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Reaction Velocities of DPN-linked Lactic Dehydrogenase

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Lineweaver-Burk plots of the reaction velocities of crystalline lactic dehydrogenase from ox heart gave straight lines with low concentrations of the reactants. Relationships between Michaelis constants, maximum velocities and the equilibrium constant have been used for selecting a possible mechanism.

The relationships between Michaelis constants, maximum velocities and the equilibrium constant developed for certain mechanisms of enzyme reactions [1–3] have been useful in the determination of the probable mechanism of liver alcohol dehydrogenase [4] and a possible mechanism of yeast alcohol dehydrogenase [5]. In the present study, an attempt has been made to establish a possible reaction mechanism for lactic dehydrogenase from ox heart (LDH) **. The reaction is as follows:

$$\text{pyruvate} + \text{DPNH} + \text{H}^+ \rightleftharpoons \text{lactate} + \text{DPN}^+$$

This enzyme is of particular interest in this connection because the dissociation constant of LDH—DPNH, like that of liver ADH—DPNH, has been determined spectrophotometrically in a single reaction step [6].

MATERIALS AND METHODS

The materials and methods used in this study were the same as those applied in a recent investigation of the essential groups in LDH [7]. L-Lactic acid was procured from Pfahnstil.

RESULTS AND DISCUSSION

Several investigators have determined Michaelis constants and maximum reaction velocities in the DPN-linked LDH system [8–13]. However, the experiments have been carried out under different conditions and with LDH from

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** The following abbreviations are used: LDH: DPN-linked lactic dehydrogenase from ox heart, DPN: diphosphopyridine nucleotide, DPNH: reduced diphosphopyridine nucleotide, ADH: alcohol dehydrogenase.

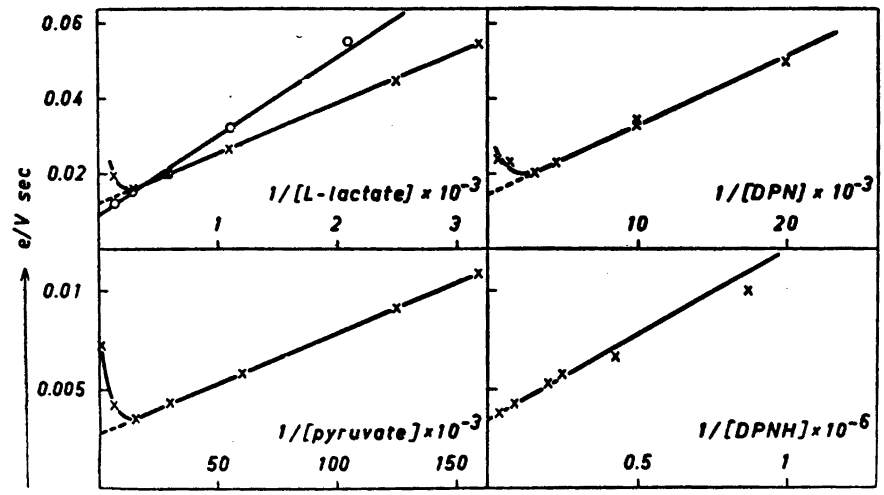

Fig. 1. Reaction velocities of LDH at pH 7.15. Temp. 23° C. Phosphate buffer, μ = 0.1 + M/1 000 versene. O O O DL-*lactate.*

different sources. None of the investigations are extensive enough for calculations of the type described below.

Figs. 1 and 2 illustrate the reaction velocities obtained for LDH at pH 7.15 and 9.0. Straight line Lineweaver-Burk plots were obtained with dilute solutions of the reactants. However, with high concentrations of L-lactate,

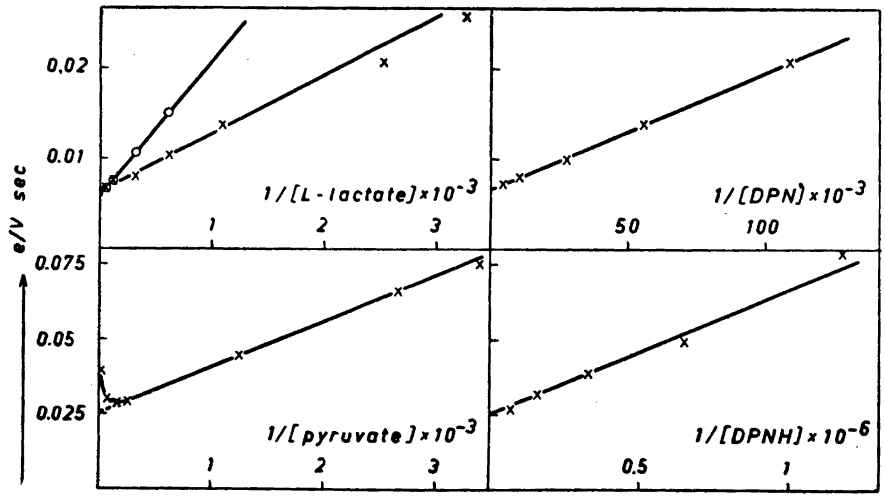

Fig. 2. Reaction velocities of LDH in 0.1 M glycine buffer at pH 9.0. Temp. 23° C. O O O DL-*lactate.*

Table 1. Michaelis constants and maximum reaction velocities for lactic dehydrogenase at 23°. Buffers: phosphate, μ 0.1 + M/1 000 versene, pH 7.15, and 0.1 M glycine, pH 9.0.

Concentration of second reactant		Kinetic Constants		
pH 7.15	pH 9.0	Michaelis constants	pH 7.15	pH 9.0
μM	μM		μM	μM
65	6 600	DPNH	2.5	1.6
12.9	8.9	Pyruvate	18	620
3 300	40 000	DPN	98	22
1 250	416	L-Lactate	1 100	1 100
228	—	»	900	—
1 250	193	DL-Lactate	5 900	5 000
		V_f, sec.$^{-1}$ *	83	177
		V_r sec.$^{-1}$	400	440

* V_f is maximum velocity of lactate oxidation.
V_r is maximum velocity of pyruvate reduction.

pyruvate and DPN at pH 7.15 and with high pyruvate concentration at pH 9.0, the velocities passed through a maximum and then decreased. In all the experiments illustrated in Figs. 1 and 2 the concentration of the second reactant (which was kept constant) was selected so as to be just below the inhibiting concentration of this reactant. At pH 7.15 the inhibiting pyruvate concentration was only three times its Michaelis constant. However, the affinity constants of DPNH and pyruvate at pH 7.3 have been found to be practically independent of the concentration of the second reactant [10].

Table 1 summarizes the maximum velocities (V_f and V_r**) and Michaelis constants calculated from Figs. 1 and 2. In the determination of V_f and V_r the straight lines were extrapolated to infinite concentration and the calculation was made on the basis of infinite concentration of both reactants. All the experimental points in the figures are the average of at least three determinations, which did not vary more than ± 5 % except at the lowest concentrations of DPNH. The velocity determinations with low concentrations of the reactants are less accurate. For that reason, the least square method has not been used in drawing the lines. The kinetic constants in Table 1 are determined within ± 5 % accuracy with the exception of the Michaelis constant of DPNH. This constant is estimated to be within ± 10 %. In Table 2 the data from Table 1 are inserted into formulae representing different reaction mechanisms. Included in the table are the numbers obtained when the errors involved are added up in one direction.

** V_f: maximum velocity of pyruvate reduction.
V_r: maximum velocity of lactate oxidation.

Table 2. Kinetic data from Table 1 inserted into formulae representing different reaction mechanisms.

	I The intramolecular transformation of ternary complex is rate-limiting. General mech. of Alberty[3]	II Special case of I. Affinity of coenzyme for LDH not influenced by substrate, or *vice versa* [3]	III A group in the enzyme is ox/red by substrate or coenzyme with the formation of enzyme-substrate or enzyme-coenzyme intermediates [3]	IV Dissociation of enzyme-coenzyme or enzyme-substrate is rate-limiting [2,3]. Probable mechanism of liver ADH [4]
pH	$K_{eq} = \frac{V_f}{V_r} \cdot A$ ** $\times 10^{12}$	$K_{eq} = \frac{V_f}{V_r} \cdot B$ *** $\times 10^{12}$	$K_{eq} = \left(\frac{V_f}{V_r}\right)^2 \cdot B$ $\times 10^{12}$	$K_{eq} = \left(\frac{V_f}{V_r}\right)^3 \cdot B$ $\times 10^{12}$
7.15	—	6.2 4.1—9.0	1.3 0.76—2.1	0.27 0.14—0.47
9.0		15.6 11—23	5.9 4.0—10.2	2.2 1.4—4.5

$K_{eq} = 4.6 \times 10^{-12}$ over the whole pH range [14].

** $A = \frac{K_m \cdot pyr_{DPNH} \cdot (H^+)}{K_m \cdot lactate_{DPN}}$ For the definition of these constants, see Alberty [3]. A has not been determined, but becomes identical with B when the affinity constants are independent of the concentration of the second reactant.

*** $B = \frac{K_{mDPN} \cdot K_m \text{ pyr} \cdot (H^+)}{K_{mDPN} \cdot K_m \text{ lactate}}$

The mechanism described in column 2, Table 2, agrees rather well with the experimental data. Furthermore, the Michaelis constant of L-lactate at pH 7.15 is nearly independent of the concentration of DPN (see Table 1), and the data of Schwert and Hakala [10] indicate that the affinity constants of DPNH and pyruvate are nearly independent of the concentration of the second reactant at pH 7.3. LDH may therefore follow mechanism 2 at pH 7.15, or nearly so. Mechanisms 3 and 4 are excluded. At pH 9.0, mechanism 2 is excluded and 4 is highly unlikely. Mechanism 3 is improbable for other reasons [7,15]. Experiments by Gibson *et al.*[12] indicate that around this pH the binding of DPN affects the binding of substrate strongly. Mechanism 1 has not been investigated at this pH because of the difficulties in obtaining accurate measurements at very low concentrations of DPNH.

If mechanism 1 or 2 is followed, the rate-limiting reaction in the reduction of pyruvate is the transformation of the ternary complex:

$$\text{LDH—DPNH-pyruvate} + H^+ \rightarrow \text{LDH—DPN-lactate}$$

If the hydrogen ion were taken from the solution, V_r should be proportional to H^+, but V_r was observed to be independent of pH. The enzyme molecule could instead deliver the proton.

If mechanism 2 is followed at pH 7.15, the Michaelis constants represent the dissociation constants of the reactants at this pH. The Michaelis constant of DPNH is 2.5×10^{-6} (23°), as compared to 7×10^{-6} obtained of Chance and Neilands [6] by direct measurements. In view of the uncertainty of the direct measurements [6], the disagreement is not too serious. The 34-fold increase in K_M for pyruvate from pH 7.15 to 9.0 could reflect the discharge of the protein binding site of this substrate, which could be an imidazol group. K_M for lactate is the same at pH 7.15 and 9.0, and may therefore be bound to another site.

The dissociation of Enzyme-DPNH is likely to be the rate-limiting step in the oxidation of alcohol by liver alcohol dehydrogenase [2,4]. LDH oxidizes lactate about 50 times faster than ADH oxidizes alcohol, and the dissociation constant of LDH—DPNH is more than 10 times higher than that of ADH—DPNH. From these observations Chance and Neilands [6] suggested that the dissociation of Enzyme-DPNH was also rate-limiting in the oxidation of substrate in the LDH system, and that a high dissociation constant for Enzyme-DPNH may in general reflect a high rate of oxidation. However, it is evident from Table 2 that mechanism 4, which is probably followed by liver ADH, is not possible for LDH, at least not at pH 7.15. This is supported by the dissociation constant calculated for LDH—DPNH on the assumption that mechanism 4 is followed. It comes out to be $K = \frac{V_f}{V_r} K_{M\ DPNH} = 0.52 \times 10^{-6}$, which is 13—14 times smaller than the measured dissociation constant, and about equal to the dissociation constant of liver ADH found from overall reaction velocities [4].

The fact that DL-lactate has a higher Michaelis constant than has the L-isomer alone suggest that the D-form can also combine on the substrate site without being oxidized, as shown previously [11]. However, the observations that DL gives a higher maximum rate than L alone and that no inhibition is observed with high concentrations of DL show that the D-form also in some other way effects the kinetic constants.

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