Equilibrium Reaction Rates and the Mechanisms of Bovine Heart and Rabbit Muscle Lactate Dehydrogenases*

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Almost all enzyme kinetic studies have been based on measurement of net chemical reaction. Isotopic tracer techniques make possible other types of kinetic measurements, including the rate of interconversion of reactants or interchange of atoms between substrates at equilibrium.1 Only very limited studies have been reported of such equilibrium rate measurements with known concentrations of all reactants (1-5). Studies by Boyer, Mills, and Fromm (4) and by Graves and Boyer (5) of the glutamine synthetase reaction at equilibrium have shown marked inequalities of exchange rates, interpretable in terms of substrate dissociation steps as rate-limiting, and have revealed oxygen transfer patterns which give insight into spatial selectivity among oxygen atoms of bound phosphoryl and carboxyl groups.

The studies with glutamine synthetase (4) served as the stimulus for the development by Boyer of theoretical relationships governing equilibrium rates in enzyme reaction systems (6). Alberty et al. (7) and Cleland (8) have subsequently presented other methods for development of such relationships for limiting cases with ordered addition of reactants, and Morales, Horovitz, and Botts (9) have considered transient states affecting isotope distribution when the concentration of enzyme-bound intermediates is appreciable. More recently, Boyer and Silverstein (10) have extended theoretical treatments to include random or nonlinear as well as linear routes to the formation of two interconvertible ternary complexes in two-substrate, two-product systems. Fortunately, some complete equations are inherently simpler than initial rate equations because the concentrations of all intermediates in equilibrium reaction rate studies are governed by the respective equilibrium constants.

The theoretical considerations suggest that equilibrium rate measurements should provide a powerful tool for detection of compulsory substrate binding orders and of rate-limiting ternary complex interconversion. Excellent net reaction rate and other measurements with the bovine heart (11-13) and skeletal muscle lactate dehydrogenase (14, 15) have given strong evidence for a compulsory or ordered sequence of substrate binding. The lactate dehydrogenase system was therefore chosen for the experimental studies on equilibrium reaction rates presented herein. The results support a compulsory order of substrate binding, and establish the usefulness of equilibrium rate measurements for elucidation of substrate binding patterns. Other important inferences also arise from the data.

EXPERIMENTAL PROCEDURE

Substrates, Enzymes, and Special Chemicals—Zinc 1-lactate was obtained from Pfannstiel Laboratories, Waukegan, Illinois; sodium pyruvate and nicotinamide from Nutritional Biochemicals Corporation, Cleveland; NAD and disodium NADH from Pabst Laboratories, Milwaukee, and Sigma Chemical Company, St. Louis; DEAE-cellulose from Sigma Chemical Company; and Triton X-100 (a nonionic detergent) from Rohm and Haas, Philadelphia.

Sodium pyruvate-1-14C was obtained from California Corporation for Biochemical Research, Los Angeles (1.5 mc per mmole) and Nuclear-Chicago (4.5 mc per mmole); lactic acid-1-14C (3 mc per mmole) and nicotinamide-(carboxyl)-14C (6.5 mc per mmole) from Calbiochem; and sodium pyruvate-2-14C (3.6 mc per mmole) and sodium lactate-1-14C (5 mc per mmole) from Nichem, Bethesda, Maryland.

Crystalline bovine heart lactate dehydrogenase was obtained from Worthington Biochemical Corporation, Freehold, New Jersey; crystalline rabbit muscle lactate dehydrogenase from Boehringer und Sohne, Mannheim, Germany; and a lyophilized preparation of yeast alcohol dehydrogenase from Worthington Biochemical Corporation. Lactate dehydrogenase solutions were prepared by centrifugation of small volumes of the crystalline suspensions, careful removal of the supernatant solution by absorbent tissue, and dissolution in appropriate buffer.

Trace metal contaminants from distilled water used for reagent preparation were removed by passage through an Amberlite MB-3 (Rohm and Haas) mixed bed resin. A commercial sample of NAD-3-14C (Schwarz BioResearch, Mount Vernon, New York) was found to be grossly impure by enzymic tests. Hence NAD-14C was prepared from nicotinamide-14C by means of the exchange reaction between NAD and nicotinamide cata-
been added. When NADH-1% (in 0.1 M NaNO₃) was used, pyruvate-1⁴C solution was similarly made in 0.1 M NaNO₃. Solutions made fresh in water to which 1 drop of benzene had been added were mixed. After 30 minutes at room temperature, the solutions were made with a few grains immediately prior to use. Pyruvate-1⁴C with a purity of about 98% was routinely prepared immediately prior to use by enzymic conversion of NAD-1⁴C as follows: 1.6 ml of 10 mm glycine (sodium salt), pH 10.5, 0.15 ml of ethanol, 0.05 ml of yeast alcohol dehydrogenase (2 mg per ml in 10 mm phosphate buffer, pH 7.7), and a few grains of NAD-1⁴C were mixed. After 30 minutes at room temperature, 0.03 ml of 10 mm AgNO₃ was added to inactivate the enzyme, and the solution was transferred to a DEAE-cellulose-NaNO₃ column, 0.5 x 1 cm. The column was washed with 20 ml of 1 mm NaNO₃ and NADH-1⁴C then was eluted with 1.5 to 2.0 ml of 0.1 mm NaNO₃. NADH-1⁴C counts enzymically convertible to NAD-1⁴C were determined as follows: 0.2 ml of 0.3 m acetaldehyde, 0.3 ml of 0.1 m imidazole (pH 6.9), 0.05 mg of yeast alcohol dehydrogenase, and 0.1 ml of NADH-1⁴C solution were incubated for 15 to 30 minutes, after which 0.1 ml of 10 mm AgNO₃ was added. The NAD and NADH fractions were separated on DEAE-cellulose-HCO₃, and the NADH fraction was counted. The radioactivity converted to NADH-1⁴C was obtained by comparison of counts in the NADH fraction of a similar mixture to which AgNO₃ was added prior to isotope. The radiopurity of pyruvate-1⁴C was measured by quantitative conversion to lactate and 2,4-dinitrophenylhydrazine precipitation as described elsewhere (17). Special precaution was taken to prevent an unsuspected, marked decomposition of pyruvate-1⁴C in aqueous solution (17).

Sodium pyruvate-1⁴C was stored as a powder in a vacuum desiccator at -15°, and solutions made fresh in water to which 1 drop of benzene had been added. When NADH-1⁴C (in 0.1 mm NaNO₃) was used, pyruvate-1⁴C solution was similarly made in 0.1 mm NaNO₃.

The radiopurity of lactate-1⁴C was assessed by precipitation of pyruvate-2,4-dinitrophenylhydrazine (17) with and without prior quantitative conversion of lactate-1⁴C to pyruvate-1⁴C with NAD and lactate dehydrogenase at pH 10.5 (16). Substrate concentrations were checked by enzymic assay based on the absorption of NADH at 340 μM (ε = 6.22 x 10⁴) (18). No pyruvate was found in the lactate preparations used when checked with NADH and lactate dehydrogenase. Pyruvate and NADH were quantitatively converted to their respective products by the lactate dehydrogenase reaction at pH 7. NAD was quantitatively reduced by yeast alcohol dehydrogenase at pH 10 under appropriate conditions. Sodium l-lactate of high molarity (up to 6.81 M) was prepared from zinc l-lactate by forming the acid with a Dowex 50-H⁺ slurry, followed by treatment with charcoal to remove a slight yellow color. Residual Zn⁺⁺ was removed by pouring the slurry atop a Dowex 50-H⁺ column. The column eluate was concentrated under partial vacuum in a rotating flask at 50°. The colorless, viscous liquid was neutralized with NaOH of high known molarity to give a neutral lactate solution of known molarity.

Separations and Radioactivity Determinations—Radioactivity was measured with gas flow counters by conventional techniques. ¹⁴C solutions were dried on glass disks which held up to 1 ml of sample, or in aluminum planchets containing lens paper cut to fit the planchet. Suitable self-absorption corrections were made with all samples.

Simple procedures for separation of NAD and NADH in small quantities were essential. NAD and NADH in lactate dehydrogenase reaction mixtures were separated on DEAE-cellulose-HCO₃ (Fig. 1) or Dowex 1-HCO₃ columns as described more fully elsewhere (16, 17). The latter columns were used when the high ionic strength of the solutions precluded use of DEAE-cellulose. With conditions as used for the data of Fig. 1, all the NADH was obtained in the first 5-ml aliquot after change of the eluent and prior complete elution of NAD in two 5-ml aliquots.

Pyruvate was separated from lactate by precipitation as the 2,4-dinitrophenylhydrazine as described elsewhere (17).

AgNO₃ was used to stop the enzymic reaction (19). The adequacy of this procedure was shown by the apparently instantaneous inhibition of lactate dehydrogenase by silver ions in initial rate measurements, and by establishing in exchange experiments that the counts obtained by termination of the reaction with strong acid (final acid concentration, 1.3 N HCl) and with AgNO₃ (10⁻⁴ M) were identical within experimental error. The use of AgNO₃ facilitated separation of NAD and NADH by keeping ionic strength low for application to DEAE-cellulose columns, and by avoiding acid decomposition and resultant loss of the identifying absorbance of NADH at 340 μM encountered in other separations at acid pH (16).

Bovine heart lactate dehydrogenase and NAD were found to be sufficiently stable at both 25° and 15° at pH 10.5, where Kₑq is only 10⁻⁴, for accurate measurements during the short time period of the equilibrium experiments. Equilibrium rate measurements were usually done at pH 9.7 and 7.9, where stability would be expected to be still greater.

Design of Experiments on Effect of Substrate Concentration on Equilibrium Reaction Rates—Convenient ratios of the two sets of substrates (NADH: NAD, pyruvate to lactate) were chosen to satisfy the apparent equilibrium constant, Kₑq. The Kₑq values noted in our experiments are in reasonable agreement with those reported recently (20). At pH 7.9 the NAD ⇔
NADH reaction was measured from the NADH side with NADH-14C. Accurate measurement could therefore be made with low ratios of both pyruvate to lactate and NAD: NADH, which were necessary to satisfy the K_m at that pH. At pH 9.7 the NADH:NAD ratio was held close to unity because isotopic measurement was made with NAD-14C; the pyruvate to lactate ratio was adjusted to satisfy K_m. In all these experiments the NADH and NAD concentrations were held constant at or above reported K_m values, while the pyruvate and lactate concentrations were held at a constant ratio but varied in absolute amount. The lowest value chosen for pyruvate and lactate concentration was below reported K_m values for either substrate, while the highest was more than 10 times the K_m value for either substrate.

Reaction mixtures containing all components, except enzyme and isotope, were prepared in sufficient volume so that duplicate pipettings of 0.4 to 0.5 ml for both pyruvate = lactate and NAD = NADH measurements could be made. The buffer was 167 mM glycine (sodium salt), pH 9.7, or 145 mM Tris-NO_3, pH 7.9. Amounts of enzyme and of radioactivity appropriate for a particular experiment were determined by preliminary trials. Initial rate measurements were also made with appropriate substrates. The A_{440} for each reaction mixture was taken before enzyme addition and then afterward until the reading was stable. The equilibrium concentrations used for reaction rate calculations were corrected on the basis of any A_{440} change noted. For the experiments done at pH 9.7, where higher enzynme levels were needed to saturate the enzyme (13, 20), the A_{440} was read with the aid of a precision silica cell spacer. To the quadruplicate aliquot of each reaction mixture in a thermostated water bath at 25°, the respective 14C-labeled substrate (50 μl) in duplicate was added at scheduled intervals, and the reaction stopped by the addition of 0.1 ml of 10 mM AgNO_3 at a time interval sufficient usually for 20 to 80% of radioactivity to appear in the product. For control “zero time” samples, AgNO_3 was added to duplicate reaction mixtures containing the lowest concentration of substrates for a particular series, prior to addition of the 14C compound.

Reaction rates were calculated from the equilibrium concentration of substrate, reaction time, and fraction of isotope incorporated into product (6). Appropriate corrections were made for the decrease in observed exchange rate as isotopic equilibrium was approached (6).

The somewhat laborious nature of the equilibrium rate measurements made the use of a wide variety of substrate concentrations and reaction conditions impractical. Experiments performed were thus chosen carefully as those which might give the most pertinent information about the catalytic reaction.

**RESULTS**

**Adequacy of Rate Measurements and Reaction Conditions**—
The equilibrium reaction rates (pyruvate = lactate, NAD = NADH) were observed to be proportional to the time of reaction and the enzyme concentration (16). This gave confidence that methodology was satisfactory, and also permitted a choice of suitable different time periods for measurement of different exchange rates. Use of different time periods was particularly desirable when marked disparity of rates was encountered.

Starting with either pyruvate-14C or lactate-14C as radiotracer in the lactate dehydrogenase system at equilibrium, a similar value for the pyruvate = lactate equilibrium reaction rate was found as noted in Table I. Such checks help to establish the reliability of procedures used.

To assess the possible importance of ionic strength variation, or the presence of nonsubstrate anions, the effect of increasing NaCl concentration was measured. The pyruvate-lactate equilibrium reaction rate was found to be inversely proportional to NaCl concentration; a 30% inhibition resulted with 4 M NaCl (16). Inhibition by NaCl was similarly found in the initial rate of NADH oxidation, and NaCl gave 36% inhibition. Competitive inhibition of lactate dehydrogenase has been noted with many anions (20). On the basis of these results and our findings, we had to forgo an otherwise desirable maintenance of constant ionic strength by addition of other salts in place of NaCl.

The initial rate from the pyruvate-NADH side (1.51 x 10^{-6} M min^{-1}; 25°, 7.02 mM sodium pyruvate, 191 μM NADH, and 688 μM lactate) was 20 times that of pyruvate-1-K or lactate-14C.

<table>
<thead>
<tr>
<th>Reaction measured</th>
<th>Isotopic compound used</th>
<th>Equilibrium reaction rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate = lactate</td>
<td>Pyruvate-14C</td>
<td>2.75</td>
</tr>
<tr>
<td>Lactate = pyruvate</td>
<td>Lactate-14C</td>
<td>2.05</td>
</tr>
</tbody>
</table>

The reaction mixture, in a 0.5-ml final volume at 25°, contained 688 μM NAD, 15.9 μM NADH, 688 μM pyruvate, 6.7 mM glycine (sodium salt), pH 9.8, and 5 μl of bovine lactate dehydrogenase solution. Samples were incubated for 10 minutes after addition of trace quantities, equivalent to 2800 c.p.m., of lactate-1-14C or, in a separate experiment, 500 c.p.m. of pyruvate-1-14C; the reaction was stopped by addition of 2 ml of 0.5% 2,4-dinitrophenylhydrazine in 4 N HCl, and separations were made as described in “Experimental Procedure.”

**Effect of Substrate Concentration on Equilibrium Reaction Rates with Bovine Heart Lactate Dehydrogenase**—The equilibrium reaction rates at pH 9.7 (0.167 M glycine, sodium salt) for a representative experiment are plotted in Fig. 2. The marked disparity between the pyruvate = lactate and NAD = NADH rates is readily apparent. Of particular interest is the pronounced reduction in the NAD = NADH rate observed with increasing pyruvate and lactate concentrations and constant NAD and NADH concentrations. In these experiments, lactate levels up to 3.3 mM were used to reach concentrations in excess of 10 times the reported K_m values to try to attain near complete saturation with substrate.

The initial rate from the pyruvate-NADH side (1.51 x 10^{-3} M min^{-1}; 25°, 7.02 mM sodium pyruvate, 191 μM NADH, and 140 mM glycine (sodium salt), pH 9.7) was 20 times that of maximal NAD = NADH equilibrium rate and 3.3 times the maximal pyruvate = lactate rate.

A marked disparity between the pyruvate = lactate and NAD = NADH rates was also found at pH 7.9 (0.145 M Tris-NO_3), as shown in Fig. 3. Increasing concentrations of pyruvate and lactate resulted in an initial maximum rate followed by progressive fall to minimal levels in the NAD = NADH rate, while the pyruvate = lactate rate rose to a plateau without the moderate dip noted at pH 9.7. The NAD = NADH rate depression is much more complete than at pH 9.7. Maximal and half-maximal pyruvate = lactate rates are observed at lower pyruvate-lactate levels than at pH 9.7.

The initial rate from the pyruvate-NADH side (2.6 x 10^{-5} M min^{-1}; 25°, 5 mM pyruvate, 130 μM NADH, and 0.1 M Tris-
FIG. 2. The effect of lactate and pyruvate concentrations on equilibrium reaction rates with bovine heart lactate dehydrogenase at pH 9.7. Reaction mixtures contained 0.99 to 1.07 mM NAD, 1.21 to 1.23 mM NADH, and pyruvate and lactate at the concentrations as shown, in 167 mM glycine (sodium salt) buffer at pH 9.7 and 25°. In these and subsequent experiments, small differences in equilibrium concentrations of NAD and NADH were noted, as indicated by the ranges given. These probably result largely from a small shift in equilibrium or a difference in the activity of pyruvate or lactate (or both) as concentration is increased.

FIG. 3. The effect of lactate and pyruvate concentrations on equilibrium reaction rates with bovine heart lactate dehydrogenase at pH 7.9. Reaction mixtures contained 1.61 to 1.68 mM NAD, 41.9 to 57.9 µM NADH, and pyruvate and lactate as shown, in 145 mM Tris-NO₃ buffer at pH 7.9 and 25°. NO₃ was 3.33 times the maximum pyruvate ⇋ lactate rate and 14.9 times the maximum NAD ⇋ NADH rate.

Effect of Substrate Concentration on Equilibrium Reaction Rates with Rabbit Muscle Lactate Dehydrogenase—Rabbit muscle lactate dehydrogenase showed rate behavior qualitatively similar to the bovine heart enzyme. Rates of the NAD ⇋ NADH and pyruvate ⇋ lactate reactions as pyruvate and lactate concentrations are increased at pH 7.9 are shown in Fig. 4. The disparity between the maximal rates of pyruvate ⇋ lactate compared to NAD ⇋ NADH was not as marked, and the substrate concentrations at which the NAD ⇋ NADH depression was first observed were higher than with bovine heart lactate dehydrogenase. The pyruvate ⇋ lactate rate was similar to the NAD ⇋ NADH rate at the lowest substrate level studied.

The initial rate (3.84 × 10⁻⁴ M min⁻¹; 4.6 mM pyruvate, 168 µM NADH, and 0.1 M Tris-NO₃, pH 7.9) was 5.09 times the maximum pyruvate ⇋ lactate rate and 11.9 times the maximum NAD ⇋ NADH rate.

DISCUSSION

The most important aspect of the present findings is probably the convincing demonstration of the usefulness of equilibrium reaction rate measurements for the assessment of the order of addition of substrates to an enzyme. As mentioned earlier, considerable evidence indicating that coenzyme addition must precede pyruvate or lactate addition with both bovine heart and skeletal muscle lactate dehydrogenase (11-15) has accrued. The results of the equilibrium reaction rate measurements are in clear concordance with these earlier studies, and thus both validate the equilibrium rate approach and add to the conclusions on binding order reached previously.

The present studies, in common with kinetic approaches, do not give absolute proof of a compulsory binding order. If interconversion of lactate and pyruvate requires interconversion of NAD and NADH, a compulsory binding order provides the simplest and most logical mechanism for lack of measurable NAD ⇋ NADH at high lactate and pyruvate concentrations. Interconversion of NAD and NADH at a rate at least equal to the rate of the measured lactate ⇋ pyruvate interconversion occurs, but the NAD ⇋ NADH interconversion at high concentrations of lactate and pyruvate would be limited to the forms remaining bound to the enzyme. We thus conclude that studies of the present type by themselves will suffice to give very strong evidence for compulsory binding mechanisms,²

²A compulsory order as used herein means that substrates can only add to or dissociate from an enzyme in a prescribed order. Random order means that the presence of one bound substrate does not influence the binding or dissociation of another substrate. Compulsory and random represent two extremes of binding patterns, and "partially compulsory" or "partially random" binding may occur. The term "alternative" order has been suggested for such cases (10).
The interconversion of NAD and NADH at equilibrium, $R$, is given as

\[ E \cdot \text{NAD} \xrightleftharpoons[k_{-2}]{k_2(\text{lactate})} E \cdot \text{NAD} \cdot \text{lactate} \]

\[ E \cdot \text{NADH} \xrightleftharpoons[k_{-3}]{k_3(\text{pyruvate})} E \cdot \text{NADH} \cdot \text{pyruvate} \]

**Scheme 1**

or allow conclusive exclusion of such a possibility depending upon exchange patterns observed.

The system which corresponds most closely to a compulsory order of substrate addition is that of bovine heart lactate dehydrogenase at pH 7.9. As noted in Fig. 3, the presence of excess lactate and pyruvate almost completely prevents exchange of NAD and NADH even though the interconversion of lactate and pyruvate continues at a rapid rate. This is a logical result of the prevention of dissociation of NAD or NADH or both from the enzyme by the high concentration of the other substances. These results are in accord with the theoretical relationships governing such a pathway (10).

For a reaction sequence as given by Scheme 1, the relationship for the rate of interconversion of NAD and NADH at equilibrium, $R$, is given by Equation 1, and for the lactate $\rightarrow$ pyruvate rate, $R'$, by Equation 2, where $P$, $L$, NAD, and NADH are the concentrations of pyruvate, lactate, oxidized coenzyme, and reduced coenzyme, respectively, and $E_i$ is the total molarity of enzyme active sites. By use of these relationships, it may be shown that reasonable approximations for the various rate constants will give patterns for $R$ and $R'$ similar to those depicted in Fig. 3.

If the lactate dehydrogenase equilibrium were not markedly in favor of lactate and NAD formation, additional data to take further advantage of the relative simplicity of Equation 2 would be more readily obtainable. For example, by appropriate measurements, minimal values could be obtained for the equilibrium constants $K_2$ and $K_3$. The present data do suffice for estimation of a minimal value for $K_3$. Because of the high lactate concentration, variation in $R'$ as observed may arise only from a change in the $K_3/P$ term of Equation 2. If so, the ratio of the slope to intercept of a 1/$R'$ against 1/($P$) plot gives $K_3/(1 + K'/k)$.

Such an estimation gives a value of about $1 \times 10^{-4}$ M, which may be compared to value of $4 \times 10^{-4}$ M for the Michaelis constant for pyruvate at pH 7.9 from the data of Winer and Schwert (20). This implies that the Michaelis constant may be greater than the dissociation constant, as is the situation with many enzyme-substrate interactions.

Changes in dissociation constants with pH probably form the basis for an important difference in the behavior of the bovine heart enzyme at pH 7.9 as contrasted to that at pH 9.7. The achievement of half-maximal rates for the pyruvate-lactate reaction at lower levels of substrate at pH 7.9 implies smaller dissociation constants for pyruvate or lactate or both. This is in harmony with lower values for the Michaelis constants (13) at the lower pH.

The data with rabbit muscle lactate dehydrogenase at pH 7.9 (Fig. 4) are also quite consistent with a compulsory pathway in which coenzyme binds first. An unusual feature is shown by this enzyme at pH 7.9, and by the bovine heart enzyme at pH 9.7. This is the decrease in the pyruvate $\Rightarrow$ lactate reaction rate to an approximate plateau at high pyruvate and lactate concentrations. Such behavior is not predictable from the generalized equation for four-component systems or from any limiting cases therefrom. One possibility is a change in conformation of the protein or binding of lactate or pyruvate at other than the catalytic site with a resultant decrease in one or more rate constants. An inhibition of initial reaction velocity by binding of a second pyruvate molecule has been suggested in early studies, as discussed by Schwert and Winer (18). An effect of ionic strength seems unlikely because this would give a continued depression in rate as concentration increases. Various anions will compete for substrates (13), and this is a possible explanation for the inhibition noted in the presence of high NaCl concentration. Such inhibition cannot explain the fall in the lactate $\Rightarrow$ pyruvate rate noted in Fig. 4 because the ratio of lactate to pyruvate is held constant as concentration is increased. This assures equal competitive effects between these two substrates at all concentrations.

Whatever modification causes the depression in the pyruvate $\Rightarrow$ lactate rate discussed in the preceding paragraph may also cause a corresponding depression of the NAD $\Rightarrow$ NADH rate. The decrease in the NAD $\Rightarrow$ NADH rate on a percentage basis is much greater than that of the pyruvate $\Rightarrow$ lactate rate. As discussed elsewhere, this can readily be explained in terms of compulsory or partially compulsory binding.

At pH 0.7, an interesting phenomenon is apparent with the bovine heart enzyme; i.e. the reaction appears not to be compulsory. Even at high lactate and pyruvurate concentrations, a low but readily measurable NAD $\Rightarrow$ NADH reaction occurs. The lack of a complete inhibition of the NAD $\Rightarrow$ NADH exchange could result from a lack of saturation by lactate and pyruvurate. This seems unlikely; however, the pyruvurate concentration greatly exceeds its reported $K_m$ values at this pH and the highest lactate concentration, 3.3 M, is more than 10 times its reported $K_m$ (13). In addition, the approach of the pyruvate $\Rightarrow$ lactate rate to a maximum with concentration increase implies that saturation has occurred. Such behavior would result if the presence of lactate or pyruvurate considerably decreased but did not prevent coenzyme dissociation. Weak binding of lactate or pyruvurate or both must prevail in the absence of coenzyme.

The behavior of the bovine heart enzyme at higher pH is of interest in relation to the report by Winer (21) of a discrepancy
above pH 8.1 between dissociation constants for beef heart lactate dehydrogenase determined fluorometrically and those calculated from kinetic data on the basis of a compulsory binding order. Data reported herein show that a compulsory binding order is not operative at the higher pH values, and may thus explain the apparent discrepancy.

Only limited information arises from comparison of equilibrium reaction rates with maximum initial velocities. For the reaction pathway of Scheme 1, the maximum initial velocity with excess pyruvate and NADH is given by

$$V_{\text{max}} = \frac{E_i}{k + \frac{1}{k_{-2}} + \frac{k}{k'k_{-2}} + \frac{1}{k_{-1}}}$$

The maximum rate of pyruvate $\rightleftharpoons$ lactate at equilibrium with excess concentration of all substrates is given by

$$R_{\text{max}} = \frac{E_i}{k' + \frac{1}{k_{-2}} + \frac{k'}{kk_{-2}} + \frac{1}{k_{-3}} + \frac{k}{kk_{-3}}}$$

Equilibrium reaction rates were observed to be considerably less than maximal initial velocities, which means that $1/k_{-1}$ is considerably less than

$$\frac{1}{k} + \frac{1}{k_{-2}} + \frac{k'}{kk_{-2}}$$

Some additional information about comparative values of rate constants arises from inequalities of the pyruvate $\rightleftharpoons$ lactate and NAD $\rightleftharpoons$ NADH rates. As is readily apparent from examination of Equations 1 and 2, $R$ will become equal to $R'$ as the concentration of lactate and pyruvate approaches zero. Equality of $R$ and $R'$ will result when $k_1$ (pyruvate) becomes $\ll k_{-4}$ and $k_3$ (lactate) becomes $\ll k_{-2}$. With rabbit muscle lactate dehydrogenase, the rates approached equality at the lowest concentrations studied, while a considerable discrepancy in rates remained for the bovine heart enzyme at pH 7.9. Equality of rates would be observed at lactate and pyruvate concentrations considerably above dissociation constant values if the chemical transformation step (interconversion of ternary complexes) were sufficiently slow or, more specifically, if

$$k' \ll k_{-4} + \frac{K_{k_{-4}}}{(\text{pyruvate})}$$

and

$$k \ll k_{-2} + \frac{K_{k_{-2}}}{(\text{lactate})}$$

The relatively low lactate and pyruvate concentrations at which inequality of rates are observed means that such conditions are not approached for the dehydrogenases studied. A tendency to approach these limitations more readily for the rabbit muscle than for the bovine heart enzyme could explain why rates are more nearly equal and higher lactate and pyruvate concentrations are required for inhibition of NAD $\rightleftharpoons$ NADH rates with the rabbit muscle enzyme.

The preceding discussion does not consider possible effects of formation of abortive ternary complexes, and our experiments were not designed to detect such complexes. The most likely forms of abortive ternary complexes are $E'NAD\text{-}\text{pyruvate}$. Formation of such complexes could be detected by increases in the NAD and pyruvate or the NADH and lactate concentrations while maintaining equilibrium. The formation of the complexes would deplete enzyme from catalytically active complexes and depress all exchange rates.

**SUMMARY**

Isotopic techniques are described for measurement of the rates of interconversion of lactate and pyruvate and of nicotinamide adenine dinucleotide and its reduced form in free solution at equilibrium in the presence of catalytic quantities of lactate dehydrogenase. At pH 7.9 with bovine heart lactate dehydrogenase, an increase in the lactate-pyruvate concentration while maintaining equilibrium results in a continued increase in the lactate $\rightleftharpoons$ pyruvate rate, and in a rise, followed by a fall to near zero, in the NAD $\rightleftharpoons$ NADH rate. These data, in the light of recent theoretical considerations, give strong evidence that a compulsory binding order exists in which the presence of NAD or NADH is essential for the binding of lactate or pyruvate. Such results are in harmony with previous conclusions of others based on substrate binding and initial velocity studies, and give validity to the equilibrium rate measurements as a powerful new probe for study of enzyme mechanisms.

Bovine heart lactate dehydrogenase at pH 9.7 shows a partially compulsory binding of substrates, characterized by a marked decrease but not complete inhibition of the NAD $\rightleftharpoons$ NADH rate at high concentrations of lactate and pyruvate. Rabbit muscle lactate dehydrogenase conformed to a compulsory binding order at pH 7.9. With the rabbit muscle enzyme at pH 7.9 and the bovine heart enzyme at pH 9.7, but not at 7.9, an unexplained decrease in the lactate $\rightleftharpoons$ pyruvate rate was observed at high concentrations of lactate and pyruvate.

The lactate $\rightleftharpoons$ pyruvate rate at equilibrium exceeded the NAD $\rightleftharpoons$ NADH rate at all except the lowest substrate concentrations, particularly with the bovine heart enzyme. At high substrate concentrations, the lactate $\rightleftharpoons$ pyruvate rate was still considerably less than maximal initial velocities without products present. These results allow inferences about the relative values of certain rate constants and approximation of some substrate dissociation constants.

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