Rat Liver Lactate Dehydrogenase

III. KINETICS AND SPECIFICITY*

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Crystalline rat liver lactate dehydrogenase was first prepared in this laboratory in 1953 (1) and characterized in some detail (2). A simple graphical method for the calculation of kinetic parameters for two-substrate enzyme systems (3) and a review of the determination of apparent dissociation constants for such systems have been published (4). Recent studies of metal ion status indicate that this enzyme does not contain stoichiometric quantities of heavy metal ion (5). The present report presents the results of mechanism studies and a survey of the specificity requirements for rat liver lactate dehydrogenase. The following is an outline of studies reported in this paper.

1. Alberty's product inhibition analysis (6) is applied to the kinetics of rat liver lactate dehydrogenase. The possible role of abortive ternary complexes (7, 8) is examined.

2. A series of kinetic experiments for the forward and reverse reactions, at various pH values, is reported. Dalziel's criteria (9) and Alberty's formulation (10) are applied to the data. The role of hydrogen ion in the reaction is investigated.

3. Detailed specificity studies of substrate analogues and of coenzyme analogues are presented.

4. Kinetic studies under maximum velocity conditions to determine the activation energy for the reaction from the lactate-diphosphopyridine nucleotide side are reported.

Similar kinetic analyses have been applied to bovine (11) and rabbit muscle lactate dehydrogenase (8), yeast alcohol dehydrogenase (12, 13), liver alcohol dehydrogenase (13-16), ribitol dehydrogenase (7), and pig heart malic dehydrogenase (12, 13). The role of hydrogen ion in the reaction is investigated.

5. Detailed specificity studies of substrate analogues and of coenzyme analogues are presented.

6. Kinetic studies under maximal velocity conditions to determine the activation energy for the reaction from the lactate-diphosphopyridine nucleotide side are reported.

Improved Hsieh procedure (18), was used in these studies. The enzyme was stored at -10° in 0.1 m ammonium sulfate-0.1 m potassium phosphate-0.001 m β-mercaptoethanol, pH 7.8. Small quantities of the frozen solution were dissolved in ice-cold 0.4 m NaCl-0.018 m NaHCO3 and kept on ice before use. Standard assays of lactate dehydrogenase were carried out in 1-cm cuvettes in the Beckman model DU or Cary model 15 spectrophotometer. The final concentrations in the cuvette were 0.11 m sodium D-lactate, 2.0 × 10^-4 m DPN+, 0.03 m sodium Veronal, pH 8.60, and 0.07 m NaCl-0.003 m NaHCO3. One unit of lactate dehydrogenase is defined as that amount in the cuvette which will effect a change in absorbance of 0.001 per second per ml at 25°. The specific activity is the ratio of enzyme concentration (in units per ml) to the absorbance of the enzyme at 280 μA. Pure lactate dehydrogenase has a specific activity of 8,400, a molecular weight of 126,000, and a molar extinction coefficient of 280 μA of 1.58 × 10^4 m^-1 cm^-1 (1). An enzyme concentration of 1 unit per ml is equivalent to 7.55 × 10^-15 m. Lactate dehydrogenase used in these studies had a specific activity of 6,600 to 5,400. In each experiment, the enzyme concentration was determined by four or five standard assays.

DPN+, TPN+, DPNH, and TPNH were purchased from the Sigma Chemical Company. 3-Acetylpyridine-DPN+, 3-pyridinealdehyde-DPN+, deaminopyridine-DPN+, 3-acetylpyridine-deaminopyridine-DPN+, and 3-pyridinealdehyde-deaminopyridine-DPN+ were purchased from Pabst Laboratories. The thionicotinamide analogue of DPN+ was a gift from Professor Bruce Anderson. DPN+ was assayed with yeast alcohol dehydrogenase (19). It was 96 to 100% pure by this assay. The other analogues were assayed in 1 m KCN, pH 10 (20). All except 3-pyridinealdehyde-DPN+ were 85 to 100% pure. The pyridinealdehyde analogue was only 70% pure.

Dalziel (21, 22) has reported that nucleotide impurities in DPN+ and DPNH lead to erroneous kinetic data. Therefore, the DPN+ for a control experiment was chromatographically purified on diethylaminoethyl cellulose according to Dalziel (21). DPNH was then enzymatically prepared from the purified DPN+ by the method of Rafter and Colowick (23).

L-Lactic acid was obtained from Sigma, D-lactic acid from the J. T. Baker Chemical Company, and calcium D-lactate and calcium L-lactate from the California Corporation for Biochemical Research. Studies reported in this paper indicate that D-lactate has no effect as either a substrate or inhibitor. "A" grade sodium pyruvate was obtained from the California Corporation for Biochemical Research; it was 95% pure by
enzymatic assay. Sodium pyruvate, 90 to 100% pure by enzymatic assay and microanalysis, was prepared by the method of Price and Levintow (24).

Sodium glyoxylate monohydrate, barium dl-α-hydroxybutyrate, sodium α-ketoglutarate, α-ketobutyric acid, α-ketosacrylic acid, and calcium dl-glyceral were purchased from the Sigma Chemical Company. Lithium hydroxypyruvate was obtained from the Nutritional Biochemicals Corporation. α-Hydroxyisobutyric acid, α-hydroxyvaleric acid, α-hydroxysacrylic acid, tartaric (hydroxymalonic) acid, and mesoxalic (ketomalonic) acid came from the California Corporation for Biochemical Research. Thiolactic acid was donated by Evans Chemetics. Lactamide was prepared according to Kleinberg and Audrieth (25). These materials were all characterized by microanalyses or neutralization equivalents or both. Materials of unsatisfactory purity were subjected to repeated recrystallizations until the purity was satisfactory.

Calcium glycerate and calcium lactate were converted to the sodium salts with sodium carbonate. Barium α-hydroxybutyrate was converted to the sodium salt with sodium sulfate. Free hydroxy acids were diluted with distilled water, boiled for 15 minutes, standardized with NaOH, and neutralized with 5 M NaOH. Free keto acids were neutralized in the cold with NaHCO₃ and maintained at pH 6 to 7 until use. Hydroxypyruvate was used as the lithium salt.

**Temperature Control**—The cuvette compartment of a Cary model 15 or Beckman model DU spectrophotometer was maintained at a constant temperature by means of circulating water from a constant temperature bath. All reagents, except enzyme, were kept immersed in the bath. Filled cuvettes were allowed to equilibrate for 5 minutes in the cuvette compartment before initiation of the reaction by addition of coenzyme. Except for studies of the activation energy, all experiments reported in this paper were performed at 25.0 ± 0.5°C.

**Buffers**—Studies of the effect of pH on kinetic parameters were performed in the pH range 7.8 to 10 in a Tris-acetate buffer system in which the ionic strength was kept constant at 0.01 M (26). The specificity studies, product inhibition studies, and activation energy studies were performed in 0.03 M sodium Veronal, standardized to pH 8.60 at the temperature of the kinetic study. Equilibrium constant determinations were performed in the Tris-acetate and Veronal buffer systems.

**Rate Measurements**—The same general techniques as were used in the enzyme assay apply. Reactions were initiated by rapid addition of coenzyme, with mixing, from a syringe. The first measurements were obtained within 10 to 15 seconds of the addition of coenzyme. Linear rates were obtained for a period of 30 to 150 seconds, depending on conditions.

**Determination of K**<sub>eq</sub>**—**Preliminary studies in which mixtures of DPN<sup>+</sup> and lactate were allowed to come to equilibrium in the presence of rat liver lactate dehydrogenase gave a value for K<sub>eq</sub> of 1.7 × 10⁻¹² M. Hakala, Glaid, and Schwert (11) have reported that the presence of pyruvic acid as a trace contaminant of lactic acid leads to low values of the experimentally measured K<sub>eq</sub>. Therefore, we did a number of K<sub>eq</sub> determinations with systems to which known amounts of pyruvate, in large excess of any reasonable pyruvate contamination, had been added. The basis of this method is that the contaminating pyruvate will represent only a small fraction of the total pyruvate present. Since the pyruvate concentration is accurately known, one can determine accurate values for K<sub>eq</sub>.

**RESULTS**

Initial velocity studies of rat liver lactate dehydrogenase are shown in Figs. 1 and 2. The lines calculated by the use of the kinetic constants fit the data reasonably well, thus indicating that the initial reaction velocities follow a rate law of the following form (10).

\[
\frac{V_I}{v_I} = 1 + \frac{K_D}{[D]} + \frac{K_L}{[L]} + \frac{K_{DL}}{[D][L]}
\]

V<sub>I</sub> is the maximum velocity for the “forward” reaction, K<sub>L</sub> is the Michaelis constant for lactate, K<sub>D</sub> is the Michaelis constant for DPN<sup>+</sup>, and K<sub>DL</sub> is a complex constant defined by Alberty (10). The reverse reaction is represented by an analogous equation in which V<sub>r</sub> is the maximum velocity, DH represents DPNH, and P represents pyruvate. The values for these constants appear in Table I.

1 In this paper, all velocities are expressed as Δ[product] (m) / Δtime (seconds) × [lactate dehydrogenase](m).
TABLE I

<table>
<thead>
<tr>
<th>Lactate-DPN+ system</th>
<th>Pyruvate-DPNH system</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>$K_D$</td>
</tr>
<tr>
<td>$10^4$</td>
<td>$10^4$</td>
</tr>
<tr>
<td>7.8</td>
<td>1.1</td>
</tr>
<tr>
<td>8.6</td>
<td>1.6</td>
</tr>
<tr>
<td>8.7</td>
<td>2.6</td>
</tr>
<tr>
<td>9.5</td>
<td>3.2</td>
</tr>
</tbody>
</table>

*Expressed as moles of product per second per mole of lactate dehydrogenase.

Alberty indicates that the following equation best describes the inhibition (6).

$$V_I = \frac{1}{v_0} = \frac{K_D}{[D]} + \frac{K_L}{[L]} \left( 1 + \frac{k_{+a} [P]}{k_3 + k_4} \right)$$

That is, comparison of these data with Figs. 1 and 2 indicates that the apparent maximum velocity is changed in the presence of pyruvate and that the apparent values of $K_D$, $K_L$, and $K_{DL}$ are also altered. Only the ordered sequence mechanism with catalytically significant ternary complexes leads to this type of product inhibition.

Alberty did not consider the possible formation of abortive ternary complexes in his derivations of the product inhibition rate equations. Fromm and Nelson (7) have extended Alberty’s equations to include possible abortive complex formation. From Alberty’s original equations, it is predicted that pyruvate would give linear competitive inhibition when lactate is the variable of DPNH. The apparent value of $K_L$ remains unchanged whereas the apparent values of $K_D$ and $K_{DL}$ are altered by the factor $(1 + (K_P/K_{DLP}) [DH])$. The value of $K_{DLP}/K_P$ (the dissociation constant for the reaction $E - DPNH = E + DPNH$), calculated from the product inhibition data, is $1.3 \times 10^{-5}$ M, a value which agrees well with the constant obtained from simple pyruvate-DPNH kinetics at pH 8.6 (Table I).

Alberty has shown that both the ordered sequence ternary complex mechanism and the Theorell-Chance mechanism lead to this equation when DPNH serves as the product inhibitor (6). The random sequence mechanism leads to an equation of a different form. Thus these data allow one to eliminate tentatively the random sequence mechanism.

In Figs. 5 and 6 are presented the results of similar studies performed in the presence of pyruvate as product inhibitor. Comparison of these data with the original equations derived by Alberty (6) is possible to distinguish among these three mechanisms by the analysis of product inhibition data.

Figs. 3 and 4 indicate the results of product inhibition studies performed from the lactate-DPN+ side in the presence of DPNH. Comparison of these figures with Figs. 1 and 2 indicates that the inhibition data fit an equation of the following form (6).

$$V_I = \frac{1}{v_0} = \frac{K_D}{[D]} + \frac{K_L}{[L]} \left( 1 + \frac{k_{+a} [P]}{k_3 + k_4} \right)$$

The apparent maximum velocity is unaffected by the presence...
substrate for systems following the Theorell-Chance or random sequence mechanisms. Pyruvate would give linear noncompetitive inhibition when lactate is the varied substrate for systems which follow an ordered sequence ternary complex mechanism. However, Fromm et al. (7, 8) have shown that abortive complex formation leads to nonlinear inhibition; i.e., plots of the slopes of the double reciprocal plots against inhibitor concentration are nonlinear.

When the DPN⁺ concentration was held constant at 10⁻³ M, pyruvate concentrations between 3 × 10⁻⁴ M and 10⁻³ M were found to give linear noncompetitive inhibition with respect to lactate. Fig. 7 contains data obtained with pyruvate as the variable substrate and DPN⁺ as the product inhibitor. The nonlinearity of the double reciprocal plot at pyruvate concentrations greater than 3 × 10⁻³ M may be interpreted as evidence for abortive complex formation at excessive pyruvate concentrations (8).

Similar experiments to detect a lactate dehydrogenase-DPNH-lactate complex indicated that no significant amounts of abortive complex were formed. When the concentration of DPNH was held constant at 5.0 × 10⁻⁵ M, lactate concentrations of 0.05 M and 0.10 M gave simple linear noncompetitive inhibition with respect to pyruvate.

Table I and Fig. 8 summarize the results of detailed kinetic studies completed from both sides of the reaction at various pH values. It is of value to apply Dalziel’s criteria (9) to the data to see whether or not these experiments confirm the conclusions of the product inhibition studies. The fact that the ratios $K_{p}K_{DPP}V_{p}/K_{DPP}V_{f}$ and $K_{DHP}K_{p}V_{f}/K_{DHP}V_{p}$ are less than unity signifies that the dissociation of coenzymes is not rate-limiting (9).

Dalziel has reported that the presence of nucleotide impurities in DPN⁺ and DPNH leads to anomalous values of the above ratios at lower pH values (21, 22). Unreported studies performed at pH 6 and 7 with rat liver lactate dehydrogenase re-
resulted in similar anomalous values. Since most of the experiments reported in this paper were performed at pH 8.6, it was decided that a control experiment with purified DPNH and purified DPN' should be run at this pH. The results of this control experiment substantiated the previous results obtained at pH 8.6 with untreated coenzymes.

Analysis of the Haldane relationships (equations relating the kinetic constants to the equilibrium constant) was of some help in defining the mechanism of the action of this enzyme. The following relationship (10) is valid for all of the three mechanisms considered in this discussion.

\[ K_{eq} = \frac{V_D K_{DP}}{V_K D_L} [H^+] \]

A value of \(3.0 \times 10^{-12} \text{M}\) was calculated from this equation, and the data were obtained at pH 8.6.

For the Theorell-Chance mechanism, an additional relationship is applicable (10).

\[ K_{eq} = \frac{V_D K_{DP} \frac{K_P}{K_D}}{V_K D_L} [H^+] \]

Application of this equation to the data obtained at pH 8.6 leads to a value of \(1.7 \times 10^{-12} \text{M}\) for \(K_{eq}\).

The equilibrium constant determined experimentally for mixtures of lactate, DPN', pyruvate, and DPNH in the presence of rat liver lactate dehydrogenase is 2.7 \(\pm\) 0.3 \(\times 10^{-12} \text{M}\) (Table II). This value agrees well with the equilibrium constant determined by Hakala, Glaid, and Schwert (11) for the beef heart enzyme.

The maximum velocity from both sides of the reaction increases with pH over the very narrow pH range of 7.8 to 9.5. \(K_D, K_L, K_{DL}, \text{and } K_{DR}\) are slightly pH-dependent in this pH range. In contrast, \(K_P\) and \(K_{DP}\) are markedly pH-dependent. Plots of \(pK_P\) and \(pK_{DP}\) (\(pK_P = -\log K_P\)) against pH lead to references with slopes approximately equal to \(-1\) (Fig. 8). This value for the slopes suggests that 1 hydrogen ion is involved in the equilibria described by these constants (27). Winer and Schwert have reported similar experiments for the beef heart enzyme (28).

Experiments at 11, 18, 25, 33, and 40°C were performed from the lactate-DPN+ side at pH 8.6 to determine the activation energy for the reaction catalyzed by rat liver lactate dehydrogenase. Fig. 9 indicates the results. The apparent activation energy of the rate-limiting step (or steps) from the lactate-DPN+ side, pH 8.6, is 13 \(\pm\) 2 kcal. \(K_D, K_L, \text{and } K_{DL}\) did not vary significantly over this temperature range. Hakala et al. have reported effects of temperature on the kinetic constants for beef heart lactate dehydrogenase (11). Calculations made from their data indicate that the beef heart enzyme system has a similar apparent activation energy.

**SPECIFICITY STUDIES**

To obtain information regarding structural requirements of the active site for the \(\alpha\)-keto acid or \(\alpha\)-hydroxy acid substrate, several substrate analogues were tested for activity as substrates or inhibitors. Table III lists some of the kinetic parameters of analogues having substrate activity.

4 Preliminary studies of \(\alpha\)-hydroxybutyrate were performed by Jerome M. Gess as part of his undergraduate research (1955).

**TABLE II**

<table>
<thead>
<tr>
<th>pH</th>
<th>Initial DPN+</th>
<th>Initial l-lactate</th>
<th>Initial added pyruvate</th>
<th>(\Delta\text{Mno})</th>
<th>(K_{eq})</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.06*</td>
<td>1.00</td>
<td>8.0</td>
<td>0.20</td>
<td>0.461</td>
<td>2.7</td>
</tr>
<tr>
<td>8.09</td>
<td>2.00</td>
<td>7.5</td>
<td>2.0</td>
<td>0.127</td>
<td>2.3</td>
</tr>
<tr>
<td>8.20</td>
<td>2.00</td>
<td>7.0</td>
<td>1.0</td>
<td>0.466</td>
<td>2.7</td>
</tr>
<tr>
<td>8.29</td>
<td>1.00</td>
<td>8.0</td>
<td>1.5</td>
<td>0.232</td>
<td>2.5</td>
</tr>
<tr>
<td>8.44</td>
<td>2.00</td>
<td>5.0</td>
<td>2.0</td>
<td>0.282</td>
<td>3.5</td>
</tr>
<tr>
<td>8.47</td>
<td>1.25</td>
<td>3.0</td>
<td>0.4</td>
<td>0.320</td>
<td>2.9</td>
</tr>
<tr>
<td>8.76*</td>
<td>2.00</td>
<td>8.0</td>
<td>2.0</td>
<td>0.803</td>
<td>3.2</td>
</tr>
<tr>
<td>9.10*</td>
<td>1.00</td>
<td>5.0</td>
<td>4.0</td>
<td>0.300</td>
<td>2.8</td>
</tr>
</tbody>
</table>

* These values were determined in 0.1 M Tris-acetate; the remaining values were measured in 0.03 M sodium Veronal.

Fig. 9. Arrhenius plot for rat liver lactate dehydrogenase from the lactate-DPN+ side, pH 8.6 (\(T^\circ\) is in degrees Kelvin). The slope of this curve leads to a value of 12 kcal for \(E^+\).

\(V_P\) and \(V_v\) depend on the hydroxy acid or keto acid used. This dependence indicates that the hydroxy acid or keto acid participates in the rate-limiting step (or steps) of the reaction sequence. Substrate analogues shown to have no significant activity as substrates include l-lactate, glycolate, \(\alpha\)-hydroxyisocaproate, \(\alpha\)-\(\alpha\)-hydroxyisovalerate, \(\alpha\)-\(\alpha\)-hydroxyvalerate, hydroxymalonic, L-malate, \(\alpha\)-ketoisovalerate, \(\alpha\)-ketoisocaproate, ketomalonate, \(\alpha\)-thiolactate, \(\alpha\)-lactamate, \(\alpha\)-trifluorolactate, and \(\alpha\)-hydroxyisobutyrate. Thiolactate is a competitive inhibitor with respect to lactate and a noncompetitive inhibitor with respect to pyruvate. Lactamide, L-lactate, trifluorolactate, and \(\alpha\)-hydroxyisobutyrate are not inhibitors.

Kinetic parameters of various coenzyme analogues are summarized in Table IV. 3-Acetylpyridine-DPN+ and 3-acetylpyridine-deamino-DPN+ exhibit remarkable properties. Lactate gives very pronounced substrate inhibition, which is not observed with DPN+ (Fig. 1), with each of these analogues (Fig. 10). Kaplan et al. have analogous data for lactate dehydrogenases from lobster heart (29), beef heart (29), and chicken heart (30). Preliminary studies of coenzyme analogue specificity with rat liver lactate dehydrogenase were made by Karne (31).

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TABLE III

Kinetic parameters obtained for \(\alpha\)-hydroxy and \(\alpha\)-keto acids

The tests were done at pH 8.6, 25°. The coenzyme was DPN\(^+\) or DPNH.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(V_1^*)</th>
<th>(K_L)</th>
<th>(K_D)</th>
<th>(K_{DL})</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Lactate</td>
<td>7.0 \times 10^2</td>
<td>2.3 \times 10^2</td>
<td>1.5 \times 10^2</td>
<td>1.1 \times 10^2</td>
</tr>
<tr>
<td>L-(\alpha)-Hydroxybutyrate</td>
<td>2.6</td>
<td>19</td>
<td>6.2</td>
<td>16</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(V_1^*)</th>
<th>(K_P)</th>
<th>(K_{DH})</th>
<th>(K_{DH})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>1.5 \times 10^6</td>
<td>1.6</td>
<td>1.4</td>
<td>2.8</td>
</tr>
<tr>
<td>Hydroxypyruvate</td>
<td>1.4</td>
<td>6.3</td>
<td>1.9</td>
<td>14</td>
</tr>
<tr>
<td>(\alpha)-Ketobutyrate</td>
<td>0.21</td>
<td>13</td>
<td>1.4</td>
<td>29</td>
</tr>
<tr>
<td>Glycerate</td>
<td>0.13</td>
<td>20</td>
<td>1.3</td>
<td>47</td>
</tr>
<tr>
<td>(\alpha)-Ketovalerate</td>
<td>0.01</td>
<td>33</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Expressed as moles of DPNH per second per mole of lactate dehydrogenase.

TABLE IV

Rat liver lactate dehydrogenase coenzyme analogue studies, pH 8.6, 25°

<table>
<thead>
<tr>
<th>Coenzyme analogue</th>
<th>(V_1^*)</th>
<th>(K_D)</th>
<th>(K_D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPN(^+)</td>
<td>7.0 \times 10^2</td>
<td>1.5 \times 10^2</td>
<td>2.3 \times 10^2</td>
</tr>
<tr>
<td>Deamino-DPN(^+)</td>
<td>10.0 \times 10^4</td>
<td>8.3 \times 10^4</td>
<td>1.7 \times 10^4</td>
</tr>
<tr>
<td>Thionicotinamide analogue of DPN(^+)</td>
<td>3.5 \times 10^6</td>
<td>4.1 \times 10^6</td>
<td>1.8 \times 10^6</td>
</tr>
<tr>
<td>3-Pyridinealdehyde-DPN(^+)</td>
<td>1.7</td>
<td>5.0</td>
<td>3.5</td>
</tr>
<tr>
<td>3-Pyridinealdehyde-deamino-DPN(^+)</td>
<td>0.8 \times 10^2</td>
<td>5.0 \times 10^2</td>
<td>7.1 \times 10^2</td>
</tr>
<tr>
<td>3-Acetylpyridine-DPN(^+)</td>
<td>0.16 \times 10^6</td>
<td>0.2 \times 10^6</td>
<td>0.3 \times 10^6</td>
</tr>
</tbody>
</table>

* Expressed as moles of product per second per mole of lactate dehydrogenase.

DISCUSSION

Product inhibition analysis indicates that an ordered sequence mechanism with one or more catalytically significant ternary complexes best describes the action of rat liver lactate dehydrogenase. This conclusion is supported by the application of Dalziel's criteria to the kinetic data. Takenaka and Schwert (32) have concluded from direct measurements of substrate binding that this mechanism also describes the behavior of the beef heart enzyme. Zewe and Fromm (8), however, have concluded from their product inhibition studies that the Theorell-Chance mechanism is consistent with the kinetics of rabbit muscle lactate dehydrogenase. By the use of product inhibition analysis, the following enzymes have also been shown to obey ordered sequence mechanisms: yeast alcohol dehydrogenase (13), liver alcohol dehydrogenase (13, 16), ribitol dehydrogenase (7), and pig heart malic dehydrogenase (17). Thus it appears that an ordered sequence mechanism is commonly followed by a number of dehydrogenases.

Analysis of the product inhibition data indicates that an abortive rat liver lactate dehydrogenase-DPN\(^+\)-pyruvate complex is formed at high pyruvate concentrations. An abortive lactate dehydrogenase-DPNH-lactate complex was not detected. Fromm et al. have obtained kinetic (5), spectrophotometric (33), and spectrophotofluorometric (34) evidence for both types of abortive complexes of rabbit muscle lactate dehydrogenase.

Additional discussions of product inhibition have been written by Bloomfield and Alberty (35), Cleland (36, 37), and Walter and Frieden (38).

The variations of the kinetic constants with pH and with temperature are similar in many respects to the results of earlier experiments reported by Schwert et al. (11, 28) for beef heart lactate dehydrogenase. It is concluded that 1 hydrogen ion is somehow involved in the equilibria described by \(K_P\) and \(K_{DH}\). The exact significance of these studies is uncertain until more is learned about the mechanism.

It is seen that rat liver lactate dehydrogenase has certain rigid requirements for its substrates. Apparently the anionic carboxyl group of lactate is involved in binding since lactamide has no effect on reaction rates. \(\alpha\)-Hydroxyisobutyrate, in which C-2 is blocked by a methyl group, is not an inhibitor. The enzyme is specific for \(\alpha\)-lactate. \(\alpha\)-Lactate is neither substrate nor inhibitor. Thus \(\alpha\)-lactate gives results identical with those obtained with \(\alpha\)-lactate. This same stereospecificity was assumed to apply to glycerate and \(\alpha\)-hydroxybutyrate, since racemic mixtures of these compounds were studied. The total chain length of the substrate is restricted from a minimum of 2 to a maximum of 4 or 5 carbon atoms. Data obtained with \(\alpha\)-ketovalerate are of dubious significance since trace contamination in the enzyme or substrate could lead to these results. No branching of the substrate is allowed. Glycerate gives reaction rates which are less than 10% of the velocities observed with lactate. The failure of glycolate to give rates comparable to those obtained with glyoxylate is a result of the very small \(K_{eq}\) \((2 \times 10^{-18} M)\) (27). Mixtures of glycolate and acetylpyridine-DPN\(^+\), which has a more positive oxidation-reduction potential...
than DPN+ (39), have been found to give significant reaction rates in the presence of rat liver lactate dehydrogenase.

Comparison of these results with experiments performed by Meister (40) for beef heart, hog heart, and pigeon breast muscle lactate dehydrogenase suggests that the lactate enzyme is of more limited specificity than are certain other types of lactate dehydrogenase.

It is of interest to note that the kinetically determined dissociation constants for rat liver lactate dehydrogenase-DPN+ and lactate dehydrogenase-DPNH interaction do not agree well with the dissociation constants determined by fluorescence and spectrophotometric titrations. This problem has been discussed by Vestling (4).

SUMMARY

1. Product inhibition analyses and Dalziel's criteria have been applied to the kinetics of the catalysis by rat liver lactate dehydrogenase. The data indicate that the mechanism involves a mandatory sequence of addition of substrates to the enzyme with the formation of one or more ternary complexes. The dissociation of coenzyme product appears not to be rate-limiting. Kinetic data show that hydrogen ion is involved in the steps in which pyruvate interacts with the enzyme-coenzyme complexes. 3. Preliminary experiments indicate an apparent activation energy of 13 kcal from the lactate-DPN+ side at pH 8.6. 4. Specificity studies indicate that rat liver lactate dehydrogenase has a specificity restricted to unbranched substrates whose chain length is 2 to 4 or 5 carbon atoms. The enzyme is stereospecific for L-lactate. These experiments indicate that the α-hydroxy and α-keto acids participate in the rate-limiting step (or steps) of the reaction sequence.

5. The enzyme is specific for DPN+ and DPNH. TPN+ and TPNH have no activity as substrate or inhibitors. 3-Acetylpyridine-DPN+ is unique in several of its properties. The Michaelis constant for the acetylpyridine analogue is about one order of magnitude smaller than that for DPN+. L-Lactate gives appreciable substrate inhibition when the acetylpyridine derivative serves as coenzyme.

REFERENCES
