Studies on the Mechanism of Enzyme-Catalyzed Oxidation Reduction Reactions. III. A Characterization of the Mechanism of the Liver Alcohol Dehydrogenase Reaction*

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The mechanism of the reaction catalyzed by liver alcohol dehydrogenase (LADH) has been investigated by studying its kinetics: (a) by the method of initial steady state velocities; (b) by determining the kinetic isotope effect on these rates produced by the stereospecific introduction of deuterium into DPNH and ethanol; (c) by determining the effect of product inhibition on these rates; and (d) by the method of continuous steady state kinetics. The data so obtained were treated by means of the digital computer program described previously. The most likely mechanism, on the basis of the Haldane, Dalziel, and product inhibition criteria, is one requiring the compulsory binding of the coenzyme as the first step in the reaction. This conclusion is confirmed and extended by the isotope effect and continuous rate studies. These suggest in addition the occurrence of one (or more) ternary complexes as a required intermediate.

In the case of those oxidative enzymes which catalyze the actual transfer of hydrogen between reductant and oxidant a useful and powerful tool of physical organic chemistry has become available to a study of mechanisms, *viz.*, the application of kinetic deuterium isotope effects. With deuterium substitution in appropriate positions of reduced substrate or coenzyme a kinetic isotope effect can be expected in all those steps of a particular mechanism where a carbon-hydrogen bond is loosened or broken (for reviews see Wiberg, 1955; Streitwieser, 1960; Melander, 1960). This technique has been applied to the elucidation of the mechanism of many organic and some enzymatic reactions[1] (Rieder and Rose, 1959; Abeles *et al.*, 1960; Rose, 1961) and has been used in our laboratory for a preliminary survey of the yeast alcohol dehydrogenase reaction (Mahler and Douglas, 1957; Shiner *et al.*, 1960).

In this paper we shall describe the application of this technique to a different enzymatic reaction, *i.e.*, the one catalyzed by liver alcohol dehydrogenase. Two stereochemically distinct forms of the deuterium-containing reduced coenzyme,[2] both prepared enzymatically, have been employed. From the first one, the "α-" or A-form, deuterium is transferred in the reaction catalyzed by liver alcohol dehydrogenase and hence a primary isotope effect may be expected in the transfer step proper. From the second, the isomeric "β-" or B-form, hydrogen is transferred while deuterium is retained in the oxidized coenzyme; here only a secondary isotope effect can be expected. In addition, other criteria for the selection of an appropriate mechanism, discussed in the previous paper (Baker and Mahler, 1962), have been employed.

Horse liver alcohol dehydrogenase was chosen for the following reasons: (1) The enzyme is readily obtainable in highly purified and active form (Bonnichsen and Brink, 1958; Dalziel, 1958). (2) The reaction involves the reversible oxidation and reduction of pyridine nucleotides and can be followed conveniently and accurately by a variety of optical methods (Theorell *et al.*, 1955; Boyer and Theorell, 1956; Theorell and Winer, 1959). (3) The reaction is stereospecific with respect to both the substrate and the coenzyme (Vennesland and Westheimer, 1954; Levy and Vennesland, 1957; Vennesland, 1958). (4) Largely as a result of comprehensive investigations in Theorell's laboratory (Theorell, 1956, 1958), a great deal of information concerning the enzyme itself is available: (a) It is exceedingly stable on storage. (b) Its electrophoretic mobility, molecular weight, and UV extinction coefficient have been determined with great accuracy (Ehrenberg and Dalziel, 1958). (c) The number of equivalent, active sites has been determined to be two (Theorell, 1956, 1958; Vallee and Coombs, 1959). (d) Extensive information has been gathered on the binding of the coenzymes (Theorell and Winer, 1959; Van Eys *et al.*, 1958; Vallee *et al.*, 1959), and some data have been obtained on the binding of the substrates (Vallee *et al.*, 1959; Theorell and Winer, 1959). (e) The reaction catalyzed by the enzyme was postulated to obey the classical, simple Theorell-Chance (1951) mechanism, although in recent years there have been reports which cast some doubt on the

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[1] Additional but by no means complete citations may be found in the contributions by Shiner *et al.* (1960), Collins (1960), and Englard (1960) to a symposium on this topic (*Ann. N. Y. Acad. Sci. 84*, 573–881, 1960).

[2] The stereospecificity of the hydrogen transfer in pyridine-nucleotide–requiring dehydrogenase reactions is discussed by Vennesland and Westheimer (1954) and Vennesland (1958).

validity of this conclusion (Theorell, 1958; Theorell and Winer, 1959; Winer, 1958; Theorell, 1961). (f) The substrate specificity has been investigated (Winer, 1958; Merritt and Tompkins, 1959), but only by measurement of pseudomaximal velocities and not by detailed study of the kinetics of the over-all reaction.

Experimental

Enzyme.—The twice-recrystallized enzyme was shipped to us at dry ice temperatures by Worthington Biochemical Corporation, Freehold, N. J. This preparation when stored at −20° retained all its activity over a 5-month period. For storage of stock solutions, we recrystallized the enzyme two additional times, recovering 97% of the units present originally, with specific activities of $\geq 100\%$ based on the assay of Bonnichsen and Brink (1958). This solution was approximately 5×10^{-4} M with regard to enzyme. The concentration of ammonium sulfate was brought to 38% (38 g/100 ml buffer). The solution was readjusted to pH 7.5 by the addition of ammonium hydroxide. When stored at −11°, it retained its full activity for more than 45 days. For kinetic runs, up to 5 mg (approximately 0.2 ml of the storage stock solution) of protein was placed on a 10-cc Sephadex G-50 columm (Pharmacia, Uppsala, Sweden) and eluted with 2.7 ml of 0.10 M, pH 7.0 phosphate buffer with no loss of activity. This secondary stock solution was approximately 1×10^{-5} M with regard to protein and was stored at 0° with no loss of activity over the period of a kinetic run. Just prior to any such run the solution was assayed according to the method of Bonnichsen and Brink (1958); for our calculation we employed a value for the turnover number of $1.33 \times 10^6 \Delta$ OD_{340}/min./mole of enzyme, the last value based on the most recent values for the molecular weight and OD_{280} of the enzyme (Ehrenberg and Dalziel, 1958).

Reactants

A. Diphosphopyridine Nucleotide, Oxidized Form (DPN^+).—DPN^+ was obtained commercially (Sigma Chemical Company, St. Louis, Mo.). It was found to be 95.0% pure, by weight, on the basis of its OD_{260}, using the molar extinction coefficient 1.80×10^4 cm^2/mole (Kornberg and Pricer, 1953). Enzymatic assay in the presence of excess ethanol at pH 9.6 showed it to be 92.5% active.

B. Diphosphopyridine Nucleotide, Reduced Form (DPNH).—DPNH was obtained commercially (Sigma Chemical Company). It was found to be 89.7% pure by weight from its OD_{340}, using the the molar extinction coefficient 6.22×10^3 cm^2/mole (Horecker and Kornberg, 1948). It was determined to be 100% enzymatically active on the basis of its oxidation in the presence of excess acetaldehyde at pH 7.5.

C. Acetaldehyde.—Acetaldehyde was obtained commercially and redistilled prior to each use in a 4° cold room. The fraction boiling at 20.0 ± 0.2° was collected, the first few ml of distillate being discarded. With a micropipet, 0.005 ml of the acetaldehyde was delivered into 10 ml of buffer to make our standard stock solution.[3] This solution is stable and does not change in concentration over periods up to 5 hours when stored at 4°.

D. Ethanol.—Ethanol, 100% pure, was obtained from B. Murr of this department, and stock solutions were prepared by delivering 10 μl portions from a micropipet at room temperature into 50 ml of buffer.

E. Dideuteroethanol (CH_3CD_2OH).—The dideuteroethanol was prepared by the method of Shiner (1952); except that Diethyl Carbitol was used as solvent. The $LiAlD_4$ (98.5 atom % D) used as the reducing agent for the conversion of acetic anhydride to the alcohol was obtained from Metal Hydrides, Inc., Beverley, Mass. The azeotropic mixture (95.5% ethanol + 4.5% water by weight) was collected at 78.4° with a yield of 77.8%. The deuterium content was 1.969 ± 0.013 atoms of deuterium per molecule, as determined by Shiner's density column method.

F. 4-α-Deutero-DPNH.—The procedure of Rafter and Colowick (1958) was modified for the use of 2 g of DPN^+ and 2.3 g of dideuteroethanol. A 60% yield of the yellow solid α-D-DPNH was obtained. Using the molecular extinction coefficient for DPNH given above, it was found to be 82.7% pure by weight; 100% of this material was enzymatically active. For kinetic runs, the barium salt of α-D-DPNH was converted to the sodium salt by addition of Na_2SO_4 followed by centrifugation and decantation. Deuterium determination (density column) showed 0.952 ± 0.018 atoms of deuterium per molecule of product disodium salt.

G. 4-β-Deutero-DPNH.—The β-deuterated coenzyme was prepared by Dr. R. Suzue in our laboratory by reaction (1) catalyzed by the enzyme, liver glucose dehydrogenase. The deuterated co-

$$\text{1-deutero-}\beta\text{-D-glucose} + DPH^+ \rightleftharpoons \text{D-gluconolactone} + \beta\text{-D-DPNH} \quad (1)$$

enzyme was found to be 81.0% pure by weight and 100% enzymatically active. For kinetic runs, the β-D-DPNH was treated in a manner analogous to that described for the α-D-DPNH. The β-D-DPNH was found to contain 0.946 ± 0.021 atoms of deuterium per molecule (density column).

H. Buffer.—The buffer used in all of the initial rate runs was 0.10 M glycylglycine–sodium hydroxide, pH 8.58 ± 0.02. It was prepared from C.P. glycylglycine obtained from the Aldrich Chemical Co., Milwaukee, Wis. All of the solutions were made up in doubly distilled water, the second distillation using an all-glass apparatus. For the continuous rate runs the buffer was 0.10 M imidazole-HCl, pH 7.58, prepared in an analogous manner from C.P. imidazole obtained from the same source. All pH determinations were performed with a Beckman pH meter, Model GS.

Kinetic Runs.—All of the kinetic runs were followed spectrophotometrically in a Cary Model 11 spectrophotometer (Applied Physics Corporation, Pasadena, Calif.) fitted with a specially

[3] The 5-μl pipet was calibrated by weight of water delivered to sealed vials at 4° to be 0.00500 ± 0.00034 g. The density of acetaldehyde at 4° is 0.7996 g/cc.

constructed thermostated cell carriage for the sample cuvet. The cell carriage, substrate solutions, and washing solutions were held at the same constant temperature with a Wilkens-Anderson constant temperature bath set at 27 ± 0.02°. A tungsten lamp was employed, and measurements were made at 340 mμ. With the 0.1 absorbancy slide wire, the slit control was set at 0.7-mm slit width to maintain a noise level less than ± 0.0003 OD units. Under these conditions the resolution of the spectrophotometer is ± 2 mμ.

TABLE I

CONDITIONS FOR INITIAL RATE RUNS

Temperature 27° ± 0.02, pH 8.58, ionic strength 0.10 (glycylglycine NaOH)	
Acetaldehyde + DPNH	
enzyme	8.46×10^{-8} site equivalents/liter
acetaldehyde	1.17×10^{-5} to 9.01×10^{-5} M
DPNH[a]	8.95×10^{-7} to 6.93×10^{-6} M
Ethanol + DPN^+	
enzyme	2.82×10^{-7} site equivalents/liter
ethanol[a]	2.20×10^{-4} to 1.70×10^{-3} M
DPN^+	1.52×10^{-5} to 1.18×10^{-4} M

[a] Slightly higher concentrations, leading to initial rates of the same magnitude, were used when deuterium-containing reactants were employed.

The reaction vessels were 3-ml quartz cuvets with a light path of 1.00 cm. The cuvets were filled with 1.5-ml portions of the reactants by means of 2-cc Luer-Lok syringes (Becton, Dickinson and Company, Rutherford, N. J.) calibrated with a buffer solution to deliver 1.50 ± 0.01 ml. After the cuvets were placed in their compartment, the recording system was turned on, the enzyme solution was pipetted by means of a 50-μl pipet into a specially designed lucite mixing spoon, and the reaction was started by a few rapid up and down motions of the spoon inside the cuvet. Complete mixing was achieved and the spectrophotometer responded to the reaction within 3 seconds.[4] To obtain initial rates, the observed rates on the Cary graph were extrapolated to time zero by means of a Keuffel and Esser french curve, model 1864-60. The slopes of the resulting extrapolated lines were determined and employed as initial rates.

The substrate concentrations used in the initial rate runs (Table I) varied over a range of 7.75 fold. As mentioned previously (Baker and Mahler, 1962), up to ten different concentrations of A and B can be treated by the IBM program. Although such a complete matrix containing 100 points would undoubtedly be exceedingly useful, we decided

[4] The completeness of the mixing was checked at 280 mμ by the introduction of concentrated enzyme solutions into the cuvet.

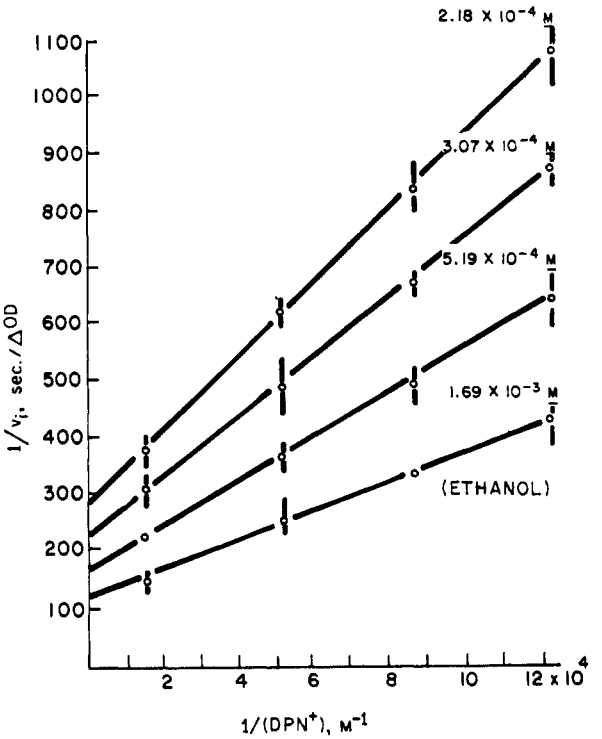

FIG. 1.—A typical initial steady state rate run.

that some reiterative method would perhaps lend itself better to a statistical analysis of the problem. A matrix of four concentrations of A and B was chosen to be run in triplicate. In this way, we could not only observe the experimental variation for any particular value but also have the choice of discarding any point in a set of three if it seemed to fall far outside the mean determined by the other two. Table II summarizes the statistics of the matrices used. The rates gathered at the lower concentrations of reactants appear to be slightly less reliable. However, this fact, if true, became apparent only after *all* the points had been collected. Our understanding of the errors involved did not allow us to make an *a priori* prediction, a prerequisite for most computations of weighting factors. This then suggests the use of the computed deviations of one particular kinetic run as weighting factors for this particular run. In essence we have done this in the following way: After the data for a run were collected and the errors involved computed, the best values falling within the standard deviations were selected so as to give a linear response in our IBM program. This is represented in Figure 1. The open circles

TABLE II

STATISTICS OF SUBSTRATE CONCENTRATION-VELOCITY MATRIX

(per cent of triplicate sets used—per cent range between points used)

substrate (decreasing concentrations) ↓	Coenzyme (decreasing concentrations) →			
	(90.2–4.2)	(78.7–4.9)	(81.1–5.9)	(83.3–5.3)
	(85.6–5.8)	(80.0–6.3)	(80.0–5.8)	(79.4–6.0)
	(86.3–5.4)	(80.8–5.9)	(74.7–5.3)	(76.0–6.3)
	(80.8–5.1)	(79.4–5.7)	(74.2–5.8)	(72.0–5.0)

Over-all average for the sets
(80.2 ± 3.4–5.53 ± 0.44)

TABLE III
CONDITIONS FOR CONTINUOUS STEADY STATE RATE RUNS
Buffer, 0.1 M imidazole, pH 7.58; enzyme, 6.98×10^{-7} site equivalents/liter; temperature, 27°

Acetaldehyde + DPNH $\rightleftharpoons$		Ethanol + DPN$^+$ $\rightleftharpoons$	
Acetaldehyde	1.62×10^{-5} M	Ethanol	8.56×10^{-4} M
DPNH	1.26×10^{-5} M	Dideuteroethanol	8.30×10^{-4} M
α-D-DPNH	1.22×10^{-5} M	DPN$^+$	8.17×10^{-4} M

represent the points actually used on the IBM-650, while the bars represent the observed deviations. The conditions used for the continuous steady state runs are summarized in Table III.

RESULTS

1. Initial Rate Kinetics.—The experimental conditions used have been summarized in Table I, while the average φ_p values obtained are tabulated in Table IV. Also recorded are the standard deviations of the actual values for sets of separate kinetic runs, and the mean of the computed standard errors for the sets.

Since the theory of errors in enzyme kinetics has not yet been developed to the point of permitting a critical appraisal of values such as those of Table IV, we make no attempt to explain their magnitude. The relative magnitude of the two types of error estimates shown is, however, of some interest. In nearly all cases the mean of the standard errors, a measure of the reliability of the individual φ_p value, was equal to or greater than the mean of the deviations between runs, an approximate measure of their reproducibility. Therefore — especially since the deviations were calculated on the basis of only two or three runs, while the standard errors were computed from the large number of data involved in a single run— we have used only the latter statistic for our calculations.

The results shown in Table IV were then subjected to the methods for mechanism characterization described previously (Baker and Mahler, 1962; see Table IV in that paper for numbered list of mechanisms).

2. Haldane Relations (Relations Between $\varphi_{12}'/\varphi_{12}$ and $\varphi_0\varphi_1'\varphi_2'/\varphi_0'\varphi_1\varphi_2$ or $\varphi_0'\varphi_1'\varphi_2'/\varphi_0\varphi_1\varphi_2$).

$K_{eq} =$	Hydrogen	Deuterium
$\varphi_{12}'/\varphi_{12} \times 10^{-2}$	3.56 ± 0.24	3.41 ± 0.28
$\varphi_0\varphi_1'\varphi_2'/\varphi_0'\varphi_1\varphi_2 \times 10^{-1}$	3.71 ± 0.82	2.05 ± 0.70
$\varphi_0'\varphi_1'\varphi_2'/\varphi_0\varphi_1\varphi_2 \times 10^{-3}$	1.08 ± 0.24	0.42 ± 0.14

A comparison of the ratio $\varphi_{12}'/\varphi_{12}$ with the other two ratios shown indicates that the first is *not* equal to either of the other two except possibly for $\varphi_0'\varphi_1'\varphi_2'/\varphi_0\varphi_1\varphi_2$ in the deuterium example. This criterion is sufficient to eliminate mechanisms Ib, IIc, and IId; it permits no choice between the allowed mechanisms Ia, IIa, and IIb.

3. Dalziel (1958) Relations (Relations Between $\varphi_1\varphi_2/\varphi_{12}$ and φ_0 or φ_0').

	Hydrogen	Deuterium
$\varphi_1\varphi_2/\varphi_{12} \times 10^1$	3.35 ± 0.38	6.47 ± 0.92
$\varphi_1'\varphi_2'/\varphi_{12}' \times 10^1$	1.88 ± 0.15	1.76 ± 0.22
$\varphi_0 \times 10^2$	6.79 ± 0.40	10.8 ± 1.7
$\varphi_0' \times 10^1$	3.67 ± 0.13	4.86 ± 0.04

$\varphi_1\varphi_2/\varphi_{12} \neq \varphi_0$ and $\varphi_1'\varphi_2'/\varphi_{12}' \neq \varphi_0'$ in all cases. Therefore, mechanism Ib is excluded. $\varphi_1\varphi_2/\varphi_{12} \leqslant \varphi_0'$ in the hydrogen case, but $\geqslant \varphi_0'$ in the deuterium example; similarly $\varphi_1'\varphi_2'/\varphi_{12} \leqslant \varphi_0$ in both the hydrogen and deuterium examples. This is the reverse of the Dalziel relation, and is not exhibited by any of the mechanisms proposed by him; the magnitude of this effect, particularly in the case of the data involving ethanol plus DPN, is significant, and suggests that the actual mechanism must be a *modified* Type II mechanism.

4. Product Inhibition.—Our studies of product inhibition were not too fruitful owing to the dif-

TABLE IV
SUMMARY OF RESULTS FOR INITIAL RATE RUNS

Reactants	Number of Experiments[a]		φ_p Values	Standard Deviation (Experimental) (%)	Computed Mean of Standard Errors (%)
Acetaldehyde + DPNH	3	φ_0	6.79×10^{-2}	3.1	6.0
		φ_1	2.85×10^{-7}	12.	3.5
		φ_2	1.41×10^{-5}	6.8	2.3
		φ_{12}	1.20×10^{-11}	2.8	5.6
Ethanol + DPN$^+$	2	φ_0'	3.67×10^{-1}	11.	4.3
		φ_1'	4.21×10^{-6}	1.7	4.3
		φ_2'	1.91×10^{-4}	1.6	2.6
		φ_{12}'	4.27×10^{-9}	0.12	1.3
Acetaldehyde + α-D-DPNH	2	φ_0	1.08×10^{-1}	20.	16.
		φ_1	4.63×10^{-7}	6.5	8.5
		φ_2	4.02×10^{-5}	0.12	0.97
		φ_{12}	2.87×10^{-11}	1.7	3.0
Dideuteroethanol + DPN$^+$	2	φ_0'	4.86×10^{-1}	1.8	0.84
		φ_1'	4.36×10^{-6}	6.5	5.0
		φ_2'	3.94×10^{-4}	1.3	2.4
		φ_{12}'	9.79×10^{-9}	4.3	5.2
Acetaldehyde + β-D-DPNH	2	φ_0	1.00×10^{-1}	18.	13.
		φ_1	2.94×10^{-7}	5.5	5.5
		φ_2	1.41×10^{-5}	3.6	2.3
		φ_{12}	2.77×10^{-11}	6.9	2.3

[a] Four concentrations each of A and B, each set run in triplicate. The units of φ are: φ_0, sec./site; φ_1, sec.M/site; φ_2, sec. M/site; and φ_{12}, sec. M^2/site.

ficulties inherent in the required alteration of substrate concentrations to obtain suitable values for velocity measurements. Some of the results and our conclusions are tabulated in Table V.

Although the data are not completely unambiguous, largely because of the rather large errors involved in the determination of the Δ values, it is clear that the bulk of the evidence suggests one of the ordered sequence (compulsory binding) Type II mechanisms, with the coenzyme as the leading substrate. The ambiguities shown might be overcome, perhaps, by some modification of the Type II mechanism not previously considered in the derivation (Alberty, 1958; Baker and Mahler, 1962).

5. Isotope Effects (Baker and Mahler, 1962).—The following relationships may be computed from the data of Table IV, excluding for the moment the data for β-D-DPNH (*i.e.*, the isomer from which deuterium cannot be removed in this reaction).

$\bar{\varphi}_0' = 1.32 \pm 0.06$ $\quad \bar{\varphi}_0 = 1.59 \pm 0.36$
$\bar{\varphi}_1' = 1.04 \pm 0.10$ $\quad \bar{\varphi}_1 = 1.62 \pm 0.19$
$\bar{\varphi}_2' = 2.06 \pm 0.10$ $\quad \bar{\varphi}_2 = 2.84 \pm 0.09$
$\bar{\varphi}_{12}' = 2.29 \pm 0.15$ $\quad \bar{\varphi}_{12} = 2.39 \pm 0.20$
$\bar{\varphi}_1\bar{\varphi}_2/\bar{\varphi}_{12} = 1.93 \pm 0.46$

The identity $\bar{\varphi}_2' \equiv \bar{\varphi}_{12}'$ is common to all mechanisms and is satisfied by the data. The identity $\bar{\varphi}_0' \equiv \bar{\varphi}_1'$ is satisfied, but $\bar{\varphi}_0 \neq \bar{\varphi}_2$ and $\bar{\varphi}_1 \neq \bar{\varphi}_{12}$, and mechanisms Ia and Ib are therefore excluded. Since φ_1' equals unity, Ia or Ib are again impossible, but all other mechanisms are possible. Since $\bar{\varphi}_0 \neq 1$ and probably $\bar{\varphi}_0' \neq \bar{\varphi}_1\bar{\varphi}_2/\bar{\varphi}_{12}$, mechanisms IIc and d are held to be less likely than mechanisms IIa or IIb.

6. Continuous Steady State Kinetics.—Investigations of continuous steady state kinetics were run under the conditions summarized in Table III. These four continuous rate experiments were used to provide over 100 separate observations on concentrations and their rate of change, each determined in triplicate. These were placed on the computer, which then calculated some 300 rates and their respective errors, providing the data for a test of equation (23) of Baker and Mahler (1962) and a computation of its coefficients. For brevity, only one of the actual experiments will be shown (Table VI). All four are summarized in Table VII. The substrates employed for the data of Table VII were (1) dideuteroethanol, (2) ethanol, (3) α-D-DPNH, and (4) DPNH. The errors given are the observed standard errors calculated for each point taken in triplicate, the standard errors computed from the closeness of fit, and the average absolute fitting errors (positive

TABLE V
SUMMARY OF RESULTS FOR SUBSTRATE INHIBITION RUNS

	φ_p	Δ_p	Mechanism Type (See Table V, Mahler and Baker, [1962])
Acetaldehyde + DPNH ⟶ ethanol (5.65×10^{-4} M)			
φ_0	$8.24 \times 10^{-2} \pm 0.79$	1.21 ± 0.19	Probably II (d) ?
φ_1	$2.40 \times 10^{-7} \pm 0.04$	0.84 ± 0.05	
φ_2	$2.39 \times 10^{-5} \pm 0.06$	1.69 ± 0.08	
φ_{12}	$2.26 \times 10^{-11} \pm 0.05$	1.88 ± 0.15	
Acetaldehyde + DPNH ⟶ DPN^+ (2.51×10^{-5} M)			
φ_0	$1.15 \times 10^{-1} \pm 0.16$	1.68 ± 0.33	Indeterminate; some support for I
φ_1	$3.31 \times 10^{-7} \pm 0.22$	1.16 ± 0.12	
φ_2	$1.26 \times 10^{-5} \pm 0.02$	0.90 ± 0.04	
φ_{12}	$5.37 \times 10^{-11} \pm 0.04$	4.47 ± 0.28	
Ethanol + DPN^+ ⟶ DPNH (9.82×10^{-7} M)			
φ_0'	$3.98 \times 10^{-1} \pm 0.12$	1.08 ± 0.07	Indeterminate
φ_1'	$8.07 \times 10^{-6} \pm 0.21$	1.92 ± 0.13	
φ_2'	$1.29 \times 10^{-4} \pm 0.05$	0.67 ± 0.04	
φ_{12}'	$3.20 \times 10^{-9} \pm 0.13$	0.75 ± 0.04	
Acetaldehyde + α-D-DPNH ⟶ dideuteroethanol (5.45×10^{-4} M)			
φ_0	$1.67 \times 10^{-1} \pm 0.03$	1.54 ± 0.28	Indeterminate
φ_1	$2.09 \times 10^{-7} \pm 0.23$	0.45 ± 0.09	
φ_2	$4.38 \times 10^{-5} \pm 0.03$	1.08 ± 0.02	
φ_{12}	$5.39 \times 10^{-11} \pm 0.10$	1.87 ± 0.09	
Acetaldehyde + α-D-DPNH ⟶ DPN^+ (2.42×10^{-5} M)			
φ_0	$8.81 \times 10^{-2} \pm 0.63$	0.82 ± 0.19	Probably II
φ_1	$1.85 \times 10^{-6} \pm 0.02$	4.00 ± 0.38	
φ_2	$5.25 \times 10^{-6} \pm 0.02$	1.30 ± 0.02	
φ_{12}	$1.84 \times 10^{-10} \pm 0.01$	6.40 ± 0.23	
Dideuteroethanol + DPN^+ ⟶ α-D-DPNH (1.63×10^{-6} M)			
φ_0'	$5.18 \times 10^{-1} \pm 0.05$	1.06 ± 0.02	Probably II
φ_1'	$1.40 \times 10^{-5} \pm 0.02$	3.20 ± 0.21	
φ_2'	$1.51 \times 10^{-4} \pm 0.09$	0.38 ± 0.03	
φ_{12}'	$2.29 \times 10^{-8} \pm 0.03$	2.34 ± 0.15	
Ethanol + DPN^+ ⟶ β-D-DPNH (3.06×10^{-6} M)			
φ_0'	$4.09 \times 10^{-1} \pm 0.29$	1.11 ± 0.12	Probably II
φ_1'	$1.20 \times 10^{-5} \pm 0.04$	2.84 ± 0.21	
φ_2'	$1.79 \times 10^{-4} \pm 0.10$	0.94 ± 0.08	
φ_{12}'	$1.08 \times 10^{-8} \pm 0.01$	2.53 ± 0.06	

The units of φ are: φ_0, sec./site; φ_1, sec. M/site; φ_2, sec. M/site; and φ_{12}, sec. M^2/site.

TABLE VI

DATA FROM EXPERIMENT ON CONTINUOUS STEADY-STATE KINETICS

Reaction: DPN^+ + dideuteroethanol (continuous rate runs)

Observed Rates, Site/Sec.	Computed Rates: All Constants	$b_1 = 0$	b_1 and $b_2 = 0$
0.1205	0.1190	0.1163	0.1050
0.1033	0.1035	0.1049	0.0957
0.0910	0.0925	0.0944	0.0875
0.0825	0.0836	0.0849	0.0795
0.0756	0.0760	0.0764	0.0726
0.0710	0.0686	0.0863	0.0659
0.0637	0.0622	0.0615	0.0601
0.0580	0.0562	0.0553	0.0548
0.0495	0.0504	0.0495	0.0498
0.0430	0.0457	0.0449	0.0457
0.0369	0.0376	0.0372	0.0387
0.0311	0.0308	0.0308	0.0327
0.0253	0.0252	0.0255	0.0276
0.0207	0.0210	0.0214	0.0237
0.0169	0.0174	0.0180	0.0203
0.0146	0.0148	0.0154	0.0177
0.0123	0.0124	0.0130	0.0152
0.0100	0.0105	0.0110	0.0131
0.0096	0.0089	0.0093	0.0114
0.0088	0.0075	0.0079	0.0099
0.0069	0.0063	0.0066	0.0085
0.0054	0.0054	0.0055	0.0074
0.0042	0.0039	0.0039	0.0056
0.0031	0.0028	0.0026	0.0043
0.0023	0.0022	0.0018	0.0035
0.0019	0.0016	0.0012	0.0028

TABLE VII

CURVE-FITTING TO EQUATION (23) OF BAKER AND MAHLER (1962)

	Experiment 1	2	3	4
Observed error	3.12	4.24	3.99	3.50
All Constants Used				
Standard error	4.04	4.48	4.65	5.85
Absolute error	1.94	1.41	0.97	1.07
b_1 is Zero				
Standard error	6.39	5.27	3.80	610.0
Absolute error	2.62	3.39	1.02	582.0
b_1 and b_2 Are Zero				
Standard error	17.1	29.8	58.3	60.9
Absolute error	13.2	27.7	55.5	57.9

errors plus negative errors).

In all of the examples except (3), the standard error for the points computed with the use of all the coefficients is less than that found when any of these are set equal to zero. Even in this one case, as in all of the others, the absolute error, a measure of the over-all fit, is smaller. In case (4), the extreme error in the fit with b_1 set equal to zero is caused by a discontinuity of the computed points. Therefore, it can be surmised that the best fit is obtained by the use of all the coefficients. Hence, mechanisms IIa, b, and c are considered the most likely.[5]

DISCUSSION

From the foregoing we conclude that under the particular conditions described in this paper the most likely mechanism for the reaction catalyzed by liver alcohol dehydrogenase is a steady-state one involving a compulsory order of binding of reactants, with the coenzyme bound first in an obligatory manner. The mechanism probably involves one or more ternary complexes, and the likelihood that the enzyme forms complexes other than those involved in the simple, linear scheme

$$E + A \rightleftharpoons EA \overset{+B}{\rightleftharpoons} EXY \underset{+B'}{\rightleftharpoons} EA' \underset{+A'}{\rightleftharpoons} E + A'$$

must be entertained.

[5] The constants derived have not been presented, as they are simple fitting parameters with the possibility of being complicated groups of rates. No attempt has been made in the treatment of these numbers to solve for the individual rates involved.

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REFERENCES

Abeles, R. H., Frisell, W., and Mackenzie, C. (1960), *J. Biol. Chem. 235*, 835.
Alberty, R. (1958), *J. Am. Chem. Soc. 80*, 1777.
Baker, R. H., Jr., and Mahler, H. R. (1962), *Biochemistry 1*, 35.
Bonnichsen, R. K., and Brink, N. G. (1958), *in* Methods of Enzymology, vol. I, Colowick, S. P., and Kaplan, N. O., ed., New York, Academic Press, Inc., p. 495.
Boyer, P. D., and Theorell, H. (1956), *Acta Chem Scand. 10*, 447.
Collins, C. J. (1960), *Ann. N.Y. Acad. Sci. 84*, 603.
Dalziel, K. (1958), *Acta Chem. Scand. 12*, 459.
Ehrenberg, A., and Dalziel, K. (1958), *Acta Chem. Scand. 12*, 465.
Englard, S. (1960), *Ann. N.Y. Acad. Sci. 84*, 695.
Horecker, B. L., and Kornberg, A. (1948), *J. Biol. Chem. 175*, 385.
Kornberg, A., and Pricer, W. E., Jr. (1953), *Biochem. Prep. 3*, 20.
Levy, H. R., and Vennesland, B. (1957), *J. Biol. Chem. 228*, 85.
Mahler, H. R., and Douglas, J. (1957), *J. Am. Chem. Soc. 79*, 1159.
Melander, L. (1960), Isotope Effects on Reaction Rates, New York, The Ronald Press.
Merritt, A. D., and Tomkins, G. M. (1959), *J. Biol. Chem. 234*, 2780.
Rafter, G. W., and Colowick, S. P. (1958), *in* Methods of Enzymology, Colowick, S. P., and Kaplan, N. O., ed.. vol. III, New York, Academic Press, Inc., p. 887.
Rieder, S. V., and Rose, I. A. (1959), *J. Biol. Chem. 234*, 1007.
Rose, I. A. (1961), *J. Biol. Chem. 236*, 603.
Shiner, V. J., Jr. (1952), *J. Am. Chem. Soc. 75*, 2929.
Shiner, V. J., Jr., Mahler, H. R., Baker, R. H., Jr., and Hiatt, R. R. (1960), *Ann. N. Y. Acad. Sci. 84*, 583.
Streitwieser, A., Jr. (1960), *Ann. N. Y. Acad. Sci. 84*, 576.
Theorell, H. (1956), *in* Currents in Biochemical Research, Green, D. E., ed., New York-London, Interscience, p. 293.
Theorell, H. (1958), *in* Advances in Enzymology, vol. 20, Nord, F. F., ed., New York-London, Interscience, p. 31.
Theorell, H. (1961), *Fed. Proc. 20*, in press.
Theorell, H., and Bonnichsen, R. K. (1951), *Acta Chem. Scand. 5*, 1105.
Theorell, H., and Chance, B. (1951), *Acta Chem. Scand. 5*, 1127.
Theorell, H., Nygaard, A. P., and Bonnichsen, R. K. (1955), *Acta Chem. Scand. 9*, 1148.
Theorell, H., and Winer, A. D. (1959), *Arch. Biochem. Biophys. 83*, 291.
Vallee, B. L., and Coombs, J. L. (1959), *J. Biol. Chem. 234*, 2615.

Vallee, B. L., Williams, R. J. P., and Hoch, F. L. (1959), *J. Biol. Chem. 234*, 2621.
Van Eys, I., Stolzenbach, F. E., Sherwood, L., and Kaplan, N. O. (1958), *Biochim. Biophys. Acta 27*, 63.
Vennesland, B. (1958), *Fed. Proc. 17*, 1150.
Vennesland, B., and Westheimer, F. H. (1954), *in* The Mechanism of Enzyme Action, McElroy, D., and Glass, B., ed., Baltimore, The Johns Hopkins Press p. 357.
Wiberg, K. B. (1955), *Chem. Revs. 55*, 713.
Winer, A. D. (1958), *Acta Chem. Scand. 12*, 1695.

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

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

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

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

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

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

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

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

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

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

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

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‡ Contribution No. 1030.

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