On the Origin of the Lactate Dehydrogenase Induced Rate Effect†

John W. Burgner, II,* and William J. Ray, Jr.

ABSTRACT: To evaluate the ability of lactate dehydrogenase to facilitate the bond making/breaking steps for both the addition of pyruvate enol to NAD (pyruvate adduct reaction) and the normal redox reaction, the ability of the enzyme to facilitate the tautomerization of bound pyruvate is assessed. In addition, the equilibrium constants for the adduct reaction are obtained for both bound and free reactants from the ratio of the rate constants in the forward and reverse reactions (at pH 7). The latter comparison indicates that the enzyme facilitates bond making/breaking in the (forward) pyruvate adduct reaction by a factor of about 10^{11} M. Similar comparisons suggest that reactant immobilization accounts for about 1000 M of this 10^{11} M rate effect. Since the (pH-independent) rate constant for the ketonization of bound pyruvate enol assisted by the external buffer, imidazolium ion, is 2×10^7 M^{-1} s^{-1} and the corresponding rate constant for free pyruvate enol, again assisted by imidazolium ion, is 35 M^{-1} s^{-1} [Burgner, J. W., II, & Ray, W. J., Jr. (1978) *Biochemistry 17*, 1664], the enzyme facilitates the bond making/breaking steps associated with the conversion of bound HO–C< to bound O=C< by a factor of about 10^6-fold. The product of the above two rate enhancement factors and the rate factor suggested previously for the environmental effect on NAD produced by its binding to lactate dehydrogenase, 100-fold, is 10^{11} M, and it accounts for the bond making/breaking effects exerted by the enzyme in the pyruvate adduct reaction. The rate constant for oxidation of ethanol (a model for lactate) by 1-methylnicotinamide (a model for NAD) is about 5×10^{-12} M^{-1} s^{-1} at 25 °C in pure ethanol ($\Delta H^{\ddagger}$ for this reaction is about 30 kcal/mol). The ratio of the rate constants for E·NAD·Lac → E·NADH·Pyr and the above model reaction is estimated as about 10^{14} M in water; i.e., the LDH-induced rate effect is about 10^{14} M. The product of the values for the above rate factors for the *normal* redox reaction is about 10^{12} M. Although the value of this product is less certain than that for the adduct reaction, these rate factors do account for much of the LDH-induced rate effect.

In comparing enzymic and nonenzymic reactions, one must make a choice between comparing the efficiency of an enzyme in facilitating bond breaking/making and the efficiency of the enzyme as a catalyst. Thus, the efficiency of an enzyme in facilitating chemical transformations is reflected in the rate constant, k_e, for a discrete bond breaking/making step, e.g., as in

$$\text{E·A·B} \xrightarrow{k_e} \text{E·P·Q}$$

By contrast, its efficiency as a catalyst is reflected in $k_{cat}/(K_{iA}K_B)$, e.g., as in

$$\text{A} + \text{B} \xrightarrow{\text{(Enz)}} \text{P} + \text{Q}$$

Both of the above constants can be compared with the bimolecular rate constant, k_{ne}, for the analogous uncatalyzed reaction. In most cases, the ratio of $k_{cat}/(K_{iA}K_B)$ to k_{ne} will be enormous because a substantial intrinsic binding energy is available to facilitate the enzymic reaction (Jencks, 1975, 1980). Part of this energy is used solely for binding, as in the case of phase-transfer catalysts [cf. Jones (1976)], and the remainder to facilitate bond making/breaking. On the other hand, the ratio k_e/k_{ne} reflects the intrinsic binding energy that is *used* during the bond making/breaking step(s). In fact, the basis of the transition-state binding paradigm is that noncovalent binding interactions are responsible for the large values of k_e/k_{ne} that usually are observed. But, this paradigm is so all inclusive that it is not useful as a detailed molecular explanation of *how* intrinsic binding energy is used to accelerate bond making/breaking steps (Ray & Long, 1976). Since the mechanism for using intrinsic binding energy is our primary interest, we concentrate on the ratio k_e/k_{ne} for the oxidation of lactate by NAD in the presence and absence of LDH.[1]

To provide even a semiquantitative rationale for the large value for k_e/k_{ne} of about 10^{14} M (see Discussion), the various factors that contribute to k_e must be identified and evaluated. This is particularly difficult since alterations that probe the importance of one factor also may affect the contributions of others. But, there seems to be no alternative other than to proceed in this manner, and we proceed cautiously by using as many internal comparisons as possible.

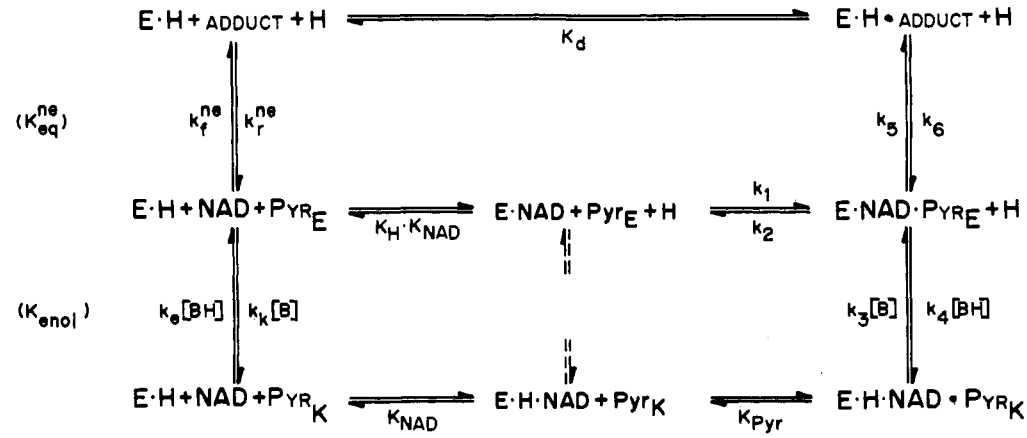
Previously, we suggested that the pyruvate adduct reaction is a useful model for studying the normal reaction for the following reasons. (1) Both reactants are substrates of the enzyme, although not the normal pair. (2) The same groups on the enzyme probably are involved in both reactions. (3) To a first approximation, the addition of the pyruvate enol (and/or the enolate) to NAD mimics the addition of the hydride ion to NAD. (4) The bond-making step in the enzymic adduct reaction actually is faster than the hydride-transfer step. (5) The enzyme is an efficient bond maker/breaker. In combination, these considerations suggest that providing a description for the basis of the large value of k_e/k_{ne} in the adduct reaction should provide a basis for explaining the somewhat larger value of k_e/k_{ne} in the normal redox reaction. Hence, this paper is the third in a series where nucleophilic additions to the 4 position of NAD bound to lactate de-

† From the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907. *Received November 9, 1983.* This investigation was supported by a research grant from the National Science Foundation (GB012576).

[1] Abbreviations: LDH, lactate dehydrogenase (the dogfish A_4 isozyme unless otherwise specified); E·X, complex of LDH with X; Pyr_K, Pyr_E, and $Pyr_{\bar{E}}$, the keto, enol, and enolate forms of pyruvate; NAD, nicotinamide adenine dinucleotide; APAD, 3-acetylpyridine adenine dinucleotide; NAD–Pyr and APAD–Pyr, the covalent adducts of pyruvate with NAD and APAD; K_{eq}, an equilibrium constant; K_X, the dissociation constant for the complex E·X; NAD–Lac, a covalent complex of NAD and lactate where the methyl group of lactate is attached via a methylene bridge at the 5 position of the nicotinamide ring of NAD.

0006-2960/84/0423-3636$01.50/0 © 1984 American Chemical Society

Scheme I



hydrogenase are examined in an attempt to evaluate those factors that contribute to the efficiency of the normal reaction.

Previous studies also have established that an external buffer acts as a general catalyst during the pyruvate adduct reaction, that the step in the overall reaction subject to general catalysis is the pyruvate enol–keto tautomerization involving *bound* pyruvate, and that the rate of this step is accelerated significantly by the enzyme (Burgner & Ray, 1978, 1984b). This system provides a unique opportunity for estimating the importance of general catalysis in an enzymically catalyzed reaction. Because of the unexpectedly large rate enhancement observed for general catalysis, 10^6 M (see Discussion), several of its aspects are compared and contrasted for the enzymic and nonenzymic processes.

The various rate and equilibrium constants in the enzymic and nonenzymic adduct reactions involving pyruvate are shown in Scheme I, where the reactions are presented so that analogies with the normal enzymic reaction are maximized. This paper compares the two nonenzymic reactions in boldface type on the left-hand side of Scheme I with the analogous enzymic reactions in boldface type on the right. In these two reactions, the buffer-assisted ketonization of free Pyr_E (lower left) is compared with the buffer-assisted ketonization of enzyme-bound Pyr_E (lower right), and the formation of the bond between Pyr_E and NAD (upper left) is compared with the analogous process when both reactants are bound to the enzyme (upper right).

Materials and Methods

Most materials and assay procedures are described in previous papers (Burgner et al., 1978; Burgner & Ray, 1974, 1978, 1984a,b). Only dogfish A_4 LDH is used in these experiments. The temperature for all assays unless otherwise noted is 15 °C, and the pH values for all buffers refer to the final assay conditions. When APAD is substituted for NAD, it is used at the same concentrations that were used previously for NAD. Spectral measurements are made in a Perkin-Elmer Model 575 spectrophotometer equipped with an electronic temperature controller; fluorescent measurements are made in an instrument described elsewhere (Burgner & Ray, 1978).

The APAD–Pyr and APAD–acetone adducts are prepared in the absence of enzyme by dissolving 10 μmol of APAD and 100 μmol of either acetone or pyruvate into 1 mL of a solution containing equal volumes of dimethyl sulfoxide and 0.1 M sodium carbonate buffer, pH 11, and allowing the mixture to stand for 1 h at room temperature. 1-Methyl-1,4-dihydronicotinamide was prepared by the method of Karrer & Blumer (1947), and its identity and purity were checked by NMR spectroscopy.

The stoichiometry of the APAD–Pyr enzyme complex was determined by using the quenching of enzymic fluorescence as an assay for bound adduct [cf. Burgner & Ray (1978)]. The adduct was prepared (see above) so that its final concentration was about 10^4-fold greater than that of the enzymic subunits, about 1.2 μM, and immediately prior to the addition, an aliquant of the stock adduct was diluted in ice-cold water so that 1–5 μL would give the desired concentration when added to the 1-ml assay mixture. Each assay solution containing enzyme was used for only two additions of the adduct, and the enzyme was exposed to the adduct only for about 2 min. Other conditions are given in Figure 1.

The rate and equilibrium position of the nonenzymic reactions of APAD and pyruvate are measured by following the appearance of the near-UV absorption band of APAD–Pyr at 350 nm. The reactions typically were initiated by adding a small aliquant of 1 M sodium pyruvate to the assay mixture. The reverse nonenzymic reaction was studied by diluting the adduct reaction mixture by 100-fold (initial pH 11) into a reaction mixture containing the appropriate buffer (lower pH). The disappearance of the near-UV absorbance band was followed.

The reverse enzymic adduct reaction was examined either by addition of a 2-fold excess of APAD–Pyr to the enzyme at pH 7.0 or after removal of excess reactants (APAD and pyruvate) from an equilibrium mixture by rapid molecular sieving at 4 °C (Penefsky, 1977); see also Burgner & Ray (1984a).

For inhibition studies, both product–time and initial velocity assays of the normal redox reaction in the presence of APAD–Pyr are initiated by adding the apoenzyme to the assay mixture *immediately* after adding the APAD–Pyr. The enzyme concentration was increased with increasing inhibitor concentration so that a measurable initial slope was obtained in all cases. Product–time data are analyzed either graphically, as described in Figure 2, or numerically, as in Burgner et al. (1978).

The reduction of 1-methylnicotinamide by "neat" ethanol, which was freshly dried over molecular-sieving beads (4 Å), was detected by the change in absorbance at 340 nm as a function of time (0.2 absorbance scale; 1-cm light path). A Teflon-stoppered cuvette, which was wrapped at the top with Parafilm, prevented the loss of solvent. To detect acetaldehyde, several test tubes containing 50 mg of 1-methylnicotinamide plus 5 mL of freshly dried ethanol were frozen, evacuated, and sealed with a flame. One tube was stored in liquid nitrogen as a control. After 5 days at 55 °C, one tube was analyzed by gas chromatography (Westcott et al., 1980). The other four tubes were combined and flushed continuously with ni-

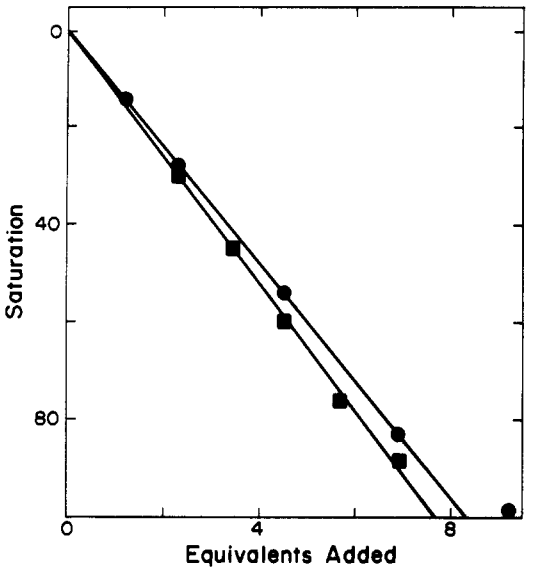


FIGURE 1: Titration of LDH with APAD–Pyr: equivalents of APAD–Pyr added per equivalent of active sites. The initial concentration of active sites was 1.25 (●) and 2.50 μM (■) in 0.3 M imidazole hydrochloride pH 7.0. The titration was followed by the decrease in fluorescence of the enzyme and completed within 2 min to prevent a significant loss of APAD–Pyr. The percent saturation was calculated by Holbrook's method [cf. Burgner & Ray (1983b)]; the solid lines were drawn by eye.

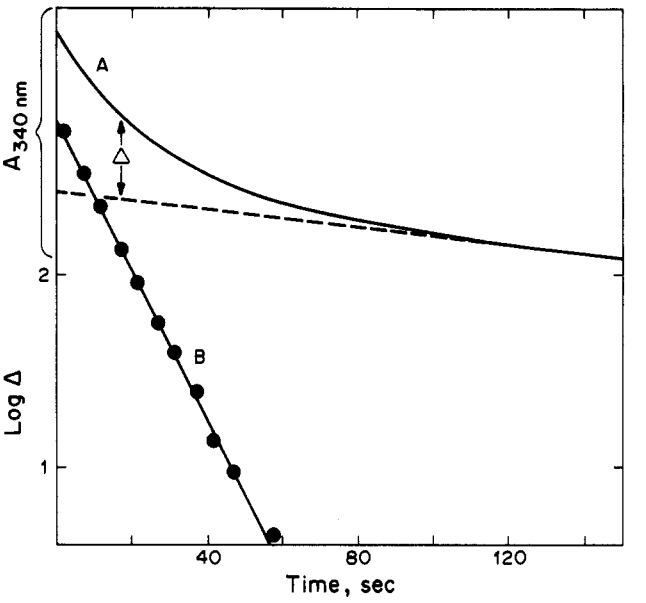


FIGURE 2: Effect of APAD–Pyr on a product–time plot for the normal enzymic reaction (Pyr → Lac). The initial concentrations of NADH, Pyr, and APAD–Pyr were 1.51×10^{-4}, 6×10^{-4}, and 14.4×10^{-9} M, respectively, in 0.3 M imidazole hydrochloride, pH 7.0. The reaction was initiated by adding enzyme to a concentration of 1.5×10^{-9} N sites. Curve A is a (semilog) plot of A_{340} against time. The dashed line represents the product–time course of the steady-state phase extrapolated back to zero time. Curve B is a (semilog) plot of the difference in the above two lines, Δ, as a function of time; k_{obsd} = 0.04 s^{-1}.

trogen (while maintaining the solution at 21–22 °C), and the gas was passed through a cold trap equilibrated with liquid nitrogen. The presence of acetaldehyde in the trap was detected qualitatively by the change in absorbance at 340 nm on addition of an aliquant of the trapped material (in water) to an assay mixture containing 0.15 mM NADH and 10 nM alcohol dehydrogenase in a phosphate buffer (pH 7 and 0.1 M).

Results

The nonenzymic adduct reaction involving pyruvate and NAD is difficult to compare with the corresponding reaction catalyzed by LDH because the NAD–Pyr adduct cyclizes during the nonenzymic reaction (Ozols & Marinetti, 1969). Hence, the pyruvate adduct of acetylpyridine adenine dinucleotide, APAD, is substituted for the corresponding adduct of NAD in many comparisons because the former adduct does not cyclize. Differences produced by this substitution are compensated for by comparing adduct reactions of APAD and NAD where the product does not cyclize, e.g., reactions involving the addition of CN^- or SO_3^{2-}.

Stoichiometry of the E·APAD–Pyr Complex. To demonstrate that the binding stoichiometry of APAD–Pyr and LDH is the same as that for NADH, both the decrease in enzymic fluorescence as a function of added APAD–Pyr and the amount of APAD–Pyr required to saturate the enzyme are measured. In Figure 1, the fractional saturation of the enzyme with APAD–Pyr is plotted against the moles of adduct added per mole of active sites. The horizontal intercept indicates an 8:1 equivalency between adduct and enzyme. Because the APAD–Pyr adduct is prepared nonenzymically (see Materials and Methods), an approximately equal distribution of A and B isomers of the adduct is present (Arnold & Kaplan, 1974), and the A isomer should bind much more tenaciously than its enantiomer. Hence, the observed 8:1 equivalency is as expected when each subunit binds only one of the two adduct isomers. To demonstrate that only half of the chemically prepared adduct present at the equivalence point actually binds tightly to the enzyme, a rapid molecular-sieving technique was used to remove any unbound APAD–Pyr (see Materials and Methods). Since the enzyme is almost completely recovered (>95%) and since the absorbance at 340 nm is decreased 2-fold by the sieving procedure, the enzyme must bind only one of the two isomers present. Hence, the stoichiometry of APAD–Pyr binding is the same as that for the binding of NADH [cf. Holbrook et al. (1975)].[2]

Competitive Binding between APAD–Pyr and NADH. The linearity of the titration in Figure 1 up to nearly the equivalence point indicates the dissociation constant for the E·APAD–Pyr complex must be substantially less than the concentration of enzyme used in that titration: 1.25 μM. The dissociation constant for this complex was estimated by measuring the competitive binding between APAD–Pyr and NADH with a steady-state assay involving the normal redox reaction. The basis for this assay, which was described previously (Burgner et al., 1978), is shown in Figure 2, plot A. This product–time plot describes the disappearance of NADH during the reduction of pyruvate and occurs when the enzyme is added to an assay mixture containing 15 nM APAD–Pyr, half-saturating pyruvate, and saturating NADH. In the absence of APAD–Pyr, the product–time plot is linear with a slope identical with the initial slope in Figure 2. The initial burst of product prior to the steady state in the presence of 1–10 nM concentrations of APAD–Pyr is caused by the relatively slow formation of the steady-state level of the dead-end E·APAD–Pyr complex, as is shown in the following section.

[2] A similar fluorometric titration also was attempted with the nonenzymically prepared NAD–Pyr adduct, since this adduct also inhibits the enzyme (Everse et al., 1971). However, no significant decrease in enzyme fluorescence was noted when 4 equiv (~4 nmol/mL) of this adduct was added to the enzyme. Since in the absence of enzyme the NAD–Pyr adduct undergoes an additional cyclization [cf. Everse et al. (1971)] that involves the α-carbon of the pyruvate moiety and the carboxamide nitrogen, this additional cyclization apparently reduces the strength of the binding interaction by a large factor.

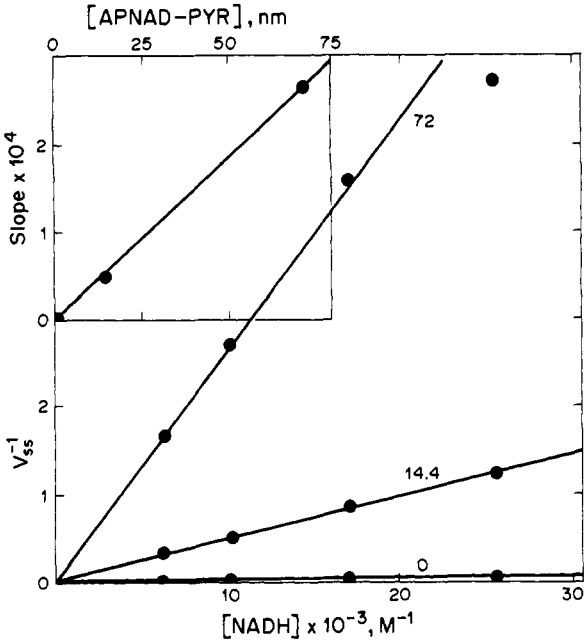
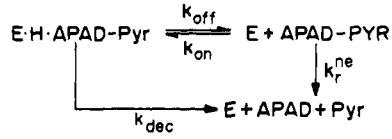


FIGURE 3: Effect of APAD–Pyr on the steady-state velocity of the normal LDH reaction (Pyr → Lac). The conditions and assay procedures are the same as those in Figure 2, except that the enzyme concentration was 7.5 nM for assays at 72 nM APAD–Pyr. The other conditions are shown on the graph. (Inset) A plot of the slopes of the lower plots against the inhibitor concentration; a value of 9×10^{-11} M is calculated from the slope to intercept ratio of the inset plot by using the measured slope in the absence of APAD–Pyr as the intercept value in this ratio.

Scheme II



Hence, the slope of the dashed line in Figure 2 describes the velocity of the normal enzymic reaction after the steady-state level of E·APAD–Pyr is reached. Figure 3 shows a plot of the steady-state velocity against 1/[NADH] in the presence and absence of APAD–Pyr; the concentrations shown are those for the adduct isomer that binds to LDH, i.e., half the total adduct concentration. (These experiments are not designed to show that this adduct is competitive with NADH; i.e., an intersection of the lines in the third quandrant cannot be ruled out. In fact, the experimental design is dictated by both the slow, spontaneous decomposition of the inhibitor and its small dissociation constant—see below as well as the following section.) The inset of Figure 3 shows a replot of the slopes of the lines in the figure against the inhibitor concentration. The intercept/slope ratio of the replot, 9×10^{-11} M, is the apparent inhibition constant for APAD–Pyr: K_I^{app}. [The vertical intercept in this replot is taken as the *measured* slope in the absence of inhibitor (not shown)—not the extrapolated value at [APNAD–Pyr] = 0 obtained from the regression intercept.] As is shown below, this apparent inhibition constant is nearly equal to the true dissociation constant K_d for the E·APAD–Pyr complex even though the APAD–Pyr adduct can both dissociate from the enzyme and decompose while bound to the enzyme as well as decompose spontaneously when free in solution (cf. Scheme II). Thus, in the present case, $K_I^{app} = (k_{off} + k_{dec})/k_{on}$, and K_I^{app} will equal K_d when $k_{off} \gg k_{dec}$. This inequality holds under the conditions used in Figure 3 for the E·H·APAD–Pyr complex but not for the E·H·NAD–Pyr complex where $k_{dec} \gg k_{off}$.[3]

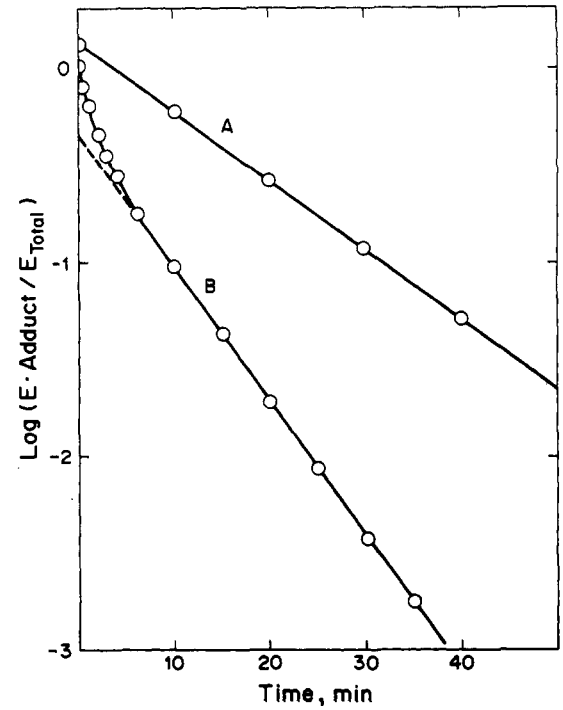


FIGURE 4: Effect of enzyme concentration on course of the reverse adduct reaction at pH 7.0. In both reactions, the buffer was 0.3 M imidazole hydrochloride. For curve A, the assay initially contained 26 μN E·APAD–Pyr and 1 M potassium oxalate; for curve B, the assay initially contained 9×10^{-10} N E·APAD–Pyr (see text for additional details). The values of k_{obsd} are 1.1×10^{-4} s^{-1} and 7×10^{-4} s for curves A and B, respectively. The lines were drawn by eye.

The attachment of the pyruvate moiety to the 4 position of the pyridinium ring of APAD increases the strength of the binding interactions by about (5×10^4)-fold, since the equilibrium dissociation constant for APADH is about 5 μM and, as noted above, that for APAD–Pyr is about 10^{-10} M. (Because the chemistry of these adducts is dihydropyridine-like, we compare their dissociation constants with those for APADH rather than APAD—see also Discussion.) In addition, the presence of enzyme stabilizes APAD–Pyr, since the rate constant for decomposition of E·H·APAD–Pyr, 1×10^{-4} s^{-1},

[3] A simple approach is used to determine whether k_{dec} or k_{off} controls the steady-state concentration of APNAD–Pyr; the observed rate constant, k_{obsd}, for the disappearance of E·APNAD–Pyr must be sensitive to the initial concentration of E·adduct if $k_{off} \geq k_{dec}$. The steady-state rate equation describing Scheme II is

$$\frac{d[E\cdot APAD\text{–}Pyr]}{dt} = (k_{dec} + k_{off}k_r^{ne})/(k_{on}[E] + k_r^{ne})[E\cdot APAD\text{–}Pyr]$$

When $k_{on}[E] \gg k_r^{ne}$ and $[E] \gg k_{off}/k_{on}$ (i.e., $[E] \gg K_{APAD\text{–}Pyr}$), only k_{dec} will be significant in this equation. Likewise, when $k_r^{ne} \gg k_{on}[E]$, k_{obsd} will equal $k_{dec} + k_{off}$. (Obviously, when $k_{on} \ll k_r^{ne}$, k_{obsd} equals $k_{dec} + k_{off}$ regardless of the value of k_{ne}; however, this condition is unobtainable here.) Figure 4, curve A, shows a semilog plot of the fraction of the E·APNAD–Pyr remaining against time where $k_{on}[E] \gg k_r^{ne}$ and $[E] \gg K_{APAD\text{–}Pyr}$. The observed value for the disappearance of adduct is 1.1×10^{-4} s^{-1}, and this value must approach k_{dec} in Scheme II. Alternatively, at an initial concentration of E·APNAD–Pyr about 10-fold larger than the value of $K_{I,APNAD\text{–}Pyr}$ (i.e., $[E] \gg K_{APAD\text{–}Pyr}$), a semilog plot of the fraction of the enzyme containing adduct against time is biphasic (curve B, Figure 4). Since the value of k_{obsd}, 7×10^{-3} s^{-1}, for the linear phase is 7-fold larger than the value of k_{obsd} at high initial [E], curve A, it follows that k_{off} must be larger than k_{dec}, that $k_r^{ne} \gg k_{off}[E]$, and that $K_I^{app} \simeq K_{APNAD\text{–}Pyr} \simeq 10^{-10}$ M. (A lower limit for $K_{APAD\text{–}Pyr}$ of 6×10^{-11} was calculated from the magnitude of the burst phase, 2×10^{-10} M, in curve B as an internal test of consistency.) A similar set of experiments also was conducted with E·NAD–Pyr. With the latter adduct complex, the decomposition step k_{dec} must be faster than the dissociation step k_{off} in contrast with E·APAD–Pyr (cf. the preceding paper).

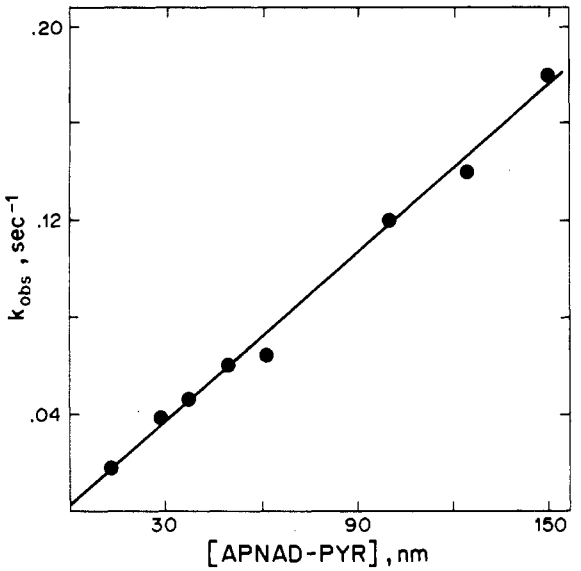


FIGURE 5: Effect of APAD–Pyr on the rate of approach to steady state. The conditions are the same as those for Figure 2. The line was drawn by eye, with a slope of 2.4×10^6 M^{-1} s^{-1} and intercept of 0.002 s^{-1} (see text for further details).

is about 0.07 of that observed in the absence of enzyme, 1.5×10^{-3} s^{-1}.

Estimation of the Dissociation Constant for the Complex of APAD–Pyr and LDH by Measuring the Rate Constants for the Binding and Dissociation Steps. As was shown above, product–time plots obtained with the normal enzymic assay are biphasic when the reaction is initiated by adding enzyme to an assay mixture containing APAD–Pyr—see Figure 2. When Δ is defined in the manner shown in Figure 2, a plot of log Δ against time will result that is linear for approximately 3 halftimes. Thus, the rate equation describing the approach to steady state is first order in enzyme concentration. This first-order dependence is further demonstrated by the insensitivity of the rate constant describing the burst phase, k_{burst}, to a 10-fold change in enzyme concentration (not shown). In addition, a plot of k_{burst} against [APAD–Pyr] is essentially linear (Figure 4); thus, the rate equation describing the burst phase also must be first order in APAD–Pyr. Since k_{burst} represents the approach to steady state, k_{burst} should be related to the sum of the on- and off-rates for APAD–Pyr, after correction for the rate of the nonenzymic decomposition—see Scheme II. Hence, $k_{burst} = k_{on}^{app}$[APAD–Pyr] + $k_{off} + k_r^{ne}$ when the [APAD–Pyr] does not change significantly (<10%) during the approach to steady state. Since [APAD–Pyr] is essentially constant within the time interval of these experiments at all but the *lowest* concentration used (~6 nM), the plot of k_{burst} vs. [APAD–Pyr] in Figure 5 can be analyzed[4] to give a value of k_{on} of about 2×10^7 M^{-1} s^{-1}. This value is somewhat smaller than the values of $(5–10) \times 10^7$ M^{-1} s^{-1}, usually given for the k_{on} of NADH [cf. Holbrook et al. (1975), but this difference does not appear to constitute a serious problem. A value of about 1×10^{-3} s^{-1} for k_{off} also can be

Table I: Estimates of the Rate Constants for the Enzyme-Catalyzed NAD–Pyr Reaction[a]

const		const	
k_1 (M^{-1} s^{-1})	1×10^7	k_5 (s^{-1})	2×10^5
k_2 (s^{-1})	3×10^5	k_6 (s^{-1})	3×10^{-3}
k_3 (M^{-1} s^{-1})	30	K_{enol}[b]	4×10^6
k_4 (M^{-1} s^{-1})	2×10^7		

[a] The constants, which are identified in Scheme I, were evaluated in the manner described under Results from experiments at pH 7 and 15 °C. [b] From Burgner & Ray (1974).

obtained[4] from the data in Figure 4, and the ratio k_{off}/k_{on}, 5×10^{-11} M, is in reasonable agreement with the value of 9×10^{-11} M for the measured equilibrium dissociation constant of APAD–Pyr (see above).

Nonenzymic Pyruvate Adduct Reaction with APAD. At constant concentrations of APAD (1.7×10^{-3} M), pyruvate (0.04 M), and Tris base (0.05 M), a stoichiometric conversion of APAD to its pyruvate adduct does not occur in the pH range 7–9, and values of the pH-dependent equilibrium constant K_{eq}^{app} can be estimated from the absorbance of the equilibrium mixture and the molar extinction coefficient for the adduct at 360 nm, 1×10^4 cm^2 mol^{-1} (DiSabato, 1968), where K_{eq}^{obsd} is equal to [APAD–Pyr]/([APAD][Pyr_K]). A plot (not shown) of K_{eq}^{app} vs. [OH] is linear ($r^2 = 0.99$) in the pH range 7–9, with a value of 8.8×10^{-3} M^{-1} at pH 7 and an intercept value indistinguishable from zero. The corresponding pH-independent equilibrium constant K_{eq}^{app}[H], calculated from the slope of this plot, is $(9.1 \pm 0.6) \times 10^{-10}$. When [$Pyr_K$] in the denominator of this constant is replaced by [Pyr_E], the value of this constant becomes 2.4×10^{-4} ($K_{enol} = 4 \times 10^{-6}$; Burgner & Ray, 1974).

Like the reaction of NAD with pyruvate, the APAD reaction is first order in both APAD and pyruvate (Griffin & Criddle, 1970). In the presence of excess pyruvate, the pseudo-first-order rate constant k_f^{ne} for formation of this adduct can be determined from the increase in absorbance (360 nm) with time, while k_r^{ne} (for the reverse reaction) can be evaluated from the decrease in absorbance after addition of a sample of the adduct (previously prepared at high pH) to an acidic reaction mixture (pH ≤6). When the pH of the reaction mixture is altered, both k_f^{ne} and k_r^{ne} change according to the following equations:

$$k_f^{ne} = k_f^W + [OH]k_f^{OH} + [B]k_f^B$$

$$k_r^{ne} = k_r^W + [H]k_r^H + [BH]k_r^{BH}$$

Here, [B] and [BH] designate buffer concentrations; the superscript W refers to water. Thus, within the pH range 7–9, a plot of k_f^{ne} vs. [OH] at a constant concentration of Tris base (50 mM) is linear, with an intercept value (equal to $0.05k_f^B + k_f^W$) of $(1 \pm 0.6) \times 10^{-4}$ M^{-1} s^{-1} and a slope, k_f^{OH}, of 100 ± 3 s^{-1} (constants calculated for the addition of pyruvate ketone—data not shown). Because of the substantial uncertainty in the intercept term, k_f^W was evaluated indirectly (see following paragraph). A plot of k_r^{ne} vs. [H^+] from pH 3 to 1 also is linear for the acetone adduct reaction[5] (not shown),

[4] The slope of the line in Figure 4 is a function of k_{on} and the fraction of the total enzyme in the free form, whereas the intercept term is the sum of k_r^{ne}, and k_{dec}. The fraction of total enzyme initially present as free enzyme in these experiments was estimated at about 0.06 from the distribution equation for an ordered bi-bi mechanism (the dissociation constant $K_{i,NADH}$ and the Michaelis constants K_{NADH} and K_{Pyr} were taken as 10^{-5} M, 10^{-5} M, and 10^{-3} M, respectively; Burgner et al., 1978). Hence, k_{on} must be about 2×10^7 M^{-1} s^{-1} (i.e., slope/0.06). In addition, k_{off} must be about 1×10^{-3} s^{-1}, since the intercept, k_{dec}, and k_r^{ne} values are 2×10^{-3} s^{-1}, 1×10^{-4} s^{-1}, and 1×10^{-3} s^{-1}, respectively.

[5] The protonation of the carboxylate group of APAD–Pyr interferes with an accurate assessment of k_r^H, because protonation of this group, $pK_a \simeq 2.5$, occurs just as k_r^H appears to become significantly greater than the water-catalyzed rate. Since the value for the water-catalyzed rate constant, 1×10^{-3} s^{-1}, for APAD–Pyr is essentially the same as that for the acetone adduct of APAD, 1.2×10^{-3} s^{-1}, our use of the value of k_r^H from the latter adduct reaction in place of that for the former seems reasonable.

Table II: Estimated Values of Rate Constants for the Various Reactions Catalyzed by LDH and Their Corresponding Nonenzymic Reactions[a]

comparison	reaction	constant at pH 7	ratio
A	$E{\cdot}NAD{\cdot}Pyr_E \xrightarrow{k_5} E{\cdot}H{\cdot}adduct$	$2 \times 10^5\ s^{-1}$	
			2×10^{11} M
	$NAD + Pyr_E \xrightarrow{k_f^W} adduct + H$	$1 \times 10^{-6}\ M^{-1}\ s^{-1}$	
B	$E{\cdot}H{\cdot}adduct \xrightarrow{k_6} E{\cdot}NAD{\cdot}Pyr_E$	$3 \times 10^{-3}\ s^{-1}$	
			3×10^4
	$adduct \xrightarrow{k_r^H} NAD + Pyr_E$	$1.2 \times 10^{-7}\ s^{-1}$	
C	$NAD + Pyr_E \underset{}{\overset{K_{eq}^{ne}}{\rightleftharpoons}} adduct + H$	$25\ M^{-1}$	
			4×10^6 M
	$E{\cdot}NAD{\cdot}Pyr_E \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E{\cdot}H{\cdot}adduct$	10^8	
D	$E{\cdot}NAD{\cdot}Pyr_E \xrightarrow{k_4[BH]} E{\cdot}H{\cdot}NAD{\cdot}Pyr_K$	$2 \times 10^7\ M^{-1}\ s^{-1}$ [b]	
			6×10^5
	$Pyr_E \xrightarrow{k_{BH}[BH]} Pyr_K$	$35\ M^{-1}\ s^{-1}$	
E	$E{\cdot}H{\cdot}NAD{\cdot}Pyr_K \xrightarrow{k_3[B]} E{\cdot}NAD{\cdot}Pyr_E + H^+$	$30\ M^{-1}\ s^{-1}$ [b]	
			2×10^5
	$Pyr_K \xrightarrow{k_B[B]} Pyr_E$	$1.4 \times 10^{-4}\ M^{-1}\ s^{-1}$	

[a] Values for constants in reactions involving NAD were estimated from those measured for the corresponding reactions involving APAD in the manner described under Results. Constants for the enzymic reactions are defined in Scheme I, and values are from Burgner & Ray (1984b). [b] The constants are bimolecular rate constants for the buffer-assisted process.

with a slope, k_r^H, equal to 0.067 ± 0.001 $M^{-1}\ s^{-1}$ and an intercept, k_r^W, equal to (1.2 ± 0.1) × $10^{-3}\ s^{-1}$ ([BH] = 0).

The pH-independent equilibrium constant K_{eq}^{app} for the pyruvate adduct reaction involving APAD and Pyr_K is equal to $(k_f^{OH} \times 10^{-14})/k_r^W$ and is about 8×10^{-10} M on the basis of the values of the rate constants given above. This value is in good agreement with that obtained from equilibrium measurements, 8.8×10^{-10} M (see above). Since K_{eq} also is equal to k_f^W/k_r^H, an estimate for k_f^W, $6 \times 10^{-11}\ s^{-1}$, can be obtained from the measured value of K_{eq} and the independently measured value of k_r^H, where pyruvate ketone is taken as the reactant (see above). When the enol is considered as the reactant, k_f^W becomes $1.5 \times 10^{-5}\ s^{-1}$.

Estimation of Constants for the Pyruvate Adduct Reaction with NAD. Values of the various rate and equilibrium constants in Scheme I for the enzymic reaction are given in Table I; these are compared in Table II with the nonenzymic reaction. In this section, the procedures used in estimating values for these constants are described; those for nonenzymic constants are described first.

The values of k_f^W and k_r^H for the reaction of pyruvate with APAD (see the preceding section) must be corrected for the difference in the effect of the acetyl and acetamido groups on the reactivity of the pyridinium ring so that the enzymic adduct reaction (involving NAD) and the nonenzymic adduct reaction (involving APAD) can be compared. An approximate correction for both rate and equilibrium effects is made by comparing the addition of CN^- to NAD and APAD (see also paper 1 in this series; Burgner & Ray, 1984a). Since the nonenzymic reaction of CN^- with NAD is $^1/_6$ the rate of that with APAD (data not shown), k_f^W for the reaction of pyruvate enol with NAD (comparison A, Table II) is taken as about $^1/_6$ of that for its reaction with APAD, or $2.5 \times 10^{-6}\ M^{-1}\ s^{-1}$. Since the reaction of Pyr_E with NAD will be compared with the enzymic reaction where only A-side addition occurs, the value of k_f^W in Table II (comparison A) is reduced by an additional 2-fold.

To estimate k_r^H for the reverse NAD–Pyr adduct reaction (comparison B, Table II), the measured value of k_r^H for the reverse APAD–Pyr adduct reaction, 0.067 $M^{-1}\ s^{-1}$, is multiplied by 17, since NAD–CN decomposes 17-fold faster than does APAD–CN.[6]

The equilibrium constants for the adduct reactions involving CN^- and APAD or NAD are 2.9×10^4 and 2.5×10^2, respectively (Wallenfels & Diekman, 1958), and the relative values of the equilibrium constants involving SO_3^{2-} are similar (J. W. Burgner, II, and W. J. Ray, Jr., unpublished results). Hence, NAD exhibits about 1% of the tendency of APAD toward nucleophilic addition at the 4 position, and K_{eq}^{app} (pH 7.0) for the reaction of pyruvate with NAD is about 100-fold less than the measured value for the corresponding reaction with APAD, or about $10^{-4}\ M^{-1}$ (see preceding section). But, the latter constant applies to the reaction of NAD with pyruvate ketone. When the equilibrium constant for the enolization of pyruvate ($K_{enol} = 4 \times 10^{-6}$; Burgner & Ray, 1974) is factored out, the equilibrium constant for the addition of pyruvate enol to NAD becomes 25 M^{-1} (comparison C, Table II).

The value of k_6, the rate constant for the enzyme-catalyzed breaking of the adduct linkage (comparison B, Table II), is taken from the second paper of this series: $3 \times 10^{-3}\ s^{-1}$ (Burgner & Ray, 1984b). Values of the other rate constants in Table I, viz., k_4 and k_5, may be estimated in the following way from data reported in the above paper and this paper. The value of K_{eq} for the process $E{\cdot}H{\cdot}NAD{\cdot}Pyr_K \rightarrow E{\cdot}H{\cdot}adduct$ is 5×10^{-6} at pH 7 (Burgner & Ray, 1984b), and K_{eq} is related to various constants in Scheme I in the following way:

[6] The ratio of the equilibrium constants for formation of APAD–CN and NAD–CN is 100 (see below), and the ratio of the rate constants for the decomposition of NAD–CN and APAD–CN is approximately 100/6 or 17, where 6 is the ratio of the respective rate constants for the addition of CN^- to APAD and NAD.

$$K_{eq} = \frac{k_5 k_1 K_{enol} K_{Pyr} K_H}{k_6 k_2}$$

Since estimates are available for K_{enol} (4×10^{-6}; Burgner & Ray, 1978) and K_{Pyr} and K_H (0.005 M and $10^{-7.1}$, respectively; Burgner & Ray, 1984b), k_5 (comparison A, Table II) can be approximated if the ratio k_1/k_2, which is the binding constant for Pyr_E to E·NAD·Pyr_E, is known. Because this constant cannot be measured directly, we have used an estimate of 50 M^{-1}, which is based on a model described in the following section. With this value for k_1/k_2, all of the equilibrium constants in the small thermodynamic cycle in the lower right-hand corner of Scheme I are either known or can be calculated. (These values are summarized in Table I.) From such a calculation, we estimate that the apparent equilibrium constant, k_3/k_4, for the buffer-assisted enolization of bound pyruvate ketone is about 10^{-6}. The individual values for k_3 and k_4 in the imidazole-catalyzed tautomerization of bound pyruvate, 30 M^{-1} s^{-1} and $10^{7.3}$ M^{-1} s^{-1}, respectively, are listed in Table II. Since the reactions involving k_3 and k_4 (comparisons D and E, Table II) represent the transfer of a proton to and from (free) imidazole and the methyl carbon of pyruvate, respectively, it follows that k_3/k_4 is equal to K_a/K_{IM}, or the ratio of two ionization constants, where K_{IM} is the normal dissociation constant for imidazolium and K_a is the effective dissociation constant for bound Pyr_K acting as a carbon acid. On the basis of a pK_a of 7.3 for imidazole, the pK_a for the β-hydrogen of bound pyruvate ketone must be about 13, which is about 6 units lower than that expected for the unbound ketone [cf. Hegarty & Jeneks (1975)]. From estimates for k_4 and k_4/k_5, we conclude that k_5 is about 2×10^5 s^{-1} and that the equilibrium constant for formation of E·H·adduct from E·NAD·Pyr_E is about 10^8 (see comparison C, Table II). As an internal check on these results, we have evaluated the product of the equilibrium constants for each of the cycles involving the dissociation of E·H·adduct in Scheme I. In each case, the products were within 1 order of magnitude of unity, which is a reasonable range, given in error in each of the constants for the various steps in Scheme I: 2–3-fold. Hence, the estimates given in Table I for the enzymic process are reasonable.

Dissociation Constant for E·NAD·Pyr_E. Although a direct assessment of the equilibrium dissociation constant for bound pyruvate enol cannot be made, the value of this constant can be estimated by comparing it with those for appropriate models: glycolate and lactate. Glycolate, although a poor substrate, acts as a competitive inhibitor with respect to lactate: K_i^{app} = 0.1 M at pH 7.0; K_I = 0.02 M after correction for competitive binding of chloride ([Cl^-] = 0.3 M; $K_{I_2,Cl}$ = 0.07 M; Burgner & Ray, 1984b). A similar value, 0.15 M, for the apparent dissociation constant of lactate from the central complexes in the normal steady-state enzymic reaction can be estimated from the data of Zewe & Fromm (1965) (rabbit A_4 enzyme, pH 7.15, in 0.6 M chloride). When a correction is made for competitive binding of chloride and for the approximately 1:1 distribution of the central complexes (Sudi, 1974; Whitaker et al., 1974; J. W. Burgner, II, unpublished data for the dogfish enzyme), the equilibrium constant for dissociation of lactate from E·NAD·Lac is the same as that for E·NAD·glycolate, 0.02 M. This observation suggests that E·NAD·Lac and E·NAD·glycolate serve as an adequate models for estimating the dissociation constant of E·NAD·Pyr_E, which we take as about 0.02 M under the above conditions.

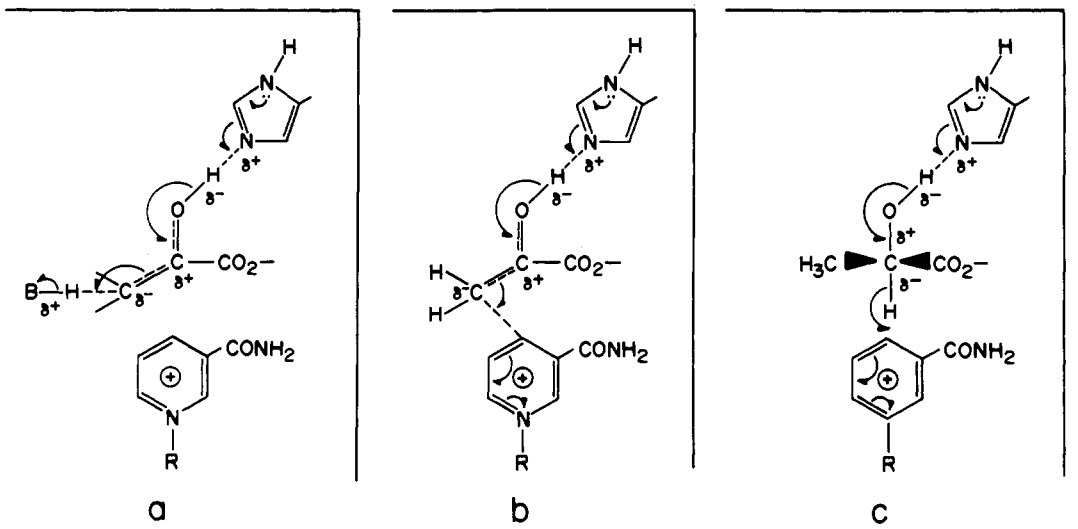
A Model for the Normal LDH Redox Reaction. When 1-methylnicotinamide chloride (about 0.01 M) is dissolved in dry, freshly distilled ethanol, a spectral peak with a maximum at 357 nm slowly appears. This peak is identical in shape and position with the peak produced by dihydro-1-methylnicotinamide prepared by the method of Karrer & Blumer (1947) and dissolved in ethanol. To establish that acetaldehyde also is a product of the above reaction, a reaction mixture containing 1-methylnicotinamide in excess of saturation in dry ethanol was incubated for 5 days at 55 °C in the absence of air. Part of this mixture was distilled under nitrogen at 21–22 °C into a cold trap (see Materials and Methods). The addition of an aliquot of this distillate to a mixture of alcohol dehydrogenase and NADH produced an immediate decrease in the optical density at 340 nm, which indicates the presence of an aldehyde. The distillate from a control reaction lacking 1-methylnicotinamide produced no detectable change under the same conditions. A gas chromatographic procedure (Westcott et al., 1980) also was used to estimate the amount of acetaldehyde present, about 0.02 μmol/L, in a sample volatilized under nitrogen at 65 °C. From the ΔA_{357} and an extinction coefficient of 6×10^3 cm^{-1} M^{-1} for the dihydro form of 1-methylnicotinamide, approximately 0.1 μmol/L of the 1-methylnicotinamide was reduced under the above conditions. Hence, only 20% of the acetaldehyde expected on a mole-per-mole basis was detected by gas chromatography. (Part of the aldehyde could have been lost either through an aldol condensation or by a further reduction to the ethyl ester, or both.) From product–time plots of the absorbance at 357 nm, the rate constant for oxidation of ethanol by 1-methylnicotinamide must be about 5×10^{-12} M^{-1} s^{-1} at 25 °C. An Arrhenius plot involving four temperatures in the range of 35–65 °C appears to be linear with a $\Delta H^{\ddagger}$ of about 30 kcal/mol (data not shown).

Discussion

There are several properties of lactate dehydrogenase that recommend it for studies on enzymic catalysis. (1) It is a very "effective" catalyst: $[k_{cat}/(K_{NAD}K_{Lac})]/k_{ne}$ is about 10^{19} M^{-1}.[7] (2) It facilitates hydride transfer with a high efficiency: k_e/k_{ne} is about 10^{14} M (see Results). (3) It accelerates a number of addition reactions that in some respects mimic hydride transfer but are easier to examine in detail (see earlier papers in this series). (4) Its molecular structure is known (Grau et al., 1981). In fact, we depend heavily on the latter two properties in providing a partial rationale for the huge size of k_e/k_{ne}. Our rationale is based on and restricted to a consideration of factors that contribute to catalysis in simple systems and that can be evaluated, at least *semi*quantitatively, by studying reactions catalyzed by lactate dehydrogenase *other than* its normal redox reaction. For example, our assessment of the contribution of general catalysis to the normal enzymic reaction is obtained by using the enzyme as a general catalyst in a different and somewhat simpler reaction—as opposed to evaluating the possible contribution of general catalysis from studies of nonenzymic systems. With this approach, we have identified and obtained *independent* minimal estimates for the magnitudes of three factors that contribute to catalysis in the normal reaction: an equilibrium binding effect, general catalysis, and reactant immobilization. We also show that the

[7] The ratio of k_e/k_{ne}, 10^{14} M, and estimates for K_{NAD}, 10^{-3} M, and K_{Lac}, 10^{-2} M, are from Results. Similar calculations indicate that the catalytic efficiency of LDH in the pyruvate, sulfite, and cyanide adduct reactions is on the order of 10^{15} M^{-1}, 10^7 M^{-1}, and 10^6 M^{-1}, respectively. The first of these values represents a respectable efficiency, but even it does not proceed at a significant rate relative to the normal redox reaction in the presence of saturating reactants—because its catalytic efficiencies as well as those for the other adduct reactions are produced primarily by large binding affinities between the enzyme and the reactants.

Scheme III



product of these contributions accounts for much of the effectiveness of the enzyme in facilitating hydride transfer. Before considering the basis for these estimates, two aspects of the normal hydride transfer process are described: the interactions that occur between the enzyme and reactants in the normal enzyme–substrate complex and the extent that LDH accelerates this reaction.

Structural Studies on Complexes of Lactate Dehydrogenase. Structural studies using diffraction techniques have been conducted on four different crystalline complexes: E·H·NAD·oxalate, E·H·NAD·oxamate, E·H·NAD–Pyr, and E·NAD·Lac [cf. Holbrook et al. (1975) and Grau et al. (1981)]. For the reactive E·NAD·Lac complex, these structural models imply that lactate and NAD are positioned so that direct hydride transfer can occur between them, that the imidazole(ium) side chain of His-195 is positioned so that general catalysis of hydride transfer to pyruvate and from lactate can occur, and that one face of the nicotinamide ring of NAD(H) is buried in a hydrophobic environment with perhaps a hydrogen bond between the carboxamide of NAD and the enzyme.

Efficiency of the Enzyme in Facilitating Hydride Transfer. The best reaction for estimating the magnitude of the denominator in the ratio k_e/k_{ne} in the LDH system would involve the reduction of NAD by lactate in water. Because of technical problems, we have used an alternate process: the reduction of 1-methylnicotinamide hydrochloride by ethanol in the neat solvent (see Results). Although this reaction is quite slow, enough products can be generated that both 1,4-dihydro-1-methylnicotinamide and acetaldehyde can be identified by proton NMR and gas chromatography, respectively. Although the effects of solvent on the rate of this reaction are not known with any accuracy, a preliminary study indicates that in 10% ethanol–water the rate constant for oxidation of ethanol is *reduced* only by about 5-fold relative to that observed in pure ethanol. On the other hand, NAD is *more reactive* than 1-methylnicotinamide toward addition of CN^- in aqueous media, and both are about equally reactive in methanol (Burgner & Ray, 1984a). Since some solvent properties apparently favor the redox reaction in water and others favor it in alcohol, we use the observed value 5×10^{-12} M^{-1} s^{-1} at 25 °C from the ethanol study in place of the value for the ideal nonenzymic reaction. By contrast, Sudi (1974) has estimated from fast-reaction kinetic studies that the rate constant for oxidation of lactate in the E·NAD·Lac complex is about 600 s^{-1}. The ratio of these constants, 10^{14} M, seems to be a reasonable estimate for the bond making/breaking effect produced by LDH, i.e., k_e/k_{ne}.

Pyruvate Adduct Reaction Catalyzed by Lactate Dehydrogenase. Both steps of the NAD–pyruvate adduct reaction (right-hand vertical reaction in Scheme I) provide information about the origin of the huge value of k_e/k_{ne}, above. These steps are the tautomerization of bound pyruvate (steps k_3 and k_4) and the reversible addition of bound pyruvate enol to the 4 position of the nicotinamide ring of NAD (steps k_5 and k_6). The values of the rate constants for these reactions as well as the equilibrium constants for the small thermodynamic cycle in the lower right-hand corner of Scheme I are given in Table I and compared with their corresponding nonenzymic reactions in Table II.

The relationships between the presumed transition states for the tautomerization of bound pyruvate, the NAD–Pyr adduct reaction, and the normal redox reaction are emphasized by the comparisons in Scheme III. In all three schematics, the side chain of His-195 acts as a general base. For the first two, proton removal from the 2-OH group by this imidazole side chain facilitates competing electrophilic reactions: attack at C_3 of pyruvate by BH^+ (ketonization of the enol) or by NAD (adduct reaction). In the last one, proton removal facilitates electrophilic attack by NAD at the 2-H of lactate (normal redox reaction). We exploit certain similarities in these transition states in the following discussion where the origin and magnitude of the rate enhancement factors cited above are considered. Subsequently, these factors are used to provide a rationale of k_e/k_{ne} for the NAD–Pyr adduct reaction and a rationale for a large part of k_e/k_{ne} for the normal redox reaction.

General Catalysis of Pyruvate Tautomerization by Lactate Dehydrogenase. When a general base catalyzes the conversion of a ketone to its tautomeric enol, the base participates in the removal of an α-hydrogen. The reverse process, conversion of the enolate to the ketone, is subject to general acid catalysis, and proton transfer again involves the α-hydrogen. During *concerted* general catalysis of such reactions, one catalyst functions at the α-carbon and the other at the hydrogen that either is or will become the enolic hydrogen. But, participation of a general catalyst at the ketone/enol oxygen in concert with a general catalyst at the α-carbon is observed only infrequently in model systems [e.g., Hegarty & Jencks (1975)], even though one criterion for such participation, a large difference in pK_a between the reactant and product (Jencks, 1972)—here the enol and the protonated ketone—obviously is satisfied. In fact,

the increased catalytic efficiency usually observed for the action of a general catalyst at the ketone/enol oxygen *in concert* with a general catalyst at the α-carbon is marginal in the few simple aqueous systems where it has been observed (Hegarty & Jencks, 1975)—even when the general acid catalyst is incorporated into the reactant so that it could act intramolecularly (Page & Jencks, 1972).

When pyruvate ketone/enol is bound to lactate dehydrogenase (in the presence of bound NAD), a rapid interconversion of the tautomeric forms of pyruvate can be observed. The general catalyst acting at the 3-carbon of pyruvate in this process has been referred to as the "external" catalyst since this catalyst does not reside on the enzyme [cf. Burgner & Ray (1978)]. The behavior of several external amine catalysts is described in an accompanying paper (Burgner & Ray, 1984b) along with the relevant pK_a relationships and deuterium isotope effects. However, only imidazole/imidazolium ion is used as the external general catalyst in the studies described here.

In the above system, pyruvate binds with its carbonyl group close to and probably H bonded to the imidazolium side chain of His-195 in the active site of lactate dehydrogenase (Holbrook et al., 1975). On the basis of this structure and on case C in Jencks' (1976) review of general catalysis, the enzyme almost certainly functions as a general catalyst during the interconversion of the bound ketone/enol by facilitating proton transfer at the ketone/enol oxygen in concert with the action of the external catalyst at the α-carbon—as in Scheme IIIa. To assess the increased catalytic efficiency provided by the participation of the enzyme in the tautomeric interconversion of bound pyruvate, we compare the rate of ketonization of bound pyruvate enol by imidazolium ion (an external general acid) and the *unprotonated* form of the enzyme—reaction designated as BH/E—with that involving free pyruvate enol and imidazolium ion in water: BH/H_2O (comparison D, Table II). We also compare the rate of enolization of bound pyruvate ketone involving imidazole (an external general base) and the *protonated* form of the enzyme, B/E·H, with that of free pyruvate ketone and imidazole in water: B/H_2O (comparison E, Table II). The rate enhancement in either direction for bound pyruvate, $10^{5.8}$-fold for ketonization and $10^{5.3}$-fold for enolization, is quite large for this type of reaction [cf. Page (1981) and Lipscomb (1981)]. The value for the ketonization of the enol will be used, below, in our attempt to provide a rationale for the efficiency of LDH in facilitating the bond making/breaking that occurs during the oxidation of lactate since there are similarities in the locations of the developing charges on the reactants in the expected transition states for the ketonization of bound pyruvate enol, Scheme IIIa, and for the oxidation of bound lactate by NAD, Scheme IIIc.

Equilibrium Binding Effects. In the first paper of this series (Burgner & Ray, 1984a), we show that in the cyanide adduct reaction the equilibrium constant involving bound reactants and products, eq 1, is about 200-fold larger than that for free reactants and products, eq 2. This difference is not unexpected, since the NAD–CN adduct is "NADH-like" and the CN group of the bound NAD–CN adduct does not appear to interact directly with the enzyme (Burgner & Ray, 1984a). Thus, the reaction in eq 1 is something like the conversion of bound NAD to bound NADH, and NADH binds some 300-fold more tenaciously to LDH than does NAD (Stinson & Holbrook, 1973). These considerations suggest that the equilibrium binding effect probably results from an environmental effect at the nicotinamide/dihydronicotinamide region of the binding site for the coenzyme.

$$\text{E·NAD} + \text{CN} \rightleftharpoons \text{E·NAD–CN} \quad (1)$$

$$\text{NAD} + \text{CN} \rightleftharpoons \text{NAD–CN} \quad (2)$$

In the cyanide adduct reaction, this equilibrium binding effect essentially alters only the rate constant for the forward (bond-forming) process. Thus, bound NAD reacts with (free) cyanide some 100–200-fold faster than does free NAD, while cyanide dissociates from bound and free NAD–CN at essentially the same rate. It is not obvious how binding interactions that increase the equilibrium constant for a reaction can be channeled by an enzyme so that only the rate of the reaction in *one* direction (here the bond-making direction) is altered. However, these interactions appear to involve only the nicotinamide/dihydronicotinamide moiety of the coenzyme, and the same equilibrium binding effect probably can increase the rate of other reactions by about 100-fold when a nucleophile adds to the bound NAD moiety to produce either NADH or a NADH-like moiety.

Reactant Immobilization and the Chelate Effect. Page & Jencks (1971) point out the enormous entropic advantage that unimolecular reactions can have over the corresponding bimolecular reactions where the reacting groups do not reside on the same molecule and when such reactions are compared in the gas phase. They also show that a large part of this advantage can be expected for reactions in solutions [see also Jencks (1975, 1980, 1981)]. One requirement for observing such a huge advantage is a transition state with a highly restricted geometry or structure i.e., a "tight" transition state, or one with a much lower standard entropy than the ground state. Thus, one way for an enzyme to facilitate a bimolecular process with a "tight" transition state would be to minimize the (standard) ground-state entropy of the reactants, i.e., the entropy of A and B in the E·A·B complex, prior to bond making. Jencks (1975, 1980) points out that the extensive immobilization of a reactant requires that the enzyme exhibit a large intrinsic binding potential with respect to the reactant. Thus, from an intuitive standpoint, extensive immobilization seems more feasible for large substrates with their many potential interaction sites than for small ones. But, even with very small substrates, one cannot rule out an immobilization mechanism on the basis of a presumed insufficiency of intrinsic binding energy, since, by definition, intrinsic binding energy is whatever it takes in terms of a Gibbs energy change to rationalize the observed phenomenon. In fact, a direct experimental test of an immobilization mechanism is difficult to devise for substrates of any size since a valid test must be capable also of providing evidence that could negate that mechanism. But, Ray & Long (1976) point out that in some cases the presence of an immobilization mechanism might be substantiated by estimating the magnitude of "chelate effects" associated with substrate binding. To estimate the size of such chelate effects, the sequential binding of two substrates, A and B, is compared with the binding of A–B. Barring complications, if A and B are extensively immobilized during binding, A–B should bind more tenaciously than A plus B (Jencks, 1981). Thus, if much of the translational and rotational entropy of A and B is lost during the binding step, the unfavorable effect of this loss on the binding process can be greatly reduced by forming the A–B bond *prior* to the binding step—again barring the complications considered below. Ray & Long (1976) refer to the increased binding of A–B relative to A plus B as a chelate effect, by analogy with the classical effect obtained for the binding of bidentate ligands, as opposed to monodentate ligands, to a metal ion. But, a metal ion represents a sterically less demanding binding site than the

Table III: Binding Interactions Involving Lactate Dehydrogenase That Give an Estimate of the Chelate Effect[a]

part	reaction	equilibrium const	estimated chelate effect
A	$E{\cdot}H{\cdot}NAD{\cdot}Pyr_K \rightleftharpoons E{\cdot}H{\cdot}NAD + Pyr_K$	5×10^{-3} M	
	$E{\cdot}H{\cdot}NAD + Pyr_K \rightleftharpoons E{\cdot}H + NAD + Pyr_K$	2×10^{-3} M	
	$E{\cdot}H + NAD{-}Pyr \rightleftharpoons E{\cdot}H{\cdot}NAD{-}Pyr$	2×10^{10} M^{-1}	
	$E{\cdot}H{\cdot}NAD{\cdot}Pyr_K + NAD{-}Pyr \rightleftharpoons E{\cdot}H{\cdot}NAD{-}Pyr + NAD + Pyr_K$	2×10^{5} M	400[b]
B	$NAD{-}Pyr \rightleftharpoons Pyr_E^- + NAD$	2×10^{-8}	
	$E{\cdot}H{\cdot}NAD{\cdot}Pyr_E^- \rightleftharpoons E{\cdot}H{\cdot}NAD{-}Pyr$	1×10^{12}	
	$E{\cdot}H{\cdot}NAD{\cdot}Pyr_E^- + NAD{-}Pyr \rightleftharpoons E{\cdot}H{\cdot}NAD{-}Pyr + NAD + Pyr_E^-$	2×10^{4}	200[c]
C	$NAD{-}SO_3^- \rightleftharpoons NAD + SO_3^{2-}$	3×10^{-2} M	
	$E{\cdot}H{\cdot}NAD{\cdot}SO_3^{2-} \rightleftharpoons E{\cdot}H{\cdot}NAD{-}SO_3^-$	2×10^{6} M	
	$E{\cdot}H{\cdot}NAD{\cdot}SO_3^{2-} + NAD{-}SO_3^- \rightleftharpoons E{\cdot}H{\cdot}NAD{-}SO_3^- + NAD + SO_3^{2-}$	6×10^{4}	600[d]

[a] The values of the various equilibrium constants in parts A and B are from this paper; those in part C are from Parker et al. (1978); all refer to pH 7.0. [b] Because pyruvate may not interact with enzyme to the same extent in $E{\cdot}H{\cdot}NAD{\cdot}Pyr_K$ as it does in E·H·NAD–Pyr because of differences in the chemical nature of A and because pyruvate appears to bind about 5-fold tighter to E·H·NADH than to E·H·NAD, in part A we have *further* reduced the product of equilibrium constants by a factor of 5-fold to account for increased binding of the pyruvate moiety in E·H·NAD–Pyr, as opposed to E·H·NAD·Pyr. The ratio of the association constants for the binding of oxamate, which is a nonreacting structural analogue of pyruvate ketone, to E·H·NADH and E·H·NAD under the same conditions is about 5 (Burgner, 1973). [c] The increased binding of the pyruvate moiety in the product complex (E·H·NAD–Pyr) likely is offset to a first approximation by the loss of the negative charge originally present on the enolate anion. [Because of interactions with Arg-171 and the protonated form of His-195, dianions are expected to bind tighter to a given form of the enzyme than monanions, but the data of Novoa et al. (1959) show that in the binding of pyruvate analogues, the oxalate dianion binds to E·H·NAD to approximately the same extent as the oxamate monoanion binds to E·H·NADH.] Hence we use only a factor of 100-fold for increased binding interactions of the NAD moiety in the adduct complex in estimating the residual chelate effect. (It should be pointed out that the structures of $E{\cdot}H{\cdot}NAD{\cdot}Pyr_K$, $E{\cdot}H{\cdot}NAD{\cdot}Pyr_E^-$, and E·H·NAD–Pyr in parts A and B probably are quite similar. Presumably, the $-CO_2^-$ group of free pyruvate, both keto and enol forms, as well as that of NAD–Pyr binds at Arg-171; moreover, pH studies suggest that both the >C=O of pyruvate ketone and the –C–O⁻ of the enol anion of pyruvate interact with the protonated from of His-195 [cf. Holbrook et al. (1975)].) [d] Both SO_3^{2-} and the $-SO_3^-$ moiety of the adduct of probably interact with the protonated form of His-195 (Parker et al., 1978), and we assume that differences in the binding of SO_3^{2-} (reactant) and the $-SO_3^-$ moiety (product) are largely compensated by tighter binding to the E·H·NADH-like form of the enzyme (product) than to the E·H·NAD form (reactant) as in part B, above. Hence, only the factor used in compensating for the increased binding of NAD is used in estimating.

active site on an enzyme. In fact, a site that employs multiple binding interactions of a substantially directional nature cannot bind *both* A–B and A plus B optimally (Haldane, 1930; Jencks, 1975). Because of this, the true chelate effect either can be over- or underestimated in A–B/(A + B) systems. Ray & Long (1976) attempted to circumvent this problem by comparing the binding of $A{-}CH_2{-}O{-}B$ with that of AH plus HB. Since that approach is not feasible here, we have assessed the chelate effect, experimentally, in the only way that seems feasible in the present system and will consider, later, whether our estimate is valid.

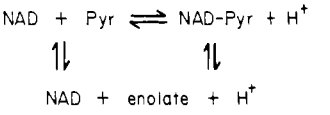
It should be pointed out that because a thermodynamic cycle is involved, a comparison of the binding of A + B and A–B to an enzyme, as above, is precisely equivalent to a comparison of the equilibrium constants for (A + B) ⇌ A–B for the free solution process and for the same reaction involving bound reactants and product. If the equilibrium constant for bound reactants and products is much larger than for free reactants and products, there is a correspondingly large chelate effect involved in the binding of A–B, relative to A + B. A sort of composite comparison of this type involves the displacement of bound A and bound B by A–B:

$$E{\cdot}A{\cdot}B + A{-}B \rightleftharpoons E{\cdot}A{-}B + (A + B) \qquad (3)$$

Below we use the value of the equilibrium constant for processes of this type as an estimate of the size of the chelate effect for the binding of A–B.[8]

In Table III, parts A–C, the sums of the individual reactions define equilibria for which there are potential chelate effects. Thus, the sum of the reactions in (A) is governed by the relative binding affinity of NAD + Pyr_K and NAD–Pyr. In (B), Pyr_E^- instead of Pyr_K is considered, and in (C), the species are NAD + SO_3^{2-} and $NAD{-}SO_3^-$. In each of these systems, which are discussed in detail in the supplemental material (see paragraph at end of paper regarding supplementary material), the product of the equilibrium constants for the individual reactions defines a process analogous to eq 3. But, this product contains both the above chelate effect *plus* any effects caused by changes in the nature of the binding interactions of the product, A–B, relative to the reactants, A + B, i.e., because of chemical changes that accompany the process A + B → A–B. In a previous section, we point out that the NAD part of NAD–CN (or NAD–Pyr) is more like NADH, chemically, than NAD. Since we already have identified the effect of this difference as an equilibrium binding effect, we must factor 100-fold from the equilibrium constants for each of the summed reactions in this table in order not to count this effect twice. The equilibrium constants for these summed reactions also are corrected for the small differences in binding of pyruvate (group) in the reactant and product complexes that are caused by the chemical differences in reactant and product coenzyme (see footnotes to Table III). In each case, the estimated magnitude of the chelate effect is between 100 M and 1000 M. Thus, we are able to verify only a relatively modest chelate effect for various A–B/(A + B) systems that involve the same or similar groups that one finds in the normal enzyme–substrate complexes, and we will use a value of 1000-fold for the reactant immobilization factor in the following discussion.

Scheme IV



Efficiency of LDH in Facilitating the Pyruvate Adduct Reaction. The *nonenzymic* NAD–pyr adduct reaction, Scheme IV, involves the addition of both the enolic form (upper pathway) and the enolate form (lower pathway) of pyruvate to NAD. The reaction involving the *enolate* anion is subject to catalysis by water in both directions. The reaction involving the *enol*, which is subject to catalysis by water in the forward direction and to specific acid catalysis in the

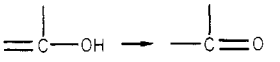
[8] Although this *approach* differs somewhat from that suggested by Jencks (1981), in the present system where the coenzyme and pyruvate bind sequentially, we feel that the equilibrium in eq 3 provides a more equitable estimate of the chelate effect.

reverse direction, is compared with the enzymic reaction that proceeds in only one way: a direct addition of the enol to NAD within the E·NAD·Pyr_E complex.[9] In the forward direction, k_e/k_{ne} is about 10^{11} M (comparison A, Table II); i.e., the enzyme increases the rate constant for addition of bound enol to NAD by a factor of 10^{11} M relative to that for the uncatalyzed (water-catalyzed) reaction. By contrast, in the reverse reaction, the enzymic process is faster than the nonenzymic process only by a factor of about $10^{3.5}$ (comparison B, Table II).

Of the three rate factors discussed above, the equilibrium binding effect involving the NAD/NADH pair, which was estimated from the cyanide adduct reaction (Burgner & Ray, 1984a), could account for a rate increase of about 100-fold for the forward pyruvate adduct reaction. In addition, reactant immobilization, which was estimated from the chelate effect on the equilibrium constants of both the pyruvate and sulfite adduct reactions, could contribute in additional rate increase of up to 1000 M. Finally, general catalysis, which was evaluated from the tautomerization of bound pyruvate, could account for a further rate increase of 10^6-fold. Since the product of these three factors is in the neighborhood of 10^{11} M and since the overall rate effect on the adduct reaction induced by lactate dehydrogenase also is 10^{11} M, the role of the enzyme in the adduct reaction can be rationalized in terms of the above concepts, although these concepts, in turn, may need to be rationalized in terms of simpler concepts (see below). Finally, the agreement of the product of these three factors with the observed efficiency of LDH as a bond maker/breaker in the pyruvate adduct reaction does not provide a completely rigorous test of the validity of any one factor, even though each factor was evaluated independently. But, this agreement does require that any increase in one factor must be accompanied by a decrease in one or both of the other factors. Since the estimates of our factors were made conservatively, we believe that all three estimates are in the correct range for the present system.

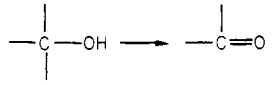
Origin of the Rate Effect for the Normal Redox Reaction Catalyzed by LDH. In a previous section (see Scheme III), we make a tentative case for similarities among features of the transition states for (a) the various adduct reactions, (b) the tautomerization of bound pyruvate, and (c) the normal redox reaction catalyzed by LDH. In fact, the similarity for the former two reactions apparently is sufficiently close that the enzymic enhancement of the tautomerization reaction is related directly to this enhancement of the adduct reaction. The closeness of this similarity is indicated by the agreement between the measured enhancement for the adduct reaction, 10^{11}-fold, and the product of the component enhancements, which include the enhancement for tautomerization, although this argument is somewhat circular—see above. In view of this similarity, we attempt to extrapolate our conclusions about the pyruvate adduct reaction to the normal redox reaction, although much less is known about the latter. For example, there is general agreement that the normal LDH reaction *probably* is a two-electron-transfer process [cf. Blankenhorn (1977)], even though most such arguments are based on a *lack* of evidence for two single-electron-transfer steps and fall short of being entirely conclusive. However, in view of the facility of the LDH-catalyzed adduct reaction, which is a two-electron-transfer process, we also favor the two-electron-transfer process for the redox reaction and further assume that general catalysis, the equilibrium binding effect, and reactant immobilization all contribute to the overall rate effect, as is discussed below.

General Catalysis in the Normal Redox Reaction. In simple systems, general catalysis of the removal of a hydroxyl hydrogen may not be observed, because of the efficiency of specific base catalysis, e.g., as in the ketonization of acetone enol, which involves specific base–general acid catalysis (Hegarty & Jencks, 1974). In the present system, where hydroxide ion presumably is excluded from the hydroxyl group of bound lactate, it is not surprising that LDH accepts a proton from this hydroxyl group as part of the hydride-transfer process, given the way in which this group interacts with the enzyme: see previous section and case C in Jencks (1976). What is unexpected is the increased efficiency of the conversion (in the ketonization of pyruvate enol)



when LDH, as opposed to water, "solvates" this group.[10] It may well be that electrostatic effects contribute significantly or even overwhelmingly to what we have designated above as general catalysis. In any case, it seems likely that those factors that contribute to the ease of

also contribute to



in the normal redox reaction. The question is, "How much?"

We are unable to answer this question with any degree of confidence, since such an answer would require a knowledge of the relative timing of proton transfers during the two processes. For example, in the general base catalyzed enolization of acetone, the product of the rate-limiting step is the enolate (Hegary & Jencks, 1974), and the transition state for forming the enolate likely occurs late in the overall process (see also above and the supplemental material). On the other hand, in the case of enzyme-bound pyruvate ketone, the transition state leading to pyruvate enol probably occurs much earlier because the enzyme acts as a general acid. In fact, maximal catalysis of this enolization should occur if the extent of bond breaking and making is approximately equal in the transition state. Unfortunately, we do not know whether this occurs or not. But, in the normal redox reaction, where the equilibrium constant for E·H·NADH·Pyr ⇌ E·NAD·Lac approaches 1 and no proton release steps are involved, the degree of bond breaking probably is nearly equal to the degree of bond making.[11] Hence, if the extent of bond breaking and making in the transition state for the enolization of enzyme-bound pyruvate ketone differs substantially from that of the normal reaction, especially with regard to the O···H···N bond involving His-195, the later group acting as a general catalyst might accelerate the normal redox reaction to a significantly greater

[9] Although Grau et al. (1981) observe that the keto oxygen of the pyruvate moiety of the adduct may not be H bonded to the active site histidine, M. G. Rossmann (personal communication) indicates that the electron-density maps are relatively weak in this region and that such an H bond may indeed be present.

[10] The enyzme pyruvate kinase also increases the rate of ketonization of pyruvate enol although only by about 10^3-fold (Kuo et al., 1979). The importance of an external general acid during this process does not seem to be known.

[11] At pH 7, where His-195 is approximately half-protonated, E·H binds Pyr_K only about 4-fold tighter than E binds Pyr_E: see Results.

extent than it accelerates the enolization reaction, vis., greater than 10^6-fold.

Reactant Immobilization in the Normal Redox Reaction. The factor of 1000 M for ground-state immobilization that was suggested, above, for the LDH-facilitated pyruvate adduct reaction is much smaller than the maximal value 10^8 M for such an effect (Page & Jencks, 1971) as well as operational values for this effect that have been suggested for nucleophilic substitution at a carbonyl group (Moore & Jencks, 1982). The reduced value in the adduct reaction may arise from a basic difference between it and the reaction of a carbonyl group with a nucleophile. Thus, in carbonyl substitution reactions, partial bond formation between the reactant groups, one of which is frequently the side chain of an enzyme, could occur in the ground state, i.e., the enzyme–substrate complex, and facilitate immobilization of the carbonyl-containing substrate. By contrast, because of differences in the chemical bonding of the free reactants in the adduct reaction, there is no reason to suspect that juxtaposition of the methyl group of Pyr_E and the C–H group at the 4 position of NAD, alone, would lead to partial bond formation. If this difference does provide a rationale for the relatively small value observed for reactant immobilization in the present system, it seems probable that the bond making/breaking steps in other enzymic reactions where partial bond formation between reacting groups can contribute relatively little to reactant immobilization in the ground state may rely less heavily on this type of an effect than might be anticipated solely on the basis of theory. Of course, one could argue that partial bond formation between two reactants bound at the active site of an enzyme is not an absolute requirement for realizing a ground-state immobilization factor much larger than 1000-fold. On the other hand, the rigidity (lack of low-energy motional states) of the enzyme–substrate binding interactions plus the rigidity of the enzyme itself that would be required for a very large immobilization factor is as yet unverified.

Similar arguments should apply to the normal redox reaction catalyzed by LDH, and any interaction between the α-C–H group of lactate and the 4-carbon of the nicotinamide ring produced solely by their juxtaposition must be relatively weak, at best. Although the immobilization of these two reactants in the normal redox reaction might contribute a factor greater than 1000 M to the bond breaking/making process, as a conservative estimate we will use this value, which apparently suffices for the adduct reaction.

Equilibrium Binding Effect in the Normal Redox Reaction. In the cyanide adduct reaction, the equilibrium binding effect of about 200-fold is channeled by the enzyme to produce a rate effect of about 100-fold on the bond-forming process (Burgner & Ray, 1984a). In the normal redox reaction, the equilibrium binding effect, about 10^4-fold, is somewhat larger, since the equilibrium constant for the interconversion of bound reactants and products (E·NAD·Lac $\rightleftharpoons$ E·H·NADH·Pyr) is close to 1 (Sudi, 1974; Whitaker et al., 1974) while the equilibrium constant for the free reactants and products is about 10^4 (at pH 7, where His-195 in both the apoenzyme and the E·NAD complex is approximately half-protonated).[12] Hence, 10^4-fold represents the equilibrium binding effect for the normal redox reaction (in the presence of excess buffer). Presumably, this equilibrium binding effect is the product of the effects for the coenzyme, 200-fold (see above), and the pyruvate/lactate pair. The latter may well originate from the increased stability of the interaction $>N^+—H\cdots O{=}C\ \delta- <$, relative to that of $>N\cdots H–O–C<$. Although we have shown that the equilibrium binding effect caused by the NAD/NADH conversion is used almost entirely to produce a rate enhancement in a nucleophilic adduct reaction involving cyanide, we are less certain about the remainder of the 10^4-fold equilibrium binding effect in the normal redox reaction. But, it seems likely that at least part of this effect can be used to produce a rate enhancement during oxidation of lactate. Hence, as a first approximation, we use 10^3-fold as the rate effect in the normal redox reaction that arises from equilibrium binding effects.

Other Factors. Although we apparently have been successful in rationalizing the efficiency of the bond breaking/making steps in the pyruvate adduct reaction (in terms of the product of factors for general catalysis, a rate effect arising from equilibrium binding effects, and ground-state immobilization), the value of the product of these factors for the normal redox reaction, 10^{12} M, is substantially smaller than the observed 10^{14} M rate effect induced by LDH in the later reaction. A basic question is whether we have failed to identify a rate effect used by the enzyme or whether our estimates of rate factors are too conservative. At present, we are unable to distinguish between these possibilities, although we favor the latter possibility—especially in view of our comments, above, on the size of the rate factor that was assigned to general catalysis. There also may be a "putting-it-all-together" factor. Thus, in isolation, each rate factor may provide a smaller rate effect than when all operate simultaneously. But, this is simply a different way of stating that our rate factors may be too small. In any case, it seems unlikely that a yet unidentified rate effect (mechanism) makes an overwhelming contribution to the bond making/breaking process in the LDH reaction.

Supplementary Material Available

Discussion of the reactions of part B of Table III (5 pages). Ordering information is given on any current masthead page.

References

Arnold, L. J., Jr., & Kaplan, N. O. (1974) *J. Biol. Chem. 249*, 652.

Blankenhorn, G. (1977) in *Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., Ed.) pp 185–199, de Gruyter, New York.

Burgner, J. W., II (1973) Ph.D. Thesis, Purdue University, West Lafayette, IN.

Burgner, J. W., II, & Ray, W. J., Jr. (1974) *Biochemistry 13*, 4229.

Burgner, J. W., II, & Ray, W. J., Jr. (1978) *Biochemistry 17*, 1654.

Burgner, J. W., III, & Ray, W. J., Jr. (1984a) *Biochemistry* (first paper of three in this issue).

Burgner, J. W., III, & Ray, W. J., Jr. (1984b) *Biochemistry* (second paper of three in this issue).

Burgner, J. W., II, Ainslie, G. R., Jr., Cleland, W. W., & Ray, W. J., Jr. (1978) *Biochemistry 17*, 1646.

DiSabato, G. (1968) *Biochem. Biophys. Res. Commun. 33*, 688.

Grau, U., Trommer, W., & Rossmann, M. G. (1981) *J. Mol. Biol. 151*, 289.

Griffin, J. H., & Criddle, R. S. (1970) *Biochemistry 9*, 1195.

[12] A thermodynamic cycle can be constructed to show that, since lactate binds to E·NAD and pyruvate binds to E·H·NADH, proton release in the redox reaction involving free reactants and products will not make a contribution to the measured equilibrium, if the protonated group in E·H·NADH (His-195) has the same pK_a as in E·H [see Burgner & Ray (1984b)] and if one operates with excess buffer at a pH equal to the pK_a.

Grimshaw, C. W., & Cleland, W. W. (1980) *Biochemistry 19*, 3153.
Haldane, J. B. S. (1930) *Enzymes*, Longmans, Green and Co., London.
Hegarty, A. F., & Jencks, W. P. (1975) *J. Am. Chem. Soc. 97*, 7185.
Holbrook, J. J., Lijas, H., Steindel, S. J., & Rossmann, M. G. (1975) *Enzymes, 3rd Ed.* 191-292.
Jencks, W. P. (1972) *Chem. Rev. 72*, 705.
Jencks, W. P. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 219.
Jencks, W. P. (1976) *Acc. Chem. Res. 9*, 425.
Jencks, W. P. (1980) *Mol. Biol., Biochem. Biophys. 32*, 3-25.
Jencks, W. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4046.
Jones, R. A. (1976) *Alrichimica Acta 9*, 35.
Karrer, P., & Blumer, F. (1947) *Helv. Chim. Acta 30*, 1157.
Kuo, D. J., O'Connell, E. L., & Rose, I. A. (1979) *J. Am. Chem. Soc. 101*, 5025.
Lipscomb, W. N. (1981) in *Structural and Functional Aspects of Enzyme Catalysis* (Eggerer, H., & Huber, R.) pp 17-23 Springer-Verlag, New York.
Moore, S. A., & Jencks, W. J. (1982) *J. Biol. Chem. 257*, 2689.
Novoa, W. B., Winer, A. D., Glaid, A. J., & Schwert, G. W. (1959) *J. Biol Chem. 234*, 1143.
Ozols, R. F., & Marinetti, G. V. (1969) *Biochem. Biophys. Res. Commun. 34*, 712.
Page, M. I. (1981) *Chem. Ind. (London)*, 144.
Page, M. I., & Jencks, W. P. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1678.
Page, M. I., & Jencks, W. P. (1972) *J. Am. Chem. Soc. 94*, 8818.
Parker, D. M., Lodola, A., & Holbrook, J. J. (1978) *Biochem. J. 173*, 959.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891.
Ray, W. J., Jr., & Long, J. W. (1976) *Biochemistry 15*, 4018.
Stinson, R. A., & Holbrook, J. J. (1973) *Biochem. J. 131*, 719.
Sudi, J. (1974) *Biochem. J. 139*, 261.
Whitaker, J. R., Yates, D. W., Bennett, N. G., Holbrook, J. J., & Gutfreund, H. (1974) *Biochem. J. 139*, 677.
Zewe, V., & Fromm, H. J. (1965) *Biochemistry 4*, 782.

Gene Heterogeneity: A Basis for Alternative 5.8S rRNA Processing†

S. Dave Smith, Nandita Banerjee, and Thomas O. Sitz*

ABSTRACT: Two bands of 5.8S rRNA were observed when the total RNA isolated from rat or mouse tissue was separated by electrophoresis on high-resolution polyacrylamide gels under denaturing conditions. The minor form, with a lower mobility, represented 15-35% of the total 5.8S rRNA, depending on the source of the tissue. Sequence analysis and the kinetics of formation showed that this minor form is elongated at the 5′ end and is not a precursor. The sequence of the minor form was found to be p(C)CGAUA[CG-, five or six nucleotides longer than the major form. The minor 5.8S rRNA constituent also formed a more stable junction complex with 28S rRNA than the shorter major sequence. The rat DNA sequence that corresponds to the additional nucleotides at the 5′ end of 5.8S rRNA has been reported to be -CCGTACG- [Subrahmanyam, C. S., Cassidy, B., Busch, H., & Rothblum, L. I. (1982) *Nucleic Acids Res. 10*, 3667-3680], a sequence which does not contain the extra adenylic acid residue at position 4 found in the minor form. This suggests that the rodent rRNA genes are heterogeneous and that the insertion of an A residue in the ribosomal precursor RNA can generate an alternate processing site.

The 5.8S ribosomal RNA (rRNA) in eucaryotic cells is hydrogen bonded to its cognate high molecular weight rRNA (25-28 S) in the large ribosomal subunit (60 S) (Pene et al., 1968; Weinberg & Penman, 1968). The 5.8S rRNA along with the two high molecular weight rRNAs, 18S and 28S rRNA, is processed from the 45S precursor RNA in the nucleolus. The signals which identify the processing sites for specific endo- and exonucleolytic cleavages are probably a combination of specific sequences and secondary and tertiary structure (Perry, 1976) and may also be influenced by ribosomal proteins (Nazar, 1982). For example, the sequence -ACGPuPu- has been postulated to be a nuclease recognition site at the 3′ end of the 18S rRNA region in the precursor (Subrahmanyam et al., 1982) while a secondary structure has been proposed as the recognition site for the processing of 5.8S rRNA in *Xenopus* (Nazar, 1982). Recently, a binary complex between U3 RNA and the 32S precursor rRNA has been postulated to signal the processing at the 3′ end of the 5.8S rRNA (Bachellerie et al., 1983; Crouch et al. 1983). The precise roles of these inter- and intramolecular interactions in processing remain to be elucidated.

In previous studies (Sitz et al., 1978, 1981), we made use of naturally occurring differences in nucleotide sequences of 5.8S rRNA to study the role of specific nucleotides in the intermolecular interactions of this RNA with other 5.8S rRNAs and with 28S rRNA and found that single nucleotide changes had dramatic effects. In the present study, we have characterized an elongated form of 5.8S rRNA, which could be derived from a subpopulation of precursor 45S rRNA which

† From the Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061 (S.D.S. and T.O.S.), and the Department of Chemical Sciences, Old Dominion University, Norfolk, Virginia 23508 (N.B.). *Received January 9, 1984.* This work was partially supported by a grant (CA-23632) from the U.S. Public Health Service. Taken in part from the M.S. thesis of S.D.S. This work was presented in preliminary form at the 186th National Meeting of the American Chemical Society (Smith & Sitz, 1983).

0006-2960/84/0423-3648$01.50/0 © 1984 American Chemical Society