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Kinetic Studies of Rabbit Muscle Lactate Dehydrogenase. II. Mechanism of the Reaction*

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ABSTRACT: The rabbit muscle lactate dehydrogenase enzyme system has been investigated at *p*H 7.15 in tris(hydroxymethyl)aminomethane-chloride buffer. On the basis of information obtained from product inhibition studies, the relationship among the ϕ constants, and the Haldane relationship, it was concluded that a modification of the Theorell-Chance mechanism, involving the isomerization of at least the enzyme-oxidized coenzyme complex, is in harmony with the experimental data. Oxalate inhibition studies indicated that a primarily ordered pathway of substrate addition to the enzyme appears to be operative with the coenzyme substrates adding first. The maximum dissociation constant for the enzyme-oxidized coenzyme-oxalate complex was calculated. Oxalate at higher concentrations may interact with the free enzyme. This effect can be detected when pyruvate and reduced nicotinamide-adenine dinucleotide are substrates, but not when nicotinamide-adenine dinucleotide and L-lactate are used, because in the latter instance it is not possible to obtain measurable velocities in the presence of high concentrations of inhibitor.

Rabbit muscle lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase, EC 1.1.1.27) has been studied kinetically (Zewe and Fromm, 1962) and fluorometrically (Fromm, 1963) in our laboratory. In a previous report (Zewe and Fromm, 1962) it was concluded, on the basis of product inhibition studies, that the reaction catalyzed by rabbit muscle lactate dehydrogenase may be represented by a simple Theorell-Chance (Theorell and Chance, 1951) mechanism in which ternary enzyme-substrate complexes are short-lived relative to the binary complexes (Alberty, 1953).

Recently Thomson and co-workers (Thomson and Darling, 1962; Thomson *et al.*, 1964) have questioned the validity of the assignment of the simple Theorell-Chance mechanism to rabbit muscle lactate dehydrogenase on the basis of their studies with deuterated NADH. They proposed that another mechanism, perhaps involving one or more ternary complexes, or one of the modifications of the Theorell-Chance mechanism proposed by Mahler *et al.* (1962), was applicable. The purpose of this report will be to examine the mechanism of this enzyme in greater detail in light of additional experimental data obtained from a study of the reaction at various *p*H values (V. Zewe and H. J. Fromm, in preparation) in the presence of reaction product, and in the presence of oxalate.

The simplest plausible reaction scheme for rabbit muscle lactate dehydrogenase appears to be a modification of the Theorell-Chance mechanism in which the enzyme-oxidized coenzyme complex undergoes isomerization. Reasons for arriving at this conclusion will be treated more fully here.

Experimental Procedure

Materials. Lactate dehydrogenase from rabbit muscle was purchased from California Corp. for Biochemical Research and was purified according to the procedure reported by Fromm (1963). Zinc L-lactate was a product of Pfanstiehl Laboratories, Inc. NAD^+ was purchased from Pabst Laboratories and was purified by column chromatography on DEAE-cellulose (Dalziel, 1963a) using Tris-chloride buffer, *p*H 7.6 (0.2 M, reservoir, 0.005 M, mixer), as the eluting buffer. The nucleotide was isolated as the barium salt. NADH was prepared

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from the chromatographed NAD^+ by reduction with yeast alcohol dehydrogenase. Oxalic acid was a Mallinckrodt analytical grade reagent, and Tris was a product of the Sigma Chemical Co.

Determinations. Sodium pyruvate was assayed with excess NADH and lactate dehydrogenase at *p*H 6.9, and pyruvate concentration was calculated from the disappearance of NADH at 340 mμ, using 6.22×10^3 M^{-1} cm^{-1} as the molar extinction coefficient for the nucleotide (Horecker and Kornberg, 1948). The sodium pyruvate used in these studies was 99–100% pure on a weight basis. Pyruvate solutions were prepared immediately before use.

Zinc L-lactate was converted to the acid form with Dowex 50 (H^+) (Brin, 1953), neutralized with sodium hydroxide, and lyophilized. The concentration was determined colorimetrically using enzymatically prepared L-lactate as a standard (Barker and Summerson, 1941). The sodium salt of NAD^+ was prepared by addition of sodium sulfate to an acidified solution of barium NAD^+, followed by centrifugation.

Prior to each kinetic study approximately 20 μmoles of sodium NAD^+ were treated with excess ethanol and yeast alcohol dehydrogenase in unneutralized Tris buffer and incubated at room temperature. When the reaction was complete, as determined spectrophotometrically, the entire reaction mixture was pipetted onto a water-cooled (4°) DEAE-cellulose column (1 × 8 cm) previously washed with 0.2 M Tris-chloride buffer, *p*H 7.6, and 0.005 M Tris-chloride buffer, *p*H 7.6. Gradient elution with 0.005 M Tris-chloride buffer, *p*H 7.6 (mixer), and 0.2 M Tris-chloride buffer, *p*H 7.6 (reservoir), followed, and 10-ml fractions were collected. NADH prepared in this manner had a ratio (A_{260}/A_{340}) of 2.25.

The kinetics of the reaction were investigated in Tris-chloride buffer, *p*H 7.15, because this was the buffer selected for the *p*H studies (V. Zewe and H. J. Fromm, in preparation). Potassium chloride was added to give a final ionic strength of 0.6 μ; final buffer concentration in the reaction mixture was 0.15 μ.

The enzyme was appropriately diluted in 0.1 M sodium phosphate buffer, *p*H 6.9, containing 1 mg/ml bovine serum albumin (Armour Co.), and was maintained at 0° prior to use.

Samples were incubated at 28° and kinetic measurements were carried out at 28° in the Cary spectrophotometer, 0–0.1 slide wire, using 2.5-cm cuvets. Velocity measurements were corrected to a constant amount of enzymic activity; velocity is expressed as the molar concentration of NADH formed or disappearing per 80 seconds.

Results

The values for the kinetic parameters for the rabbit muscle lactate dehydrogenase system in Tris-chloride buffer, *p*H 7.15, are given in Table I. These are quite similar to those obtained in the previous case when sodium phosphate buffer was used (Zewe and Fromm, 1962).

TABLE I: Kinetic Parameters of the Lactate Dehydrogenase System in Tris-Chloride Buffer, *p*H 7.15.[a]

V_f	3.58×10^{-6} M/80 sec
K_{NAD}	1.69×10^{-4} M
$K_{L\text{-lactate}}$	1.09×10^{-2} M
$K_{NAD\text{-}L\text{-lactate}}$	5.10×10^{-6} M^2
V_r	16.7×10^{-6} M/80 sec
K_{NADH}	7.43×10^{-6} M
$K_{pyruvate}$	2.09×10^{-4} M
$K_{NADH\text{-}pyruvate}$	1.14×10^{-9} M^2

[a] The values for the kinetic parameters were determined from the original Lineweaver-Burk (1934) plots according to the method of Florini and Vestling (1957).

The mode of enzyme and substrate interaction in the lactate dehydrogenase system was reexamined, using as criteria the correlation between the experimentally determined equilibrium constant and that calculated from the various parameters (Alberty, 1953), the Dalziel relationships (Dalziel, 1957), which were observed experimentally over the *p*H range from 5.8 to 9 (V. Zewe and H. J. Fromm, in preparation), and the product inhibition patterns (Alberty, 1958; Fromm and Nelson, 1962).

Haldane Relationship. Alberty (1953) has described in detail the value of correlating the apparent equilibrium constant determined experimentally with the kinetic parameters obtained for an enzymatically catalyzed reaction of the type, $A + B \rightleftarrows C + D$.[1] According to the Haldane relationship, mechanisms which are consistent with equation (1)

$$V_f/v = 1 + K_A/A + K_B/B + K_{AB}/AB \qquad (1)$$

should obey the following relationship:

$$K_{app} = V_f K_{CD}/V_r K_{AB} \qquad (2)$$

Furthermore, if the simple Theorell-Chance mechanism is applicable, the following condition should also be fulfilled:

$$K_{app} = V_f{}^3 K_C K_D/V_r{}^3 K_A K_B \qquad (3)$$

At *p*H 7.15, the apparent equilibrium constant is 6.04×10^{-5} (V. Zewe and H. J. Fromm, in preparation), and the values obtained by substitution of the appropriate kinetic parameters into equations (2) and (3) are 5.93×10^{-5} and 9.35×10^{-6}, respectively. The discrepancy between the apparent equilibrium constant and equation

[1] A, B, C, and D are substrates involved in the general reaction, $A + B \rightleftarrows C + D$; K_A, K_B, K_C, and K_D are the corresponding apparent Michaelis constants; K_{AB} and K_{CD} are complex constants defined by Alberty (1953); V_f and V_r are the maximal reaction velocities for the forward and reverse directions, respectively. In this report, K_A is K_{NAD}, K_B is $K_{L\text{-lactate}}$, K_C is K_{NADH}, and K_D is $K_{pyruvate}$.

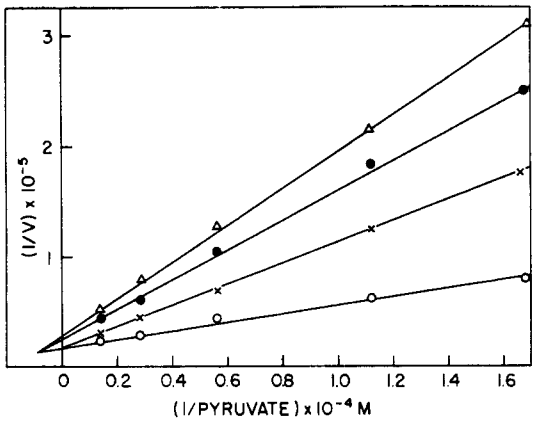

FIGURE 1: Plot of reciprocal of initial reaction velocity (v) versus the reciprocal of the molar concentration of pyruvate in the absence and presence of L-lactate. The concentrations of L-lactate are: (○), none; (×), 3.60×10^{-2} M; (●), 7.20×10^{-2} M; and (△), 9.60×10^{-2} M. NADH concentration was maintained constant at 3.82×10^{-5} M, and pyruvate was varied in the range from 5.95×10^{-5} to 7.15×10^{-4} M. Velocity is expressed as the molar concentration of NADH formed in, or disappearing from, the reaction mixture in 80 seconds after the addition of enzyme. Kinetic measurements were made in the Cary spectrophotometer, 0–0.1 slide wire, in 2.5-cm cuvets. Tris-chloride buffer, pH 7.15, was used. Other experimental details are described under Experimental Procedure.

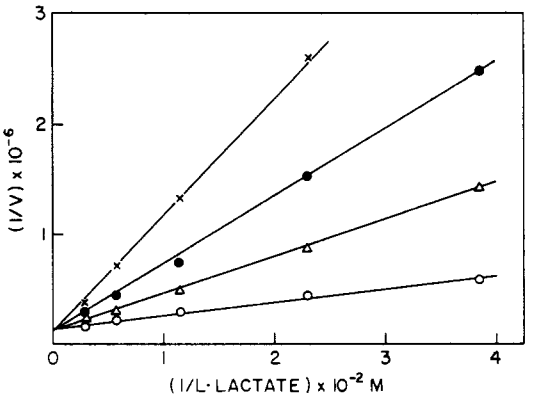

FIGURE 2: Plot of reciprocal of initial reaction velocity (v) versus the reciprocal of the molar concentration of L-lactate in the absence and presence of pyruvate. The concentrations of pyruvate are: (○), none; (△), 3.90×10^{-5} M; (●), 7.80×10^{-5} M; and (×), 1.56×10^{-4} M. NAD^+ concentration was maintained constant at 5.89×10^{-4} M, and L-lactate varied in the range from 2.60×10^{-3} to 3.48×10^{-2} M. Experimental conditions and the expression for velocity are given in Figure 1. Other details are described under Experimental Procedure.

(3) is apparent not only at this pH, but throughout the pH range 6–8.9 (V. Zewe and H. J. Fromm, in preparation). This is one indication that the simple Theorell-Chance mechanism previously proposed (Zewe and Fromm, 1962) may not be applicable to the lactate dehydrogenase system. In the previous case it was felt that the discrepancy between the apparent equilibrium constant calculated from equations (2) and (3) and the experimentally observed value was such that the Haldane relationship could not be employed to permit a choice of mechanism to be made from among those which are consistent with equation (1). Part of this difficulty may be traced to the fact that, in the former instance (Zewe and Fromm, 1962), the value used for the apparent equilibrium constant at 28° was obtained from a Van't Hoff plot of the effect of temperature on the apparent equilibrium constant (Hakala *et al.*, 1956), and, as such, the value was subject to considerable error of estimation. In the present case, the equilibrium constant was measured under experimental conditions similar to those employed for the kinetic studies and no such extrapolation was required.

Dalziel Relationship. Another criterion useful in the elucidation of reaction mechanisms is the relationship among the various ϕ constants (Dalziel, 1957). Throughout the pH range studied, the product of the appropriate constants from the pyruvate side of the reaction ($\phi_1'\phi_2'/\phi_{12}'$) was consistently less than the corresponding ϕ_o of the forward direction (V. Zewe and H. J. Fromm, in preparation). Considered in itself, such a relationship is indicative of a reaction scheme in which ternary enzyme-substrate complexes are kinetically significant. However, when the ϕ relationship was considered from the lactate side of the reaction, different results were obtained. Now $\phi_1\phi_2/\phi_{12}$ was greater than ϕ_o'. This was true in the pH range from 6 to 7.6, inclusive. Since none of the mechanisms considered by Dalziel (1957) gives a relationship of this type, the possibility was raised that an unsymmetrical mechanism, possibly involving inactive or isomeric binary complexes, was operative (Mahler *et al.*, 1962). A second indication of the occurrence of coenzyme isomerization was the discrepancy between the magnitude of the maximal velocity term and the value of the smallest rate constant in the reaction sequence. This has been shown to be the case with many of the dehydrogenase systems which have been studied to date (Cleland, 1963). The possibility of isomerization in the rabbit muscle lactate dehydrogenase system was indicated by the fact that in the previous report (Zewe and Fromm, 1962), the expression for V_r was 1.00×10^{-5} M/10 min, while k_2, the rate constant for the dissociation of the enzyme-NAD^+ complex, was 7.63×10^{-5} M/10 min. No such discrepancy existed when V_f and k_5 were compared. Similarly, calculation of rate constants, assuming the simple Theorell-Chance mechanism to be operative throughout the pH range studied, revealed that from pH 6 to 7.6 V_r was consistently greater than k_2 (V. Zewe and H. J. Fromm, in preparation). No evidence for isomerization of the enzyme-NADH complex was indicated, for V_f was always less

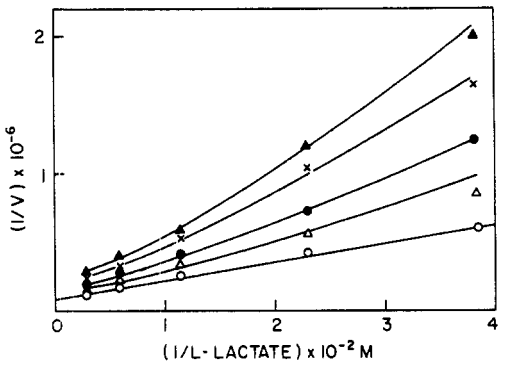

FIGURE 3: Plot of reciprocal of initial reaction velocity (v) versus the reciprocal of the molar concentration of L-lactate in the absence and presence of NADH. The concentrations of NADH are: (○), none; (△), 3.98 × 10^{-6} M; (●), 7.88 × 10^{-6} M; (×), 1.58 × 10^{-5} M, and (▲), 2.25 × 10^{-5} M. NAD^+ concentration was maintained constant at 5.35 × 10^{-4} M, and L-lactate was varied in the range from 2.58 × 10^{-3} to 3.40 × 10^{-2} M. Experimental conditions and the expression for velocity are given in Figure 1. Other details are described under Experimental Procedure.

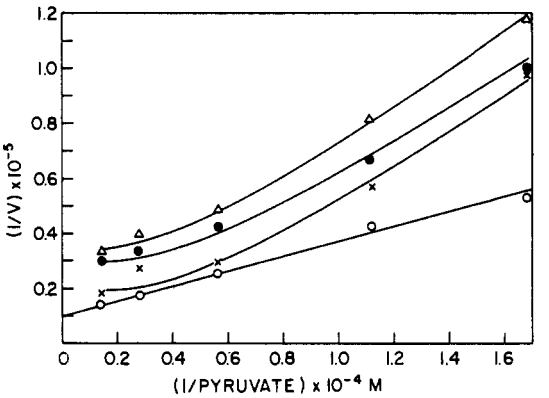

FIGURE 4: Plot of reciprocal of initial reaction velocity (v) versus the reciprocal of the molar concentration of pyruvate in the absence and presence of NAD^+. The concentrations of NAD^+ are: (○), none; (×), 3.80 × 10^{-4} M; (●), 1.51 × 10^{-3} M; and (△), 2.06 × 10^{-3} M. NADH concentration was maintained constant at 3.20 × 10^{-5} M, and pyruvate was varied in the range from 5.90 × 10^{-5} to 7.15 × 10^{-4} M. Experimental conditions and the expression for velocity are given in Figure 1. Other details are described under Experimental Procedure.

than any of the rate constants calculated for the sequence, assuming the simple Theorell-Chance mechanism.

Product Inhibition Studies. Valuable information concerning the reaction mechanism may also be obtained from kinetic studies conducted in the presence of reaction product (Alberty, 1958; Fromm and Nelson, 1962). The experimental results are shown in Figures 1 to 4. Experimental conditions are indicated in the legends to the figures. These patterns differ from those reported previously (Zewe and Fromm, 1962) in only one important respect. When the reciprocal of pyruvate concentration is plotted in Lineweaver-Burk fashion (Lineweaver and Burk, 1934) in the absence and presence of product L-lactate at a constant level of NADH, the lines intersect, not on the $1/v$ axis, but to its left (see Figure 1). This same effect (intersection of the Lineweaver-Burk plots to the left of the $1/v$ axis) was obtained in the pH range from 6 to 8.5, and thus cannot be dismissed as a function of the hydrogen ion concentration. It should be noted, however, that this effect occurs at the higher concentrations of L-lactate. At the lowest level of L-lactate shown in Figure 1, the inhibited and uninhibited lines appear to converge at a common point on the $1/v$ axis. In an earlier report (Zewe and Fromm, 1962) similar data were obtained (Figure 9); however, the significance of this observation was not recognized at that time.

Mechanism of the Reaction.[2] Possible reaction schemes were considered in an effort to satisfy the experimentally observed Haldane relationship, the ϕ relationships, and the product inhibition patterns. The results are summarized in Table II. Cursory examination of mechanisms I to III in Table II reveals that the ternary complex and isoternary complex mechanisms are not compatible with the experimental data, for neither the product inhibition patterns (mechanism II) nor the ϕ relationship and the product inhibition patterns (mechanisms I and III) agree with the kinetic results. Although it is conceivable that the effect of product D on the intercept of a 1/B plot is so small that it cannot be seen experimentally, the fact that a negative value for a rate constant was consistently calculated whenever the ternary complex mechanism or one of its modifications was considered suggested that perhaps another explanation could be found for the experimental data.

When the simple Theorell-Chance mechanism (Table II, mechanism IV) was assumed operative at pH values from 5.8 to 9 (V. Zewe and H. J. Fromm, in preparation) all six rate constants could be calculated, although the V_r–k_2 discrepancy was present. Therefore, steady-state rate equations for various modifications of the Theorell-Chance mechanisms were derived and compared with the experimental results.

One of the first possibilities considered involved the rapid and reversible formation of inactive enzyme-NAD^+ and enzyme-NADH complexes. This mechanism had been previously treated by Mahler *et al.* (1962) and Dalziel (1963b) (Table II, mechanism V). The ϕ relationships agree with those obtained experimentally. However, when acid product is included along with substrates, the steady-state rate equations predict no effect of inhibitor on the intercepts of the Lineweaver-Burk plots when 1/acid substrate is varied

[2] For those mechanisms cited below in which ternary complexes are not kinetically significant (Alberty, 1953), they are deleted in order to simplify the derivations.

TABLE II: Theorell-Chance and Ternary Complex Mechanisms and Modifications.

Mechanism	Steps Included in Sequence[a]	ϕ Relationships		Acid Product Inhibition Patterns[b]
		Forward	Reverse	
(I)	1,4,7,9; ternary	$\phi_1\phi_2/\phi_{12} < \phi_o'$	$\phi_1'\phi_2'/\phi_{12}' < \phi_o$	Forward—1;[c] reverse—3[c]
(II)	1,2,3,6,9; ternary, EA′ reactive	$\phi_1\phi_2/\phi_{12} > \phi_o'$	$\phi_1'\phi_2'/\phi_{12}' < \phi_o$	Forward—1;[c] reverse—3[c]
(III)	1,2,4,7,9; ternary, EA reactive	$\phi_1\phi_2/\phi_{12} < \phi_o'$	$\phi_1'\phi_2'/\phi_{12}' < \phi_o$	Forward—1;[c] reverse—3[c]
(IV)	1,4,7,9; simple T-C[d]	$\phi_1\phi_2/\phi_{12} = \phi_o'$	$\phi_1'\phi_2'/\phi_{12}' = \phi_o$	Forward—2;[c] reverse—4[c]
(V)	1,1a,4,7,9,9a; T-C,[d] EX and EY inactive	$\phi_1\phi_2/\phi_{12} > \phi_o'$[e]	$\phi_1'\phi_2'/\phi_{12}' < \phi_o$[e]	Forward—2;[f] reverse—3[f]
(VI)	1,2,3,6,9; T-C,[g] EA′ reactive	$\phi_1\phi_2/\phi_{12} > \phi_o'$	$\phi_1'\phi_2'/\phi_{12}' < \phi_o$	Forward—2;[h] reverse—3[h]
(VII)	1,2,4,7,9; T-C,[d] EA reactive	$\phi_1\phi_2/\phi_{12} = \phi_o'$	$\phi_1'\phi_2'/\phi_{12}' = \phi_o$	Forward—2;[f] reverse—3[f]
(VIII)	1,2,3,5,8,9; T-C,[i] EA′ and EC′ reactive	$\phi_1\phi_2/\phi_{12} > \phi_o'$[j]	$\phi_1'\phi_2'/\phi_{12}' < \phi_o$[j]	Forward—1;[k] reverse—3[k]

[a] Steps in sequence are as follows: (1) $E + A \underset{2}{\overset{1}{\rightleftharpoons}} EA$; (1a) $E + A \rightleftharpoons EX$, K_1; (2) $EA \underset{4}{\overset{3}{\rightleftharpoons}} EA'$; (3) $EA' + B \underset{6}{\overset{5}{\rightleftharpoons}} EA'B$; (4) $EA + B \underset{8}{\overset{7}{\rightleftharpoons}} EAB$; (5) $EA'B \underset{10}{\overset{9}{\rightleftharpoons}} EC' + D$; (6) $EA'B \underset{12}{\overset{11}{\rightleftharpoons}} EC + D$; (7) $EAB \underset{14}{\overset{13}{\rightleftharpoons}} EC + D$; (8) $EC' \underset{16}{\overset{15}{\rightleftharpoons}} EC$; (9) $EC \underset{18}{\overset{17}{\rightleftharpoons}} E + C$; (9a) $EY \rightleftharpoons E + C$, K_2. [b] (1) 1/A and 1/B—D affects both slope and intercept. (2) 1/A—D affects slope and intercept; 1/B—D affects slope, no effect on intercept. (3) 1/C and 1/D—B affects slope and intercept. (4) 1/C—B affects slope and intercept; 1/D—B affects slope, no effect on intercept. [c] Abortive complex EAD assumed in forward; ECB in reverse. [d] $k_{13} \gg k_{17}$ and $k_8 \gg k_2$. [e] ϕ relationship depends upon the relative values of k_1K_1 and $k_{18}K_2$. [f] Abortive complex EAD assumed in forward; ECB and EB in reverse. [g] $k_{11} \gg k_{17}$ and $k_6 \gg k_2$. [h] Abortive complex EA′D assumed in forward; ECB and EB in reverse. [i] $k_9 \gg k_{17}$ and $k_6 \gg k_2$. [j] ϕ relationship depends upon the relative values of k_3 and k_{18}. [k] Abortive complexes EAD and EA′D assumed in forward; ECB and EC′B in reverse.

in the presence of acid product. This is the case even when the abortive ternary complexes EAD and ECB are assumed. It should be pointed out that this mechanism is the same as that derived by Dalziel (1962) to take into account the effect of inhibitors present in the coenzyme preparations. In view of the fact that all coenzyme preparations used in these studies have been purified by chromatography prior to use in the kinetic experiments, it seems reasonable to assume that inhibitors are not present. For the same reason it can be assumed that a limiting case of mechanism V in which only one inactive enzyme-coenzyme complex is formed is probably not applicable to the experimental data.

A second type of iso-Theorell-Chance mechanism, analogous to the type already discussed for the ternary complex (mechanism III), involves the formation of an unreactive EA′ complex (see Table II, mechanism VII). Since the Dalziel relationships predict that the products $\phi_1\phi_2/\phi_{12} = \phi_o'$ and $\phi_1'\phi_2'/\phi_{12}' = \phi_o$, and these results were not obtained experimentally, this mechanism was eliminated from consideration.

Another possibility treats of the formation of an isomeric enzyme-oxidized coenzyme complex, while in the last iso-Theorell-Chance mechanism to be considered, the assumption is made that both enzyme-oxidized coenzyme and enzyme-reduced coenzyme complexes undergo isomerization. The rate constant–maximal velocity discrepancy is seen experimentally only when the maximal velocity for the reverse direction and the rate constant for the dissociation of the enzyme-oxidized coenzyme complex are compared; a similar result is not obtained when the maximal velocity for the forward direction and the rate constant for the breakdown of the enzyme-reduced coenzyme complex are compared. However, it is conceivable that both isomerizations do occur, although only one can be detected experimentally in the presence of the other.

The simpler situation will be treated first (Table II, mechanism VI):

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA$$

$$EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA'$$

$$EA' + B \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EC + D$$

$$EC \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + C$$

Here E, A, EA, EA′, B, EC, D, and C represent free enzyme, NAD^+, enzyme-NAD^+ complex, enzyme-isomerized NAD^+ complex, L-lactate, enzyme-NADH complex, pyruvate, and NADH, respectively. The steady-state rate equations in the forward and reverse directions are obtained by setting D and B equal to zero in equations 4 and 5 in the Appendix. Consideration of the Dalziel relationship (Dalziel, 1957) leads to the conclusion that the product of $\phi_1\phi_2/\phi_{12}$ is equal to

$(k_2 + k_3)(k_3 + k_4)/k_2k_3k_4$, while ϕ_o' is $(k_2 + k_3 + k_4)/k_2k_4$. The former expression contains a $1/k_3$ term not present in the latter. Thus, $\phi_1\phi_2/\phi_{12}$ will be greater than ϕ_o', which is the experimental observation. When the ϕ relationship from the reverse direction is considered, $\phi_1'\phi_2'/\phi_{12}'$ is equal to $1/k_7$, while the corresponding ϕ_o is equal to $(k_3 + k_7)/k_3k_7$. It is apparent that the relationship $\phi_1'\phi_2'/\phi_{12}'$ will be less than ϕ_o, a result which is obtained experimentally.

This mechanism also fulfills another of the criteria employed in the elucidation of reaction mechanisms—the Haldane relationship (Alberty, 1953). The ratio of ϕ_{12}'/ϕ_{12} or V_fK_{CD}/V_rK_{AB} leads to the expression $k_1k_3k_5k_7/k_2k_4k_6k_8$, which is the equilibrium constant for the reaction. Substitution of the appropriate kinetic parameters into equation (3) leads to a complex expression of rate constants which is not equal to the expression for equilibrium.

Inspection of the product inhibition pattern (Figure 2) reveals that, when 1/L-lactate is varied in the presence of a constant amount of NAD^+ at several concentrations of pyruvate, the lines intersect at a common point on the $1/v$ axis. A common intercept is obtained even when the concentration of pyruvate is increased four times over the highest concentration shown in Figure 2. However, in order to obtain measurable velocities, it is necessary at the same time to increase the L-lactate concentration 3-fold. When 1/pyruvate is varied in the presence of L-lactate, somewhat different results are obtained. At the lowest level of L-lactate, the inhibited and uninhibited lines appear to intersect at the same point on the $1/v$ axis. As the concentration of L-lactate is increased, it becomes apparent that these lines intersect the uninhibited line at a common point to the left of the $1/v$ axis (Figure 1).

The existence of the abortive ternary complexes enzyme-NAD^+-pyruvate and enzyme-NADH-L-lactate has been demonstrated spectrophotometrically (Fromm, 1961) and fluorometrically (Fromm, 1963). In order to explain the experimental data, further consideration of enzyme-substrate complexes is necessary. One of the most obvious possibilities in the above mechanism is the formation of an unreactive EAB complex. Although this assumption would readily explain the product inhibition data when 1/pyruvate is varied in the presence of L-lactate, the steady-state rate equation calculated, using this assumption, is at variance with the experimental data obtained when 1/L-lactate is varied in the absence and presence of pyruvate. In this case the equation would predict an effect of pyruvate concentration on both the slope and intercept terms of a 1/B double-reciprocal plot. In the presence of an EAB complex, the B term of equation (4) in the Appendix would be modified as follows: $(k_6D + k_7)[(1 + D/K_1) + k_4(1 + B/K)/k_3]/k_5k_7B$, where K is the dissociation constant for the EAB complex. It is readily apparent upon simplification that the B terms in the numerator and denominator will cancel, leaving an intercept term containing D. Since EAB would be formed in the absence of product, it would be reasonable to assume that at high concentrations of L-lactate a decreased initial reaction velocity would be observed. Such an effect could not be demonstrated at *p*H 7.15 with L-lactate concentrations up to 0.13 M at a NAD^+ concentration of 5.22×10^{-4} M.

Evidence is available from equilibrium isotope exchange studies that the reactants can also bind in reverse order. It has been shown (Silverstein and Boyer, 1964) that at equilibrium with high levels of pyruvate and L-lactate the NAD^+-NADH exchange rate is not completely depressed, but occurs at approximately 0.01 times that of the pyruvate-L-lactate exchange. Since the pyruvate-L-lactate exchange under these conditions takes place along the preferred pathway [E-NAD^+ $\rightleftarrows$ intermediate complexes $\rightleftarrows$ E-NADH], while the NAD^+-NADH exchange represents the alternate pathway [E-L-lactate $\rightleftarrows$ intermediate complexes $\rightleftarrows$ E-pyruvate], it is possible to suggest that a small percentage of the reaction takes place along the alternate pathway. Although such a pathway may not be of kinetic significance ordinarily, stress conditions could accentuate its importance. Thus, when high concentrations of second substrate are present, the occurrence of E–second substrate complexes may be of kinetic importance. With the support of this experimental evidence, an alternate explanation for the product inhibition data involves the formation of an enzyme-L-lactate complex at high concentrations of L-lactate.

An indication of enzyme-inhibitor complex formation is also available from kinetic studies conducted in the presence of oxalate. Oxalate is a competitive inhibitor of L-lactate and acts uncompetitively with respect to NAD^+ (see Figures 5 and 6); this is compatible with a compulsory order of substrate addition to the enzyme with the coenzyme adding first (Fromm, 1964). When the oxalate used to inhibit the reverse reaction is in the same concentration range as that used to inhibit the forward reaction, the dicarboxylic acid also acts uncompetitively with respect to NADH (see Figure 8). At higher concentrations, however, both slope and intercept terms of a 1/NADH plot are affected by the inhibitor concentration. Such a result is interpreted in terms of an EI complex. Novoa *et al.* (1959) have postulated the formation of an enzyme-oxalate complex in their studies with the beef heart enzyme.

In light of the kinetic data the steady-state rate equations in the presence of reaction product were derived and the formation of these abortive complexes were postulated:

$$EA' + D = EA'D; \quad K_1$$

$$EC + B = ECB; \quad K_2$$

$$E + B = EB; \quad K_3$$

The K's represent the dissociation constants for the complexes. If the assumption is made that only the reactive form of the enzyme-oxidized coenzyme complex is capable of reacting with acid, the EAD complex does not exist. This assumption takes precedence in the fact that the EAB complex apparently is not kinet-

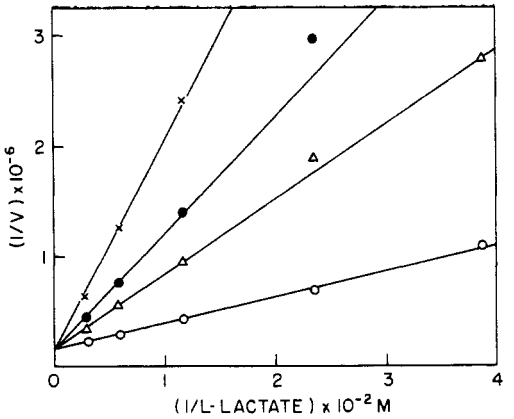

FIGURE 5: Plot of reciprocal of initial reaction velocity (v) versus the reciprocal of the molar concentration of L-lactate in the absence and presence of oxalate. The concentrations of oxalate are: (○), none; (△), 0.87×10^{-4} M; (●), 1.74×10^{-4} M; and (×), 3.47×10^{-4} M. NAD^+ concentration was maintained constant at 5.39×10^{-4} M, and L-lactate varied in the range from 2.58×10^{-3} to 3.40×10^{-2} M. Experimental conditions and the expression for velocity are given in Figure 1. Other details are described under Experimental Procedure. Maximal values for the dissociation constant for the enzyme-NAD^+-oxalate complex calculated from equation (9) are 1.48×10^{-3} M, 1.67×10^{-3} M, and 1.65×10^{-3} M at oxalate concentrations of 0.87×10^{-4} M, 1.74×10^{-4} M, and 3.47×10^{-4} M, respectively.

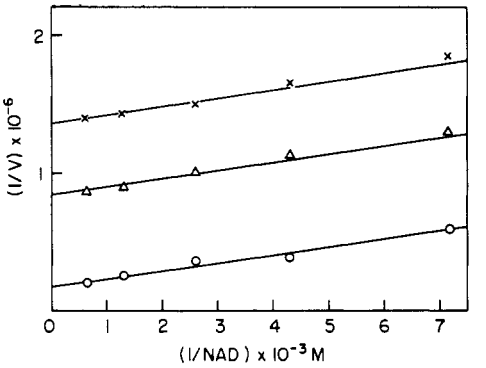

FIGURE 6: Plot of reciprocal of initial reaction velocity (v) versus the reciprocal of the molar concentration of NAD^+ in the absence and presence of oxalate. The concentrations of oxalate are: (○), none; (△), 1.74×10^{-4} M; and (×), 3.47×10^{-4} M. L-Lactate concentration was maintained constant at 1.27×10^{-2} M, and NAD^+ was varied in the range from 1.40×10^{-4} to 1.56×10^{-3} M. Experimental conditions and the expression for velocity are given in Figure 1. Other details are described under Experimental Procedure. Maximal values for the dissociation constant for the enzyme-NAD^+-oxalate complex calculated from equation (9) are 1.57×10^{-3} and 1.76×10^{-3} M at oxalate concentrations of 1.75×10^{-4} and 3.47×10^{-4} M, respectively.

ically significant. In view of the fact that the concentrations of pyruvate used as inhibitor never exceeded 1.57×10^{-4} M, an ED complex was not included. However, under experimental conditions in which the concentration of pyruvate would be much higher, an ED complex may be of kinetic significance. The steady-state rate equation in the presence of product D is given in the Appendix (equation 4). Visual inspection of equation (4) reveals that in a 1/B plot product D affects only the slope term of a double-reciprocal plot. This result agrees with the experimental data (see Figure 2). The equation in the presence of product L-lactate is given in the Appendix, equation (5). This equation predicts an effect of product B on both the slope and intercept terms of the Lineweaver-Burk plots. This is due to the fact that, because of the high concentrations of L-lactate required to produce inhibition, the L-lactate also reacts with the free enzyme, and this effect is seen on the intercept term, $1/k_8C$, in a 1/D plot.

It must be pointed out that the assumption of an EB complex would render the product inhibition equations of mechanisms V and VII compatible with the kinetic results. However, the Dalziel relationship of mechanism VII is not compatible with the experimental results, so this assumption still would not reconcile this mechanism with the data. The ϕ relationships predicted from mechanism V are obtained experimentally. However, in view of the fact that the dead-end enzyme-coenzyme complexes result from the presence of inhibitor along with the coenzyme, and purified coenzyme preparations were used in the studies, this mechanism does not seem as likely as mechanisms VI and VIII. It is not possible with the kinetic techniques used currently to distinguish among mechanisms V, VI, and VIII.

The steady-state rate equations in the presence of coenzyme product are given as equations (6) and (7) in the Appendix. Both equations predict that the double-reciprocal plots of 1/coenzyme substrate in the absence and presence of coenzyme product share a common intercept on the $1/v$ axis (see Figures 11 and 13 in Zewe and Fromm, 1962), while plots of 1/acid substrate in the presence of coenzyme should be nonlinear (Figures 3 and 4). As the figures indicate, these predictions are compatible with the experimental results.

The possibility exists that the opposite situation can explain the kinetic results, i.e., isomerization of the enzyme-NADH complex, but not the enzyme-NAD^+ complex. Since the ϕ relationships predicting employing such a mechanism are the opposite of those obtained experimentally, this mechanism was not considered further.

The last possibility involves the formation of two isomeric enzyme-coenzyme complexes (Mahler *et al.*, 1962; Dalziel, 1963b) and may be represented as follows (Table II, mechanism VIII):

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA$$

$$EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA'$$

$$EA' + B \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EC' + D$$

$$EC' \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EC$$

$$EC \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E + C$$

Here E, A, EA, EA′, B, EC′, D, EC, and C represent free enzyme, NAD^+, enzyme-NAD^+ complex, enzyme-isomerized NAD^+ complex, L-lactate, enzyme-isomerized NADH complex, pyruvate, enzyme-NADH complex, and free NADH, respectively. The steady-state rate equation for this mechanism is obtained by setting D equal to zero in equation (8) in the Appendix. Since this is a symmetrical mechanism, the equation for the reverse direction is obtained simply by substitution of the rate constants for the reverse direction into equation (8). The Dalziel (1957) and Haldane (Alberty, 1953) relationships for this mechanism are also compatible with the experimental results.

In the product inhibition equation given in the Appendix (equation 8), the abortive complexes EAD and EA′D in the forward direction and ECB and EC′B in the reverse were included.

$$EA + D = EAD; \quad K_1$$

$$EA' + D = EA'D; \quad K_2$$

$$EC + B = ECB; \quad K_3$$

$$EC' + B = EC'B; \quad K_4$$

The equation for the reverse direction is obtained by the substitution of the appropriate rate constants for the reverse direction, B/K_3 for D/K_1, and B/K_4 for D/K_2. Equation (8) predicts the effect of product D on the intercept term of a 1/B plot. This result was not obtained experimentally (see Figure 2), but the equation may be made compatible with the experimental data by assuming any of the following conditions to be present. First of all, it is entirely possible that pyruvate combines only with the reactive EA′ complex. In this case EAD would not exist and all K_1-containing terms could be deleted from the equation. An alternate explanation involves the relative magnitude of the D/k_3K_1 term compared with the rest of the intercept term. If its contribution is slight, the effect of D on the intercept will not be detected, regardless of how high the concentration of pyruvate becomes. A third explanation involves the existence of the enzyme-NAD^+-pyruvate complex under the experimental conditions employed. The dissociation constant for this complex calculated from fluorescence is 0.3 mM (Fromm, 1963). Since the highest pyruvate concentration employed to effect inhibition was only 0.157 mM, it is reasonable to assume either that the complex is not formed at all or that the amount present is so small that it is not kinetically significant. All of these explanations appear to be valid, and any one of them will render the steady-state rate equation compatible with the experimental data. The equation for the reverse direction predicts an effect of B concentration on the intercept term of a 1/D plot; in this case it is not necessary to postulate the interaction of the free enzyme with B. The effect of B on the intercept term results from the formation of an abortive ECB complex. The dissociation constant for this complex calculated from fluorescence titration is 38 mM (Fromm, 1963). As illustrated in Figure 1, the effect of B on the intercept of the double-reciprocal plot does not begin to become apparent until its concentration is in excess of the dissociation constant for the complex. As the concentration of inhibitor B is increased, its effect on the intercept becomes more apparent. This, too, appears to be a valid interpretation of the product inhibition patterns.

The rate equations accounting for the presence of coenzyme products are of the same form as equations (6) and (7) and are therefore compatible with the experimental data.

Oxalate Inhibition Studies. The effect of oxalate as an inhibitor has been briefly discussed. Such studies were undertaken in an effort to provide experimental evidence which would shed additional light on the reaction mechanism. In Figures 5–8 are Lineweaver-Burk plots of the reaction in both forward and reverse directions showing the effect of oxalate as inhibitor. As was the case with the beef heart enzyme (Novoa *et al.*, 1959) oxalate apparently functions as a competitive inhibitor of L-lactate, while inhibition with respect to NAD^+ appears uncompetitive. Thus,

$$EA' + I = EA'I; \quad K_i$$

If the combination of oxalate with the enzyme-NADH complex is kinetically significant in this direction, the intercept of the double-reciprocal plot should be affected when L-lactate concentration is varied in the presence of different concentrations of oxalate. The fact that there is a common intercept on the $1/v$ axis indicates that this compound is not readily discerned experimentally (Figure 5). The combination of oxalate with the unreactive EA complex does not appear to be kinetically significant for the same reason, for an effect of oxalate concentration on the intercept should be detectable. The steady-state rate equation in the forward direction showing the effect of oxalate as inhibitor is given in the Appendix, equation (9). Because not all the values for rate constants can be calculated, the value for the dissociation constant for the EA′I complex can be obtained only as the product k_5K_i. Since the value for k_5 can be no smaller than the maximal velocity in this direction, the maximum value for the dissociation constant of the EA′I complex at *p*H 7.15 is 1.58×10^{-3} M.

In the reverse direction (Figure 8) oxalate at high concentrations affects both the slope and intercept terms of a 1/NADH plot, while at concentrations comparable to those used to effect inhibition in the forward direction oxalate inhibition with respect to NADH appears uncompetitive. In a 1/pyruvate plot, at high and low

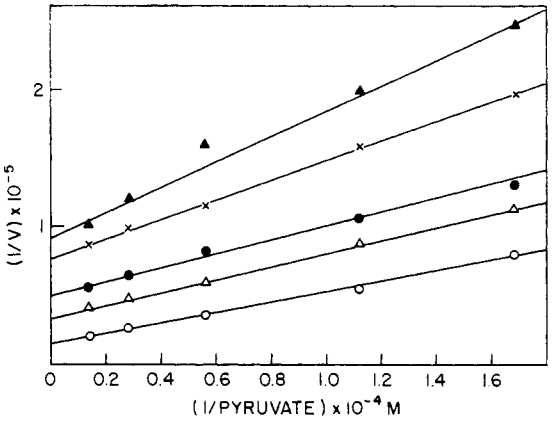

FIGURE 7: Plot of reciprocal of initial reaction velocity (v) versus the reciprocal of the molar concentration of pyruvate in the absence and presence of oxalate. The concentrations of oxalate are: (○), none; (△), 8.34×10^{-4} M; (●), 1.39×10^{-3} M; (×), 2.78×10^{-3} M; and (▲), 4.16×10^{-3} M. NADH concentration was maintained constant at 2.80×10^{-5} M, and pyruvate was varied in the range from 5.95×10^{-5} to 7.15×10^{-4} M. Experimental conditions and the expression for velocity are given in Figure 1. Other details are described under Experimental Procedure.

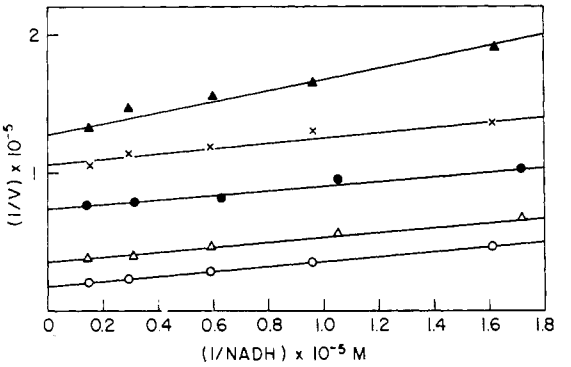

FIGURE 8: Plot of reciprocal of initial reaction velocity (v) versus the reciprocal of the molar concentration of NADH in the absence and presence of oxalate. The concentrations of oxalate are: (○), none; (△), 6.95×10^{-4} M; (●), 2.78×10^{-3} M; (×), 4.16×10^{-3} M; and (▲), 6.25×10^{-3} M. Pyruvate was maintained constant at 3.58×10^{-4} M and NADH was varied in the range from 6.44×10^{-6} to 6.65×10^{-5} M. Experimental conditions and the expression for velocity are given in Figure 1. Other details are described under Experimental Procedure.

concentrations of oxalate, both slope and intercept terms appear to be affected (Figure 7). The results in the reverse direction may be explained by assuming the formation of an EI complex (E + I = EI; K_{ii}). This is a reasonable assumption, for higher concentrations of oxalate were required for inhibition in the reverse direction than in the forward. The effect of inhibitor concentration on the slope and intercept terms of Figure 7 may be explained by assuming the formation of an ECI complex (EI + C = ECI; K_{iii}). In the steady-state rate equation derived assuming this complex (equation 10), the inhibitor concentration affects both slope and intercept terms of 1/C and 1/D plots. Results obtained in the forward direction (Figure 5) indicate that the formation of an ECI complex via the pathway EC + I = ECI apparently is not kinetically significant. A simplier explaination of data of Figures 7 and 8 would be to assume that ECI is not formed, in whice case terms containing K_{iii} would be deleted in equation 10.

In summary, the use of oxalate as a competitive inhibitor of L-lactate leads to the conclusion that with rabbit muscle lactate dehydrogenase a compulsory order of substrate addition appears to predominate, with the coenzymes adding first (Fromm, 1964). The results in the reverse direction cannot be interpreted simply and the interaction of oxalate with the free enzyme may occur.

Discussion

Evidence is available from independent studies that the reaction catalyzed by rabbit muscle lactate dehydrogenase involves a compulsory order of substrate addition to the enzyme, with the coenzymes adding first. These include equilibrium isotope exchange kinetics (Silverstein and Boyer, 1964), fluorescence titration experiments (Fromm, 1963), and studies with deuterated coenzymes (Thomson *et al.*, 1964). The purpose of the present report is to study the reaction mechanism in more detail in an effort to determine if kinetically significant ternary complexes are formed. Conclusions reached concerning the reaction mechanism were based upon the relationship between the experimentally determined equilibrium constant and the value calculated from the various kinetic parameters (Alberty, 1953), the Dalziel relationship (Dalziel, 1957), and the unsymmetrical product inhibition pattern with respect to acid substrate and acid product in the forward and reverse directions. For the reasons enumerated, it is thought that the simple Theorell-Chance mechanism is not applicable to the enzyme system under consideration, but that an iso-Theorell-Chance mechanism can adequately explain the experimental data. Several of the iso-Theorell-Chance mechanisms considered are kinetically indistinguishable and appear in harmony with the experimental data; thus a choice cannot be made among them. Although mechanism V (Table II), which postulates the formation of inactive enzyme-coenzyme complexes, could be made compatible with the experimental data by assuming the formation of an EB complex, its applicability to the enzyme system under discussion is considered less likely than mechanisms VI or VIII because purified coenzymes were used in all studies.

The iso-Theorell-Chance mechanisms discussed

(mechanisms VI and VIII) fulfill the criteria proposed by Thomson and co-workers (1964) which they said are indicative of a pathway involving kinetically significant ternary complexes. Because the mechanisms proposed in this report predict the ϕ relationships observed at neutral *p*H by both the authors and Thomson *et al.* (1964), i.e., $\phi_1\phi_2/\phi_{12} > \phi_o'$ and $\phi_1'\phi_2'/\phi_{12}' < \phi_o$, such a criterion may not be presented as unequivocal evidence that kinetically significant ternary complexes are formed. Furthermore, the primary isotope effect detected in the ϕ_o' term may be reasonably explained in two ways. The first explanation treated in a previous report (Fromm, 1963) involves the effect of deuterated NADH on the magnitude of the rate constants and will not be discussed further here. An alternate explanation is based upon the argument that Thomson and co-workers (Thomson and Darling, 1962; Thomson *et al.*, 1964) have presented no experimental evidence indicating that the reaction mechanism is the same in the presence of the deuterated coenzyme and its hydrogen analog. Since the sole difference between the Theorell-Chance mechanism and the ternary complex mechanism is the lifetime of the ternary enzyme-substrate complex, an effect on the rate constant could make the ternary complex become kinetically significant.

Finally, the possibility remains that Thomson's group (Thomson and Darling, 1962; Thomson *et al.*, 1964) is using an isozyme of the rabbit muscle enzyme with catalytic properties different from those presented in this report. In a recent report (Thomson *et al.*, 1964) is shown a graph of the effect of *p*H on lactate dehydrogenase, and the *p*H optimum from the pyruvate side of the reaction was observed at 7.4. Under our experimental conditions, with both Tris-chloride and phosphate buffers, the *p*H optimum from the pyruvate side of the reaction occurred at 6.25. We were unable to duplicate the results of Thomson *et al.* (1964) although we used their experimental conditions and an enzyme preparation from the same source (Sigma Chemical Co.). In view of the lack of agreement on such a comparatively self-evident property as the *p*H optimum, the authors feel that the comparison of something as subtle as the lifetime of a ternary complex cannot be validly drawn.

Intensive investigation of the lactate dehydrogenase system was not extended to other *p*H values, and it is entirely possible that in more alkaline regions a mechanistic change has occurred. However, the results obtained from studies conducted at *p*H 7.15 appear to be compatible with an iso-Theorell-Chance mechanism.

Appendix

Equations (4) to (10) were derived according to the steady-state method for the mechanisms discussed in the text. For the sake of completeness, the abortive ternary complexes enzyme-reduced coenzyme-L-lactate in the forward direction and enzyme-oxidized coenzyme-pyruvate in the reverse direction are included in equations (4) and (5). Since the enzyme-reduced coenzyme L-lactate complex in the forward direction and the enzyme-oxidized coenzyme-pyruvate complex in the reverse direction are formed whether or not product is present, the terms are common to both the inhibited and uninhibited equations. The kinetic significance of these abortive complexes is indicated by a decreased initial reaction velocity at high concentrations of the acid substrate. In the substrate concentration range employed in the kinetic studies, this effect was not detected in the absence of reaction product. When coenzyme product is present along with the reactants, the enzyme-reduced coenzyme-L-lactate complex in the forward direction and the enzyme-oxidized coenzyme-pyruvate complex in the reverse direction do become kinetically significant and are the cause of the nonlinear product inhibition data shown in Figures 3 and 4.

$$E_o/v = [k_3(1 + B/K_2) + k_7]/k_3k_7 + (k_2 + k_3)/k_1k_3A + (k_6D + k_7)[(1 + D/K_1) + k_4/k_3]/k_5k_7B + k_2k_4(k_6D + k_7)/k_1k_3k_5k_7AB \quad (4)$$

$$E_o/v = [(k_2 + k_3)(1 + D/K_1) + k_4]/k_2k_4 + (1 + B/K_3)/k_8C + (1 + B/K_2)[1 + k_5B(k_2 + k_3)/k_2k_4]/k_6D + k_7(1 + B/K_3)[1 + k_5B(k_2 + k_3)/k_2k_4]/k_6k_8CD \quad (5)$$

$$E_o/v = 1/k_3 + (1 + B/K_2)/k_7 + (k_2 + k_3)[1 + k_8C(1 + B/K_2)/k_7]/k_1k_3A + (k_3 + k_4)/k_3k_5B + k_2k_4[1 + k_8C(1 + B/K_2)/k_7]/k_1k_3k_5AB \quad (6)$$

$$E_o/v = [k_4 + (1 + D/K_1)(k_2 + k_3)]/k_2k_4 + [1 + k_1A/k_2 + k_1k_3A(1 + D/K_1)/k_2k_4]/k_8C + 1/k_6D + k_7[1 + k_1A/k_2 + k_1k_3A(1 + D/K_1)/k_2k_4]/k_6k_8CD \quad (7)$$

For the sake of completeness the abortive ternary complexes ECB and EC'B are included in the rate equation in the forward direction although they apparently exert no effect on the kinetics of the system in the absence of the coenzyme product.

$$E_o/v = (1 + D/K_1)/k_3 + (1 + B/K_3)/k_9 + (k_8 + k_9)(1 + B/K_4)/k_7k_9 + (k_2 + k_3)/k_1k_3A + (1 + D/K_2)[1 + k_6D(k_8 + k_9)/k_7k_9]/k_5B + k_4(1 + D/K_1)[1 + k_6D(k_8 + k_9)/k_7k_9]/k_3k_5B + k_2k_4[1 + k_6D(k_8 + k_9)/k_7k_9]/k_1k_3k_5AB \quad (8)$$

Oxalate inhibition:

$$E_o/v = [k_3(1 + B/K_2) + k_7]/k_3k_7 + (k_2 + k_3)/k_1k_3A + [k_3(1 + I/K_1) + k_4]/k_3k_5B + k_2k_4/k_1k_3k_5AB \quad (9)$$

$$E_o/v = [k_4 + (k_2 + k_3)(1 + D/K_1)(1 + I/K_i)]/k_2k_4 + I/k_8K_{ii}K_{iii} + (1 + I/K_{ii})/k_8C + [1 + k_7I/k_8K_{ii}K_{iii}]/k_6D + k_7(1 + I/K_{ii})/k_6k_8CD \quad (10)$$

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