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Rate of Isotope Exchange in Enzyme-Catalyzed Reactions*

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ABSTRACT: The methodology of treating the kinetics of isotope exchange catalyzed by enzymes is discussed. A relatively simple way of deriving the equations relating velocity at equilibrium of an enzymatic reaction with the kinetic parameters of the reaction is described. The following general relations are helpful. The equilibrium rate of n consecutive reactions is given by

$$1/V_e = \sum_1^n 1/V_i$$

The equilibrium rate of n parallel reactions is

$$V_e = \sum_1^n V_i$$

These relations also make possible the calculation of exchange rates through coupled enzymatic reactions which occur in metabolizing systems. In the experimental part the rate of tritium equilibration between position 4A of reduced nicotinamide–adenine dinucleotide and position 2 of L-lactate is measured. Series of measurements in which lactate is varied between 0.37 and 37 mM at constant pyruvate and reduced nicotinamide–adenine dinucleotide concentrations, as well as series in which pyruvate is varied between 4.3 and 86 μM at constant lactate and pyruvate concentrations, are reported. The results, in conjunction with the equations derived in the first part, make possible the evaluation of three rate constants involved in the lactate dehydrogenase reaction. These constants are summarized in Table II, and are shown to be close to the ones obtained from initial rate studies.

When a labeled substrate is added to an actively metabolizing system the label may appear in a particular product either as a result of net synthesis or because the product is in a state of dynamic equilibrium with the initially labeled metabolite. The latter process falls into the class of isotope-exchange reactions; for example, when L-[2-^{3}H]lactate is infused into an isolated perfused rat liver, tritium appears in the liver malate (Hoberman, 1965). It is important to know whether the labeling of malate results from an exchange between lactate and endogenous malate or by net formation of malate. The labeling by exchange may be attributed to coupling of the lactate and malate dehydrogenase systems (eq 1–3). In the normal rat liver these reactions appear to be in a state of equilibrium (Bücher and Klingenberg, 1958). When an isotopic compound is used to measure the

L-[2-^{3}H]lactate + NAD $\rightleftharpoons$ pyruvate + [4A-^{3}H]NADH + H$^+$ (1)

oxalacetate + [4A-^{3}H]NADH + H$^+$ $\rightleftharpoons$ L-[2-^{3}H]malate + NAD (2)

L-[2-^{3}H]lactate + oxalacetate $\rightleftharpoons$ L-[2-^{3}H]malate + pyruvate (3)

metabolic throughput of a particular process, it is essential to evaluate the contribution of the isotopic-exchange reaction to the observed rate of appearance of the labeled product. In order to evaluate this contribution it is necessary to derive and evaluate an expression for the rate of the exchange at equilibrium of each of the coupled enzymatic reactions in terms of the rate constants of the individual steps.

The kinetics of isotope-exchange reactions catalyzed by enzymes have been discussed by a number of investigators (Koshland, 1955; Boyer, 1959; Alberty *et al.*, 1962). The subject has recently been reviewed by Rose (1966) and Cleland (1967). The present communication describes a simplified way to derive the relations between the measured exchange rates at thermodynamic equilibrium and the kinetic parameters on which the rates depend. Using the derived equations three of the rate constants characterizing the reaction catalyzed by

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lactate dehydrogenase are evaluated from experimental data. The constants are shown to have values close to those obtained from initial rate studies (Schwert *et al.*, 1967). Having demonstrated the validity of the present analysis, the methodology may be applied to estimate the rates of exchange through coupled reactions in intact cells.

Theoretical Considerations

The Equilibrium Velocity. An important concept in the treatment of isotopic exchange is the equilibrium velocity, V_e. Consider reactions 1 or 2 after they have reached thermodynamic equilibrium. In the presence of the enzyme the reactants on the right-hand side of the equation are continually converted into the reactants shown on the left-hand side while an equal number of the reactants on the left side undergo transformation to those on the right. At any instant the number of molecules flowing in one direction is equal to the number flowing in the opposite direction. The rate of these equal flows is termed the equilibrium velocity. McKay (1938; see also Frost and Pearson, 1961) has shown that when a trace amount of a labeled reactant is added to a system containing a second compound which may become labeled, the rate of appearance of the label in the other reactant is described by the expression

$$-\ln (1 - f) = V_e \frac{(a + b)}{ab} t \tag{4}$$

where a is the total concentration of the initially labeled reactant, A; b is the total concentration of the reactant, B, which receives the label; and f is the extent of isotopic equilibration, defined by

$$f = \frac{a_0^* - a^*}{a_0^* - a_\infty^*} = \frac{b^*}{b_\infty^*} \tag{5}$$

where a_0^*, a^*, and a_∞^* are the concentrations of isotopically labeled A at time 0, t, and infinity, respectively, and b_0^*, b^*, and b_∞^* are the concentrations of labeled B at times 0, t, and infinity. It may be seen that f varies from 0 at $t = 0$ to 1 at $t = \infty$. We assume that initially b is unlabeled, *i.e.*, $b_0^* = 0$ in eq 5.

If one plots the experimentally obtained values of $-\ln (1 - f)$ *vs.* t, one obtains a straight line with the slope $V_e(a + b)/ab$. V_e may then be calculated from the slope and the known values of a and b. V_e may also be evaluated with the use of eq 6

$$V_e = \frac{ab}{a + b} \frac{\ln 2}{t_{1/2}} \tag{6}$$

The value of $t_{1/2}$, the time for half-equilibration, is easily obtained from the plot of $-\ln (1 - f)$ *vs.* t. The McKay relationships (4 and 6) hold regardless of the number of steps involved or other details of mechanism so long as only a single atom on A and B exchange the label (Frost and Pearson, 1961). Intermediates will rapidly become labeled during the time that the steady state is established, *i.e.*, just as in initial velocity experiments the concentrations of enzyme-bound intermediates are low relative to the concentrations of unbound intermediates and a few exchanges will suffice to introduce the isotope label. Relationships 4 and 6 make possible the calculation of the equilibrium velocity from the observed rate of isotopic equilibration.

The Equilibrium Velocity of Complex Reactions. To establish relationships between V_e and the rate constants of individual steps of a multistep reaction the following rules are helpful (V_i is the equilibrium velocity of the ith step). (I) The equilibrium velocity of a reaction proceeding by n consecutive steps is

$$1/V_e = \sum_1^n 1/V_i \tag{7}$$

(II) The equilibrium velocity of a reaction proceeding by n parallel steps is

$$V_e = \sum_1^n V_i \tag{8}$$

The second relationship is obvious. To verify I we consider a reaction proceeding by two consecutive steps

$$A_1 \underset{}{\overset{V_1}{\rightleftharpoons}} A_2 \overset{V_2}{\rightleftharpoons} A_3 \tag{9}$$

In each unit of time V_1 moles of A_2 are formed from A_1. Of this amount the fraction $V_2/(V_1 + V_2)$ is transformed to A_3. Accordingly the amount of A_3 formed from A_1 per unit time is

$$V_e = V_1V_2/(V_1 + V_2) \tag{10}$$

On rearrangement this relationship is

$$1/V_e = 1/V_1 + 1/V_2 \tag{11}$$

This argument is readily extended to three consecutive steps, and so on, to give rule I. Amore rigorous proof of eq 7 is given in the Appendix.

With the use of rules I and II the relationship between the equilibrium velocity and the specific rate constants of a given enzymatic reaction is readily expressed as shown below. The exact form of the equation for a single enzyme is determined by the particular reaction scheme which one applies to describe the course of the enzymatic reaction. When rules I and II are applied to some commonly assumed pathways, the equations derived by Boyer (1959), Alberty *et al.* (1962), and Cleland (1967) are obtained with relative ease. In the section which follows we illustrate the method by deriving the relation between the equilibrium velocity and the specific rate constants of a reaction catalyzed by a two-substrate dehydrogenase assumed to obey kinetics of the Theorell–Chance type.

Equilibrium Velocity of a Reaction Proceeding by the Theorell–Chance Pathway. In the Experimental Section

Symbols	Reaction steps	Equilibrium velocity	
$E + D \rightleftharpoons ED$	$E + NAD \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot NAD$	V_1	(12)
$ED + L \rightleftharpoons EH + P$	$E\cdot NAD + \text{lactate} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E\cdot NADH + \text{pyruvate}$	V_2	(13)
$EH \rightleftharpoons E + H$	$E\cdot NADH \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + NADH$	V_3	(14)

we interpret the exchange kinetics in the reaction catalyzed by bovine heart lactate dehydrogenase in terms of the pathway first suggested by Theorell and Chance (1951) for liver alcohol dehydrogenase and later shown to apply also to skeletal muscle lactate dehydrogenase (Zewe and Fromm, 1962) and malate dehydrogenase (Raval and Wolfe, 1962). This is further discussed in a later section. The Theorell–Chance scheme for the lactate dehydrogenase reaction is given in eq 12–14.

In principle three equilibrium rates may be obtained from measurements of rates of isotopic exchange in the above reaction. (a) Pyruvate–lactate exchange using isotopic carbon in either compound. In this case the equilibrium rate is simply

$$1/V_e = 1/V_2 \tag{15}$$

(b) Exchange of isotopic hydrogen between position 2 of lactate and the 4A position of NADH. In this case

$$1/V_e = 1/V_2 + 1/V_3 \tag{16}$$

(c) NAD–NADH exchange, *i.e.*, the nucleotide is labeled in a position in the molecule other than as in b. In this case

$$1/V_e = 1/V_1 + 1/V_2 + 1/V_3 \tag{17}$$

Exchanges a and c have been studied by Silverstein and Boyer (1964a,b) in the reactions catalyzed by lactate and alcohol dehydrogenases. In the following, we develop the relationships required for the evaluation of b.

The equilibrium velocities of reaction steps 13 and 14 are

$$V_2 = k_4[EH][P] \text{ and } V_2 = k_6[E][H] \tag{18}$$

In the reciprocal form, this is

$$1/V_e = 1/V_2 + 1/V_3 = 1/k_4[EH][P] + 1/k_6[E][H] \tag{19}$$

To evaluate [EH] we use the equilibrium relationship for eq 14

$$[EH] = k_6/k_5[E][H]$$

which, substituted in eq 19, gives

$$\frac{1}{V_e} = \frac{1}{E}\left[\frac{k_5}{k_4k_6[H][P]} + \frac{1}{k_6[H]}\right] \tag{20}$$

The total enzyme concentration, $[E_t]$, is

$$[E_t] = [E] = [EH] + [ED] \tag{21}$$

The equilibrium equations 13 and 14 combine to give for [ED]

$$[ED] = \frac{k_4k_6[H][P]}{k_3k_5}\frac{}{[L]}[E] \tag{22}$$

and

$$[E_t] = [E]\left[1 + \frac{k_6}{k_5}[H] + \frac{k_4k_6[H][P]}{k_3k_5\,[L]}\right] \tag{23}$$

Substituting eq 23 in 20 gives

$$1/V_e = \frac{1}{[E_t]}\left[\frac{k_5}{k_4k_6}\frac{1}{[H][P]} + \frac{1}{k_6[H]}\right] \times \left[1 + \frac{k_6[H]}{k_5} + \frac{k_4k_6[H][P]}{k_3k_5}\frac{}{[L]}\right] \tag{24}$$

This equation may also be written as

$$\frac{[E_t]}{V_e} = \frac{1}{k_6}\frac{1}{[H]} + \frac{1}{k_4}\left[1 + \frac{k_5}{k_6[H]}\right] \times \frac{1}{[P]} + \frac{1}{k_3}\left[1 + \frac{k_4[P]}{k_5}\right]\frac{1}{[L]} + \frac{1}{k_5} \tag{25}$$

This expression contains four terms, one in 1/[H], one in 1/[L], one in 1/[P], and a term independent of reactant concentration. The coefficients of each of these terms may be evaluated by varying the concentrations of one reactant while holding the concentrations of the others constant and high enough to make negligible the contribution of terms containing their reciprocals. It will be noted that [D](NAD) does not appear in eq 25. This makes possible the use of NAD as a regulating reactant, *i.e.*, when the concentration of lactate is arbitrarily increased, the concentration of NAD can be correspondingly decreased. An examination of the equilibrium expression $K = [P][H]/[L][D]$ shows that the concentrations of [P] and [H] remain constant.

Accordingly a series of measurements of V_e keeping the concentrations of [L] and [H] constant should yield a linear relationship between $[E_t]/V_e$ and 1/[P] provided that the concentrations of [H] and [L] are high enough to make terms in 1/[H] and 1/[L] negligible in comparison with the term in 1/[P]. The slope of the straight line

obtained in this manner is $1/k_4$ and the intercept, $1/k_5$. Holding the concentrations of [P] and [H] constant while varying the concentration of [L] should give a linear plot of $[E_t]/V_e$ *vs.* 1/[L]. The slope of this line gives the value of $1/k_3$ (provided that $k_4[P]/k_5 \ll 1$). Finally a plot of $[E_t]/V_e$ *vs.* 1/[H] at constant [P] and high [L] will give the value of k_6. From the foregoing considerations it may be seen that all four rate constants involved in the exchange can be evaluated. In the present study experiments were carried out for the evaluation of three of these constants for the lactate dehydrogenase system.

Materials and Methods

Lactate Dehydrogenase. Twice-recrystallized heart muscle lactate dehydrogenase suspended in $(NH_4)_2SO_4$ (Worthington Biochemical Corp.) was dialyzed against 0.05 M sodium phosphate buffer (pH 6) for 24 hr at 5°. The dialyzed solution was then fractionated on a DEAE-Sephadex column following the procedure of Wachsmuth and Pfleiderer (1963). The enzyme preparation used for the exchange is isozyme V which was eluted from the column in the last enzymatically active fraction. This fraction comprises about 70% of the total amount of enzyme added to the column. The specific activity of the preparation was generally 41,000 moles of NADH oxidized/mole of enzyme per min, assuming a molecular weight of 135,000 for the enzyme. The preparation of Winer and Schwert (1958) was reported to have a specific activity of 46,000 at pH 8.05. More recently a preparation with a specific activity of of 80,000 and a molecular weight of 144,000 has been reported (Schwert *et al.*, 1967).

[4-^{3}H]NADH. This was prepared by reduction of NAD with [2-^{3}H]ethanol in the presence of yeast alcohol dehydrogenase. A typical preparation was as follows: 10 mmoles of glycine, 5 mmoles of semicarbazide, 250 μmoles of unlabeled ethanol, 200 μCi of [2-^{3}H]ethanol, and 100 μmoles of NAD were separately added to a small volume of distilled water, adjusting the pH to 10.5 after each addition. The volume was made to 10 ml with distilled water, 2 mg of alcohol dehydrogenase was added, and the mixture was incubated at 23°. The reaction, which was followed by observing the adsorption at 340 mμ of appropriately diluted aliquots, was found to be complete in 10 min. The enzyme was precipitated by placing the vessel containing the reaction mixture in a boiling-water bath for 3 min. After removing coagulated protein [4A-^{3}H]NADH was isolated from the resulting solution by chromatography on DEAE-cellulose using a modification of the procedure of Pastore and Friedkin (1961). The NADH fraction selected for use was 97% pure as estimated by the extent of disappearance of absorbancy at 340 mμ on addition of pyruvate and lactate dehydrogenase. The 340/290 ratio was 4.05. The initial fractions of the NADH peak did not give a satisfactory assay and were discarded. In a typical run, 45 μmoles of NADH was formed with a specific activity of 0.35μCi/μmole. [4A-^{3}H]NADH was freshly prepared for use for each series of kinetic measurements.

Lactate Dehydrogenase Assay. Enzymatic activity was determined by following the change in absorbancy at 340 mμ in the presence of 10^{-3} M pyruvate and 10^{-4} M NADH in Tris-KCl at pH 8.03 and an ionic strength of 0.2 M. The rate was calculated from the initial velocity of the reaction. Activity is expressed in terms of nanomoles of NADH oxidized per second per milliliter of reaction mixture (ϵ 6.22).

Kinetic Experiments. Reaction mixtures were prepared by adding appropriate amounts of NAD, [4A-^{3}H]-NADH, and L-lactate to 0.2 M Tris buffer (pH 8.03). NaCl was added to bring the ionic strength of the solution to 0.1. The final volume was 5 ml. When the solution reached constant temperature (28.5°), the exchange was started by addition of enzyme. At suitable times, aliquots of the reaction mixture were removed and the reaction was stopped. Three methods were used for this purpose: (a) the aliquot was added to five volumes of boiling water; (b) 100 μl of reaction mixture was added to 25 μl of a sodium sulfite solution (pH 8), and (c) 10 μl of reaction mixture was placed on a paper strip and rapidly dried with a current of warm air. Procedures b and c were used when radioactive NADH and lactate were separated by paper strip chromatography while a was employed when the separation was carried out by column chromatography. No significant differences in rates of exchange were observed as a result of the different methods used to stop the reaction.

Separation of Reactants and Evaluation of $(1 - f)$. In the early experiments the reactants were separated on DEAE-cellulose ion-exchange columns. This procedure makes possible the determination of specific activity of each fraction, thus giving a firmer check on the progress of the exchange. The procedure was a modified version of Pastore and Friedkin (1961), employing a gradient of 0–0.5 M acetate at pH 8.0 in a 1 mM glycylglycine buffer. The amount of tritium in each fraction was determined on a Packard Tri-Carb liquid scintillation counter, using Bray's counting solution. In the experiments used to evaluate the rate constants, separations were performed by paper chromatography. This permits simultaneous processing of a great number of samples, thus improving the uniformity of the results. The sample was placed on a strip of Whatman No. 1 filter paper. The strips were developed by ascending chromatography at room temperature. The solvent system was acetone–water (55:45). After drying at room temperature the strips were scanned for radioactivity using a Vanguard strip scanner with 4π geometry. The [^{3}H]NADH and [^{3}H]lactate peaks were well separated. The output of the Geiger chambers was either led to a rate meter giving a chart recording or the Vanguard integrator was preset for 1 min counts and the total number of counts in the peak determined by summation and background subtraction. The latter procedure is the more reliable one since the relatively long time constant of the rate meter results in distortion of the recorded peaks.

The fraction not exchanged, $(1 - f)$, is determined as follows. The number of counts in the lactate fraction, c, and in the NADH fraction as well as the sum of the two fractions, c_t, were obtained. Designating c_∞ as

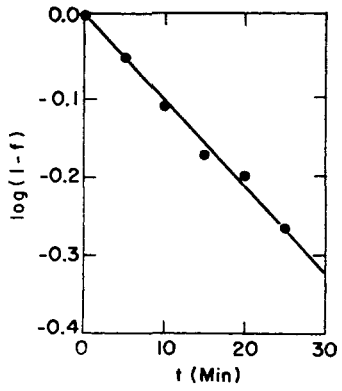

FIGURE 1: Plot of ln $(1 - f)$, extent of isotopic equilibrium, *vs.* time.

the number of counts in the lactate fraction at isotopic equilibrium, $(1 - f)$ is calculated from the equation

$$(1 - f) = \frac{c_\infty/c_t - c/c_t}{c_\infty/c_t} \qquad (26)$$

This is of course equal to $(b_\infty^* - b^*)/b^\infty$ and offers a correction for losses during the processing.

Results

As anticipated, bovine heart lactate dehydrogenase (EC 1.1.1.27) was observed to catalyze an exchange of hydrogen between [4A-^{3}H]NADH and lactate. When ln $(1 - f)$ is plotted *vs.* time, straight lines are obtained as illustrated in Figure 1. From these straight lines values of $t_{1/2}$ for the exchange were obtained and are listed in column 7 of Table I. With these data the equilibrium velocity of the reaction was calculated by eq 6 and the values of the equilibrium velocity obtained, V_e', are shown in column 8 of Table I. The prime on V_e is used because the observed equilibrium velocities must be corrected for the kinetic isotope effect. This correction is discussed in a later section.

In the first five experiments NAD was varied over a 20-fold range of concentration thereby causing parallel changes in the pyruvate concentration. The concentrations of lactate and NADH were held constant and relatively high so that the first and third terms of eq 25 make only a small contribution to V_e'. When the reciprocal values of V_e' are plotted against 1/[P], the straight line shown in Figure 2 is obtained. The slope of this line is 200 sec. It may be seen, using data of Schwert *et al.* (1967), that k_5/k_6[H] is well below unity. Accordingly the slope of the straight line illustrated in Figure 2 is essentially equal to $1/k_4$[E$_t$] so that k_4[E$_t$] = 5×10^{-8} sec^{-1}. To obtain k_4 the enzyme concentration must be known. In order to avoid uncertainties caused by possible differences among enzyme preparations, the value of k_4[E$_t$] is divided by the observed value of k_2[E$_t$], which is the activity of lactate dehydrogenase in the system as determined by our method of assay. Results are therefore expressed in terms of k/k_2. As shown in column 5 of Table I, the average enzyme activity in expt 1–5 was 13.5×10^{-6} M sec^{-1}. Accordingly k_4/k_2 is 372 M^{-1}. This value is listed in column 2 of Table II.

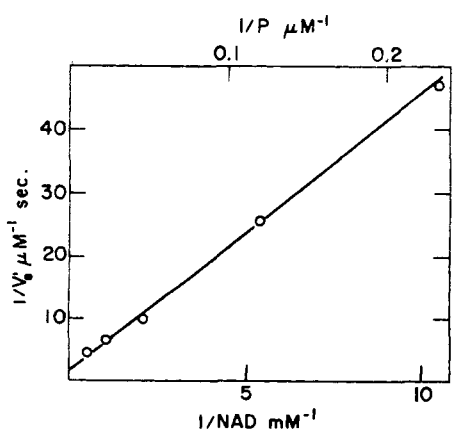

FIGURE 2: Plot of $1/V_e'$ (expt 1–5) *vs.* 1/[P] and 1/[D].

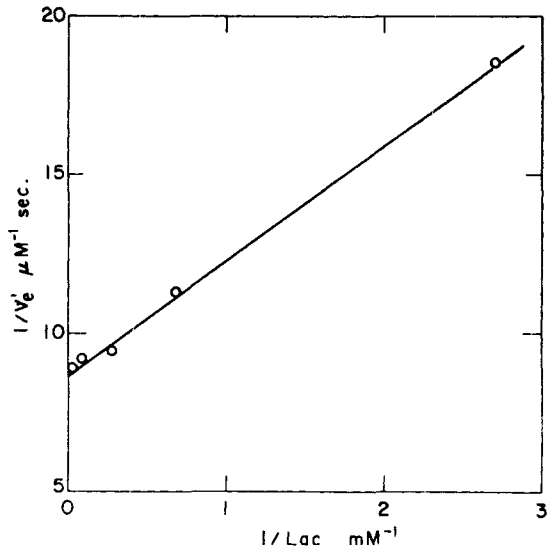

FIGURE 3: Plot of $1/V_e'$ (expt 6–10) *vs.* 1/[L].

The intercept of the straight line of Figure 2 with the $1/V_e'$ axis has a value of 1.5 sec/μM. While the term in 1/[H] is negligible, an estimation based on our final experimental data (Table I and II) shows that the term in 1/[L] contributes 0.3 to this intercept. Accordingly the value of $1/k_3$[E$_t$] is 1.2 sec/μM making k_3[E$_t$] = 0.82×10^{-6} M sec^{-1}. The ratio of k_5/k_2, also shown in Table II, is thus 0.061.

The data of expt 6–10 lead to a value of k_3/k_2. The concentration of lactate was varied, while NADH and pyruvate were held constant by adjusting the concentration of NAD. Values of $1/V_e'$ are plotted against 1/[L] in Figure 3. A straight line results, with a slope of 3.6×10^3 sec^{-1}. Although the contribution to the slope of the term in k_4[P]/k_5 is not negligible, it is readily calculated from the values of k_4/k_2 and k_5/k_2 already obtained. The correction is 0.22, *i.e.*, the slope of the straight line of Figure 3 is $1.22/k_3$[E$_t$]. Accordingly k_3[E$_t$] = 0.34×10^{-3} sec^{-1}. The average enzyme

TABLE I: Half-Lives of Isotopic Equilibration As Well As Uncorrected Equilibrium Velocities, at Several Compositions of Equilibrium Mixture of the Reaction Catalyzed by Lactate Dehydrogenase.

	Lactate (mM)	NAD (mM)	NADH (mM)	Pyruvate[a] (mM)	Lactate Dehydrogenase[b] (units)	$t_{1/2}$ (sec)	V_e' (μM sec^{-1})
1	18.4	0.095	0.525	0.0043	13.6	16,200	0.0213
2	18.4	0.190	0.525	0.0086	13.5	9,200	0.0387
3	18.4	0.475	0.525	0.0216	14.1	3,550	0.101
4	18.4	0.950	0.525	0.0430	10.8[c]	2,280	0.157
5	18.4	1.900	0.525	0.0860	13.0	1,680	0.213
6	0.37	38.0	0.515	0.0354	10.7[c]	2,750	0.054
7	1.48	9.50	0.515	0.0354	13.8	3,000	0.088
8	3.70	3.80	0.515	0.0354	15.5	3,000	0.104
9	14.80	0.95	0.515	0.0354	16.0	3,180	0.108
10	37.00	1.90	0.515	0.0354	15.5	3,180	0.111

[a] Calculated from the equation $[P] = K_{equil}[L][D]/[H]$ where $K_{equil} = 1.32 \times 10^{-3}$ (as reported by Schwert *et al.*, 1967). [b] One unit equals 1 nmole of NADH oxidized per ml of reaction mixture per sec. The conditions of assay were: pH 8.03; I = 0.2 M Tris-KCl; T = 28.5°. [c] These values were not included in the calculation of the average.

activity, $k_2[E_t]$, in expt 6–10 was 15.2×10^{-6} M sec^{-1} so that $k_3/k_2 = 22.5$ M^{-1}.

The ratio k_6/k_2 although determinable in principle was not obtained because [4A-^{3}H]NADH with a specific activity high enough to achieve a full range of measurement of V_e' was not available to us at that time.

Correction for Isotope Effect. It has been shown (Harris, 1951; see also Melander, 1960) that if one assumes the isotope effect is constant throughout the exchange process, the McKay relationship becomes

$$-\ln(1-f) = \alpha V_e \frac{(\beta/\alpha)a + b}{ab} t \qquad (27)$$

where α and β are the isotope effect on the forward and backward rates, respectively. The observed linearity of the relationship between $-\ln(1-f)$ and t (Figure 1) is evidence of constancy of α and β during the course of the exchange reaction.

An exact correction for this isotope effect cannot be made, but an estimate can be arrived at as follows. If we neglect the relatively small equilibrium isotope effect, *i.e.*, $\beta/\alpha = 1$, we have simply: $V_e' = \alpha V_e$ (compare eq 4 with eq 27). To obtain an estimate of α use may be made of eq 20

$$1/V_e' = 1/[E]\left[\frac{k_5' + k_4'[P]}{k_4'k_6'[H][P]}\right] \qquad (28)$$

where the primes are used to denote rate constants for the tritiated molecules. [E] is not primed because the absolute concentrations of the tritiated substrates are negligibly small so that the equilibrium between free and bound forms of the enzyme is not appreciably changed. Bearing in mind that almost all experiments were conducted so that $k_5 \gg k_4[P]$, eq 20 may be divided by eq 28, giving

$$\alpha = \frac{k_4'}{k_4}\frac{k_6'}{k_6}\Big/\frac{k_5'}{k_5} \qquad (29)$$

Measurements of the effect of deuterium substitution on the heart-type lactate dehydrogenase reaction have been conducted by Thomson and Nance (1965). In their terminology, this ratio is simply $1/\alpha = \Phi_{12}^{[T]}/\Phi_{12}^{[H]}$. The value they report for this ratio for deuterium substitution is $\Phi_{12}^{[D]}/\Phi_{12}^{[H]} = 2.12$. To convert into tritium rate, we use the relation (Melander, 1960)

$$k_{[D]}/k_{[H]} = (k_{[T]}/k_{[H]})^{1.44} \qquad (30)$$

This leads to a value of 2.95 for $1/\alpha$. The observed ratios of rate constants were multiplied by this factor to give the corrected ratios shown in column 3 of Table II. These can now be compared with constants obtained from initial rate studies shown in the last column of the table.

Discussion

The present communication develops a method of analysis of the kinetics of enzyme-catalyzed reactions in a state of equilibrium. Although the subject has been treated before (*loc. cit.*) the present method of analysis makes readily possible the derivation of equations in a form convenient for use in (a) obtaining numerical values of rate constants of individual steps, when the reaction pathway is known to assumed, and (b) calculation of the equilibrium velocity from data made available by initial rate studies. The equation particular to

TABLE II: Rate Constants for the Reaction Catalyzed by Lactate Dehydrogenase.

	Observed	Corrected[a]	From Initial Velocity[b]
k_3/k_2 (M^{-1})	22.5	66	93
k_4/k_2 (M^{-1})	372	1090	3130
k_5/k_2	0.06	0.18	0.22

[a] Corrected for the isotope effect as explained in the text. [b] The values were calculated from Figures 3 and 4 of Schwert *et al.* (1967) as follows (mol wt 36,000): $k_2 = V_r = 355\ sec^{-1}$, $k_3 = V_f/K_L = 3.3 \times 10^4\ M^{-1}\ sec^{-1}$, $k_4 = V_r/K_P = 1.1 \times 10^6\ M^{-1}\ sec^{-1}$, and $k_5 = V_f = 79\ sec^{-1}$.

the Theorell–Chance scheme is employed here to describe the kinetics of lactate dehydrogenase of bovine heart muscle.

A detailed study of the kinetics of the lactate dehydrogenase reaction by initial rate measurements was performed by Winer and Schwert (1958) and later by Schwert *et al.* (1967). They find that, in order to explain the data over a wide range of pH, ternary complexes must be assumed to be kinetically significant intermediates in the reaction. On inspection of their data, however, it may be seen that, in the region of pH 8, the Theorell–Chance mechanism is a satisfactory model of the reaction. This is indicated by applying the criteria formulated by Alberty (1953, 1968) and Dalziel (1957); at pH 8, $V_f(80) = V_R K_{RP}/K_R K_P$ (112) and $V_r(355) = V_f K_{OL} K_O(310)$. The V's and K's are as defined by Schwert *et al.* (1958, 1967) and the numbers in parentheses are values based on the graphical data, Figures 3 and 4 of Schwert *et al.* (1967). The values seem close enough to equality to justify the adoption of the Theorell–Chance scheme at pH 8 instead of one of greater complexity.

The data of Table II compare the values of k/k_2 observed in the present studies with those calculated from data derived from initial rate studies. It may be seen that two of the rate constants, *i.e.*, k_3, characterizing the transfer of hydrogen from lactate to NAD, and k_5, the specific rate of dissociation of NADH from the enzyme, have values which are similar to those obtained by conventional kinetic analyses. The presently observed value of k_4 deviates significantly from the value calculated from the initial velocity data of Schwert *et al.* (1967). Our value of k_4 is obtained from the slope of a plot of $1/V_e'$ *vs.* 1/[P]. This slope depends upon the pyruvate concentration which in turn is calculated with the use of K_{equil}. There is as yet no general agreement regarding the absolute value of K_{equil} for the reaction catalyzed by lactate dehydrogenase. We have elected to use the relatively high value of 1.32×10^{-2} at pH 8 given by Schwert *et al.* (1967). On the basis of their extensive studies, these authors conclude that "the accumulated errors of estimation of kinetic parameters yield a value for equilibrium constant which differs by a factor of 2 to 3 from the true value." The use of a lower value of K_{equil} to calculate pyruvate concentrations would obviously improve the level of agreement between our value of k_4 and that calculated from the data of initial velocity studies.

On the whole the range of values of the three rate constants evaluated at equilibrium are in reasonable accord with those derived from initial rate studies. This indicates that, under our conditions, the presence of the reactants in equilibrium concentration does not influence the course of the enzymatic reaction. (It is conceivable that the formation of abortive complexes with products will lead to a different rate at equilibrium than with only initial reactants present.) The determination of equilibrium velocities by means of isotope-exchange reactions or other means thus offers an alternative method for evaluation of kinetic constants.

Several authors, notably Boyer *et al.* (1959) and Silverstein and Boyer (1964a,b), as well as Morrison and Cleland (1966), utilized measurements of exchange rates in order to distinguish among various possible pathways for a particular enzymatic reaction. Silverstein and Boyer have shown that the rate of the lactate–pyruvate exchange is considerably faster than the NAD–NADH exchange thereby demonstrating that the coenzyme is added to the enzyme before interaction with lactate or pyruvate. Although the present experiments were not designed to bring out differences among alternative pathways, several calculations were carried out based on schemes other than that of Theorell–Chance for the derivation of equations using the rules and other details described in the theoretical section of this paper. The results obtained on assuming that the slow step is the interconversion of ternary complexes were so far from observed values of k_3, k_4, and k_5 as to rule out the possibility that the slow step in the lactate dehydrogenase reaction is determined by the above interconversion.

Perhaps the most useful aspect of the present analysis is its application for calculating isotope-exchange rates from known values of the specific rate constants of a reaction as obtained from conventional initial rate measurements. Knowing the enzymatic activity in a particular tissue or organ which is in a state of equilibrium or quasiequilibrium, the equilibrium velocity may be calculated and compared with measured rates (Hoberman, 1965). For example, the equilibrium velocity of the transfer of hydrogen from lactate to malate, as described by eq 1 and 2, is related to the equilibrium velocities of the separate reaction by application of rule I

$$1/V_e = 1/V_{LDH} + 1/V_{MDH} \qquad (31)$$

where the subscripts refer to the reactions catalyzed by lactate dehydrogenase and malate dehydrogenase, respectively.

V_{MDH} may be calculated with the use of eq 25 and known values of the kinetic constants for the reaction

catalyzed by MDH so that V_e can be estimated at any set of conditions. This extension of the analysis to coupled redox systems is in progress, and preliminary results are consistent with predictions based on the present treatment.

Appendix

A general proof of eq 7 is presented in this section. We consider a reaction proceeding by n consecutive steps

$$A_0 \underset{}{\overset{V_1}{\rightleftharpoons}} A_1 \overset{V_2}{\rightleftharpoons} A_2 \overset{V_3}{\rightleftharpoons} \cdots \overset{V_{i-1}}{\rightleftharpoons} A_{i-1} \overset{V_i}{\rightleftharpoons} A_i \overset{V_{i+1}}{\rightleftharpoons} A_{i+1} \overset{V_{n-1}}{\rightleftharpoons} \cdots \overset{V_n}{\rightleftharpoons} A_n$$

The equilibrium rate is the rate of transfer of an atom, or group of atoms, from A_0 to A_n, when all consecutive reactions are at thermodynamic equilibrium. This rate is classically measured by introducing a labeled reactant (as done in this paper), but can also be measured without the introduction of label by nuclear magnetic resonance and other relaxation methods. More than one reactant may participate in each step, but only the one containing the "exchangeable" atom(s) designated A_i need to be considered.

If the last sojourn of a molecule containing an exchangeable atom (or atoms) was in A_0 and not in A_n at the end of a sequence, we say that the molecule originates in A_0. The molecules of any intermediate A_i are comprised of two populations: one, A_i^0, originating at A_0 and the other, A_i^n, originating at A_n (the contribution of a third population, those shuttling back and forth without seeing A_0 or A_n, becomes zero if we look far enough back in time). We shall designate X_i the fraction of the molecules in A_i originating in A_0, *i.e.*, $X_i = A_i^0/A_i$.

A_i^0 can be divided into further subpopulations, according to the time each molecule last reached A_i and according to the direction from which it came (it should be remembered that each atom can shuttle back and forth along the system so that an atom, even if it originates on the left, may finally arrive at any A_i from the right).

We define a_ϑ as the number of molecules originating in A_0 and arriving in A_i in the time interval d_ϑ.

$$A_i^0 = \int_0^\infty a_\vartheta d\vartheta$$

Since all molecules in an intermediate are chemically identical the rate at which a_ϑ molecules leave A_i in either direction is proportional to their fraction in the population

$$-\frac{da_\vartheta}{dt} = V_i\frac{a_\vartheta}{A_i} + V_{i+1}\frac{a_\vartheta}{A_i}$$

This expression can be integrated between $t = \vartheta$ and $t = 0$, to give an expression for a_ϑ

$$-\int_{a_\vartheta^{in}}^{a_\vartheta}\frac{da_\vartheta}{a_\vartheta} = \frac{V_i + V_{i+1}}{A_i}\int_\vartheta^0 dt$$

a_ϑ^{in}, which stands for the original value of a_ϑ at $t = -\vartheta$, is simply $a_\vartheta^{in} = V_iX_{i-1} + V_{i+1}X_{i+1}$ (all a_ϑ molecules came from A_{i-1}, A_{i+1}; none had yet time to leave A_i). We get thus

$$a_\vartheta = (V_iX_{i-1} + V_{i+1}X_{i+1})e^{-(V_i+V_{i+1})\vartheta/A_i}$$

To obtain A_i^0 we integrate a_ϑ over the whole range of ϑ ($\vartheta = 0$ to ∞)

$$A_i^0 = \int_0^\infty a_\vartheta d\vartheta = (V_iX_{i-1} + V_{i+1}X_{i+1})\int_0^\infty e^{-(V_i+V_{i+1})\vartheta/A_i}d\vartheta$$

which gives

$$X_i = \frac{A_i^0}{A_i} = \frac{V_iX_{i-1} + V_{i+1}X_{i+1}}{V_i + V_{i+1}}$$

This last expression gives us a relation between neighboring populations of molecules originating in A_0. There are $n - 1$ equations of this type, one for each intermediate. In addition, $X_0 = 1$ and $X_n = 0$, by definition. We can obtain an expression for each X_i by solving the set of equations. To do this we rewrite the last equation

$$V_i(X_{i-1} - X_i) = V_{i+1}(X_i - X_{i+1})$$

or in full

$$V_1(1 - X_1) = V_2(X_1 - X_2)$$
$$V_2(X_1 - X_2) = V_3(X_2 - X_3)$$
$$\vdots \qquad \vdots$$
$$V_{i-1}(X_{i-2} - X_{i-1}) = V_i(X_{i-1} - X_i)$$
$$V_i(X_{i-1} - X_i) = V_{i+1}(X_i - X_{i-1})$$
$$\vdots \qquad \vdots$$
$$V_{n-1}(X_{n-2} - X_{n-1}) = V_nX_{n-1}$$

It is immediately seen that all the terms, on the right as well as on the left, are equal. For each, it can be written

$$V_i(X_{i-1} - X_i) = V_nX_{n-1}$$

or

$$X_i = X_{i-1} - V_nX_{n-1}/V_i$$

This means that each X_i can be obtained from the next lower one, by subtracting the term V_nX_{n-1}/V_i. Since

$X_0 = 1$, we can sum up all differences to give

$$X_i = 1 - V_n X_{n-1} \sum_{1}^{i} 1/V_i$$

and specifically

$$X_{n-1} = 1 - V_n X_{n-1} \sum_{1}^{n-1} 1/V_i$$

which can be rearranged to give finally for X_{n-1}

$$X_{n-1} = 1/\left(1 + V_n \sum_{1}^{n-1} 1/V_i\right) = \frac{1}{V_n \sum_{1}^{n} 1/V_i}$$

The equilibrium rate is the rate at which atoms originating at A_0 arrive at A_n. This rate is given by the rate at which the atoms of the last intermediate originating at A_0 proceeds to A_n

$$V = V_n X_{n-1}$$

We introduce the expression derived for X_{n-1}, and this gives the final expression (eq 7) for the equilibrium rate of n consecutive reactions.

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