Biochem. J. (1970) **118**, 405–408
Printed in Great Britain

Mammalian D-2-Hydroxy Acid Dehydrogenase

EFFECT OF INHIBITORS AND REACTION SEQUENCE

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(*Received* 4 *March* 1970)

1. The reaction of D-2-hydroxy acid dehydrogenase with D-lactate and 2,6-dichlorophenol-indophenol (DCIP) at pH 8.6 yields reciprocal plots of 1/rate versus 1/[D-lactate], at different DCIP concentrations, which appear to be parallel. However, at pH 7.55, or in the presence of the competitive inhibitor oxalate at pH 8.6, the plots are convergent. This is inconsistent with the mechanism previously proposed for this enzyme. 2. The pattern of inhibition by the product, pyruvate, is consistent with either an Ordered mechanism or an Iso Theorell–Chance mechanism. 3. The observation that the enzyme forms a complex with D-lactate favours the Ordered reaction. In this, first D-lactate and then DCIP bind to the enzyme to form a ternary complex, from which pyruvate and reduced DCIP dissociate in that order.

D-2-Hydroxy acid dehydrogenase [D-2-hydroxy acid–(acceptor) oxidoreductase, EC 1.1.99.6) is an enzyme present in many animal tissues that catalyses the oxidation of a wide range of D-2-hydroxy acids to the corresponding keto acids, and uses a number of artificial acceptors (Tubbs & Greville, 1961). The acceptor used by the enzyme *in vivo* is not known. The enzyme has been purified from rabbit kidney (Cammack, 1969); it has a molecular weight of approx. 100000 and contains FAD. It is usually assayed by using D-lactate as substrate and the blue dye DCIP† as acceptor. It was proposed by Tubbs (1962) that the mechanism of this reaction was of the type classified by Dalziel (1957) as type IV (i) and by Cleland (1963*a*) as Ping Pong Bi Bi, in which the enzyme is alternately reduced by the substrate and reoxidized by the acceptor; both compounds do not bind to the enzyme at the same time. The mechanism was proposed on the basis of two observations. (1) The reciprocal plots of 1/rate versus 1/[D-lactate] at different values of [DCIP] were parallel, i.e. the K_m/V ratio for D-lactate was independent of the concentration of acceptor. The Ping Pong mechanism is the only common mechanism in which this happens. (2) The inhibition of the reaction by the product, pyruvate, was found to be approximately competitive with DCIP and approximately uncompetitive with D-lactate.

Subsequent studies on the kinetics of the purified enzyme have shown that the reciprocal plots for D-lactate and DCIP are not always parallel, and the previous observations were re-examined. The new results are inconsistent with the Ping Pong mechanism and in this paper an alternative reaction sequence is proposed.

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† Abbreviation: DCIP, 2,6-dichlorophenol-indophenol.

MATERIALS AND METHODS

Chemicals. Tris (Trizma base) was from Sigma (London) Chemical Co., London S.W.6, U.K.; sodium pyruvate was from Boehringer Corp. (London) Ltd., London W.6, U.K.; calcium D-lactate was from Calbiochem Ltd., London W.1, U.K.; other chemicals were the purest products of Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.

Standard potassium D-lactate was prepared by passing a solution of calcium D-lactate through a small column of Zeo-Karb 225 (Permutit Ltd., London W.1, U.K.) in the H^+ form, and immediately titrating the eluted D-lactic acid to pH 7 with standard KOH solution. DCIP was purified by a modification of the method of Savage (1957) and stored as the dry solid at 4°C. It was found that DCIP solution after storage for several weeks contained a compound that inhibited D-2-hydroxy acid dehydrogenase competitively with DCIP. Thus for these studies a fresh solution of DCIP was prepared daily. The molar extinction of DCIP at 600nm was taken as 22000 (Armstrong, 1964).

D-2-Hydroxy acid dehydrogenase was purified from rabbit kidneys as previously described (Cammack, 1969), and dissolved in 25mM-tris–chloride buffer, pH 8.6. The solution contained 0.60 unit/ml.

Assay conditions. The assay medium contained 100μmol of tris–chloride buffer, pH 8.6, various quantities of potassium D-lactate and DCIP and 25–50μl of enzyme solution, final volume 2.0ml, in a 1cm light-path cell. The assay

temperature was 30°C. The decrease of E_{600} with time was followed in a Beckman DK 2A recording spectrophotometer, and the initial rate measured.

RESULTS AND DISCUSSION

Fig. 1 shows reciprocal plots of 1/rate versus 1/[D-lactate] at various DCIP concentrations for purified rabbit kidney D-2-hydroxy acid dehydrogenase at pH 8.6. The lines appear to be parallel, in agreement with the results of Tubbs (1962). However, it was found that at lower pH values the lines were not parallel, but tended to converge to a point in the third quadrant. Fig. 2 shows this effect at pH 7.55. The plots of 1/rate versus 1/[DCIP] show a similar convergence.

Such convergent reciprocal plots are not consistent with the Ping Pong mechanism proposed by Tubbs (1962):

$$E+S \rightleftharpoons ES \rightleftharpoons E'P \rightleftharpoons E'+P \qquad \text{(Mechanism 1)}$$
$$E'+A \rightarrow E+A'$$

where E and E′ are oxidized and reduced forms of the enzyme, A and A′ are the oxidized and reduced forms of the acceptor, and S and P are the substrate and oxidized substrate, respectively. The general rate equation for a reaction involving two reactants S and A, giving reciprocal plots that are straight lines, may be written:

$$v = \frac{V}{1+\frac{K_S}{[S]}+\frac{K_A}{[A]}+\frac{K_{AS}}{[A][S]}} \qquad (1)$$

For the plots of $1/v$ versus $1/[S]$ at different values of [A] to be parallel the term in $K_{AS}/[A][S]$ must be negligible compared with the other terms in the denominator. In the rate equation for mechanism 1 this term is absent: the Ping Pong mechanism is the only simple bimolecular mechanism in which this occurs. However, in other types of mechanism it is

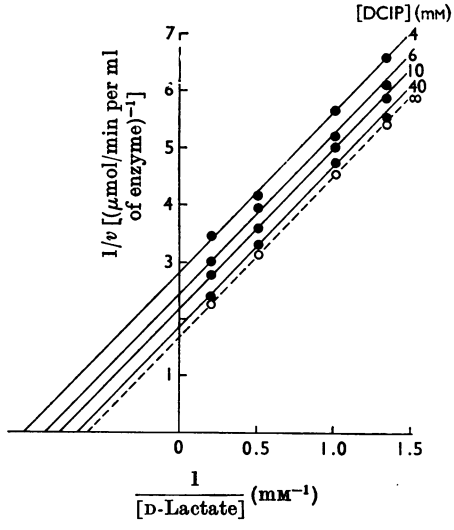

Fig. 1. Reciprocal plots of $1/v$ versus 1/[D-lactate] at pH 8.6; ●, at the fixed DCIP concentrations indicated; ○, extrapolated to infinite DCIP concentration.

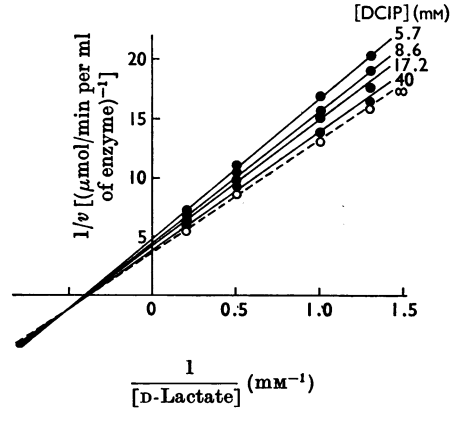

Fig. 2. Reciprocal plots of $1/v$ versus 1/[D-lactate] at pH 7.55; ●, at the fixed DCIP concentrations indicated; ○, extrapolated to infinite DCIP concentration.

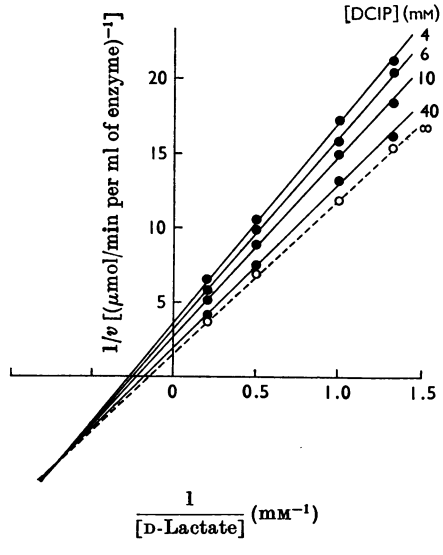

Fig. 3. Reciprocal plots of $1/v$ versus 1/[D-lactate] at pH 8.6 in the presence of 10 μM-oxalate; ●, at the fixed DCIP concentrations indicated; ○, extrapolated to infinite DCIP concentration.

possible for the term to be small, so that the reciprocal plots are approximately parallel.

Reciprocal plots in the presence of a competitive inhibitor. In order to increase any convergence of the reciprocal plots at pH 8.6, they were redetermined in the presence of oxalate, an inhibitor which is competitive with D-lactate (Tubbs, 1962). The effect of this is to increase both K_S and K_{AS} (if it exists) by a factor $(1+[I]/K_I)$, where [I] is the inhibitor concentration and K_I the dissociation constant of the enzyme–inhibitor complex (Cleland, 1963*b*). Fig. 3 shows that the plots became significantly convergent. This is inconsistent with Mechanism 1, since compounds that form dead-end complexes with the enzyme or one of the enzyme–substrate complexes cannot introduce a term in 1/[A][S] into the denominator of eqn. 1 (Cleland, 1963*b*).

Succinate dehydrogenase (EC 1.3.99.1) and D-amino acid oxidase (EC 1.4.3.3) were shown not to react by the Ping Pong mechanism because the reciprocal plots converged in the presence of malonate (DerVartanian, Zeijlemaker & Veeger, 1966; Zeijlemaker, DerVartanian, Veeger & Slater, 1969) and benzoate (Koster & Veeger, 1968) respectively. In each case it was proposed that the reaction mechanism was of the Theorell–Chance type (Theorell & Chance, 1951) in which the

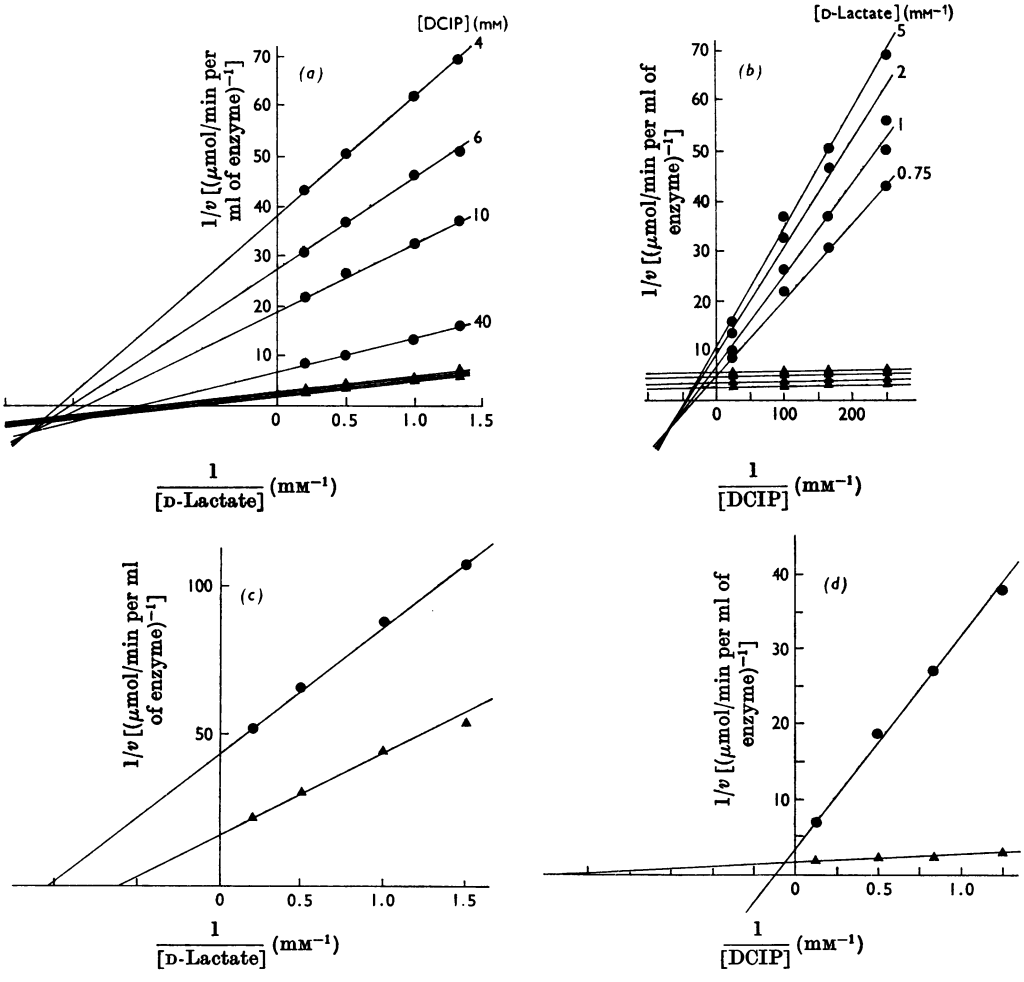

Fig. 4. Reciprocal plots determined at pH 8.6; ▲, in the absence of pyruvate; ●, in the presence of 10 mM-pyruvate. (*a*) 1/*v* versus 1/[D-lactate] at the DCIP concentrations indicated. (*b*) 1/*v* versus 1/[DCIP] at the D-lactate concentrations indicated. (*c*) 1/*v* versus 1/[D-lactate] extrapolated to infinite DCIP concentration. (*d*) 1/*v* versus 1/[DCIP] extrapolated to infinite D-lactate concentration.

enzyme–substrate complex is oxidized by the acceptor so rapidly that the ternary complex ESA is kinetically insignificant.

Fig. 4 shows the effect of pyruvate on the reciprocal plots for D-lactate and DCIP at pH 8.6. It may be seen that at finite concentrations of one reactant the inhibition is of a mixed type with respect to the other reactant. At infinite concentration of D-lactate the inhibition is of a mixed type towards DCIP, and at infinite concentration of DCIP the inhibition is very nearly uncompetitive towards D-lactate. The deviation from purely uncompetitive inhibition might be due to a small affinity of pyruvate for the D-lactate site on the oxidized enzyme, causing a small element of competitive inhibition towards D-lactate. A similar effect has been reported in yeast L-lactate–cytochrome *c* oxidoreductase (EC 1.1.2.3) (Hinkson & Mahler, 1963).

Cleland (1963*a*) has tabulated the pattern of product inhibition that would be expected for ten different types of reaction mechanism. Table 2 of his paper shows that the pattern of inhibition seen in the present case is inconsistent with the Ping Pong and Theorell–Chance mechanisms. However, it is consistent with particular forms of two other mechanisms, referred to as Ordered Bi Bi and Iso Theorell–Chance. The reaction sequences may be written:

(Ordered Bi Bi) $E+S \rightleftharpoons ES$ (Mechanism 2)

$$ES+A \rightleftharpoons EA'P \rightleftharpoons EA'+P$$

$$EA' \rightarrow E+A'$$

The Iso Ordered Bi Bi mechanism also gives the same pattern of product inhibition. Therefore in this case any isomerization of the free enzyme could not be detected kinetically.

(Iso Theorell–Chance) $E+A \rightleftharpoons EA$ (Mechanism 3)

$$EA+S \rightarrow EP+A'$$

$$EP \rightleftharpoons E^*+P$$

$$E^* \rightleftharpoons E$$

E* is a modified form of the oxidized enzyme.

The type of inhibition by pyruvate observed in the present case could only be obtained with this mechanism if the acceptor became bound to the enzyme before the substrate. Moreover, the mechanism would require that (1) the substrate must bind to the EA complex, become oxidized, and cause release of the reduced acceptor, all within a time so short that the ternary complex ESA is kinetically insignificant; (2) the enzyme undergoes an isomerization after completing each cycle of the reaction. These requirements are so restrictive that Mechanism 2 is the most likely reaction scheme for D-2-hydroxy acid dehydrogenase.

The observation that D-lactate alone can form a complex with the enzyme, producing a change in its flavin spectrum (Cammack, 1969) suggests that D-lactate is the first compound to bind to the enzyme, and thus favours the Mechanism 2. This evidence is not conclusive, however, since it has not so far been shown that this enzyme–lactate complex can be formed sufficiently rapidly to be a part of the enzyme reaction. To do this it would be necessary to follow the kinetics of its formation by rapid-reaction techniques. The same type of Ordered mechanism (Mechanism 2) was proposed by Hinkson & Mahler (1963) for yeast L-lactate–cytochrome *c* oxidoreductase with DCIP or ferricyanide as acceptor.

I thank Professor M. Dixon for his advice and encouragement during this work. The work was supported by a grant from the Science Research Council.

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