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Studies on the Mechanism of Enzyme-Catalyzed Oxidation-Reduction Reactions. VI.* Kinetic Studies with Yeast L(+)-Lactate Dehydrogenase

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The kinetics of the reaction catalyzed by crystalline L-lactate dehydrogenase of yeast have been investigated in a sodium pyrophosphate–acetic acid buffer at *p*H 7.5 and 20°, with L-lactate as the substrate and ferricyanide, cytochrome *c*, or 2,6-dichlorophenolindophenol as the acceptor. The observed rate-law is of the form $E_t/v_0 = \phi_0 + \phi_1/S + \phi_2/A + \phi_{12}/SA + P(\phi_0' + \phi_1'/S + \phi_2'/A + \phi_{12}'/SA)$, where E_t, S, A, and P are the stoichiometric concentrations in moles/liter of enzyme, lactate, acceptor, and pyruvate, and the ϕ's are empirical parameters. With relatively concentrated ferricyanide (0.24–0.97 mM) the reaction is shown to be consistent with the following mechanism: $E \underset{k_2}{\overset{k_1S}{\rightleftharpoons}} ES \underset{k_4 \simeq 0}{\overset{k_3A}{\rightleftharpoons}} EXY \underset{k_6P}{\overset{k_5}{\rightleftharpoons}} EZ \xrightarrow{k_7} EZ' + A_{red}$; $EZ' \xrightarrow{k_9A} E + A_{red}$; $E \underset{k_{12}}{\overset{k_{11}P}{\rightleftharpoons}} EP$. With indophenol as acceptor the above mechanism is satisfied but with $k_9 \simeq 0$. With ferricyanide at low concentration and with cytochrome *c* a different mechanism appears to be involved: $E \underset{k_2}{\overset{k_1S}{\rightleftharpoons}} ES \xrightarrow{k_3} EX + P$; $EX \xrightarrow{k_5A} EY + A_{red}$; $EY \xrightarrow{k_7A} E + A_{red}$; $E \underset{k_{11}}{\overset{k_9P}{\rightleftharpoons}} EP$. The dissociation constants for the enzyme-substrate complex have been calculated for all these cases and were found to be acceptor-independent and equal to $\sim 6 \times 10^{-4}$ M.

Yeast L(+)-lactate dehydrogenase, commonly referred to as cytochrome b_2, was first crystallized by Appleby and Morton (1954, 1959a), in whose laboratories many of the properties of the enzyme were characterized (Appleby and Morton, 1959a,b, 1960; Appleby *et al.*, 1960; Armstrong *et al.*, 1960; Morton *et al.*, 1961; Morton and Armstrong, 1961; Morton, 1961). In an effort to learn more about the mechanism of action of this enzyme which possesses both riboflavin-5′-monophosphate and protoheme as prosthetic groups (Appleby and Morton, 1954, 1959b; Boeri *et al.*, 1955), the kinetics of the enzyme-catalyzed reaction have been investigated in some detail. These kinetic studies were inaugurated (*a*) to decide whether during an enzyme-catalyzed reaction employing the same oxidative enzyme a different sequence of reactions must be postulated when different acceptors are used to oxidize the substrate, (*b*) to decide whether this oxidation proceeds *via* a ternary complex, (*c*) to obtain some indication concerning the numerical values of certain critical equilibrium and rate constants, and (*d*) if possible to choose between or eliminate previously postulated sequences of reactions postulated for the enzyme-catalyzed reaction (Morton *et al.*, 1961; Morton and Armstrong, 1961). Such a study of this particular enzyme seemed desirable, since kinetic and mechanistic parameters established in this instance could have implications for other flavoenzymes and even serve as a soluble model system for the particulate enzymes involved in electron transport. As a basis for these kinetic studies the procedure developed by Dalziel (1957) for two-substrate systems was utilized for an analysis of the data. It has also proved helpful to study the effects of added product upon the kinetic parameters, as suggested by Alberty (1958).

With the use of Dalziel's notation the following modified general rate equation may be formulated as shown in equation (1), where E_t = total molarity of

$$\frac{E_t}{v_0} = \phi_0 + \frac{\phi_1}{S} + \frac{\phi_2}{A} + \frac{\phi_{12}}{S \cdot A} + P\left(\phi_0' + \frac{\phi_1'}{S} + \frac{\phi_2'}{A} + \frac{\phi_{12}'}{S \cdot A} + \frac{\phi_3 P}{S \cdot A}\right) \quad (1)$$

the enzyme; v_0 = initial velocity of the enzyme-catalyzed reaction; ϕ = empirical constants, generated by combinations of particular rate constants characteristic of any postulated mechanism; S =

* For paper V of this series see Fernandez *et al.* (1962).

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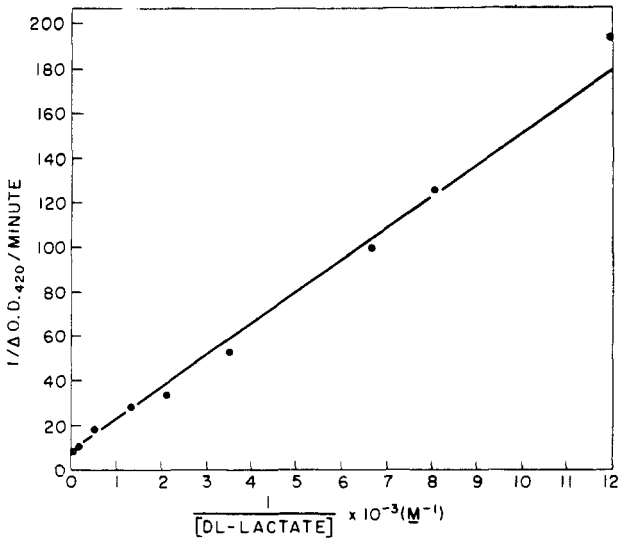

FIG. 1.—Lineweaver-Burk plot for a wide range of DL-lactate concentrations. Sodium DL-lactate was varied from 8.3×10^{-5} M to 5.35×10^{-2} M. Potassium ferricyanide was used as the acceptor and was 6.7×10^{-4} M. All experiments were done in sodium pyrophosphate–acetic buffer (260 μmoles in 3.0 ml) and at 20°.

substrate concentration in moles/liter; A = concentration of second substrate or acceptor in moles/liter; P = concentration of the product formed from S in moles/liter. Initially, when $P = 0$, equation (1) simplifies to the simple Dalziel equation since all terms multiplied by P vanish.

Morton's group (Morton *et al.*, 1961; Morton and Armstrong, 1961) have previously shown that ϕ_{12} is equal to zero when cytochrome *c* is used as acceptor and has a finite and positive value when ferricyanide is used. The present investigations corroborate and extend their findings.

Experimental Procedure

Reagents.—Horse heart cytochrome *c* was obtained from Sigma Chemical Co., St. Louis, Mo. The calcium salt of L(+)-lactate was obtained from the California Corporation for Biochemical Research, Los Angeles. After removal of the calcium by precipitation with sodium pyrophosphate the *p*H of the resulting solution was adjusted to the desired value with either 0.1 M acetic acid or 0.1 M sodium pyrophosphate. The disodium salt of ethylenediaminetetraacetate (EDTA, Versene) was white-label-grade material from Eastman Organic Chemicals Division, Distillation Products Industries, Rochester, N. Y. Practical-grade acetone was redistilled over potassium permanganate. The fraction boiling between 55° and 56° was collected and used for the enzyme preparation. *n*-Butanol was redistilled from practical-grade butanol, and the fraction boiling from 115–117° was utilized for the enzyme preparation. Sodium pyruvate was C.P. grade from Schwarz Laboratories, Inc., Mt. Vernon, N. Y. All other reagents were reagent-grade materials available from commercial sources. Crystalline yeast L(+)-lactate dehydrogenase was prepared from air-dried Red Star baker's yeast according to the procedure given by Appleby and Morton (1959a). Unless stated otherwise, stock enzyme solutions which were stored in 0.5 M DL-lactate under nitrogen at −20° were utilized for all experiments. Prior to use, the enzyme solution was frequently diluted 20- to 50-fold into 0.1 M pyrophosphate-acetate buffer, *p*H 7.5, to reduce the level of the lactate concentration in the solution. This dilution allowed a greater range of lactate concentrations to be used in these studies.

Reaction Velocity Determinations.—All velocity determinations were performed at room temperature, in an air-conditioned room controlled to a temperature of about 20°, and performed spectrophotometrically by means of a Cary Model 11 recording spectrophotometer equipped with a slide wire suitable for the absorbancy range 0–0.1. Plümpers (polyethylene enzyme spoons) were a gift of California Corporation for Biochemical Research. All reagents were placed into a 1.0-cm light path absorption cell and brought to a total volume of 3 ml. The reaction was initiated by the addition of 5–25 μl of enzyme solution stirred into the reaction mixture with either a polyethylene or a lucite enzyme spoon (see Baker, 1960, for details of construction). All calculations involving spectrophotometric determinations utilized the following mM extinction coefficients: 1.04 at 420 mμ for ferricyanide (Appleby and Morton, 1959a), 8.9 at 550 mμ for oxidized cytochrome *c*, 29.9 at 550 mμ for reduced cytochrome *c*, 21 at 550 mμ for the differential extinction coefficient (reduced minus oxidized) for cytochrome *c* (Massey, 1959), 38.8 at 557 mμ and 232 at 424 mμ for reduced YLDH,[1] 199 at 265 mμ for YLDH, 23.9 at 557 mμ for the differential extinction coefficient of YLDH (Appleby and Morton, 1959b), and 21 at 600 mμ for 2,6-dichlorophenolindophenol (Steyn-Parve and Beinert, 1958).

Graphic Determinations.—All ϕ values were graphically determined by the method of Dalziel (1957) and represent the averages of the duplicate values obtained by that method. The ϕ_0 and ϕ_1 values in the duplicate determinations with ferricyanide as acceptor (0.102 mM–1.02 mM) were within ±3% and ±9%, respectively, of the average values. ϕ_2 values varied from ±26% to ±70% of the average values, while duplicate values of ϕ_{12} ranged from ±5% to ±30% of the average. When the acceptor was cytochrome *c*, ϕ_1 and ϕ_2 were within ±5% and ϕ_0 was within ±30% of the average values.

Results

Kinetics of Reaction with Ferricyanide as Acceptor.—The most extensive investigations were performed when potassium ferricyanide was employed as the final electron acceptor. As seen from Figure 1 the reciprocal of the velocity of the enzyme-catalyzed reaction as a function of the reciprocal substrate concentration is linear over a wide range (8.3×10^{-5} M to 5.35×10^{-2} M) of L-lactate concentrations. Although not evident from the plot shown in Figure 1, there is a slight substrate inhibition at the highest lactate concentrations used.

When the reciprocal velocity of the enzyme-catalyzed reaction is plotted as a function of the reciprocal of the ferricyanide concentration (Fig. 2), a biphasic curve is obtained. This suggests the possibility that two different mechanisms are operating which depend upon the concentration of ferricyanide employed as the acceptor. A further indication that two different mechanisms may be operating was obtained after determination of the Dalziel kinetic parameters. As shown in Table I, ϕ_{12} has vastly different values which depend upon the concentration range of acceptor employed. In the more concentrated ranges of ferricya-

[1] The following abbreviations will be used in this paper: YLDH, crystalline yeast L(+)-lactate dehydrogenase; EDTA, ethylenediaminetetraacetate; OD, optical density; TN, μmoles acceptor reduced per minute per mole of enzyme bound heme.

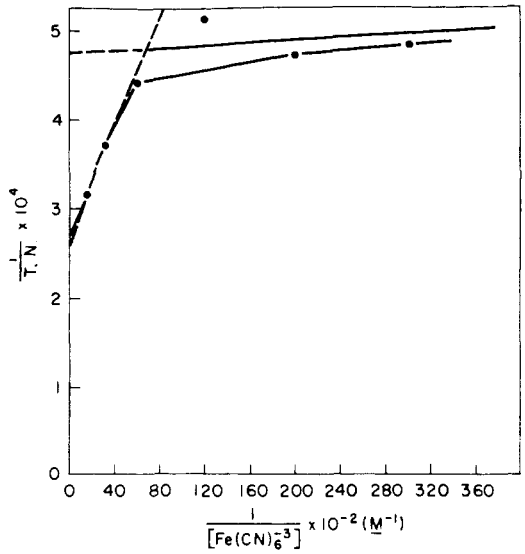

FIG. 2.—Lineweaver-Burk plot as ferricyanide is varied. The reaction mixture contained 250 μmoles of sodium pyrophosphate–acetic acid buffer and 12 μmoles of sodium DL-lactate at *p*H 7.5 in a total volume of 3.0 ml. The temperature was 20°.

nide (0.102 mM to 1.02 mM; see experiments 1–3) ϕ_{12} has a finite positive value, while with the more dilute concentrations (6.03×10^{-2} mM to 0.24 mM; see experiment 4) ϕ_{12} is virtually equal to zero. Thus, with one acceptor two different mechanisms may be operating.

TABLE I
COMPARISON OF DALZIEL KINETIC PARAMETERS AT *p*H 7.5[a]
(ferricyanide as acceptor)

Expt. No.	Conc. of Ferricyanide (mM)	ϕ_0 (sec. $\times 10^{3}$)	ϕ_1 (M sec. $\times 10^{6}$)	ϕ_2 (M sec. $\times 10^{7}$)	ϕ_{12} (M^2 sec. $\times 10^{10}$)
1	0.102–1.02	6.2	4.8	3.3	Uncertain
2	0.102–1.02	6.6	4.2	6.6	0.66
3[b]	0.102–1.02	6.0	4.6	2.5	2.4
4	0.06 –0.24	7.2	6.6	1.3	<0.01

[a] L(+)-Lactate was varied from 0.31 mM to 1.57 mM. Four concentrations of L(+)-lactate and four concentrations of ferricyanide were used. Enzyme was approximately 4.6×10^{-8} M for all experiments. Tenth molar acetate-pyrophosphate buffer, *p*H 7.5, was employed throughout this study. All values are for room temperature, *ca.* 20°. [b] A different enzyme preparation from that used for experiments 1, 2, and 4 was used for these experiments.

As seen from the data for experiments 1–3 of Table I the values of the kinetic parameters were quite reproducible at the higher concentration ranges of ferricyanide. This was especially true for the parameters ϕ_0 (reproducibility within 10%) and ϕ_1 (reproducibility within 14%), even when different preparations of enzyme were used.

Effects of Pyruvate on Lactate → Ferricyanide Reaction.—To obtain additional information concerning possible mechanisms of action of this enzyme-catalyzed reaction the effects of added product upon the reaction were investigated. As seen from Figure 3 the reciprocal of the velocity of the reaction plotted against pyruvate concentration is linear over a considerable range of concentrations of the latter. Data illustrated by Figure 4 show that inhibition caused by pyruvate is neither of a simple competitive nor of a simple non-

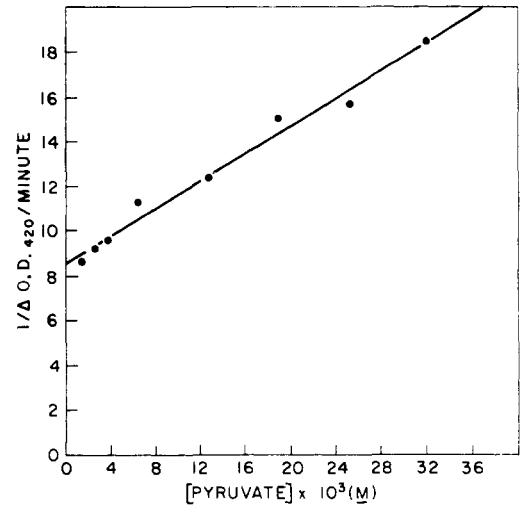

FIG. 3.—Effects of pyruvate on reaction velocity. The reaction mixture contained 12 μmoles of sodium DL-lactate, 2.0 μmoles of potassium ferricyanide, and 250 μmoles of sodium pyrophosphate–acetic acid buffer, *p*H 7.5, in a total volume of 3.0 ml.

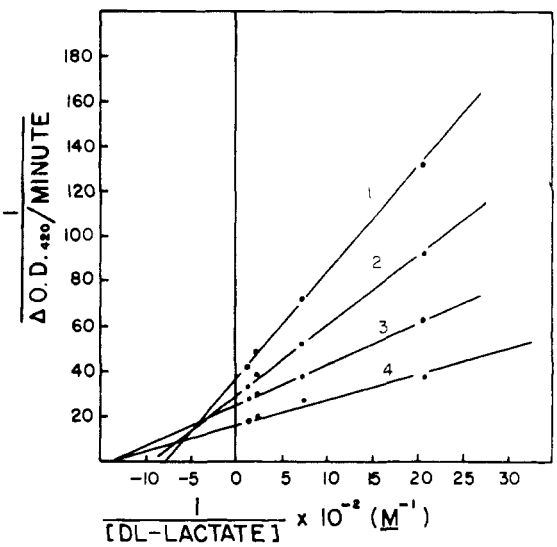

FIG. 4.—Lineweaver-Burk plot when pyruvate is used as inhibitor. Curve 1, 53.3 mM sodium pyruvate; curve 2, 26.6 mM sodium pyruvate; curve 3, 13.3 mM sodium pyruvate; curve 4, no pyruvate. 0.67 mM ferricyanide served as acceptor, the buffer used was 250 μmoles of sodium pyrophosphate–acetic acid, *p*H 7.5; temperature 20°.

competitive type. This behavior of pyruvate inhibition has been checked and verified three different times. It therefore appears that pyruvate has a rather complex effect upon the kinetics of the reaction. Krupka and Laidler (1961) have shown recently that such a complex plot will result if an inhibitor exhibits its action at two different intermediate stages of the enzyme-catalyzed reaction. On the other hand, ferrocyanide, the other product of the reaction, even when added in considerable excess, in no way affected the initial velocity of the reaction.

In Table II are shown the effects of pyruvate concentrations upon the kinetic parameters when the more concentrated ranges of acceptor were used. ϕ_0, ϕ_1, and ϕ_{12} showed apparent increases as the pyruvate concentration was increased, but there was little or no effect on ϕ_2. The most important observation is that there was an effect of the product upon ϕ_0, since it has

TABLE II

COMPARISON OF THE KINETIC PARAMETERS AS PYRUVATE CONCENTRATION IS INCREASED AT *p*H 7.5[a]

Pyruvate Molarity ($\times 10^3$)	ϕ_0 (sec. $\times$ $\times 10^3$)	Δ_0[b]	ϕ_1 (M sec. $\times 10^6$)	Δ_1[b]	ϕ_2 (M sec. $\times 10^7$)	Δ_2[b]	ϕ_{12} (M^2 sec. $\times 10^{10}$)	Δ_{12}[b]
0	6.6	1.0	4.7	1.0	2.9	1.0	?	—
2.46	7.2	1.2	5.7	1.2	3.0	1.0	1.4	—
6.15	9.0	1.5	6.6	1.4	2.1	0.73	1.4	—
12.3	11.0	1.7	7.8	1.7	2.4	0.83	1.8	—

[a] Ferricyanide was varied from 0.102 mM to 1.02 mM. L(+)-Lactate was varied from 0.33 mM to 1.57 mM. Four concentrations of each of the substrates were used. Enzyme was *ca.* 4.6×10^{-8} M. Tenth molar acetate-pyrophosphate buffers were employed. All values are for room temperature, *ca.* 20°. [b] $\Delta_m = \frac{\phi_m \text{ (with inhibitor)}}{\phi_m \text{ (without inhibitor)}}$ (see Baker, 1960).

been demonstrated (Alberty, 1958; Baker and Mahler, 1962) that this parameter is affected only when a ternary complex is formed during the course of the enzyme-catalyzed reaction.

A Possible Mechanism for the Lactate → Ferricyanide Reaction.—A mechanism for the enzyme-catalyzed reaction, with ferricyanide at a fairly elevated level as the electron acceptor, must be consistent with the following experimental facts:

1. The reciprocal of the initial velocity must be a linear function of: (*a*) the reciprocal of the substrate concentration (Fig. 1), (*b*) the concentration of the product formed from the substrate (Fig. 3), and (*c*) the reciprocal of the acceptor concentration (Fig. 2).
2. Inhibition caused by the product must appear to be neither of the simple competitive nor of the simple noncompetitive types (Fig. 4).
3. The initial velocity must not be affected by the reduced acceptor.
4. A ternary complex must be formed (Table II).

Considering all of the above relationships, the sequence of reactions shown in equation (2) for the dehydrogenation of lactate by ferricyanide in the presence of yeast L(+)-lactate dehydrogenase provides a simple system which may be modified to yield a steady state rate equation which agrees with the experimental findings.

$$(a)\ E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad (2)$$

$$(b)\ ES + A \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EXY$$

$$(c)\ EXY \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EZ + P$$

$$(d)\ EZ \xrightarrow{k_7} EZ' + A_{red}$$

$$(e)\ EZ' + A \underset{}{\overset{k_9}{\rightleftharpoons}} E + A_{red}$$

$$(f)\ E + P \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} EP$$

The general steady-state rate law in the direction of product formation derived from this proposed sequence is the same as equation (1), where now

$$\phi_0 = \frac{1}{2k_5} + \frac{1}{2k_7}$$

$$\phi_1 = \frac{1}{2k_1}$$

$$\phi_2 = \frac{k_4}{2k_3k_5} + \frac{1}{2k_3} + \frac{1}{2k_9}$$

$$\phi_{12} = \frac{k_2}{2k_1k_3}\left(\frac{k_4 + k_5}{k_5}\right)$$

$$\phi_3 = \frac{k_2k_4k_6k_{11}}{2k_1k_3k_5k_7k_{12}}$$

$$\phi_0' = \frac{k_6}{2k_5k_7}$$

$$\phi_1' = \frac{k_{11}}{2k_1k_{12}}$$

$$\phi_2' = \frac{k_4k_6}{2k_3k_5k_7}$$

$$\phi_{12}' = \frac{k_2}{2k_1k_3}\left(\frac{k_4k_{11}}{k_5k_{12}} + \frac{k_{11}}{k_{12}} + \frac{k_4k_6}{k_5k_7}\right)$$

The factor of 1/2 shown in the values of the ϕ constants is necessary to convert the observed initial velocity (based upon a one-electron acceptor) to the initial velocity based on substrate oxidation. Some important relationships between these constants are shown in (3).

$$(a)\ \frac{\phi_1}{\phi_1'} = \frac{k_{12}}{k_{11}} = \text{dissociation constant of } EP \text{ complex} = K_{EP}^{diss} \qquad (3)$$

$$(b)\ \frac{\phi_{12}}{\phi_1} = \frac{k_2}{k_3}\left(\frac{k_4 + k_5}{k_5}\right)$$

$$(c)\ \Delta_0 = \frac{\phi_0 \text{ (with inhibitor)}}{\phi_0 \text{ (without inhibitor)}} = \frac{\phi_0 + \phi_0'P}{\phi_0} \neq 1$$

$$(d)\ \Delta_1 = \frac{\phi_1 \text{ (with inhibitor)}}{\phi_1 \text{ (without inhibitor)}} = \frac{\phi_1 + \phi_1'P}{\phi_1} \neq 1$$

$$(e)\ \Delta_2 = \frac{\phi_2 \text{ (with inhibitor)}}{\phi_2 \text{ (without inhibitor)}} = \frac{\phi_2 + \phi_2'P}{\phi_1} \neq 1$$

$$(f)\ \Delta_{12} = \frac{\phi_{12} \text{ (with inhibitor)}}{\phi_{12} \text{ (without inhibitor)}} = \frac{\phi_{12} + \phi_{12}'P}{\phi_{12}} \neq 1$$

When $k_4 = 0$,

$$(g)\ \phi_2' = 0, \text{ therefore } \Delta_2 = 1$$

$$(h)\ \frac{\phi_{12}}{\phi_2} = \frac{k_2k_9}{k_1(k_3 + k_9)};\ \text{if } k_9 \gg k_3,\ \frac{\phi_{12}}{\phi_2} = \frac{k_2}{k_1} = K_{ES}^{diss};\ \text{if } k_9 = k_3,\ \frac{\phi_{12}}{\phi_2} = \frac{k_2}{2k_1}$$

$$(i)\ \phi_3 = 0$$

Since, as it stands, equation (1) is nonlinear with respect to the concentration of P (the product), a modification of this mechanism must be made to eliminate ϕ_3. Of all of the rate constants which contribute to ϕ_3 only k_4 may be considered to be negligibly small. This assumption eliminates ϕ_3 without adversely affecting the other rate parameters. Since it is not unreasonable that an acceptor such as ferricyanide with a high

oxidation-reduction potential (E_0' = 360 mv at *p*H 7.0) would accept electrons in an irreversible process as a consequence of its interaction with the *ES* complex, considering k_4 to be vanishingly small is probably justified. This mechanism is quite similar to one previously derived by Morton *et al.* (1961) for the same enzyme when similar concentrations of acceptor were used.

2,6-Dichlorophenolindophenol as Acceptor.—The enzyme-catalyzed reaction with this acceptor has not been investigated as thoroughly as that with the concentrated ranges of ferricyanide. Figure 5 indicates that the reciprocal of the velocity of the reaction is linear with the reciprocal of the acceptor concentration over a wide range of concentrations (1.06×10^{-6} M to 1.6×10^{-4} M). In contrast to the plot obtained when ferricyanide is used as the acceptor, the curve is monophasic.

TABLE III
KINETIC PARAMETERS FOR 2,6-DICHLOROPHENOLINDOPHENOL AS ACCEPTOR[a]

ϕ_0 (sec.)	ϕ_1 (M sec.)	ϕ_2 (M sec.)	ϕ_{12} (M^2 sec.)
4.0×10^{-2}	4.2×10^{-6}	3.9×10^{-7}	1.9×10^{-10}

[a] The dye was varied from 4.94×10^{-6} M to 4.94×10^{-5} M. L(+)-Lactate was varied from 1.3 mM to 8.1 mM. 0.1 M pyrophosphate-acetate buffer, *p*H 7.5, was used. All determinations were made at room temperature, *ca.* 20°.

As seen in Table III, the values for ϕ_{12} in the case of the dye as acceptor have real meaning and significance. Thus, it is probable that a mechanism similar to that previously proposed for the reaction with ferricyanide at high concentration as acceptor may also be applicable for the description of the reaction when this dye is used instead. A proposed sequence for the enzyme-catalyzed reaction in this instance and the accompanying rate equation derived from this sequence are shown in equation (4).

$$(a)\ E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad (d)\ EZ \xrightarrow{k_7} E + H_2A \qquad (4)$$

$$(b)\ ES + A \xrightarrow{k_3} EXY \qquad (e)\ E + P \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} EP$$

$$(c)\ EXY \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EZ + P$$

Rate Equation

$$(f)\ \frac{E_t}{v_0} = \phi_0 + \frac{\phi_1}{S} + \frac{\phi_2}{A} + \frac{\phi_{12}}{SA} + P\left(\phi_0' + \frac{\phi_1'}{S} + \frac{\phi_{12}'}{SA}\right)$$

$$\phi_0 = \frac{k_5 + k_7}{k_5 k_7} \qquad \phi_0' = \frac{k_6}{k_5 k_7}$$

$$\phi_1 = \frac{1}{k_1} \qquad \phi_1' = \frac{k_{11}}{k_1 k_{12}}$$

$$\phi_2 = \frac{1}{k_3} \qquad \phi_{12}' = \frac{k_2 k_{11}}{k_1 k_3 k_{12}}$$

$$\phi_{12} = \frac{k_2}{k_1 k_3}$$

Cytochrome c as Acceptor.—It was hoped that an approach similar to that utilized with the higher ranges of ferricyanide would help to clarify the possible sequence of reactions for cytochrome *c* as acceptor. As seen from Tables IV and V, rather ambiguous results were

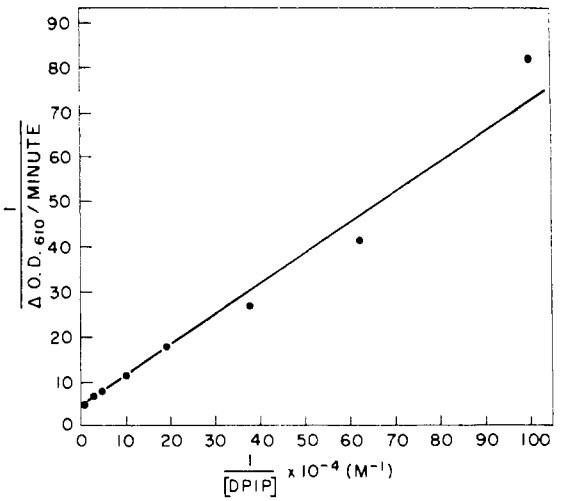

FIG. 5.—Lineweaver-Burk plot for concentrations of 2,6-dichlorophenolindophenol varying from 1.06×10^{-6} M to 1.6×10^{-4} M. All reactions were at *p*H 7.5, in the buffer described in the legend to Fig. 4. The concentration of DL-lactate was 4×10^{-3} M.

obtained. Within the limits of the experimental accuracy it appeared quite uncertain whether $\phi_{12} \neq 0$ either in the presence or in the absence of the product, pyruvate. Since ϕ_{12}, if not equal to zero, would be expected to be sensitive to the presence of product, the lack of a definite effect suggests rather strongly that this parameter is indeed virtually zero. Reduced cytochrome *c* had no effect upon the velocity of the reaction, even when it was present in concentrations equal to one-fourth that of the oxidized cytochrome *c*. In fact, reduction of the residual oxidized cytochrome

TABLE IV
COMPARISON OF THE VALUES OF THE KINETIC PARAMETERS WITH CYTOCHROME *c* AS ACCEPTOR[a]

Expt. No.	ϕ_0 (sec.)	ϕ_1 (M sec.)	ϕ_2 (M sec.)	ϕ_{12}[b] (M^2 sec.)
1	4.8×10^{-3}	5.2×10^{-6}	5.5×10^{-7}	$\sim 3.1 \times 10^{-11}$
2[c]	3.9×10^{-3}	9.0×10^{-5}	7.2×10^{-7}	$\sim 1.8 \times 10^{-11}$

[a] Oxidized cytochrome *c* was varied from 4.4 mM to 44.0 mM. L(+)-Lactate was varied from 0.33 mM to 1.57 mM. Enzyme was *ca.* 6.3×10^{-9} M. All experiments were done at room temperature, *ca.* 20°. 0.1 M acetate-pyrophosphate buffer, *p*H 7.5, was used. [b] Experimental error was very high. Therefore, this parameter could also be zero in all instances. [c] Determined in 0.133 M NaCl.

c present in cytochrome *c* reduced by H_2/Pd still occurred even though, presumably, most of the cytochrome *c* was in the reduced form. Only one of the enzyme reaction sequences proposed by Dalziel (1957) gives a rate equation in which $\phi_{12} = 0$. Thus, the following proposed sequence of reactions is consistent (within experimental error) with the results reported in this study. To allow for a slight inhibition caused by the presence of pyruvate in the highest concentrations used (12.3×10^{-3} M) and the apparent irreversibility of the reaction, this scheme (equation 5) has been slightly modified from those proposed by Dalziel (1957) and Morton *et al.* (1961).

TABLE V

COMPARISON OF THE KINETIC PARAMETERS AS PYRUVATE CONCENTRATION IS INCREASED, *p*H 7.5, CYTOCHROME *c* AS ACCEPTOR[a]

Pyruvate Molarity ($\times 10^3$)	ϕ_0 (sec. $\times 10^3$)	Δ_0[b]	ϕ_1 (M sec. $\times 10^6$)	Δ_1[b]	ϕ_2 (M sec. $\times 10^7$)	Δ_2[b]	ϕ_{12} (M^2 sec. $\times 10^{11}$)	Δ_{12}[b]
0	3.9	1.0	9.0	1.0	7.2	1.0	1.8	1.0
2.46	16.0	4.0	6.6	0.74	5.6	0.78	9.6	5.3
6.15	8.4	2.1	12.0	1.3	7.2	1.0	−1.3	−0.74
12.3	Uncertain	—	25.0	2.7	1.4	0.19	−13.0	−7.4

[a] All parameters were determined when 0.13 M NaCl was present in the reaction mixture to minimize the effects of changes in ionic strength known to affect the reaction with this acceptor (Boeri and Tosi, 1956). Other conditions were the same as described for Table IV. [b] See Table II, footnote *b*, for definition.

$$(a)\ E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \qquad (5)$$

$$(b)\ ES \xrightarrow{k_3} E' + P$$

$$(c)\ E' + A \xrightarrow{k_5} E'' + A_{red}$$

$$(d)\ E'' + A \xrightarrow{k_7} E + A_{red}$$

$$(e)\ E + P \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} EP$$

Rate Equation

$$(f)\ \frac{E_t}{v_0} = \phi_0 + \frac{\phi_1}{S} + \frac{\phi_2}{S} + \frac{\phi_1'}{S} \times P$$

$$\phi_0 = \frac{1}{2k_3} \qquad \phi_1' = \frac{k_9}{k_{10}}\left(\frac{k_2 + k_3}{2k_1k_3}\right)$$

$$\phi_1 = \frac{k_2 + k_3}{2k_1k_3}$$

$$\phi_2 = \frac{k_5 + k_7}{2k_5k_7}$$

Some important relationships between these constants are shown in equation (6).

$$(a)\ \frac{\phi_1'}{\phi_1} = \frac{k_9}{k_{10}} = K_{EP}^{diss} \qquad (6)$$

$$(b)\ \frac{\phi_1}{\phi_0} = \frac{k_2 + k_3}{k_1}$$

$$(c)\ \Delta_1 = \frac{\phi_1 \text{ (with inhibitor)}}{\phi_1 \text{ (without inhibitor)}} = \frac{\phi_1 + \phi_1'P}{\phi_1} \neq 1$$

when $k_2 = k_3$

$$(d)\ \frac{\phi_1}{\phi_0} = \frac{2k_2}{k_1} = 2K_{ES}^{diss}$$

ϕ_{12} vanishes also when ferricyanide at low concentration is used as the acceptor. Therefore, it is likely that the same sequence of reactions is operative under these conditions as well.

DISCUSSION

As with all kinetic studies mechanisms may not be assigned unambiguously, but certain ones may be eliminated. From those studies in which a high level of ferricyanide was employed as the acceptor those mechanisms which have been eliminated include (*a*) mechanisms not containing a ternary complex ($\Delta_0 \neq 1$), (*b*) mechanisms in which the acceptor is released prior to the product ($\Delta_0 \neq 1$), and (*c*) compulsory ordered mechanisms to which equilibrium conditions apply (E_t/v_0 is dependent upon substrate concentration; see Segal, 1959, for a complete discussion of this point). These data do not unequivocally eliminate a *non*compulsory pathway for binding of the two substrates (Segal, 1959), even though the reciprocal velocity is a linear function of either the reciprocal substrate or acceptor concentrations.

When cytochrome *c* serves as the acceptor (and presumably also when a low concentration of ferricyanide is used) only one of the nine mechanisms studied by Dalziel (1957) is permissible (mechanism IV*i*). In addition to the other eight mechanisms of Dalziel, mechanisms in which a reduced acceptor is released prior to the product are also eliminated.

Unfortunately, the possibility that the first binary complex is formed from the combination of free enzyme with acceptor is not excluded by the present experimental data for either acceptor. However, there is no evidence that the acceptor combines with the free enzyme in the absence of the substrate, but it is well known that lactate will reduce cytochrome b_2 in the absence of acceptor (Appleby and Morton, 1959a). The latter observation lends support to the present postulate that binding of lactate to the enzyme to form an enzyme-lactate complex precedes the binding of the acceptor. This observation, of course, does not unequivocally prove the postulate to be true. The mechanisms proposed above, while consistent with the present experimental data (and therefore possibly describing the actual reaction sequences involved), are to be considered purely hypothetical for the moment.

As seen from the proposed sequences (2) and (4), ϕ_{12}/ϕ_2 either is approximately equal to the dissociation constant of the *ES* complex (concentrated ferricyanide employed as the acceptor) or equals this dissociation constant exactly (2,6-dichlorophenolindophenol used as the acceptor). Others (Frieden, 1957; Baker and Mahler, 1962) have pointed out analogous relationships in other instances. From Table VI it is seen that the dissociation constants calculated in this way are in fair agreement for the reaction with two different acceptors. Since the dissociation constant for the *ES* complex should be independent of the acceptor employed, this agreement gives added evidence that the proposed sequences for the partial reactions are correct.

Assuming the proposed mechanisms to be correct for the reaction when 2,6-dichlorophenolindolphenol and the concentrated ranges of ferricyanide were used as acceptors and assuming that $k_4 = 0$, both k_1 and k_2 may be evaluated as follows:

From ferricyanide data:

$$k_1 = \frac{1}{2\phi_1} = 6.7 \times 10^6\ M^{-1}\ min.^{-1}$$

$$= 1.1 \times 10^5\ M^{-1}\ sec.^{-1}$$

$$k_2 = \frac{\phi_{12}k_1}{\phi_2} = 4.8 \times 10^3\ min.^{-1} = 80\ sec.^{-1}$$

From 2,6-dichlorophenolindophenol data:

TABLE VI

COMPARISON OF THE DISSOCIATION CONSTANTS WITH THE DIFFERENT ACCEPTORS

Acceptor	$K_{lactate} = \frac{k_2}{k_1}$ M	Assumptions for the Calculations
$Fe(CN)_6^{-3}$ (0.10 mM to 1.0 mM)	7.2×10^{-4}	From equations (2) & (3) $\frac{\phi_{12}}{\phi_2} \simeq \frac{k_2}{k_1}$; $\phi_1 = \frac{1}{2k_1}$; $k_4 = 0$ $k_3 \gg k_2$
2,6-Dichlorophenolindophenol	4.9×10^{-4}	From equation (4) $\frac{\phi_{12}}{\phi_2} = \frac{k_2}{k_1}$; $\phi_1 = \frac{1}{k_1}$
Cytochrome *c*	7.4×10^{-4}	From equations (5) & (6) $\frac{\phi_1}{2\phi_0} \simeq \frac{k_2}{k_1}$; $k_2 = k_3$; $\phi_0 = \frac{1}{2k_3}$
$Fe(CN)_6^{-3}$ (0.06 mM to 0.24 mM)	4.6×10^{-4}	Same

$$k_1 = 1.4 \times 10^7 \text{ M}^{-1} \text{ min.}^{-1} \text{ sec.}^{-1} = 2.4 \times 10^5 \text{ M}^{-1} \text{ sec.}^{-1}$$

$$k_2 = \frac{\phi_{12} k_1}{\phi_2} = 6.9 \times 10^3 \text{ min.}^{-1} = 110 \text{ sec.}^{-1}$$

From the postulated mechanism with cytochrome *c* as the acceptor, k_3 for sequence (4) may be calculated as follows:

$$k_3 = \frac{1}{2\phi_0} = 6.25 \times 10^3 \text{ min.}^{-1} = 100 \text{ sec.}^{-1}$$

Since the values of both k_1 and k_2 should be acceptor-independent it is legitimate to compare these two parameters evaluated from sequences (2) and (4) with the k_3 of sequence (5). In this comparison it is seen that $k_2 \simeq k_3$. Assuming these rate constants to be approximately equal in sequence (4), $k_2 \ll k_1$, *i.e.*, the dissociation constant of the *ES* complex, may again be calculated (when either cytochrome *c* or dilute ranges of ferricyanide serve as acceptor). Again the fair agreement (see Table VI) for the dissociation constants calculated in this manner with those calculated previously adds some support to the hypotheses that the assumptions and proposed sequences of reactions are correct.

Morton and Armstrong (1961) have reported independently similar kinetic studies, and it seems worthwhile to compare the presently determined kinetic parameters with those determined in their laboratories (see Table VII). It is seen that the kinetic parameters V and K_A compare favorably, while K_B and K_{AB} are in only fair agreement.

Calculation of V_{max}[2] (μmoles of substrate oxidized/minute/mole of enzyme) shows that the relative efficiencies of the various acceptors determined by us are very similar to those found by Morton's group, *viz.*, 2,6-dichlorophenolindophenol ($V_{max} = 1.5 \times 10^3$) < ferricyanide ($4.8 \times 10^3$ for the concentrated ranges and 4.2×10^3 for the dilute ranges) < cytochrome *c* (6.3×10^3).

The biphasic curve shown when ferricyanide is used as the acceptor and the difference in the values for ϕ_{12} strongly suggest that ferricyanide reacts with two different sites of the enzyme. Two different mechanisms appear to be obeyed: one, not involving a ternary complex but involving a modified form of the non-complexed enzyme, operative with ferricyanide at low concentration and with cytochrome *c;* the other, involving a ternary complex but no modified free enzyme, characteristic for ferricyanide at higher concentrations and for 2,6-dichlorophenolindophenol. This result lends some support to the previous suggestion along these lines by Morton *et al.* (1961). Singer and his group (Minakami *et al.*, 1962; Guiditta and Singer, 1959) have also observed a biphasic dependence of the reciprocal velocities of DPNH and succinic dehydrogenase on the reciprocal of the ferricyanide concentrations, and suggested that this dependence results from the interaction of ferricyanide at two different sites in their systems. It is therefore possible that ferricyanide reacts with either the flavin or the protoheme or both of YLDH, depending upon what concentration is used, or that, alternatively, it might even be capable of interacting with yet another site not identical with either. Previous investigators (Morton and Armstrong, 1961) have suggested that ferricyanide in high concentrations reacts with the flavin moiety and that cytochrome *c* and ferricyanide in low concentration react with the protoheme moiety. Thus, the ternary complex would be represented by lactate-enzyme-bound-FMN-ferricyanide (or 2,6-dichlorophenolindophenol), with the flow of electrons proceeding from left to right. The modified free enzyme present during the reaction with a low ferricyanide concentration or with cytochrome *c* could be represented by enzyme which has had its protoheme reduced by lactate (see Morton and Armstrong, 1961). Our data would suggest, in contrast to previous suggestions (Morton *et al.*, 1961; Hasegawa and Ogura, 1961) that with 2,6-dichlorophenolindophenol and high ferricyanide concentrations the same

[2] $1/\phi_0 = V_{max}$ for two-electron acceptor (Dalziel, 1957).

TABLE VII

COMPARISON OF KINETIC PARAMETERS WITH MORTON AND ARMSTRONG'S DATA

Kinetic Parameters[a]	Values Determined: High $Fe(CN)_6^{-3}$ — Morton & Armstrong (1961)	High $Fe(CN)_6^{-3}$ — Present	Cytochrome *c* — Morton & Armstrong[b] (1961)	Cytochrome *c* — Present
$V = 1/\phi_0$ (min.$^{-1}$)	10.5×10^3	9.5×10^3	13.0×10^3	12.5×10^3
$K_A = \phi_1/\phi_0$ (M)	5.46×10^{-4}	7.1×10^{-4}	5.36×10^{-4}	8.7×10^{-4}
$K_B = \phi_2/\phi_0$ (M)	3.1×10^{-4}	3.4×10^{-5}	6.47×10^{-5}	8.1×10^{-6}
$K_{AB} = \phi_{12}/\phi_0$ (M^2)	9.5×10^{-7}[c]	2.5×10^{-8}	0	0

[a] See Dalziel (1957) for verification of these relationships. [b] Average of two cited determinations. [c] The value quoted in the cited reference (9.8×10^{-4}) is incorrect and should be as cited above (personal communication with J. M. Armstrong, 1962).

mechanisms are operating, namely, that a ternary complex is formed.

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Studies on the Mechanism of Enzyme-Catalyzed Oxidation-Reduction Reactions. VII.* *p*H Effects on the Kinetics of the Reaction Catalyzed by Yeast L(+)-Lactate Dehydrogenase

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The effects of *p*H upon the kinetics of binding of L(+)-lactate, D(−)-lactate, and pyruvate by crystalline yeast L(+)-lactate dehydrogenase have been studied by means of initial velocity measurements. These studies implicate a group on the free enzyme with a pK_a' of 6.5–7.0, which is acceptor independent and appears to be concerned with binding the groups attached to the α-carbon of the substrate and the product. The dissociation constants for the complexes of the free enzyme with L-lactate, caprylate, D-lactate, and pyruvate are 7.2×10^{-4} M, 4.2×10^{-3} M, 7.2×10^{-3} M, and 1.5×10^{-2} M, respectively, all at *p*H 7.5 and 20°.

From studies of the *p*H dependence of an enzyme-catalyzed reaction inferences may be made concerning the dissociation constants (pK_a values) of group(s) on the free enzyme involved in the binding of the substrate and/or product. Dixon (1953a) has presented a method for evaluating such data which has been used in the present investigations.

In the companion paper (Hinkson and Mahler, 1963) we have shown that the oxidation of lactate by ferricyanide (at concentrations ≥2 mM) and cytochrome *c* with YLDH[1] obeys the rate laws of equations (1) and (2), respectively,

$$E_t/v_0 = \phi_0 + \phi_1/S + \phi_2/A + \phi_{12}/S{\cdot}A + P(\phi_0' + \phi_1'/S + \phi_2'/A + \phi_{12}'/S{\cdot}A) \quad (1)$$

$$E_t/v_0 = \phi_0 + \phi_1/S + \phi_2/A + \phi_1'/S{\cdot}A \quad (2)$$

where the ϕ_n's are experimentally determined parameters, interpretable in terms of mechanism-dependent specific rate constants, and E_t, S, A, and P are the stoichiometric concentrations of enzyme, lactate, acceptor, and pyruvate, all in moles/liter. It appeared appropriate to determine the effect of *p*H on v_0 and

* For paper VI of this series see Hinkson and Mahler (1963).

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[1] The following abbreviations will be used in this paper: YLDH = crystalline yeast L(+)-lactate dehydrogenase (cytochrome b_2); $p\phi_n$ = negative $\log_{10}$ of the kinetic parameter ϕ_n; FMN = riboflavin-5'-phosphate; OD = optical density.