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Bimodal Substrate Inhibition of Lactate Dehydrogenase. Factors Affecting the Enzyme in Vivo†

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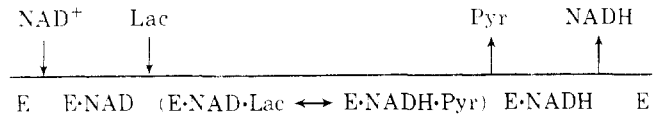
ABSTRACT: In the presence of NAD, pyruvate inhibits various isozymes of lactate dehydrogenase via (1) the rapidly reversible formation of a dead-end, (abortive) ternary complex, E·NAD·pyruvate, and (2) the slowly reversible formation of a binary enzyme·inhibitor complex in which the inhibitor is the adduct of pyruvate and NAD, NAD-Pyr. Thus, pyruvate-induced inhibition patterns obtained from *initial* velocity studies of the normal enzymic reaction, NADH + pyruvate → NAD + lactate, are caused solely by the dead-end ternary complex (E·NAD·pyruvate). Because of weak binding, it is unlikely that this complex is important in vivo in mammalian systems. The curvature of the product–time plots obtained with saturating substrate concentrations and product concentrations far from equilibrium is produced by the slow formation of the binary E·NAD-Pyr complex ($t_{1/2}$ = 5 min at 15 °C for dogfish A_4 enzyme). In the *limiting steady state,* at pH 7, and at *saturating NADH* concentration formation of the adduct complex reduces the fraction of active enzyme only by about 50%. But as the system approaches equilibrium a much greater reduction in active enzyme can be observed. In addition, the decomposition of the adduct complex is slow ($t_{1/2}$ = 1 to 5 min under pseudophysiological conditions). However, significant amounts of the adduct complex cannot be detected in extracts of rat heart that were rapidly prepared under conditions where the adduct complex is relatively stable. Hence, the adduct complex, as well, is unlikely to be important, physiologically, in mammalian systems.

The NAD^+-dependent lactate dehydrogenases (LDH)[1] of vertibrates are present in vivo largely in two isomeric forms: the A isozyme, which is obtained from skeletal muscle, and the B isozyme, which is isolated from cardiac muscle. (There also are three additional isomeric forms that are present at substantially lower concentrations; see Masters & Holmes, 1974.) This paper deals only with isozymes that have a uniform subunit composition: A_4 and B_4.

The biological significance of the A and B isozymes and their distribution in various tissues often is correlated with the oxidative capacity of the tissues (cf., Everse & Kaplan, 1973, 1975). Thus, the B form, found principally in "oxidative" tissue, may be primarily a lactate oxidase, because it has a smaller Michaelis constant for lactate than the A form; conversely, the A form may be primarily a pyruvate reductase, because it is found principally in "glycolytic" tissue and because of the presumed inability of voluntary muscle to oxidize lactate. In addition, metabolic control of the B form of the enzyme might be exerted by pyruvate in the Pyr → Lac reaction, since this form of the enzyme is subject to pyruvate inhibition at significantly lower concentrations than the A form (see below). However, comparisons between tissue levels of pyruvate and its inhibitor constant do not lend support to the latter suggestion and, as an alternative, Stambaugh & Post (1966) suggest that "product inhibition" may be an important factor. Furthermore, Rose & Rose (1969) suggest that such metabolic control of the B form is unlikely, since perfusion experiments with rat hearts (Williamson, 1965) indicate that the lactate/pyruvate ratio is at its equilibrium value, except when abnormally high pyruvate concentrations (10 mM) are used in the perfusate.

The results of in vitro studies involving initial velocity, product inhibition, isotope exchange, and rapid mixing experiments with isozymes from several different organisms (Hakala et al., 1965; Zewe & Fromm, 1962, 1965; Anderson et al., 1964; Silverstein & Boyer, 1964; Stambaugh & Post, 1966; Urban, 1969; Holbrook & Gutfreund, 1973; Boland & Gutfreund, 1975) are consistent with an ordered sequential process (both substrates add before release of either product):



(1)

Substrate inhibition of either the forward or reverse reactions by lactate or pyruvate, respectively, where these are present at concentrations greater than their respective Michaelis constants, also is well documented (see Everse & Kaplan,

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[1] Abbreviations used: LDH, lactate dehydrogenase; E, a subunit of the LDH tetramer; Pyr, pyruvate; Pyr_K and Pyr_E, the keto and enol pyruvate forms, respectively; Lac, lactate; APAD and APADH, the oxidized and reduced forms of 3-acetylpyridine analogue of NAD, respectively; NAD-Pyr and APAD-Pyr or adduct, the covalent addition product of the oxidized coenzyme and pyruvate produced by LDH.

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1973). Early investigations suggested that this inhibition was caused by a rapidly reversible dead-end "abortive ternary" complex of either E·NADH·Lac or E·NAD·Pyr_K (Winer et al., 1957; Zewe & Fromm, 1962); more recent studies suggested that inhibition by pyruvate is caused by the enzymic formation of a tightly bound inhibitor which produces an E·NAD-Pyr or abortive "binary" complex[2] (see Burgner & Ray, 1978; as well as Lee et al., 1965, 1966; DiSabato, 1968; Gutfreund et al., 1968; Everse et al., 1971). However, recognition that *both* modes of inhibition can be important in in vitro assays does not appear to be widespread, and this paper is primarily concerned with assessing the relative importance of each as well as their in vivo importance. The accompanying paper (Burgner & Ray, 1978) describes additional studies on the mechanism of the adduct reaction that suggest its use as a model for the normal enzymic reaction.

Experimental Section

Materials

Lithium lactate and sodium pyruvate were obtained from Sigma, and APADH, NADH, and NAD were obtained from P-L labs. The best available grades of other chemicals were used. Solutions of substrates were prepared and, where necessary, adjusted to pH 7 immediately before use. Purified beef and pig isozymes of LDH were obtained from either Boehringer-Mannheim or Sigma; dogfish M_4 LDH was purified and stored according to procedures described previously (Burgner & Ray, 1974). Stock solutions of dogfish LDH were prepared by dialyzing ammonium sulfate suspensions against four changes of sodium phosphate buffer (pH 7.0) at 4 °C (μ = 0.15) containing 1×10^{-4} dithiothreitol. Stock solutions of the beef and pig enzymes were obtained by diluting 10 to 20 μL of ammonium sulfate suspension into 2.0 mL of deionized water. Untrained Wister strain rats (female), maintained on Wayne Lab Block (Allied Mills, Chicago, Ill.), were used.

Methods

Standard Assays. Routine assays of dogfish LDH were performed at 25 °C in the manner described previously (Burgner & Ray, 1974); assays of beef and pig LDH were conducted by the method described in the catalog of the Boehringer Mannheim Co. Prior to the assay, stock solutions of enzyme were diluted into the appropriate buffer containing 0.1 mg/mL bovine serum albumin and 1.0×10^{-3} M dithiothreitol, so that 10 to 20 μL of enzyme sample was sufficient for the assay. Enzyme concentrations were estimated by using a molar extinction coefficient of 2×10^5 M^{-1} at 280 nm (Pesce et al., 1967) and are reported in terms of the normality of active sites, [E].

Kinetic Studies. Initial velocity and product-time experiments were conducted at 15 or 25 °C by measuring the appearance or disappearance of NADH or APADH at 340 or 363 nm, respectively, either with a Cary Model 15 spectrophotometer or an apparatus described elsewhere (Wratten & Cleland, 1965). Multiple chart speed and absorbance scales were used to maintain line slopes near 45°; compensation for the initial absorbance of the sample was made either electronically or with a reference blank. The initial slopes of the lines were estimated by eye, either directly or by linearly extrapolating plots of $[P]/t$ against t to $t = 0$ (Alberty & Koerber, 1957).

Decomposition of the adduct complex at low enzyme concentration (10^{-9} to 10^{-10} N) was measured by (a) rapidly diluting a solution containing the enzyme·adduct complex (see Burgner & Ray, 1978) into the standard assay mixture for the Pyr → Lac reaction (see above), and (b) initiating the assay, after the adduct complex had been exposed to excess NADH for various time intervals, by addition of pyruvate; the initial velocity was estimated after each interval. The fraction of adduct-inhibited enzyme that remained was fitted to a function describing a first-order process. A control, which involved a mixture identical with that used to prepare the enzyme·adduct complex, except that pyruvate was not present, was compared with the "infinity point", above.

Cardiac Muscle Extracts. Rats were killed by a sharp blow to the back of the head, and the heart were removed as rapidly as possible and placed in ice cold isotonic saline for 30 s. (The rats were maintained in a darkened enclosure until just before use.) One-half of the heart was homogenized immediately at 4 °C in cold distilled water, 0.05 M sodium phosphate buffer, adjusted to pH 7.0 at 4 °C or 0.1 M sodium phosphate, pH 7.5 at 4 °C. The extract either was assayed immediately (total lapsed time subsequent to excising the heart, about 3 min) or was centrifuged for 2 min at 4 °C prior to the assay (see below). An alternate procedure occasionally was used in which the hearts were frozen immediately after removal between two aluminum blocks (230 g each) previously cooled in liquid N_2 and were stored in liquid nitrogen for later analysis. The frozen tissue was lightly pulverized just before use and was homogenized and extracted, as above, without prior thawing.

Enzymic Assay. The activity of LDH was measured at 4 °C in the presence of 1×10^{-3} M NAD^+ and 5×10^{-3} M lithium lactate in 2 mL of 0.1 M sodium phosphate, pH 7.5, by following the production of NADH, fluorometically, with the instrument described elsewhere (Burgner & Ray, 1978), except that an Oriel 150 W Hg–Xe lamp excitation source was substituted for the deuterium source; the wavelength of excitation radiation was 340 nm, and Corning filters 3–72 and 4–72 were used for the emission radiation. Under these conditions, the assay is linear in the absence of adduct for the entire span of the recorder, which represents a total NAD^+ to NADH conversion of approximately 0.1%, viz., about 1 μM.

The *effective catalytic efficiency* of the LDH in heart muscle extracts was obtained by diluting the extract 100-fold into an ice-cold sodium phosphate buffer, 0.05 M, pH 7.0, containing 0.01 M potassium oxalate and 1×10^{-4} M NAD^+, and further diluting the mixture, immediately, by 33.3-fold into the same buffer without NAD^+ and oxalate. Enzymic activity was measured after adding 30 μL of the twice-diluted extract to the above assay mixture. The *total catalytic capacity* was obtained by incubating the *initial* dilution for 40 min at room temperature (25 °C) and assaying as above.

Synthesis of the Adduct Complex of LDH in a Crude Rat Heart Extract. The homogenate from one-half of a frozen rat heart and 1 mL of 0.1 M sodium phosphate, pH 7.5, was centrifuged at 18 000g, the supernatant made 1×10^{-3} M in NAD^+ and 0.02 M in pyruvate, and the mixture incubated for 15 min at room temperature. The mixture either was used within 15 to 30 min or stored frozen.

Data Processing. Reciprocal velocities were plotted against reciprocal substrate concentration and, when the results appeared to be linear, the computer programs of Cleland (least-squares method with an equal variance for measured velocities) were used to obtain estimates for the various kinetic constants and their standard errors (Cleland, 1967). When

[2] The complex with the stoichiometry, enzyme plus adduct, i.e., E·NAD-Pyr or E·adduct is referred to as the "abortive binary complex"; "abortive ternary complex" is used for the complex where stoichiometry is enzyme, plus product-coenzyme, plus reactant substrate, i.e., E·NAD-Pyr_K or E·NADH·Lac, where there is no covalent bond between the coenzyme and substrate molecules.

TABLE I: Kinetic Constants for Lactate Dehydrogenases.[a]

	Dogfish A_4[b]	Pig A_4[c]	Rabbit A_4[d]	Beef A_4[c]	Pig B_4[c]	Beef B_4[c]
V_1/E_t (min^{-1})	3 000 ± 300	18 000 ± 2000		16 800 ± 900	5 300 ± 300	4 600 ± 60
K_{NAD} (mM)	0.11 ± 0.02	0.19 ± 0.06	0.17	0.02 ± 0.01	0.04 ± 0.02	0.02 ± 0.002
K_{iNAD} (mM)	0.35 ± 0.03	0.5 ± 0.1	0.5	0.6 ± 0.2	0.5 ± 0.1	2 ± 1.0
K_{Lac} (mM)	60.0 ± 10.0	10.0 ± 3.0	11.0	5.0 ± 1.0	2.3 ± 0.5	0.3 ± 0.1
V_2/E_t (min^{-1})	13 800 ± 2000	14 000 ± 2000		23 000 ± 2000	10 100 ± 3000	12 900 ± 500
K_{NADH} (μM)	30 ± 0.5	5.0 ± 3.0	7.0	2.8 ± 0.9	5.0 ± 0.7	3.7 ± 0.5
K_{iNADH} (μM)	8.1 ± 0.1	6.0 ± 3.0	6.0	7.0 ± 3.0	14.0 ± 2.0	5.0 ± 1.0
K_{Pyr} (mM)	0.67 ± 0.5	7.0 ± 2.0	0.2	5.0 ± 1.0	2.2 ± 0.2	1.7 ± 0.8

[a] The values in this table were obtained by fitting initial velocity data as a function of substrate and coenzyme concentration to eq 2. [b] The buffer was 0.1 M potassium phosphate; pH 7.5 at 15 °C. [c] The buffer was 0.50 M Tris-Cl, pH 9.0 at 25 °C. [d] Zewe & Fromm (1965); Tris-Cl, pH 7.15 at 28 °C.

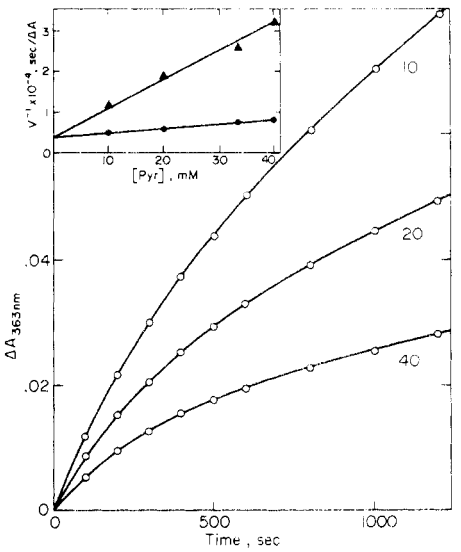


FIGURE 1: The effect of pyruvate concentration on the product-time curves of the reverse reaction. A plot of ΔA_{363} against time is shown for reactions at 15 °C in 0.15 M imidazole·HCl, pH 7.0. The assay mixture (3 mL) initially contained 1.5×10^{-4} M APADH, 0.3 mg of bovine serum albumin, 2×10^{-4} M dithiothreitol plus the indicated pyruvate concentrations and 1×10^{-10} N enzyme. The data points represent the average of three or more determinations, and the solid line was calculated from eq 5 and the numerically estimated values for v_0 and v_1 (see the inset) and for k' of 3.7×10^{-3}, 3.0×10^{-3}, and 2.4×10^{-3} s^{-1}, respectively, at 40, 20, and 10 mM pyruvate. (Inset) The reciprocals of the numerically estimated values for v_0 and v_{lim} from the above product-time data are plotted against the pyruvate concentration. The solid lines are least-squares fits of the data to eq 3 and 4 (see Results).

initial velocity patterns were affected by substrate inhibition, data were fitted to eq 3 or eq 8, and, when product-time plots were analyzed, eq 4 was used; fitting procedures analogous to those of Cleland were employed except that initial estimates for the relevant constants were obtained graphically.

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (2)$$

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB + AB^2/K_I} \quad (3)$$

A program which used initial estimates and a least-squares procedure to obtain values for the initial steady-state velocity, the limiting steady-state velocity and the rate constant for the transition between the initial and limiting velocities was provided by Dr. William Finkelstadt, Department of Chemistry, Purdue University. The nomenclature of Cleland (1963a,b) is used throughout.

Results

Steady-State Kinetic Constants. For each of the enzymes used in this study, the initial velocity patterns both for the forward and the reverse reaction under conditions where substrate inhibition is insignificant are described by the equation for a sequential process (eq 2). These results are in accord with those obtained for lactate dehydrogenases isolated from other types of tissue which react by an ordered Bi Bi mechanism (Anderson et al., 1964; Zewe & Fromm, 1965; Urban, 1969). Values for the kinetic constants obtained from these studies appear in Table I and are similar to published constants for other lactate dehydrogenases (Zewe & Fromm, 1962, 1965; Anderson et al., 1964; Schwert et al., 1967; Urban, 1969). Hence, an ordered Bi Bi mechanism (eq 1) probably describes the normal catalytic process for all of these enzymes, and conclusions about the mechanisms of inhibition by pyruvate or lactate (see below) probably are general for all lactate dehydrogenases where this mechanism holds.

Product-Time Curves for the Normal Enzymic Reaction at Inhibitory Pyruvate Concentrations. Time plots (Figure 1) of the disappearance of APADH (or NADH) during the reaction of dogfish A_4 LDH at various inhibitory levels of pyruvate show that the rate, d[P]/dt, decreases with time from its *initial* steady-state value, v_0, to a *limiting* steady state value, v_{lim}. Moreover, such plots show that v_0, v_{lim}, and the relaxation constant, $1/t_{1/2}$, for the approach of v_0 to v_{lim} are inversely dependent on the pyruvate concentration. (The results also are independent of the order of mixing.) In addition, after preincubating the enzyme for 30 min in the presence of 20 mM pyruvate and the appropriate concentration (depending on the source of the enzyme) of NAD and APAD, the transient phase of the product-time curves disappears, and v_0 is reduced to v_{lim}, where v_{lim} is the same as that observed without preincubating the enzyme, even though the assay mixture in both cases contains the same concentrations of all reactants. Since E-adduct is formed during the preincubation step (see Burgner & Ray, 1978), the process in Figure 1 by which v_0 is transformed into v_{lim} is identified. Similar results, from a qualitative standpoint, also were obtained in the pH range 7 to 9 for the LDH isozymes from pig, beef, rat, and frog.

Plots (inset, Figure 1) of the reciprocal of the *initial* and *limiting* steady-state velocities against the pyruvate concentration for the dogfish A_4 isozyme, as well as for several of the other enzymes (not shown), are linear; these demonstrate that the overall pyruvate inhibition must involve an interaction with the enzyme in addition to that which occurs in the E-adduct complex. Initial velocity studies where the NADH is varied at fixed levels of pyruvate indicate that inhibition of the initial steady-state velocity by pyruvate is uncompetitive with respect to NADH (Figure 2) and that, for an ordered Bi Bi reaction

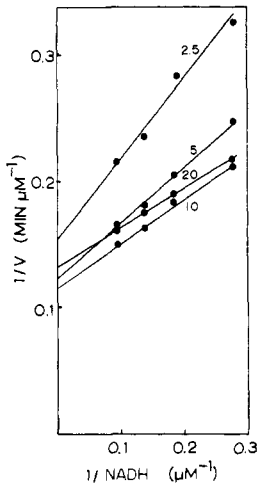


FIGURE 2: The effect of NADH and pyruvate concentrations on the initial velocity of the Pyr → Lac reaction. A double-reciprocal plot of initial velocity against NADH at the fixed pyruvate concentrations (in millimolar) noted on the figure is shown for beef B_4. The reaction mixtures also contained 2.7×10^{-10} M enzyme and 0.05 M Tris-Cl, pH 9.0 at 25 °C. The data were fitted numerically to eq 3, which describes uncompetitive substrate inhibition, and the lines were calculated from the estimates for the kinetic constants in Table I and a value for $K_{I(Pyr)}$ of 27 ± 4 mM.

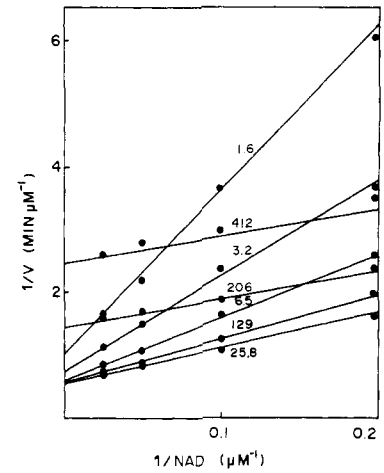
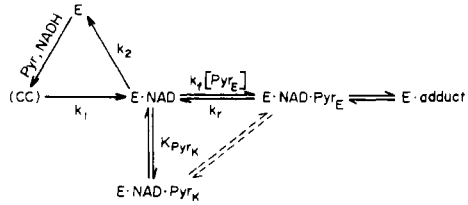


FIGURE 3: The effect of NAD and lactate concentrations on the initial velocity of the Lac → Pyr reaction. A double-reciprocal plot of initial velocity against NAD at the fixed lactate concentrations (in millimolar) noted in the figure is shown for beef B_4. The reaction mixtures also contained 3.2×10^{-10} M enzyme and 0.1 M potassium phosphate, pH 7.5 at 25 °C. The data were fitted numerically to eq 3, and the estimates for the constants (V_1/E_t, 2000 ± 80 min^{-1}; K_{NAD}, 11 ± 1 μM; K_{iNAD}, 35 ± 8 μM; K_{Lac}, 2.6 ± 0.4 mM; $K_{I(Lac)}$, 79 ± 5 mM) were used to calculate the lines.

scheme, inhibition must occur after NADH and pyruvate have added to the enzyme. Hence, the obvious explanation for the inhibitory effect on the *initial* steady-state velocity, by pyruvate, observed in Figures 1 and 2 involves the *rapidly* reversible formation of an abortive ternary complex with the stoichiometry E, NAD, and Pyr_K.[3] An alternative, but less attractive, explanation involves combination of pyruvate in a dead-end fashion with the central complexes so that either catalysis or product release is inhibited (cf., Uhr et al., 1974). Similarly, an analogous complex with the stoichiometry E, NADH, and lactate probably exists, since at high concentrations lactate is an uncompetitive inhibitor of the Lac → Pyr reaction (Figure 3). However, with lactate, v_0 always is equal to v_{lim}. Values of inhibition constants estimated by standard procedures (see Experimental Section) appear in Table II and are considered below.

Analysis of the Initial and Limiting Steady-State Velocities in the Reverse Reaction. For convenience, eq 1 was reformulated (Scheme I) to include the inhibitory pyruvate complexes,

SCHEME I: The Mechanism for Pyruvate Inhibition of LDH.



E·adduct and E·NAD·Pyr_K, and to exclude E·NADH which is negligible with respect to the concentrations of the other enzyme forms at the saturating reactant concentrations noted in Figure 1. (The free enzyme, E, also is negligible, but is included in this scheme only for descriptive purposes.) In Scheme I pyruvate enol (Pyr_E) reacts with E·NAD to form the E·adduct complex. (The adduct complex also can be formed by conversion of E·NAD·Pyr_K to E·NAD·Pyr_E (dashed arrows); however, since the concentration of E·NAD·Pyr_E is negligible, relative to other enzyme forms and since in the present inhibition studies only the extent of but not the mechanism by which E·NAD is converted to E·adduct is important, we will not distinguish between these alternative pathways, here (cf., Burgner & Ray, 1978).) Additional reaction steps—dissociation of E·adduct and dissociation of NAD from E·NAD·Pyr_K—do not contribute significantly and are not included (see Burgner & Ray, 1978, and the results, below).

An expression (eq 4) describing the inhibitory effect of pyruvate on the initial velocity (v_0) of the Pyr → Lac reaction under the above conditions was formulated for the case where the concentrations of E·NADH, E·NADH·Pyr, and E·NAD·Pyr_K reach their steady-state levels prior to the onset of measurements (viz., within a second or so—cf., Holbrook & Gutfreund, 1973) and where a negligible fraction of the total enzyme is converted to E·adduct prior to this time (Burgner & Ray, 1974).

TABLE II: Michaelis and Inhibition Constants.[a]

	Substrate	pH	K_M (mM)	K_I (mM)
Pig B_4[b]	Pyruvate	7.0	0.05	2.0
Pig A_4[b]	Pyruvate	7.0	0.50	43.0
Dogfish A_4[b]	Pyruvate	7.0	0.20	11.0
Beef B_4[c]	Pyruvate	7.5	0.08	9.6
Beef A_4[c]	Pyruvate	7.5	0.23	35.0
Beef B_4[c]	Pyruvate	9.0	4.0	6.0
Beef A_4[c]	Pyruvate	9.0	9.2	71
Beef B_4[d]	Lactate	7.5	1.6	180
Beef A_4[d]	Lactate	7.5	12	800
Beef B_4[d]	Lactate	9.0	1.6	180
Beef A_4[d]	Lactate	9.0	12	800

[a] The values in this table were obtained by fitting initial velocity data as a function of substrate concentration to eq 8. [b] The NADH concentration was 0.13 mM; the buffer was 0.1 M potassium phosphate. [c] The NADH concentration was 0.1 mM; the buffer was 0.1 M potassium phosphate at pH 7.5 and 0.05 M Tris-Cl at pH 9.0. [d] The NAD concentration was 0.85 mM; the buffer was 0.1 M potassium phosphate at pH 7.5 and 0.05 M Tris-Cl at pH 9.0.

[3] Tienhaara & Meany (1973) suggest that the hydrated form of pyruvate is an inhibitor of LDH; however, we are unable to demonstrate a significant *inhibition* of dogfish A_4 LDH by using their procedures (J. W. Burgner II & W. J. Ray, Jr., unpublished observations, 1975).

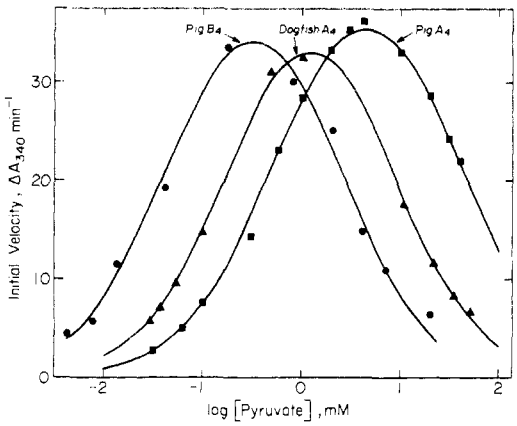


FIGURE 4: The effect of pyruvate on the initial velocity of the reverse enzymic reaction. A plot of the initial velocity against the logarithm of the pyruvate concentration is shown for pig B_4 (O) and A_4 (□) and dogfish A_4 (Δ). The initial velocity, ΔA_{340}/min, was estimated graphically from product-time plots analogous to those in Figure 1 and obtained under the same conditions (except that NADH, 1.33×10^{-4} M, was substituted for the APAD). The solid lines were calculated from eq 8 by using a value for the maximum velocity of 44 OD/min at 340 min and values for the Michaelis and inhibition constants from the first three rows in Table II.

$$\frac{1}{v_0} = \frac{1}{V_{max}}\left[1 + \frac{[Pyr_K]}{K_{Pyr_K}(1 + k_2/k_1)}\right] \quad (4)$$

In this expression, k_1 and k_2, respectively, describe the dissociation of lactate from the central complexes, (CC), and of NAD from E·NAD, while K_{Pyr_K} is the dissociation constant for E·NAD·Pyr_K. The rate equation (eq 5) describing the limiting steady-state velocity, v_{lim}, was formulated in an analogous manner for the case where E·adduct has reached its steady-state level prior to the accumulation of a significant amount of product (since, under the conditions used in Figure 1, the change in concentration of the substrates is negligible).

$$\frac{1}{v_{lim}} = \frac{1}{V_{max}}\left\{1 + \frac{[Pyr_K](1 + K_{Pyr_K}k_f/k_r)}{K_{Pyr_K}(1 + k_2/k_1)}\right\} \quad (5)$$

Equations 4 and 5 require that both v_0^{-1} and v_{lim}^{-1} be linear functions of pyruvate concentration, as observed in the inset of Figure 1. Since these equations adequately describe the *trends* in the velocity data in Figure 1, at least two different inhibitor complexes must be involved. Unfortunately, only an upper estimate of the value for K_{Pyr_K} can be determined from this analysis (see figure legend), since the intercept/slope ratio for the initial velocity plot is equal to $(1 + k_2/k_1)K_{Pyr_K}$, and this expression only approaches K_{Pyr_K} when the rate of dissociation of lactate from the central complex is much greater than that for NAD from E·NAD (i.e., $k_1 \gg k_2$). Since this condition does not always hold for LDH (Borgmann et al., 1974), the quantitative accuracy of eq 4 and 5 cannot be verified independently.

An Analysis of the Transient Phase of the Product–Time Curves for the Forward Reaction. An integrated rate equation (eq 6) was formulated for Scheme I by using the steady-state approximation (i.e., by assuming that the concentration *ratios* of (CC) to E·NAD to E·NAD·Pyr_K are constant over the time course of the plots in Figure 1) and by using the following expression for the appearance of product: $[P] = k_{cat}\int_0^\infty [E\cdot NAD]dt$, where $k_{cat} = (V_{max})/[E_t]$.

$$[P] = v_{lim}t + \left[\frac{v_0 - v_{lim}}{k'}\right](1 - e^{-k't}) \quad (6)$$

Here, k', which describes the observed rate constant for the process in which v_0 becomes equal to v_{lim} (see Figure 1), is given by eq 7 (see Scheme I for definition of constants).

$$k' = [k_f Pyr/(1 + k_2/k_1 + Pyr/K_{Pyr_K})] + k_r \quad (7)$$

Estimates for the values of k', v_0, and v_{lim} in eq 6 were obtained from the data in Figure 1, both by numerical and graphical procedures (see Experimental Section), and the values thus obtained were used to construct the solid lines in this figure. Clearly, Scheme I adequately describes the system under the specified conditions. In addition, from eq 4, 5, and 6, the rate constant for E·adduct decomposition, k_r, is defined by the expression v_{lim}/v_0k'; an average value for k_r, $7 \pm 1 \times 10^{-4}$ s^{-1}, was estimated from this expression and the values of the constants in legend of Figure 1, as well as from additional data (not shown). The agreement of this value with that obtained by directly measuring E·adduct decomposition, 5×10^{-4} s^{-1} (see below), provides additional support for Scheme I.

The Rate of Decomposition of the Adduct Complex. To determine the value of the rate constant (k_r) for decomposition of the adduct complex under conditions similar to those used in Figure 1 (viz., at 10^{-10} N enzyme), an initial velocity assay (see Methods) was used to estimate the concentration of adduct complex that remained after a given time interval in a reaction mixture where the adduct complex was treated with excess NADH and where the concentration of pyruvate was much lower than was used for the formation of E·adduct. In these studies $(v_\infty - v_t)/v_\infty$ was taken as equal to the $[E\cdot adduct]_t/[E\cdot adduct]_0$, where v represents the initial, *assay* velocity and the subscripts indicate the *reaction time* at which aliquots were removed and assayed. Semilog plots of the fraction of adduct complex remaining against time were linear to at least 4 half-times (except for a small, unidentified initial "burst phase" in the case of E·APAD–Pyr, but not E·NAD–Pyr (see Burgner & Ray, 1978), which accounted for approximately 10% of the total reaction and which was ignored). The k_r value, obtained for E·APAD–Pyr, 5×10^{-4} s^{-1}, is the same as the value estimated at higher E·adduct concentrations (10^{-7} N) using the oxamate trapping procedure described previously (Burgner & Ray, 1978).

Assessment of the Inhibiton Constant for Pyruvate. Figure 4 is a plot of the initial velocity for the reverse enzymic reaction (Pyr → Lac) against the logarithm of [Pyr] at saturating NADH. (A log plot was used because of the large range of concentrations used.) Equation 8 is the expected substrate-velocity equation for Scheme I at saturating [NADH], when the inhibitor complex, E·NAD·Pyr_K, also is taken into account.

$$\frac{1}{v_0} = \frac{1}{V_{max}}\left(1 + \frac{[Pyr]}{K_{I(Pyr_K)}} + \frac{K_{Pyr}}{[Pyr]}\right) \quad (8)$$

Estimates for the values of the maximum velocity, V_{max}, the Michaelis constant for pyruvate, K_{Pyr}, and the inhibition constant of pyruvate, $K_{I(Pyr_K)}$ (which is equal to $K_{Pyr_K}(1 + k_2/k_1)$, were obtained numerically (see Table II, first three rows) by using an unweighted least-squares procedure and the data in Figure 4; the solid lines in the figure were constructed by using these estimates and eq 8. Additional data were obtained with beef A_4 and B_4 LDH at pH 7.5 and 9.5 by using the same procedures; estimates for the Michaelis and inhibitor constants are given in Table II. Analogous substrate inhibition data for lactate also are summarized in Table II. Qualitatively, these results suggest that, since the initial velocity approaches zero at high, inhibitory levels of either pyruvate or lactate, dissociation of the product coenzyme from its abortive ternary complex is not kinetically important under these conditions.

An Assay for the LDH–Adduct Complex. An assay for the

LDH·adduct complex was designed on the basis that a *slowly dissociating* inhibitor will decrease enzymic activity in an *initial velocity assay* in proportion to the enzyme–inhibitor complex present *prior to the assay*, even though under the conditions of the assay the enzyme–inhibitor complex is (thermodynamically) unstable. Hence, the *effective catalytic efficiency* will refer to the initial velocity (conversion of NAD^+ to NADH) produced by LDH plus LDH·adduct mixtures, while *total catalytic capacity* will refer to the initial velocity after dissociation of the LDH·adduct complex. In the present case, total capacity assays were performed after the adduct was displaced by incubating with NAD plus oxalate, since oxalate is a *rapidly dissociating inhibitor* whose effect can be eliminated, even in an initial velocity assay, simply by dilution (see Methods). Under the conditions employed, *at least* 90% of the adduct could be displaced prior to the assay without using an oxalate concentration that was high enough to affect the initial velocity the subsequent assay. Hence, such an assay will *correctly assess* the amount of the LDH·adduct complex present if this fraction is either 0 or 1 and will *underestimate* the amount of the LDH·adduct complex present by an amount equal to substantially *less than 10%* of the *total* LDH present when this fraction is in the intermediate range. (In order to facilitate the handling of samples, the effective catalytic efficiency sometimes was measured after addition of oxalate, but before a significant fraction of the LDH·adduct complex had time to dissociate; the lack of an oxalate effect on the rate of dissociation of the adduct was verified in crude extracts as well as in solutions prepared from purified LDH.) The ability to use the above assay to detect the adduct complex in samples obtained by in vitro treatment with NAD and pyruvate was substantiated by using "completely inhibited" samples of LDH, prepared either from the purified enzyme or in crude extracts by treatment with excess NAD^+ and pyruvate (see Methods). In both cases the ratio of effective efficiency to total capacity was found to be less than 5%.

The rate constant for decomposition of the LDH·adduct complex at 4 °C was assessed by following the increased catalytic efficiency with time. Since a half-time of 19 min was estimated in this manner, most of the adduct complex that originally was present in a rat heart would remain undissociated during the time (3 min maximum) that was required for homogenization and dilution of the homogenate for the assay (steps which were conducted at 0 °C).

Finally, a recovery experiment was performed in the following way. Completely inhibited pig heart LDH, equivalent to the total LDH in one-half of a rat heart, was added to the tissue extraction buffer just prior to the homogenization step. (The NAD and pyruvate in the sample of inhibited enzyme were diluted by 100-fold in this step; hence, no additional formation of adduct complex was possible.) Upon completion of the extraction procedures (see Methods), the *effective catalytic efficiency* was 60% of the *total catalytic capacity* for the sum of the two enzymes. Hence, the adduct complex was relatively stable during the extraction step.

The Adduct Complex in Vivo. The effective catalytic efficiency and the total catalytic capacity were determined for six rats hearts homogenized in cold 0.1 M phosphate buffer, pH 7.5, and prepared for assay with no more than a 3-min interval between excision of the heart and initiation of the assay (see Methods); the average value obtained for the ratios of these efficiencies, 0.9 ± 0.07, is not significantly different from unity—which is the value expected if no adduct complex was present in the tissue. Other experiments in which different homogenization media were used (cold distilled water, and 0.05 M phosphate, pH 7) provided analogous results.

Since these results are in contrast with those of Everse & Kaplan (1975), which indicated the presence of significant concentrations of the adduct complex in chicken-heart extracts, additional experiments were performed to determine whether the adduct complex could be produced in mammalian systems under "physiological conditions" and under "forcing conditions". In the former case, purified pig B_4 LDH plus substrates and products (the latter two at equilibrium), each at its physiological concentration, were incubated at 37 °C in 0.1 M sodium phosphate, pH 7.0, until a constant concentration of adduct complex was obtained. At the (total) reactant concentrations indicated by Williamson (1965) for rat hearts perfused with 0.01 M glucose (3×10^{-3} M NAD, 1.1×10^{-3} M lactate, 2×10^{-4} M pyruvate and 8.8×10^{-7} M NADH) and at 0.25 mg/mL of enzyme, approximately 30% of the total pig B_4 LDH was converted to the adduct complex, as was indicated by a comparison of effective efficiency and total capacity assays. Since substantially less adduct complex was observed in rat heart extracts (see above), intracellular conditions and/or concentrations of reactants (in pig heart) apparently differ significantly from these in vitro conditions. (The choice of reactant concentrations is somewhat arbitrary, since only total concentrations (bound plus free) are known, in vivo, and since the measured lactate/pyruvate and NAD/NADH ratios are not equivalent; because of this problem and because NADH is associated primarily with mitochondria (Williamson, 1965) its equilibrium concentration in these experiments was defined by the total concentrations observed for the other reactants and the equilibrium constant, 1.9×10^4, for the overall reaction (Honorst et al., 1961).)

Williamson (1965) also indicated that the lactate–pyruvate couple might not be at its mass action equilibrium when rat hearts were perfused with 50 times the physiological concentration of pyruvate (0.01 M), i.e., under forcing conditions. (Since the concentrations of lactate and pyruvate were not measured intracellularly, they may in fact be closer to equilibrium than is suggested by the data.) To determine whether a decreased efficiency of LDH under forcing conditions, which is caused by formation of the adduct complex, might be responsible for the apparent nonequilibrium value of the lactate—pyruvate couple, purified pig-heart B_4 LDH was incubated in vitro in the presence of (a) the total NAD concentration (3×10^{-3} M) within the cell and (b) the lactate and pyruvate concentrations detected in the above perfusate (10^{-3} M and 10^{-2} M, respectively). Although approximately 90% of the enzyme was converted to the adduct complex under these in vitro conditions, both fresh rat and previously frozen pig heart *tissue slices* incubated at 37 °C for 15 to 60 min in 0.01 M pyruvate, prior to extraction, as well as *extracts* of rat and pig hearts prepared and incubated in 3×10^{-3} M NAD^+ and 0.01 M pyruvate (see Methods) contain only limited amounts (10 to 30%) of adduct complex. (In both cases, under these conditions, the amount of the adduct complex—but not the total catalytic capacity—begins to disappear after 15 to 30 min; because treatment of extracts with additional NAD^+, but not with additional pyruvate, causes a reappearance adduct complex (to a level equal to about 70% of the maximal level), the concentration of free NAD^+ must slowly decrease (fate unknown) during this incubation.)

Discussion

The initial velocity data obtained at noninhibitory substrate concentrations show that the reaction sequence is sequential for the lactate dehydrogenases used here (see Table I). By comparison with the results of more extensive studies on other lactate dehydrogenases (see introductory section), an ordered

addition of substrates and release of products (eq 1) is suggested. This suggestion is supported by the similarity of the constants in Table I to those of the more thoroughly studied enzymes, rabbit A_4 and beef B_4, especially when comparisons are made between enzymes of the same isomeric type. The involvement of either a minor alternative sequence of substrate addition and product release (cf., Holbrook & Gutfreund, 1973) or of isomeric enzyme–substrate complexes should not effect any of the conclusions in the following discussion to a significant extent.

At concentrations greater than five times their Michaelis constants, pyruvate and lactate reduce the flux through the normal catalytic cycle of the heart and muscle isozymes of pig and beef LDH as well as the muscle form of dogfish LDH. Product-time curves (Figure 1) at inhibitory pyruvate levels show that this inhibition arises partly from an inhibitor complex that forms during the "dead time" of the assay (typically 3–5 s) and partly from an additional inhibitor complex that forms more slowly ($t_{1/2} \geq 25$ s). (Although the APAD coenzyme was used in Figure 1, similar results were obtained with NAD (see Results); hence, for qualitative conclusions, NAD and APAD are used interchangeably.) With lactate as the substrate, no comparable slowly formed inhibitor complex is observed. The rapidly produced substrate inhibition is of the linear uncompetitive type, *vis á vis* the coenzyme, and for reasons noted in the Results both substrates must produce this effect by adding to the alternative enzyme·coenzyme complex, viz., to give E·NAD·Pyr_K or E·NADH·Lac. (Pyr_K is specified here since the concentrations of its enol and *gem*-diol forms are negligible at pH 7 (Teinhaara & Meany, 1973; Burgner & Ray, 1974).)

That the slowly formed inhibitor complex produced in the enzymic assay at high pyruvate concentration is the E·adduct complex is substantiated primarily by the observation that the expected change in the ratio of initial to limiting steady-state velocities is produced when successively larger fractions of the enzyme are converted to E·adduct in a preassay step (by treatment with NAD and pyruvate). The identity of rate constants for decomposition of E·adduct obtained from direct measurement of this process and from an analysis of product–time curves in enzymic assays (at high pyruvate concentrations) provides additional support for this view (see Results).

Since both types of inhibition are obtained with each of the five different lactate dehydrogenases that were studied, we suggest that both the E·NAD·Pyr_K and E·adduct complexes will be important in the in vitro assays of all lactate dehydrogenases when (a) *product–time plots* for the reverse reaction at saturating substrate concentrations show a fall-off in excess of that required by approach to equilibrium and (b) when the *initial velocity* decreases with increasing concentration of the C-3 acid substrate.

A previous analysis, based on *initial velocity* assays similar to those in Figure 2 (Vessell & Pool, 1966; Stambaugh & Post, 1966), suggested that substrate inhibition by pyruvate or lactate is unimportant, physiologically, because cellular concentrations of these substrates are insufficient to produce significant inhibition, i.e., to produce significant amounts of the E·NAD·Pyr_K and E·NAD·Lac ternary complexes. This is confirmed by a comparison of (a) the estimates of the Michaelis constants for pyruvate and lactate (Table I) which are similar, respectively, to the pyruvate and lactate levels measured in vivo (cf., Vessel & Pool, 1966), and (b) the inhibitor constants for these compounds (Table II), which are substantially larger than these concentrations. In fact, the physiological unimportance of this type of inhibition seems inescapable, even though estimates of the constants were obtained under conditions that were somewhat different from physiological conditions (i.e., temperature and pH).

The adduct complex has been designated as a metabolic regulator of LDH activity on the basis of (a) preassay incubation studies conducted with physiological concentrations of LDH reactants and products, and (b) the slow generation of increased LDH activity in chicken heart tissue extracts (cf., Everse & Kaplan, 1973, 1975). However, this interpretation does not take into account the likelihood that pyruvate and lactate are at equilibrium or near equilibrium levels in vivo, as was suggested by Rose & Rose (1969). Thus, results obtained with rat hearts perfused with 5 mM glucose (Williamson, 1965), with rat liver after various durations of ischemia (Honorst et al., 1961; Krebs & Veech, 1970), and with human gastrocnemius muscle at rest (Berström et al., 1971) suggest that the ratio of reductant/oxidant of certain NAD-coupled metabolic systems viz., Lac/Pyr, malate/oxalacetate, and glycerol-1-phosphate/dihydroxyacetone phosphate, are at their mass-action equilibrium position. Hence, although the in vitro studies presented here and elsewhere (Everse & Kaplan, 1973, 1975) show that, under physiological conditions and at concentrations of reactants and products in the physiological range, LDH exhibits a capacity for forming *small amounts* of the adduct complex, amounts sufficient to produce a physiologically significant reduction in the effective catalytic efficiency of LDH cannot be observed under such conditions. Moreover only a small fraction of the total LDH can be present in the form of its adduct complex in rapidly prepared tissue extracts from the hearts of resting rats. Since the assay procedure for detecting the LDH·adduct complex has been tested under a variety of conditions and since the assay responds to the presence of the adduct produced artificially under highly abnormal conditions, from a physiological standpoint, both in tissue extracts and in tissue slices, the failure to detect significant amounts of the adduct complex (10% or so of the total enzyme extracted) in hearts of resting rats indicates either that there is little or no adduct complex actually present in the heart under these conditions or that, if present, this form of the enzyme is inefficiently extracted. In the latter case, a decrease in total catalytic capacity of LDH extracts from tissue slices incubated under "forcing conditions" (see Results) should have been observed (because of increased conversion to the adduct complex). Since such was not observed, we conclude that there are no valid in vivo experiments supporting the contention that the LDH·adduct complex is physiologically important in rat heart (with respect to the effective catalytic efficiency of LDH) and that, in conjunction with the data of Williamson on the "physiological levels" of NAD and pyruvate, there is no reason to expect this complex to be important, based on a 30% inhibition of LDH in in vitro experiments under his conditions. Similar conclusions were reported recently by Bailey & Lim (1977) for salmon and beef heart LDH.[4]

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[4] Bailey & Lim (1977) report that NADH in less than stoichiometric amounts induces an immediate and substantial dissociation of the enzyme·adduct complex and interpret this observation as indicating that the adduct complex is unimportant in regulating LDH activity in vivo. We are unable to show an analogous effect with pig heart LDH by using a catalytic efficiency assay to detect the increase in active sites that should accompany such a process. In fact, we find no evidence that NADH limits formation of the adduct complex in any way other than that expected the low of mass action. Hence, we question the experimental basis for the conclusions of Bailey & Lim.

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