References
Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5631.
Bender, M. L., Clement, G. E., Gunter, C. R., & Kézdy, F. J. (1964) *J. Am. Chem. Soc. 86*, 3697.
Bizzozero, S. A., Baumann, N. K., & Dutler, H. (1975) *Eur. J. Biochem. 58*, 167.
Burgen, A. S. V., Roberts, G. C. K., & Feeney, J. (1975) *Nature (London) 253*, 753.
Caldin, E. F. (1964) *Fast Reactions in Solution*, pp 10–11, Wiley, New York.
Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry*, pp 916–921, W. H. Freeman, San Francisco.
Caplow, M. (1969) *J. Am. Chem. Soc. 91*, 3639.
Carapellucci, P. A. (1975) *J. Phys. Chem. 79*, 2768.
Cerjan, C., & Barnett, R. E. (1972) *J. Phys. Chem. 76*, 1192.
Cleland, W. W. (1975) *Acc. Chem. Res. 8*, 145.
de Jersey, J., Willadsen, P., & Zerner, B. (1969) *Biochemistry 8*, 1959.
Dixon, G. H., & Neurath, H. (1957) *J. Biol. Chem. 225*, 1049.
Dunford, H. B., & Hewson, W. D. (1977) *Biochemistry 16*, 2949.
Fastrez, J., & Fersht, A. R. (1973a) *Biochemistry 12*, 1067.
Fastrez, J., & Fersht, A. R. (1973b) *Biochemistry 12*, 2025.
Hess, G. P. (1971) *Enzymes, 3rd Ed. 3*, 218–219.
Hinkle, P. M., & Kirsch, J. F. (1971) *Biochemistry 10*, 2717.
Hirohara, H., Philipp, M., & Bender, M. L. (1977) *Biochemistry 16*, 1573.
Jencks, W. P. (1980) in *Chemical Recognition in Biology* (Chapeville, F., & Haenni, A.-I., Eds.) Springer-Verlag, New York.
Kunugi, S., Hirohara, H., Nishimura, E., & Ise, N. (1978) *Arch. Biochem. Biophys. 189*, 298.
Loo, S., & Erman, J. E. (1977) *Biochim. Biophys. Acta 481*, 279.
Makinen, M. W., & Fink, A. L. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 301.
Nakatani, H., & Dunford, H. B. (1979) *J. Phys. Chem. 83*, 2662.
Philipp, M., & Bender, M. L. (1973) *Nature (London), New Biol. 241*, 44.
Renard, M., & Fersht, A. R. (1973) *Biochemistry 12*, 4713.
Rosenberg, S., & Kirsch, J. F. (1979) *Anal. Chem. 51*, 1379.
Sawyer, C. B., & Kirsch, J. F. (1975) *J. Am. Chem. Soc. 97*, 1963.
Schonbaum, G. R., Zerner, B., & Bender, M. L. (1961) *J. Biol. Chem. 236*, 2930.
Schowen, R. L. (1977) in *Isotope Effects on Enzyme Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrup, D. B., Eds.) pp 65–67, University Park Press, Baltimore, MD.
Shia, D. D. F., & Sturtevant, J. H. (1969) *Biochemistry 8*, 4910.
Šolc, K., & Stockmayer, W. H. (1973) *Int. J. Chem. Kinet. 5*, 733.
Zannis, V. (1975) Ph.D. Thesis, University of California, Berkeley, CA.

Steady-State Kinetic Studies on D-Lactate Dehydrogenase from *Megasphera elsdenii*†

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ABSTRACT: Initial rate measurements were made of the oxidation of D-lactate and D-α-hydroxybutyrate by oxygen and potassium ferricyanide, catalyzed by D-lactate dehydrogenase from *Megasphera elsdenii*. The detailed kinetic work indicates a "ternary complex" type mechanism, with a complex of keto acid and reduced enzyme reacting with the electron acceptor at pH 8. However, as the pH is lowered, the double-reciprocal plots become nonlinear, with a downward curvature. This seems to be due to negative interactions within the protein rather than to a complexity of the kinetic mechanism. The variation of initial rate parameters at pH 8 with temperature yields nonlinear Arrhenius plots with a greater activation energy above the break point than below. This type of behavior has been previously reported only for fumarase (Massey, 1953). Studies with deuterated D-lactate show only a small isotope effect on ϕ_0 and ϕ_1 (K_M/V_{max} for lactate) but a large effect on ϕ_2 (K_m/V_{max} for ferricyanide).

The first enzyme in lactate metabolism of the anaerobic bacterium *Megasphera elsdenii* is a flavoprotein, D-lactate dehydrogenase (EC 1.1.99.6) (Baldwin & Milligan, 1964; Brockman & Wood, 1975).

This enzyme belongs to a class of oxidation–reduction flavoproteins termed C–N transhydrogenase (Massey & Hemmerich, 1980). It catalyzes the transfer of reducing equivalents from CH substrates, in this case D-lactate, to the flavin N(1)–N(5) center of an electron transferring flavoprotein (Brockman & Wood, 1975; Whitfield & Mayhew, 1974). This electron transferring flavoprotein in turn passes the reducing equivalents on to butyryl-CoA dehydrogenase, another flavoprotein (Engel & Massey, 1971). Many bacteria grown anaerobically possess soluble D-lactate dehydrogenases (Snoswell, 1966; Molinari & Lara, 1960) as does yeast (Cremona, 1964). A mammalian enzyme has also been described (Tubbs & Greville, 1959; Cammack, 1969).

Previous workers (Stachiewicz et al., 1961; Ghiretti-Magaldi et al., 1961) found that the yeast enzyme appears to require zinc as well as FAD for activity. Olson & Massey (1979) showed conclusively that *Megasphera elsdenii* D-lactate dehydrogenase contains one essential zinc per FAD. The role

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Table 1: Kinetic Coefficients Describing the Oxidation of D-Lactate and α-Hydroxybutyrate by D-Lactate Dehydrogenase and Molecular Oxygen at 25 °C in Potassium Phosphate Buffer[a]

substrate	pH	μ[b]	ϕ_0 (min) × 10^3	ϕ_1 (mM min) × 10^2	ϕ_2 (μM min)	ϕ_1/ϕ_0 (mM)	ϕ_2/ϕ_0 (μM)
D(−)-lactate	7	0.23	4.25	2	0.75	4.7	176
D(−)-α-hydroxybutyrate	7	0.23	4.1	1.6	1.8	3.9	440
D(−)-lactate[c]	7	0.23	5.2	2.8	0.79	5.4	150
D(−)-lactate (ionic strength increased with L(+)-lactate)	7	0.43	4.25	4.75	1.3	11.2	306
D(−)-lactate	7	0.52	4.5	6	1.7	13.3	377
D(−)-lactate[c]	7	0.43	1.9	2.8	1.3	15	690
D(−)-lactate	8	0.23	4.5	2.9	1.25	6.4	278
D(−)-α-hydroxybutyrate	8	0.23	4.6	1.52	2.2	3.3	470

[a] The kinetic coefficients shown are those in the reciprocal initial rate equation $e/v = \phi_0 + \phi_1/[S_1] + \phi_2/[S_2]$ where e is the concentration of active sites based on the flavin concentration, S_1 is D-lactate or D-α-hydroxybutyrate, and S_2 is oxygen. [b] ϕ_1/ϕ_0 is the Michaelis constant for D-lactate or D(−)-α-hydroxybutyrate, ϕ_2/ϕ_0 is the Michaelis constant for oxygen, and μ is the ionic strength of the buffer. [c] Data taken from Olson & Massey (1979).

of the essential zinc will be discussed in a following paper (Morpeth & Massey, 1982b).

The purpose of this report is to describe the steady-state kinetic properties of D-lactate dehydrogenase when molecular oxygen or potassium ferricyanide are the electron acceptors. We consider it important to be able to understand the interactions of D-lactate dehydrogenase with relatively simple electron acceptors before any attempt is made to investigate the dynamics of electron transfer to the electron transferring flavoprotein.

Earlier reports on the steady-state kinetics of D-lactate dehydrogenase using these nonphysiological electron acceptors have shown that with oxygen the enzyme exhibits Michaelis–Menten behavior while with dyes it does not (Brockman & Wood, 1975; Olson & Massey, 1979). With either potassium ferricyanide or dichlorophenolindophenol (DCPIP), double-reciprocal plots have downward curvature at 25 °C and pH 7.

We are also interested in comparing the mechanism of D-lactate dehydrogenase with those of other flavoenzymes, especially those which react with lactate. Both L-lactate oxidase (Lockridge et al., 1972; Massey et al., 1980) and L-lactate flavocytochrome b_2 (Capeillere-Blandin et al., 1975; Pompon et al., 1980) have been extensively studied.

Experimental Procedures

Materials. All reagents were purchased from Sigma Chemical Co. D-Lactate dehydrogenase was prepared and assayed as described previously (Olson & Massey, 1979). Deuterated substrate, D-[α-^{2}H]lactate, was a gift from Dr. Sandro Ghisla, University of Konstanz, Konstanz, West Germany.

Initial Rate Measurement of Alcohol Oxidation. Enzyme turnover with oxygen as an acceptor was monitored as described previously (Olson & Massey, 1979), using a stopped-flow apparatus and linking the production of pyruvate to the disappearance of NADH with rabbit muscle L(−)-lactate dehydrogenase. Since Olson & Massey (1979) showed that L-lactate does not inhibit D-lactate dehydrogenase, the L-lactate produced does not interfere.

When potassium ferricyanide was the electron acceptor, the reaction was followed by the decrease in ferricyanide absorbance at 420 nm. All assay mixtures were purged with oxygen-free nitrogen for several minutes before being tightly stoppered and allowed to equilibrate to the assay temperature. The assays were initiated by injecting 1–5 μL of a suitable dilution of enzyme.

The stock enzyme was stored in the presence of 1 mM dithiothreitol, which after dilution and addition to the assay had no effect on the rate. Rates were converted to turnover numbers by using $\xi_{340} = 6220\ M^{-1}\ cm^{-1}$ for NADH (Horecker & Kornberg, 1948) and $\xi_{420} = 1000\ M^{-1}\ cm^{-1}$ for potassium ferricyanide (Loach, 1968).

All initial rate experiments were carried out in phosphate buffer, ionic strength = 0.23, and for linear double-reciprocal plots the kinetic coefficients in eq 1 (Dalziel, 1957) were

$$\frac{e}{V_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \quad (1)$$

estimated by the direct graphical method of Eisenthal & Cornish-Bowden (1974). All data are shown in double-reciprocal plots for presentation purposes. In eq 1, e is the concentration of enzyme active sites, S_1 and S_2 are the electron donor and acceptor, respectively, and V_0 is the specific initial velocity (micromolar [D-lactate] consumed per second). Initial rate assays were performed in duplicate and were generally reproducible to within 10% at worst. At least two complete experiments were performed with each donor/acceptor pair. In experiments where the ionic strength was varied, oxygen concentrations were suitably corrected by use of a nomogram (Hitchman, 1978). ϕ_0 was found to be reproducible to within 10% and ϕ_1 and ϕ_2 generally to within 20%.

Determination of D-Lactate Concentration. Stock D-lactate and α-hydroxybutyrate solutions were assayed routinely at 25 °C and 420 nm by using D-lactate dehydrogenase. The assay mixture contained potassium phosphate buffer, pH 8 (100 μmol), potassium ferricyanide (1 μmol), and D-lactate dehydrogenase (2–5 nmol). The reaction was allowed to reach completion, and the concentration of D-lactate was found from the decrease in A_{420} of ferricyanide.

Results and Discussion

Oxygen Kinetics. At pH 7, 25 °C, in phosphate buffer, ionic strength = 0.23, a series of parallel Lineweaver–Burk plots is obtained upon varying D-lactate at different fixed oxygen concentrations (Olson & Massey, 1979). When the electron donor was α-hydroxybutyrate, similar results were seen. At pH 8 both substrates again gave parallel line kinetics. Figure 1a shows the results at pH 8 with D-lactate as the variable substrate. Both the primary and secondary plots were linear over the concentration of substrates used, as were the alternative primary plots, which also yielded a series of parallel lines. Values for the kinetic coefficients of eq 1 are given in Table I. All the parameters were corrected to an activity to flavin ratio of 130. Also included in Table I are values for the initial rate parameters of D-lactate oxidation at pH 7 and high ionic strength, using L-lactate or potassium phosphate to increase the ionic strength. Olson & Massey (1979) found

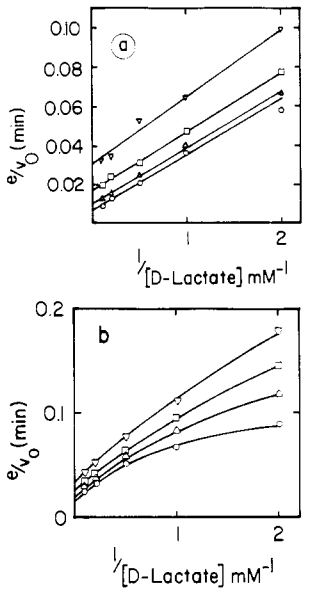


FIGURE 1: Determination of the initial rate parameters of D-lactate oxidation. The primary plots (a) show the variation of the initial specific rate at 25 °C in potassium phosphate buffer, pH 8, ionic strength = 0.23, with the reciprocal of D-lactate concentration between 0.5 and 10 mM at several concentrations of oxygen. The oxygen concentrations were (○) 560, (△) 280, (□) 118, and (▽) 56 μM. Primary plots (b) show the variation of the initial specific rate at 25 °C in potassium phosphate buffer, pH 6, ionic strength = 0.23, with the reciprocal of D-lactate concentration at several concentrations of oxygen. The oxygen concentrations were (○) 560, (△) 280, (□) 118, and (▽) 56 μM.

that on increasing the ionic strength from 0.224 to 0.442, the V_{max} ($1/\phi_0$) for D-lactate oxidation increased from 190 to 530 min^{-1}. In several experiments, however, we were unable to reproduce this ionic strength dependent increase in the V_{max}. Indeed over the ionic strength range we used, the V_{max} did not vary while ϕ_{lac} and ϕ_{02} increased with ionic strength. The data of Olson & Massey (1979) are also presented in Table I; their results at low ionic strength (μ = 0.23) are in excellent agreement with those found in this study.

Parallel line kinetics due to the absence of a ϕ_{12} term are usually associated with a simple "ping-pong" mechanism (Scheme I). For the ping-pong mechanism, the kinetic

Scheme I

$$E + \text{D-Lac} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-Lac} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_{red}\text{-Pyr}$$

$$E_{red}\text{-Pyr} \xrightarrow{k_5} E_{red} + \text{Pyr}$$

$$E_{red} + O_2 \xrightarrow{k_6} E_{ox} + H_2O_2$$

coefficients are $\phi_0 = 1/k_3 + 1/k_5$, $\phi_1 = (k_3 + k_2)/(k_1k_3)$, and $\phi_2 = 1/k_6$. Thus ϕ_2 should be independent of the first substrate. Inspection of Table I shows that at both pH 7 and 8 the ϕ_2 values for α-hydroxybutyrate and lactate differ significantly. Thus ϕ_2 is dependent upon the nature of the reductant, suggesting that a simple ping-pong mechanism is not in operation.

Dalziel (1975) and Palmer & Massey (1968) have pointed out that parallel line kinetics may also occur with a compulsory order mechanism or a "ternary complex" mechanism, when ϕ_{12} is vanishingly small. We feel it is chemically unlikely that D-lactate dehydrogenase would operate by the classical compulsory order mechanism in which both the electron donor and acceptor bind before flavin reduction. However, the ternary complex mechanism (Scheme II) in which the reduced enzyme–pyruvate complex reacts with the electron acceptor

Scheme II

$$E + \text{D-Lac} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-Lac} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_{red}\text{-Pyr}$$

$$O_2 + E_{red}\text{-Pyr} \xrightarrow{k_7} E_{ox}\text{-Pyr-}H_2O_2$$

$$E_{ox}\text{-Pyr-}H_2O_2 \xrightarrow{k_8} E_{ox}\text{-Pyr} + H_2O_2$$

$$E_{ox}\text{-Pyr} \xrightarrow{k_9} E_{ox} + \text{Pyr}$$

(rather than reduced enzyme alone) is a reasonable possibility. Indeed this mechanism is used predominately by the amino acid oxidases (Massey & Gibson, 1964; Bright & Porter, 1976) and also by L-lactate oxidase (Lockridge et al., 1972).

The kinetic coefficients for the ternary complex mechanism shown in Scheme II are $\phi_0 = 1/k_3 + 1/k_8 + 1/k_9$, $\phi_1 = (k_3 + k_2)/(k_1k_3)$, $\phi_2 = (k_3 + k_4)/(k_3k_7)$, and $\phi_{12} = (k_2k_4)/(k_1k_3k_7)$.

With this mechanism ϕ_2 is no longer independent of the nature of the reductant, and there is also no reason why k_7 should be the same for varying reductants since the species now reacting with oxygen will be dependent on the nature of the primary product. Thus the substrate dependence of ϕ_2 can be rationalized, making a ternary complex mechanism a more appealing choice than the ping-pong mechanism. The requirement that ϕ_{12} be small can be readily accounted for if $k_2k_4 \ll k_1k_3k_7$.

It is also notable that at both pH 7 and pH 8 ϕ_0 is identical for both D-lactate and D-α-hydroxybutyrate. This suggests that the rate-limiting step may be independent of the nature of the reductant. Thus when oxygen is the electron acceptor, it is possible that the rate-limiting step is a product-release step (k_8 or k_9).

When the kinetics of D-lactate oxidation were investigated at pH 6, the primary plots varying D-lactate were no longer linear. Figure 1b shows the data from one such experiment. At the highest concentrations of oxygen (560 μM), the double-reciprocal plots are curved downward, becoming linear at lower oxygen concentrations.

Kinetics with Ferricyanide as Acceptor. When potassium ferricyanide is the electron acceptor at pH 6 or 7, a series of roughly parallel curves is seen in either primary plot (Figure 2). Varying the pH changes the curvature of the plots. At pH 6 the double-reciprocal plots are more curved than at 7, and at pH 8 they appear to be linear (Figure 3) with respect to both lactate and potassium ferricyanide. Similar behavior was seen when D-α-hydroxybutyrate was used as reductant.

Detailed initial rate data at pH 8 and 25 °C are shown in Figure 3 and Table II. Both the primary and secondary plots are linear over the concentration ranges used. From Table II it can be seen that the ϕ_2 values for D-lactate and D-α-hydroxybutyrate are different (at pH 8 and 25 °C). Using the same arguments as before, we propose that D-lactate dehydrogenase operates by a mechanism in which a reduced enzyme–pyruvate complex reacts with 2 equiv of ferricyanide. This mechanism is shown in Scheme III, and the kinetic

Scheme III

$$E + \text{D-Lac} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{-Lac} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_{red}\text{-Pyr}$$

$$2A + E_{red}\text{-Pyr} \xrightarrow{k_7} E_{ox}\text{-Pyr} + 2A_{red}$$

$$E_{ox}\text{-Pyr} \xrightarrow{k_9} E_{ox} + \text{Pyr}$$

coefficients are the same as for the ternary complex mechanism shown in Scheme II except $\phi_0 = 1/k_3 + 1/k_9$.

Table II: Kinetic Coefficients Describing the Oxidation of D-Lactate and α-Hydroxybutyrate by D-Lactate Dehydrogenase and Potassium Ferricyanide at 25 °C in Potassium Phosphate Buffer, Ionic Strength = 0.23 at pH 8[a]

substrate	temp (°C)	ϕ_0 (min) × 10^4	ϕ_1 (mM min) × 10^2	ϕ_2 (mM min) × 10^4	ϕ_1/ϕ_0 (mM)[b]	ϕ_2/ϕ_0 (mM)	ϕ_{12} (mM^2 min)
D(−)-lactate	25	12	1.9	1.3	15.8	0.11	
D(−)-α-hydroxybutyrate	25	9	2.2	4.3	24.4	0.48	
D(−)-lactate	4	27	7.1	17.8	26.3	0.66	1.6
D(−)-lactate	10	24	6.45	15.2	26.9	0.63	0.6
	15	21	5.6	9.7	26.6	0.462	
	20	16.2	2.7	2.4	16.6	0.15	
	30	7	1.35	2.6	19.2	0.37	
	35	5.5	0.94	4.2	17.1	0.77	
D(−)-[α-^{2}H]lactate	4	25	1.46	140	58.4	5.6	4
	25	18	2.6	6.3	14.4	0.35	

[a] The kinetic coefficients shown are those in the reciprocal initial rate equation $e/v = \phi_0 + \phi_1/[S_1] + \phi_2/[S_2] + \phi_{12}/([S_1][S_2])$ where e is the concentration of active sites based on the flavin concentration, S_1 is D-lactate or D(−)-α-hydroxybutyrate, and S_2 is ferricyanide. [b] ϕ_1/ϕ_0 is the Michaelis constant for D-lactate or D(−)-α-hydroxybutyrate, and ϕ_2/ϕ_0 is the Michaelis constant for ferricyanide.

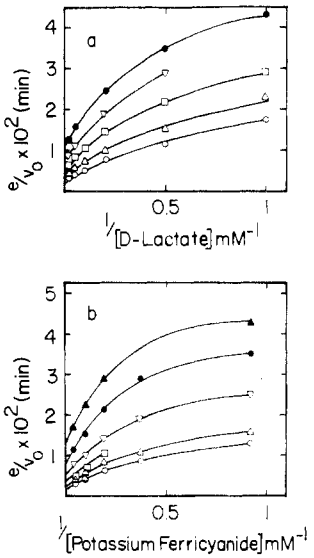


FIGURE 2: (a) Primary plots showing the variation of the reciprocal of the specific initial rate at pH 6, 25 °C, with the reciprocal of the D-lactate concentration at constant potassium ferricyanide concentrations. The potassium ferricyanide concentrations were (○) 2.2, (△) 1.1, (□) 0.55, (▽) 0.21, and (●) 0.11 mM. (b) Primary plots showing the variation of the reciprocal of the specific initial rate at pH 6, 25 °C, with the reciprocal of the potassium ferricyanide concentration at several D-lactate concentrations. The D-lactate concentrations were (○) 40, (△) 20, (□) 10, (▽) 5, (●) 2, and (▲) 1 mM.

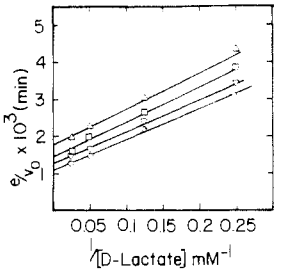
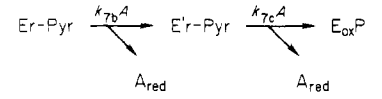


FIGURE 3: Primary plots showing the variation of the initial specific rate at 25 °C in potassium phosphate buffer, pH 8, ionic strength = 0.23, with the reciprocal of D-lactate concentration between 4 and 40 mM at several concentrations of potassium ferricyanide. The potassium ferricyanide concentrations were (○) 2.9, (▽) 1, (□) 0.4, and (△) 0.2 mM.

This position is supported by the steady-state data obtained at pH 8 and 4 °C, varying both D-lactate and ferricyanide concentrations. Under these conditions converging sets of Lineweaver–Burk plots are obtained, thus precluding a ping-pong mechanism, where ϕ_{12} must be zero.

We have proposed in the previous section that when oxygen is the electron acceptor with D-lactate dehydrogenase, a product-release step is rate limiting. Since ϕ_0 for the reaction with ferricyanide is much less than ϕ_0 for oxygen, the rate-limiting step in the oxidase activity cannot be pyruvate release (k_9) since this will be the same in both cases. Thus we suggest that the rate-limiting step in the oxidase activity of D-lactate dehydrogenase is release of the other product, hydrogen peroxide (k_8).

The nonlinearity at pH 7 was first observed by Brockman & Wood (1975) and confirmed by Olson & Massey (1979). The latter workers suggested the nonlinearity was possibly due to ferricyanide being a one electron acceptor, oxidizing the reduced enzyme in two discrete steps, while oxygen, a two electron acceptor, could oxidize the enzyme in a single step. This explanation is not tenable, however. If k_7 in Scheme III consists of k_{7b} and k_{7c}



then ϕ_2/A becomes $[k_3(k_{7b} + k_{7c}) + k_4k_{7c}]/(k_3k_{7b}k_{7c}A)$ which still predicts linear double-reciprocal plots. The parameters ϕ_0 and ϕ_1 do not change, and $\phi_{12} = (k_2k_4)/(k_3k_{7b}k_1)$.

The tendency of the double-reciprocal plots to approach linearity at alkaline pH has not been reported previously. Double-reciprocal plots of the type seen in Figure 2 are often attributed to negative interactions between the protomers in an oligomeric enzyme. However nonlinear double-reciprocal plots of this type can also be explained by classical steady-state kinetics without involving allosteric interactions. A mechanism involving a nonequilibrium random order of substrate binding predicts nonlinear Lineweaver–Burk plots (Ferdinand, 1966). This is, however, chemically unlikely in the case of D-lactate dehydrogenase, where the flavin prosthetic group must first be reduced by D-lactate before it can react with the electron acceptor.

More plausible is an enzyme substitution mechanism, with the reaction being diverted at a faster rate through a reduced enzyme–substrate (E_{red}–lactate) complex at high substrate concentrations. However, this possibility is also unlikely, since the abortive complex (E_{red}–lactate) should be most in evidence at high substrate concentrations. Thus, in the primary plots of the ferricyanide reaction at pH 6 (Figure 2), the Lineweaver–Burk plots with ferricyanide as variable substrate should be markedly less curved at low lactate concentrations.

One of the few other flavoproteins to display nonlinear steady-state kinetics is L-lysine monooxygenase from *Pseudomonas fluorescens* (Flashner & Massey, 1974). Like D-lactate dehydrogenase this enzyme catalyzes the initial step in the utilization of the sole carbon source for the organism.

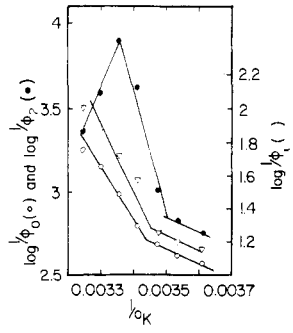


FIGURE 4: Arrhenius plots for the reciprocal of the parameters in eq 1. The parameters were obtained in full kinetic experiments such as those shown in Figure 3 in potassium phosphate buffer, pH 8, ionic strength = 0.23.

Unlike D-lactate dehydrogenase, the double-reciprocal plots with lysine monooxygenase have an upward curvature. Flashner and Massey showed that this is due to a second molecule of lysine binding at a regulatory site. They also found that certain substrate analogues could bind at this regulatory site and cause the double-reciprocal plots to become linear. Again, it is unlikely that a situation of this type is in operation with D-lactate dehydrogenase, since the substrate analogues butyrate and oxalate, even at high concentrations (50 mM), do not affect the curvature of the double-reciprocal plots. Finally, Bright & Porter (1976) report that L-amino acid oxidase oxidizing phenylalanine yields nonlinear double-reciprocal plots when oxygen is varied. In this case two species of reduced enzyme, E_{red} and the complex, E_{red}–product, react with the electron acceptor. For D-lactate dehydrogenase, this possibility is unlikely since both sets of primary plots are curved, a result inconsistent with this mechanism. Thus, we may discard some of the more likely kinetic explanations. However, Henderson (1968) has shown that with an enzyme-substitution mechanism when either of the substrates acts as a modifier then the rate equation also contains squared terms in the substrate which is not acting as modifier. This predicts that both sets of primary plots will be curved as is the case with D-lactate dehydrogenase. The results presented in this paper therefore suggest that the nonlinear plots seen at pH 6 with D-lactate dehydrogenase are probably due to negative interactions rather than to a complexity of the kinetic mechanism.

These nonlinear curves with ferricyanide help us understand the family of curves seen with D-lactate and oxygen at pH 6 (Figure 1b). These plots varying D-lactate concentration are curved at high concentrations of oxygen and become linear as the oxygen concentration is decreased. This is due possibly to the reductive half-reaction being rate limiting at high oxygen concentrations, but as the oxygen concentration is lowered, its reaction becomes limiting, and the double-reciprocal plots approach linearity.

Temperature Dependence. Since pH has such a profound effect on the kinetics of D-lactate dehydrogenase, we decided to investigate also the effect of temperature on the initial rate parameters, using D-lactate/ferricyanide as the electron donor–acceptor pair (pH 8, ionic strength = 0.23). All the double-reciprocal plots at the various temperatures were linear. The data are shown in Table II and Figure 4 as Arrhenius plots. For both $1/\phi_0$ and $1/\phi_1$ the Arrhenius plots were biphasic, showing a sharp break around 17 °C with a greater activation energy above the break point than below.

The activation energies per mole above and below the transition temperature were 14.6 and 3.6 kcal for $1/\phi_0$, 20 and 4 kcal for $1/\phi_1$, and 8.4 kcal for $1/\phi_2$ (below the transition temperature only). Discontinuous Arrhenius plots are not uncommon, especially with membrane bound enzymes (Londesborough, 1980). However, it is usually found that the activation energy below the transition temperature is greater than that above. The only other enzyme which has been reported to give nonlinear Arrhenius plots in which the activation energy below the transition point is less than that above it is fumarase (Massey, 1953; Dixon & Webb, 1980). This has been attributed to fumarase existing in different conformations above and below the transition temperature. We suggest a similar explanation for the present results.

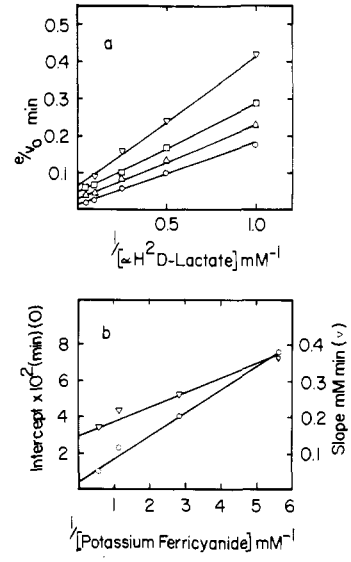


FIGURE 5: (a) Primary plots showing the variation of the initial specific rate at 4 °C in potassium phosphate buffer, pH 8, ionic strength = 0.23, with the reciprocal of D-[α-^{2}H]lactate between 1 and 10 mM at several concentrations of potassium ferricyanide. The potassium ferricyanide concentrations were (○) 1.8, (△) 0.9, (□) 0.35, and (▽) 0.18 mM. (b) The secondary plots show the variation of the intercepts (○) and slopes (▽) of the primary double-reciprocal plots with the reciprocal of the potassium ferricyanide concentration.

Turnover with Deuterated D-Lactate. The results of detailed initial rate experiments with deuterated D-lactate and potassium ferricyanide at 4 and 25 °C in potassium phosphate buffer, pH 8, ionic strength = 0.23, are presented in Table II. Figure 5 shows the converging lines obtained at 4 °C. The parameters ϕ_0 and ϕ_1 show only a minor isotope effect; ϕ_2 on the other hand is significantly larger when deuterated D-lactate is the substrate, increasing 7.9-fold at 4 °C and 4.8-fold at 25 °C.

These results suggest that the lactate-derived hydrogen is retained by the reduced enzyme after lactate is oxidized (probably on the flavin) and is transferred during the oxidative half-reaction. The rationale behind this statement is that for Scheme II, rapid reaction studies have shown that the ϕ_0 parameter is equal to k_3 (Morpeth & Massey, 1982a). Thus since ϕ_0 shows only a minor isotope effect on changing to the deuterated substrate and a decrease in k_4 cannot cause an increase in ϕ_2, then the isotope effect is probably due to k_7 (the second-order rate constant for the reaction between the reduced enzyme complex and potassium ferricyanide). These results suggest that there is direct hydrogen transfer from D-lactate to flavin in D-lactate dehydrogenase. There is evidence for direct hydrogen transfer in other flavoproteins (Drysdale et al., 1961; Louie & Kaplan, 1970; Brüstlein & Bruice, 1972; Jorns & Hersh, 1974; Spencer et al., 1976; Schopfer & Massey, 1979).

In summary, at alkaline pH values (pH 8) with either oxygen or ferricyanide as electron acceptor, D-lactate de-

hydrogenase seems to operate by a ternary complex mechanism such as that used by the amino acid oxidases. As the pH is lowered, the kinetics become much more complex, probably due to one or both of the substrates acting also as a modifier. This point is being investigated in greater detail and will be discussed in a later communication.

It is tempting to believe that the complex behavior we have described at low pH is due to negative interactions. In vivo D-lactate dehydrogenase catalyzes the first step in two pathways which are of utmost importance to *Megasphera elsdenii* (Brockman, 1971) and so is an ideal point for metabolic control. D-Lactate dehydrogenase produces pyruvate for the pyroclastic reaction and is involved in the expulsion of excess reducing equivalents from the cell. Thus it would be to the advantage of the bacteria if this enzyme were able to work at maximum efficiency over the widest possible concentration range of substrate. This would be the effect of negative cooperativity.

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References

Baldwin, R. L., & Milligan, L. P. (1964) *Biochim. Biophys. Acta 92*, 421–432.

Bright, H. J., & Porter, D. J. T. (1976) *Enzymes, 3rd Ed. 13*, 89–173.

Brockman, H. L., Jr. (1971) Ph.D. Thesis, Michigan State University, East Lansing, MI.

Brockman, H. L., Jr., & Wood, W. A. (1975) *J. Bacteriol. 124*, 1454–1461.

Brüstlein, M., & Bruice, T. C. (1972) *J. Am. Chem. Soc. 94*, 6548–6549.

Cammack, R. (1969) *Biochem. J. 115*, 55–61.

Capeillere-Blandin, C., Bray, R. C., Iwatsubo, M., & Labeyrie, F. (1975) *Eur. J. Biochem. 54*, 549–566.

Cremona, T. (1964) *J. Biol. Chem. 239*, 1457–1465.

Dalziel, K. (1957) *Acta Chem. Scand. 11*, 1706–1723.

Dalziel, K. (1975) *Enzymes, 3rd Ed. 11*, 1–60.

Dixon, M., & Webb, E. C. (1980) *Enzymes*, 3rd ed., pp 164–182, Academic Press, New York.

Drysdale, G. R., Spigel, M. J., & Strittmatter, P. (1961) *J. Biol. Chem. 236*, 2323–2328.

Eisenthal, R., & Cornish-Bowden, A. (1974) *Biochem. J. 139*, 715–720.

Engel, P. C., & Massey, V. (1971) *Biochem. J. 125*, 879–887.

Ferdinand, W. (1966) *Biochem. J. 98*, 278–283.

Flashner, M., & Massey, V. (1974) *J. Biol. Chem. 249*, 2587–2592.

Ghiretti-Magaldi, A., Cremona, T., Singer, T. P., & Bernath, P. (1961) *Biochem. Biophys. Res. Commun. 5*, 334–338.

Henderson, J. F. (1968) *Can. J. Biochem. 46*, 1381–1396.

Hitchman, M. L. (1978) *Measurement of Dissolved Oxygen*, p 28, Wiley, New York.

Horecker, B. L., & Kornberg, A. (1948) *J. Biol. Chem. 175*, 385.

Jorns, M., & Hersh, L. (1974) *J. Am. Chem. Soc. 96*, 4012–4014.

Loach, P. A. (1968) *Handbook of Biochemistry* (Sober, H. A., Ed.) J-29, Chemical Rubber Co., Cleveland, OH.

Lockridge, O., Massey, V., & Sullivan, P. A. (1972) *J. Biol. Chem. 248*, 7012–7017.

Londesborough, J. (1980) *Eur. J. Biochem. 105*, 211–215.

Louie, D., & Kaplan, N. O. (1970) *J. Biol. Chem. 245*, 5691–5698.

Massey, V. (1953) *Biochem. J. 53*, 72–79.

Massey, V., & Gibson, Q. H. (1964) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 23*, 18–29.

Massey, V., & Hemmerich, P. (1980) *Biochem. Soc. Trans. 8*, 246–257.

Massey, V., Ghisla, S., & Kiescke, K. (1980) *J. Biol. Chem. 255*, 2796–2806.

Molinari, R., & Lara, F. J. S. (1960) *Biochem. J. 75*, 57–65.

Morpeth, F. F., & Massey, V. (1982a) *Biochemistry* (second paper of three in this issue).

Morpeth, F. F., & Massey, V. (1982b) *Biochemistry* (third paper of three in this issue).

Olson, S. T., & Massey, V. (1979) *Biochemistry 18*, 4714–4724.

Palmer, G., & Massey, V. (1968) in *Biological Oxidations* (Singer, T. P., Ed.) p 263, Interscience, New York.

Pompon, D., Iwatsubo, M., & Lederer, F. (1980) *Eur. J. Biochem. 104*, 479–488.

Schopfer, L. M., & Massey, V. (1979) *J. Biol. Chem. 254*, 10634–10643.

Snoswell, A. (1966) *Methods Enzymol. 9*, 321–327.

Spencer, R., Fisher, J., & Walsh, C. (1976) *Biochemistry 15*, 1043–1053.

Stachiewicz, E., Labeyrie, F., Curdel, A., & Slonimski, P. P. (1961) *Biochim. Biophys. Acta 50*, 45.

Tubbs, P. K., & Greville, G. D. (1959) *Biochim. Biophys. Acta 34*, 290–291.

Whitfield, C. D., & Mayhew, S. G. (1974) *J. Biol. Chem. 249*, 2801–2810.