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The Lactate Dehydrogenase Catalyzed Pyruvate Adduct Reaction: Simultaneous General Acid–Base Catalysis Involving an Enzyme and an External Catalyst†

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ABSTRACT: The pH dependence of the reaction catalyzed by lactate dehydrogenase, where pyruvate adds covalently to NAD to yield a NAD–Pyr adduct, together with published data on the pH dependence of parameters in the *normal* redox reaction suggests similar binding modes for enolpyruvate and lactate in their complexes with E·NAD (where E is one-fourth of the tetramer), for ketopyruvate in its complexes with the protonated species, E·H·NAD and E·H·NADH, and for the NAD–Pyr adduct and NADH plus pyruvate in their complexes with E·H. These similarities, together with previous data, suggest a reaction scheme for the formation of the enzyme–adduct complex that includes the relevant proton-transfer steps. Seven different amine chloride buffers were used in a study of the reverse adduct reaction, i.e., the decomposition of E·H·NAD–Pyr. These act with varying efficiencies as *external* general acid catalysts; the enzyme apparently acts as a (internal) general base. The involvement of the amine chloride buffers as external general catalysts is supported by the concentration dependence of the buffer effect, by a Brönsted plot, and by solvent deuterium isotope effects. The involvement of the enzyme as an internal general catalyst is inferred from the pH dependence of the reaction and the identities of the nearby groups in the E·H·NAD–Pyr complex (from crystallographic studies). The dependence of the adduct reaction on chloride concentration indicates the presence of dead-end inhibitor complexes of E·H·Cl and E·H·NAD·Cl. Chloride also accelerates the decomposition of the adduct in the complex E·H·NAD–Pyr by binding to this complex.

In the previous paper of this series (Burgner & Ray, 1984a), we compare the nucleophilic addition of cyanide to both free NAD[1] and the complex of NAD with lactate dehydrogenase, E·NAD. Cyanide was used in that comparison, because it is a small anion. The object of that study was to assess the extent that binding of NAD to LDH increases the susceptibility of the nicotinamide ring toward nucleophilic attack at its 4 position.

Pyruvate also acts as a nucleophile toward free NAD, and the analogous adduct reaction[2] with bound NAD also is catalyzed by LDH. But, both the enzymic and the nonenzymic adduct reactions involving pyruvate are more complex than

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[1] Abbreviations: NAD, nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase (the dogfish A_4 enzyme unless otherwise specified); E, a subunit of an LDH tetramer; Pyr, pyruvate; Pyr_K and Pyr_E, the enol and keto forms of pyruvate, respectively; NAD–Pyr or adduct, the product from a covalent addition of the β-carbon of pyruvate enol at the 4 position of the nicotinamide ring of NAD; E·adduct, E·H·adduct, or E·NAD–Pyr, the adduct complex. Equilibrium dissociation constants are designated by K, with appropriate subscripts. k_r^{obsd} is equivalent to k_{obsd}^r used in a previous paper (Burgner & Ray, 1978). In this paper, the formal charges on NAD, H, and BH are omitted.

[2] The adduct reaction refers to the enzymically catalyzed *formation* of the adduct complex (E·H·adduct) from NAD and Pyr; the "reverse" reaction refers to the process E·H·adduct → $E{\cdot}NAD{\cdot}Pyr_E$ → E·NAD + Pyr.

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those with cyanide because of the additional proton-transfer steps that are required, i.e.

$$(E)NAD + CN^- \rightarrow (E)NAD\text{–}CN$$

$$(E)NAD + Pyr \rightarrow (E)NAD\text{–}Pyr + H$$

In fact, previous studies suggest that both the keto and enol forms of Pyr are reactants in the LDH-catalyzed adduct reaction and that the enzyme facilitates the required proton-transfer steps accompanying the enolization/ketonization of bound pyruvate during this reaction (Burgner & Ray, 1978). In the present paper, we provide a basis for evaluating the importance of general catalysis by the enzyme in the enolization/ketonization process (see subsequent paper; Burgner & Ray, 1984b). Evaluating the efficiency of general catalysis by the enzyme is important because a proton transfer also occurs during the *normal* redox reaction catalyzed by LDH [cf. Holbrook et al. (1975)]. LDH almost certainly acts as a general acid in that reaction (and as a general base in the reverse process), and such a modus operandi surely contributes to its efficiency as a catalyst.

Our previous study (Burgner & Ray, 1978) suggests not only that LDH acts as a general catalyst in the adduct reaction but also that an external (unbound) buffer molecule participates as a general catalyst during the reaction. This participation occurs during the tautomerization of enzyme-bound pyruvate, which constitutes one of the two alternative pathways leading to and from the bound NAD–Pyr adduct. Since this suggestion was based only on concentration effects produced by one general base, imidazole, the present study was initiated to provide a firmer basis for this mechanism by assessing the effects of pH, other general catalysts, and solvent deuterium on the adduct reaction. Because we will relate some features of the adduct reaction to the normal LDH reaction in the third paper of this series (Burgner & Ray, 1984b), we also demonstrate that there are parallel pH effects on both processes. It should be emphasized that in these studies the adduct reaction is used primarily to trap the reactive Pyr_E (forward adduct reaction) and as a source of bound Pyr_E (reverse adduct reaction) and not as a model, per se, of the normal enzymic reaction.

Materials and Methods

Most materials and assay procedures are described in previous papers (Burgner et al., 1978; Burgner & Ray, 1974, 1978). Only dogfish A_4 LDH is used in these experiments. The temperature in all assays is 15 °C, and the pH values for the buffers refer to the final assay conditions. The anion concentration is varied by addition of the potassium form of the appropriate salt to the assay mixture before adjusting the pH. For the solvent isotope experiments, the adduct reaction mixture (Burgner & Ray, 1978) and the piperazine and imidazole buffers are prepared in water at the appropriate pH; equal volumes are lyophilized, and the appropriate solvent is added to the remaining powder. For *N*,*N*-dimethylhydroxylamine, the hydrochloride is dissolved in D_2O and adjusted either to a pD of 6.0 or to a pD that produces a concentration of BD^+ equal to the BH^+ concentration in H_2O at pH 6.0. The adduct complex of the enzyme is prepared in D_2O by centrifuging a small volume of an ammonium sulfate slurry of precipitated enzyme, washing once with an equal volume of 65% ammonium sulfate in D_2O, and recentrifuging the precipitate. The centrifuged precipitate is dissolved at room temperature in the appropriate volume of adduct reaction mixture prepared in D_2O, and this solution is allowed to stand for at least 1 h before use.

Analysis of Product–Time Curves for the Enzymic Reaction. The binding of the adduct or NADH to the active site of the enzyme is monitored by following the quenching of fluorescence from enzymic tryptophanes. Tryptophan fluorescence is measured, and the results are quantitated in the manner described previously (Burgner & Ray, 1978).

Data Processing. Some of the generalized constants employed in this section are given appropriate superscripts under Results and Discussion. Values for k_f^{obsd}, the experimentally obtained pseudo-first-order rate constant in the forward reaction, are fitted to a general equation (eq 1), which describes

$$k_f^{obsd} = \frac{k_f}{f(NAD,Pyr,Cl)} + k_r^{app} \quad (1)$$

the relationship between k_f^{obsd}, k_r^{app}, and the fractional concentrations of the various enzyme species present where k_r^{app} refers to the rate constant for the reverse reaction.[3] If there are no E·Pyr or enzyme–chloride complexes present

$$f(NAD,Pyr,Cl) = 1 + \frac{K_{Pyr}}{[Pyr]} + \frac{K_{NAD}K_{Pyr}}{[NAD][Pyr]} \quad (2)$$

If an E·Pyr complex is present

$$f(NAD,Pyr,Cl) = 1 + \frac{K_{Pyr}}{[Pyr]} + \frac{K_{NAD}K_{Pyr}}{[Pyr][NAD]}\left(1 + \frac{[Pyr]}{K_{I,Pyr}}\right) \quad (3)$$

If an E·Pyr complex as well as the chloride complexes E·Cl and E·NAD·Cl is present

$$f(NAD,Pyr,Cl) = 1 + \frac{K_{Pyr}}{[Pyr]}\left(1 + \frac{[Cl^-]}{k_{I_2,Cl}}\right) + \frac{K_{Pyr}K_{NAD}}{[Pyr][NAD]}\left(1 + \frac{[Pyr]}{K_{I,Pyr}} + \frac{[Cl^-]}{K_{I_1,Cl}}\right) \quad (4)$$

Values for k_r^{app} in the reverse reaction are fitted to eq 5 with

$$k_r^{app} = k_r^0 + \frac{(k_r^{max} - k_r^0)[Cl]}{K_{Cl} + [Cl]} \quad (5)$$

data obtained at multiple chloride concentrations. Here, k_r^0 and k_r^{max} are the values of k_r^{app} at zero and saturating concentrations of chloride, respectively. Experimental data are fitted to eq 2, 3, and 5 by nonlinear regression analysis [cf. Burgner & Ray (1978)] by assuming equal variance for the values of the rate constants and using initial estimates of the constants obtained graphically. For eq 4, a linear regression procedure is used by considering reciprocal concentrations of NAD and pyruvate and by weighing according to the fourth power of the difference, $k_f^{obsd} - k_r^{app}$ (Wilkinson, 1961).[4]

Results and Discussion

Before the NAD–Pyr adduct reaction can be used to examine features of the normal redox reaction of LDH (see the following paper), the bond-making and -breaking steps in the

[3] The superscript obsd designates the observed parameter under the conditions specified; app is used to denote constants that have been corrected for *competitive* anion binding (by extrapolation to saturating reactants) but that retain a pH dependence. Since there can be no *competitive* anion binding in the reverse reaction (the E·H·adduct complex is the reactant), the observed rate constant for this process is designated as k_r^{app}.

[4] Although the fractional errors in k_r^{app} and k_f^{obsd} are not substantially different, the variances of k_r^{app} are considerably less than those for k_f^{obsd}; hence, k_r^{app} in eq 1 and 5 is treated as a constant by using the appropriate experimental value. This procedure substantially reduces the complexity of the regression analysis that employs these equations.

Table I: Estimates for the Observed Constants Describing the Forward Reaction at Constant Chloride[a]

pH	k_f^{app} (s^{-1}) expt	k_f^{app} (s^{-1}) calcd[b]	K_{NAD}^{obsd} (mM) expt	K_{NAD}^{obsd} (mM) calcd	K_{Pyr}^{obsd} (M) expt	K_{Pyr}^{obsd} (M) calcd	$K_{I,Pyr}^{obsd}$ (M) expt	$K_{I,Pyr}^{obsd}$ (M) calcd	k_r^{app} (s^{-1})
6	0.04		0.3		0.05		0.06		9.3×10^{-3}
6.5	0.06	0.06	0.1	0.4	0.09	0.04	0.01	0.03	5.1×10^{-3}
7	0.07	0.10	1.7	1.0	0.03	0.05	0.10	0.06	3.2×10^{-3}
7.5	0.1		3.0		0.04		0.29		2.5×10^{-3}
8.0	0.14	0.14	2.6	2.8	0.07	0.07	0.17	0.12	2.2×10^{-3}

[a] The chloride concentration is 0.3 M; hence, all constants except k_f^{app} include a competitive chloride dependence. Except for pH, the other experimental conditions are given in Figure 7. [b] The calculated values of the constants are obtained by correcting for chloride effects with the appropriate pH-independent estimates of inhibition constants in Table II and the following relationships: $K_{Pyr}^{obsd} = K_{Pyr}^{app}(1 + [Cl]/K_{I_2,Cl})$; $K_{NAD}^{obsd} = K_{NAD}^{app}(1 + [Cl]/K_{I_2,Cl})/(1 + [Cl]/K_{I_2,Cl})$; $K_{I,Pyr}^{obsd} = K_{I_1,Pyr}(1 + [Cl]/K_{I_2,Cl})$.

Table II: Estimates for the Apparent Constants for the Forward Reaction Obtained by Varying the Chloride Concentration[a]

pH	K_f^{app} (M^{-1} s^{-1})	K_{NAD} (mM)	K_{Pyr}^{app} (M)	$K_{I_1,Pyr}$ (M)	$K_{I_2,Cl}^{app}$ (M)	$K_{I_1,Cl}$ (M)
6.5	0.06	2.5	0.005	0.03	0.04	0.07
7.0	0.10	2.4	0.01	0.03	0.07	0.1
8.0	0.14	0.6	0.04	0.02	0.37	0.06

[a] The estimates of the constants are obtained from least-squares fits of eq 1 and 4 to the data in Figure 7 and other data (not provided). The values of k_r^{app} are from Figure 2. In terms of the proton-dependent constants in Scheme I, $K_{Pyr}^{app} = K_{Pyr}(1 + K_H/[H])$ and $K_{I_2,Cl}^{app}(1 + K_H/[H])$.

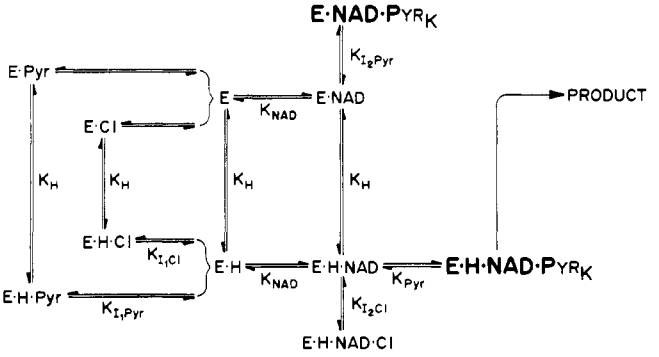
former reaction must be described with sufficient accuracy to validate such a comparison. But first, it is necessary to consider the reactant binding steps, both because a correct evaluation of these is necessary in obtaining valid kinetic parameters for subsequent steps and because the effect of pH on some of these steps reflects the prototropic equilibrium for the catalytically competent complex(es) in the normal reaction.

Kinetic Scheme for the Adduct Reaction. The LDH-catalyzed adduct reaction between pyruvate and NAD is conducted at reactant concentrations below saturation to eliminate a number of technical difficulties [cf. Burgner & Ray (1978)]. In addition, enzyme, reactant, and buffer concentrations are adjusted so that the appearance of product is always first order in enzyme. Under such conditions (and at low enzyme concentrations), the *fraction* of enzyme present in a reactant or potentially reactant form, E_R, can be specified in terms of the concentration of all reactants and inhibitors present, provided the identities of all important complexes together with their dissociation constants are known. Previously (Burgner & Ray, 1978), we showed that the concentration of the reactive, ternary complex with the stoichiometry E, NAD, and Pyr_E (pyruvate enol) is negligible with respect to the other enzymic species and that under constant assay conditions the conservation equation for this system can be represented as

$$E_T = E + E{\cdot}NAD + E{\cdot}NAD{\cdot}Pyr_K + E{\cdot}adduct \quad (6)$$

But, this equation is incomplete, and a number of additional species are detected when pH and anion concentration are varied. The present study shows that chloride binding produces E·Cl and E·NAD·Cl complexes and that the formation of the first of these is not significantly pH dependent in the neutral pH range; i.e., chloride binds almost as well to E as to E·H [see also Anderson (1981)]. Pyruvate also binds nearly equally well to both E and E·H to produce two additional complexes. [Evidence for an E·Pyr complex in the normal LDH redox reaction also was provided by Wang (1977).] Furthermore, the formation of a binary complex involving NAD is essentially independent of pH (in the neutral range), so both E·NAD and E·H·NAD are present [cf. Stinson & Holbrook (1973)]. Finally, in the presence of bound NAD, pyruvate (as well as the structurally similar ligands L-2-hydroxysuccinamate and L-lactate) binds to both the protonated and unprotonated complexes E·NAD and E·H·NAD to give E·NAD·Pyr and E·H·NAD·Pyr (Burgner et al., 1978; Grimshaw & Cleland, 1980; see also the section, below, on equilibrium effects). This behavior is in contrast with that observed when NADH instead of NAD is bound to the enzyme, where protonation of a group on the enzyme with a pK_a near 7 controls the formation of ternary complexes such as E·H·NADH·Pyr_K and E·H·NADH·oxamate; viz., the unprotonated complexes E·NADH·Pyr_K and E·NADH·oxamate form either weakly or not at all [cf. Schwert et al. (1967), Adams et al. (1973), Holbrook et al. (1975), Whitaker et al. (1974), and Holbrook & Stinson (1973)].

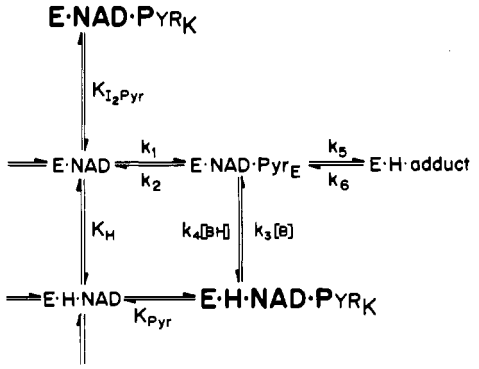
Scheme 1: Minimal Mechanism for the Forward Adduct Reaction



Scheme I is the minimal reaction scheme for the forward adduct reaction that provides a rationale for both the above published data and the present observations regarding the effects of reactants, pH, and chloride on the rate of the reaction. From left to right and bottom to top, this scheme specifies that *within the neutral pH* range (a) the binding of anions is competitive with NAD and is pH independent, (b) the binding of NAD is pH independent, (c) the binding of chloride to E·H·NAD is competitive with the binding of pyruvate and (the former) is pH dependent, and (d) the binding of pyruvate to E·H·NAD produces a reactive complex and to E·NAD gives an unreactive complex.

Estimates for the various constants that are obtained from substrate velocity and inhibition studies described in the Appendix are given in Tables I and II. These studies confirm that we have appropriately extrapolated the rates of the adduct reaction to *saturating reactants*. In fact, all critical parameters for the adduct reaction are evaluated by extrapolating our observations to *saturating* concentrations of NAD and pyruvate, where *only* the *two* species shown in *boldface* in Scheme

Scheme II: Forward Adduct Reaction at Saturating Reactants



I, E·NAD·Pyr$_K$ and E·H·NAD·Pyr$_K$, initially are present at significant concentrations.

Bond-Making and Bond-Breaking Steps: The Forward Reaction. To describe the bond-making and -breaking steps in the forward adduct reaction, the pathway in Scheme I leading from E·H·NAD·Pyr$_K$ to E·H·adduct (i.e., PRODUCT) must be expanded to include the steps shown in Scheme II so that the following observations from earlier studies conducted at constant [Cl$^-$] and pH (Burgner & Ray, 1974, 1978) are accounted for. (a) The reactive complex in the adduct reaction involves the enol form of pyruvate, viz., the E·NAD·Pyr$_E$ complex. (b) Two different pathways lead to E·NAD·Pyr$_E$ (center, Scheme II) depending on whether E·NAD or E·H·NAD is involved: (1) enolization of Pyr$_K$ prior to binding, viz.

$$\mathrm{Pyr_K} \rightarrow \mathrm{Pyr_E} + \mathrm{E{\cdot}NAD} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{E{\cdot}NAD{\cdot}Pyr_E} \qquad (7)$$

(upper line, Scheme II) and (2) enolization of *bound* Pyr$_K$, viz., E·H·NAD → E·H·NAD·Pyr$_K$ → E·NAD·Pyr$_E$ + H$^+$ (lower horizontal and right vertical lines, Scheme II). (c) Pathway 1 does not involve a proton transfer and is independent of buffer effects (general acid or general base catalysis) as long as the process Pyr$_E$ ⇌ Pyr$_K$ is at equilibrium. (d) Pathway 2 involves a proton transfer and a buffer effect. In this pathway LDH acts as a general acid together with with an external (general) base to facilitate enolization of bound Pyr$_K$. This external base, B, participates in the *reverse* adduct reaction as a general acid BH$^+$ (see subsequent section). As in Scheme I, the *only* two species that initially are present at significant concentrations in the presence of saturating NAD and pyruvate are indicated in boldface: E·NAD·Pyr$_K$ and E·H·NAD·Pyr$_K$.

Equilibrium Constant for the Adduct Reaction: pH and Anion-Binding Effects. The following analysis shows that the apparent equilibrium constant for the adduct reaction varies with pH and suggests that the group whose protonation controls the binding of pyruvate to E·NADH in the normal enzymic reaction probably controls the adduct reaction also. In other words, the equilibrium indicated by K_H in Scheme II, which determines whether the binding of Pyr$_K$ produces reactive E·H·NAD·Pyr$_K$ or unreactive E·NAD·Pyr$_K$, probably is the same as the equilibrium that controls the binding of Pyr to E·NADH in the normal enzymic reaction.

The true equilibrium constant for the adduct reaction, K_{eq}, is defined as [E·H·adduct][H]/[E·H·NAD·Pyr$_K$]; at a fixed pH, the apparent equilibrium constant is [E·H·adduct]/([E·H·NAD·Pyr$_K$] + [E·NAD·Pyr$_K$]). On the basis of Schemes I and II, $K_{eq}^{app} = K_{eq}/(H + K_H^{app})$. Thus, K_{eq}^{app} should plateau at a pH greater than pK_H^{app} where $K_H^{app} = K_H K_{I_2Pyr}$ and $K_{eq} = k_1k_5K_{enol}K_{Pyr}K_H/(k_2k_6) = k_3k_5k_a/(k_2k_6)$. Values for K_{eq}^{app} in Figure 1 are determined both kinetically, from

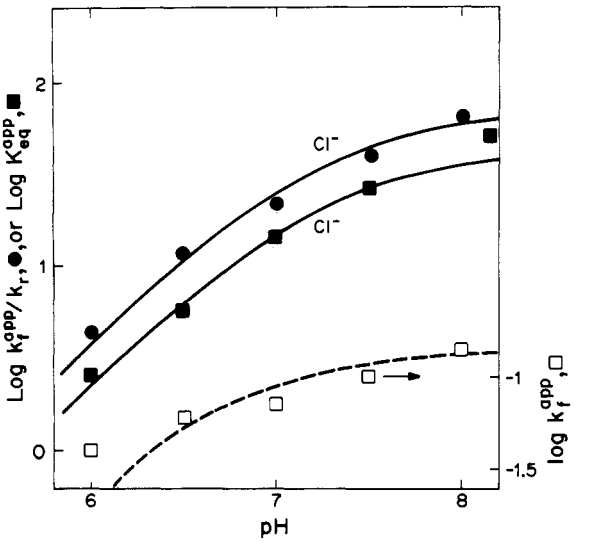


FIGURE 1: Effect of pH on the apparent equilibrium constant of the adduct reaction. Values of k_f^{app} from Tale I and k_r^{app} from Figure 2 at 0.3 M chloride are used to calculate the values for the equilibrium constant (●). The other estimates (■) for the equilibrium constant are measured by a photometric procedure, corrected for substrating concentrations of reactants, and corrected for inhibition effects by chloride (see text). The solid lines are calculated as described in the text and values for k_{eq} of 5×10^{-6} (top line) and 3×10^{-6} (middle line) and for pK_H^{app} of 7.1. In addition, values of k_f from Table I are plotted against the pH (open symbols). The dashed line is calculated from values of k_2/k_5 and k_4/k_5 at pH 7, respectively, with a pK_a for imidazole of 7.3, a K_{eq}^{app} of 5×10^{-6} M, and a pK_H^{app} of 7.1.

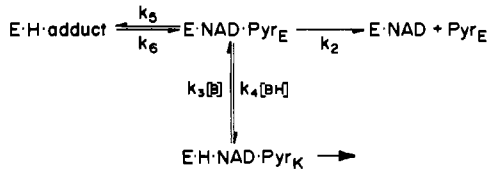
k_f^{app}/k_r^{app}, and more directly from equilibrium values of [E·H·adduct]/[E$_R$]. In the former case, the experimental values of k_f^{app} in Table I and estimates for k_r^{app} obtained from the data in Figure 2 (see below) at 0.3 M chloride were used. In the latter case, the concentration of E·H·adduct present at equilibrium was measured by a photometric procedure (Burgner & Ray, 1978) under conditions where the reactant concentrations are nearly saturating and at a chloride concentration of 0.3 M; subsequently, a small correction to account for the lack of saturation was made (so that E_R = E·H·NAD·Pyr + E·NAD·Pyr).[5] In Figure 1, the equilibrium constants obtained from both the kinetic procedure (●) and equilibrium ratio measurements (■) are plotted against pH. In both cases, the solid line, which mimics the trends quite well,[6] shows the expected variation of K_e^{app} with pH if the pK_H^{app} of the group controlling the adduct reaction is 7.1.[7] Throughout the neutral pH range, the value of K_{eq} determined by the equilibrium procedure is only slightly less than that determined from the ratio of k_f^{app}/k_r^{app}. The lack of a significant difference between values obtained by such disperate methods provides excellent support for the validity of both our approach and the proto-

[5] Equation 3 and the values of the constants in Table I were used for the correction, since these constants were obtained at the same chloride concentrations, 0.3 M, that were used for the equilibrium experiments. This treatment increases the observed values by about 3–4-fold.

[6] A process requiring the protonation of a specific group and also liberating a proton from a much weaker acid will exhibit a plateau above the pK_a^{app} of the stronger acid, as in Figure 1.

[7] The value of 5×10^{-6} M^{-1} for K_{eq} that was used to construct the curve through "●" in Figure 1 is different from the value previously reported for "K_e" (Burgner & Ray, 1978) because the former value was calculated for a scheme that contained too few intermediates. The present value, the values of other parameters given in the legend of Figure 1, the definitions given above, and eq 8, which describes the reverse reaction, are used to calculate the dashed line in this figure. The agreement between the open symbols, which are values of k_f from Table I, and this line suggest that k_f behaves essentially as expected from our model.

Scheme III: Reverse Adduct Reaction



tropic equilibria given in Scheme II, i.e., that Pyr_K binds to both E·NAD and E·H·NAD but that only E·H·NAD·Pyr_K reacts to give E·H·adduct (with the release of a proton).

The group that controls the binding of Pyr_K to E·H·NADH in the normal LDH reaction has a pK_a in the range of 6.3–7.3 [cf. Holbrook et al. (1975) Parker et al. (1978), and Grimshaw & Cleland (1980)]. This group is thought to be His-195 [cf. Grau et al. (1981)], and Pyr_K apparently is H bonded to the protonated form of this group in the reactive ternary complex. On this basis, one might expect that Pyr_K should bind preferentially to E·H·NAD and only weakly to E·NAD and that pK_H (absence of pyruvate) should be much less than pK_H^{app} (presence of pyruvate). But in the normal LDH reaction, the substrate inhibition constant for pyruvate is relatively constant over the pH range of 7–9, which shows that pyruvate actually binds nearly as well to E·NAD as to E·H·NAD and thus that $pK_H \approx pK_H^{app}$ (although the precise interactions between pyruvate and the enzyme need not be the same in the protonated and unprotonated forms of this complex).[8] If $pK_H \approx pK_H^{app}$, then pK_H must be near 7.1. In fact, H bonding between Pyr_K and the protonated form of His-195 probably is required to produce the *reactive* E·H·NAD·Pyr complex, and K_H would then be the ionization constant for this group. Additional support for this viewpoint is provided by the observation that in the crystalline product, the carbonyl group of the pyruvate moiety in NAD–Pyr is in the vicinity of His-195 (Grau et al., 1981).

The Reverse Reaction. (A) General Considerations. Previously, we suggested that decomposition of the E·H·adduct complex in the reverse adduct reaction, i.e., E·H·adduct → E·H + reactants, was sensitive to buffer concentration at constant pH (7.0) and that no single step was rate limiting for this process, even under conditions where the reverse process is effectively irreversible (Burgner & Ray, 1978). Scheme III, a partial mirror image of Scheme II, summarizes the kinetically important steps for the reverse process. According to our prevous results, the equilibrium (E·NAD·Pyr_E]/[E·H·adduct] or k_6/k_5 is far to the left, and the buffer, [BH], affects the observed rate constant for the reverse reaction, k_r^{app}, in a hyperbolic manner (at constant ionic strength and pH).

In the present study, we vary the pH, solvent, and identity of the participating buffer, as well as its concentration. All of the rate effects observed in these studies are *qualitatively* consistent with our earlier suggestion that an external buffer, [BH], can facilitate the ketonization of *bound* pyruvate enol, as above. But, we also find that the effect of both pH and buffer concentration on the rate of the reverse adduct reaction also depends on the identity and concentration of *nonbuffer anions* in the reaction mixture. Hence, any *quantitative* rationale for both the buffer effect on the reverse adduct reaction and the solvent deuterium isotope effect on k_4, discussed in subsequent sections, requires a reasonably accurate evaluation

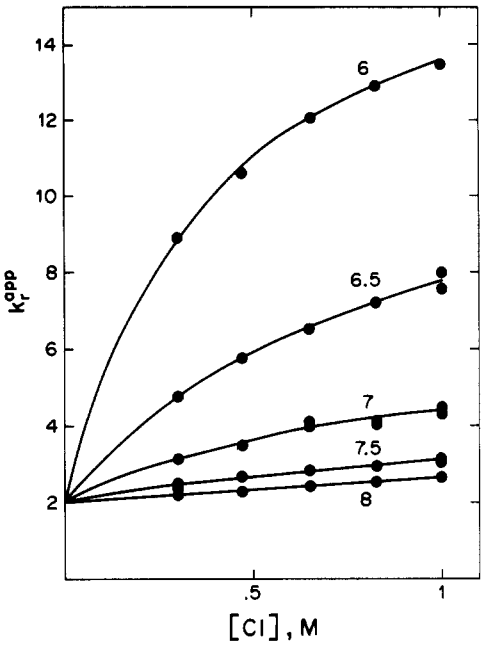


FIGURE 2: Effect of chloride and pH on the observed rate constant for the decomposition of E·adduct. The E·adduct complex is prepared and values of the rate constant for its decomposition are estimated by procedures described under Materials and Methods. The initial concentration of E·adduct is 1 μN, the total imidazole concentration is held constant at 0.3 M, and the pH of the reaction mixture, which is indicated on the graph, is adjusted to the appropriate value for each chloride concentration. The solid lines are calculated from the estimates of the constants in Table III and eq 5.

Table III: Effect of Chloride on the Reverse Adduct Reaction[a]

pH	$k_r^{max} - k_r^0$ ($\times 10^{-3}$ s^{-1})	k_r^0 ($\times 10^3$ s^{-1})	K_M (M)
6.0	16.0 ± 0.4	1.9 ± 1.7	0.4 ± 0.02
6.5	11.0 ± 1.	2.4 ± 1.3	0.9 ± 0.2
7.0	4.5 ± 0.6	1.7 ± 0.8	0.9 ± 0.2
7.5	2.5 ± 0.3	2.1 ± 0.1	1.3 ± 0.3
8.0	*b*	2.0 ± 0.1	*b*

[a] The constants in this table are calculated by nonlinear regression from the data in Figure 2 and eq 5. [b] Value not significant.

of this nonbuffer anionic effect.

(B) Anion-Binding Effects. The structural models obtained from X-ray diffraction studies of the crystalline adduct complex provide a framework for interpreting anion binding effects. These models show an anion binding site close to the bound adduct in the E·H·adduct complex. They also show that the side chain of Arg-173, which constitutes part of the anion binding site, is half a turn around an α-helix from Arg-171, which forms an ion pair with the carboyxlate group of the bound adduct (Grau et al., 1981). Thus, it is not surprising that an anion bound at this site could affect the adduct reaction.[9]

Kinetic experiments show that anions bind weakly to the adduct complex and that these bound anions produce changes in the rate of the reverse adduct reaction under conditions where this process is effectively irreversible (E·H·adduct → E + reactants). In experiments involving chloride, the bound anion not only increases the rate of the reverse adduct reaction

[8] In a somewhat similar system, L-2-hydroxysuccinate, a poorly reacting analogue of lactate, which should be capable of H bonding only to the *unprotonated* form of His-195 in E·NAD, appears to bind with nearly equal affinity to E·H·NAD and E·NAD (Grimshaw & Cleland, 1980).

[9] Difference electron density maps between the various complexes, E·H·adduct, E·H·NADH·oxamate, and E·H·NAD·oxalate, exhibit structural differences in the vicinity of the anion binding site (White et al., 1976); however, the possible corollary that differences in the identity of the anion bound at this site produce structural differences that extend to the catalytic site has not been studied.

but also increases the effect of pH on this process—see Figure 2, which shows the effect of both chloride and pH on k_r^{app} at a constant concentration of 0.3 M imidazole but at a varying ionic strength. The estimates obtained from these data by regression analysis according to eq 5, which describes a rectangular hyperbola, are given in Table III, and the trends in the estimates for k_r^0 (zero chloride) and k_r^{max} (saturating chloride) suggest that a bound anion causes k_r^{app} to increase significantly with decreasing pH. Furthermore, as shown in Figure 3, the type of anion present also affects k_r^{obsd}. In fact, k_r^{obsd} probably decreases slightly with increasing phosphate or sulfate concentrations rather than increasing, as with chloride. Unfortunately, we are unable to specify precisely how anion binding to the E·H·adduct complex alters its rate of conversion to E·NAD (or E·H·NAD) + Pyr; but we can specify that an anion-dependent increase in rate of dissociation of the adduct from E·H·adduct is not the cause of these effects [cf. Burgner & Ray (1984b)].[10] On the other hand, anions are known to alter the *structural* stability of free LDH [e.g., as in hybridization experiments; see Chilson et al. (1964, 1965)] as well as the affinity of LDH for NADH (Anderson, 1981); hence, the possibility that anion binding alters the reactivity (*kinetic* stability) of the E·H·adduct complex seems reasonable.[11]

The results from studies on the effect of anions (particularly chloride) on the reverse adduct reaction suggest the following conclusions. An effective study of the buffer effect on the reverse adduct reaction can be accomplished only by (a) carefully choosing the counterion for the buffer, (b) working at as low a pH as possible, (c) maintaining a constant anion concentration as the buffer concentration and pH are varied, and (d) correcting for general chaotropic effects of amines on the adduct complex (see below). Hence, the studies described below are conducted at pH 6 (the adduct complex is unstable below this pH at high ionic strength), and most involve chloride. The latter choice was made because our earlier studies were conducted with this counterion and because bound chloride produces a relatively high kinetic sensitivity to buffer effects without inducing an extreme response toward general chaotropic effects. (Although bromide and perchlorate ions produce larger effects on the reverse adduct reaction than does chloride, these ions induce such a high sensitivity to general chaotropic effects at pH 6 that studies of the buffer effect with amine buffers in the presence of these ions are unprofitable.)

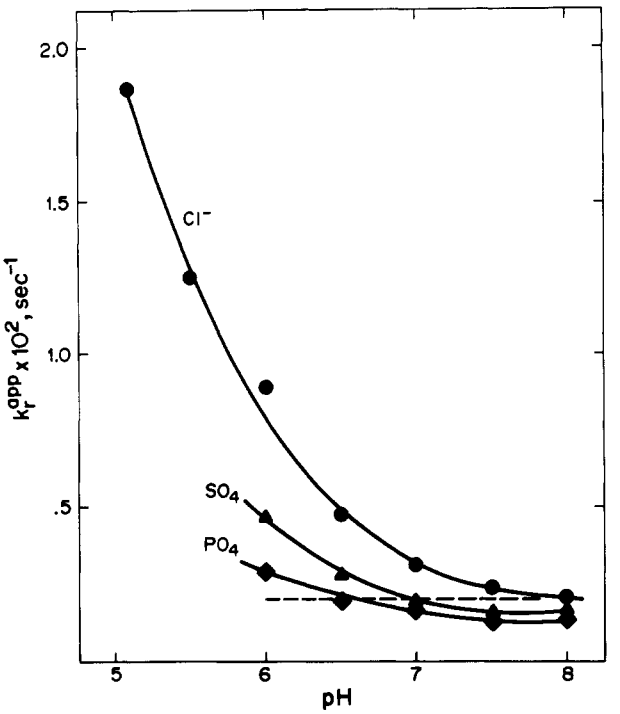


FIGURE 3: Effect of pH upon the decomposition of E·adduct in the presence of different anions. The total imidazole concentration is 0.3 M in all cases, and the total chloride, sulfate, and phosphate concentrations are 0.3, 0.15, and 0.64 M, respectively. The other conditions are described under Material and Methods. The lines are drawn by eye to indicate trends in the data.

In most studies, the concentration of chloride was maintained at 1 M to allow as much variation as possible in the buffer concentration.

(*C*) *Buffer-Specific Aspects of General Catalysis.* A study of the buffer sensitivity of the reverse reaction was undertaken to veryify that an external buffer facilitates ketonization of bound Pyr_E by LDH. According to a previous study (Burgner & Ray, 1978), such an effect can be represented by step k_4[BH] in Scheme III. According to this scheme, the effect of such a step on k_r^{app} is given by the following general relationship, where k_6 is the rate constant for formation of E·NAD·Pyr_E from E·H·adduct and k_5 refers to the reverse of the k_6 process, k_2 to dissociation of the enol, and k_4 to ketonization of bound Pyr_E:

$$k_r^{app} = \frac{k_6(k_2/k_4 + [BH])}{(k_5 + k_2)/k_4 + [BH]} \quad (8)$$

From this relationship, k_r^{app} should increase hyperbolically to a limiting value, equal to k_6, as the concentration of the general acid, BH, is increased to a point where essentially none of the E·NAD·Pyr_E reverts to E·H·adduct—at which point production of E·NAD·Pyr_E (via k_6) becomes rate limiting. This indeed is the case for the buffer piperazine chloride, (▲) in Figure 4A. But, all other buffers tested fail to produce such clean-cut results, and with some E·H·adduct preparations, even piperazine chloride occasionally produced a slight upward trend [(▲) Figure 4B]. Thus, when chloride is used as the buffer anion, the effect on k_r^{app} produced by amine buffers such as imidazole, pyridine, and *N*,*N*-dimethylhydroxylamine chlorides fails to level off. The behavior of the dimethylhydroxylamine chloride [(▲) Figure 4A] suggests that its overall effect on k_r^{app} is the sum of a hyperbolic response (due to general acid catalysis) superimposed on a roughly linear response (because of a general chaotropic effect on k_6), and the value of k_6 calculated on the basis of this assumption is the same as that observed in the case of piperazine chloride.

[10] On the basis of eq 8, the enhanced sensitivity of k_r^{app} in the presence of bound chloride might be rationalized as (a) an increase in the value of k_4 (increased sensitivity to general acid catalysis of ketonization), (b) a decrease in the value of k_5 (a decreased rate of bond making in the forward adduct reaction), (c) an increase in k_6 (an increased rate of bond breaking in the reverse reaction), or (d) any combination of the above.

[11] In most studies of the effects produce by anions other than chloride on k_r^{app}, no attempt was made to separate the observed effect into k_r^0 and k_r^{max}, as in eq 5. But, at a constant concentration of 0.3 M imidazole, 0.3 M anion, and pH 6.0, the effect on k_r^{app} was perchlorate > bromide > chloride > fluoride ~ acetate > sulfate > phosphate. Likewise, although it is essentially impossible to obtain values of k_r^0 (absence of anions) that are statistically significant, k_r^0 seems to be relatively unaffected by the concentration of imidazolium ion, even at pH 6 where imidazolium ion has a large effect on the reverse adduct reaction in the presence of bound chloride. Several different explanations can be posed for these observations in terms of Scheme III, but none is unique, mostly because k_r^{app} is equal to a relatively complex combination of the constants: see eq 8. On the other hand, at the phenomenological level, it seems reasonable to point out that the relative sensitivity of k_r^{app} toward imidazolium ion induced by anions parallels their relative effect on the stability of LDH, as is indicated by the anion-dependent hybridzation efficiencies involving the A_4 and B_4 isozymes: chloride > fluoride > acetate > sulfate > phosphate (Chilson et al., 1964). Thus, the effect of anions on the rate of the reverse adduct reaction seems to be related to subtle structural effects induced by anion binding.

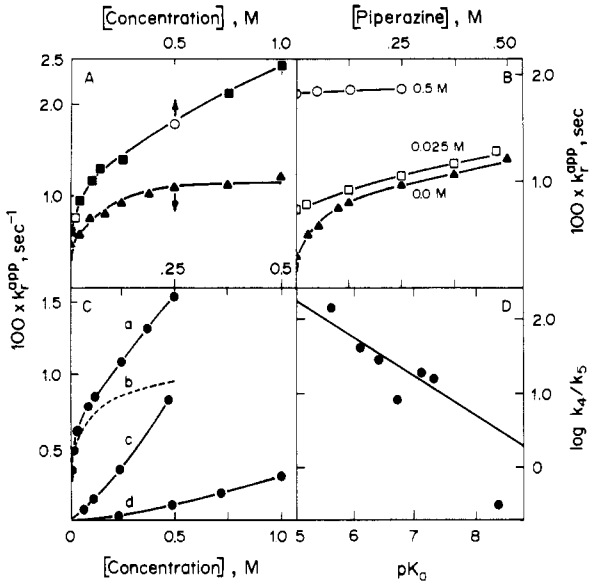


FIGURE 4: Effect of different conditions on the reverse adduct reaction. In each part of this figure, the pH and Cl^- and initial E·adduct concentration were held constant to 6.0, 1 M, and 1 μN, respectively. (Part A) A plot of k_r^{app} against *N,N*-dimethylhydroxylamine (upper curve) and piperazine (lower curve). The solid lines are generated from eq 8 and the data in Table IV. (Part B) The effect of piperazine on k_r^{app} in the presence of the molar concentration of *N,N*-dimethylhydroxylamine shown on the graph. The lines are drawn by eye. (Part C) The effect of *N*-methylimidazole (a), guanidine hydrochloride (c), and *N,N'*-dimethylimidazole (d) on k_r^{app}. Curve b represents the data of curve a for *N*-methylimidazole after correction for chaotropic effects (see text). The dashed line for b is calculated with eq 8 and from the data in Table IV. The other lines are drawn by eye. The reaction mixtures for curves c and d were buffered slightly with 0.05 M *N*-methylimidazole, and its effect on the rate for these plots was removed by subtraction of the value in the absence of either *N,N*-dimethylimidazole or guanidine from all other values. (Part D) A Brönsted plot of the relationship betwen the pK_a of general acids and their effect on the rate constant for the reverse adduct reaction. The values of k_4/k_5 are from Table IV. The points represent from left to right *N,N*-dimethylhydroxylamine, piperazine, histamine, *N,N,N',N'*-tetramethylethylenediamine, *N*-methylimidazole, imidazole, and Tris. The line has a slope of 0.5 and is given only as a reference.

(D) Correction of k_6 for Chaotropic Effects in the Chloride System. The dual nature of the overall effect produced by most amine chloride buffers (including *N,N*-dimethylhydroxylamine chloride) is shown by three different lines of investigation. The first involves the response of k_r^{app} to added amine chloride buffers at two different but constant concentrations of *N,N*-dimethylhydroxylamine chloride (at constant pH and ionic strength). In Figure 4B, piperazine chloride acting only as a general acid increases k_r^{app} (▲) when the concentration of *N,N*-dimethylhydroxylamine chloride, 0.025 M, is within the range where the hydroxylamine chloride is acting primarily as a general acid [(□) Figure 4A]. In contrast, added piperazine chloride has no effect on k_r^{app} [(●) Figure 4B] when the concentration of the hydroxylamine chloride, 0.5 M, "saturates" the general acid effect [(○) Figure 4A]. Thus, the "posthyperbolic" response of k_r^{app} to *N,N*-dimethylhydroxylamine chloride and other amine chlorides probably arises from general chaotropic effects.

A second indication of the dual nature of the overall effect by amine chlorides is the observation that under the conditions used in Figure 4A, piperazine chloride produces no effect on the activity of LDH, while *N,N*-dimethylhydroxylamine chloride inactivates the enzyme in the absence but not in the presence of its substrates. Other amine buffers, such as imidazole chloride, also inactivate LDH under these conditions, even in the presence of *saturating* substrate concentrations—see below. Moreover, purely chaotropic agents that cannot act as general acids such as guanidine hydrochloride and *N,N*-dimethylimidazolium chloride do affect k_r^{app}, but in a qualitatively different manner (plots c and d, Figure 4C); i.e., they produce not a hyperbolic response but a response that is slightly concave upward. Finally, when the overall effect of amine chloride concentration on k_r^{app} is separated into the sum of a general acid catalyzed response, $(k_r^{app})_{ga}$, plus an experimentally determined chaotropic response, $(k_r^{app})_c$,[12] (as in plots a–c, Figure 4C), the maxima for the hyperbolic effects for six of the amine buffers that are used are essentially the same, as would be expected from eq 8, i.e., were equal to k_6—see discussion of Table IV, below.

Table IV: Effect of Different General Acids on the Rate Constants for Reverse Adduct Reaction[a]

buffer (pK_a)[b]	k_6 ($\times 10^2$ s^{-1})	k_2/k_5	k_4/k_5[c]
Me_2NOH (5.6)[d]	1.3	0.28	143
piperazine (6.1)	1.3	0.26	43
histamine (6.4)	1.0	0.43	30
TEMED (6.7)	1.3	0.31	8
N-methylimidazole (7.1)	1.0	0.42	18
imidazole (7.3)	1.1	0.35	16
imidazole (7.3)[e]	0.3	1.3	98
Tris (8.4)		0.39	0.3

[a] Reactions were conducted in 1 M chloride at pH 6.0 unless otherwise specified. [b] The approximate pK_a under the conditioins given in Figure 4. [c] The buffer-dependent effects on k_4/k_5 are corrected to 1 M buffer acid by using the listed pK_a values. [d] *N,N*-dimethylhydroxylamine. [e] The reaction was conducted in 0.3 M chloride at pH 7.0.

(E) Comparison of Constants Obtained in the Chloride System. When the (corrected) experimental values of $(k_r^{app})_{ga}$ for the various amine chloride buffers (see above) are equated with k_r^{app} and the parameters of eq 8 are evaluated by nonlinear regression analysis (see Materials and Methods), the values for k_6, k_2/k_5, and k_4/k_5, given in Table IV, are obtained. (The lower line in Figure 4A is a plot of this equation for piperazine; the curved dashed line in Figure 4C is the analogous plot for *N*-methylimidazole). Because k_6, formation of E·H·NAD·Pyr_E from the adduct, should be independent of buffer composition, the relatively constant value obtained for k_6, (1.2 ± 0.2) ×

[12] Chaotropic effects of the various amines on the reverse adduct reaction are corrected for in the following manner. Values for k_r^{app} are obtained under the general conditions used in Figure 4A and in the presence of 0.5 M *N,N*-dimethylhydroxylamine chloride, where the hyperbolic phase of the effect on k_r^{app} induced by this buffer is saturated. The response of k_r^{app} to various amine chloride buffers, which are added to the above reaction mixture in a series of different assays, is measured at three concentrations of the added buffers (see below). As is indicated above, piperazine chloride produces no change in k_r^{app} under these conditions, while added *N,N*-dimethylhydroxylamine produces an essentially linear increase. All other amine buffers tested produce increases that exhibit small but significant upward curvatures with increasing concentration. The following relationship describes the observed increase:

$$(k_r^{app})_c = 1 + b[BH^+] + e[BH^+]^2 \quad (9)$$

Here $(k_r^{app})_c$ is that part of k_r^{app} caused by the chaotropic effect of amine chlorides and is taken as the difference between k_r^{app} in the presence of 0.5 M *N,N*-dimethylhydroxylamine chloride plus another amine chloride and k_r^{app} in the presence of the hydroxylamine chloride only. These data were fit to eq 9 by weighted nonlinear regression techniques. Obviously, for piperazine chloride, $b = e = 0$, and for *N,N*-dimethylhydroxylamine chloride, $e = 0$, while finite values of e are obtained for all other buffers. From the assumption that $k_r^{app} = (k_r^{app})_c + (k_r^{app})_{ga}$, the latter constant can be obtained as the difference between *measured* values for k_r^{app} and values for $(k_r^{app})_c$ that are *interpolated* from the experimental data in conjunction with eq 9. Plot a in Figure 4C shows uncorrected values of k_r^{app} as a function of 1-methylimidazole concentration, and plot b shows these values after correction for chaotropic effects. The dashed line in plot b is calculated from eq 8 and the data in Table IV.

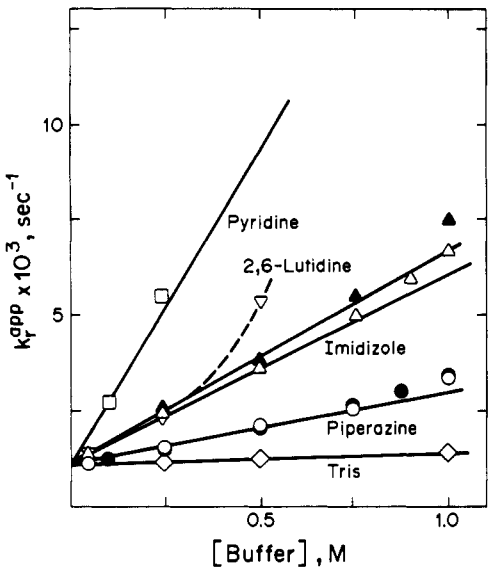


FIGURE 5: Effect of different acids on the decomposition of E·adduct in the presence of sulfate. The pH and anion and E·adduct concentrations were 6.0, 1 M, and 1.8 μM, respectively. The solid symbols (▲ and ●) represent *N*-methylimidazole and *N,N,N',N'*-tetramethylethylenediamine, respectively. All lines are drawn by eye to represent trends.

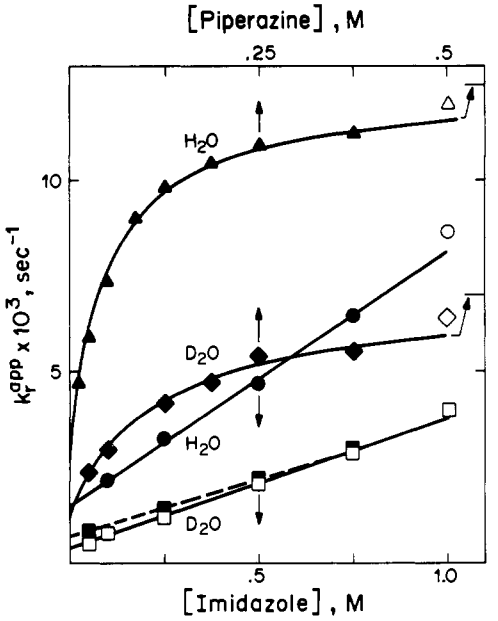


FIGURE 6: Deuterium oxide isotope effects on buffer catalysis of the decomposition of the E·adduct at pH 6.0. The preparation of the buffer solutions and of E·adduct is described under Materials and Methods. The initial concentration of E·adduct was 1 μN. Estimates of kinetic constants describing the decomposition of E·adduct in piperazine–chloride (▲, ♦) and imidazole–sulfate (●, ■) are given in Table IV, and these values are used to draw the lines in the figure. The data represented by the unfilled symbols designate studies where the E·H·adduct complex was prepared in D_2O.

10^{-2} s^{-1}, tends to substantiate our approach. Similarly, since k_2/k_5 represents the partition of E·NAD·Pyr$_E$ between dissociation and return to E·H·adduct, this ratio also should be insensitive to reaction composition, and the values of k_2/k_5 in Table IV are relatively constant: 0.36 ± 0.06. Only in the case of k_4/k_5 does buffer identity produce a large effect. In fact, values of k_4/k_5 differ by nearly 20-fold among those buffers producing a hyperbolic response and are larger than 400-fold if the buffer Tris [tris(hydroxymethyl)aminomethane] is included. (Because of its low catalytic efficiency, a hyperbolic effect was not observed even at concentrations of up to 1 M Tris-HCl.) Since the ratio k_2/k_5 is essentially constant (see above), we assume that the variation in k_4/k_5 is caused primarily by in changes buffer as is shown by the Brönsted plot in Figure 4D. However, the only conclusion drawn from this plot is that the trends observed are those expected for general catalysis of the reverse adduct reaction by an external acid—our primary reason for examining the effect of different acids on the reverse adduct reaction in the first place.

Buffer Effects with Other Anions. As noted above, the sensitivity of k_r^{app} toward both general catalysis and chaotropic effects is markedly reduced by replacing the anion, chloride (1 M), with sulfate (0.5 M). The results of such studies with five different amine sulfate buffers is shown in Figure 5. As opposed to the results with chloride (Figure 3), there is no obvious saturation effect with any of these buffers, but one cannot correct for chaotropic effects (cf. footnote 12) that seem evident at higher concentrations of 2,6-lutidine and pyridine (data at higher pyridine concentrations not shown) and seem to occur with imidazole. Although such (chaotropic) effects might partially mask the curvature of a hyperbolic response, it appears that in the presence of sulfate the fractional contribution of general catalysis to the reverse adduct reaction is smaller than that in the presence of chloride.

Since Scheme III should account for the important features of the reverse adduct reaction, regardless of the identity of the anion used, the expression for k_r^{app} in eq 8 presumably still holds. This expression does provide a rationale for a response of k_r^{app} on [BH] that does not readily saturate, if k_5 becomes much larger than k_4[BH] because of either a decrease in k_4 or an increase in k_5 or both. An analysis of the data obtained with sulfate buffers on this basis produces values of k_4/k_5 that are sensitive to buffer effects to an extent roughly parallel to those obtained in chloride (Table III), but the results are not presented here (analysis available on request).

Table V: Kinetic Solvent Isotope Effects on the Reverse Adduct Reaction[a]

	k_6 ($\times 10^2$ s^{-1})	k_2/k_5	k_4/k_5 (M^{-1})[b]
piperazine			
pH 6.0	1.27 ± 0.02	0.28 ± 0.04	81 ± 11
pD 6.4	0.84 ± 0.05	0.25 ± 0.1	23 ± 6
k^{H_2O}/k^{D_2O}	1.5 ± 0.09	[c]	3.5 ± 1.0
N,N-dimethylhydroxylamine			
pH 6.0	1.28 ± 0.04	0.26 ± 0.06	117 ± 13
pD 6.0	1.01 ± 0.09	0.21 ± 0.13	83 ± 22[d]
k^{H_2O}/k^{D_2O}	1.27 ± 0.12	[c]	1.4 ± 0.4

[a] Data from Figure 6 and similar studies using piperazine and *N,N*-dimethylhydroxyamine 1 M in chloride. [b] Corrected to 1 M general acid. [c] Value not significant. [d] Corrected for an increase in the pK_a for the hydroxylamine of about 0.35 in D_2O.

The Solvent Isotopic Effect on the Reverse Reaction. The reverse adduct reaction also was conducted in both H_2O and D_2O with the buffers piperazine chloride and *N,N*-dimethylhydroxylamine chloride. The results, Figure 6, show an isotopic effect, and the values of k_4/k_5 in Table V, which are corrected for chaotropic effects, exhibit solvent isotopic effects greater than unity, which is the expected direction if step k_4 is subject to general acid catalysis and that designated by k_5 is not. In addition, the size of the isotopic effect, about 3-fold for piperazine chloride, is in the range that might be expected for a general acid catalyzed reaction [cf. Jencks (1969)] and is in accord with our previous conclusions (Burgner & Ray, 1978). Unexpectedly, k_6 also exhibits a solvent deuterium isotopic effect of about 1.5 as is indicated by the difference in vertical intercepts for the plots in Figure 6. Figure 6 also shows that solvent isotope effects are obtained

in the presence of other anions such as sulfate. A possible rationale for the latter effects are noted in the following section.

The Reverse Adduct Reaction: Summary. The reverse adduct reaction was investigated at as low a pH as possible (pH 6) to increase the magnitude of the effect of buffer concentrations on k_r^{app} to a workable range. (This occurs because k_6 increases with decreasing pH—at least in the presence of imidazole, see Table III.) The adduct complex in 1 M chloride at pH 6 is a marginally stable stystem, and we encountered substantial difficulties under these conditions that were not encountered in more limited studies under less extreme conditions (Burgner & Ray, 1978) although the more limited studies were not nearly as informative. Presumably, the solvent deuterium isotopic effect on the bond-breaking step of the reverse adduct reaction, k_6 (see the previous section), is one example of a problem that arises because of the relatively extreme conditions used here. Thus, replacing H_2O with D_2O produces an (unexpected) effect on k_6 (the bond-breaking step) that is in the same direction as replacing the counterion chloride by sulfate; i.e., the adduct complex is stabilized, kinetically, both by replacing Cl^- with SO_4^{2-} and by replacing H_2O with D_2O. Such stabilization is opposite in direction to the chaotropic effects induced by some of the amine buffers that were studied. But in spite of the numerous corrections that are required to allow a semiquantitative interpretation of our results, the involvement of a buffer molecule as a general acid in the reverse adduct reaction seems inescapable. Since buffers are involved as general acids in the *reverse* adduct reaction, they also must be involved as general bases in the forward reaction, and it was in the forward reaction at pH 7 that we first obtained evidence for such an involvement (Burgner & Ray, 1974, 1978). Thus, the *sole* purpose of this paper is to validate our previous interpretation of how the general base imidazole is involved in the enolization reaction of bound Pyr_K under much less extreme conditions, pH 7 and 0.3 M chloride, since that interpretation appears to have no precedent. In fact, because of the general instability of the system at pH 6 in the absence of bivalent anions, *none* of the rate constants obtained at this pH are used in our subsequent analysis of the enolization of bound Pyr_K—only values at pH 7 and 0.3 M chloride are used. These values are essentially the same as those reported previously, except that a minor correction for the chaotropic effect of 0.3 M chloride at pH 7 has been made, which is based on the present studies—see Table IV.

Significance. The studies described here, together with models of the LDH–adduct complex from X-ray diffraction studies, provide a sufficiently detailed picture of the adduct reaction to allow certain features of this process to be compared with the normal enzymic reaction [see the following paper (Burgner & Ray, 1984b)]. Thus, the present kinetic and equilibrium studies show that the acidic form of a group with a pK_a of about 7 controls the adduct reaction involving Pyr_K. Control by such a group seems quite reasonable in terms of the prototropic changes that must accompany the addition of Pyr_K to NAD in the adduct reaction, as is suggested in Scheme II. In terms of molecular models, the active site histidine, His-195, is the group that would be expected to provide this proton. Thus, although Pyr_K appears to bind to the E·NAD complex whether His-195 is protonated or not, it reacts with bound NAD at a detectable rate only if the former group is protonated. The question of whether His-195 can be protonated subsequent to the binding of Pyr_K is moot, as is the question of whether Pyr_E can bind both to E·NAD and its protonated form. But, binding of Pyr_E primarily to the unprotonated form seems most reasonable since, by analogy, such binding should involve H bonding to His-195.

Appendix

Substrate–Velocity and Inhibition Studies of the Adduct Reaction in the Forward Direction: Substrate–Velocity Studies. We define the reactant enzyme complexes E_E as the sum of all the enzyme species shown in Scheme I. In accord with our data (see below) and for practicality, we assume, as shown in Scheme I, that the *same dissociation constant*, K_H, applies to the protonation of *all binary complexes*, viz., E·Pyr, E·Cl, and E·NAD. The rate equation for Scheme I under conditions where the steady state approximation holds is formulated by procedures analogous to those used previously [Appendix in Burgner & Ray (1978)]:

$$\frac{d[E_R]}{dt} = -\frac{k_f[E_R]}{f(NAD,Pyr,Cl^-,H)} + k_r^{app}[E\cdot adduct] \qquad (10)$$

Here, k_f and k_r^{app} are collections of rate constants (cf. eq 8 for the collection of constants describing k_r^{app}) and

$$f(NAD,Pyr,Cl^-,H^+) = 1 + \frac{K_{Pyr}K_H}{K_{I_2,Pyr}[H]} + \frac{K_{Pyr}}{[Pyr]}\left(1 + \frac{[Cl^-]}{k_{I_1,Cl}} + \frac{K_H}{[H]}\right) + \frac{K_{Pyr}K_{NAD}}{[Pyr][NAD]}\left(1 + \frac{[Pyr]}{K_{I_1,Pyr}} + \frac{[Cl^-]}{K_{I_2,Cl}}\right)\left(1 + \frac{K_H}{[H]}\right) \qquad (11)$$

Since the forward reaction does not go to completion under most of the conditions used, the observed constant k_f^{app} for the first-order disappearance of reactant enzyme (E_R) in eq 10 includes the constant k_r^{app}, which is the first-order rate constant for the reverse adduct reaciton under the same conditions.

Figure 7 shows typical rate data for the formation of the adduct complex as a function of reactant concentreations at pH 7, 0.3 M imidazole, and $\mu = 0.3$. Here, the reciprocal rate is plotted against the reciprocal pyruvate concentration at different nonvarying NAD concentrations, in accord with the linear transform of eq 2 and 3. The value for k_r^{obsd} was obtained in an independent experiment under the same conditions of temperature, pH enzyme, imidazole concentrations, and ionic strength—see Figure 2. The data in this figure are fitted by nonlinear regression to eq 3 and 2, which describe, respectively, the expected concentration dependence of k_f^{obsd} when E·Pyr is and is not kinetically significant. On the basis of a variance ratio test of the sums of squares of the differences between observed and calculated values of k_f^{obsd}, eq 3 provides a better description of the trends in the data than does eq 2 ($\alpha = 0.99$). Hence, E·Pyr appears to be present in the adduct reaction mixture in kinetically significant amounts and thus is included in Scheme I. The solid lines in Figure 7 are drawn according to eq 3 with the constants, obtained from the above regressions, given in Table I. [Analogous studies (not shown) at other pH values indicate that the concentration of the E–pyruvate complex also is kinetically significant throughout the pH range of 6–8, and the estimates of the constants from these studies also appear in Table I.] The pH-induced changes in the values of the kinetic constants shown in Table I will be considered after examining the effect of anion concentration on the adduct reaction.

Inhibition of the Adduct Reaction by Anions. Although no *systematic* study of the effect of anions of the adduct reaction has been conducted previously, studies on the *normal* enzymic reaction (Rivedal & Sanner, 1979; Grimshaw & Cleland, 1980; Anderson, 1981; Ward & Wingor, 1982) and scattered observations on the adduct reaction (Griffin &

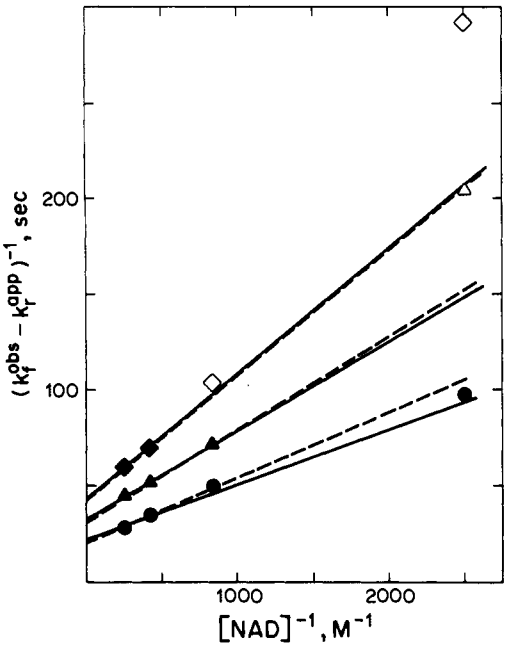


FIGURE 7: Effect of pyruvate and NAD on the observed rate constant for the forward reaction. Values for the rate constants are estimated by procedures described under Materials and Methods. The reaction mixture initially contained pyruvate, NAD (at the millimolar concentrations given on the graph), and 0.3 M imidazole buffer at pH 7.0 with a total chloride concentration of 0.3 M. Sufficient enzyme was added to generate a final enzyme concentration of 1 μN in the assay. The solid lines in this figure are drawn from eq 1 and 4, the values of the kinetic constants in Table II at pH 7.0, and the appropriate value k_r^{app} in Figure 4. The dashed lines are drawn from eq 1 and 3, the values of the kinetic constants in Table I at pH 7.0, and the appropriate value of k_r^{app} in Figure 4.

Criddle, 1970) suggest that anions bind to LDH and significantly influence the values of its kinetic parameters. To correct for the effect of the anion (chloride) present in the adduct reaction, we evaluated chloride inhibition patterns as a function of the pyruvate and NAD concentrations, respectively. These inhibition patterns show that the binding of chloride is competitive with respect to pyruvate and noncompetitive with respect to NAD (these data are available on request). Similar studies (not shown) at pH 6.5 and 8 provide analogous patterns for chloride inhibition. [Denaturation of the enzyme at high chloride concentration and pH 6 (and below) and "rapid" dissociation of the adduct at pH 9, and above, limit the pH range studied.] The internal consistency of the linear regression estimates for *chloride*-independent constants in Table II (viz., k_f^{app}, K_{Pyr}^{app}, K_{NAD}, and $K_{I_2,Pyr}^{app}$) was tested by calculating the corresponding values of the constants that should be observed at 0.3 M chloride and comparing the calculated and experimental values at each pH value—see Table I as well as the dashed lines in Figure 7, which are calculated from the constants in Table II. Note that K_A or K_A^{app} designates the constant obtained after correcting values of K_A^{obsd} for *competitive* chloride binding; if a pH dependency remains, K_A^{app} is used, and if not, K_A is indicated. In the case of k_f, k_f^{app} is used after correction of k_f^{obsd} for reactant equilibrium effects only (see eq 1), since Scheme I requires that the observed value of k_f^{obsd} be independent of *competitive* chloride effects at "infinite" reactant concentrations. The reasonable agreement of the "exptl" and "calcd" values in Table I over a range of 2 pH units where other parameters change considerably indicates that chloride does indeed bind as a dead-end inhibitor to two different enzymic species; i.e., it binds to E·H·NAD competitively with pyruvate (both enol and keto forms) and to the apoenzyme competitively with NAD as in Scheme I.

Only a limited amount of published data on the effect of chloride in the normal enzymic reaction is available for comparison with the values given in Table II, and none of these data provide a complete estimate of the type, number, or magnitude of the anionic interactions with LDH, although the electron-density maps of the apoenzyme and the binary E·H·NAD complex indicate the presence of a sulfate at the active site (Adams et al., 1973). But, the values at pH 7.0 for $K_{I_1,Cl}$ (0.1 M) and $K_{I_2,Cl}^{app}$ (0.07 M), which are the apparent dissociation constants for E·H·Cl (or E·Cl) and for E·H·NAD·Cl, respectively, are in the required range to reproduce the chloride inhibition data that are available [cf. Lovell & Winsor (1974) and Rivedal & Sumner (1978)]. In addition, the increase pH (Table II) is in accord with eq 10 and 11, where these constants are described in terms of their pH-independent constants. By contrast, the absence of a pH effect on the binding of Cl^- and Pyr to the apoenzyme, i.e., on $K_{I_1,Cl}$ and $K_{I_1,Pyr}$, argues that protonation of the group that controls the adduct reaction (see Results and Discussion) does not significantly influence the binding of chloride or pyruvate to the apoenzyme. Hence, Scheme I adequately represents those enzyme species that are kinetically important in the forward adduct reaction at subsaturating NAD and Pyr and provides a reasonable rational for the variation of k_f^{obsd} with conditions.

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