Effect of pH on the Liver Alcohol Dehydrogenase Reaction†

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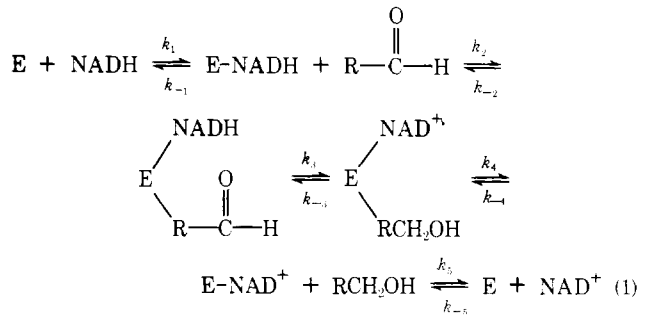
ABSTRACT: New transient kinetic methods, which allow kinetics to be carried out under conditions of excess substrate, have been employed to investigate the kinetics of hydride transfer from NADH to aromatic aldehydes and from aromatic alcohols to NAD^+ as a function of pH. The hydride transfer rate from 4-deuterio-NADH to β-naphthaldehyde is nearly pH independent from pH 6.0 to pH 9.9; the isotope effect is also pH independent with $k_H/k_D \simeq 2.3$. Likewise, the rate of oxidation of benzyl alcohol by NAD^+ changes little with pH between pH 8.75 and pH 5.9; the isotope effect for this process is between 3.0 and 4.4. Earlier substituent effect studies on the reduction of aromatic aldehydes were consistent with electrophilic catalysis by either zinc or a protonic acid. The pH independence of hydride transfer is consistent with electrophilic catalysis by zinc since such catalysis by protonic acid (with a pK between 6.0 and 10.0) would show strong pH dependence. However, protonic acid catalysis cannot be excluded if the pK_a of the acid catalyst in the ternary NADH-E-RCOH complex were <6.0 or >10.0. The two kinetic parameters changing significantly with pH are the kinetic binding constant for ternary complex formation with aromatic alcohol and the rate of dissociation of aromatic alcohols from enzyme. This is consistent with base-catalyzed removal of a proton from alcohol substrates and consequent acid catalysis of protonation of a zinc–alcoholate complex. The equilibrium constant for hydride transfer from benzaldehyde to benzyl alcohol at pH 8.75 is $K_{eq} = k_H/k_{-H} = 42$; this constant has important consequences concerning subunit interactions during liver alcohol dehydrogenase catalysis.

The role of the catalytically active groups at the active site of liver alcohol dehydrogenase has been under vigorous discussion recently. The enzyme catalyzes the following reaction by a compulsary ordered mechanism (eq 1) (Theorell and Chance, 1951; Wratten and Cleland, 1963). There are two zinc atoms per monomer. One of these is easily removed from the enzyme by dialysis; the resulting apozinc enzyme formed is catalytically inactive. The second zinc atom seems to serve a structural role and is less easily removed from the enzyme (Drum et al., 1969). It is also clear that at least one proton is released from the protein during formation of alcohol–NAD^+ ternary complex (Shore et al., 1974); the overall equation describing catalysis also contains a proton as follows:



$$E + NADH \rightleftharpoons E\text{-}NADH + R{-}C(=O){-}H \rightleftharpoons$$

$$E(NADH)(O{=}C{-}R{-}H) \rightleftharpoons E(NAD^+)(RCH_2OH) \rightleftharpoons E\text{-}NAD^+ + RCH_2OH \rightleftharpoons E + NAD^+ \quad (1)$$

$$R{-}C(=O){-}H + NADH + H^+ \rightleftharpoons RCH_2OH + NAD^+ \quad (2)$$

Recent studies indicate that 1 equiv of proton is taken up during aldehyde reduction. Base catalysis by a group with pK_a = 6.5 has also been observed for hydride transfer during ethanol oxidation (Brooks et al., 1972). The unusually small substituent effect during reduction of aromatic aldehydes is consistent with electrophilic catalysis of hydride transfer; however, such catalysis could result from protonation of substrate by a protonic acid–base catalyst or of complexation by zinc (Jacobs et al., 1974).

The role of zinc in formation of binary and ternary complexes has been under intense investigation. Observation of a large red shift during formation of a slowly reacting ternary complex is consistent with formation of a covalent aldehyde–zinc bond; this would support the role of zinc as the electrophilic catalyst for hydride transfer (Dunn and Hutchinson, 1973). Furthermore, an inactive binary complex formed with a chromophoric aldehyde substrate of liver alcohol dehydrogenase shows a similar large red shift which is also observed in the zinc complex of this chromophoric substrate (McFarland et al., 1974). Laser-Raman investigation of a chromophoric-inhibitor bound to the coenzyme binding site of liver alcohol dehydrogenase indicates zinc complexation of the inhibitor (McFarland et al., 1975). All these oservations are consistent with the presence of zinc at the active site and indicate that a zinc atom may act as the electrophilic catalyst; the recent X-ray structure of liver alcohol dehydrogenase shows that zinc is located near the coenzyme binding site in the crystalline enzyme (Brändén et al., 1973). However, this zinc atom is not necessary for coenzyme binding, since apo zinc enzyme binds coenzymes tightly (Iweibo and Weiner, 1972).

One recent experiment indicates that, at least in the cobalt enzyme, the metal atom is not located at the active site; fluorescence quenching experiments locate the "catalytic" cobalt atom at least 18 Å from the coenzyme binding site (Takahisi and Harvey, 1973).

Two step kinetic behavior has been interpreted as consistent with a subunit interaction mechanism for liver alcohol dehydrogenase (Bernhard et al., 1970; McFarland and Bernhard, 1972); however, it has also been suggested that an equilibrium constant of unity for the hydride transfer

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reaction could lead to such biphasic kinetic behavior in the absence of subunit interactions (Luisi and Favilla, 1972).

A new method of studying the transient kinetics of aromatic alcohol oxidation and aldehyde reduction (McFarland and Bernhard, 1972) has been applied to the liver alcohol dehydrogenase reaction. These studies are helpful in answering the question of whether zinc or a protonic acid acts as the acid catalyst at the active site, and they establish a numerical value for the hydride transfer equilibrium constant.

Experimental Section

Preparation of Reagents. The preparation of enzyme has been reported previously (Bernhard et al., 1970; McFarland and Bernhard, 1972). Enzyme was obtained from C. F. Boehringer and Sohne and purified by gel filtration chromatography on Bio-Gel P-30.

The preparation of 4-deuterio-NADH has been reported previously (McFarland and Bernhard, 1972).

1,1-Dideuteriobenzyl alcohol was synthesized by the reduction of benzoyl chloride (Baker Analyzed Reagent) with an excess of lithium aluminum deuteride >98% deuterium (Ventron Inorganics) in diethyl ether. The ether layer was extracted with Na_2CO_3 to remove benzoic acid and the ether was evaporated leaving the desired product. The product was decolorized by treatment with activated charcoal. The product showed a single gas chromatographic peak with nearly the same retention time as an authentic sample of benzyl alcohol on a 5-ft column of 5% SE-52; both benzoic acid and benzaldehyde were well separated from the alcohol on this column. The nmr spectrum was consistent with the structure of the proposed product; the uv spectrum was identical with the spectrum of an authentic sample of benzyl alcohol. Steady-state kinetic analysis indicated an isotope effect, $k_H/k_D = 1.21$. This is a secondary isotope effect as expected since hydride transfer is not the rate-limiting step in alcohol oxidation by NAD^+ under steady-state conditions; this is taken as an indication that there are no inhibitors in the deuteriobenzyl alcohol which result in the observed primary isotope effect on the transient kinetics.

β-Naphthaldehyde (β-NAPA)[1] was obtained from Aldrich Chemical and purified by vacuum sublimation.

Benzyl alcohol was obtained from Aldrich Chemical, determined by gas chromatography to be pure, and used without further purification.

NADH and NAD^+ (P-L Biochemical "Chromatopure") were used without further purification.

Buffers used were as follows: pH 5.9–8.2 buffers were 0.05 *M* phosphate; pH 8.2–8.75 buffers were 0.05 *M* pyrophosphate; and pH 9.9 buffer was 0.1 *M* CO_3^{2-}.

Transient Kinetic Experiments. All stopped-flow experiments were carried out on a Durrum stopped-flow spectrophotometer using a tungsten lamp and an air activated pushing device. Phototube voltages were stored in a Northern Scientific time averaging computer modified to collect data at two scanning speeds in each of two 1024 memory cell storage units. The data were then plotted on a Houston Instrument X–Y recorder.

Reduction of β-NAPA. 4-Deuterio-NADH (used to reduce reaction rates to easily observed values) ($5 \times 10^{-5} M$) and enzyme (1×10^{-5} *N*, as determined by pyrazole titration) were mixed in the stopped-flow mixing chamber with 0.01 *M* pyrazole and 1×10^{-5}–1×10^{-4} *M* β-NAPA. Two exponential processes were observed as reported previously between pH 7.5 and 9.9; these two processes were easily separable visually since reaction rates for the two varied by at least 20-fold. For pH 6.0 and 7.0, an approximate method for separation of two steps was employed. In this case the two steps were separated by factors of 5–10; the end point of the first reaction was successively approximated until the best single exponential plot was obtained. Both rate processes were fit to single exponential functions; at each pH value the reciprocal of the first-order rate constant was plotted against the reciprocal of substrate concentration. Linear regression analysis was carried out on these reciprocal plots; Table I reports these values. Reported errors are calculated from standard deviations in slope and intercepts.

Oxidation of Benzyl Alcohol. In this experiment 8×10^{-6} *N* liver alcohol dehydrogenase premixed with 0.1 *M* isobutyramide was mixed in the stopped-flow spectrophotometer with NAD^+ (1×10^{-4} or 1×10^{-3} *M*) and benzyl alcohol (1×10^{-5}–1×10^{-3} *M*). A single exponential process was observed at 340 nm where the production of NADH is observed. The reciprocal of the rate constants from this single exponential were then plotted against the reciprocal of substrate concentration. Linear regression analysis was used to derive the kinetic parameters in Table II; errors are derived from standard deviations of slopes and intercepts in the linear regression procedure.

Steady-State Kinetic Experiments. All steady-state experiments were carried out on a Cary 16 spectrophotometer equipped with a Varian recorder. All measurements were made at 340 nm where the appearance of NADH in alcohol oxidation or the disappearance during aldehyde reduction was followed. Initial velocities were determined and reciprocal plots were made. Values in Tables I and II are from linear regression analysis of these reciprocal plots.

Results

The reduction of β-naphthaldehyde (β-NAPA) by NADH was studied by rapid kinetic techniques described previously (McFarland and Bernhard, 1972) between pH 6.0 and 9.9. The procedure used consists of the addition of pyrazole to NAD^+ produced during reaction of aldehyde and NADH; formation of the tight ternary complex at a rate faster than the rate-limiting step for turnover ensures that enzyme undergoes only a single reaction before being removed as the E–NAD^+–pyrazole ternary complex. Two step kinetic behavior and hyperbolic substrate concentration dependence for the first kinetic step (which exhibits a primary isotope effect with 4-deuterio-NADH) has been previously observed at pH 8.75. It seemed quite likely that these same studies would be possible at more acidic pH values provided that the pyrazole addition rate were faster than the turnover number at a given pH. With fixed concentration of pyrazole (0.02 *M*) (this is the concentration used in our single turnover studies), NAD^+ concentration dependence of the rate of ternary complex formation is hyperbolic. The extrapolated maximum rate constant for pyrazole addition at pH 7.0 is 102 sec^{-1}; at pH 8.75 the value is also 102 sec^{-1}, i.e., the rate constant for pyrazole addition is pH independent. At pH 7.0 the turnover number, V_{max}/E_0, for β-NAPA reduction is 11.2 sec^{-1} (Table I); therefore the experiment for limiting enzyme to single turnover should be feasible at more acidic pH's than 8.75. It is clear that there are two kinetic processes observed at pH 7.0

[1] Abbreviations used are: β-NAPA, β-naphthaldehyde; IBA, isobutyramide.

Table I: Reduction of β-NAPA by NADH in 0.05 *M* Phosphate Buffer.

pH	K_{app} ($M \times 10^6$)[a]	k_D (sec^{-1})[a]	k_H/k_D[a]	k_2 (sec^{-1})[a]	K_m ($M \times 10^6$)[b]	V_{max}/E_0 (sec^{-1})[b]
6.0	9.4 ± 1.0	133 ± 13	2.6	31	1.8	30
7.0	9.1 ± 0.6	143 ± 10		11	1.6	11.2
7.7	12 ± 1.3	173 ± 19	2.3	6	1.7	5.5
8.2	33 ± 4.0	180 ± 21		4.5		
8.2[c]	13 ± 1.2	170 ± 16				
8.75[c]	33 ± 4.0	300 ± 40		0.6	1.5	0.5
9.9[d]	48 ± 10	245 ± 49	2.2	0.2	1.0	0.25

[a] Transient conditions: liver alcohol dehydrogenase, 1.0×10^{-5} *N*; NADH–NADD, 5.0×10^{-5} *M*; pyrazole, 0.02 *M*. [b] Steady-state conditions: variable substrate; enzyme, $1\text{–}10 \times 10^{-8}$ *N*; NADH, 1×10^{-4} *M*. [c] 0.05 *M* pyrophosphate buffer. [d] 0.1 *M* carbonate buffer.

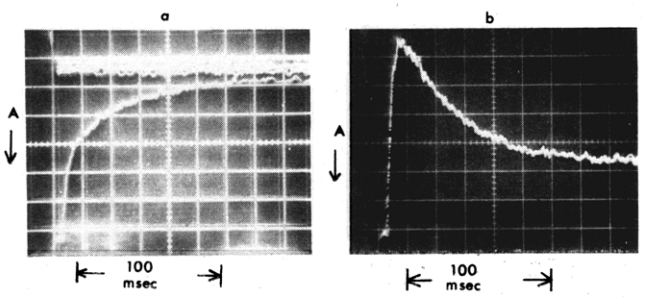


FIGURE 1: (a) The time course of the reduction of β-NAPA by NADH in the presence of liver alcohol dehydrogenase and pyrazole at pH 7.0 observed at 330 nm. [β-NAPA] = 2.9×10^{-5} *M*; [NADD] = 5.1×10^{-5} *M*; [liver alcohol dehydrogenase], 1.0×10^{-5} *N*; [pyrazole], 0.02 *M*. (b) The time course of formation of enzyme–NAD^+–pyrazole complex observed at 300 nm during reduction of β-NAPA by NADH.

(Figure 1); it is also possible to analytically separate the two rate processes which differ by an order of magnitude at saturating concentrations of substrate. As Figure 1 also illustrates, the reaction is limited to a single enzyme equivalent; the β-NAPA concentration dependence of the first kinetic process at pH 7.0 is hyperbolic as it is at pH 8.75 and the primary isotope effect for the fast kinetic process establishes that the process is at least partially limited by hydride transfer, k_3 (eq 1). Table I reports the single turnover and steady-state kinetic results of reduction of β-NAPA by NADH as a function of pH. K_{app} and k_D are the apparent binding constant and the maximum rate constant for the first process in the transient kinetic experiment described earlier. The isotope effect on this first rate process is given as k_H/k_D and k_2 is the rate constant for the second kinetic process observed during our transient kinetic experiments. Finally the steady-state Michaelis constant K_m and turnover number, V_{max}/E_0, are reported in the last two columns of Table I. Several interesting results are observed. (1) The first kinetic process (observed with deuterio-NADH to slow the rate to values easily observed in our stopped-flow spectrophotometer) shows a primary isotope effect throughout the pH range studied. This process, therefore, represents hydride transfer, k_3 (eq 1). (2) The rate constant for hydride transfer, k_D, shows no profound pH dependence; there is only a slight 2–3-fold increase in rate with increasing pH. (3) k_2 and the steady-state turnover number, V_{max}/E_0, show similar numerical values throughout the pH range and exhibit an acid-catalyzed behavior. These two rates represent the same kinetic process—desorption of alcohol from the enzyme surface, k_4 (eq 1) (Jacobs et al., 1974).

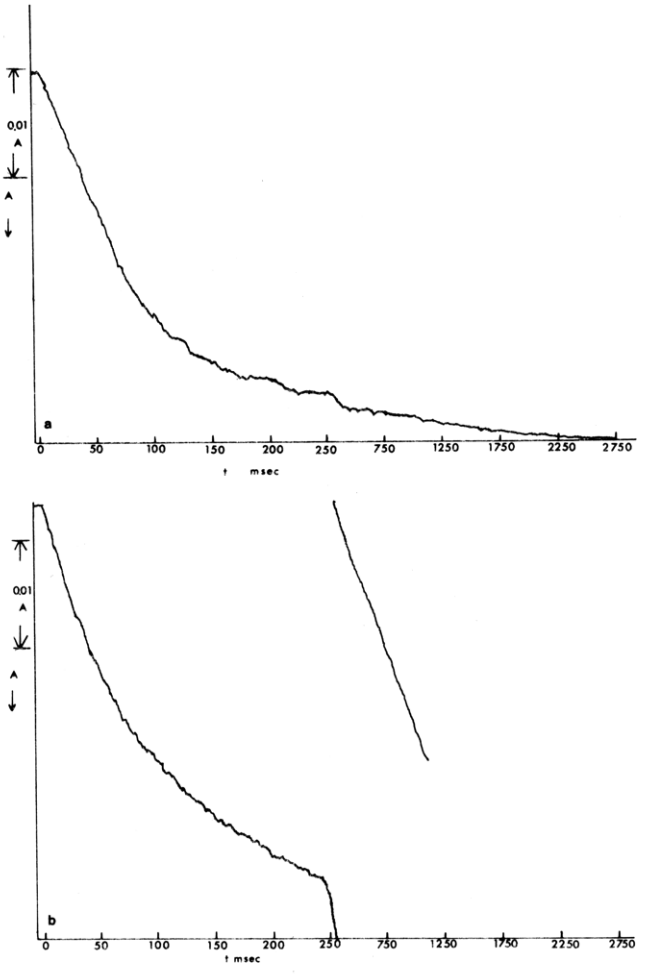


FIGURE 2: (a) The time course of the reaction of benzyl alcohol and NAD^+ in the presence of liver alcohol dehydrogenase and isobutyramide. [Benzyl alcohol], 6.0×10^{-4} *M*; [NAD^+], 1.0×10^{-4} *M*; [liver alcohol dehydrogenase], 8.0 μ*N*; [IBA], 0.1 *M*. Buffer—pH 8.0, 0.05 *M* phosphate. (b) The time course of the reaction of benzyl alcohol and NAD^+ in the presence of liver alcohol dehydrogenase. [Benzyl alcohol], 6.0×10^{-4} *M*; [NAD^+], 1.0×10^{-4} *M*; [liver alcohol dehydrogenase], 8.0 μ*N*. Buffer—pH 8.0, 0.05 *M* phosphate.

In order to further test the conclusion that there is no decrease in rate between pH 8.75 and 9.9 as the result of decreased acid catalysis of hydride transfer, the rate of reduction of benzaldehyde by NADD was observed. The rate constants for this reaction at pH 8.75 and pH 9.9 were 128 and 133 sec^{-1}, respectively; these rate constants were determined under conditions of saturating concentrations of

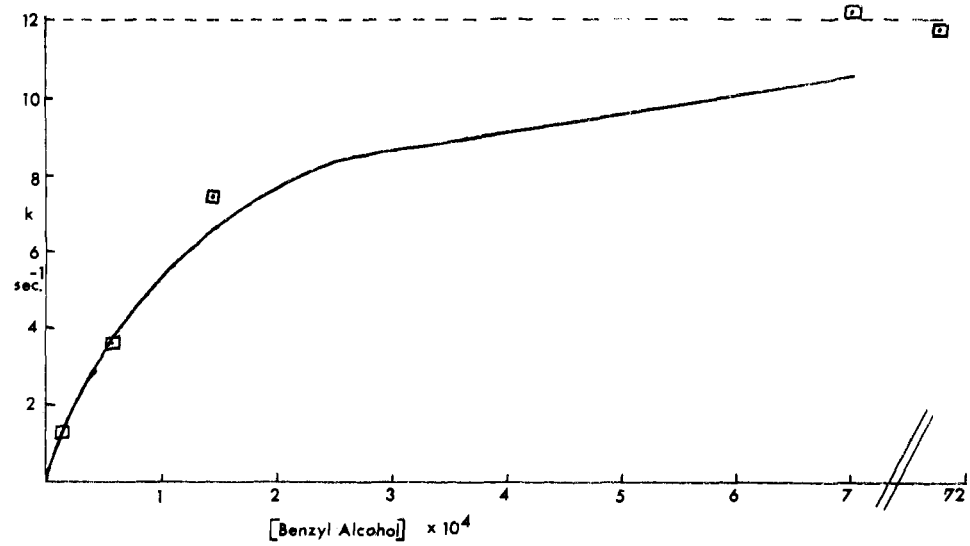


FIGURE 3: Concentration dependence of benzyl alcohol oxidation by NAD^+ in the presence of liver alcohol dehydrogenase and isobutyramide. [Liver alcohol dehydrogenase], 8.0 μN; [NAD^+], 1×10^{-4} M; [IBA], 0.1 M. Buffer—pH 8.0, 0.05 M phosphate. Solid line is best-fit hyperbole.

Table II: Oxidation of Benzyl Alcohol by NAD^+ in 0.05 M Phosphate Buffer.

pH	K_{app} (M)[a]	k_H[a] (sec^{-1})	k_H/k_D[a]	K_m(M)[b]	V_{max}/E_0 (sec^{-1})
5.9	$3.0 \pm 0.5 \times 10^{-3}$	3.6 ± 0.6	4.2[c]	1.9×10^{-4}	0.9
6.5	$7.6 \pm 1.4 \times 10^{-4}$	4.2 ± 0.8			
7.0	$5.5 \pm 0.8 \times 10^{-4}$	10.5 ± 1.6			
7.5	$3.0 \pm 0.4 \times 10^{-4}$	13.0 ± 2.0			
8.0	$1.0 \pm 0.1 \times 10^{-4}$	11.8 ± 0.8			
8.75[d]	$3.2 \pm 0.8 \times 10^{-5}$	8.0 ± 2.0	3.3[e]	2.0×10^{-5}	3.9
	$1.7 \pm 0.10 \times 10^{-5}$[f]	2.4 ± 0.1[f]			

[a] Transient kinetics: liver alcohol dehydrogenase, 8.4μN; NAD^+, 1×10^{-4} M; IBA, 0.1 M. [b] Steady-state kinetics: NAD^+, 1×10^{-4} M; variable substrate (see Experimental Section). [c] Single substrate concentration used in this determination. [d] 0.05 M pyrophosphate buffer. [e] Rates are extrapolated to infinite substrate concentrations. [f] D-Benzyl alcohol.

benzaldehyde. This confirms our conclusion from the extrapolated rates of Table I that there is no acid catalysis of hydride transfer from NADH to aldehyde over the pH range investigated.

A similar pH study of transient kinetics of oxidation of aromatic alcohol substrates would give insight into the mechanism of action of the enzyme in both directions. We have used isobutyramide (IBA) to limit alcohol oxidation to a single enzyme reaction; formation of the tight ternary E-NADH-IBA complex ($K_{EIR} = 5.5 \times 10^{-3}$ μM) limits reaction to 1 molar equiv of enzyme. Using the procedure we observe a single exponential process (Figure 2a) for the appearance of NADH during the NAD^+ oxidation of benzyl alcohol. As shown, the kinetic process is limited to 0.87 enzyme equiv of NADH produced; this is based on an extinction coefficient of 4.2×10^3 l./(M cm) for the E-NADH-IBA complex at 340 nm (M. F. Dunn and J. T. McFarland, unpublished data). The behavior in the absence of IBA is normal steady-state kinetic behavior in which a kinetically rapid "burst" is followed by steady-state turnover. Figure 2b shows the time course of the reaction; the portion which reappears at the top of the chart after having gone "off scale" consists of data which are "rolled over" by the computer. These data represent voltage readings which are actually lower than those shown at the bottom of the chart; therefore, these values represent continued NADH production. However, it is difficult to separate the burst because the rate constant for hydride transfer at pH 8.0 is 12 sec^{-1} while the turnover number is $V_{max}/E_0 \simeq 3.0$ sec^{-1} (Table II). The initial rates of reaction in the presence or absence of IBA are nearly the same as can be seen in Figure 2; it is our conclusion, therefore, that IBA does not significantly inhibit reaction rates during alcohol oxidation. The 1,1-dideuteriobenzyl alcohol was also subjected to NAD^+ oxidation; the rate of this enzyme-catalyzed reaction indicates the presence of a primary isotope effect of 3–4 on the single exponential observed under these transient kinetic conditions (Table II). Figure 3 illustrates the concentration dependence of the hydride transfer rate constant during alcohol oxidation at pH 8.0; again as with aldehyde reduction this rate shows hyperbolic concentration dependence (Figure 3) allowing the linear reciprocal extrapolation of maximum rate and binding constant shown in Table II. The first two columns of this table report the apparent binding constant and the maximum rate constant from the transient kinetic experiments dealing with the oxidation of benzyl alcohol by NAD^+. The final two columns report the Michaelis constant K_m and the turnover number for the same process.

Several results are of interest. (1) There is a large primary isotope effect of 4.2 at pH 5.9 and 3.3 at pH 8.75. Therefore, we believe we are observing hydride transfer, k_{-3} (eq 1). (2) There is nearly the same threefold decrease in the rate constant for hydride transfer, k_H, observed in aldehyde reduction but no large pH dependence. (3) The apparent binding constant for alcohol, K_{app}, undergoes a large (two orders of magnitude) decrease with decreasing pH; that is, there is strong dependence on a basic group on the

enzyme surface.

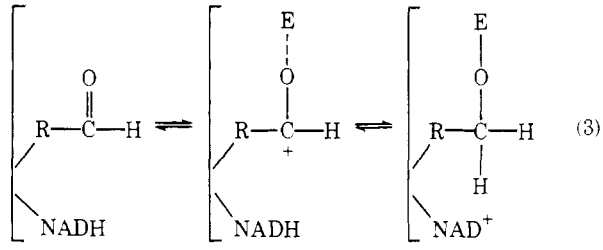
We are unable to study the rate of hydride transfer at more acidic pH values because our enzyme preparation undergoes rapid loss of activity below pH 5.9

Discussion

Previous investigations showing biphasic rate behavior during transient kinetic reduction of aromatic aldehydes by NADH catalyzed by liver alcohol dehydrogenase have been interpreted as an indication that the two monomers in the dimer do not act independently, i.e., show subunit interactions (Bernhard et al., 1970; McFarland and Bernhard, 1972). The types of interaction suggested were such that one subunit remained inactive during catalysis at the "active" subunit. It has been pointed out that biphasic rate behavior would also result from an equilibrium constant for hydride transfer, $K_{eq} = k_3/k_{-3}$ (see eq 1), which is unity for all substrates (Luisi and Favilla, 1972). Such a circumstance would result in a hydride transfer reaction with an amplitude of one-half the total enzyme sites, and a second, slower process.

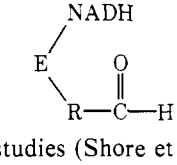
Our data now indicate (Figure 1) that biphasic reaction persists to pH 7.0 during reduction of aromatic aldehydes and is not confined only to basic pH values. This lends more credibility to the postulate for subunit interaction; furthermore, our data now allow us to estimate the magnitude of the equilibrium constant for hydride transfer. At pH 8.75 the rate constant for hydride transfer, k_3 (eq 1), from NADH to benzaldehyde has been previously reported as 339 sec^{-1} (Jacobs et al., 1974); our present determination of the rate constant for hydride transfer from benzyl alcohol to NAD^+ (k_{-3}, eq 1) is 8.0 sec^{-1} (Table II). Therefore, the reduction of aromatic aldehydes is a spontaneous process, $K_{eq} = k_3/k_{-3} = 42.4$; this precludes any equilibrium explanation for the biphasic kinetic behavior during reduction of aromatic aldehydes.[2] Taken together these two facts strengthen the case for one-half site reactivity in liver alcohol dehydrogenase.

As mentioned previously the catalytic mechanism is not clear; zinc and at least one protonic acid are involved in catalysis but their roles have not been established. Our data have some bearing on this question. Previous studies in this laboratory have shown an abnormally small substituent effect for the liver alcohol dehydrogenase catalyzed reduction of para-substituted benzaldehydes. This would be expected if hydride transfer were subject to electrophilic catalysis by either proton or zinc atom as shown in eq 3, where E is the



(3)

electrophile. Such catalysis would be expected to result in a transition state quite similar to that of the starting material complexed with the electrophile (Hammond, 1955). Since little reorganization of bonding would be expected, the stabilization of substituent in the transition state would be quite similar to the stabilization of the electrophile–aldehyde complex and little effect of substituent on reaction rate would result. Such an expectation is borne out by the decrease in Hammett ρ for acid-catalyzed addition of semicarbazide to substituted benzaldehydes (Jencks and Anderson, 1960). Therefore, the small substituent effect for the enzyme reaction is consistent with electrophilic catalysis of hydride transfer. However, such electrophilic catalysis could result from transfer of proton from an amino acid side chain, transfer of a proton from a water molecule bound to zinc at the active site, or by direct catalysis by zinc. The pK_a for a labile proton at the active site has been estimated from several previous experiments. Ethanol oxidation shows base catalysis with $pK_a = 6.5$ (Brooks et al., 1972); therefore, if a proton serves as the electrophilic catalyst for hydride transfer to aromatic aldehydes we should observe acid catalysis with $pK_a = 6.5$. Other studies indicate that binding of trifluoroethanol and NAD^+ in an inactive ternary complex results in release of a proton from a group with $pK_a = 7.5$; in the absence of NAD^+, the same group is thought to have a $pK_a = 9.6$ (Shore et al., 1974). A proton is taken up during aldehyde reduction; this is consistent with a pK_a perturbation of an enzyme residue on change in nucleotide oxidation state (Dunn, 1974). Therefore, if a proton were acting as an electrophilic catalyst we should expect the reduction of aldehydes to show acid catalysis with a pK_a between 6.5 and 9.6, and alcohol oxidation to show base catalysis with a $pK_a = 6.6$–7.6. Tables I and II show that neither of these expectations is obtained; reduction of aromatic aldehydes shows no decrease in rate with increasing pH (acid catalysis), but is indeed 2–3-fold slower at acid pH values. Likewise we do not observe base catalysis of benzyl alcohol oxidation; there is a 2–3-fold decrease in rate at acidic pH but this is quite analogous to the rate decrease at acidic pH for aldehyde reduction. Our conclusion, therefore, is that zinc (or a protonic acid with $pK_a < 6.0$ or > 10.0) serves the role of electrophilic catalyst since neither foreward (k_3) nor reverse (k_{-3}) rates for hydride transfer show acid-base catalysis. Furthermore, if the pH dependence for reduction of β-NAPA is the same as that for benzaldehyde, the ratio of k_3 to k_{-3} (the equilibrium constant for hydride transfer) increases less with pH than does either rate process since both values decrease concomitantly with decreasing pH; this indicates that no proton is involved in the hydride transfer equilibrium constant. Since our kinetic experiments are carried out under conditions in which enzyme is present as ternary complex, we are in effect studying the transformation of this complex to products. Therefore protonic acid catalysis cannot be excluded by our data if the pK_a of the group acting as catalyst were not between 6.0 and 10.0 in the ternary



complex. Previous studies (Shore et al., 1974, Dunn, 1974) have suggested sizable changes in pK_a of an enzyme residue on binary complex formation; similar perturbation on formation of ternary complex could result in a modified pK_a for a protonic acid removing this pK_a from the range of our experiments.

Since it is clear from earlier studies that a proton must be involved in the liver alcohol dehydrogenase reaction, our data should show pH dependence for some rate or binding

[2] This result is confirmed by independent experiments reported elsewhere (Luisi and Bignetti, 1974).

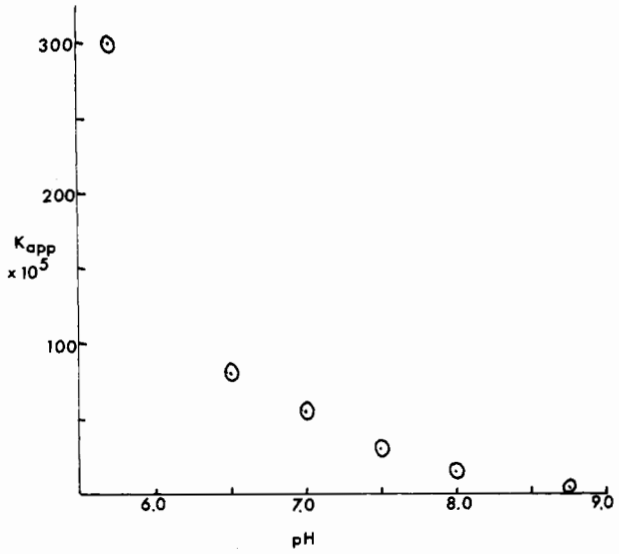


FIGURE 4: pH dependence of K_{app} for benzyl alcohol oxidation. [Liver alcohol dehydrogenase], 8.0 μN; [NAD^+], 1×10^{-4} M; [IBA], 0.1 M. Buffers—pH 5.7–8.0, 0.05 M phosphate; pH 8.75, 0.05 M pyrophosphate.

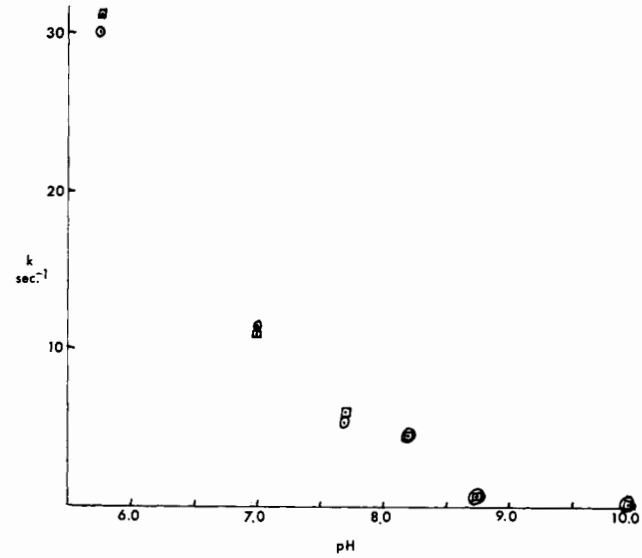
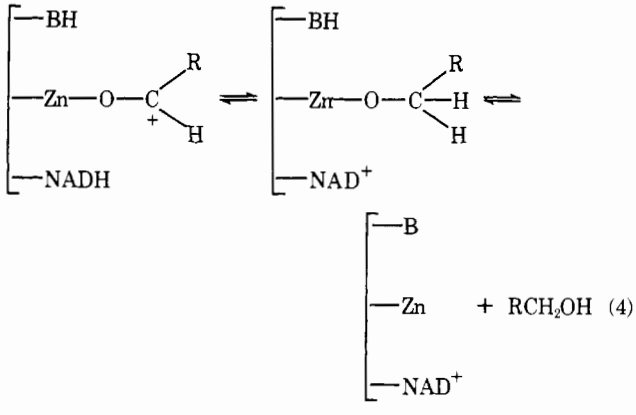


FIGURE 5: pH dependence of V_{max}/E_0 and k_2 for β-NAPA reduction by NADD in the presence of liver alcohol dehydrogenase. (⊙) V_{max}/E_0 measured from steady-state kinetics; (⊡) k_2 measured from transient kinetics; [NADD], 5×10^{-5} M; [liver alcohol dehydrogenase], 1×10^{-5} N; [pyrazole], 0.02 M. Buffers—pH 6.0–8.2, 0.05 M phosphate; pH 8.75, 0.05 M pyrophosphate.

process in the overall reaction scheme. Tables I and II show that the binding and rate constants showing significant pH dependences are the apparent binding constants for benzyl alcohol (showing two orders of magnitude variation) and the rate constant for alcohol desorption from the enzyme surface (again showing greater than two orders of magnitude change). Figures 4 and 5 show the pH dependence graphically; qualitatively the two figures suggest involvement of a base with $pK_a < 6.5$ for alcohol binding and involvement of an acid $pK_a < 7.0$ for alcohol desorption. We cannot make quantitative assignments from our data because of enzyme denaturation below pH 5.9.

Two catalytic mechanisms appear to fit the data. The first is one in which a base on the enzyme surface acts to remove a proton from alcohol substrate permitting substrate to form a zinc alcoholate (eq 4). This is in keeping with the increase in the apparent binding constant for alcohol with increases in concentration of basic form of an amino acid side chain (eq 4).

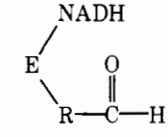


(4)

Furthermore, alcohol desorption under this mechanism should show acid catalysis with the same pK_a since the protonated form of the amino acid side chain would be required to donate a proton to the zinc alcoholate complex before alcohol desorption can take place. Alternatively, the observed pH dependence may result from a conformational change of the enzyme in which the conformation adopted when a particular amino acid side chain is protonated has a much smaller affinity for alcohol substrate.

It should be noted that there may be more than one acid–base catalyst involved in formation of aromatic alcoholate complex. The rate of desorption of alcohol does not decrease in direct proportion to pH change; this may suggest that two side chain groups may be capable of donating the necessary proton.

In conclusion, the pH independence of hydride transfer either from NADH to aromatic aldehyde or from aromatic alcohol to NAD^+ indicates that electrophilic catalysis of hydride transfer (Jacobs et al., 1974) results from catalysis by zinc or from a protonic acid with a $pK_a < 6.0$ or > 10.0 in the ternary



complex.

One possible mechanism consistent with the large pH dependence of the apparent binding constant for aromatic alcohols and the desorption rate for aromatic alcohol is that a general acid-base catalyst on the enzyme with $pK_a < 7.0$ acts to remove a proton from alcohol permitting formation of zinc alcoholate. In the opposite direction, the protonated form of the base donates a proton to zinc alcoholate (eq 4).

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Nonidentical Alkylation Sites in Rabbit Muscle Glyceraldehyde-3-phosphate Dehydrogenase†

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ABSTRACT: These studies establish the specificity of 3,3,3-trifluorobromoacetone for reaction with the active site cysteines of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase and suggest the potential use of trifluoroacetonyl groups as ^{19}F nuclear magnetic resonance probes for study of symmetry relations between the four protomers of the enzyme. The alkylation of the holoenzyme follows biphasic kinetics and indicates either preexistent or induced nonequivalence among the sites; these effects are not predisposed by a low coenzyme/enzyme ratio. Two additional alkylation sites not at the active centers are created by acylation with β-(2-furyl)acryloyl phosphate; it is concluded that pseudosubstrates cause an intramolecular rearrangement which exposes two sulfhydryl functions besides those of the active site (Cys-149).

Glyceraldehyde-3-phosphate dehydrogenase is a key enzyme in the glycolytic cycle where it catalyzes the oxidation and subsequent phosphorylation of its substrate aldehyde to the corresponding acyl phosphate. The enzyme was first crystallized from yeast (Warburg and Christian, 1939) and has since been obtained from a variety of sources including human, beef, chicken, pheasant, halibut, sturgeon, and lobster (Allison and Kaplan, 1964) tissues.

In mammals the primary structure of GPD[1] is highly conserved (Perham, 1969) resulting in very similar catalytic and molecular characteristics. Since yeast GPD is adapted to quite different physiological conditions, its contrasting physical data (Velick and Udenfried, 1953; Allison and Kaplan, 1964) and mechanistic properties as revealed by (a) the mode of coenzyme binding (Conway and Koshland, 1968; Cook and Koshland, 1970; Kirschner, 1971) and (b) inhibition by alkylating agents (MacQuarrie and Bernhard, 1971a,b, Stallcup and Koshland, 1972) are not surprising.

The active site of GPD involves Cys-149 (Cys-148 in the case of the lobster enzyme) which in the presence of NAD^+ and substrate aldehyde forms a thiol ester intermediate (acyl enzyme). The low pH value found in muscle cells requires Cys-149 to possess considerable nucleophilic activity well below its pK_a value of 8.0–8.1 (Behme and Cordes, 1967; MacQuarrie and Bernhard, 1971a) which may be provided by H-bond formation with a residue of pK_a = 4.6–6.8 (Cseke and Boross, 1970). Francis *et al.* (1973) present evidence that the "activating" group may be His-38 and emphasize its additional role as an acyl acceptor during catalysis.

Two other amino acids seem to be a common feature of the active center region. A specific lysine (Lys-182 or -183 respectively; Davidson, 1970) with a suggested role in the enzyme–coenzyme interaction (Mathew *et al.,* 1967) occurs close to Cys-149 as demonstrated by the irreversible S → N transfer of an acetyl group between these residues (Park *et al.,* 1965). A tryptophan close to the active site can be detected by fluorescence (Velick, 1958; Keleti, 1968). Its involvement in the catalytic process has often been suggested, most recently by Heilmann and Pfleiderer (1974).

GPD has a molecular weight of 144,000 and is tetrameric. The complete primary structure of the lobster (Harris

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[1] Abbreviations used are: GPD, glyceraldehyde-3-phosphate dehydrogenase; NAD^+, nicotinamide adenine dinucleotide; TFA, 3,3,3-trifluorobromoacetone; FA-P, β-(2-furyl)acryloyl phosphate; $GPD(TFA)_2$, trifluoroacetonylglyceraldehyde-3-phosphate dehydrogenase (dialkylated); $(FA)_2GPD$, furylacryloyl-glyceraldehyde-3-phosphate dehydrogenase (diacylated); Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, the $R_2C_6H_3S$ residue derived from Nbs_2.