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Mechanisms of Enzyme-catalyzed Oxidation–Reduction Reactions. I. An Investigation of the Yeast Alcohol Dehydrogenase Reaction by Means of the Isotope Rate Effect[1,2]

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The kinetics of the reversible dehydrogenation of ethanol by diphosphopyridine nucleotide, catalyzed by the alcohol dehydrogenase from yeast, have been examined in dilute phosphate buffer *p*H 7.6. Both protonated and deuterated reactants, *viz.* 1,1′-dideuteroethanol and DPND were used. The occurrence of appreciable isotope effects on the Michaelis constants for these two components (but not for their reaction partners) and for the extrapolated maximal initial velocity in either direction has been demonstrated. The results of this and previous investigations are interpreted in terms of a mechanism which includes independent binding of substrate and coenzyme by Zn^{++} and protein at the active site and a rate-limiting step (at high reactant concentration) with a transition state analogous to that postulated for the Meerwein–Pondorff–Oppenauer reaction.

The mechanism of action of certain pyridine-nucleotide requiring dehydrogenases has been the subject of a number of recent papers, and attempts at elucidation have made use of a variety of experimental and conceptional devices. These have taken the form of exhaustive kinetic studies employing both steady state[3–7] and transient-state[8] techniques, studies on the rate, nature and extent of binding of substrates, coenzymes and inhibitors,[3–10] non-enzymic model reactions[11,12] and a clear-cut demonstration that in the reaction catalyzed by these enzymes

$$SH_2 + Co^+ \underset{}{\overset{\text{enzyme}}{\rightleftharpoons}} S + CoH + H^+ \quad (1)[13]$$

the transfer of hydrogen between substrate and the *para* position of the pyridine ring[14] is direct and stereospecific.[15] In addition various possible overall mechanisms for reaction 1 have been discussed and the applicable steady state rate equations derived.[16,17] As a result of these investigations, considerable inroads have been made into the area of uncertainty concerning the mechanism of the overall reaction.

On the other hand, relatively little is known concerning the mechanism of the hydrogen (or electron) transferring step proper and the transition state through which this transfer takes place. It occurred to us that the hydrogen isotope rate effect might be utilized to gain some information about the rate-limiting steps in enzyme-catalyzed oxidation–reduction reactions. For it has been demonstrated experimentally and can be shown to hold from first principles at least to a first approximation that, at room temperature, a breaking of a C–H bond in the rate-limiting step will lead to a ratio $k_H/k_D \approx 7$; that a transition state in which the bonding to the hydrogen in the transition state is about as strong as that in the initial state will give a smaller ratio for $k_H/k_D \approx 1.4$, while so-called secondary isotope effects in reactions which do not involve the transfer of hydrogen at all will be smaller still.[18] Although the method has been employed successfully for the elucidation of the mechanism of organic oxidation–reduction reactions,[19] no application to isolated enzymatic reactions of this type has so far been reported.

For our initial investigation we chose the alcohol dehydrogenase of yeast for the following reasons: (1) The enzyme can be obtained easily in crystalline form[20] and is commercially available. (2) The kinetics of the enzyme-catalyzed reaction and the binding of the reaction partners by the enzyme have been the subject of detailed investigations[3,6] which showed that (a) the kinetics are reasonably simple and straightforward, (b) the apparent dissociation constants for DPNH, DPN^+ and acetaldehyde are approximately equal to the experimentally determined Michaelis constants and (c) the enzyme can bind four molecules of coenzyme

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(2) Some of the experimental findings described in this report have been presented previously, at the 129th Meeting of the American Chemical Society, Dallas, Texas, April, 1956, and at the 47th Annual Meeting of the American Society of Biological Chemists, Atlantic City, N. J., April, 1956 (*Fed. Proc.*, **15**, 307 (1956)).

(3) H. Theorell and R. Bonnichsen, *Acta Chem. Scand.*, **5**, 1105 (1951); A. P. Nygaard and H. Theorell, *ibid.*, **9**, 1300, 1551 (1955).

(4) H. Theorell, A. P. Nygaard and R. Bonnichsen, *ibid.*, **9**, 1148 (1955).

(5) J. E. Hayes and S. F. Velick, *J. Biol. Chem.*, **207**, 225 (1954).

(6) M. T. Hakala, A. J. Glaid and G. W. Schwert, *ibid.*, **221**, 191 (1956).

(7) J. B. Neilands, *ibid.*, **199**, 373 (1952); B. Chance and J. B. Neilands, *ibid.*, **99**, 383 (1952).

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

(9) N. O. Kaplan and M. M. Ciotti, *J. Biol. Chem.*, **201**, 785 (1953); N. O. Kaplan, M. M. Ciotti and F. E. Stolzenbach, *ibid.*, **211**, 419 (1954); N. O. Kaplan and M. M. Ciotti, *ibid.*, **211**, 431 (1954).

(10) C. S. Vestling, H. Terayama, J. R. Florini and J. N. Baptist, *Fed. Proc.*, **15**, 375 (1956).

(11) A. J. Swallow, *Biochem. J.*, **61**, 197 (1955); G. Stein and G. Stiassny, *Nature*, **176**, 734 (1955).

(12) R. H. Abeles and F. H. Westheimer, *Fed. Proc.*, **15**, 208 (1956).

(13) The following abbreviations will be used: S, substrate of the general structure $R_1R_2C{=}O$, here $=CH_3CHO$; Co^+-DPN, diphosphopyridine nucleotide; CoH-DPNH, reduced diphosphopyridine nucleotide; SHD, CH_3CD_2OH; DPND, paradeutero DPNH; ϵ, molar extinction coefficient; E, enzyme; k_1, rate constants; K_i, Michaelis and equilibrium constants; k_D, K_D, constants for reactants containing deuterium; k_H, K_H, constants for reactants containing protium.

(14) M. E. Pullman, A. San Pietro and S. P. Colowick, *J. Biol. Chem.*, **206**, 129 (1954).

(15) For a review see B. Vennesland and F. H. Westheimer in "The Mechanism of Enzyme Action," ed. by W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1954.

(16) H. L. Segal, J. F. Kachmar and P. D. Boyer, *Enzymol.*, **15**, 187 (1952).

(17) R. A. Alberty, THIS JOURNAL, **75**, 1928 (1953); *Advances in Enzymology*, **17**, 1 (1956).

(18) A recent review is given by K. B. Wiberg, *Chem. Revs.*, **55**, 713 (1955).

(19) F. H. Westheimer and N. Nicolaides, THIS JOURNAL, **71**, 25 (1949); F. H. Westheimer, *Chem. Revs.*, **45**, 419 (1949).

(20) E. Racker, *J. Biol. Chem.*, **184**, 313 (1950).

per molecule of protein at equivalent sites; the binding sites for DPN^+ are identical with those for DPNH. (3) Hydrogen transfer between substrate and coenzyme has been investigated by the use of deuterium-labeled reactants; it was shown to be direct and stereospecific.[15,21] (4) The enzyme has been shown to contain four gram-atoms of zinc per mole of protein. The metal has been tentatively implicated in enzymatic activity and coenzyme-binding.[22] These latter results introduce an added parameter which must be taken into account when formulating a reaction mechanism. The enzyme catalyzes the reaction

$$RCH_2OH + DPN^+ \rightleftarrows RCHO + DPNH + H^+ \quad 1(a)$$

(R = CH_3 in this investigation)

By means of kinetic investigations using 1,1'-dideuteroethanol and DPND, it has been possible to confirm and extend some of the findings just described and to propose a structure for the transition state and a mechanism for the reaction consistent with all the experimental data so far available. These investigations form the basis for the present report.

Experimental

Enzyme.—The enzyme used in these studies was a crystalline, commercial product (Worthington Biochemical Corp.) recrystallized two additional times in this Laboratory.[5] The stock suspension of the enzyme was kept at −15° and diluted for kinetic experiments with a diluting medium to yield the following final concentrations: enzyme protein, 10–40 μ g. per ml.; crystalline bovine serum albumin (Armour or Pentex), 1 mg. per ml.; phosphate 0.001 *M* and cysteine 0.01 *M*, all adjusted to *p*H 7.6. Enzyme made up in this manner is reasonably stable at 0°. New dilutions were made every hour; rate runs under identical conditions showed the same initial rate of DPNH formation or disappearance at the beginning and end of this period. Enzyme concentrations were determined spectrophotometrically at 280 mμ using the molar extinction coefficient (ϵ) (1.89×10^5 cm.^{2} mole^{-1}) and molecular weight (150,000) reported by Hayes and Velick.[5]

Reactants.—The DPN was purchased from the Sigma Chemical Co. It was found to be 98% pure from the optical density at 260 mμ,[23] using $\epsilon = 18.0 \times 10^6$ cm.^{2} mole^{-1}. The purity of the sample used was 95% as estimated from hydrosulfite reduction and 85% as estimated from enzymatic reduction in the presence of excess alcohol, alcohol dehydrogenase and semicarbazide at *p*H 7.6, or in the absence of semicarbazide at *p*H 10.0. Similar results were also obtained with excess lactate and lactic dehydrogenase at *p*H 7.6. It seems likely that this apparent discrepancy is referable to the presence of approximately 10% enzymatically inactive α-isomer of DPN.[24] All measurements of DPNH formation or disappearance were made at 340 mμ, $\epsilon^{25} = 6.22 \times 10^6$ cm.$^2 \times$ mole^{-1}. Solutions of acetaldehyde were prepared fresh and standardized prior to use by reduction of aliquots in phosphate buffer *p*H 7.0 in the presence of excess DPNH and alcohol dehydrogenase.

The DPND used was prepared from the DPN sample indicated above by chemical reduction by the method of Fisher, *et al.*[21] The reduction was performed in 100-mg. lots in 99.5% pure D_2O (Stuart Oxygen Co.) and could be shown to be essentially complete. The yield of isolated, purified disodium DPND varied between 65 and 70% of starting DPN on a weight basis. The dry weight purity of the reduced material varied between 70 and 75% based on light absorption at 340 mμ. Of this absorption 9.5–10.5% was not destroyed by enzymatic reoxidation of the reduced coenzyme by excess acetaldehyde and alcohol dehydrogenase at *p*H 7.6, or by pyruvate and lactic dehydrogenase or by cytochrome-c plus DPNH-cytochrome reductase. Since similar results were also obtained on analysis of the starting DPN and of DPNH prepared from the same sample, this discrepancy may be again referable to the presence of the α-isomer in the starting material. The DPND prepared in this manner was found not to reduce either 2,6-dichlorophenolindophenol or cytochrome-c non-enzymatically at a significant rate as determined by measuring the change in light absorption at 600 and 550 mμ, respectively, and is thus relatively free of inorganic or organic reducing impurities. In view of the reduction method employed in which hydrogen might be introduced only from the solid, anhydrous reagents, the completeness of the reduction under these conditions and the demonstration that the DPND so formed contains one non-exchangable deuterium atom per molecule,[15] the final product may be estimated to be contaminated by <5% DPNH, but no deuterium analysis was performed. The dry, solid DPND was stored in a desiccator over $CaCl_2$ at −20° and solutions were made up as needed and kept at 0° or frozen at −20°. Their concentration was determined by the enzymic oxidation of the reduced coenzyme in aliquots in the manner already indicated. DPNH used in this investigation was prepared by the method described for DPND but substituting H_2O for D_2O in the reduction step. Yields and purity were determined in a similar manner, and comparable data were obtained. When the DPNH isolated in this manner was compared to a commercial DPNH sample of high purity (≥90%), initial rates in the alcohol dehydrogenase reaction at several different concentrations of substrate and coenzyme were found to be sensibly identical.

The dideuteroethanol was synthesized by Prof. V. J. Shiner, Jr., by the already published method which involves reduction of acetic anhydride by $LiAlD_4$ (95% pure, obtained from Metal Hydrides, Inc.) in dibutyl Cellosolve and distillation of the product.[26] Dilute solutions of the alcohol were prepared as needed and stored frozen at −20°. Concentrations were determined in aliquots by measuring the reduction of excess DPN in the presence of alcohol dehydrogenase, with added semicarbazide at *p*H 7.6 or in its absence at *p*H 10. The ethanol employed had similarly been prepared by $LiAlH_4$ reduction of the anhydride. Its determination, etc., was performed as indicated for the deuterated counterpart. Initial reaction rates observed in the alcohol dehydrogenase reaction with an authentic ethanol solution of equal concentration did not differ significantly from the rates observed with ethanol prepared in this manner at several different concentrations of substrate and coenzyme in the reaction mixture.

Kinetic Runs.—All kinetic experiments were performed in a Beckman recording spectrophotometer, model DK-1, kept in a room maintained at a temperature of 22°. The reaction mixtures contained in a total volume of 0.98 ml. of substrate and coenzyme at the desired concentration, 2 μmoles of Na_2HPO_4 and 0.5 mg. of crystalline bovine serum albumin all adjusted to *p*H 7.6. If the reaction was to be studied in the direction of alcohol oxidation, 10 μmoles of semicarbazide hydrochloride adjusted to the same *p*H was added as well. The optical density at 340 mμ was then determined after temperature equilibration for 2 minutes against a blank containing all the components except the pyridine nucleotide in 1-ml. quartz cuvettes with a light path of 1.00 cm. The reactions were started by adding sufficient enzyme in 0.020 ml. to give optical density changes between 0.010 and 0.1 per minute. The enzyme was added from a micropipet in the form of a drop placed in a spoon made from a flattened out stirring rod, and mixing was achieved by rapid up and down motions of the spoon inside the cuvette. This operation was completed in three seconds or less. Reaction was then allowed to proceed for 60–90 seconds and the change in optical density recorded on a strip chart allowed to travel at a rate of 2 inches (4 scale divisions) per minute. The straight lines (zero-order rates) or smooth curves obtained (first or intermediate order) were extrapolated graphically to zero time and the initial rate, *v*, per

(21) H. F. Fisher, E. E. Conn, B. Vennesland and F. H. Westheimer, *J. Biol. Chem.*, **202**, 687 (1953).

(22) B. L. Vallee and F. L. Hoch, THIS JOURNAL, **77**, 821 (1955); *Proc. Natl. Acad. Sci.*, **41**, 327 (1955).

(23) A. Kornberg and W. E. Pricer, Jr., *Biochem. Prep.*, **3**, 20 (1953); Pabst Laboratories, ultraviolet absorption spectra of 5'-ribonucleotides, Milwaukee, Wisc., 1955.

(24) A. O. Kaplan, M. M. Ciotti, F. E. Stolzenbach and N. R. Bachur, THIS JOURNAL, **77**, 815 (1955).

(25) B. L. Horecker and A. Kornberg, *J. Biol. Chem.*, **175**, 385 (1948).

(26) V. J. Shiner, Jr., THIS JOURNAL, **75**, 2929 (1952).

minute taken as 4 times this extrapolated change in optical density for the first 15 seconds and converted to a change in reduced pyridine nucleotide concentration per minute by use of the appropriate extinction coefficient. v was found to be proportional to enzyme concentration for values leading to optical density changes ranging from 0.010 to 0.200 per minute. All the data are expressed as $v/(E_t)$, *i.e.*, initial rate per mole of enzymatically active sites (4 per molecule enzyme of mol. wt. 150,000) and are thus in the units of min.$^{-1}$.

Results

Derivations of Rate Law.—The kinetic data obtained at various substrate and coenzyme concentration in reaction 1 in both directions are plotted in Figs. 1 to 4 by means of the reciprocal

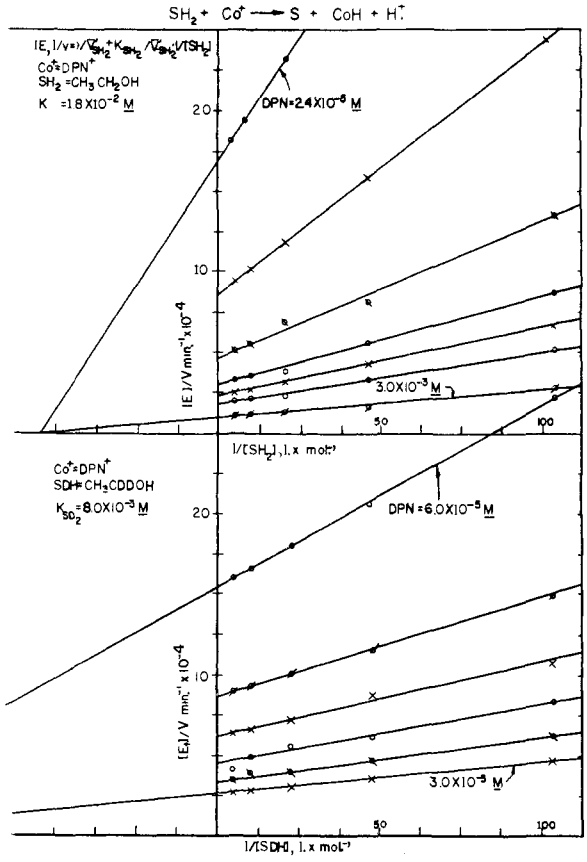


Fig. 1.—Variation of initial rate of ethanol oxidation with ethanol concentration. Top plots: substrate ethanol, varied as indicated; DPN^+ constant at concentrations in order: O, 2.4×10^{-5} *M*; ×, 5.0×10^{-5} *M*; ○, 1.0×10^{-4} *M*; ●, 2.5×10^{-4} *M*; ×, 5.0×10^{-4} *M*; O, 1.0×10^{-3} *M*; ○, 3.0×10^{-3} *M*. Bottom plots: substrate dideuteroethanol, varied as indicated; DPN^+ constant at concentrations in order: O, 6.0×10^{-5} *M*; ○, 1.2×10^{-4} *M*; ×, 2.4×10^{-4} *M*; O, 5×10^{-4} *M*; ○, 1.0×10^{-3} *M*; ×, 3.0×10^{-3} *M*. All other conditions as indicated in the text.

method of Lineweaver and Burk.[27] To obtain the data of Figs. 1 and 2 representative of reaction 1 in the direction of alcohol oxidation, initial rates were obtained at seven different DPNH concentrations varying from 3.0×10^{-3} to 2.4×10^{-5} *M* and five different alcohol concentrations between $1.92 \times$

(27) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

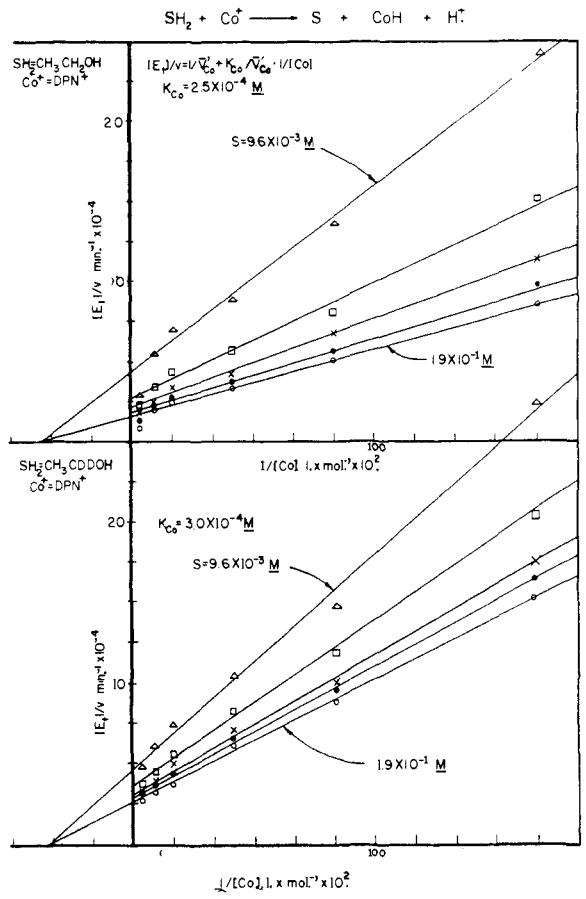


Fig. 2.—Variation of initial rate of ethanol oxidation, with DPN^+ concentration. Top plots: substrate DPN^+, varied as indicated; ethanol constant at concentrations in order: △, 9.6×10^{-3} *M*; □, 1.9×10^{-2} *M*; ×, 3.8×10^{-2} *M*; ●, 9.6×10^{-2} *M*; O, 1.9×10^{-1} *M*. Bottom plots: substrate DPN^+, varied as indicated; dideuteroethanol constant at concentrations in the same order as above. All other conditions are described in the text.

10^{-1} and 9.6×10^{-3} *M*, a total of 2×35 experiments. Similarly for the data of Figs. 3 and 4, which depict the situation obtaining on reduction of acetaldehyde by DPNH, the concentrations of the former were varied from 1.0×10^{-3} *M* to 5.0×10^{-5} *M* in five steps and those of the latter from 2.0×10^{-5} *M* to 2.5×10^{-6} *M* in four steps for a total of 2×20 experiments. In all cases the top plots are those for the protonated pairs, CH_3CH_2OH + DPN^+ and CH_3CHO + DPNH, respectively, while the bottom plots are for the deuterated pairs CH_3CD_2OH + DPN^+ and CH_3CHO + DPND, respectively.

It is evident from the data presented that in agreement with previous results obtained under different conditions[5] the initial rates for reaction 1 obey the relationship

$$v_f = \frac{\bar{V}'_{SH_2}(E)_0}{1 + K'_{SH_2}/(SH_2)} = \frac{\bar{V}'_{Co}(E)_0}{1 + K'_{Co}/(Co)} \quad (2)$$

$$v_r = \frac{\bar{V}'_{S}(E)_0}{1 + K'_{S}/(S)} = \frac{\bar{V}'_{CoH}(E)_0}{1 + K'_{CoH}/(CoH)} \quad (3)$$

where the quantities in parentheses refer to total concentrations of component added (in moles

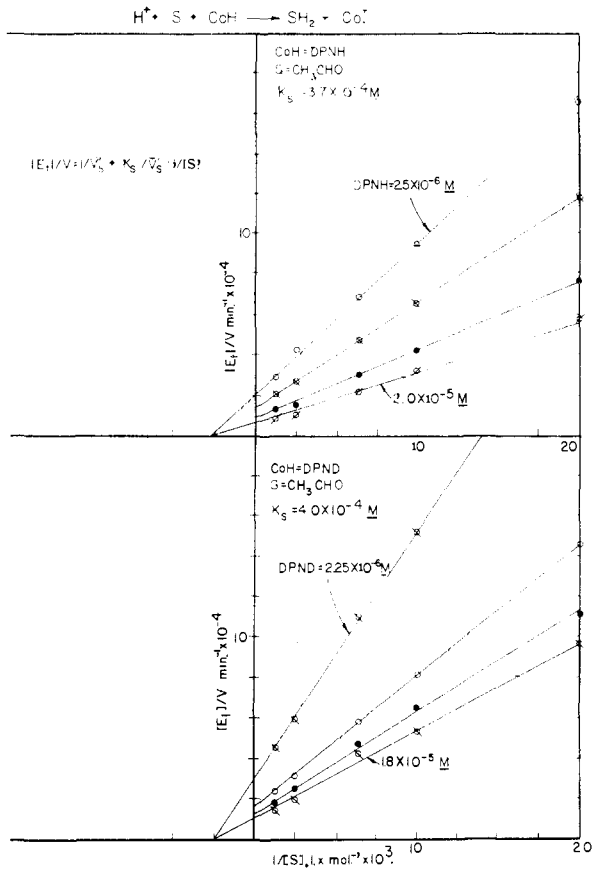


Fig. 3.—Variation of initial rate of acetaldehyde reduction with acetaldehyde concentration. Top plots: substrate acetaldehyde, varied as indicated, DPNH constant at concentrations in order: ○, 2.5×10^{-6} *M*; ◔, 5.0×10^{-6} *M*; ●, 1.0×10^{-5} *M*; ◴, 2.0×10^{-5} *M*. Bottom plots: substrate acetaldehyde, varied as indicated; DPND constant at concentrations in order: ◔, 2.25×10^{-6} *M*; ○, 4.5×10^{-6} *M*; ●, 9.0×10^{-6} *M*; ◴, 1.8×10^{-5} *M*. All other conditions are described in the text.

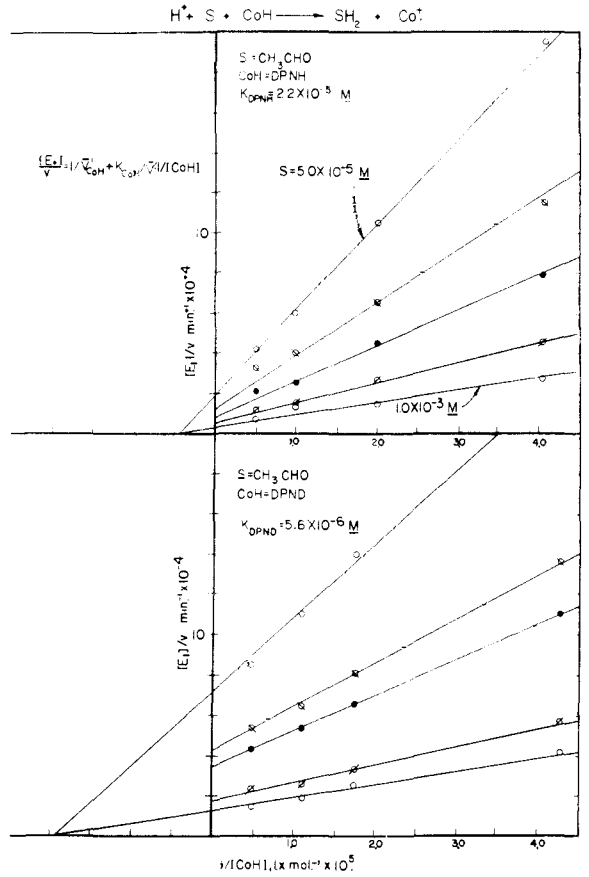


Fig. 4.—Variation of initial rate of acetaldehyde reduction with coenzyme concentration. Top plots: substrate DPNH, varied as indicated; acetaldehyde constant at concentrations in order: ○, 5.0×10^{-5} *M*; ◔, 1.0×10^{-4} *M*; ●, 2.5×10^{-4} *M*; ◴, 5.0×10^{-4} *M*; ○, 1.0×10^{-3} *M*. Bottom plots: substrate DPND, varied as indicated; acetaldehyde constant at concentrations described above. All other conditions are described in the text.

l.$^{-1}$), v_f and v_r are the observed initial steady state rates for the reaction in the forward and reverse direction, and the various $\bar{V}'_i$s and K'_is are experimental constants defining the straight line plots. Alberty has shown[17] that a variety of possible mechanisms for the reaction in question will give linear plots of this type, but that in general the K'_i's will be dependent on the concentration of the other component (*e.g.*, K'_{Co} determined from the reciprocal plot of v *vs.* (Co) will depend on the (SH_2) at which the experiment is performed). In the present case the K'_is are sensibly constant over the range tested as indicated by the constancy of the x-intercepts, and thus they are characteristic constants for the reactant in question under the experimental conditions employed and may be referred to as true K_i's or "Michaelis" constants, *i.e.*

$$K'_i = K_i \tag{4}$$

It also has been shown that for all reactions for which equations 2 and 3 hold the following relations also obtain[17]

$$\bar{V}'_{SH_2} = \frac{\bar{V}_f}{1 + K_{Co}/(Co)} \tag{5}$$

$$\bar{V}'_{Co} = \frac{\bar{V}_f}{1 + K_{SH_2}/(SH_2)} \tag{6}$$

$$\bar{V}'_S = \frac{\bar{V}_r}{1 + K_{CoH}/(CoH)} \tag{7}$$

$$\bar{V}'_{CoH} = \frac{\bar{V}_r}{1 + K_s/(S)} \tag{8}$$

Here the constants $\bar{V}_f$ (a rate constant) and K_{SH_2}, K_{Co} for the forward reaction and $\bar{V}_r$, K_S and K_{CoH} for the reverse are true constants characteristic of the reaction and may be called the turnover numbers in the two directions and the Michaelis constants for the four reactants. These relationships are tested in Fig. 5 for the forward and in Fig. 6 for the reverse reaction. It will be noted that (a) equations 5 and 6 require that the straight lines for $1/\bar{V}'_{SH_2}$ *vs.* 1/(Co) and $1/\bar{V}'_{Co}$ *vs.* 1/(SH_2) have the same y-intercept *viz.* $1/\bar{V}_f$, and that an analogus relation hold for $1/\bar{V}'_S$ *vs.* 1/(CoH) and $1/\bar{V}'_{CoH}$ *vs.* 1/(S); and (b) that the K_i's obtained from Figs. 1–4 and equations 2–4 are in satisfactory agreement with the K_i's derived from Figs. 5 and 6 and equations 5–8.

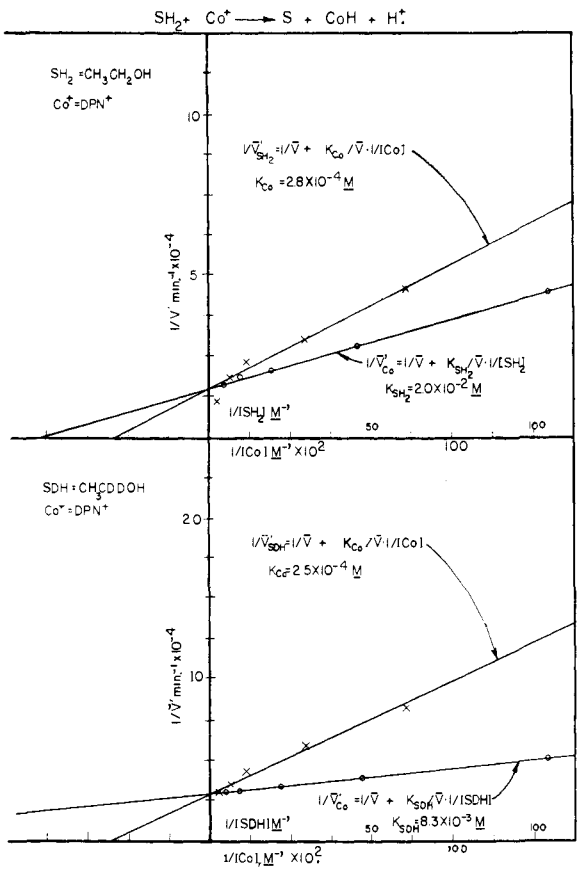


Fig. 5.—Determination of $\bar{V}_f$ and K_i. The data of Figs. 1 and 2 are plotted according to equations 5 and 6 and the constants determined; top plots for ethanol and DPN^+; bottom plots for dideuteroethanol and DPN^+.

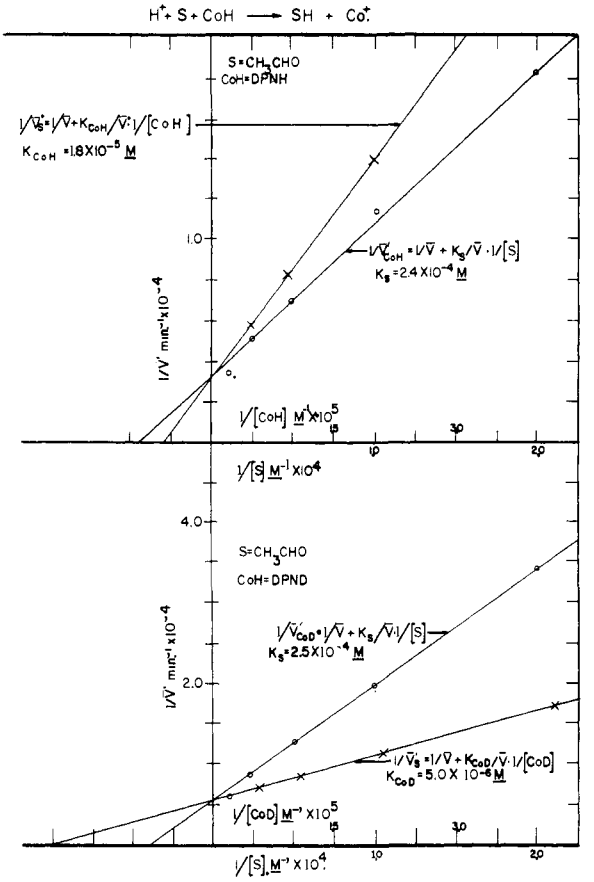


Fig. 6.—Determination of $\bar{V}_r$ and K_i. The data of Figs. 3 and 4 are plotted according to equations 7 and 8 and the constants determined. Top plots for acetaldehyde and DPNH; bottom plots for acetaldehyde and DPND.

The over-all rate laws for the forward and reverse direction are then

$$v_f/(E)_0 = \frac{\bar{V}_f}{(1 + K_{SH_2}/(SH_2))(1 + K_{Co}/(Co))} \quad (9)$$

$$v_r/(E)_0 = \frac{\bar{V}_r}{(1 + K_S/(S))(1 + K_{CoH}/(CoH))} \quad (10)$$

again in complete agreement with the results obtained by Hayes and Velick[5] for the forward reaction in pyrophosphate–glycine buffer at *p*H 7.9 and 26° and those of Negelein and Wulff[28] over a very narrow range of reactant concentrations under similar conditions.

Mechanism of Over-all Reaction.—On the basis of equilibrium and steady state analysis a mechanism consistent with the rate law observed has been derived[16,17,29] which postulates: no compulsory order of binding of reactants to the enzyme, oxidized and reduced form of each reactant are bound at the same site, but the extent of binding of substrate is uninfluenced by the presence of coenzyme at an adjacent site, and all steps are rapid and reversible except the actual transformation step (eq. 11 (e) below) as

$$E + SH_2 \overset{K_1}{\rightleftarrows} ESH_2 \qquad K_1 - K_4,\ K_6 - K_9 \text{ are dissocn. constants} \quad (11)(a)$$

$$E + Co^+ \overset{K_2}{\rightleftarrows} ECo \quad (b)$$

$$ECo + SH_2 \overset{K_3}{\rightleftarrows} E^{Co}_{SH_2} \qquad K_1 = K_3 = K_{SH_2} \quad (c)$$

$$ESH_2 + Co^+ \overset{K_4}{\rightleftarrows} E^{Co}_{SH_2} \qquad K_2 = K_4 = K_{Co} \quad (d)$$

$$E^{Co}_{SH_2} \underset{k_{5-}}{\overset{k_5}{\rightleftarrows}} E^{CoH}_S + H^+ \qquad \bar{V}_f = k_5;\ \bar{V}_r = k_{5-} \quad (e)[30]$$

$$E^{CoH}_S \overset{K_6}{\rightleftarrows} ES + Co^+ \qquad K_6 = K_8 = K_{CoH} \quad (f)$$

$$E^{CoH}_S \overset{K_7}{\rightleftarrows} ECoH + S \qquad K_7 = K_9 = K_S \quad (g)$$

$$ECoH \overset{K_8}{\rightleftarrows} E + CoH \quad (h)$$

$$ES \overset{K_9}{\rightleftarrows} E + S \quad (i)$$

(28) E. Negelein and H. J. Wulff, *Biochem. Z.*, **289**, 436 (1937).

(29) K. J. Laidler and I. M. Socquet, *J. Phys. and Colloid Chem.*, **54**, 530 (1950); G. W. Schwert and M. T. Hakkala, *Arch. Biochem. Biophys.*, **28**, 55 (1952).

(30) Dissociation of the proton from SH_2 may take place either in step (e) as indicated or in steps (a) and (c) leading to complexes of the type ESH and E^{SH}_{Co}, *i.e.*, those involving the conjugate base of the alcohol. Determination of the *p*H dependence of $\bar{V}_f$ and K_{SH_2} may lead to a means of distinguishing between the alternatives. It is even possible that dissociation does not take place until steps (g) or (i), *i.e.*, in that case the complexes would involve the conjugate acid of the carbonyl compound.

Effects of Deuterium Substitution.—The introduction of deuterium for hydrogen in CH_3CH_2OH to give CH_3CD_2OH (SH_2 and SDH) and in DPNH to yield DPND (CoH and CoD) leads to the results shown in the lower portions of Figs. 1-6 and summarized in Table I. It will be observed that isotope effects of approximately equal extent are observed for $\bar{V}_f = k_5$ and $\bar{V}_r = k_{5-}$, *i.e.*, the specific rate constants for the reaction (obtained by extrapolating the observed rate to infinite concentration of both reactants); and for K_{SH_2} and K_{CoH} constants involving the reactants containing deuterium, but *not* on K_S and K_{Co}, which are constants for reactants which do not.

TABLE I

CRYSTALLINE YEAST ALCOHOL DEHYDROGENASE
SUMMARY OF KINETIC CONSTANTS

1. Reduction of acetaldehyde by reduced DPN

	DPNH	DPND	$r = \frac{k \text{ or } K_H}{k \text{ or } K_I}$
K_S	3.0×10^{-4} *M*	3.2×10^{-4} *M*	0.97
K_{Co}	2.0×10^{-5} *M*	5.3×10^{-6} *M*	3.7
k_{-5}(per site)	8250	4500	1.8

2. Oxidation of ethanol by DPN^+

	CH_3CH_2OH	CH_3CD_2OH	
K_{SH_2}	1.9×10^{-2} *M*	8.2×10^{-3} *M*	2.3
K_{Co}	2.7×10^{-4} *M*	2.7×10^{-4} *M*	1.0
k_5(per site)	3650	2000	1.8

Tests for Suggested Mechanism.—In addition to the adherence to the rate law exemplified by equations 9 and 10, the proposed mechanism would also be expected to lead to the following consequences: (a) Independently determined dissociation constants should be numerically equal to the Michaelis constants: this has been demonstrated by Hayes and Velick[5] for DPN and DPNH (K_2 and K_8) by means of binding studies and for acetaldehyde (K_9) by equilibrium shift experiments. The present research adds further support to this contention because

(b) The deuterium isotope effect on the K'_i's shows the following characteristic features: (1) its magnitude is independent of (S) for K'_{CoH}/K'_{CoD} and independent of (Co) for K'_{SH_2}/K'_{SHD}; (2) it is equal to unity for K'_{Co} and K'_S and independent of the concentration of the deuterium-containing reactants (SD_2 and CoD). This observation is most easily explained by assuming that the only rate constants which in product, sum or ratio make up these K_i's are those exclusively for reactants involving deuterium when an isotope effect is observed and exclusively those not involving deuterium in the absence of an isotope effect. This means $K_{Co} = k_{-2}/k_2 = K_2$, $K_S = k_{-9}/k_9$, etc.

(c) The combination of positive isotope effects for $\bar{V}_f$ and $\bar{V}_r$, positive and constant isotope effect for K_{SH_2} and K_{CoH} regardless of the concentration of the other component, and absence of any effect of deuterium substitution in the reaction partner on K_{Co} and K_S can be shown by inspection[31] to be unique among the mechanisms described by Alberty for the general reaction here under consideration.[17] Thus these criteria provide an additional *necessary* (but by no means sufficient) condition for the proposed mechanism.

(d) Alberty has extended the Haldane relationship,[32] relating the over-all equilibrium constant for an enzyme-catalyzed reaction to the kinetic constants for this reaction, to include a variety of mechanisms for the enzyme–coenzyme–substrate case.[17] All mechanisms lead to equations of the form

$$K_{eq} = \frac{K_{CoH}K_S(H^+)}{K_{Co} \times K_{SH_2}}\left(\frac{\bar{V}_f}{\bar{V}_r}\right)^n = \frac{(S)(CoH)(H^+)}{(Co^+)(SH_2)} \quad (12)$$

where (H^+) is the total hydrogen ion concentration and n is dependent on the mechanism; for the one proposed $n = 1$. Table II shows a comparison of the data obtained in this investigation with some previously reported, for the various constants of equation 12.

TABLE II

CHARACTERISTIC CONSTANTS FOR THE ALCOHOL DEHYDROGENASE REACTION

	This investigation	Hayes and Velick[5]	Negelein and Wulff[28]	Theorell, *et al.*[3]		
*p*H	7.6	7.9	7.9	7.15	8.0	6.0
t, °C.	22	26	26	23	20	23
$K_{Co} \times 10^4$, *M*	2.7	1.7	0.9	23		1.6
$K_{CoH} \times 10^5$, *M*	2.0	2.3	3.0	1.0		0.43
$K_{CoD} \times 10^6$, *M*	0.53					
$K_S \times 10^4$ *M*	3.0	1.8	1.1	1.4		0.54
$K_{SH_2} \times 10^2$, *M*	1.9	1.3	2.4	7.4		10.0
$K_{SHD} \times 10^2$, *M*	0.82					
$V_f \times 10^{-3}$, min.$^{-1}$	3.65	6.7	9.3	0.92		0.57
$V_f \times 10^{-3}$, min.$^{-1}$[a]	2.00					
$V_r \times 10^{-3}$, min.$^{-1}$	8.25		15.6	7.36		5.57
$V_r \times 10^{-3}$, min.$^{-1}$[b]	4.50					
K_{eq}(exptl.) $\times 10^{11}$		1.3[d]	1.15	1.11	0.71	0.9
K_{eq}(eq. 12) $\times 10^{11}$	1.3	1.6	0.6	1.1		0.81
K_{eq}(eq. 12) for deuterated cpds. $\times 10^{11}$	0.85					

[a] With CH_3CD_2OH. [b] With DPND. [c] K_{eq} at *p*H 7.0 instead of 7.15, all K_{eq} at 20°; K_i's are calculated equilibrium constants. [d] See also Racker.[20] The lower rates observed in the present investigation are probably referable to a lower temperature and different reaction conditions with respect to buffer *p*H, nature and concentration.

Discussion

The Isotope Effects.—The observed isotope effects on the dissociation constants for the complexes of the enzyme with the reduced substrate (K_1 and K_3) and the reduced coenzyme (K_6 and K_8) are all positive, *i.e.*, $K_i^H/K_i^D > 1$. Thus the deuterated substrates are held more firmly by the enzyme than are their protonated counterparts. Although in general a greater number of investigations of deuterium isotope effects on rate rather than equilibrium constants have been reported,[18] in principle their interpretation should be quite similar.[33] The data then imply a smaller zero-point energy difference between the responsible carbon–hydrogen and carbon–deuterium bonds in the enzyme–

(31) A more rigorous proof of this statement will be given in a subsequent paper of this series.

(32) J. B. S. Haldane, "Enzymes," Longmans, Green and Co., London, 1930, pp. 80–82.

(33) In effect the ratio of equilibrium constants K_1^H/K_1^D is equal to the equilibrium constant for an isotope-exchange reaction of the type DPND + E-DPNH = DPNH + E-DPND. Reactions of the general nature XH + YD = XD + YH have been treated *in extenso* by Bigeleisen and Mayer (*J. Chem. Phys.*, **15**, 281 (1947)), who have shown that in the limit, at low temperatures, the free energy of the reaction can be accounted for by the zero point energy differences of reactants and products.

substrate complex than in the free substrate. Thus in going from the former to the latter, *i.e.*, in dissociation, the protonated compound gains relatively more zero-point energy than does the deuterated one. It would be premature to speculate on the implications of these findings with respect to the actual structures involved pending investigations with the individual enantiomorphic monodeutero DPN isomers rather than the chemically produced mixture of enantiomorphs used in the present study. The main conclusion that may be drawn from the latter is that there appears to be a strong, probably secondary,[34] isotope effect exerted by one or both the *p*-carbon–hydrogen bonds in the pyridine ring and the carbon–hydrogen bonds in the alcohol on the binding of these substrates by the enzyme.

An Elaboration of the Mechanism.—Any mechanism proposed must take into consideration observations madė in this and previous investigations bearing on the following points: (a) the probable mechanism for the over-all reactions catalyzed by the enzyme as indicated in equation 11 and discussed in an earlier section, (b) the direct and stereospecific transfer of hydrogen from donor to acceptor, (c) a possible association of Zn^{++} with enzymatically active sites; (d) an effect on the C–H bonds in ethanol and DPNH during the binding of these reactants by the enzyme and (e) the transition state in the interconversion of the two ternary complexes as intermediate in structure between the two, to explain the observed isotype effect on k_5 and k_{-5}.

The structures and conversions indicated in Scheme I would appear to fulfill, though by no means uniquely, these requirements. It will be observed that reactions 11(a), (d), (f) and (i), *i.e.*, those involving binding of the substrate prior to that of the coenzyme, have been omitted. This has been done solely to increase simplicity of representation. It is evident that with binding centered on the metal this attachment is indeed independent of the presence of the other reactant and may proceed in either sequence (substrate before coenzyme, or coenzyme before substrate). We have also omitted all implications of possible additional bonds between coenzymes or Zn^{++} and or substrates on the one hand, and enzyme–protein on the other, for lack of specific information. At least some of these additional interactions must of course take place to explain, for instance, stereospecificity and structural specificity with respect to the reactants. There is also evidence that –SH bonds on the enzyme protein may be of great importance in binding[35] and may possibly interact with the quaternary pyridinium nitrogen[4,8,36] present in DPN^+ and in the type of E–Zn–DPNH complex postulated here. It is also recognized that enzyme–coenzyme complexes essentially equivalent to the ones indicated may be postulated involving formulation of the metal–coenzyme bond through the amide oxygen (or N) rather than the *m*-carbon atom of the pyridine ring. This alternative will lead to an eight-membered ring (counting the H to be transferred) instead of the six-membered one of the proposed mechanism. The same type of electronic displacements are possible in that alternative and as indicated by molecular models the two carbons of donor and acceptor are spatially juxtaposed in a manner allowing direct transfer of the hydrogen between the two.[37] Thus no choice is possible between these two alternatives at the present time.

In either case the essence of the expanded mechanism is the postulation of ternary enzyme–substrate–coenzyme complexes centered around the Zn atom, allowing for an interaction of the C–H bonds with the positive centers, exemplified by Zn^{++} ion in the case of ethanol and the potential ouaternary nitrogen in the case of DPNH, and a reasonable and symmetrical means for the direct transfer of hydrogen itself.

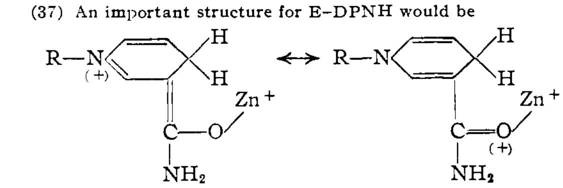
The similarity between this proposed path for the alcohol dehydrogenase reaction and that of certain organic reactions,[38] especially the Canizzaro

(34) V. J. Shiner, Jr., THIS JOURNAL, **74**, 5285 (1952); **75**, 2925 (1953).

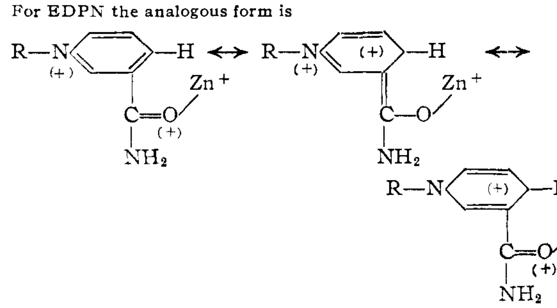
(35) See for instance the contributions by S. Velick, P. D. Boyer and E. Racker to "The Mechanism of Enzyme Action," edited by W. D. McElroy and B. Glass, The Johns Hopkins Press, Baltimore, Md., 1954.

(36) E. Racker, *Physiol. Rev.*, **35**, 1 (1955).

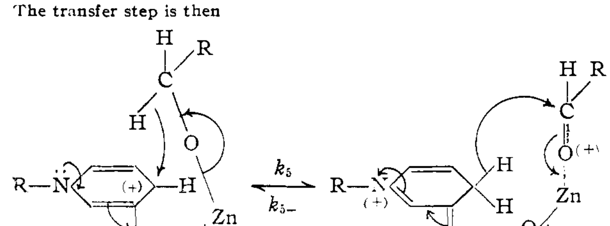
(37) An important structure for E-DPNH would be



For EDPN the analogous form is



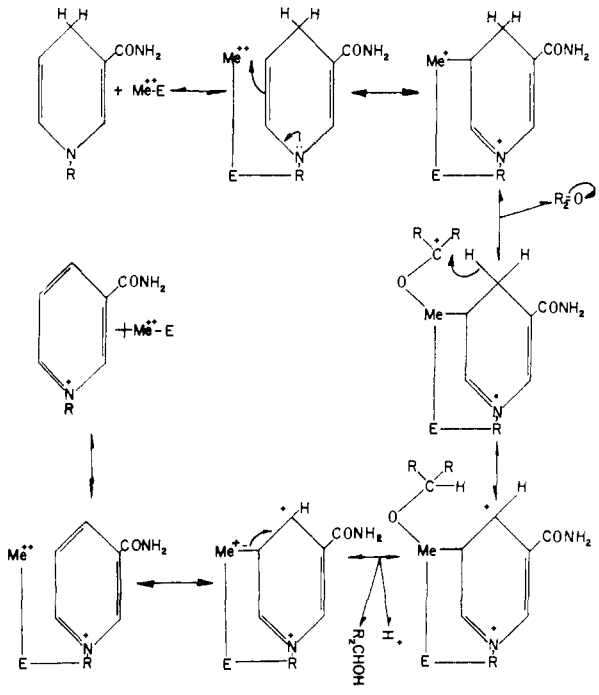
The transfer step is then



Possibly also the structure is Zn^+ –O(+)(H)←C–H instead of Zn^+ –O←C–H as indicated.

(38) See for instance the discussion of these reactions in L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940; J. Hine, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1955. A Meerwein-Pondorff type of mechanism for this reaction also has been suggested by K. Wallenfels and H. Z. Sund, *Angew. Chem.*, **67**, 517 (1955); we are indebted to one of the referees for bringing this note to our attention.

A MECHANISM FOR METALLO-PYRIDINOPROTEIN CATALYSIS



Scheme I.

and Tischchenko reactions and the Meerwein–Pondorff–Oppenauer equilibrium is rather striking. Scheme II indicates the commonly accepted mechanism for the latter reaction. It will be observed that the following similarities obtain: both reactions may be formulated as involving hydride ion transfers to carbonyl compounds; both require the participation of a strong, metallic Lewis acid. The metal in turn is linked to a base: alkoxide ion or enzyme. The transition state postulated is similar; the metal mediates but does not participate directly, in the sense of valence change, in the electronic displacements leading to the hydrogen transfer. The similarity is even more striking if we consider the great similarity in reactivity between pyridine nucleotides and carbonyl compounds, as evidenced by their interaction with CN^-, OH^-, HSO_3^- and other typical carbonyl agents.[39]

As far as additional evidence supporting the enzymatic mechanism is concerned not a great deal is known at the present time: the interaction of aldehydes with Zn^{++} appears reasonable in view of the known proclivity of —C=O to exist as $\overset{(+)}{\text{—C}}\text{—}\overset{(-)}{\text{O}}$; similarly the postulates of a complex between alcohol hydroxyl and the metal appears reasonable on theoretical grounds and is attested to by the known catalysis of alkyl halide formation from alcohols by salts of Zn^{++}.[40] No data are

MECHANISM OF MEERWEIN-PONDORFF-OPPENAUER REACTION

(WOODWARD, et al., J. Amer. Chem. Soc. 67, 1425 (1945))

Scheme II.

available concerning possible interaction of pyridine nucleotides or N-alkyl nicotinamides with metal ions.[41] Experiments bearing on this point and on the formal analogies alluded to above are now in progress.

Possible structures for some of the complexes here under consideration and for the transition state in the hydrogen transfer step have been described previously as involving addition of the substrate to the coenzyme in the *p*-position.[39,42] They are considered unlikely in the present case, for steric reasons, and for their inability to account for both the independent interaction of both reaction partners with the enzyme, and the possible interaction of the susceptible bond with some other center in the enzyme–reactant complex during the formation of this complex demonstrated here. A structure for the Zn^+–DPN^+ complex of liver alcohol dehydrogenase, involving the amide group, has been proposed by Theorell, *et al.*[4]

Finally it must be stressed that the results presented, and the admittedly speculative mechanism proposed, are valid at best only for one particular pyridine nucleotide-requiring dehydrogenase, catalyzing one particular reaction, under one particular set of experimental conditions.[43] Certain detailed features of the proposed mechanism may be fairly common, but its more general applicability *in toto* is considered highly unlikely in view of the fact, to mention but one example, that presently available kinetic evidence[4] and preliminary data on isotope effects on the same reaction (1a) but catalyzed by the mammalian enzyme, indicate a totally different over-all mechanism.

Acknowledgments.—We are indebted to Professor V. J. Shiner, Jr., for many helpful discussions and to Professor R. A. Alberty for a critical reading of the manuscript before submission.

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(39) For a review see N. O. Kaplan, *Rec. Chem. Progr.*, **16**, 177 (1955). A similar role for Zn is also mentioned by Vallee, *Disc. Faraday Soc.*, **20**, 263 (1955).

(40) The observed competitive (to alcohol) inhibition of this enzyme by hydroxylamine[9] may easily be explained by the present mechanism in terms of competitive binding of hydroxylamine and ethanol by Zn^{++}. Hydroxylamine is known to form Zn complexes of structures $Zn(ONH_2)_2$ and $Zn^+(ONH_2)$ (*cf.* J. W. Mellor, "A Comprehensive Treatise on Inorganic and Theoretical Chemistry," Vol. VIII, John Wiley and Sons, Inc., New York, N. Y., 1928, p. 288). It is significant that with the yeast enzyme hydroxylamine exerts its inhibitory action immediately, while with the liver enzyme which has been shown to obey a different mechanism preincubation with DPN^+ is necessary. A similar inhibition by *o*-phenantholine has recently been reported by Hoch and Vallee, *J. Biol. Chem.*, **221**, 491 (1956).

(41) But Vallee and Hoch[22] cite unpublished potentiometric data indicating formation of Zn^{++}–DPN^+ complexes.

(42) R. M. Burton and N. O. Kaplan, *J. Biol. Chem.*, **211**, 447 (1954).

(43) In a very recent study Nygaard and Theorell[3] concluded from precise kinetic measurements of the reaction catalyzed by this enzyme, in the *p*H range 6.0–7.15, at ionic strength 0.1 and in the presence of 0.001 *M* Versene that the formation of ternary complexes prior to the rate-limiting step is a likely mechanism. They found, however, that the various *K*'s were not independent of the concentration of the other reactant under these conditions.