Wang, C. C., and Shaw, E. (1972), *Arch. Biochem. Biophys. 150,* 259-268.
Weber, K., Pringle, J. R., and Osborn, M. (1972), *Methods Enzymol. 26,* 3-27.
Weiss, Y., and Gertler, A. (1976), *Isr. J. Med. Sci. 12,* 1342-1343.
Wilcox, P. E. (1970), *Methods Enzymol. 19,* 64-108.
Wold, F. (1969) *Anal. Biochem. 32,* 185-190.
Wrigley, C. W. (1968), *Sci. Tools 15,* 17-23.
Yphantis, D. A. (1964), *Biochemistry 3,* 297-317.

pH, Isotope, and Substituent Effects on the Interconversion of Aromatic Substrates Catalyzed by Hydroxybutyrimidylated Liver Alcohol Dehydrogenase†

Robert T. Dworschack‡ and Bryce V. Plapp*,§

ABSTRACT: The mechanism of hydrogen transfer catalyzed by horse liver alcohol dehydrogenase (EC 1.1.1.1) with amidinated amino groups was studied with steady-state kinetics. Hydroxybutyrimidylation of the enzyme increases the maximum velocities of the enzymatic reactions and the rates of dissociation of the enzyme-coenzyme complexes. Results of product inhibition studies with the modified and native enzymes acting on benzyl alcohol and benzaldehyde are consistent with an ordered mechanism. Primary deuterium isotope effects obtained for oxidation of pentadeuterioethanol (V_H/V_D = 3.1) and [1,1-D_2]benzyl alcohol (V_H/V_D = 3.6) and for reduction of a series of para-substituted benzaldehydes (V_H/V_D = 2.4) indicate that the turnover numbers reflect the rates of hydrogen transfer with hydroxybutyrimidylated enzyme. Isotope effects were not observed for native enzyme. The magnitudes and signs of the ρ values obtained for oxidation of para-substituted benzyl alcohols ($\rho^+ = -0.2$) and for reduction of a series of para-substituted benzaldehydes ($\rho^+ = 1.1$) catalyzed by hydroxybutyrimidylated enzyme suggest that interconversion of central complexes occurs via concerted hydride and proton transfer in which only a small amount of charge develops in the transition state. The rate of oxidation of benzyl alcohol depends upon a group with a pK of 8.4, which must be unprotonated for maximum activity but which allows partial activity in its protonated form. The rate of reduction of benzaldehyde is essentially independent of pH over the range from pH 6.0 to 9.9. The maximum velocity for benzaldehyde reduction catalyzed by native enzyme required protonation of a group with a pK of 8.0 but did not show an isotope effect, indicating that steps in the mechanism other than hydrogen transfer are rate limiting. The pH effects for both enzymes can be explained by a coherent model that is consistent with the structure of the enzyme as determined by x-ray crystallography. This model postulates that a water (or hydroxide) molecule coordinated to the zinc ion acts as a proton donor (or acceptor) and that the state of protonation of His-51 modulates the rate of transfer of hydrogen in the central complexes.

The work of Klinman (1972, 1975, 1976) on the mechanism of the interconversion of para-substituted benzyl alcohols and benzaldehydes catalyzed by yeast alcohol dehydrogenase (EC 1.1.1.1) has led to the conclusion that little or no charge develops on C-1 of the substrate during hydrogen transfer. Mechanisms involving either concerted general acid-base catalysis of a hydride transfer or a protonated radical intermediate and hydrogen atom transfer are consistent with the results. Similar studies with horse liver alcohol dehydrogenase have been more difficult and have led to differing conclusions. The main difficulty is that the chemical conversion of substrate to product catalyzed by the native liver enzyme cannot be observed using steady-state techniques, because transfer of hydrogen is not rate limiting for turnover. The rate-limiting step for the oxidation of ethanol or benzyl alcohol is dissociation of the enzyme-NADH complex (Dalziel, 1963; Wratten and Cleland, 1965), and for the reduction of benzaldehyde release of benzyl alcohol is limiting (McFarland and Bernhard, 1972). More recently, transient kinetic techniques have been used to study interconversion of ternary complexes of native enzyme. Large primary deuterium isotope effects were obtained, but the ρ values obtained from Hammett plots do not appear to be compatible with each other. Jacobs et al. (1974) found a small positive ρ value for reduction of some benzaldehydes, which led them to suggest that there was a highly positively charged transition state; from this result, a large negative ρ value for the oxidation of the benzyl alcohols is predicted. But Hardman et al. (1974) found a small negative ρ value for the alcohols and suggested that a hydride ion and a proton are transferred synchronously.

We now report effects of modification of amino groups of liver alcohol dehydrogenase on rate-limiting steps in reactions of various substrates and the applicability of modified enzymes for steady-state studies on the mechanism of hydrogen transfer. A deuterium isotope effect of 4.8 on steady-state turnover had been obtained previously for the oxidation of ethanol catalyzed by picolinimidylated enzyme (Plapp et al., 1973). Extending this approach to para-substituted benzyl alcohols and benzaldehydes, we have found that substituent effects with the liver enzyme are similar to those found for the yeast enzyme. Furthermore, we have integrated our results on the effects of pH

† From the Department of Biochemistry, The University of Iowa, Iowa City, Iowa 52242. *Received November 10, 1976.* This work was supported by Research Grant AA00279 from the National Institute on Alcohol Abuse and Alcoholism, United States Public Health Service.
‡ Supported by Training Grant GM550 from the National Institutes of Health. Present address: Department of Medicine, University of California, San Diego, La Jolla, California 92093.
§ Supported by Research Scientist Development Award (K2AA10) from the National Institute on Alcohol Abuse and Alcoholism.

on catalysis by modified enzyme with results of others to propose a new mechanism for liver alcohol dehydrogenase.

Experimental Procedure

Materials. Crystalline horse liver alcohol dehydrogenase (EE isozyme) was obtained as previously described (Dworschack and Plapp, 1977). NAD^+ (chromatographic grade I) was purchased from Boehringer-Mannheim and NADH (grade III) was purchased from Sigma. Acetone, acetaldehyde, cyclohexanone, benzyl alcohol, benzaldehyde, and *p*-dimethylaminobenzaldehyde were obtained from Fisher; *p*-chloro-, *p*-methyl-, *p*-methoxy-, and *p*-nitrobenzaldehydes were purchased from K & K Chemicals. *p*-Methyl- and *p*-methoxybenzaldehydes and benzaldehyde were distilled on the day of use. *p*-Dimethylaminobenzaldehyde was used without further purification. *p*-Nitro- and *p*-chlorobenzaldehydes were purified as bisulfite addition compounds according to Vogel (1964); *p*-chlorobenzaldehyde was also recrystallized from ethanol-water (1:1).

[1,1-D_2]Benzyl alcohol was prepared from $LiAlD_4$ (Ventron-Alfa Products) and benzoic acid according to the procedure of Mićović and Mihailovic (1953) and purified by distillation: bp 100 °C (15 mm). *p*-Nitrobenzyl alcohol was prepared by reduction of *p*-nitrobenzaldehyde with $NaBH_4$ and purified by extraction into diethyl ether and recrystallization from hot water. Both *p*-nitrobenzyl compounds absorbed light at 340 nm, $\epsilon = 510\ M^{-1}\ cm^{-1}$; the aldehyde absorbed maximally at 266 nm and the alcohol at 276 nm. *p*-Dimethylaminobenzyl alcohol was prepared by reduction of the aldehyde and obtained as a light yellow oil upon vacuum distillation: bp 106 °C (0.3 mm). *p*-Methoxybenzyl alcohol was prepared similarly: bp 96 °C (1 mm).

The sodium salt of reduced 4-deuterionicotinamide adenine dinucleotide (NADD) was prepared from NAD^+ and hexadeuterioethanol (International Chemical and Nuclear) according to the procedures of Rafter and Colowick (1957) and Dalziel (1962).

Enzyme Modification. Liver alcohol dehydrogenase was partially acetimidylated in the presence of NAD^+ and pyrazole, and the excess reagents were removed as described previously (Plapp et al., 1973). The amino groups at the active sites were modified by reaction with 100 mM 4-bromobutyramide (Fries et al., 1975) in 0.5 M triethanolamine hydrochloride buffer, pH 8.0, at 25 °C for 2 h. Assay (Plapp, 1970) of aliquots of the enzyme showed that 4-hydroxybutyrimidylation increased the activity 23-fold for the rate of oxidation of ethanol. Amino groups at the active sites of partially acetimidylated enzyme were also differentially alkylated with $NaBH_4$ and the appropriate keto compound (Means and Feeney, 1968; Zoltobrocki et al., 1974). The cyclohexyl enzyme was activated 7-fold.

Isotope, pH, and Substituent Effects. Deuterium isotope effects were determined for the oxidation of protio and deuterio alcohols with NAD^+ and reduction of aldehydes with NADH or NADD. For studies on substituent effects, the para-substituted benzaldehydes were dissolved in dioxane; the dioxane was diluted to less than 0.25% in the final assay mixture. For both studies the concentrations of coenzyme were fixed at saturating or near saturating levels, while the concentrations of substrates were varied. The observed rates were usually obtained by following the decrease in absorbance at 340 nm. However, *p*-dimethylaminobenzaldehyde strongly absorbs in that region and therefore the change in absorbance due to the formation of alcohol was observed at 281 nm, the isosbestic point for the spectra of NAD^+ and NADH. Molar extinction coefficients for the aldehyde and the alcohol were 1450 and 2570 $M^{-1}\ cm^{-1}$, respectively. The oxidation of *p*-dimethylaminobenzyl alcohol was followed by monitoring NADH formation with the Hitachi spectrofluorometer (excitation 340 nm, emission 460 nm). The initial velocities as a function of substrate concentration were fitted to the Michaelis-Menten equation (Cleland, 1967) to obtain values for the maximum velocity and the Michaelis constant. All values of maximum velocities were converted to apparent turnover numbers (i.e., these values were not corrected for saturation by the coenzyme) assuming a subunit molecular weight of 40 000 and specific activities (toward ethanol) of 2.4 and 48 units/mg protein for native and hydroxybutyrimidylated enzymes, respectively, in the standard assay (Plapp, 1970).

The same procedure was used to study the oxidation of benzyl alcohol and the reduction of benzaldehyde at 25 °C and several pH values from 6.0 to 9.9. The buffers used were all 0.1 μ sodium phosphate, containing 0.25 mM EDTA.[1] To provide adequate pH control, buffers at pH 9.0 and 9.9 contained 3.6 and 2.1 mM glycine, respectively (Dalziel, 1963). The pK values were obtained by fitting the rate constants with an iterative nonlinear least-squares program (NONLIN, C. M. Metzler, The Upjohn Co.) to equations derived for various mechanisms giving a pH dependence. The simplest mechanism giving a good fit to the data (coefficient of determination, $r^2 > 0.98$) was chosen. (The more complicated expressions were derived from the general scheme presented by Laidler and Bunting (1973).)

(a) Single ionizing group, activity only in protonated or unprotonated form.

$$HES \xrightarrow{K_a} ES \xrightarrow{k} E + P$$

$$k_{obsd} = k/(1 + [(H^+)/K_a]) \quad (1)$$

$$ES \xrightarrow{K_a} HES \xrightarrow{k} HE + P$$

$$k_{obsd} = k/(1 + [K_a/(H^+)]) \quad (2)$$

(b) Single ionizing group, activity at both extremes of pH.

$$\begin{array}{l} HES \xrightarrow{k_2} HE + P \\ \quad\uparrow K_a \\ ES \xrightarrow{k_3} E + P \end{array} \qquad k_{obsd} = \frac{k_2 + [k_3K_a/(H^+)]}{1 + [K_a/(H^+)]} \quad (3)$$

(c) Two ionizing groups, activity of two forms.

$$\begin{array}{l} H_2ES \xrightarrow{k_1} H_2E + P \qquad \text{if } k_1 = 0 \\ \quad\uparrow K_b \\ HES \xrightarrow{k_2} HE + P \\ \quad\uparrow K_a \\ ES \xrightarrow{k_3} E + P \qquad \text{if } k_3 = 0 \end{array}$$

$$k_{obsd} = \frac{k_2 + [k_3K_a/(H^+)]}{1 + [K_a/(H^+)] + [(H^+)/K_b]} \quad (4)$$

$$k_{obsd} = \frac{k_2 + [k_1(H^+)/K_b]}{1 + [K_a/(H^+)] + [(H^+)/K_b]} \quad (5)$$

[1] Abbreviation used: EDTA, ethylenediaminetetraacetic acid.

TABLE I: Survey of Isotope Effects for Native and Modified Enzymes.[a]

Enzyme	Ethanol	Acetaldehyde	Benzaldehyde
Native	1.2 ± 0.02	1.1 ± 0.03	1.1 ± 0.04[b]
Partially acetimidylated	1.3 ± 0.05	1.0 ± 0.01	1.2 ± 0.02
Acetimidylated	2.5 ± 0.1	1.4 ± 0.06	1.2 ± 0.05[b]
4-Hydroxybutyrimidylated	3.1 ± 0.2	1.5 ± 0.1	2.5 ± 0.2
Isopropylated	5.2 ± 0.2	1.1 ± 0.02	2.1 ± 0.05
Cyclohexylated	3.9 ± 0.1	2.3 ± 0.09	2.1 ± 0.06

[a] Assays were carried out at 25 °C in 33 mM sodium phosphate buffer (pH 8.0)–0.25 mM EDTA. Activity toward ethanol was determined with 2 mM NAD^+ and activity toward the other substrates was determined with 200 μM NADH. Values ± standard errors are given. [b] These values also obtained in 50 mM sodium pyrophosphate–HCl buffer, pH 8.75.

Results

Survey of Isotope Effects. Native and several modified forms of the enzyme were studied for the presence of primary deuterium isotope effects on catalytic turnover (Table I). Primary isotope effects were obtained for oxidation of ethanol and reduction of benzaldehyde by enzyme derivatives with amino groups at the active sites modified with three different substituents. Native and partially acetimidylated enzymes did not exhibit substantial isotope effects with any of the substrates tested. Based on this limited survey, the isotope effect appears to be larger when the size of the substituent on the amino groups is large. Thus, hydroxybutyrimidylated enzyme was chosen for more detailed studies.

Product Inhibition Studies. The product inhibition patterns for native enzyme acting on benzyl alcohol and benzaldehyde are classically consistent with the ordered Bi Bi mechanism; that is, NAD^+ and NADH are competitive inhibitors against one another, whereas the substrates are noncompetitive inhibitors against one another. In contrast, the hydroxybutyrimidylated enzyme is quantitatively different in that the substrates are competitive or almost so, against one another (Figures 1E, 1F). Such results would be expected if the mechanism of the modified enzyme was rapid equilibrium random (Cleland, 1963) or rapid equilibrium ordered with formation of dead-end enzyme–benzyl alcohol or enzyme–benzaldehyde complexes (Frieden, 1976). However, the noncompetitive pattern in Figure 1B and the uncompetitive pattern in Figure 1D eliminate these two simple mechanisms. Furthermore, it may be noted that the kinetic constants (K_m and K_i) for the coenzymes are not substantially altered by the second substrate; the constants obtained with the aromatic substrates in this study are similar to those obtained with ethanol and acetaldehyde (cf. Fries et al., 1975). If a random mechanism were involved, the kinetic constants for coenzymes should vary as the substrates are varied (Wratten and Cleland, 1965). We conclude tentatively that the simplest mechanism consistent with the data for the modified enzyme is the ordered Bi Bi mechanism.

On the assumption of an ordered mechanism, kinetic constants were calculated from the product inhibition studies. These are presented in Table II. Since the Haldane constants (Cleland, 1963) calculated from the kinetic constants (0.1–0.4 $\times 10^{-10}$ M) are comparable to the pH-independent equilibrium constants (0.23–0.50 $\times 10^{-10}$ M) determined by Luisi and Bignetti (1974), Bernhard et al. (1970) and Klinman (1972), it appears that the kinetic constants are reasonably accurate.

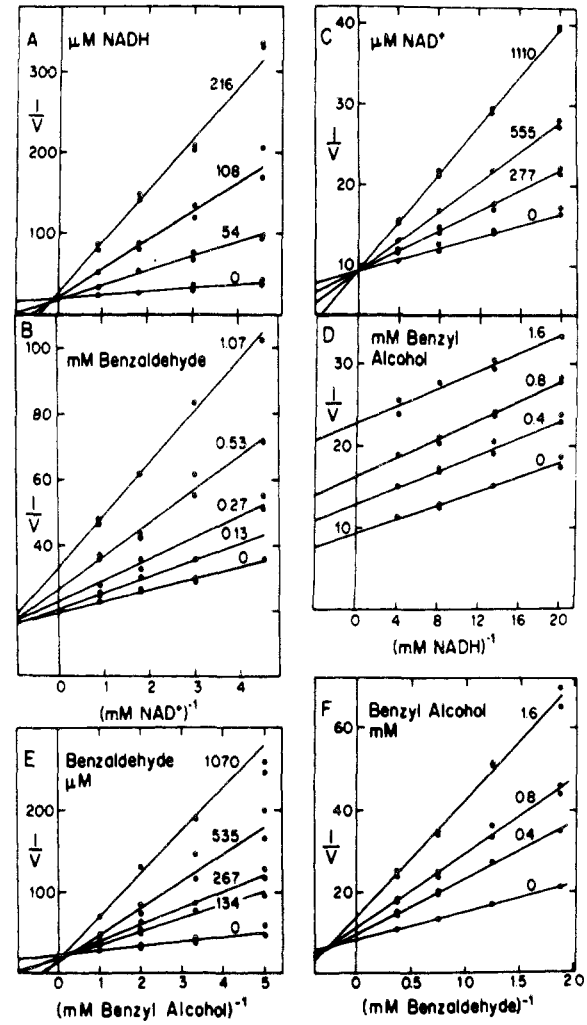


FIGURE 1: Product inhibition studies of hydroxybutyrimidylated alcohol dehydrogenase with benzyl alcohol and benzaldehyde. The experiments were performed and analyzed as described previously (Plapp, 1970); assays were performed on a Cary 118 spectrophotometer. The buffer was 33 mM sodium phosphate, pH 8.0, containing 0.25 mM EDTA. V has units of ΔA_{340} per min. The lines are computed fits to the equation for an hyperbola. (A, B) Inhibition by NADH or benzaldehyde against varied concentrations of NAD^+ at 2 mM benzyl alcohol; enzyme, 60 nN. (C, D) Inhibition by NAD^+ or benzyl alcohol against varied concentrations of NADH at 2.67 mM benzaldehyde; enzyme 4.4 nN. (E) Inhibition by benzaldehyde against varied concentrations of benzyl alcohol at 5.5 mM NAD^+; enzyme, 60 nN. (F) Inhibition by benzyl alcohol against varied concentrations of benzaldehyde at 216 μM NADH; enzyme, 4.4 nN.

Substituent Effects. If turnover with hydroxybutyrimidylated enzyme measures the rate of transfer of hydrogen in the ternary complexes, the mechanism of the transfer can be conveniently studied. The apparent Michaelis constants and turnover numbers obtained for oxidation of three para-substituted benzyl alcohols (with quite different σ^+ values) catalyzed by native and hydroxybutyrimidylated enzymes are presented in Table III. σ^+ is used, as opposed to the more general substituent constant σ, because σ^+ is adjusted for direct conjugative interactions between the para substituent and the reaction center (Hammett, 1970). The turnover numbers increase slightly as the para substituent becomes more electron donating. The value of ρ^+ is quite small (−0.2) for both modified and native enzymes.

The apparent Michaelis constants, turnover numbers, and

TABLE II: Kinetic Constants for Hydroxybutyrimidylated Enzyme Acting on Aromatic Substrates.[a]

Michaelis constants (μM)		Inhibition constants (μM)		Turnover numbers (s^{-1})	
K_a	170 ± 15	K_{ia}	360 ± 16	V_1/E_t	2.5 ± 0.1
K_b	300 ± 35	K_{ib}	1600 ± 150	V_2/E_t	80 ± 2
K_p	990 ± 50	K_{ip}	5100 ± 620		
K_q	62 ± 2	K_{iq}	15 ± 1		
		K_bK_{ia}/K_a	460 ± 32		
		K_pK_{iq}/K_q	300 ± 32		

[a] The values of the constants and their standard errors were obtained from computed fits (Cleland, 1967) to the points in Figure 1. The subscripts a, b, p, and q represent NAD^+, benzyl alcohol, benzaldehyde, and NADH, respectively. V_1 represents the maximum velocity in the reaction of NAD^+ and alcohol, and V_2 is for the reverse reaction. Activities were determined at 25 °C in 33 mM sodium phosphate buffer, pH 8.0, containing 0.25 mM EDTA.

TABLE III: Effect of the Substituent on the Apparent Michaelis Constants and Turnover Number for Native and 4-Hydroxybutyrimidylated Enzymes Acting on Para-Substituted Benzyl Alcohols.[a]

Substituent	σ^+	K_b (μM)	V_1/E_t (s^{-1})
		Native	
None (H)	0.0	30 ± 8	1.8 ± 0.1
Methoxy	−0.78	11 ± 1	2.8 ± 0.1
Dimethylamino	−1.7	3.8 ± 0.2	2.6 ± 0.1
		4-Hydroxybutyrimidylated	
None (H)	0.0	290 ± 11	3.0 ± 0.1
Methoxy	−0.78	200 ± 70	5.2 ± 0.7
Dimethylamino	−1.7	58 ± 7	5.8 ± 0.2

[a] Assays were carried out in 33 mM sodium phosphate buffer, pH 8.0, 0.25 mM EDTA, at 25 °C. NAD^+ concentrations were 5 mM and the alcohol concentrations were as follows: *p*-dimethylamino, 50–500 μM with modified enzyme and 10–100 μM with native enzyme; *p*-methoxy, 0.1–1 mM with modified enzyme and 5–50 μM with native enzyme; benzaldehyde, 100–500 μM with both enzymes. Values ± standard errors are given.

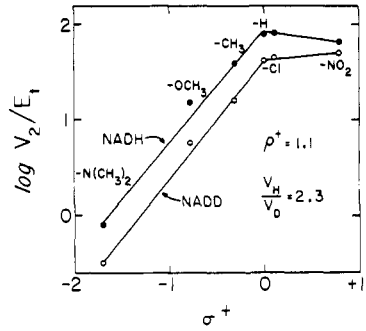


FIGURE 2: Hammett plots of the rates of reduction of para-substituted benzaldehydes catalyzed by 4-hydroxybutyrimidylated enzyme. Assay conditions are listed in Table V. The data were fitted by a least-squares program: reduction with NADH (●); reduction with NADD (○).

isotope effects obtained from the reduction of six para-substituted benzaldehydes are presented in Tables IV and V. For the series from benzaldehyde to *p*-dimethylaminobenzaldehyde, the substituents cause only slight changes in the Michaelis constants for native enzyme, whereas a larger effect is observed for the Michaelis constants for modified enzyme. ρ^+ for the reciprocal of the Michaelis constants for modified enzyme is −0.8 ± 0.1, a value which is similar to that obtained by Hansch et al. (1973) for the binding of a series of para-substituted benzamides to native enzyme and to the value obtained by Klinman (1972) for the binding of benzaldehydes to yeast alcohol dehydrogenase. Since there was no systematic isotope effect on the apparent Michaelis constants, they may actually be dissociation constants. Then we may conclude from the sign and magnitude of ρ^+ that an acidic group on the enzyme slightly polarizes the carbonyl group on the substrate, as suggested previously for yeast alcohol dehydrogenase (Klinman, 1972).

The isotope effect on turnover number observed with native enzyme is constant at 1.1, whereas the isotope effects observed with modified enzyme are larger, but decrease with *p*-chloro- and *p*-nitrobenzaldehydes. The Hammett plots for turnover with NADD and NADH by modified enzyme are illustrated in Figure 2. For the substrates *p*-dimethylaminobenzaldehyde to benzaldehyde ρ^+(H) is 1.14 ± 0.09 and ρ^+(D) is 1.19 ± 0.05. The insensitivity of the isotope effect of 2.3 ± 0.1 to variations in the para substituent is consistent with a hydride transfer mechanism (Swain et al., 1961; see also Klinman, 1972). (A linear Hammett relationship was not obtained for the reduction of the para-substituted benzaldehydes catalyzed by native enzyme; this observation agrees with the results of Blomquist (1966) and precludes an analysis of the substituent effects.) The rates of reduction of *p*-chloro- and *p*-nitrobenzaldehydes catalyzed by modified enzyme are lower than those for benzaldehyde; the nonequivalent decrease accounts for the decreasing isotope effects with these substituents. We cannot explain the anomaly at this time, except to note that it may be caused by a shift in the rate-limiting step. Biphasic Hammett plots are not unusual and have been observed in other systems: aldehyde dehydrogenase (Dietrich et al., 1962), thiaminase (Mazrimas et al., 1963), L-amino acid oxidase (Radda, 1964), chymotrypsin (Caplow and Jencks, 1962), Schiff's base formation with butylamine (Santerre et al., 1958), and semicarbazone formation (Noyce et al., 1958).

pH Effects. The turnover numbers, isotope effects, and Michaelis constants obtained for the oxidation of benzyl alcohol catalyzed by native and hydroxybutyrimidylated enzymes from pH 6.0 to pH 9.9 are listed in Tables VI and VII. With native enzyme an isotope effect of 1.5 was observed; this is apparently due to an isotope effect on the release of NADD from the enzyme (Bush et al., 1973). There was only a threefold increase in the turnover numbers and a slight decrease in the Michaelis constants for benzyl alcohol with increasing pH. The Michaelis constants for modified enzyme did not show a dependence on pH, whereas there was a substantial pH dependence for turnover (Table VII). Isotope effects for reduction of benzaldehyde catalyzed by native and hydroxybutyrimidylated enzymes were constant over the range of pH studied: native, 1.0 ± 0.1; and hydroxybutyrimidylated, 2.4 ± 0.1 (Table VIII). A reasonably large dependence of V_2/E_t on pH

TABLE IV: Effect of the Substituent on the Apparent Michaelis Constants, Turnover Numbers, and Isotope Effects for Native Enzyme Acting on Para-Substituted Benzaldehydes.[a]

Substituent	σ^+	K_p^H (μM)	K_p^D (μM)	V_2/E_t^H (s^{-1})	V_2/E_t^D (s^{-1})	V^H/V^D
Nitro	0.79	3.8 ± 0.7	3.2 ± 0.6	3.9 ± 0.2	3.7 ± 0.1	1.1 ± 0.1
Chloro	0.11	11 ± 2	23 ± 4	7.9 ± 0.6	7.7 ± 0.6	1.0 ± 0.1
None (H)	0.0	43 ± 7	53 ± 5	18 ± 1	18 ± 1	1.0 ± 0.1
Methyl	−0.31	21 ± 7	50 ± 8	15 ± 1	14 ± 1	1.1 ± 0.1
Methoxy	−0.78	28 ± 7	34 ± 5	8.5 ± 0.2	7.9 ± 0.2	1.1 ± 0.1
Dimethylamino	−1.7	25 ± 10	24 ± 10	0.099 ± 0.020	0.092 ± 0.022	1.1 ± 0.3

[a] Assays were carried out in 33 mM sodium phosphate buffer, pH 8.0, 0.25 mM EDTA. NADH and NADD concentrations were 200 μM and the benzaldehyde concentrations were as follows: *p*-nitro and *p*-dimethylamino, 40–200 μM; *p*-chloro, 100–500 μM; *p*-H, *p*-methyl, and *p*-methoxy, 0.4–2 mM. Values and their standard errors are given.

TABLE V: Effect of the Substituent on the Apparent Michaelis Constants, Turnover Numbers, and Isotope Effects for 4-Hydroxybutyrimidylated Enzyme Acting on Para-Substituted Benzaldehydes.[a]

Substituted	σ^+	K_p^H (μM)	K_p^D (μM)	V_2/E_t^H (s^{-1})	V_2/E_t^D (s^{-1})	V^H/V^D
Nitro	0.79	47 ± 6	67 ± 9	65 ± 3	50 ± 2	1.3 ± 0.1
Chloro	0.11	140 ± 12	140 ± 12	80 ± 3	45 ± 1	1.8 ± 0.1
None (H)	0.0	860 ± 100	440 ± 26	98 ± 5	42 ± 1	2.3 ± 0.1
Methyl	−0.31	720 ± 49	710 ± 55	38 ± 1	16 ± 1	2.4 ± 0.2
Methoxy	−0.78	450 ± 25	450 ± 24	15 ± 1	5.7 ± 0.2	2.6 ± 0.2
Dimethylamino	−1.7	78 ± 13	48 ± 11	0.80 ± 0.10	0.32 ± 0.03	2.5 ± 0.4

[a] Assay conditions are the same as those listed in Table IV. Values and their standard errors are given.

TABLE VI: Apparent Turnover Numbers, Isotope Effects, and Michaelis Constants for the Oxidation of Benzyl Alcohol Catalyzed by Native Enzyme from pH 6.0 to pH 9.9.[a]

pH	V_1/E_t^H	V_1/E_t^D	V^H/V^D	K_b^H	K_b^D
6.0	0.67 ± 0.02	0.48 ± 0.01	1.4 ± 0.1	51 ± 8	70 ± 14
6.5	0.84 ± 0.06			51 ± 7	
7.0	1.3 ± 0.1	0.88 ± 0.05	1.5 ± 0.1	67 ± 11	55 ± 14
7.5	1.6 ± 0.1			23 ± 3	
8.0	1.9 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	30 ± 8	36 ± 6
8.4	2.0 ± 0.1			23 ± 2	
8.7	2.1 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	9 ± 3	10 ± 4
9.0	2.1 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	19 ± 5	11 ± 4
9.9	2.4 ± 0.1			36 ± 5	

[a] Enzyme activities were determined with variable concentrations of benzyl alcohol (100 to 500 μM) and 5 mM NAD^+. The turnover numbers have units of s^{-1}; the Michaelis constants are μM. Values ± standard error are given.

for native enzyme was observed, but the modified enzyme showed only a small decrease in activity at high pH. The Michaelis constants toward benzaldehyde with both enzymes did not vary with pH: K_p^H = 90 ± 24 μM and K_p^D = 130 ± 25 μM; hydroxybutyrimidylated enzyme, K_p^H = 890 ± 100 μM and K_p^D = 780 ± 160 μM.

The data in Tables VI–VIII were used to calculate the apparent pK values for the enzyme substrate complexes and the limiting velocities for their breakdown to form products; some values are presented in Table IX.

Discussion

Enzyme Mechanism and Rate-Limiting Steps. Although the kinetic mechanisms of both native and hydroxybutyrimidylated enzymes are qualitatively similar (consistent with ordered Bi Bi), the rates of the rate-limiting steps are changed by the hydroxybutyrimidylation. Previous studies have shown that modification of the amino groups of the enzyme (in particular of Lys-228, which is at the active site) increases the activity of the enzyme by increasing the rates of dissociation of the enzyme–coenzyme complexes, (Plapp, 1970; Plapp et al., 1973; Sogin and Plapp, 1975; Dworschack et al., 1975). Similar conclusions can be drawn from the analysis of the kinetic constants in Table II as compared with native enzyme.

The most important evidence on the rate-limiting steps with hydroxybutyrimidylated enzyme are the isotope effects observed in both the forward and reverse reactions (Tables V, VII, and VIII). The magnitudes of the isotope effects indicate that transfer of hydrogen in the ternary complexes is at least partially rate limiting in the steady-state turnover. Although the isotope effects are not large, they are the same as those obtained from transient kinetic studies with the liver enzyme (Hardman et al., 1974; McFarland and Bernhard, 1972; Jacobs et al., 1974) and almost as large as those obtained from steady-state studies with the yeast enzyme (Klinman, 1972, 1975).

TABLE VII: Apparent Turnover Numbers, Isotope Effects, and Michaelis Constants for the Oxidation of Benzyl Alcohol Catalyzed by 4-Hydroxybutyrimidylated Enzyme from pH 6.0 to pH 9.9.[a]

pH	V_1/E_t^H	V_1/E_t^D	V^H/V^D	K_b^H	K_b^D
6.0	0.32 ± 0.01	0.13 ± 0.01	2.5 ± 0.1	71 ± 10	64 ± 6
6.5	0.54 ± 0.03	0.24 ± 0.01	2.2 ± 0.1	75 ± 14	88 ± 10
7.0	0.71 ± 0.02	0.33 ± 0.04	2.2 ± 0.3	29 ± 8	18 ± 4
7.5	1.4 ± 0.1	0.57 ± 0.02	2.5 ± 0.1	120 ± 14	51 ± 7
8.0	2.4 ± 0.1	0.70 ± 0.01	3.6 ± 0.2	190 ± 11	45 ± 5
8.4	3.6 ± 0.2	1.0 ± 0.1	3.5 ± 0.2	200 ± 22	82 ± 13
9.0	5.2 ± 0.2	1.4 ± 0.1	3.7 ± 0.2	150 ± 14	67 ± 6
9.9	7.0 ± 0.2	2.1 ± 0.1	3.4 ± 0.1	100 ± 6	61 ± 7

[a] Enzyme activities were determined with variable concentrations of benzyl alcohol (100 to 500 μM) and 5 mM NAD^+. The turnover numbers have units of s^{-1}; the Michaelis constants are μM. Values ± standard error are given.

TABLE VIII: Apparent Turnover Numbers and Isotope Effects for the Reduction of Benzaldehyde Catalyzed by Native and 4-Hydroxybutyrimidylated Enzymes from pH 6.0 to pH 9.9.[a]

	Native			4-Hydroxybutyrimidylated		
pH	V_2/E_t^H	V_2/E_t^D	V^H/V^D	V_2/E_t^H	V_2/E_t^D	V^H/V^D
6.0	30 ± 0.1	36 ± 1	0.9 ± 0.1	74 ± 3	30 ± 2	2.4 ± 0.2
6.5	37 ± 0.1			91 ± 11	36 ± 6	2.3 ± 0.3
7.0	35 ± 0.1	33 ± 1	1.1 ± 0.1	89 ± 4	35 ± 1	2.5 ± 0.1
7.5	29 ± 0.1	36 ± 1	0.8 ± 0.1	106 ± 5	44 ± 2	2.4 ± 0.1
8.0	18 ± 0.1	16 ± 1	1.1 ± 0.1	81 ± 6	30 ± 1	2.7 ± 0.2
8.5	12 ± 0.1	11 ± 1	1.1 ± 0.1	90 ± 3	42 ± 1	2.2 ± 0.1
9.0	6.9 ± 0.1	6.9 ± 0.1	1.0 ± 0.1	54 ± 1	23 ± 1	2.3 ± 0.1
9.9	4.3 ± 0.1	3.8 ± 0.1	1.1 ± 0.1	46 ± 1	20 ± 1	2.4 ± 0.1

[a] Enzyme activities were determined with variable concentrations of benzaldehyde (0.4 to 2.0 mM) and 200 μM NADH. The turnover numbers have units of s^{-1}. Values ± standard error are given.

Given that transfer of hydrogen is at least partially rate limiting, the mechanism of hydroxybutyrimidylated enzyme acting on benzyl alcohol may not be a compulsory order, but rather a preferred order, mechanism, as was found previously for the native enzyme acting on secondary alcohols (Dalziel and Dickinson, 1966; Dalziel, 1975). If we attempt to calculate the rate constant for the binding of NAD^+ to modified enzyme from the kinetic constants obtained with ethanol as substrate (Fries et al., 1975) or with benzyl alcohol as substrate (Table II), the values differ by about 15-fold. For the compulsory ordered mechanism, the values should be the same, but for the preferred order mechanism, the values could differ since the rates of binding of NAD^+ to the enzyme-alcohol complex could differ. Thus, the mechanism of hydroxybutyrimidylated enzyme may be partially random, which, of course, would still be consistent with the product inhibition results obtained in this study. Further studies are required to provide quantitative estimates of the extent of randomness of the mechanism and the magnitudes of the rate constants for each step. In the following discussion it should be noted that we have assumed that transfer of hydrogen is predominantly rate limiting in catalysis.

Interpretation of Substituent Effects. Hardman et al. (1974) and we have found small, negative ρ values ($\rho^+ = -0.2$ to -0.4) for the oxidation of substituted benzyl alcohols. Furthermore, Blackwell and Hardman (1975) found a value of ρ^* of -1.8 for a series of different primary alcohols. These ρ values imply that only a small amount of positive charge develops on the alcohol in the transition state, and this is consistent with the results and conclusion for yeast alcohol dehydrogenase. (See Klinman, 1975, 1976, for a full discussion of the interpretation of isotope and substituent effects.) Likewise, for the reduction of benzaldehydes, the ρ value we find ($\rho^+ = 1.1$) approaches the value for the yeast enzyme ($\rho^+ = 2.2$; Klinman, 1972), and both of these values are consistent with the development of little or no net negative charge in the transition state (Klinman, 1972). In contrast to our results with the liver enzyme, Jacobs et al. (1974) found a smaller substituent effect for the reduction of substituted benzaldehydes under pre-steady-state conditions; we have no explanation for this discrepancy. All of the substituent effects discussed here were obtained under conditions where significant deuterium

TABLE IX: Summary of pK Values and Limiting Maximum Velocities.[a]

Enzyme	Substrate	pK	Limiting V/E_t (s^{-1})		Eq
			High pH	Low pH	
Amidinated	$PhCH_2OH$	8.4 ± 0.1	6.8 ± 0.4	0.24 ± 0.01	3
Amidinated	$PhCD_2OH$	8.6 ± 0.2	2.2 ± 0.2	0.12 ± 0.02	3
Native[b]	$PhCH_2OH$	7.3 ± 0.1	2.2 ± 0.1	0.64 ± 0.02	3
Amidinated	PhCHO	9.8 ± 0.2		81 ± 6	2
Native[b]	PhCHO	8.0 ± 0.1	4.2 ± 0.2	37 ± 2	3

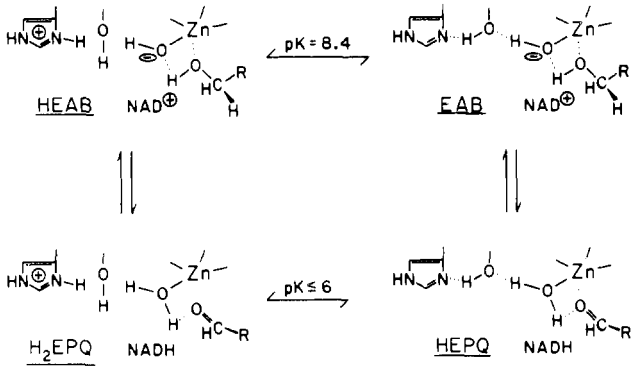
[a] Selected data in Tables VI–VIII were fitted to the simplest possible equation. (See Experimental Procedure.) Values ± standard deviation are reported. [b] Note that substantial isotope effects on turnover rates were not observed with native enzyme.

isotope effects indicated that the transfer of hydrogen was being studied.

It has been suggested that the small ρ values observed for the alcohol dehydrogenases are consistent with mechanisms in which the transfer of a hydride ion from one substrate to the other is coupled with the transfer of a proton mediated by a group on the enzyme acting as an acid-base catalyst (Hardman et al., 1974; Blackwell and Hardman, 1975; Klinman, 1975, 1976). Such a mechanism is discussed in detail below.

Mechanism of Hydrogen Transfer and Explanation of pH Effects. Based mostly on x-ray crystallographic evidence but supported by some significant chemical and kinetic results, we propose that the partial structure EAB shown in Scheme I can

Scheme I



represent the active ternary complex just before hydrogen is transferred from an alcohol to the coenzyme. The structure incorporates the hydrogen bonded system of the hydroxyl group of Ser-48 and the imidazole group of His-51 in the mechanism proposed by Brändén et al. (1975), but differs in that the alcohol is protonated (i.e., not an alcoholate) and that hydroxide ion is interposed between the alcohol and the serine hydroxyl group.

There are several reasons for the modification of the mechanism. Mechanistically, it is simpler and more reasonable to leave water on the zinc than to displace the water with the alcoholate. Displacing the water adds an extra step to the mechanism, which could involve the loss of water from the pentacoordinate structure shown in Scheme I and require rearrangement of the ligands about the zinc, and which could be relatively slow if it required conformational alterations of the protein. Thermodynamically, formation of an alcoholate would require several kilocalories (perhaps 9 kcal) of binding energy and this certainly would not facilitate formation of the active complex. Experimentally, Sloan et al. (1975) detected the presence of protons of water bound to Co^{II}, which was substituted for Zn^{II} in the enzyme, and showed that binding of coenzyme and a substrate analogue immobilized some of the protons. Finally, as discussed below, placement of a water molecule as shown in Scheme I facilitates the explanation of the observed pH dependencies.

As shown in Scheme I, the zinc ion has five ligands. (The ligands furnished by the protein to the zinc ion are the sulfur atoms of Cys-46 and Cys-174 and an imidazole nitrogen from His-67.) This is consistent with the evidence from x-ray crystallography that *o*-phenanthroline binds to the zinc ion, displaces the water, and forms a pentacoordinate complex (Brändén et al., 1975). Direct coordination of the alcohol (or aldehyde) to the zinc is supported by spectroscopic and kinetic evidence on the reactions of a chromophoric aldehyde with the enzyme (Dunn and Hutchinson, 1973; Dunn et al., 1975). On the other hand, Sloan et al. (1975) proposed that the alcohol formed a second sphere complex in which the water molecule is interposed between the alcohol and the metal ion. However, their measurements of the distance from the metal to the alcohol are somewhat uncertain because substitution of cobalt for the "rapidly exchanging" zinc atoms does not occur predominantly at the active sites (Sytkowski and Vallee, 1976; Harvey and Barry, 1976). The uncertainty about the direct, inner sphere coordination of the alcohol oxygen to the zinc is indicated by the dotted line in Scheme I, although we think that most of the evidence favors direct coordination. (See the review by Dunn (1975) for a valuable discussion of the properties of zinc complexes and their possible catalytic functions.)

Our discussion of the pH dependencies of reactions of alcohol dehydrogenase will begin with a review of relevant previous work. From the pH dependency for the binding of NAD^+ to the native liver enzyme, it may be concluded that the free enzyme has a group with a pK of about 9.0 (8.75 determined by Taniguchi et al. (1967); 9.0–9.6 estimated by Dalziel (1963)). In the enzyme–NAD^+ complex, the pK of the group has shifted to about 7.5 (6.85, Taniguchi et al. (1967); 8.0 estimated by Dalziel (1963)). (When Dalziel's data are fitted to the appropriate equation by a nonlinear least-squares procedure, pK values of 8.5 ± 0.1 for free enzyme and 6.9 ± 0.1 for the enzyme–NAD^+ complex can be calculated (Sogin, 1974).) From the pH dependence for the binding of trifluoroethanol (an inert substrate analogue) or capric acid to the enzyme–NAD^+ complex, Shore et al. (1974) determined that the enzyme–NAD^+ complex had a pK of 7.6. They also found that, when both NAD^+ and trifluoroethanol were bound to the enzyme in the pH range 5.5 to 8.5, exactly one proton was liberated per equivalent of enzyme. They proposed that the *alcohol was bound directly to the unprotonated, or basic, form of the group* that has the pK of 7.6 in the enzyme–NAD^+ complex. It follows from thermodynamic considerations of the binding data that the pK of that group in the enzyme–NAD^+–alcohol complex must be less than 4.5. This means, in other words, that the hydrogen on the hydroxyl group of the alcohol competitively displaces the proton bound to the enzyme at neutral pH. Taniguchi et al. (1967) and Shore et al. (1974) suggest that the group could be water bound to the zinc, as illustrated by EAB in Scheme I. It is then apparent that the hydroxide ion can act as the base to remove the proton from ethanol during the transfer of hydride ion from the ethanol to NAD^+. Moreover, it follows that, during reduction of an aldehyde, the protonated group, (i.e., H_2O) could act as an acid to donate a proton to the substrate, as shown by HEPQ.

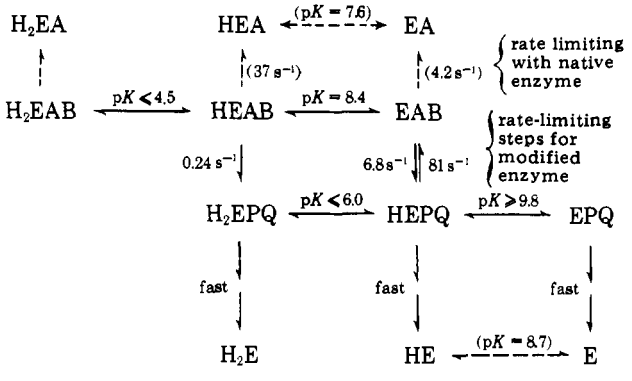
In support of this proposal is the important observation of Dunn (1974) that, after the enzyme–NADH–aldehyde complex is formed, there is no uptake of protons during the pre-steady-state reduction, but one proton is taken up for each molecule of product NAD^+ that is displaced by NADH (at pH 8.8). This result implies that a group on the enzyme–NADH–aldehyde complex has a pK above 8.8 (and is protonated at pH 8.8), whereas the group in the resulting enzyme–NAD^+–alcohol complex has a pK below 8.8 (and is unprotonated at pH 8.8). Therefore, it appears certain that there is a group on the enzyme that acts as a proton donor or acceptor, in a manner exactly analogous to the imidazole group of His-195 in lactate dehydrogenase (Holbrook et al., 1975). It is important to emphasize that the state of protonation of this group is coupled to the *binding* of substrates or substrate analogues (so that dissociation constants show pH dependencies), but that once the complex is formed it is not likely that the group changes its state of protonation over the pH range of 4.5 to 9 (so that maximum velocities could be pH indepen-

dent). From the x-ray crystallographic evidence, the water bound to the zinc ion is most likely to be the group. (Of course, in a hydrogen-bonded, "proton-relay" system, the observed pK corresponds to the pK of the overall system; mechanistically, it is simpler to assume that the proton is released from the water.)

If EAB in Scheme I represents the structure of a central, ternary complex poised for reaction, how can we explain the effects of pH on the maximum velocities of hydrogen transfer? For instance, how can the maximum velocity of benzyl alcohol oxidation catalyzed by hydroxybutyrimidylated enzyme require the unprotonated form of a group with a pK of about 8.4, when it appears that the binding of NAD^+ and alcohol already has caused deprotonation (even at pH 6) of a group at the active site? Based on the crystallographic evidence, it is possible that the state of protonation of the imidazole group of His-51 controls activity. Protonation of the imidazole would disrupt the hydrogen-bonding system (see HEAB in Scheme I) and should thereby decrease the rate of hydrogen transfer. (Protonation could also weaken the binding of the alcohol to the enzyme.) By analogy, the imidazole group should change its state of protonation in the reaction $H_2EPQ \rightleftharpoons HEPQ$, but based on evidence discussed below, we think the pK is less than 6. It may be noted that in the HEPQ complex the water molecule on the zinc is protonated, making the system neutral overall and decreasing its pK relative to the hydrogen-bonded system in the EAB complex which has a net negative charge overall.

Now we should explain in more detail the pH effects on the maximum velocities of the reactions with benzyl alcohol and benzaldehyde catalyzed by native and hydroxybutyrimidylated enzyme. We propose the mechanism in Scheme II for interpreting the facts in Table IX and some other information discussed above. The letters A, B, P, and Q represent NAD^+, benzyl alcohol, benzaldehyde, and NADH, respectively. The solid lines represent reactions determined with hydroxybutyrimidylated enzyme and the dashed lines (and numbers in parentheses) represent native enzyme.

Scheme II



An important aspect of Scheme II is that it postulates two pathways for the conversion of enzyme-NAD^+-alcohol complex to enzyme-NADH-aldehyde complex or to enzyme-NAD^+ complex. The dual pathways are required to fit the observed pH dependencies, which show limiting velocities at both extremes of pH studied. In other words, both the protonated and unprotonated forms of enzyme are catalytically active.

For the oxidation of benzyl alcohol catalyzed by hydroxybutyrimidylated enzyme, the enzyme is about 28 times more active at the limits of high pH than at low pH. McFarland and Chu (1975) have studied the transient rate of oxidation of benzyl alcohol by native enzyme. Although they found an isotope effect of 3.3, they observed only a 3.6-fold increase in oxidation rate with pH increasing from 5.9 to 7.5. This result fits the concept of dual pathways, even though the relative rate constants differ.

Brooks et al. (1972) have studied the transient oxidation of ethanol by native enzyme. In contrast to the results with benzyl alcohol, the maximum rate required the unprotonated form of a group with a pK of 6.4, and there was very little activity at low pH. The steady-state rate of oxidation of ethanol catalyzed by picolinimidylated enzyme showed an isotope effect of about 4.8 (Plapp et al., 1973), a pK of 8.0 ± 0.2, and little activity at low pH (Sogin, 1974). Thus, the oxidations of ethanol and benzyl alcohol seem to differ in the extent of activity observed at low pH. (It may also be noted that the rates for benzyl alcohol oxidation are about one-tenth the rates for ethanol oxidation.)

For the steady-state reduction of benzaldehyde by hydroxybutyrimidylated enzyme, there was only a weak pH dependence. This result is similar to that found by McFarland and Chu (1975) for the transient rate of reduction of β-naphthaldehyde by native enzyme. The pH independence only allows us to set upper and lower limits for the pK values of groups on the enzyme-NADH-aldehyde complex (Scheme II).

The steady-state reduction of benzaldehyde by native enzyme does not show an isotope effect, but, remarkably, does show dependence on a group with a pK of 8.0 (Table IX). Since McFarland and Bernhard (1972) have demonstrated that turnover for the native enzyme is largely controlled by the rate of dissociation of the enzyme-NAD^+-alcohol complex, it is reasonable to assume that the pK of 8.0 corresponds to the ionization of the enzyme-NAD^+-alcohol complex. Furthermore, it appears that the alcohol dissociates more rapidly from the protonated form, and this is consistent with the pH dependencies for the apparent K_m for benzyl alcohol determined from transient kinetics and for steady-state turnover of β-naphthaldehyde with native enzyme (McFarland and Chu, 1975). (The pH dependencies found by McFarland and Chu can be fitted to eq 5, but the data do not go to low enough pH to define the lower pK well.)

Thus, there is evidence from four different studies that there is (at least) one group in the enzyme-NAD^+-alcohol complex with a pK in the range from 6.4 to 8.4 that affects the maximum velocities of the enzymatic reactions. We have proposed that this group could be the imidazole of His-51, but we should point out that other ionizable groups in the active site, or pH-dependent conformational changes could be responsible for the pH effects. Indeed, Shore et al. (1974) suggested that the pK of 6.4 seen in the transient oxidation of ethanol could result from an isomerization step.

Further qualifications of our interpretations should be mentioned. We have assumed that the substantial isotope effects observed with hydroxybutyrimidylated enzyme indicate that transfer of hydrogen is at least partially rate limiting in the overall mechanism. But it is difficult to prove and moreover unlikely, that there is only one rate-limiting step. If, in fact, another pH-independent step were partially limiting hydrogen transfer, then the observed pK of 8.4 would be lower than the true pK (Cleland, 1970). For example, a pK of 7.3 (Table IX) for the steady-state oxidation of benzyl alcohol by native enzyme, which shows only a small isotope effect, is lower than the pK of 8.4 for the reaction catalyzed by hydroxybutyrimidylated enzyme, which shows a substantial isotope effect. A second qualification is that we used a chemically modified enzyme for our work. Although the hydroxybutyrimidyl sub-

stituent retains the net positive charge of the amino group below pH 10, it is possible that the modification distorts the protein structure. After all, the modified enzyme has markedly increased activity! Even so, the location of the amino group of Lys-228 in the protein structure makes it unlikely that the pK values of groups directly involved in the mechanism would be altered greatly, unless the changes in pK values were coupled to changes in the protein conformation (Brändén et al., 1975). On the other hand, we certainly could expect the modified enzyme to exhibit first-order rate constants for the reactions in Scheme II that differ from those given by native enzyme. It is interesting, then, that the maximum rate constants obtained with modified enzyme are only about one-fourth of those found for transient rates with native enzyme. We think that the modification interferes somewhat with the catalytic step, as observed previously with picolinimidylated enzyme acting on ethanol (Plapp et al., 1973). Finally, it should be pointed out that the data obtained in this study and previously do not demand that a water molecule be present during hydrogen transfer. Other mechanisms can explain the data. Results from more definitive investigations, such as x-ray crystallography are required to substantiate the present proposal.

Any discussion of a liver alcohol dehydrogenase should also consider knowledge of the homologous yeast alcohol dehydrogenase (Eklund et al., 1976). Klinman (1975) found that activity (V_{max}) of the native yeast enzyme on *p*-methylbenzyl alcohol required that a group with a pK of 8.25 be unprotonated for maximum activity. This result is consistent with our proposals. On the other hand, she found that the pH dependence for acetaldehyde reduction (V_{max}/K_m) required the group with the pK of 8.25 to be protonated. This result is quite different from the results with liver enzyme acting on aromatic aldehydes and points up the need to study other substrates with both enzymes. Nevertheless, Klinman's interpretation of these results in terms of concerted proton transfer is consistent with the mechanism detailed in Schemes I and II.

Acknowledgments

The technical assistance of David P. Bohlken, discussions on pH effects with David C. Sogin, and discussions with Judith P. Klinman were greatly appreciated.

References

Bernhard, S. A., Dunn, M. F., Luisi, P. L., and Schack, P. (1970), *Biochemistry 9,* 185–192.

Blackwell, L. F., and Hardman, M. J. (1975), *Eur. J. Biochem. 55,* 611–615.

Blomquist, C. H. (1966), *Acta Chem. Scand. 20,* 1747–1757.

Brändén, C.-I., Jörnvall, H., Eklund, H., and Furugren, B. (1975), *Enzymes, 3rd Ed. 11,* 104–190.

Brooks, R. L., Shore, J. D., and Gutfreund, H. (1972), *J. Biol. Chem. 247,* 2382–2383.

Bush, K., Shiner, V. J., Jr., and Mahler, H. R. (1973), *Biochemistry 12,* 4802–4805.

Caplow, M., and Jencks, W. P. (1962), *Biochemistry 1,* 883–893.

Cleland, W. W. (1963), *Biochim. Biophys. Acta 67,* 104–137.

Cleland, W. W. (1967), *Adv. Enzymol. 29,* 1–32.

Cleland, W. W. (1970), *Enzymes, 3rd Ed. 2,* 1–65.

Dalziel, K. (1962), *Biochem. J. 84,* 240–244.

Dalziel, K. (1963), *J. Biol. Chem. 238,* 2850–2858.

Dalziel, K. (1975), *Enzymes, 3rd Ed. 11,* 1–60.

Dalziel, K., and Dickinson, F. M. (1966), *Biochem. J. 100,* 491–500.

Dietrich, R. A., Hellerman, L., and Wein, J. (1962), *J. Biol. Chem. 237,* 560–564.

Dunn, M. F. (1974), *Biochemistry 13,* 1146–1151.

Dunn, M. F. (1975), *Struct. Bonding 23,* 61–122.

Dunn, M. F., Biellmann, J.-F., and Branlant, G. (1975), *Biochemistry 14,* 3176–3182.

Dunn, M. F., and Hutchinson, J. S. (1973), *Biochemistry 12,* 4882–4892.

Dworschack, R. T., and Plapp, B. V. (1977), *Biochemistry 16,* 111–116.

Dworschack, R. T., Tarr, G., and Plapp, B. V. (1975), *Biochemistry 14,* 200–203.

Eklund, H., Brändén, C.-I., and Jörnvall, H. (1976), *J. Mol. Biol. 102,* 61–73.

Frieden, C. (1976), *Biochem. Biophys. Res. Commun. 68,* 914–917.

Fries, R. W., Bohlken, D. P., Blakley, R. T., and Plapp, B. V. (1975), *Biochemistry 14,* 5233–5238.

Hammett, L. P. (1970), Physical Organic Chemistry, New York, McGraw-Hill, pp 360–362.

Hansch, C., Kim, K. H., and Sarma, R. H. (1973), *J. Am. Chem. Soc. 95,* 6447–6449.

Hardman, M. J., Blackwell, L. F., Boswell, C. R., and Buckley, P. D. (1974), *Eur. J. Biochem. 50,* 113–118.

Harvey, R. A., and Barry, A. (1976), *Biochem. Biophys. Res. Commun. 72,* 886–892.

Holbrook, J. J., Liljas, A., Steindel, S. J., and Rossmann, M. G. (1975), *Enzymes, 3rd Ed. 11,* 191–292.

Jacobs, J. W., McFarland, J. T., Wainer, I., Jeanmaier, D., Ham, C., Hamm, K., Wnuk, M., and Lam, M. (1974), *Biochemistry 13,* 60–64.

Klinman, J. P. (1972), *J. Biol. Chem. 247,* 7977–7987.

Klinman, J. P. (1975), *J. Biol. Chem. 250,* 2569–2573.

Klinman, J. P. (1976), *Biochemistry 15,* 2018–2026.

Laidler, K. J., and Bunting, P. S. (1973), The Chemical Kinetics of Enzyme Action, Oxford, Clarendon Press, pp 149–151.

Luisi, P. L., and Bignetti, E. (1974), *J. Mol. Biol. 88,* 653–670.

Mazrimas, J. A., Song, P.-S., Ingraham, I. O., and Draper, R. D. (1963), *Arch. Biochem. Biophys. 100,* 409–413.

McFarland, J. T., and Bernhard, S. (1972), *Biochemistry 11,* 1486–1493.

McFarland, J. T., and Chu, Y.-H. (1975), *Biochemistry 14,* 1140–1146.

Means, G. E., and Feeney, R. E. (1968), *Biochemistry 7,* 2192–2201.

Mićović, V. M., and Mihailovic, M. L. J. (1953), *J. Org. Chem. 18,* 1190–2000.

Noyce, D. S., Tottini, A. T., and Smith, S. G. (1958), *J. Org. Chem. 23,* 752–755.

Plapp, B. V. (1970), *J. Biol. Chem. 245,* 1727–1735.

Plapp, B. V., Brooks, R. L., and Shore, J. D. (1973), *J. Biol. Chem. 248,* 3470–3475.

Radda, G. K. (1964), *Nature (London) 203,* 936–937.

Rafter, G., and Colowick, S. (1957), *Methods Enzymol. 3,* 887–888.

Santerre, G. M., Hansrote, C. L., Jr., and Crowell, T. I. (1958), *J. Am. Chem. Soc. 80,* 1254–1257.

Shore, J. D., Gutfreund, H., Brooks, R. L., Santiago, D., and Santiago, P. (1974), *Biochemistry 13,* 4185–4190.

Sloan, D. L., Young, J. M., and Mildvan, A. S. (1975), *Biochemistry 14,* 1998–2008.

Sogin, D. C. (1974), Ph.D. Thesis, University of Iowa, Iowa City, Iowa.
Sogin, D. C., and Plapp, B. V. (1975), *J. Biol. Chem. 250,* 205-210.
Swain, C., Wiles, R., and Bader, R. (1961), *J. Am. Chem. Soc. 83,* 1945-1955.
Sytkowski, A. J., and Vallee, B. L. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 344-348.
Taniguchi, S., Theorell, H., and Åkeson, Å. (1967), *Acta Chem. Scand. 21,* 1903-1920.
Vogel, A. I. (1964), Practical Organic Chemistry, London, Longmans Co., pp 342-343.
Wratten, C. C., and Cleland, W. W. (1965), *Biochemistry 4,* 2442-2451.
Zoltobrocki, M., Kim, J. C., and Plapp, B. V. (1974), *Biochemistry 13,* 899-903.

Redox Properties of Microsomal Reduced Nicotinamide Adenine Dinucleotide-Cytochrome b_5 Reductase and Cytochrome b_5†

Takashi Iyanagi

ABSTRACT: Hepatic NADH-cytochrome b_5 reductase was reduced by 1 mol of dithionite or NADH per mol of enzyme-bound FAD, without forming a stable semiquinone or intermediate during the titrations. However, the addition of NAD^+ to the partially reduced enzyme or illumination in the presence of both NAD^+ and EDTA yielded a new intermediate. The intermediate had an absorption band at 375 nm and the optical spectrum resembled anionic semiquinones seen on reduction of other flavin enzymes. Electron paramagnetic resonance measurements confirmed the free-radical nature of the species. To explain the results, a disproportionation reaction between the oxidized and reduced NAD^+ complexes (E-FAD-NAD^+ + E-$FADH_2$-NAD^+ $\rightleftharpoons$ 2E-FADH·-NAD^+) is assumed. Potentiometric titration of NADH-cytochrome b_5 reductase at pH 7.0 with dithionite gave a midpoint potential of −258 mV; titration with NADH gave −160 mV. This difference may be due to a difference in the relative affinity of NAD^+ for the reduced and oxidized forms of the enzyme. The effects of pH on the midpoint potential of the NAD^+-free enzyme were very similar to those which have been measured with free FAD. At pH 7.0, midpoint potentials of trypsin-solubilized and detergent-solubilized cytochrome b_5 were 13 and 0 mV, respectively.

Reduced nicotinamide adenine dinucleotide-cytochrome b_5 reductase (EC 1.6.2.2) of hepatic microsomes is a flavoprotein containing 1 mol of FAD per mol of enzyme (Strittmatter and Velick, 1957). It is an electron transfer component involved in the biosynthesis of monoenoic fatty acids (Oshino et al., 1971; Enoch et al., 1976) and ethanolamine plasmalogen (Fritz et al., 1974).

The solubilized enzyme from membrane catalyzes one-electron transfer from NADH[1] to various electron acceptors such as cytochrome b_5, ferricyanide (Strittmatter and Velick, 1957), and quinones (Iyanagi and Yamazaki, 1969). The reaction mechanism has been studied by Strittmatter (1965), who concluded from spectrophotometric and kinetic data that it involves successive one-electron oxidations of reduced enzyme to which NAD^+ is bound, and that the rate of the second phase of reoxidation of cytochrome b_5 reductase is dependent on the pyridine nucleotide substituent. This suggests that a flavin semiquinone is involved as a catalytic intermediate. The intermediate observed during reoxidation of NAD^+-free reduced enzyme by ferricyanide showed the spectral characteristics of a blue (neutral) semiquinone (Strittmatter, 1965), but no EPR studies have yet been reported. Strittmatter (1965) also observed that interaction between a semiquinone intermediate and the pyridine nucleotide affects the reactivity of the flavin with electron acceptors. The redox potential of NADH-cytochrome b_5 reductase has been reported by Iyanagi et al. (1974), but has not been analyzed in terms of the interaction between oxidized or reduced enzyme and the pyridine nucleotide. In order to understand the electron-transfer mechanisms from a two-electron donor, NADH, to a one-electron acceptor, cytochrome b_5, it is important to study the redox properties of NAD^+-bound or -free enzyme. In the present paper the oxidation-reduction properties of purified NADH-cytochrome b_5 reductase and cytochrome b_5 are reported.

Materials and Methods

Enzyme Preparation. NADH-cytochrome b_5 reductase was prepared from pig livers according to the method of Takesue and Omura (1970) with some modification (Iyanagi and Yamazaki, 1969). The concentration of enzyme was determined spectrophotometrically by the use of the extinction coefficient of 10.2 mM^{-1} cm^{-1} at 460 nm (Strittmatter and Velick, 1957). Trypsin-solubilized cytochrome b_5 was prepared from rabbit microsomes by the method of Omura and Takesue (1970). Detergent-solubilized cytochrome b_5 was prepared from rabbit microsomes by a method described elsewhere

† From the Division of Biochemistry, Tsukuba University School of Medicine, Ibaraki, 300-31, Japan. *Received December 13, 1976.* A preliminary report of part of this work has been presented at the 1975 annual meeting of the Japanese Biochemical Society.

[1] Abbreviations used are: EPR, electron paramagnetic resonance; N, the number of electrochemical equivalents involved in any given oxidation-reduction process (Clark, 1960); I, ionic strength; NAD^+ and NADH, oxidized and reduced nicotinamide adenine dinucleotide; E-FAD, enzyme-bound flavin adenine dinucleotide (NADH-cytochrome b_5 reductase), oxidized form; E-FADH·, semiquinone form; E-$FADH_2$, reduced form; EDTA, ethylenediaminetetraacetic acid.