Roles of Zinc Ion and Reduced Coenzyme in the Formation of a Transient Chemical Intermediate During the Equine Liver Alcohol Dehydrogenase Catalyzed Reduction of an Aromatic Aldehyde†

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ABSTRACT: The chromophore *trans*-4-*N*,*N*-dimethylaminocinnamaldehyde (I) (λ_{max} 398 nm, ϵ_{max} 3.10×10^4 M^{-1} cm^{-1} OD) has been found to react rapidly with the enzyme–NADH complex to form a transient chemical intermediate (λ_{max} 464 nm, ϵ_{max} 6.2×10^4 M^{-1} cm^{-1} OD) which then decays in a much slower step to a final equilibrium mixture consisting primarily of intermediate, NAD^+, and *trans*-4-*N*,*N*-dimethylaminocinnamyl alcohol. At pH values above pH 9, the intermediate can be observed as a relatively stable species in the presence of high enzyme concentrations. The rapid formation of the intermediate occurs only in the presence of the enzyme–NADH complex. NAD^+ will not function in place of NADH in the process of intermediate formation. However, on the basis of the spectral properties of the intermediate, NADH is concluded to undergo no covalent change during the formation of the 464-nm transient. The rate of intermediate decay to the final equilibrium mixture, the stoichiometry of intermediate formation, and the apparent equilibrium constant for intermediate formation have been studied by both stopped-flow rapid-mixing spectrophotometry and conventional spectrophotometric techniques in the pH range 6–9.5. The rate of decay to products is subject to a small deuterium isotope effect ($k_H/k_D = 1.2$) when the reactive hydrogen of NADH is replaced by deuterium. The apparent equilibrium constant for intermediate formation, $\sim 4 \times 10^{-7}$ M, varies only slightly as a function of pH, and is found to be numerically identical with the steady-state Michaelis constant for I reduction. Data for the stoichiometry of intermediate formation (two per 84,000 daltons) has been analyzed by both the method of Scatchard and the method of Job. Owing to the rapid rate of intermediate formation, the combined stopped-flow temperature perturbation of equilibrium method has been used to investigate the kinetic behavior of the system. The results show that intermediate formation is a simple, reversible pH-independent process with $k_1 \simeq 4 \times 10^7$ M^{-1} sec^{-1}, $k_{-1} \simeq 280$ sec^{-1}, $\Delta G^\circ = -7.3$ kcal/mol, $\Delta H^\circ = 2.5$ kcal/mol, and $\Delta S^\circ = 32$ eu. It is proposed that the absolute requirement for NADH reflects a heretofore unsuspected (noncovalent) effector role for NADH in facilitating the chemical activation of the substrate for further reaction. The large spectral change, the pH independence, and the magnitudes of the rate constants which characterize the process of intermediate formation are consistent with a structure for the intermediate involving a coordination bond between the zinc ion at the enzyme active site and the carbonyl oxygen of I.

The nicotinamide adenine dinucleotide requiring enzyme, horse liver alcohol dehydrogenase (EC 1.1.1.1), catalyzes the interconversion of aldehydes and primary alcohols (see Sund and Theorell, 1962, for a review). This thoroughly characterized enzyme has been the subject of numerous steady-state kinetic studies (Theorell and Chance, 1951; Theorell and McKinley-McKee, 1961; Dalziel, 1963; Wratten and Cleland, 1963, 1965) and binding studies (Theorell and Yonetani, 1963; Taniguchi *et al.*, 1967; Sigman, 1967; Theorell and Tatemoto, 1971; Sarma and Woronick, 1972; Everse, 1973).

On the basis of these investigations, the sequential relationship between binding steps and the redox step(s) for the overall transformation can be summarized according to the preferentially ordered (Theorell–Chance) mechanism shown in eq 1–5.

$$E + 2NADH \rightleftharpoons E(NADH)_2 \quad (1)$$

$$E(NADH)_2 + 2S \rightleftharpoons E(NADH,S)_2 \quad (2)$$

$$H^+ + E(NADH,S)_2 \rightleftharpoons \cdots \rightleftharpoons E(NAD^+,P)_2 \quad (3)$$

$$E(NAD^+,P)_2 \rightleftharpoons E(NAD^+)_2 + 2P \quad (4)$$

$$E(NAD^+)_2 \rightleftharpoons E + 2NAD^+ \quad (5)$$

The use of high enzyme concentrations (10–100 μN sites) and rapid kinetic techniques has made possible the kinetic characterization of individual steps in the overall transformation of eq 1–5 (Shore, 1969; Bernhard *et al.*, 1970; Shore and Gutfreund, 1970; Dunn and Bernhard, 1971; McFarland and Bernhard, 1972; Luisi and Favilla, 1972). However, heretofore it has not been possible to define the transient bonding forces which facilitate the chemical transformation. Indeed, the participation of various site functional groups and the involvement of zinc ion in the mechanism of catalysis has been the subject of much speculation (see Coleman, 1971, for a review of this topic). A number of detailed reaction pathways which satisfy the stoichiometry requirement for a net transfer of two hydrogen nuclei and two electrons, *viz.*, the chemical step (eq 3), have been proposed (Theorell and McKinley-McKee, 1961; Wallenfels and Sund, 1957; Ables *et al.*, 1957; Hamilton, 1971; Kosower, 1962; Schellenberg, 1970).

This paper describes the reaction of the intense chromo-

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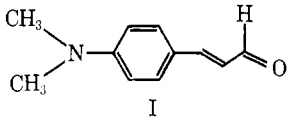


I

phore, *trans*-4-*N*,*N*-dimethylaminocinnamaldehyde (I) with the enzyme–NADH complex. As will be shown, reduction of I involves the formation of a transient chemical intermediate in the neutral pH range. At pH values greater than 9, the intermediate can be observed as a relatively stable species in the presence of high enzyme concentrations. Both stopped-flow and the combined stopped-flow temperature-jump rapid kinetic techniques (Erman and Hammes, 1966) and uv-visible spectrophotometry have been employed to investigate: (1) the spectral properties and the stability of the intermediate, (2) the stoichiometry of intermediate formation, and (3) the kinetics of intermediate formation and decay to products for the pH range 6–9.5.

On the basis of these studies, it is concluded that the essential horse liver alcohol dehydrogenase zinc ion plays a Lewis acid catalytic role in the facilitation of hydride transfer. This and other features of the horse liver alcohol dehydrogenase catalytic mechanism are discussed.

Materials and Methods

Materials. The coenzymes NAD^+ and NADH (Sigma, grades V and III, respectively, or Boehringer, grade I) were used without further purification in all experiments, except for those involving the investigation of deuterium isotope effects (see Methods). *trans*-*N*,*N*-Dimethylaminocinnamaldehyde (Aldrich) was purified by vacuum sublimation prior to use. Reagent grade buffer salts (obtained from standard chemical suppliers) were used without further purification. The buffer solutions (chloride ion free) were prepared with twice-distilled water.

Horse liver alcohol dehydrogenase (Boehringer Mannheim Corp.) was further purified in a modification of the method of Bernhard *et al.* (1970). In this modification, the clear supernatant (aqueous Na_2HPO_4–ethanol) is removed from 100 mg of the commercial preparation and discarded. The residual slurry containing the enzyme is then dissolved in 2–4 ml of 0.1 M sodium pyrophosphate buffer (pH 8.75 or 9.48) and incubated with 200 μl of a 1×10^{-2} M stock solution of 1,4-dithioerythritol overnight. Elution of the resulting enzyme solution over a 3×60 cm P-2 Bio-Gel column equilibrated with pyrophosphate buffer gives a preparation virtually free of Na_2HPO_4, 1,4-dithioerythritol, and ethanol (residual ethanol $\leq$ site normality).

The coenzyme binding titer of the resulting horse liver alcohol dehydrogenase solution was determined by the NAD^+–pyrazole spectrophotometric horse liver alcohol dehydrogenase assay method (Theorell and Yonetani, 1963). These values are reported throughout as N. The normalities generally were found to range from 90 to 95% of the value predicted by the 280-nm extinction coefficient reported for enzyme, $3.53 \times 10^4\ M^{-1}\ cm^{-1}$ OD (Dalziel, 1957).

Methods. Specifically labeled α-4-deuterio-NADH (NADD) was prepared according to a modification of the procedure of Rafter and Colwick (1957). In this modification, ethanol-d_6 is used in place of the isotopically normal material, and an alternative isolation and work-up procedure has been employed to yield an ethanol-free product. The modified isolation procedure is carried out as follows. On completion of the reaction according to the original procedure, the isolation of NADD is accomplished by the addition of 100 ml of acetonitrile/200 mg of NAD^+ starting material. The resulting two-phase system is separated by carefully withdrawing the lower phase ($\sim$2 ml) which contains the NADD with a Pasteur pipet. The NADD is then precipitated by dispersing the concentrated NADD solution into an additional 100 ml of acetonitrile. After decanting, the resulting light-yellow precipitate of NADD is washed with four 25-ml aliquots of anhydrous acetonitrile and finally with four 25-ml aliquots of anhydrous ether and then dried under high vacuum. The final product, obtained in yields of 50–60%, has a $OD_{340}/OD_{260} = 0.418$, in good agreement with a previous report (McFarland and Bernhard, 1972). NADH prepared by the same procedure has been used in comparative experiments to determine kinetic isotope effects.

The stopped-flow rapid-mixing experiments were carried out with a single-beam Durrum Model D-110 spectrophotometer equipped with a 2-cm Kel-F observation cuvette (mixing dead time, 2–3 msec). The time course of the transmittance changes occurring in the freshly mixed sample were recorded by storage of the digital equivalent of the analog signal *via* the high-speed, 8 bit X1,000 word MOS shift register memory of the Biomation 802 transient recorder. Permanent traces of the stored signal were obtained through memory output in smoothed analog form to an X–Y recorder with time base. Optical density changes have been calculated from the following relationship:

$$OD = (f/2) \log (v_{t_2}/v_{t_1}) \quad (6)$$

where v_{t_1} and v_{t_2} refer to the photomultiplier tube (transmittance) voltages at times t_1 and t_2, respectively. The experimentally determined (wavelength independent) correction factor ($f = 1.26$) corrects the observed D-110 OD values (*e.g.*, $\log (v_{t_1}/v_{t_2})$ to the OD of calibrated solutions. OD values relative to the OD of the buffer solution employed (as reference) have been calculated from the relationship:

$$OD = (f/2) \log (v_{buf}/v_t) \quad (7)$$

where v_{buf} is the transmittance voltage of the reference buffer solution.

The stopped-flow kinetic studies generally were carried out by mixing a solution of I with a solution containing preincubated enzyme and NADH. No difference in the observable reaction time course was found for the alternative preincubation condition where a solution of enzyme is mixed with a solution containing I and NADH. However, note that an appreciable amount of the reaction to form the intermediate (see Results) occurs within the mixing dead time.

All steady-state kinetic studies were carried out on a Beckman DB-GT spectrophotometer equipped with thermostatted cell compartments at 25 $\pm$ 0.2°.

The experiments involving perturbation of equilibrium after the rapid mixing of enzyme and substrates were performed with a combined Durrum stopped-flow (D-110) and Durrum temperature-jump (D-150) apparatus. This instrument is similar to the apparatus previously described by Erman and Hammes (1966).

In a typical experiment, the high-voltage capacitor, initially charged to 5 kV, is discharged over a 100-μsec interval following a delay of 200 msec from the time of mixing. The 200-msec delay time is sufficient to allow the maximum formation of intermediate (see Results). Note that the amounts of NAD^+

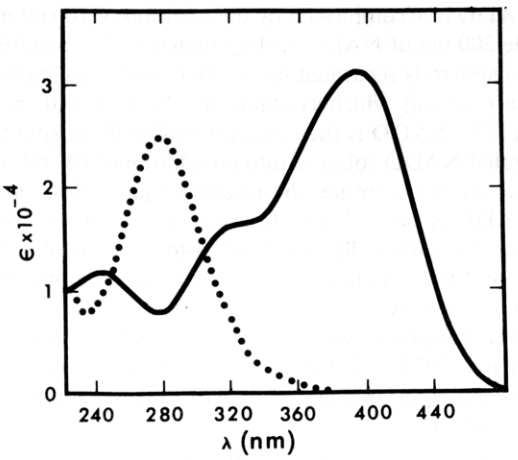


FIGURE 1: A comparison of the spectrum of I (——) with the spectrum of the corresponding alcohol, *trans*-4-*N*,*N*-dimethylaminocinnamyl alcohol (.....) in 0.1 M sodium phosphate buffer (pH 6.83) and 25.0 ± 0.2°.

and *trans*-4-*N*,*N*-dimethylaminocinnamyl alcohol formed during this time span are negligible. The width of the heating pulse and the relaxation of the cuvette to the original temperature limited the time interval accessible to investigation to between 50 μsec and 200 msec.

Since the system under investigation is relatively insensitive to ionic strength effects (Sund and Theorell, 1962), the buffer solutions employed (0.1 M sodium phosphate below pH 8, and 0.1 M sodium pyrophosphate above pH 8) were not adjusted to a constant ionic strength. The different ionic strengths of these buffers result in negligible differences in temperature rise since the 100-μsec discharge time is sufficient to allow a nearly complete discharge (>90%) of the 5-kV capacitor through the cuvette.

The temperature rise generated by the 5-kV discharge was estimated by comparing the equilibrium concentrations of the intermediate obtained at T_1 and T_2 with a calibration curve relating the amount of intermediate formed as a function of the cuvette temperature. The calibration curve was prepared by varying the temperature of the thermostatted cuvette and recording the change in the amount of intermediate formed as a function of cuvette temperature at a pH (pH 9.48) where the reaction does not proceed significantly beyond intermediate formation. Both the discharge time and the electronic noise–filter time constant were maintained $\leq (0.1)\tau$ throughout.

Values of the reciprocal relaxation time $1/\tau$ have been calculated from the traces by assuming that only a single relaxation occurs and that the relaxation adheres to an apparent first-order rate law (Eigen and de Maeyer, 1963). The relationship, $\Delta OD \propto \Delta v$, for OD changes smaller than 0.1 OD has been used throughout in the calculation of $1/\tau$ values.

Results

Stopped-Flow Rapid-Mixing Kinetic Studies. Kinetic experiments were carried out to investigate the transient kinetic behavior of the horse liver alcohol dehydrogenase catalyzed reduction of I by NADH at pH 8.75 by using the stopped-flow, rapid-mixing technique. Since the long-wavelength electronic transition of I (λ_{max} 398 nm) is completely bleached on reduction (Figure 1) the progress of the reaction initially was monitored at 400 nm.

Under the conditions [NADH] > [E] > [I] (Figure 2, upper trace), the OD time course at this wavelength is characterized by a very rapid OD decrease ($t_{1/2} \simeq 3$ msec for the particular conditions of Figure 2). Note that a significant portion of the total change in OD (>50%) occurs during the instrument mixing dead time (2–3 msec). Also note that the residual OD value 50 msec after mixing (0.09 OD) does not correspond to the expected OD (0.00 OD) for the quantitative conversion of I to the corresponding alcohol. Furthermore, this apparent end point does not correspond to completion of reaction since a further decrease in OD occurs (not shown in the figure), but at a rate which is orders of magnitude slower than the initial rapid step shown in Figure 2. This slow apparent first-order reaction proceeds to a stable end point, which, however, still does not correspond to the quantitative reduction of I.

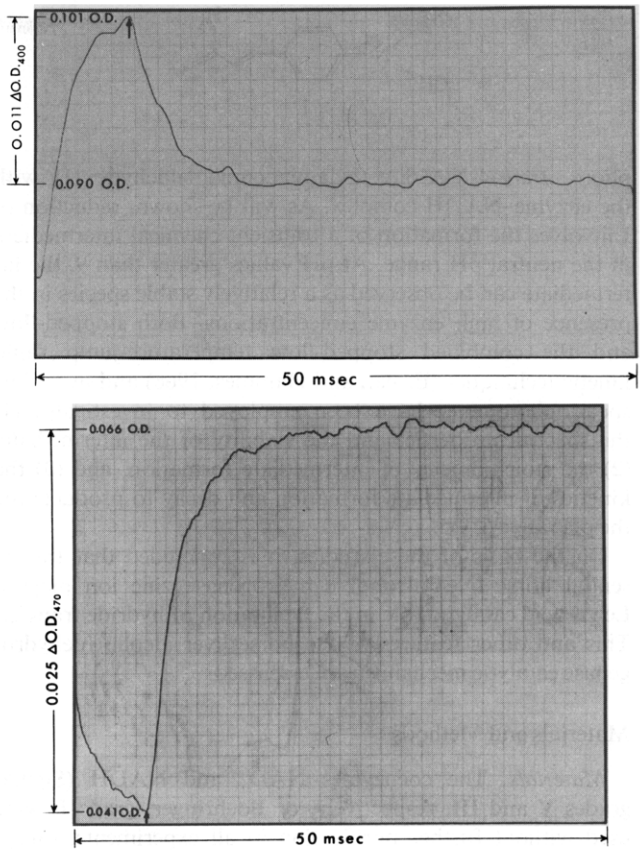


FIGURE 2: Stopped-flow rapid-mixing traces of the time course for the reaction of I with the LADH–NADH binary complex. The upper trace and lower trace compare the first 50 msec of the progress curves for the reaction monitored at 400 and 460 nm, respectively. The initial portion of each trace records the OD changes in the observation cuvette as the freshly mixed sample displaces the old solution from the cuvette before flow stops. The vertical arrows indicate the point in each trace where cessation of flow occurs. Conditions were as follows: $[E]_0$ = 7.3 μN, $[NADH]_0$ = 78.0 μM, $[I]_0$ = 2.50 μM, 0.1 M sodium pyrophosphate buffer (pH 8.75), and 25 ± 0.2°.

The visible spectrum of this final reaction mixture contains a new (unexpected) long-wavelength absorption with a λ_{max} of *ca.* 460 nm. Figure 2 (lower trace) compares the time course for the appearance of this new species with the time course in the upper trace. It is evident from this comparison that the rapid OD increase occurs on the same time scale with approximately the same half-life as the rapid OD decrease at 400 nm.

Following the initial rapid rise in OD at 460 nm (Figure 2, lower trace), the OD decreases to a final (stable end point) value in a single exponential process identical in rate with

TABLE I: Effect of Coenzyme Oxidation State on Intermediate Formation at pH 9.5, and 25°.

	Extent and App Rate of Intermediate Formation			
	Fast Step		Slow Step	
Coenzyme[a]	$t_{1/2}$[b] (msec)	ΔOD_{460}[c]	$t_{1/2}$[b] (sec)	ΔOD_{460}[c]
NADH	6	0.096		
NAD^+	30	0.004	20	0.08

[a] The concentrations used were: $[E]_0 = 4.68$ μN, $[I]_0 = 4.58$ μM, and $[NADH]_0 = 96.8$ μM or $[NAD^+]_0 = 1.0$ mM. [b] Reaction half-lives have been calculated assuming the progress curves obey a pseudo-first-order rate expression. [c] The OD values have been calculated as the total change in OD at 464 nm.

the above noted slow process observed at 400 nm. This final equilibrium mixture consists principally of the 460-nm species (hereafter referred to as the intermediate) and the products, NAD^+ and 4-*N*,*N*-dimethylaminocinnamyl alcohol. The identity of these products has been established by demonstrating that the coenzyme product functions quantitatively in the horse liver alcohol dehydrogenase catalyzed oxidation of ethanol, and that the isolated product derived from I, as judged by the comparison of mass spectra and uv spectra, is identical with that of an authentic sample prepared by reduction of I with $NaBH_4$.

Above pH 9, and in the presence of a large excess of the enzyme–NADH complex, I reacts to form the intermediate in nearly quantitative amounts. Figure 3 summarizes the spectral properties of the stable system at pH 9.36. This figure compares the spectrum of a solution containing I and concentrated horse liver alcohol dehydrogenase to the spectrum obtained after the addition of NADH (in an amount slightly in excess of the enzyme site concentration). It is clear from the comparison of these spectra that the absorption band of the intermediate is nearly twice as intense as the 398-nm transition of I. Indeed, the OD_{464}/OD_{400} ratio for these species is approximately 1.9 and, therefore, the intermediate must have an of $\epsilon_{max} \geq 6.0 \times 10^4$ M^{-1} cm^{-1} OD. Solutions stored for more than a month at 4° retain the characteristic spectrum of the intermediate with little or no loss in intensity. In fact, the spectrum of the intermediate is lost only as rapidly as the enzyme denatures and/or as rapidly as the coenzyme decomposes under these conditions.

The dependence of intermediate formation on the coenzyme oxidation state is summarized in Table I.

In these experiments the effects of NADH *vs.* NAD^+ on the time course of the appearance of the 464-nm absorption have been compared by mixing the same enzyme solution with solutions containing respectively I and NADH, and I and NAD^+. The rapid appearance of the intermediate, as per Figure 2, occurs only in the presence of the enzyme–NADH complex. NAD^+ elicits only a small increase in OD on a rapid time scale. This increase (Table I) is approximately 4% of the change obtained with the reduced coenzyme. Furthermore, the rapid process obtained with NAD^+ has an apparent rate which is fivefold slower than the NADH-mediated process. Significant amounts of the chromophore are formed (80% of the NADH change) on a much slower time scale

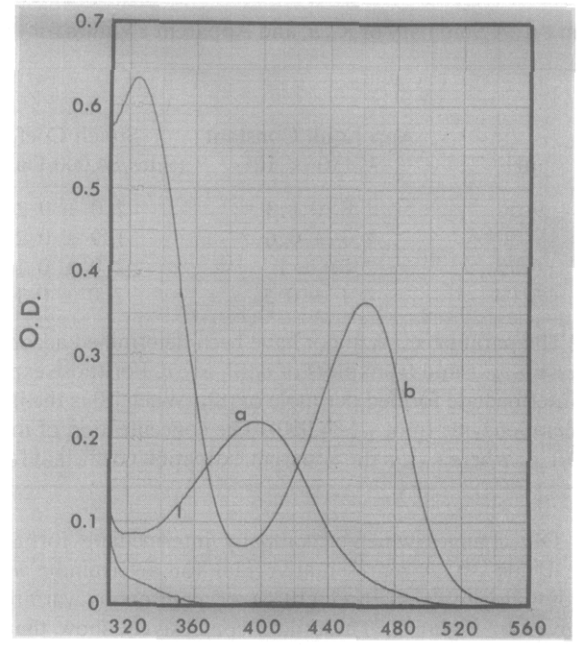


FIGURE 3: Comparison of the spectrum of I in a concentrated solution of horse liver alcohol dehydrogenase (trace *a*) with the spectrum of the intermediate formed on the addition of NADH to the cuvette (trace b) in 0.1 M sodium pyrophosphate buffer (pH 9.36) and 25.0 ± 0.2°. Trace *a* is the spectrum of a solution containing: $[I]_0 = 7.10$ μM and $[E]_0 = 98.0$ μN. Trace b is the spectrum of the reaction mixture obtained on the addition of NADH, final concentration 131 μM, to this solution: The absorption at a λ_{max} of 325 nm, is primarily the absorption contributed by the spectrum of enzyme-bound NADH. (Note that at this pH the chromophore (λ_{max} 464 nm) persists for more than a month time at 4°, see text.) The trace appearing in the lower left-hand corner of the figure is the tail of the enzyme 280-nm absorption band.

($t_{1/2} \simeq 20$ sec). However, control experiments, in which the OD at 330 nm was monitored, demonstrate that NADH in appreciable amounts is produced at the same apparent rate when enzyme and NAD^+ are mixed under these conditions in the absence of I.

Therefore, the slow rate of intermediate formation with NAD^+ appears to be an artifact resulting from the presence of NADH formed *via* the horse liver alcohol dehydrogenase catalyzed oxidation of the residual ethanol present in the preparation. The small amount of intermediate which rapidly forms in the presence of NAD^+ may be due to the presence of a trace impurity of *trans*-4-*N*,*N*-dimethylaminocinnamyl alcohol in the I preparation, or alternatively, to the presence of trace amounts of NADH.

Together these observations indicate that the 464-nm chromophore is formed in a rapid, reversible process involving enzyme, NADH and I, and that the stability of the chromophore to further reaction is dependent on the pH of the reaction mixture. Therefore, a reaction scheme of at least the complexity of eq 8 must be considered

$$E(NADH)_2 + nI \overset{K_1}{\rightleftharpoons} E(I)_n + H^+ \rightleftharpoons \cdots \rightleftharpoons E + NAD^+ + \text{alcohol} \quad (8)$$

The apparent dissociation constant, K_1, the stoichiometry coefficient, *n*, and the apparent molar extinction coefficient, ϵ, are reported in Table II for different pH values. These parameters are based on measurements at 480 nm, a wavelength where the spectrum of I makes a negligible contribution to

TABLE II: A Summary of K_1, n, and Apparent ϵ Values for the I Intermediate as a Function of pH at 25 ± 0.2°.[a]

pH	App Equil Constant K_1 (M) × 10^6	Stoich Coef, n (Sites/84,000 Daltons)	App Extinction Coef, ϵ_{app} $[S]_0/OD_{460}$ (M^{-1} cm^{-1} OD) × 10^{-4}	$[S]_0/OD_{480}$ (M^{-1} cm^{-1} OD) × 10^{-4}	$[E]_0/OD_{480}$ (M^{-1} cm^{-1} OD) × 10^{-4}
9.50	4.8 ± 1.3	2.0 ● 0.2			4.8 ± 2
9.50	4.3 ± 0.6	1.9 ± 0.2	6.2 ± 0.2	4.7 ± 1	
6.75	2.51 ± 1	2.0 ± 0.1	5.3 ± 0.2		
6.04	4.1 ± 0.5	2.0 ± 0.1	6.5 ± 0.2		

[a] The parameters K_1 and n have been determined according to the method of Scatchard (Edsell and Wyman, 1959) from plots of $v/(E)_f$ *vs.* v and from plots of $v/[S]_f$ *vs* . v. For those experiments where [E] is the independent variable, v is given by the moles of intermediate formed per mole of $[S]_t$. When [S] is the independent variable, v is given by the moles of intermediate formed per mole of enzyme (mol wt 84,000). The concentration of intermediate formed was calculated from the relationship [Intermed] = OD/ϵ_{app} where ϵ_{app} is the apparent extinction coefficient for the intermediate at λ_{max} of 460 nm.

the OD changes which accompany intermediate formation (see Figure 3). A representative plot for determining n (according to Job's method (1928) of continuous variations) is given in Figure 4. These data conclusively show that *the intermediate is formed with a stoichiometry of two per enzyme molecule* (*i.e.*, *one per coenzyme binding site*). The concentration dependence of the process adheres to a hyperbolic expression and can be fit adequately by a single hyperbolic constant of $K_1 \simeq 4.0 \times 10^{-6}$ M, independent of pH. The independence of the apparent extinction coefficient when determined by extrapolation to infinite enzyme or by extrapolation to infinite substrate, respectively, provides further conformation of these results.

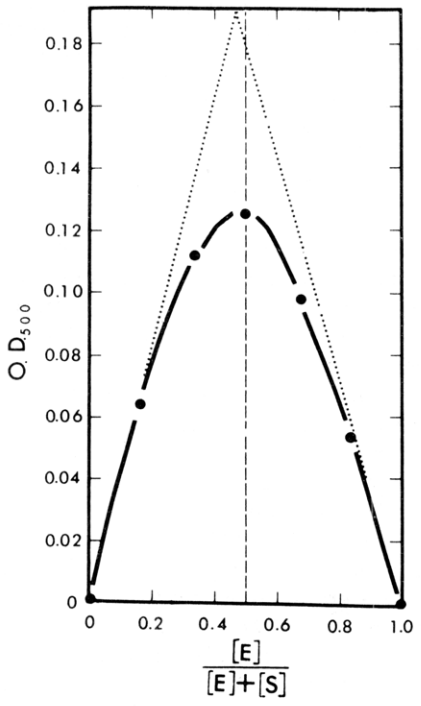


FIGURE 4: The determination of the combining stoichiometry for the formation of the intermediate according to the method of continuous variation (Job, 1928) in 0.1 M sodium pyrophosphate buffer (pH 9.36) at 25.0 ± 0.2°. The sum, ([E] + [S]) = 30.7 μM, was maintained throughout (with [E] expressed as the normality of the enzyme–coenzyme binding capacity of horse liver alcohol dehydrogenase, see Methods). The solid line is the best fit (by eye) of the experimental points. The dotted lines have been drawn as the tangents to the solid line at the limiting values (0 and 1.0) of the ratio [E]/([E] + [S]). The vertical dashed line indicates the abscissa intercept corresponding to a theoretical stoichiometry ratio of 1:1 (*i.e.*, one per enzyme site). The stoichiometry estimated from the point of intersection of the (extended) dotted lines is ~1/site (*e.g.*, 2/84,000 daltons, see text). Optical density measurements were made at 500 nm to eliminate any contribution from the spectrum of I (see Figure 3).

As mentioned above, the stability of the intermediate to further reaction is dependent on pH. An investigation of the concentration and pH dependence of the decay process indicates that in the pH range 6–7, the reaction time course (Figure 5) exhibits an apparent first-order dependence on the concentration of the intermediate, and (apparent) zero-order dependencies on the concentrations of NADH, E, and I when [NADH] > [E] > [I]. Above pH 7.5, the decay process also depends on the enzyme site concentration. Under these conditions, the apparent first-order rate constant for decay initially increases with increasing [E] and then approaches a saturated value when [E] ≫ [I]. As previously discussed, above pH 9 the reaction of I with the enzyme–NADH complex does not proceed appreciably beyond intermediate formation when [E] ≫ [I].

The decay process is subject to a small kinetic isotope effect, $k_H/k_D = 1.2$ (see Figure 5), when deuterium is substituted for

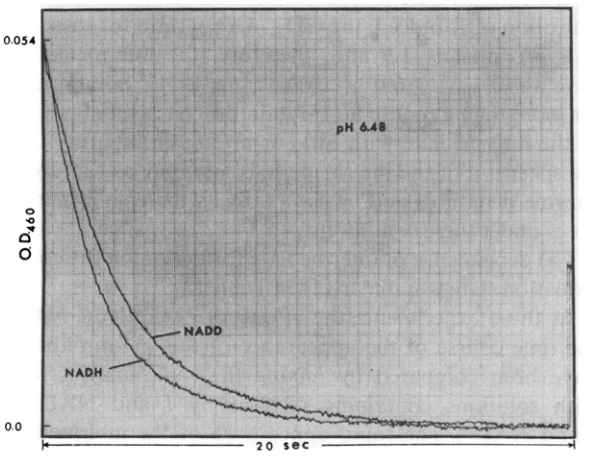


FIGURE 5: Comparison of the effects of deuterium substitution for the α-4-hydrogen of NADH on the time course of the OD change for intermediate decay. Conditions were as follows: $[E]_0 = 4.72$ μN, $[I]_0 = 2.30$ μM, $[NADH]_0 = 155$ μM, $[NADD]_0 = 1.66$ μM, 0.1 M sodium phosphate buffer (pH 6.48), and 25.0 ± 0.2° (see Table III). Note that intermediate formation, *viz.*, Figure 2, is complete during the first 50 msec after mixing. Thus, on the time-scale monitored in these traces, the time course for the formation of the intermediate is compressed against the ordinate axis.

TABLE III: A Comparison of the Effects of Deuterium Substitution for the α-4-Hydrogen of NADH on the Decay Process.

pH	k_H (sec^{-1})	k_D (sec^{-1})	k_H/k_D	Final 460-nm OD		OD^{H} [d]: OD^{D}_{460}
				NADH	NADD	
8.48[a]	$4.14 \pm 0.2 \times 10^{-2}$	$3.41 \pm 0.2 \times 10^{-2}$	1.21	0.0483	0.0396	1.22
6.48[b]	0.385 ± 0.01	0.326 ± 0.02	1.18	0[c]	0[c]	

[a] Conditions: $[E]_0 = 15.7\ \mu N$, $[S]_0 = 2.30\ \mu M$, $[NADH]_0 = 145\ \mu M$, and $[NADD]_0 = 153\ \mu M$. [b] Conditions: $[E]_0 = 4.72\ \mu N$, $[S]_0 = 1.38\ \mu M$, $[NADH]_0 = 155\ \mu M$, and $[NADD]_0 = 166\ \mu M$. [c] The reaction at pH 6.48 goes to completion. [d] The ratio of the final 460-nm OD values gives a measure of the isotope effect on the composition of the system at equilibrium. (Note that this ratio gives the ratio of final concentrations of the intermediate.)

TABLE IV: A Comparison of Steady-State Kinetic Parameters for I and for Selected Horse Liver Alcohol Dehydrogenase Substrates at 25.0 ± 0.2°.

Aldehyde Substrates	pH	K_m^S (M) × 10^6 [a]	K_m^{NADH} (M) × 10^6 [b]	k_{cat}[c] ([S]/N-sec)	K_{eq}[d] × 10^{12}
Benzaldehyde[e]	8.75	20	2.1 ± 0.1	4.0 ± 0.3	40
Azoaldehyde[f]	8.75	4	~2	2.0 ± 0.2	2
β-Naphthaldehyde[f]	8.75	1.5	~2	0.4 ± 0.2	10
Acetaldehyde[g]	8.75	210	2.1	10 ± 1	9.72
I	8.75	8.7 ± 1.5		0.4 ± 0.01	
I	8.19	6.1 ± 1.5		0.078 ± 0.02	50,000
I	7.71	4.9 ± 1.5		0.193 ± 0.05	
I	6.83	4.5 ± 1.5		0.80 ± 0.1	
I	6.13	3.9 ± 1.5	~2	1.78 ± 0.2	

[a] The Michaelis–Menten constant for substrate measured under the conditions $[NADH] \gg K_m^{NADH}$. [b] The Michaelis–Menten constant for NADH measured under the conditions $[S] > K_m^S$. [c] The specific catalytic constant for substrate turnover calculated on the basis of the enzyme normality (as determined by the pyrazole assay, see Methods). [d] Defined as: $K_{eq} = [NADH] \cdot [RCHO][H^+]/[NAD^+][RCH_2OH]$. [e] Values taken from Wratten and Cleland (1965). [f] Values taken from Bernhard *et al.* (1970). [g] Values taken from Sund and Theorell (1962).

the reactive hydrogen of NADH. The data provided in Table III demonstrate that this kinetic isotope effect is pH independent, and that deuterium substitution influences both the rate of approach to the final equilibrium mixture and the final composition of the system at equilibrium.

The steady-state kinetic parameters, K_m^S, K_m^{NADH}, k_{cat}, and the redox equilibrium constant, K_{eq}, for the I system as a function of pH are presented in Table IV along with the corresponding values taken from literature sources for acetaldehyde, benzaldehyde, β-naphthaldehyde, and azoaldehyde. Note that K_m^S and K_I (Table II) are identical within experimental error.

Furthermore, it is noteworthy that the specific rate of I turnover (k_{cat}) is orders of magnitude slower than the turnover rates observed for the other substrates listed in Table IV.

Combined Stopped-Flow Temperature-Jump Kinetic Studies. The relaxations shown in Figures 6 and 7 are typical examples of the records obtained when the combined stopped-flow temperature-jump (SF-TJ) technique is used to investigate the relaxation spectrum for the process of intermediate formation. In all SF-TJ experiments, the heating pulse has been delayed 200 msec from the time of mixing to allow the process of intermediate formation to reach a quasi-equilibrium condition. Therefore, the traces shown in Figures 6 and 7 have time zero defined to coincide with the initiation of the heating pulse.

Within the limitations of the time interval accessible to study (~50 μsec to ~200 msec) and OD resolution of the instrument (~10^{-2}–10^{-3} ΔOD), the relaxation spectrum for intermediate formation contains only a single relaxation.[1] There is no evidence for a rapid, unresolved relaxation in this system, since there is no appreciable OD change during the heating pulse.

Figure 6 compares the relaxation observed at the λ_{max} for I (400 nm) with the relaxation observed at the λ_{max} for the intermediate (460 nm) for the same experiment. The increase in the concentration of I quantitatively accounts for the decrease in the concentration of intermediate. Both progress curves adhere to a first-order (single-exponential) rate expression, and the rate constants ($1/\tau$ values) are identical within experimental error (±15%).

[1] The simplicity of the observed relaxation spectrum is in part a consequence of the experimental conditions. The NADH concentration employed (70–80 μM) is sufficiently in excess of the binary horse liver alcohol dehydrogenase–NADH dissociation constant to ensure saturation of the available enzyme sites at all pH values investigated. Since an overwhelming excess of coenzyme is present, the 13° temperature change causes a negligible perturbation of the concentration of occupied sites. For this reason, NADH dissociation will not contribute to the observed relaxation spectrum because the amplitude of this relaxation is negligible. The relaxations which conceivably could result from subsequent steps in the overall transformation have not been observed because either these relaxations are too slow, or the amplitude of these relaxations are near zero. Note that the heating pulse is timed to occur when the concentrations of the final products (NAD^+ and alcohol) are near zero, and hence, any relaxation involving these species will have a negligible amplitude.

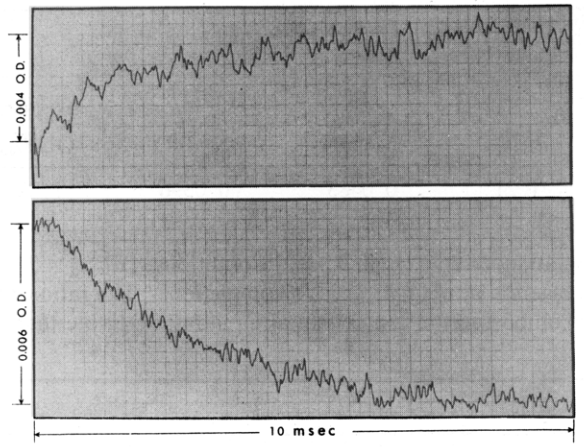


FIGURE 6: Temperature-jump traces of the time course for the change in OD of the I–enzyme–NADH system. The two traces compare the progress curves obtained at 400 nm (upper trace) and at 460 nm (lower trace) for the relaxation of the perturbed system to the new quasi-equilibrium (see text). The heating pulse in this combined stopped-flow temperature-jump experiment has been delayed 200 msec from the time of mixing to allow the freshly mixed reactants to reach an initial, quasi-equilibrium condition with respect to intermediate formation (see Methods). Conditions were as follows: $[E]_0 = 1.82$ μN, $[NADH]_0 = 78.2$ μM, $[I]_0 = 4.54$ μM, and 0.1 M sodium pyrophosphate buffer (pH 8.75). Instrument settings: delay time, 200 msec after mixing; heating pulse width, 100 μsec; capacitor charge, 4 kV; electronic time constant, 50 μsec; initial temperature 18.0 ± 0.5°; ΔT, ~10°. Note that the trace zero time coincides with the initiation of the heating pulse (200 msec after mixing).

Figure 7 documents the concentration dependence of the 460-nm relaxation. It is apparent in Figure 7 that $1/\tau$ increases with increasing I concentration, whereas the amplitude of the relaxation (the total OD change) decreases with increasing I concentration. At I concentrations significantly greater than 30 μM, the maximum I concentration employed in Figure 7, the amplitude of the relaxation becomes indistinguishable from the background electronic noise. This qualitative behavior characterizes the system for the entire pH range investigated (pH 6.13–9.48). The concentration dependence of the relaxation has been quantitated graphically by constructing plots of $1/\tau$ *vs.* the sum $[S_f] + [E_f]$, where $[S_f]$ and $[E_f]$ are respectively the quasi-equilibrium concentrations of I and the enzyme–coenzyme complex (Eigen and de Maeyer, 1963). These results, for all pH values investigated, are summarized in Figure 8. *It is apparent from Figure 3 that all of the data can be fit by the solid straight line within the limits ±25% (as indicated by the error brackets) irrespective of pH.* The data for each pH investigated are represented by a dotted line. For each such line, the spread of the observed $1/\tau$ values at any particular concentration was found to be no greater than ±18% of the mean value. (The error limits for the individual pH values have been omitted from the figure for clarity.)

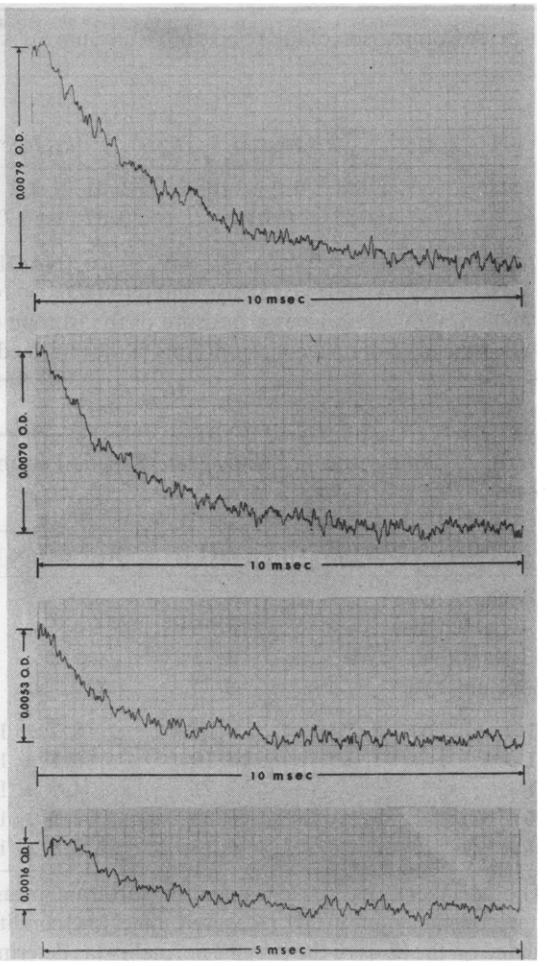


FIGURE 7: The effects of concentration on the relaxation process observed for the I–enzyme–NADH system. The traces record the time course of the OD change at 460 nm for the following I concentrations (from top to bottom): 5.78, 9.55, 18.2, and 27.8 μM. $[NADH]_0$ (76.2 μM) and $[E]_0$ (2.58 μN) were employed throughout. Instrument settings: delay time, 200 msec after mixing; heating pulse width, 100 μsec; capacitor charge, 5 kV; electronic time constant, 50 μsec; initial temperature, 18.0 ± 0.5°; ΔT, ~13.2°. Note that the trace zero time coincides with the initiation of the heating pulse (200 msec after mixing).

TABLE V: A Summary of Slope (k_1) and Intercept (k_{-1}) Values Derived from the Dependence of $1/\tau$ on ($[E_f] + [S_f]$) (See Figure 3) for the Reaction of I with the Horse Liver Alcohol Dehydrogenase–NADH Complex as a Function of pH.[a]

pH	Slope[b] (k_1) (M^{-1} sec^{-1}) × 10^{-7}	Intercept[b] (k_{-1}) (sec^{-1})	$k_{-1}/k_1 = K_1$ (M) × 10^6
6.13	3.6	268	7.4
7.21	4.4	227	5.2
7.65	5.3	280	5.3
8.19	3.8	345	9.1
9.48	5.3	222	4.2

[a] Relaxation measurements were carried out in 0.1 M sodium phosphate buffer below pH 8, and in 0.1 M sodium pyrophosphate buffers above pH 8. The system, initially at quasi-equilibrium at 18° (see text), was perturbed (by a 5-kV discharge through the sample during a 100-μsec interval) to a final temperature of 31.2°. The relaxation to the new equilibrium condition was monitored by following the change in OD at 460 nm. [b] The accuracy of the rate constants, as determined by the maximum deviation from the mean value, is estimated to be better than ±18%.

The individual slope and intercept values for each pH are summarized in Table V. Note that the variation in these values does not display a regular dependence on pH. In fact, the variations are well within the limitations of experimental reproducibility.

The kinetic consequence of substituting specifically labeled α-4-deuterio-NADH (NADD) for the isotopically normal

TABLE VI: A Comparison of the Effect of Deuterium Substitution for the α-4-Hydrogen of NADH on $1/\tau$.

pH	$1/\tau$ (sec^{-1})[c] NADH	NADD	$(1/\tau_H)/(1/\tau_D)$
9.48[a]	690 ± 75	650 ● 60	1.06
6.48[b]	453 ± 45	447 ± 50	1.00

[a] Concentrations: $[E]_0$ = 2.1 μN, $[I]_0$ = 7.50 μM, $[NADH]_0$ = 107 μM, or $[NADD]_0$ = 113 μM. [b] Concentrations: $[E]_0$ = 2.7 μN, $[I]_0$ = 2.38 μM, $[NADH]_0$ = 156 μM, or $[NADD]_0$ = 165 μM. [c] Discharge, 5 kV; electronic time constant, 50 μsec; heating pulse, 100 μsec; pulse delay, 200 msec; and wavelength; 460 nm.

coenzyme has been investigated for pH 9.48 and 6.48. The results of these experiments are summarized in Table VI.

The thermodynamic parameters for intermediate formation at 31°, estimated from the van't Hoff relationship ($\partial \ln K_{eq}/\partial T = \Delta H°/RT^2$) and the expression for $\Delta G°$ ($\Delta G° = -RT \ln K_{eq}$), are: $\Delta G° \simeq -7.3$ kcal/mol, $\Delta H°$ 2.5 kcal/mol, $\Delta S° \simeq 32$ eu. Note that the small value of $\Delta H°$ imparts only a small temperature dependence to K_{eq}, and thus negates the high sensitivity imparted to the system by the large value of the extinction coefficient for the intermediate.

Discussion and Conclusions

Role of NADH. The results of the experiments given above offer a description of the reactions between I and the enzyme–NADH complex that is fully consistent with the designation of the 464-nm transient species as an obligatory chemical intermediate for the aldehyde–alcohol transformation. The observations in support of this conclusion are as follows. (1) The reaction is a very rapid, reversible process. The reaction to form the 464-nm-absorbing species (the intermediate) occurs to a significant extent only in the presence of the reduced coenzyme–horse liver alcohol dehydrogenase complex. (2) Relative to the spectral properties of I (see Figure 3), the spectrum of the intermediate is characterized by a large (66 nm) spectral shift to longer wavelength, and by a large (*ca.* twofold increase in ϵ_{max}. (3) The stoichiometry of intermediate formation (two per enzyme molecule, see Table II) is identical with the stoichiometry of coenzyme binding. (4) The apparent numerical identity of K_m^S to K_1 (*viz.*, Tables II and IV) indicates that the parameter K_m^S is determined by the same, preequilibrium process described by K_1.

It is equally clear that since the decay rate measures the rate at which the chromophore is bleached, the chemical (redox) step follows the appearance of the intermediate, and that the decay process very likely is rate limiting for turnover in the steady state.

It is unlikely that the remarkable specificity requirement which the reaction displays for the reduced coenzyme is the result of a covalent-bonding interaction between I and NADH at the site. The large (66 nm) spectral red shift which characterizes the intermediate can only be the result of bonding interactions which *do not* qualitatively affect the bond hybridization of the carbonyl carbon of I. This constraint eliminates the possibility that the reaction involves covalent-bond formation between NADH and I (*e.g.*, hydride transfer, or nucleophilic attack of the reduced nicotinamide "enamine"

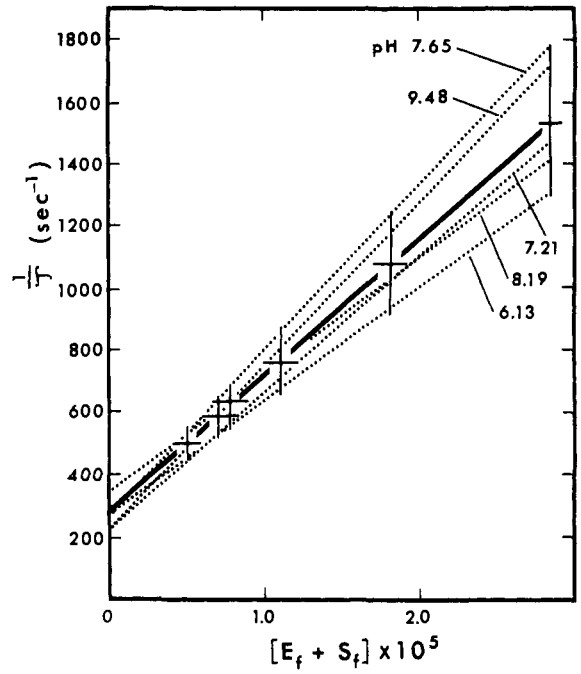


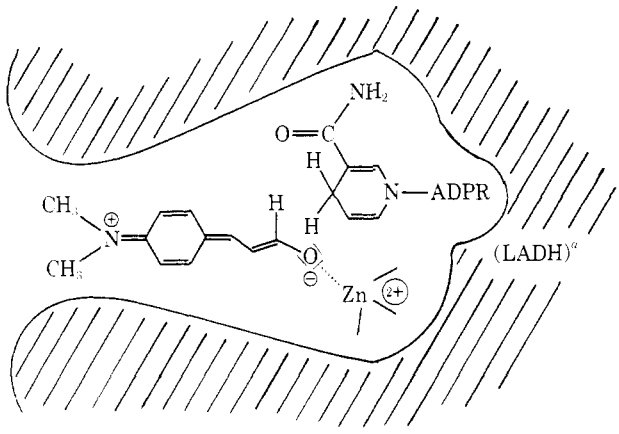
FIGURE 8: A summary of the dependence of $1/\tau$ on concentration as a function of pH. The experiments were carried out with a temperature-jump cuvette system fitted to a rapid-mixing stopped-flow spectrometer (see Methods). Observations were made at 460 nm in solutions buffered with 0.1 M sodium phosphate below pH 8, and with 0.1 M sodium pyrophosphate above pH 8. The system of I–enzyme–NADH, in quasi-equilibrium with the intermediate, was perturbed by a 5-kV discharge over a 100-μsec interval from an initial temperature of 18.0 ± 0.5° to a final temperature of 31.2 ± 0.5°. See Figure 7 for a representative set of progress curves and see the caption to Figure 7 for a description of the general conditions of concentration and the instrument settings employed in these experiments. See Table V for a summary of the slope (k_1) and intercept (k_{-1}) values calculated from these data for each pH.

system on the carbonyl), since all such processes would result in a sp^3 (tetrahedral) hybridization state for C-1, and thereby result in a bleaching of the chromophore spectrum rather than an enhancement. Therefore, specificity must be conveyed by the more subtle, weak-bonding interactions between substrate and site. *Since NAD^+ will not function in place of NADH, the bonding topography between site and substrate must depend on the oxidation state of the bound coenzyme.*

Since NADH is concluded to undergo no covalent change during the formation of the intermediate, the (absolute) requirement for NADH reveals an effector role played by NADH in facilitating site–substrate chemical-bonding interactions. It is of interest to point out that a similar, noncovalent effector role has been reported for the NAD^+-mediated activation of substrate for the glyceraldehyde-3-phosphate dehydrogenase system (Racker and Krimsky, 1952; Malhotra and Bernhard, 1968; Trentham, 1971). Coenzyme-mediated spectral changes which are similar to those described here for the I–horse liver alcohol dehydrogenase system have been reported for a chromophoric substrate analog (β-2-furylacryloylphosphate) when covalently linked to the active sulfhydryl of sturgeon muscle glyceraldehyde-3-phosphate dehydrogenase (Malhotra and Bernhard, 1973).

The selective advantage of providing for both binding and/or bonding specificity on the basis of substrate oxidation state and for the chemical activation of the substrate for further reaction, illustrated by those two systems, suggests that the phenomenon of "noncovalent activation of substrate chemical bonds by effector molecules" (Malhotra and Bernhard, 1973)

SCHEME I



[a] LADH = horse liver alcohol dehydrogenase.

may be a general characteristic of NAD^+-requiring dehydrogenases.

Role of Zinc Ion. The combined SF-TJ kinetic experiments identify the process of intermediate formation as a reversible reaction which proceeds with the stoichiometry of eq 9

$$E(NADH)_2 + 2I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E(I)_2 \qquad (9)$$

$$k_1/k_1 = K_1$$

Over the pH range 6–9.5, the kinetic behavior of this reaction is pH independent. Furthermore, the substitution of deuterium for the reactive hydrogen of NADH has a negligible effect on the kinetics of this process.

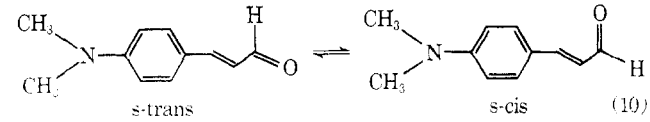
The pH independence of the process, the magnitude of the red shift in the chromophore spectrum, the rapid formation rate, k_1, and the absence of a deuterium isotope rate effect, together place severe restrictions on the possible chemical identity of the intermediate. Indeed, *these findings are consistent only with a structure for the intermediate involving a coordination bond between the essential zinc ion at the enzyme active site and the carbonyl oxygen of I*, as depicted in Scheme I.

The arguments in favor of this bonding scheme and the considerations which eliminate other alternative possibilities are presented in the following discussion.

The (66-nm) perturbation of the chromophore spectrum to longer wavelengths can be explained only if the carbonyl carbon retains a degree of saturation corresponding to sp^2 hybridization. Hydride transfer to give sp^3 hybridization at this carbon can not have occurred since such a transformation would result in complete bleaching of the visible spectrum (*viz.*, the spectrum of the alcohol). The absence of a deuterium isotope effect is consistent with this conclusion.

A cis–trans isomerization about the α,β-acryloyl double bond cannot account for the large red shift. The spectrum of the cis isomer of I has not been reported. However, the comparison of cis and trans isomers for structurally related compounds (Dolter and Curran, 1960; Charney and Bernhard, 1967) shows that cis–trans isomerization causes only a slight variation in the intensity and the position (1–2 nm) of the long-wavelength electronic transition.

An s-trans–s-cis isomerization (Charney and Bernhard, 1967; Bernhard and Lau, 1971) (eq 10) can account for a shift to longer wavelength. However, such an explanation for this system appears inadequate since the observed 66-nm



(10)

(3420 cm^{-1}) shift on intermediate formation is considerably larger than that predicted for the s-cis–s-trans isomerization of other arylacryloyl derivatives, *e.g.*, cinnamaldehyde (1400 cm^{-1}) and furylacrolein (1140 cm^{-1}) (Charney and Bernhard, 1967).

Since the spectrum of the intermediate and kinetics of intermediate formation do not depend on pH (for the pH range investigated), any explanation based on the formation of a protonated derivative of I (*e.g.*, protonation of the carbonyl oxygen) must be discarded. This is true because the transfer of a proton from a (hypothetical) enzyme-site residue (E–X–H) to a bound-substrate species within the ternary complex, according to eq 11, can only be significant if the proton

$$[E\text{–}X\text{–}H(S)] \rightleftharpoons [E\text{–}X^- (S^+\text{–}H)] \qquad (11)$$

affinity of the bound substrate species is comparable to, or greater than, the proton affinity of the site residue.

The relative proton affinities of the substrate and of the site residue in the complex can be estimated if it is assumed that the pK_a of I and the apparent pK_a of the site residue reflect relative proton affinities in the ternary complex. Compound I has a $pK_a \simeq 3$ in aqueous solution.[2] Since the kinetics of intermediate formation are pH independent over the pH range 6–9.5, the hypothetical group, –X–H, would have to have a $pK_a > 9.5$. Therefore, if the above assumptions are valid, then the transfer of a proton from –X–H to I cannot be a stoichiometrically significant process. Thus, only if the relative proton affinities of I and/or the site residue were perturbed by several orders of magnitude relative to their proton affinities in aqueous solution could the proton transfer depicted by eq 11 become stoichiometrically significant. With the possible exception of the chymotrypsin Asp(102)-His(57)-Ser(195) hydrogen-bonding system (Blow *et al.*, 1969; Robillard and Shulman, 1972) there are no precedents in the literature for enzyme-mediated alterations in apparent proton affinities of the magnitude necessary to accomodate the requirements of the intermediate.

Schiff's base formation between I and an enzyme ϵ-aminolysyl (or α-amino-terminal) residue, where the Schiff's base exists in a protonated state, can be eliminated for similar reasons. The neutral Schiff's base can also be rejected as a possibility, since model compounds, *e.g.*, the oxime of I (λ_{max} 338 nm) or the hydrazone of I (λ_{max} 336 nm) exhibit spectra with the long-wavelength transition blue shifted with respect to the spectrum of I (λ_{max} 398 nm).

The absorption spectrum of I, as expected from its extended π-bonding framework and its high dipole moment (μ = 6.4 D, Dolter and Curran, 1960), is highly sensitive to the polarity of the medium. For example, the position of the long-wavelength electronic transition shifts from 398 nm in water to 360 nm in dioxane. Since the spectrum of the intermediate is shifted to longer wavelength, the site environment must be considerably more polar than aqueous milieu.

[2] Protonation of I in aqueous solution, as indicated by the large spectral shift to shorter wavelength, occurs at the $(CH_3)_2$ amino nitrogen. Therefore, it can be concluded that the pK_a for protonation of the carbonyl oxygen of I must have an even lower pK_a value.

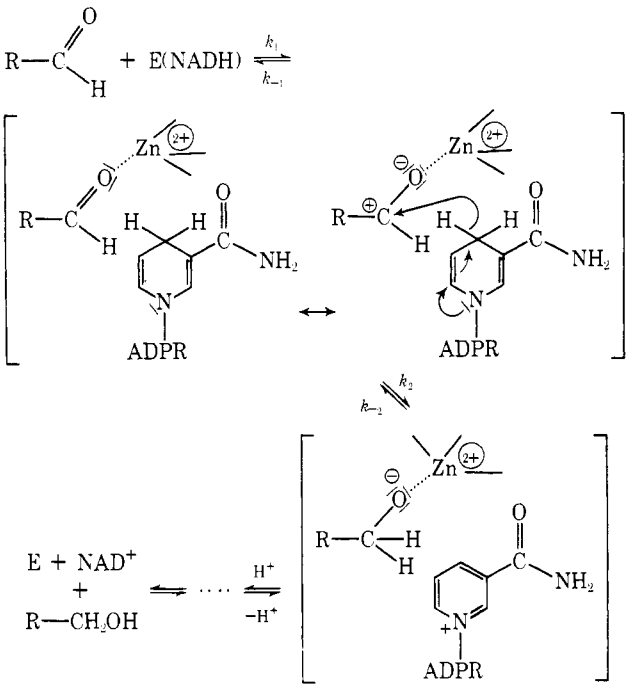
The highly polarized character of the chromophore in the charge-dipole force field resulting from the coordination of the carbonyl oxygen of I to the essential zinc ion at the site can account for the large spectral shift observed for the intermediate. The conclusion that I binds to a highly polar site is consistent with the existence of the well-characterized nonpolar (hydrophobic) pocket presumed to be adjacent to the site. (This hydrophobic pocket has been proposed to be the region responsible for binding the aliphatic or aromatic moieties of substrates and/or inhibitors (Sund and Theorell, 1962; Sigman, 1967; Sarma and Woronick, 1972; Hansch *et al.*, 1972).)

The coordination bond between the carbonyl oxygen of the aldehyde and zinc ion is envisaged to assist hydride transfer *via* activation of the carbonyl group of the substrate for reduction by NADH by increasing the electrophilicity of the carbonyl carbon, as illustrated in Scheme II.

The requirement of zinc ion for horse liver alcohol dehydrogenase activity has been well documented (Kagi and Vallee, 1960; Åkeson, 1964; Oppenheimer *et al.*, 1967; Drum *et al.*, 1969; Drum and Vallee, 1970; Iweibo and Weiner, 1972; Coleman *et al.*, 1972). The involvement of zinc ion as a Lewis acid catalyst in the catalytic mechanism has been suggested by many investigators (Ables *et al.*, 1957; Wallenfels and Sund, 1957; Theorell and McKinley-McKee, 1961; Creighton and Sigman, 1971). Nevertheless, the results presented here offer the first direct chemical evidence documenting a Lewis acid catalytic role for the essential horse liver alcohol dehydrogenase Zn^{2+}. If zinc ion has a Lewis acid function in facilitating the chemical reaction, *then the most reasonable mechanism for the redox step is a direct transfer of hydride ion from NADH to the activated carbonyl according to the mechanism of Scheme II.*

If it is assumed that the chemical mechanism of Scheme II applies to all aldehyde substrates, then the detection of the coordination complex will depend on the stability of the complex and on the velocity of the hydride-transfer step. Thus, if the reaction to form the intermediate has a favorable equilibrium constant (*i.e.*, if $k_{-1}/k_1 \leq [E]$) and if the hydride-transfer step is relatively slow (*i.e.*, if $[S]k_1 > k_2$), then during the early stage of the reaction the coordination complex will be formed in appreciable amounts as a metastable intermediate. However, if $k_2 > [S]k_1$, or if complex formation is not favorable (*i.e.*, if $k_{-1}/k_1 > [E]$), the complex will not be formed in appreciable amounts, and hence will not be detectable by direct observation. The latter seems to hold for most aldehydes whether or not they are aliphatic or aromatic. The reason the reaction with I proceeds to yield detectable amounts of the coordination complex undoubtedly has its origins in the unusual stability of the resultant complex. The dimethylamino nitrogen of I (as indicated by the previously noted large dipole moment of the molecule) is conjugated through the ring and the acryloyl double bond with the carbonyl group (Ingold, 1969). Within the I complex, the partial positive charge induced by coordination of the carbonyl oxygen to zinc ion is extensively delocalized over the π-bonding system. Accordingly, the dipolar quinodial resonance structure, *cf.* Scheme I, largely describes the electron density distribution for the chromophore π system. Since for most aldehydes extensive resonance stabilization of the positive charge is energetically unfavorable, the analogous coordination complexes must be significantly elevated in energy relative to the I complex. Indeed, the activated character of these complexes is a prerequisite for the facile transfer of hydride ion from NADH to the carbonyl carbon. Thus, the relatively

SCHEME II



slow rate of reduction exhibited by I likely is attributable to the stability of the coordination complex.

Both the kinetic behavior and the stoichiometry of the reaction (eq 9) demonstrate that the two enzyme sites function as identical and independent units in the formation of the intermediate. This result is in contrast to our previous findings which strongly suggest that the two sites become catalytically nonequivalent during a single turnover of sites (Bernhard *et al.*, 1970; Dunn and Bernhard, 1971). The initial experimental observations which led us to this conclusion have been further amplified by the work of Brändén *et al.* (1973), McFarland and Bernhard (1972), Luisi *et al.* (1972), Everse (1973), and Czeisler and Hollis (1973).

While the differences between the behavior of I and other aromatic aldehydes (*e.g.*, benzaldehyde, β-naphthaldehyde, and the azoaldehyde) is not apparent in full detail, it is apparent that the horse liver alcohol dehydrogenase catalyzed reduction of I has a free-energy profile quite different from the previously investigated systems. The intermediate formed from the reaction of I with the enzyme–NADH complex, as evidenced by the large spectral shift and the absence of an isotope effect, is concluded to be an event which is not kinetically discernible for the above-mentioned aldehydes. These aldehydes undergo a rapid (burst) reaction when mixed with the enzyme–NADH complex. This burst reaction has been shown to result in the oxidation of NADH and the formation of alcohol (Bernhard *et al.*, 1970), and the burst has been shown to be subject to a primary deuterium isotope effect when NADD is used in place of NADH (McFarland and Bernhard, 1972). According to these phenomonological differences, the two processes (intermediate formation, and burst reaction) must reflect different steps in the chemical transformation. With reference to the mechanism proposed in Scheme II, these steps would appear to be, respectively, the formation of the coordination bond to Zn^{2+}, and the transfer of hydride from NADH to the substrate.

Since the kinetic evidence for site nonequivalence is associated only with the hydride-transfer step, it can be argued that nonequivalence is manifest catalytically during the chemi-

cal transformation, and, therefore, issued from a chemically triggered event. If this is the case, then the conversion of NADH to NAD^+ is the chemical event most likely to function as the "trigger." The production of the positively charged nicotinamide ring, and the accompanying subunit structural changes necessary for the accommodation of the charge could result in conformational changes which propagate to the second site and render it inactive.

Alternatively, it may be argued that the unique properties of I so alter the mechanism that site nonequivalence is not expressed. Or, it may be that the original conclusion that the horse liver alcohol dehydrogenase sites become catalytically nonequivalent is incorrect. If this were true, *reasonable* alternative arguments would need be developed to rationalize the experimental observations on which the hypothesis of horse liver alcohol dehydrogenase site nonequivalence is based. This point will need further experimental testing, and a continuing effort to do so is underway in this laboratory.

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