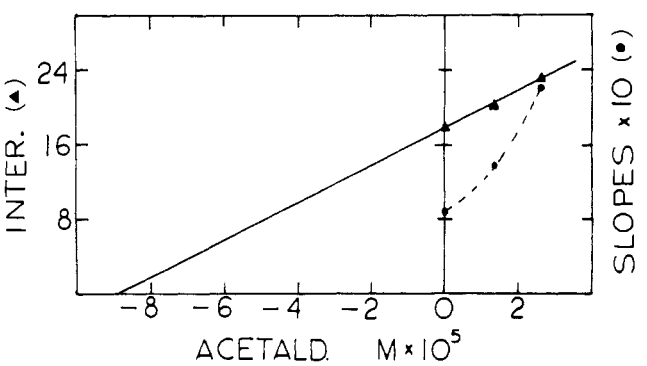


FIG. 9.—Replots of slopes and intercepts from Figure 8 versus acetaldehyde concentration.

attempts to fit the points to this type of function have not been successful because the number of points was too small and their standard errors not small enough. Thus it is not possible to say whether the slope replot is best represented by a parabola or by a 2/1 function.

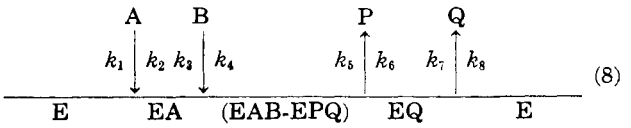
In the comparable experiment in the reverse direction, acetaldehyde gave noncompetitive inhibition with ethanol as variable substrate (Fig. 8). Again the intercepts were significantly different ($P < 0.1\%$ for the closest pair) and the replot of intercepts versus acetaldehyde concentration was linear (Fig. 9). The slope replot was consistently curved in four different experiments (Fig. 9), but the data were insufficient to indicate whether the curve was other than a parabola.

Discussion

The initial velocity studies of previous workers (Mahler and Douglas, 1957; Theorell and McKinley-McKee, 1961; Dalziel, 1962) have shown that both the liver and yeast alcohol dehydrogenases obey the initial velocity equation:

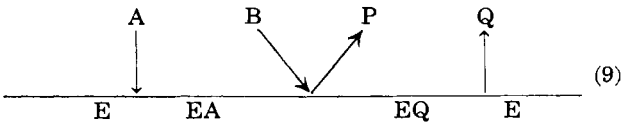
$$\frac{1}{v} = \frac{1}{V_1}\left(\frac{K_{ia}K_b}{AB} + \frac{K_a}{A} + \frac{K_b}{B} + 1\right) \qquad (7)$$

The presence of the $K_{ia}K_b/AB$ term in this equation shows that both substrates must add to the enzyme before either product can be released and the mechanism is therefore sequential. The simplest mechanism which fits the product inhibition data reported in this paper is the Ordered Bi Bi mechanism (mechanism 8),



(8)

where A is DPN, B is ethanol, P is acetaldehyde, and Q is DPNH. If k_5 is much larger than either k_6 or k_7 and k_4 is much larger than either k_2 or k_3, the ordered mechanism reduces to mechanism 9, which was first suggested for the liver enzyme by Theorell and Chance (1951).



(9)

The steady-state rate equation for the ordered mechanism (mechanism 8) can be written in the form of equation (10) (Cleland, 1963a). The rate equation

$$v = \frac{V_1\left(AB - \frac{PQ}{K_{eq}}\right)}{K_{ia}K_b + K_bA + K_aB + AB + \frac{K_{ia}K_bQ}{K_{iq}} + \frac{K_{ia}K_bK_qP}{K_{iq}K_p} + \frac{K_{ia}K_bPQ}{K_pK_{iq}} + \frac{K_bK_qAP}{K_{iq}K_p} + \frac{K_aBQ}{K_{iq}} + \frac{ABP}{K_{ip}} + \frac{K_{ia}K_bBPQ}{K_pK_{iq}K_{ib}}} \qquad (10)$$

for the Theorell-Chance mechanism is the same except that it lacks denominator terms in ABP and BPQ, while that for a Rapid Equilibrium Random mechanism [where the order of addition of A and B is not obligatory, but the rate-limiting step is solely the conversion of (EAB) to (EPQ)] lacks both of these and AP and BQ terms as well.

When equation (10) is simplified by setting either P or Q equal to zero and rearranging, we obtain equations for the two types of product inhibition experiments reported in this paper:

Vary B, inhibit with P:

$$\frac{1}{v} = \frac{K_b}{V_1}\left(1 + \frac{K_{ia}}{A}\right)\left(1 + \frac{P}{\left(\frac{K_pK_{iq}}{K_q}\right)}\right)\left(\frac{1}{B}\right) + \frac{1}{V_1}\left(1 + \frac{K_a}{A}\right)\left(1 + \frac{P}{K_{ip}\left(1 + \frac{K_a}{A}\right)}\right) \qquad (11)$$

Vary A, inhibit with Q:

$$\frac{1}{v} = \frac{K_a}{V_1}\left(1 + \frac{K_{ia}K_b}{K_aB}\right)\left(1 + \frac{Q}{K_{iq}}\right)\left(\frac{1}{A}\right) + \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right) \qquad (12)$$

Equation (11) predicts linear noncompetitive inhibition, while equation (12) predicts linear competitive inhibition. If there were no ABP term in equation (10) ($K_{ip} = \infty$), then the term containing P would drop out of the intercept term of equation (11) and competitive inhibition would be observed. If AP and BQ terms were missing as well, the equations corresponding to both equations (11) and (12) would predict competitive inhibitions in which the observed inhibition constants would rise toward infinity as the concentration of the nonvaried substrate was raised, so that the inhibitions were overcome, in contrast to the type of inhibition predicted by equation (12), where the inhibition constant is K_{iq} regardless of the concentration of B.

The experiments reported here show that as predicted by equation (11) for both the liver and yeast enzymes

TABLE I
KINETIC CONSTANTS FOR ALCOHOL DEHYDROGENASES AT *p*H 7.15

Kinetic Constant (M)	Yeast Enzyme		Liver Enzyme			
	Nygaard and Theorell (1955)	This Work[a]	Theorell *et al.* (1955)	Theorell and McKinley-McKee (1961b)	Dalziel (1962)	This Work[a]
$K_a \times 10^5$	5.7	7.4 ± 2.5[b]	1.0	0.59	0.30	1.74 ± 0.12[c]
$K_b \times 10^4$	180	130 ± 10[c]	5.9	2.6	3.0	5.5 ± 0.2[c]
$K_p \times 10^4$	5.5	7.8 ± 0.8[c]	1.1	2.4	4.4	2.44 ± 0.11[c]
$K_q \times 10^5$	3.8	10.8 ± 1.0[c]	1.0	0.67	1.3	2.68 ± 0.12[c]
$K_{ia} \times 10^4$	2.3	6.1 ± 0.5[d]	1.2	1.4	0.67	1.28 ± 0.06[d]
$K_{ib} \times 10^2$		4.3 ± 0.8[e]				1.94 ± 0.19[e]
$\frac{K_{ia}K_b}{K_a} \times 10^2$	7.4	11 ± 3[d]				
$K_{ip} \times 10^4$		6.7 ± 1.2[e]				0.87 ± 0.08[e]
$\frac{K_pK_{iq}}{K_q} \times 10^4$	1.4	1.3 ± 0.1[d]				
$K_{iq} \times 10^6$	10	18 ± 3[f]	0.43	0.28	0.43	1.50 ± 0.07[d]

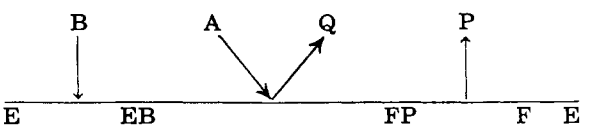
[a] Standard errors are computed from the standard errors of the estimates given by least square fits to experimental data (see text), and are probably a valid measure only of the precision rather than of the accuracy of the calculated kinetic constants. [b] Calculated from the values of $K_{ia}K_b/K_a$, K_{ia}, and K_b. [c] These values were determined from apparent K's obtained from over-all fits to equations (3), (4), or (5). For example for data conforming to equation (12), $K_a = K_{app}(1 + K_b/B)/(1 + K_{ia}K_b/(K_aB))$. Since the nonvaried substrates were almost saturating, the apparent K's are nearly equal to the Michaelis constants. [d] Slope inhibition constants from fits to equations (3) and (4). [e] These values were determined from intercept inhibition constants from fits to equations (4) or (5). For example for data conforming to equation (11), $K_{ip} = K_{it}/(1 + K_a/A)$. Since the nonvaried substrates were almost saturating, the observed K_{it}'s are nearly equal to the desired inhibition constants. [f] Calculated from the values of K_pK_{iq}/K_q, K_p, and K_q.

acetaldehyde is a noncompetitive inhibitor when ethanol is varied (the parabolic or 2/1 slope effects for the liver enzyme will be discussed below). The rate equation thus contains an ABP term. The similar experiments in the reverse direction (acetaldehyde varied, ethanol inhibiting) show that a BPQ term is also present. The presence of AP and BQ terms can be inferred from the large slope effects evident in experiments corresponding to equation (11) (where, for example, the high level of A used would essentially eliminate inhibition due only to a P term, so that an AP term must be present). The presence of a BQ term was directly proved for the liver enzyme by running the experiment shown in Figure 6 at a lower DPNH concentration (2.5×10^{-5} M). Reducing DPNH from eighty-eight times its dissociation constant to seventeen times this value made no appreciable difference in the slopes of the reciprocal plots. If the BQ term were missing from the rate equation, the slope inhibition constant should have decreased by a factor of 5. Presence of the AP term was also directly demonstrated for the liver enzyme by showing that the competitive inhibition constant for DPN did not vary when acetaldehyde was lowered from 82 times K_p to 20 times K_p. Again if the AP term were not in the rate equation, K_{ia} should have decreased by a factor of 4.

The experiments reported here thus confirm the presence of AP, BQ, ABP, and BPQ terms in the denominator of the rate equation as predicted for the Ordered Bi Bi mechanism. Analysis of the apparent Michaelis constants and inhibition constants from the various experiments allows calculation of the kinetic constants for the two enzymes. These are shown in Table I along with values reported by other workers. In general, the agreement is good.

The analysis given above eliminates the simple Theorell-Chance and Rapid Equilibrium Random mechanisms. In order to prove that the mechanisms of the alcohol dehydrogenases are really ordered, however, it is necessary to examine all other possible mechanisms giving a rate equation similar to equation (10) and eliminate them from consideration. Equation (10) is also given by a Theorell-Chance mechanism in which dead end (EAB) and (EPQ) ternary complexes are formed which do not participate in the reaction, but which can only break down again to EA and EQ, respectively. In view of the direct hydrogen transfer which occurs during the catalytic reaction (Westheimer *et al.*, 1951; Levy and Vennesland, 1957) and the high improbability of mechanisms without central complexes, this mechanism seems unlikely. If ternary complexes are present, they undoubtedly participate in the reaction sequence. Nevertheless, there is no way to distinguish this mechanism from the ordered one by steady-state kinetics.

Another mechanism giving equation (10) is an Iso Theorell-Chance mechanism, in which the order of addition of reactants is reversed, and B and P react with different forms of free enzyme:



(13)

This mechanism can be ruled out because of the known binding of DPN and DPNH in the absence of ethanol or acetaldehyde (Theorell and Bonnichsen, 1951) and, more specifically, by the recent findings of Boyer and Silverstein (1963) that isotopic exchange at equilibrium between B and P is much faster than that between A and Q.

Equation (10) is also given by a Rapid Equilibrium Random mechanism with dead end EAP, EBQ, EABP, and EBPQ complexes. While the existence of EAP and EBQ complexes is not difficult to visualize (see below), it is hard to imagine E-DPN-ethanol-acetaldehyde or E-DPNH-acetaldehyde-ethanol complexes in which the binding of the extra acetaldehyde or ethanol molecule prevents reaction between the other two bound reactants. This mechanism is also eliminated by the isotopic exchange data of Boyer and Silverstein (1963),

since the initial A-Q and B-P exchange rates must be equal for a Rapid Equilibrium Random mechanism.

Theorell has recently suggested for liver alcohol dehydrogenase that the mechanism is actually random, with either A or B able to add first, but that relationships between the rate constants are such that the rate equation resembles that of a Theorell-Chance mechanism (Theorell and McKinley-McKee, 1961b). As can be seen from Figures 6 and 8, the intercept effect is less than the slope effect, and thus it is not surprising that workers have not been able to detect the presence of the ABP and BPQ terms in the rate equation by less direct methods than the product inhibition studies reported here. A recent analysis by one of the present authors (W.W.C.) of the full rate equation for the Random Bi Bi mechanism, however, shows that it is not possible to have an appreciable contribution from the alternate pathways where the nucleotides add after the other reactants without altering the form of the rate equation from that given in equation (10). Reciprocal plots for the Random mechanism are always 2/1 functions:

$$\frac{1}{v} = \frac{a + b\left(\frac{1}{S}\right) + c\left(\frac{1}{S}\right)^2}{d + e\left(\frac{1}{S}\right)} \qquad (14)$$

where a, b, c, d, and e are functions of the other reactant concentrations and are always positive. These plots are hyperbolas which are curved near the vertical axis, but become straight (tangent to one asymptote) as $1/S$ is increased. The vertical intercept of the curve may lie below or above the intercept of the asymptote, producing curves that are concave down or up, respectively. In the latter case there may or may not be a minimum point for positive values of $1/S$. If $bde = cd^2 + ae^2$, equation (14) becomes a straight line, but this relation holds over a wide range of concentrations of the other reactants only if the rapid equilibrium assumption is made. In the present case, any appreciable contribution from the alternate pathways will lead to curvature of the reciprocal plots near the vertical axis which should be detectable. Since no evidence for this type of behavior has been found, it is probably valid to say for the two enzymes discussed here that the mechanism is predominantly ordered and that the contribution of the alternate order of addition is small.

That the reactants can add in reverse order has however recently been demonstrated by the isotopic exchange studies of Boyer and Silverstein (1963). These workers found that at equilibrium with very high levels of ethanol and acetaldehyde, the rates of DPN-DPNH exchange were about 0.02 and 0.01 times those of ethanol-acetaldehyde exchange for the yeast and liver enzymes, respectively. Since ethanol-acetaldehyde exchange under these conditions takes place along the preferred pathway [enzyme-DPN $\leftrightharpoons$ (central complexes) $\leftrightharpoons$ enzyme-DPNH], while DPN-DPNH exchange represents only the alternate pathway [enzyme-ethanol $\leftrightharpoons$ (central complexes) $\leftrightharpoons$ enzyme-acetaldehyde] because the steady-state levels of enzyme-DPN and enzyme-DPNH are nearly zero, these data suggest that only 1 or 2% of the reaction takes place along the alternate pathway. This amount of reaction would not affect the kinetics below the region of substrate inhibition by ethanol or acetaldehyde (which inhibition should result from the presence of appreciable levels of enzyme-ethanol or enzyme-acetaldehyde complexes). Although this small contribution from the alternate pathway of reaction is of great theoretical interest in connection with the arrangement of reactants at the active site, it would seem that when the flow through this pathway is too small to be detected by kinetic studies, the kinetic mechanism should be considered ordered.

For liver alcohol dehydrogenase, the inhibitions of ethanol against acetaldehyde and vice versa produced curved slope replots. If these curves were parabolas, the resulting S-parabolic I-linear noncompetitive inhibitions are readily explained by the formation of dead end E-DPN-acetaldehyde and E-DPNH-ethanol complexes (Cleland, 1963b). This type of behavior has been reported for ribitol dehydrogenase (Fromm and Nelson, 1962) and lactic dehydrogenase (Zewe and Fromm, 1962), and is actually to be expected here, since both ethanol and acetaldehyde show substrate inhibition at high concentrations with the liver enzyme. It seems logical, moreover, that this effect is noticeable with the liver enzyme, which has a broad substrate specificity, and not for the yeast enzyme, which works well only with ethanol and acetaldehyde.

The problem, however, is that the replots from the ethanol inhibition studies look more like 2/1 functions than parabolas, although the data were not sufficiently precise to allow a meaningful fit to this function (the acetaldehyde inhibition experiments did not include more than three lines, and thus no distinction can be made). This type of function can be explained if one postulates that the strong binding of ethanol which occurs somewhere other than at the active site (Theorell *et al.*, 1955) alters certain rate constants without affecting the maximum velocity. Baker indeed has reported that the kinetically determined dissociation constants of DPN and DPNH for the liver enzyme differ when different alcohols and aldehydes are used as second substrates (Baker, 1960). If this is true it is direct evidence for alteration of the enzymatic activity by combination of the second substrate at other than the active site, but preliminary experiments in this laboratory have so far failed to find significant differences in the dissociation constants for DPN and DPNH determined from both initial velocity and product inhibition studies using ethanol and butanol and the corresponding aldehydes. More detailed studies are underway in an effort to determine whether alcohols or aldehydes really can modify the enzymatic properties by combination at other than the catalytic site.

If the simple ordered mechanism (mechanism 8) is really followed by both the yeast and liver enzymes, it should be possible to calculate all the rate constants from the known kinetic constants (Cleland, 1963a). When this is attempted with data for either the liver (Theorell *et al.*, 1955) or yeast (Nygaard and Theorell, 1955) enzymes, however, it is found that at *p*H 6 or 7.15 the rate constant for dissociation of E-DPN (k_2) is calculated to be less than the turnover number for reaction of DPNH and acetaldehyde (V_2/E_t). The turnover number in a given direction is determined by the unimolecular rate constants for reaction in that direction, and cannot be less than any of these. Thus in the present case, the calculation of the rate constant is invalid. As has been pointed out by Cleland (1963a), this inconsistent calculation strongly suggests that there is isomerization of the E-DPN complex (Baker [1962] has also suggested E-DPN isomerization for the liver enzyme). The existence of more than one E-DPN complex in the mechanism does not change the form of the rate equation when written in terms of kinetic constants as in equation (10), but the expressions for the kinetic constants in terms of rate constants are more complex. The combination of kinetic constants which in the simple mechanism (mechanism 8) would equal the rate constant for dissociation of

the E-DPN complex now can have any value from zero up to the value of this rate constant (and thus can be greater than, equal to, or less than V_2/E_t). When E-DPN isomerization is detected, it is impossible to detect E-DPNH isomerization since only one rate constant for dissociation of a nucleotide can be less than the corresponding turnover number (Cleland, 1963a). It is of interest that E-DPN (or E-TPN) isomerization is indicated not only for the alcohol dehydrogenases but also for glutamic (Frieden, 1959) and lactic (Novoa *et al.*, 1959) dehydrogenases (but not for ribitol dehydrogenase [Nordlie and Fromm, 1961]). E-DPNH isomerization has not as yet been detected for any pyridine nucleotide–linked dehydrogenase.

The complete mechanism for both alcohol dehydrogenases is thus Ordered Bi Bi with probable isomerization of the E-DPN complex. Whether the two (or more) E-DPN complexes are both part of the reaction sequence (the more likely situation) or whether one is a dead end complex formed with free enzyme is such a way as to block addition of substrates cannot be told from steady-state kinetic studies. Whether the E-DPNH complex isomerizes cannot be told, and of course it is impossible to tell how many different ternary complexes are present. For the liver enzyme, either dead end E-DPN-aldehyde and E-DPNH-alcohol complexes exist, or combination of alcohols and/or aldehydes at sites other than the active site alter the catalytic properties of the protein. From such rate constant calculations as can be made from existing data, from the isotopic exchange data of Boyer and Silverstein (1963), and from the isotope effects observed with deuterium by Baker (1962), it is probable that the rate-limiting steps, particularly for the liver enzyme, are mainly the isomerizations and/or dissociations of the enzyme-nucleotide complexes, rather than the isomerizations and dissociations of the central complexes. Similar conclusions have been reached for the liver enzyme by Dalziel (1962).

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