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Rapid Kinetic Evidence for Adduct Formation between the Substrate Analog *p*-Nitroso-*N*,*N*-dimethylaniline and Reduced Nicotinamide–Adenine Dinucleotide During Enzymic Reduction*

M. F. Dunn† and Sidney A. Bernhard

ABSTRACT: In this study, the intense chromophore *p*-nitroso-*N*,*N*-dimethylaniline (NDMA) has been investigated as a substrate analog for equine liver alcohol dehydrogenase (LADH). The LADH-catalyzed reduction of NDMA by reduced nicotinamide–adenine dinucleotide (NADH) is accompanied by the formation of 1 mole of NAD$^+$/mole of NADH and NDMA consumed. The NDMA reduction product is (tentatively) assigned as *p*-*N*,*N*-dimethylaminophenylhydroxylamine on the basis of its uv spectrum, and on the forementioned stoichiometric relationships. At pH 8.75, the steady-state NDMA turnover number is 2.4-fold greater than the acetaldehyde turnover number. The Michaelis constant, $K_m^s = 7 \times 10^{-7}$ M, for NDMA is the smallest yet reported for an LADH substrate. The favorable spectral properties of this system allow the independent observation of both NDMA and NADH disappearance during reaction. The transient-state kinetic course of NDMA reduction by NADH has been investigated at pH 8.75 (0.05 M sodium pyrophosphate buffer), using a rapid-mixing stopped-flow spectrophotometer. Under the conditions of concentration [NDMA] > [E] > [NADH], the disappearance of NDMA occurs *via* two transformations which are clearly distinct in rate. NDMA disappears to the same apparent product in each transformation step in an amount equal to *one-half* of the (limiting) NADH concentration. Under the condition [NADH] > [E] > [NDMA], however, the disappearance of NDMA occurs *via a single* (apparent first-order) *kinetic process*. The rate of this transformation has a first-order dependence on the NADH-saturated enzyme-site concentration (over the accessible enzyme concentration range 4×10^{-7}–2×10^{-5} N). The assumed specific rate for this process is calculated to be 2×10^7 M^{-1} sec^{-1}. When both [NADH] and [NDMA] ≫ [E], a presteady-state "burst" reaction results in the disappearance of NDMA in an amount equivalent to *one-half* of the (limiting) concentration of enzyme sites. A comparison of the rate of NADH disappearance (at 330 nm) with the rate of NDMA disappearance (at 440 nm) under a variety of conditions demonstrates, by the nonequivalence of the two processes, an obligatory metastable intermediate. The intermediate can be formed in amounts in excess of the active-site concentration, and thus it can be released from the enzyme into free solution as a complex formed from the reactants.

A necessary condition for the comprehension of enzyme catalysis is a detailed knowledge about the transformation of reactant to product at the active site. Such knowledge includes the chemistry of any intermediates which may intervene.

In systems where a stable intermediate cannot be trapped, it has been informative to examine the transformation by combining uv–visible spectrophotometry or fluorometry with rapid kinetic techniques. In kinetic studies using the rapid-mixing stopped-flow spectrophotometer, it is useful to work under conditions of [E] > [S] (*i.e.*, the transient state). Frequently reaction is limited to a single turnover of sites under these conditions, and as a consequence, the kinetic behavior is simplified. Furthermore, these conditions favor the observation and chemical identification of individual steps in the catalytic mechanism.

In the present study, the intense chromophore, I, *p*-nitroso-*N*,*N*-dimethylaniline (NDMA), is introduced as an aldehyde substrate–analog for horse liver alcohol dehydrogenase (LADH). The use of NDMA as a substrate–analog has the advan-

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[1] Abbreviations used are: NDMA, *p*-nitroso-*N*,*N*-dimethylaniline; LADH, horse liver alcohol dehydrogenase; NADH, reduced nicotinamide–adenine dinucleotide.

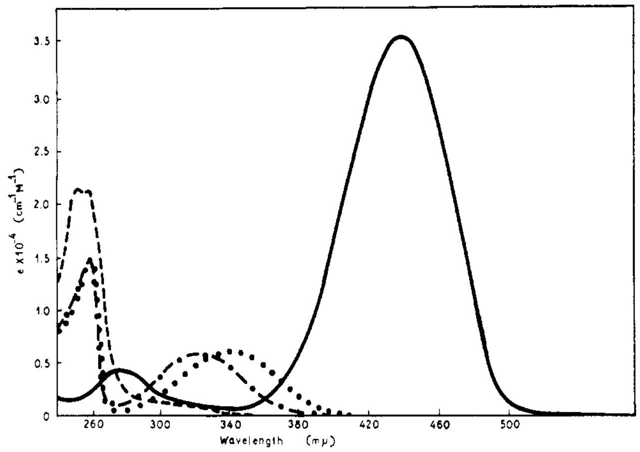
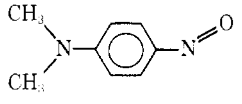


FIGURE 1: A comparison of the spectrum of NDMA (I) (——) with the spectrum of the enzymatic reduction product (II) (– – –), and with the spectrum of NADH free in solution (• • • •), and LADH bound (• – • –). These spectra were measured in 0.05 M sodium pyrophosphate buffer (pH 8.75) at 25 ± 0.1°. (See Experimental Section for a detailed description of conditions and procedures.)

tage of providing a system for study with excellent uv–visible light absorption properties (see Figure 1, Results). It should be noted that the substitution of the nitroso moiety for the aldehyde carbonyl introduces *no new steric parameters at the residue undergoing reduction.*



I

Much of the chemistry of "stable" nitroso compounds, particularly aromatic nitroso compounds, is analogous to the chemistry of aldehydes. The nitroso and carbonyl groups are both isosteric and isoelectronic. The bond dipoles of both groups have the same relative ground-state orientation (with a partial negative charge residing on oxygen). It is significant that many reactions involving the nitroso group proceed mechanistically *via* nucleophilic attack on nitrogen (Smith, 1966).

In this paper, the chemical course of the LADH-catalyzed reaction between NDMA and NADH is reported. The transient kinetic course of the reaction has been studied in detail with the use of the rapid-mixing stopped-flow apparatus. The results of the present work support and extend the results and conclusions of our previous work on aromatic aldehyde substrates for LADH (Bernhard *et al.*, 1970). In addition, evidence supporting the formation of a covalent intermediate between NDMA and NADH during catalysis is presented.

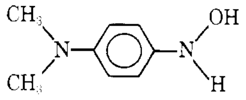
Experimental Section

Materials. The commercially available compounds acetonitrile (Baker Chemical Co., reagent grade), *p*-nitroso-*N*,*N*-dimethylaniline (NDMA) (Aldrich Chemical Co.), NADH, and NAD$^+$ (Boehringer Mannheim Corp., Grade I) were used without further purification. The concentrations of NDMA (see below), NADH, and NAD$^+$ were determined spectrophotometrically, using the appropriate molar extinction coefficients. Rabbit muscle D-glyceraldehyde 3-phosphate dehydrogenase (rabbit GPD) freshly prepared according to the method of Ferdinand (1964) was a gift from Dr. R. A. MacQuarrie.

LADH (Boehringer Mannheim Corp.) was further purified as previously described (Bernhard *et al.*, 1970). The LADH concentration was determined by the NAD–pyrazole assay procedure (Theorell and Yonetani, 1963), and is reported throughout as N, the normality of the coenzyme binding sites.

Spectral Determinations. The uv–visible spectra reported in Figure 1 were obtained with the Cary Model 14 spectrophotometer. The spectrum of NDMA was measured on a solution prepared by dilution of a 20-μl aliquot of an acetonitrile stock (1×10^{-3} M) into 3 ml of 0.05 M sodium pyrophosphate buffer (pH 8.75). The NDMA molar extinction coefficient (ϵ_{440}) was obtained from a study of the dependence of optical density (OD) on concentration. From this study, it was established that the Beer–Lambert law is obeyed over the accessible (2×10^{-7}–6×10^{-5} M) concentration range. The position and intensity of the long-wavelength π,π^* absorption band for the neutral molecule are sensitive to solvent polarity, and shifts from a visible λ_{max} in H_2O at 440 nm (ϵ 3.54×10^4 OD M^{-1} cm^{-1}) to a visible λ_{max} in dioxane at 412 nm (ϵ 3.16×10^4 OD M^{-1} cm^{-1}).

The spectrum of the NDMA reduction product, presumably *p*-*N*,*N*-dimethylaminophenylhydroxylamine (II), was obtained by the addition of excess ethanol (10 μl of 95% ethanol to 3 ml) to a reaction mixture containing 3.86×10^{-5} M NDMA and catalytic amounts of LADH (1.30×10^{-7} N) and NAD$^+$ (3.20×10^{-7} M). Under these conditions, the chemical potential of the ethanol–acetaldehyde redox reaction is used to recycle the catalytic amount of coenzyme present, and thus drive the reduction of NDMA to completion. The concentrations of the other components (LADH, coenzyme, acetaldehyde, and ethanol) remaining on completion of reaction were such that their contributions to the spectrum of II are negligible.



II

NAD$^+$ Assay. The amount of enzymatically reducible dinucleotide (NAD$^+$) produced during reaction was determined in the following way. LADH (470 μl of 1×10^{-5} N) was added to 10 ml of 0.1 M sodium pyrophosphate buffer (pH 8.80), containing 1.00×10^{-4} M NDMA and 1.02×10^{-4} M NADH. After completion of reaction, a 1-ml aliquot of the resulting solution was mixed with 2 ml of 1×10^{-2} M sodium arsenate (pH 8.9 pyrophosphate buffer) and 200 μl of 1×10^{-2} M D-glyceraldehyde 3-phosphate. This solution was then assayed for the amount of NAD$^+$ present by following the appearance of the NADH absorption band at 340 nm after the addition of 25 μl of a 10-mg/ml suspension of freshly prepared rabbit GPD. At 340 nm, an OD change of 0.185 corresponding to 2.98×10^{-5} M NADH was found as compared to an expected 2.92×10^{-5} M NADH for the stoichiometric reaction sequence. A control solution to which a known 3.09×10^{-5} M NAD$^+$ (final concentration) had been added gave an observed OD change at 340 nm corresponding to 3.06×10^{-5} M of NADH produced in the assay.

Identification of Reaction Product. The product of NDMA reduction was tentatively identified as *p*-*N*,*N*-dimethylaminophenylhydroxylamine (II) on the basis of the similarity of its spectrum to that of the close structural analog *N*,*N*-dimethyl-*p*-phenylenediamine (a spectrum markedly distinct from that of the corresponding nitroso compound, I), and on the basis of the above observations that NDMA and NADH react in equimolar amounts to give 1 molar equiv of NAD^+. The labile character of compounds of the 1,4-diaminobenzene structure toward air oxidation (Smith, 1965) has thus far prevented the isolation and characterization of the NDMA enzymic reduction product. Attempts to prepare an authentic sample of II *via* the nonenzymatic reduction of NDMA have thus far failed. NDMA is not reduced by $NaBH_4$ under mild conditions (basic aqueous media at room temperature). Under more forceful conditions ($NaBH_4$ in isopropyl alcohol at 80°) the product of reaction is *N*,*N*-dimethyl-*p*-phenylenediamine, in accord with the findings of Boyer and Ellzey (1960).

Rate Measurements. Steady-state initial velocity measurements for the determination of the Michaelis–Menten parameters V_{max}, $K_m{}^S$, $K_m{}^{NADH}$ and for inhibition studies with NAD^+ were made with the use of the Beckman DB spectrophotometer and the Sargent SRL recorder modified to allow a 0.05-OD full-scale expansion. Rate measurements for the NDMA system at 330 nm (ϵ_{330} 5.85 × 10^3 OD M^{-1} cm^{-1}) and 440 nm (ϵ_{440} 3.54 × 10^4 OD cm^{-1} M^{-1}) gave identical initial velocities, within the limitations of experimental error, for conditions of dilute enzyme ($\sim 10^{-9}$ M). Note (Figure 1) that contributions from NDMA and the final product, II, to the 330-nm extinction cancel. The NDMA and acetaldehyde turnover numbers were directly compared by measuring the changes in OD at 330 nm, as well as the changes in OD at 440 nm, using identical stocks of buffer, NADH, and LADH. In each case, to assure that the "turnover numbers" were indeed measured at concentrations of "invariant" reactant sufficient to saturate the enzyme site, the same concentration-dependent rate studies were repeated at several concentrations of invariant reactant.

The V_{max} values reported in Table I (Results) represent the average of four separate determinations each. Similarly, the K_m values in Table I and the NAD^+ product inhibition studies each are the average of three independent determinations involving triplicate initial velocity measurements at four or five coenzyme or substrate concentrations (and at four different NAD^+ concentrations for the inhibition studies). In general, concentrations were varied from values corresponding to 0.1–15 K_m. The NAD^+–inhibition studies were carried out at NAD^+ concentrations of 0, 1, 2, and 5 × 10^{-4} M.

Transient Kinetic Measurements. Stopped-flow rapid-mixing kinetic studies were generally carried out as previously described (Bernhard *et al.*, 1970). In the present study, solutions of enzyme, NADH, and buffer were premixed and reacted in the stopped-flow apparatus with the appropriate buffered NDMA solution.

Rates of reaction and concentration changes have been calculated from the stopped-flow storage oscilloscope traces as previously described (Bernhard *et al.*, 1970).

Results

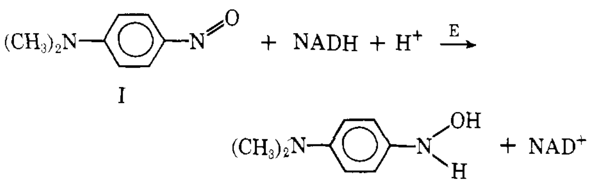
Spectral and Chemical Properties. The spectrum of *p*-nitroso-*N*,*N*-dimethylaniline (NDMA) and the corresponding enzymic reduction product, together with the spectrum of NADH (free and LADH bound), are given in Figure 1 for comparison. The excellent spectral properties of this system allow the independent observation of NADH at 330 nm and NDMA at 440 nm during reaction. The large $\Delta\epsilon_{440\,nm}$ (3.54 × 10^4 OD M^{-1} cm^{-1}) for the reduction of NDMA permits the investigation of active-site–substrate stoichiometry and rate relationships over the 0.1–20 μM concentration range.

TABLE I: A Comparison of the Steady-State Parameters of NDMA and Acetaldehyde, pH 8.75 Pyrophosphate Buffer (0.05 M), and 25 ± 1°.

Michaelis (Steady-State) Parameters	Substrate	
	NDMA[a]	Acetaldehyde
$K_m{}^S$ (M)	7 × 10^{-7}	2.1 × 10^{-4}[b]
$K_m{}^{NADH}$ (M)	2 ± 1 × 10^{-6}	2.1 × 10^{-6}[b]
V_{max}[c]	24 ± 2	10 ± 1[a]

[a] Experimental values determined in the present study (see Experimental Section). [b] Values taken from Sund and Theorell (1962). [c] V_{max} in units of molarity of substrate (converted) per sec per N, where N is the normality of enzyme sites (see Experimental Section).

Reduction of NDMA by NADH occurs only in the presence of LADH. Solutions of NDMA and LADH are stable, although an appreciable, uncatalyzed reaction between NDMA and NADH occurs at low pH. This pH dependent reaction is negligible ($t_{1/2}$ = >10 hr) at the pH values used in this study. The ultimate chemical course of the LADH-catalyzed reduction of NDMA most probably takes place according to eq 1.



(1)

The formulation of eq 1 is based on the following evidence. NDMA and NADH in the presence of catalytic (nanomolar) amounts of LADH react in equimolar proportions. The complete loss of the intense π,π* transition at 440 nm characteristic of the conjugated nitroso moiety accompanies the reaction. The spectrum of the reduction product, presumed to be II (Figure 1), is very similar to the characteristic uv spectrum of *N*,*N*-dimethyl-*p*-phenylenediamine, a close structural analog (λ_{max}^{ETOH} 250 nm, ϵ_{max} 3.0 × 10^{-4} OD M^{-1} cm^{-1}; shoulder at 313 nm, ε 4.8 × 10^3 OD M^{-1} cm^{-1}; Grammaticakis, 1951). The product of NADH oxidation is established as NAD^+ by its quantitative function in the rabbit muscle glyceraldehyde 3-phosphate dehydrogenase catalyzed oxidation of glyceraldehyde 3-phosphate (see Experimental Section).

The reverse reaction, oxidation of the hydroxylamine product by NAD^+ (eq 1), is not observed even in the presence of millimolar NAD^+. The irreversibility of this reaction likely is due to an unfavorable redox potential. The intervention of a strongly inhibitory ternary complex analogous to the hydroxylamine–NAD^+–LADH complex (Kaplan and Ciotti, 1954) can be excluded as a possibility, since pronounced product inhibition by NAD^+ is not observed (Table I).

Steady-State Kinetics. A preliminary investigation of the substrate (NDMA) and the coenzyme (NADH) Michaelis–Menten parameters, derived from initial velocity measure-

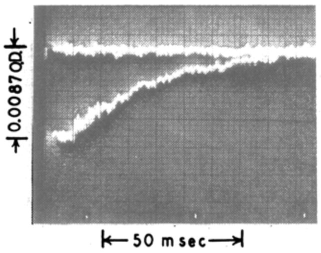


FIGURE 2: An oscilloscope trace typical of the progress curves obtained for the change in transmission at 440 nm for the LADH-catalyzed reduction of NDMA by NADH when the concentration of NDMA (I) is limiting. Conditions: $[\text{NDMA}]_0$, 2.4×10^{-7} M; $[\text{LADH}]_0$, 1.2×10^{-6} N; $[\text{NADH}]_0$, 8.0×10^{-5} M (0.05 M sodium pyrophosphate (pH 8.75) at 25 ± 0.1°). The initial OD is corrected for the instrument mixing dead time, ~3 msec.

ments, are given in Table I. As reference, the corresponding values for acetaldehyde as substrate are included. NDMA compares quite favorably with acetaldehyde as an LADH substrate. *Indeed, a 2.4-fold greater limiting velocity is attained for NDMA and rate saturation obtains at much lower substrate concentrations.* The NDMA Michaelis constant, K_m^S, of 7×10^{-7} M is the smallest to date reported for an LADH substrate. The independence of the parameter K_m^{NADH} on the structure of the substrate is notable, and in accord with the findings of others for both aliphatic and aromatic aldehyde substrates[2] (Wratten and Cleland, 1963, 1965; Theorell and McKinley-McKee, 1961; Dalziel, 1962).

Transient-State Kinetics at 440 nm. Under conditions of $[\text{NADH}] \gg [\text{E}] > [\text{NDMA}]$, *the rapid disappearance of NDMA (Figure 2) is characterized by an exponential (first-order) decay of substrate.* Completion of reaction is accompanied by an optical density change that corresponds to the total disappearance of NDMA. The apparent rate of reaction, k_{app}, has a first-order dependence on the concentration of coenzyme saturated sites over the range 4×10^{-7}–2×10^{-5} N enzyme (see Figure 3). At higher enzyme concentrations, the rate of transformation exceeds the time resolution capabilities of the instrument. Assuming the rate expression $k_{\text{app}} = k(\text{E})(\text{S})$, where k is the specific second-order rate constant, a value for k of 2×10^7 N^{-1} sec^{-1} is obtainable from the slope of the plot in Figure 3. This magnitude is typical of values generally attributed to diffusion controlled rates of reaction between substrates and enzyme active sites (Eigen and Hammes, 1963).

When NADH is the limiting reactant, *i.e.*, under conditions of $[\text{NDMA}] > [\text{E}] > [\text{NADH}]$, *the optical density changes observed at 440 nm occur via two distinct kinetic processes of equal amplitude* (Figure 4). This observation is independent of the NDMA concentration so long as $[\text{NDMA}] > [\text{E}]$ for the concentration range investigated (2×10^{-6}–2×10^{-5} M NDMA). Completion of reaction corresponds to the disappearance of NDMA in an amount equivalent to the total (limiting) NADH concentration. Under the conditions employed in Figure 4 these two kinetic processes have apparent first-order specific rates of 125 ± 25 and 4.5 ± 0.5 sec^{-1}, respectively.

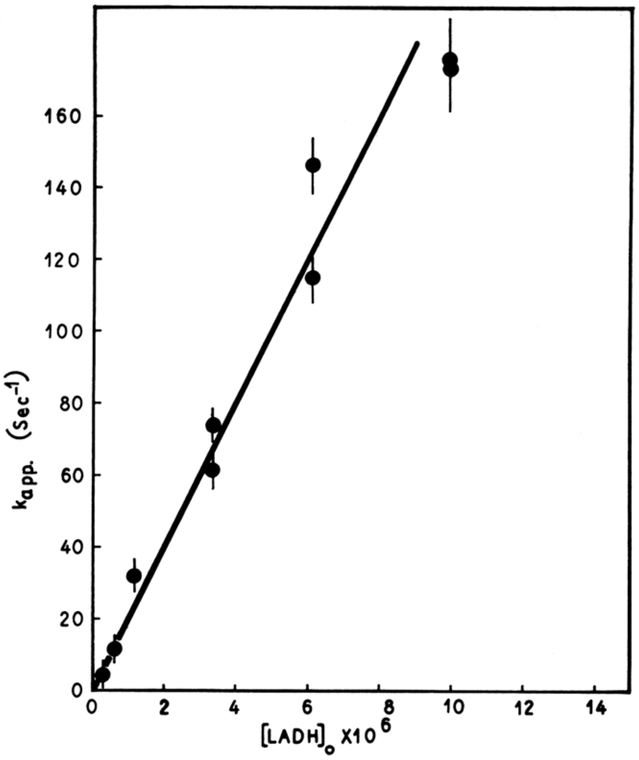


FIGURE 3: Dependence of the rate of NDMA disappearance (k_{app}) at 440 nm on the concentration of NADH-saturated enzyme sites for the LADH-catalyzed reduction of NDMA by NADH. Values of k_{app} were calculated by hand from oscilloscope traces (*e.g.*, Figure 2, see Experimental Section). Conditions: $[\text{NDMA}]_0$, 2.4×10^{-7} M; $[\text{NADH}]_0$, 8.0×10^{-5} M (0.05 M sodium pyrophosphate (pH 8.75) at 25 ± 0.1°). A second-order rate constant of 2×10^7 M^{-1} sec^{-1} is calculated from the slope of the solid-line fit.

When the amplitude of the rapid initial step (the burst), expressed as moles/l. of NDMA consumed, is measured as a function of the NADH concentration and plotted as in Figure 5, a curve analogous to a stoichiometric titration curve is obtained (Bernhard *et al.*, 1970). The ordinate intercept at the titration end point (P_{max}) is a measure of the stoichiometry relationship between the moles of reactant consumed in the burst and the enzyme-site concentration. The abscissa intercept (ϕ) is a measure of the stoichiometry relationship between the equivalents of enzyme sites required for the burst process and N the equivalents (normality) of enzyme–coenzyme binding sites (as measured by the pyrazole–NAD$^+$ assay (see Experimental Section)). Of particular note in Figure 5 is the initial slope value (m) of 0.5 mole of NDMA consumed per mole of NADH, the value of 0.5 for the number of equivalents of NDMA consumed per NADH saturated site (P_{max}/N), and the one-to-one correspondence of ϕ and the enzyme normality, N. It is clear that these features of the transient behavior of NDMA as a LADH substrate parallel those previously described for several aldehyde substrates (Bernhard *et al.*, 1970).

Transient-State Kinetics at 330 nm. The spectral band characteristic of NADH (Figure 1) is the major contributor among the reactants and catalyst (>80%) to the 330-nm extinction in this system. Therefore, the time course of the 330-nm optical density changes might be expected to primarily reflect the chemical changes taking place in the reduced nicotinamide ring during oxidation to NAD$^+$, provided that reaction proceeds strictly according to eq 1.

Transient kinetic experiments under a variety of conditions demonstrate that *the time course of the optical density changes*

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[2] In contrast to the strictly competitive inhibition of NADH by NAD$^+$ generally observed in both aromatic and aliphatic aldehyde substrate systems, NAD$^+$ inhibition of NDMA reduction by NADH is complex. NAD$^+$ is a mixed competitive inhibitor with respect to NADH in this system.

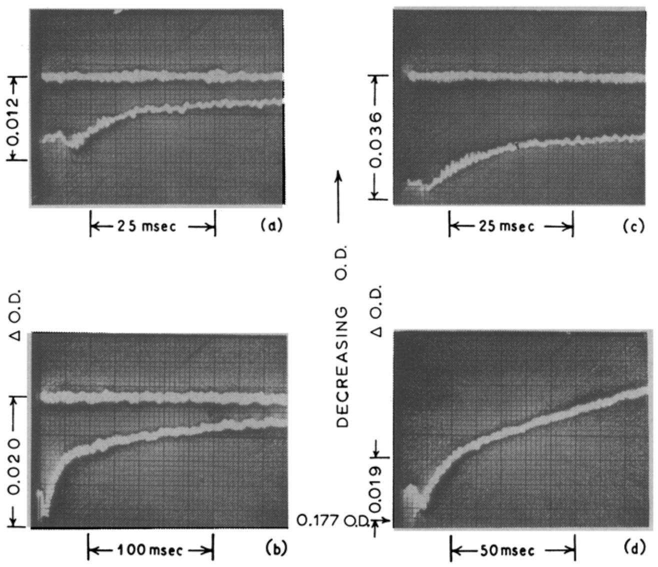

FIGURE 4: Oscilloscope traces of the progress curves for the change in transmission at 440 nm as a function of the NADH concentration for the LADH-catalyzed reduction of NDMA by NADH. Conditions: $[\text{NDMA}]_0$, 4.97×10^{-6} M; $[\text{LADH}]_0$, 1.06×10^{-6} N; $[\text{NADH}]_0$ trace (a), 3.40×10^{-7} M; (b) 5.64×10^{-7} M; (c) 1.04×10^{-6} M; and (d) 4.60×10^{-6} M (0.05 M sodium pyrophosphate (pH 8.75) at 25 ± 0.1°).

at 330 nm does not parallel the 440-nm changes. The traces in Figure 6 compare the time course of the optical density changes at 330 nm with the changes at 440 nm in the same experiment. In this experiment, there is a threefold excess of NDMA, and a sevenfold excess of NADH over the enzyme-site concentration. *It is clear from this comparison that the disappearance of NDMA and the disappearance of NADH are not concomitant processes.*

Indeed, the disappearance of absorbing material at 440 nm is complete before any appreciable decrease in optical density has occurred at 330 nm. However, the total optical density change at 330 nm on completion of reaction is that expected for the conversion of NADH to NAD^+ (in an amount equivalent to the initial NDMA concentration according to the stoichiometry of eq 1). Also, the steady-state rate ($V_{\max}$), calculated from the slope of the linear (zero-order) regions of the progress curves at 440 nm (Figure 6a), is in good agreement with the value determined under the normal steady-state conditions with dilute enzyme (Table I).

Under conditions where the enzyme site concentration exceeds either the NADH or the NDMA concentrations (*e.g.*, the transient state), the 330-nm changes occur as slow ($k_{\text{app}} \sim 10^{-1}\ \text{sec}^{-1}$) exponential (first-order) *decreases* in OD. The total OD change corresponds to the conversion of NADH to NAD^+ in amounts equivalent to the change in NDMA concentration as measured by the relatively rapid 440-nm changes (see Figures 2 and 4) in the same experiment.

Discussion

In our previous transient kinetic studies on LADH (Bernhard *et al.*, 1970) the transient reduction of aromatic aldehyde by an excess of enzyme–coenzyme was found to occur *via* two kinetically distinct processes with half the chemical transformation taking place in each step. Under steady-state conditions of concentrations ($[\text{S}]_0 \gg [\text{E}]_0$) a presteady-state burst reaction was found to yield one-half an enzyme site equivalent of product. From these observations, it was concluded that there can exist an inhomogeneity in enzyme–NADH–aldehyde sites in the dimeric enzyme. Since this site nonequivalence is manifest in the transient reaction even when

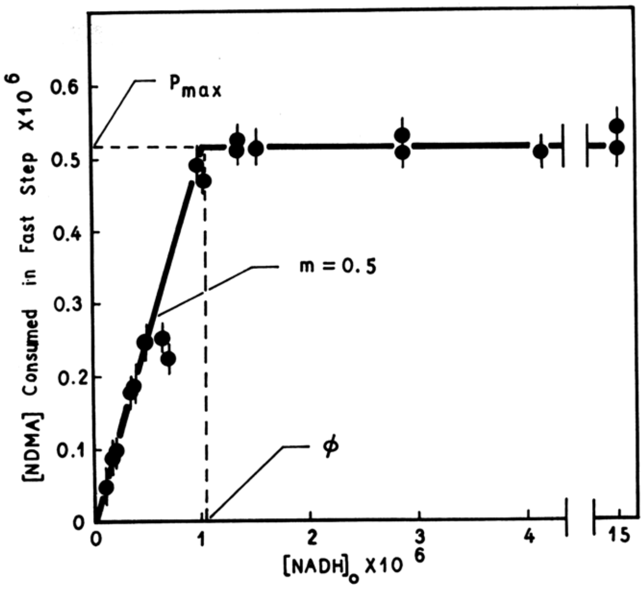

FIGURE 5: Dependence of the amount (moles per liter) of NDMA consumed in the rapid initial step (see Figure 4) as a function of the NADH concentration. These values are calculated from the optical-density changes and the molar extinction coefficient at 440 nm for the rapid process. Conditions: $[\text{NDMA}]_0$, 5×10^6 M; $[\text{LADH}]_0$, 1.06×10^{-6} N; and variable amounts of $[\text{NADH}]_0$ (0.05 M sodium pyrophosphate (pH 8.75) at 25 ± 0.1°). The initial solid line is theoretical for a slope (m) of 0.5. The ordinate and abscissa intercepts of this line with the horizontal end-point line, $P_{\max}$, and ϕ, respectively, are indicated (see text).

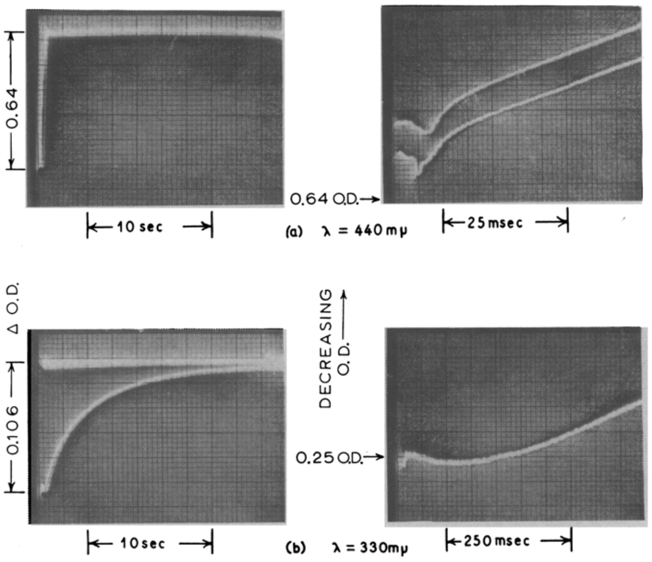

FIGURE 6: Oscilloscope traces comparing the progress curves for the changes in transmission at the wavelengths 440 nm (a) and 330 nm (b) for the LADH-catalyzed reduction of NDMA by NADH. Conditions: $[\text{NDMA}]_0$, 1.81×10^{-5} M; $[\text{NADH}]_0$, 4.00×10^{-5} M; $[\text{LADH}]_0$, 5.35×10^{-6} N (0.05 M sodium pyrophosphate (pH 8.75) at 25 ± 0.1°). Note that the burst reaction at 440 nm persists under these conditions (second photograph of a). Two separate determinations, differing only in total offset voltage, are shown in this trace to demonstrate the reproducibility of the burst.

the total enzyme-site concentration is in very great excess over the limiting reactant, it suggests that subunit interactions in the enzyme dimer play an important role in ordering the course of the catalytic process.

The optical density change at 440 nm accompanying the disappearance of NDMA provides additional evidence for the above conclusion. In the present study, two distinct (first-order) kinetic processes are observed for the disappearance of NDMA when the enzyme site concentration is in excess of [NADH]. Just as was observed for the aromatic aldehydes (Bernhard *et al.*, 1970; J. McFarland and S. A. Bernhard, 1971, in preparation), these two processes (see Figures 4 and 5) involve transformations of equal amplitude. When both NADH and NDMA are in excess of the site concentration, the disappearance of NDMA in the presteady-state burst is equivalent to one-half of the enzyme sites, as measured by the NAD$^+$–pyrazole assay.

In contrast to the above-mentioned biphasic transient kinetic behavior, only a single kinetic process is observed at 440 nm when an excess of enzyme is mixed with limiting NDMA and NADH is present in large excess. This single kinetic process follows a bimolecular rate law (Figure 3) over the accessible enzyme concentration range (4×10^{-7}–2×10^{-5} N), *viz.* (eq 2)

$$k_{\mathrm{app}} = k[\mathrm{E \cdot NADH}][\mathrm{NDMA}] \qquad (2)$$

If the equilibrium constant for the dissociation of ternary enzyme–coenzyme–reactant complex to enzyme–coenzyme complex and substrate were the same as the empirically determined "Michaelis constant" for the desorbtion of substrate from the enzyme–coenzyme complex (K_m^S), then the transient rate of reaction should exhibit a hyperbolic dependence on the concentration of NADH-saturated sites, approaching saturation when [E·NADH] > K_m^S. No such tendency toward saturation of the transient reaction velocity is observed although the known value of K_m^S (7×10^{-7} M) is orders of magnitude smaller than accessible concentrations of enzyme–coenzyme complex. This result constitutes direct evidence that intermediates (*e.g.*, III) beyond "$\mathrm{E(NADH)_2(S)_2}$" in the generalized reaction scheme of eq 3 are still more tightly bound to the enzyme site, and are directly involved in the catalyzed reaction mechanism, although such intermediates are not involved in the more rapid initial process of NDMA reduction.

$$\mathrm{E(NADH)_2(S)} + \mathrm{S} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E(NADH)_2(S)_2} \underset{\text{reaction}}{\overset{\text{fast chemical}}{\rightleftharpoons}}$$

$$\underset{\mathrm{III}}{\mathrm{E(NADH)(S)(X)}} \underset{\text{step}}{\overset{\text{slow}}{\rightleftharpoons}} \cdots \mathrm{E} + 2\mathrm{NAD}^+ + 2\mathrm{P} \qquad (3)$$

If NDMA dissociates rapidly from the ternary complex, then the occurrence of only a single kinetic process (in contrast to the biphasic kinetic behavior with aromatic aldehydes) when [E] > [NDMA] is not in conflict with the conclusions derived from the transient kinetic studies on the aromatic aldehydes. For as previously pointed out (Bernhard *et al.*, 1970), given a large excess of sites, slow ligand-dissociation rates are a necessary condition for the observation of multiple kinetic steps for processes involving identical chemical transformations. Indeed, it has been recently demonstrated that for aromatic substrates, alcohol–product dissociation is a relatively slow process following the more rapid reduction of aldehyde at the site (J. McFarland and S. A. Bernhard, 1971, in preparation).

A minimal value for the rate of dissociation of NDMA from the ternary complex can be estimated under the following assumptions. (a) The bimolecular association constant, $k = 2 \times 10^7$ N^{-1} sec^{-1}, calculated from Figure 3 places a reasonable estimate on the rate of formation of the ternary complex, k_2 of eq 3; it should not be slower and cannot be significantly faster, since this rate constant is in the range of diffusion-controlled bimolecular specific rates (Eigen and Hammes, 1963). (b) From a consideration of Figure 3, a maximal value can be placed on the magnitude of K_s; $K_s \geq 2 \times 10^{-5}$ N. This is so, because there is no way of reducing the net affinity of enzyme for substrate at stages beyond the bimolecular association process, although a variety of happenstances at these later stages could lead to an increase in the apparent affinity of enzyme for substrate.

If k_2 and k_{-2} are the forward and reverse constants, respectively, for the formation of $\mathrm{E(NADH)_2(S)_2}$ from $\mathrm{E(NADH)_2(S)}$ and S (eq 3), and defining K_s by the ternary complex–binary complex equilibrium of eq 3, *viz.*, $K_s = k_{-2}/k_2$, then a minimal value of k_{-2} is obtainable (eq 4).

$$k_{-2} = (\geq 2 \times 10^{-5}\ \mathrm{N})(2 \times 10^{7}\ \mathrm{N}^{-1}\ \mathrm{sec}^{-1}) \geq 400\ \mathrm{sec}^{-1} \qquad (4)$$

This calculated minimum value of 400 sec^{-1} is sufficiently rapid to assure equilibration of NDMA among the available enzyme sites during the transient course of reaction. Therefore, the necessary condition for two transient steps to be observable (when the NDMA concentration is limiting) does not obtain. The rapid rate of desorption of NDMA is in contrast to the relatively slow rate of alcohol product desorption following reduction of aromatic aldehyde (J. McFarland and S. A. Bernhard, 1971, in preparation).

The rate of NADH dissociation is known to be a slow process (Shore, 1969; Shore and Gutfreund, 1970) relative to the transient processes observed herein. Indeed, the rate of NADH dissociation at pH 8.75 is approximately 5 sec^{-1} as estimated from previous literature (Theorell and McKinley-McKee, 1961; Shore, 1969). This rate is very similar to the rate of the slow transient process reported herein; 4–5 sec^{-1} (see Figure 4, traces a and b).

It is noteworthy that with the exception of the somewhat faster rate of NDMA desorption from the site, the above-mentioned desorbtion rates and reaction rates are all of comparable magnitude. Possibly this is due to the loss of selective advantage for enhanced catalysis in enzymes once all catalytic processes have attained velocities comparable to the rate of nucleotide–product desorption from the enzyme site. Hence it would not be surprising that the rate of this particular catalyzed reaction could alternatively be dependent on desorbtive, electron-transfer, or proton-transfer processes, specific rate control being dependent on particular details of substrate structure.

Under conditions of [NADH] and [NDMA] ≫ [E] (conditions for maintaining a steady state), there is a presteady-state burst reaction yielding one-half the active-site concentration of product. Under these conditions, the regeneration of a reactive species [presumably $\mathrm{E(NADH)_2(S)_2}$] only occurs following the slow rate of dissociation of a burst product. With acetaldehyde (Theorell and Chance, 1951; Wratten and Cleland, 1965) and benzaldehyde (Wratten and Cleland, 1965; Dunn *et al.*, 1971, in preparation) this rate is the same as the rate of NAD$^+$ dissociation. For β-naphthaldehyde and 4-(2′-imidazolylazo)benzaldehyde (Bernhard *et al.*, 1970; Dunn *et al.*, 1971, in preparation), this rate is likely the rate of alcohol–product dissociation.

It has been concluded in the previous discussion that the difference in magnitude between K_s and K_m^s reflects the importance of enzyme-bound intermediates other than the NDMA–Michaelis complex. From an inspection of Figure 6, it is clear that an intermediate is formed, and that it can be generated in amounts *in excess* of the concentration of active sites for the following reasons. The OD changes at 440 nm due to nitroso reduction and at 330 nm (due to NADH oxidation) are not concomitant processes; disappearance of the 440-nm absorbance is virtually complete before any appreciable change occurs at 330 nm. Since the equivalents of NDMA consumed in this experiment is threefold greater than the equivalents of sites, a threefold greater concentration of the intermediate species over sites must be produced. Furthermore, since the initial 330-nm OD changes are slight, the intermediate must have a 330-nm extinction coefficient similar to that of NADH. Finally, the enzymatically formed intermediate must necessarily be released into free solution if the requirement for more than a single turnover is to be satisfied.

The magnitude of the turnover number for NDMA, 24 *vis-à-vis* 10 for acetaldehyde, is consistent with these conclusions, if the rate of substrate turnover is limited by the rate of intermediate dissociation from the enzyme site. Products, NAD$^+$ and II, are then generated from the spontaneous breakdown of intermediate in free solution. Note (Figure 6) that the rate of decomposition of intermediate to products is such that detectable concentrations of the intermediate will not be formed under the conventional laboratory steady-state conditions of dilute ($\sim 10^{-9}$ N) enzyme sites.

It is of interest to note that most prosthetic groups (cofactors or coenzymes) that participate directly in enzymic catalysis act through the intermediacy of covalent structures involving substrate. The nicotinamide–adenine dinucleotide coenzymes are unique in that heretofore no good evidence for such intermediates has been found. Therefore, it seems of considerable mechanistic interest that the evidence for intermediate formation during the LADH-catalyzed reduction of NDMA by NADH involves a labile species released into solution, thus freeing enzyme sites for additional rounds of substrate turnover at a rate significantly greater than the measured rate of NAD$^+$ dissociation (Shore, 1969; Shore and Gutfreund, 1970) from the binary enzyme–coenzyme complex.

While the chemical nature of this intermediate has not yet been defined completely, the similarity of catalytic turnover reported here to that observed for aldehydes suggests that the intermediate is catalytically relevant to the mechanism of LADH action. Detailed kinetic and structural investigations concerning the nature of the intermediate are presently under way.

Conclusions

The LADH-catalyzed reduction of NDMA by NADH is probably a close mechanistic analog of aldehyde reduction. However, in contrast to the behavior of aromatic aldehydes (Bernhard *et al.*, 1970), NDMA is shown not to exhibit a high binding affinity for the enzyme–coenzyme binary complex. Both the NDMA formation and dissociation rates ($\geq 2 \times 10^7$ M^{-1} sec^{-1} and ≥ 400 sec^{-1}, respectively) for the enzyme–coenzyme–substrate ternary reactant complex are shown to be rapid relative to the slow process observed in the transient state reaction. The fact that rapid (burst) disappearance of NDMA occurs in an amount equal to one-half the concentration of the limiting reactant only for limiting [E] or limiting [NADH] (*e.g.*, when [NDMA], [NADH] $\gg$ [E], and when [NDMA] > [E] > [NADH]) but not for limiting [NDMA] further defines the mechanistic origins of the previously reported nonequivalence of LADH sites (Bernhard *et al.*, 1970).

In particular, these findings together with the observation that NDMA disappearance when [NDMA] is limiting ([NADH] > [E] $\gg$ [NDMA]) occurs as a single exponential process satisfy the prediction that *site nonequivalence will be manifest in a two step (biphasic) kinetic sequence when substrate or coenzyme is limiting only if the dissociation rate for the limiting reactant from the complex is slow.*

The evidence for chemical intermediate formation in amounts in substantial excess of the number of enzyme sites, but having spectral properties uniquely distinct from that of final products, provides forceful arguments for the transient existence of a discrete coenzyme–substrate intermediate during the chemical transformation.

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