Catalytic Mechanism of Pig Heart Mitochondrial Malate Dehydrogenase Studied by Kinetics at Equilibrium*

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ABSTRACT: The effects of substrate concentration on the rates of interconversion of oxalacetate and malate and nicotinamide–adenine dinucleotide and reduced nicotinamide–adenine dinucleotide in solution at equilibrium in the presence of catalytic concentrations of pig heart mitochondrial malate dehydrogenase have been determined by means of isotopic exchange of substrate. At pH 8.0 increase in the concentration of oxalacetate and malate at equilibrium results in an increase toward a plateau value in the oxalacetate $\rightleftarrows$ malate rate and in an increase followed by a marked decrease to near zero in the nicotinamide–adenine dinucleotide $\rightleftarrows$ reduced nicotinamide–adenine dinucleotide rate, particularly at oxalacetate concentrations in excess of 20 mM. This result supports a compulsory binding order in which oxalacetate and malate will bind to enzyme–reduced nicotinamide–adenine dinucleotide and enzyme–nicotinamide–adenine dinucleotide, but not to enzyme devoid of bound coenzyme. The data at pH 9.0 was compatible with a partially compulsory substrate binding order in which coenzyme dissociation may occur from ternary enzyme–coenzyme–substrate complexes though at a lesser rate than from binary enzyme–coenzyme complexes. The oxalacetate $\rightleftarrows$ malate rate greatly exceeded the nicotinamide–adenine dinucleotide $\rightleftarrows$ reduced nicotinamide–adenine dinucleotide rate (up to 75-fold) except at lowest substrate concentration, suggesting that coenzyme dissociation rather than chemical transformation is rate limiting in the catalysis. Experiments with increasing concentrations of nonreactive substrate pairs suggest the formation of the abortive complex enzyme–reduced nicotinamide–adenine dinucleotide–malate but not enzyme–nicotinamide–adenine dinucleotide–oxalacetate. At pH 9.0 a decrease to a plateau value was found in the nonlimiting rate, oxalacetate $\rightleftarrows$ malate at elevated oxalacetate and malate levels suggesting a conformational change in the enzyme. Net initial rate of oxalacetate reduction exceeded oxalacetate $\rightleftarrows$ malate and nicotinamide–adenine dinucleotide $\rightleftarrows$ reduced nicotinamide–adenine dinucleotide rates while the net rate of malate oxidation was greater than the nicotinamide–adenine dinucleotide $\rightleftarrows$ reduced nicotinamide–adenine dinucleotide but less than the oxalacetate $\rightleftarrows$ malate rate. Minimum estimates of some dissociation constants were obtained.

The study of enzyme kinetics at equilibrium by isotopic exchange of substrate has been shown to be useful for elucidation of aspects of enzyme mechansim, some of which are not readily obtainable by other means. These include observation of rates which are not limiting in the catalysis, elucidation of the existence and order of compulsory pathway mechanisms, determination of whether chemical transformation or reactant dissociation is rate limiting, and estimation of minimum values for dissociation constants (Boyer, 1959; Boyer and Silverstein, 1963; Silverstein and Boyer, 1964a,b; Fromm *et al.* 1964). Investigation of these aspects of the mechanism of pig heart mitochondrial malate dehydrogenase is the subject of this report. Previous equilibrium kinetic study of dehydrogenases for lactate (bovine heart and rabbit muscle) and alcohol (horse liver and yeast) have revealed marked differences in their reaction mechanisms (Silverstein and Boyer, 1964a,b).

* From the Laboratory of Molecular Biology, Department of Medicine, State University of New York, College of Medicine, Brooklyn, New York. 11203. *Received December 2, 1968.* This work was supported in part by U. S. Public Health Service Research Grant AM 09273, Health Research Council of New York City Research Grant U1607, National Science Foundation Grant GB-8458, and University Awards Committee of State University of New York Grant S-68-12-001. A preliminary account has been presented (Silverstein and Sulebele, 1966).

Experimental Procedure

Materials. Oxalacetic acid, tricyclohexylamine phosphoenolpyruvate, nicotinamide, NAD, Na_2NADH, malic acid, and DEAE-cellulose were obtained from the Sigma Chemical Co., St. Louis; nicotinamide-^{14}C (COOH-^{14}C, 12 mCi/mmol) from Calbiochem, Los Angeles; sodium bicarbonate-^{14}C, 20.5 mCi/mmol from Nuclear-Chicago, New York; 2,4-dinitrophenylhydrazine from Eastman Kodak. Pig heart mitochondrial malate dehydrogenase was purchased from the Boehringer Mannheim Corp. and was inhibited by oxalacetate in excess of 1×10^{-4} M. Specific activity was 53 at 1° under the standard assay conditions given below.

Preparation of Oxalacetic Acid-4-^{14}C. Oxalacetic acid-4-^{14}C was prepared by carboxylation of phosphoenolpyruvate with bicarbonate-^{14}C catalyzed by spinach phosphoenolpyruvate carboxylase obtained by the method of Bandurski (1955) with a specific activity of 0.2. A mixture consisting of 0.1 M $NaHCO_3$-^{14}C (100 μCi), 0.1 M phosphoenolpyruvate, 2 mM $MgCl_2$, and 2 mg of lyophilized enzyme in 0.1 ml of 0.2 M Tris-Cl (pH 8.2) was allowed to react at 10° in a tightly stoppered tube for up to 1 hr until a maximum level of generated oxalacetate was observed by assay with malate dehydrogenase and NADH on aliquots of a duplicate nonradioactive mixture. The reaction was terminated by the addition of 4 mg of Dowex 50-H, the mixture brought to 0°, collected by centrifugation with 0.6 ml of distilled water, and acidified with concentrated HCl to 1 N. Nitrogen gas was bubbled through for 2 hr to remove CO_2-^{14}C

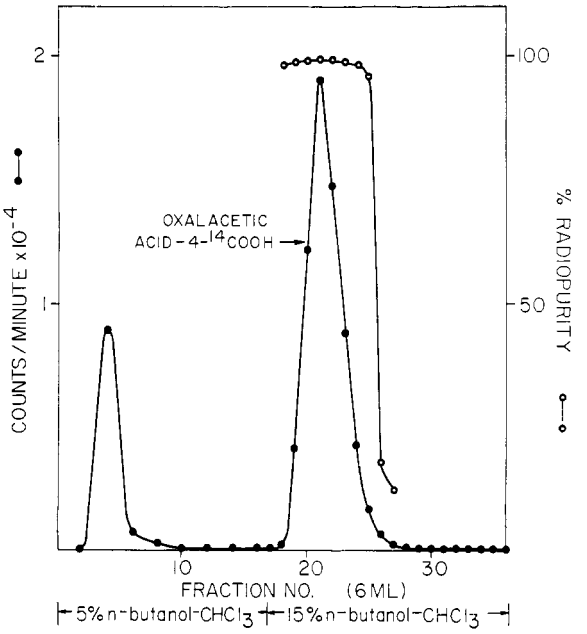

FIGURE 1: Purification of oxalacetic acid-4-COOH-^{14}C on silicic acid column (1 × 18 cm) containing 7 g of silicic acid. Stationary aqueous phase was 3 N H_2SO_4. Radiopurity of material placed on the column was 45%. A peak of radioimpurity was eluted with 5% 1-butanol-$CHCl_3$. Oxalacetic acid was extracted with 1 N HCl and radiopurity determined by 2,4-dinitrophenylhydrazone precipitation with and with out prior conversion to malate (Table I).

TABLE I: Enzymatic Test of Purity of Oxalacetic Acid-4-^{14}C by 2,4-Dinitrophenylhydrazine Precipitation.[a]

	Supernatant[b]	Precipitate[b]	% Impurity
Without conversion into malate-4-^{14}C	1,087	143,000	0.76
After conversion into malate-4-^{14}C	139,000	436	0.31

[a] Duplicate samples of oxalacetic acid-4-^{14}C were precipitated as the 2,4-dinitrophenylhydrazone without and with prior conversion into malate-4-^{14}C. The nonconverted sample was in a conversion mixture without enzyme. The percentage impurity is indicated by radioactivity not precipitable initially and by radioactivity precipitible after quantitative conversion of oxalacetic acid-4-^{14}C into malate-4-^{14}C, and is 1.07%. [b] Given in counts per minute.

The yield was 2–3 μmole of oxalacetic acid-4-^{14}C with a specific activity of 14–19 μCi/μmol. Radiopurity was 97–99% (Table I). For quantitation and separation from malate, oxalacetate-^{14}C in up to 0.5 ml was treated with 2 ml of 0.5% 2,4-dinitrophenylhydrazine in 4 N HCl for 20 min at 25° and precipitated by similar reaction with two 10–15-μmol additions of carrier oxalacetic acid (0.1 M in 0.1 N HCl; prepared just prior to use) followed by 20-min periods at 0°; 1 ml of 1% Triton X-100 and 30 or 40 μl of horse serum[1] were then added, and the precipitate obtained by centrifugation at 0–5° drained dry and washed with 4 ml of 0.25% Triton X-100 (0°). Aliquots of the 2,4-dinitrophenylhydrazine supernatant and the precipitate dissolved in pyridine were plated and radioactivity was determined in a gas-flow counter. Decrease in radiopurity from 98 to 47% on storage for 6 months in 0.1 N HCl at −15° (contrary to previous experience, DeVellis *et al.*, 1963) was prevented in 1 N HCl (6% impurity) but not by benzene addition (4, 25°) (Silverstein and Boyer, 1964c). Oxalacetate-4-^{14}C was stable for at least 2 hr at 1°, pH 8.0. Radioimpurity and residual nonradioactive pyruvate derived from unreacted phosphoenolpyruvate were removed by silicic acid chromatography at 4° (Figure 1) (Bulen *et al.*, 1952). Oxalacetic acid-^{14}C was extracted in 1 N HCl and stored at −15°.

Other Radiosubstrates, Analysis of Purity, Substrate Separations. NAD-carboxyl-^{14}C was prepared, stored, and examined for purity as described previously (Silverstein and Boyer, 1966). NADH-^{14}C was enzymically generated immediately prior to use (Silverstein and Boyer, 1964a). NADH-^{14}C counts enzymically convertible to NAD-^{14}C were determined by reaction with pyruvate and lactate dehydrogenase and ion-exchange separation. NAD and NADH were separated for assay of reaction mixtures on 1.9 × 3.0 cm DEAE-cellulose columns by NH_4HCO_3 elution (Silverstein, 1965). Immediately prior to use an aliquot of oxalacetic acid-^{14}C was thawed, neutralized with 1 N NaOH at 0°, and diluted in the buffer used for the kinetics.

Concentrations of substrate were verified by enzymic assay based on the absorbance of NADH at 340 mμ (Horecker and Kornberg, 1948). Purity of malic and oxalacetic acids was 100% as determined by quantitative conversion of product with malate dehydrogenase and coenzyme. Similar purity was readily achieved by recrystallization of a preparation of oxalacetic acid which was initially 60% pure (Pedersen, 1952). NAD concentration was checked by quantitative reduction by alcohol and yeast alcohol dehydrogenase at pH 10, while NADH concentration was checked by oxidation with pyruvate and lactate dehydrogenase at pH 7.

Equilibrium reactions were terminated with $AgNO_3$ (15 mM, final concentration) for NAD ⇄ NADH measurement, and HCl (final concentration, 3.6 N) containing 2,4-dinitrophenylhydrazine for oxalacetate ⇄ malate determination. The adequacy of termination of reaction was indicated spectrally by apparently instantaneous cessation of oxalacetate reduction by NADH upon addition of $AgNO_3$ and by equality of counts in exchange experiments obtained by termination of the reaction with strong acid and with $AgNO_3$.

Experimental Design for Kinetic Experiments at Equilibrium. Equilibrium substrate mixtures at pH 8.0[2] were prepared in water at 0° immediately prior to use. The absorbance at 340 mμ was taken at 1° before and after enzyme addition to ob-

[1] For compaction of precipitate; any protein is satisfactory.

[2] The pH values noted were determined with a glass electrode standardized at pH 7.0 with phosphate buffer and read at 25°. When standardized and read at 1, 10, and 20° the pH readings were 8.5, 8.4, and 8.2, respectively, for experiments noted at pH 8.0. Similarly, buffer which read 9.0 at 25° read 9.5 at 1°. Since similar equilibrium kinetic results were obtained at 1, 10, and 20°, it does not appear likely that the temperature related alterations in hydrogen ion activity indicated by these measurements significantly affected the catalytic mechanism of pig heart mitochondrial malate dehydrogenase.

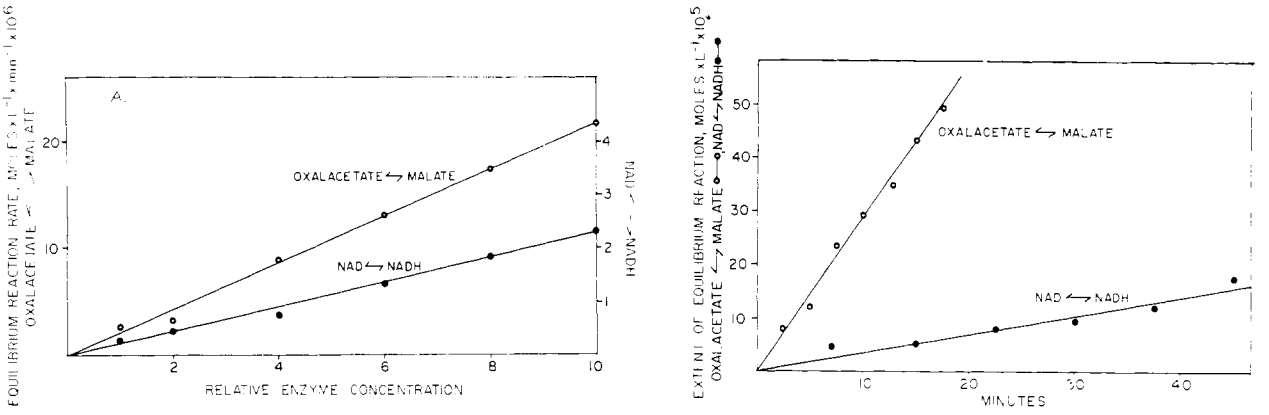

FIGURE 2: Direct proportionality studies. (A) Of equilibrium reaction rate and enzyme concentration. The reaction mixture contained 0.33–3.3 μg/ml of pig heart mitochondrial malate dehydrogenase, 4.95 mM NAD, 48 μM NADH, 23.3 mM malate, 233 μM oxalacetate, and 70 mM Tris-NO_3 at pH 8.0[2] and 1°. (B) Direct proportionality of extent of reaction at equilibrium with time. The reaction mixture contained 3.1 μg/ml of pig heart mitochondrial malate dehydrogenase, 68.5 μM NADH, 6.85 mM NAD, 32.3 mM malate, 323 μM oxalacetate, and 70 mM Tris-NO_3 at pH 8.0[2] and 1°.

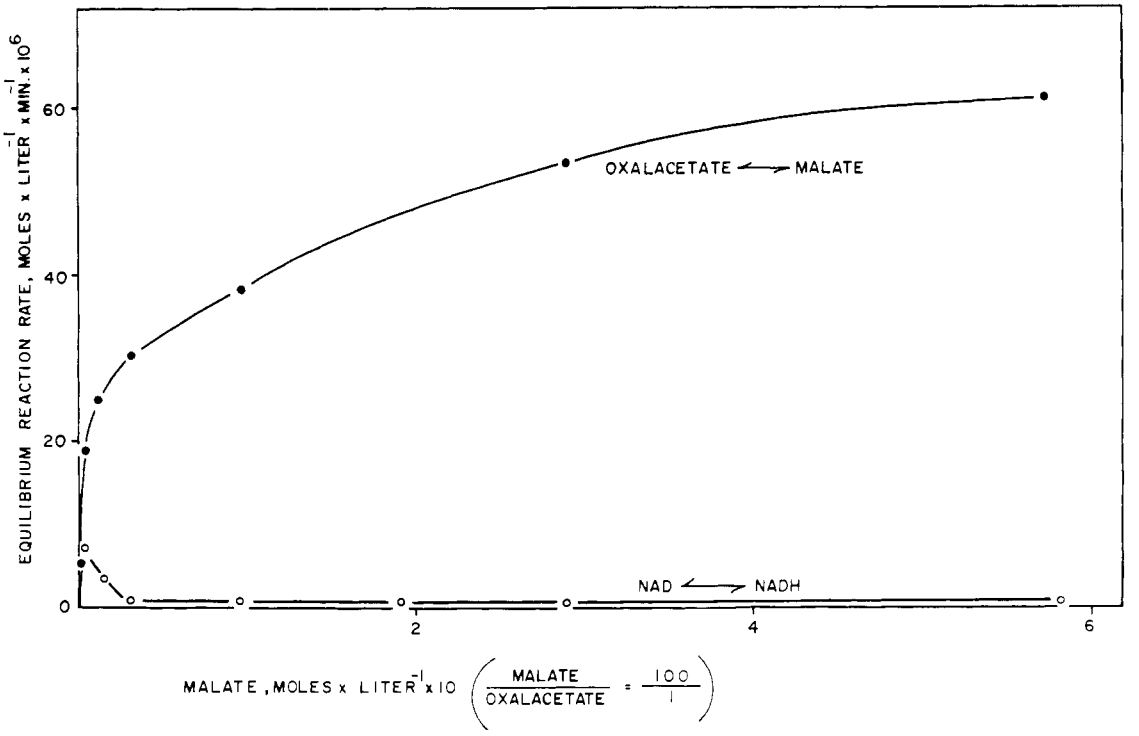

FIGURE 3: Effect of malate/oxalacetate concentration on the oxalacetate $\rightleftarrows$ malate and NAD $\rightleftarrows$ NADH reaction rates at equilibrium catalyzed by pig heart mitochondrial malate dehydrogenase. Reaction mixtures contained 7.28 mM NAD, 73.2 μM NADH, 4.2 μg/ml of enzyme, and malate and oxalacetate as shown in 70 mM Tris-NO_3 at pH 8.0[2] and 1°.

serve any substrate shift and to ensure the presence of equilibrium. Oxalacetate-^{14}C and NADH-^{14}C, respectively, in the buffer present in the reaction mixture, were added in duplicate or triplicate to separate 0.2-ml aliquots of reaction mixture generally at 1°[3] and the reaction stopped when sufficient time had elapsed for 20–80% of isotopic equilibrium to be attained. Unreacted radiosubstrate was determined in triplicate or quadruplicate by addition of the enzyme inactivating agent to an aliquot of reaction mixture prior to addition of radiosubstrate.

Reaction rates were calculated from initial and final radiosubstrate activity, reaction time, and equilibrium substrate concentration (Boyer, 1959). When not otherwise indicated, initial rate measurements were determined by the rate of change of absorbance at 340 mμ of a reaction mixture containing 0.209 μmol of NADH, 1 μmol of oxalacetate (freshly prepared), 320 μmol of Tris-NO_3 at pH 8.0, and 1° in a volume of 1.63 ml.

[3] Temperature of 1° was generally used to preclude any problems of oxalacetate decomposition during measurements.

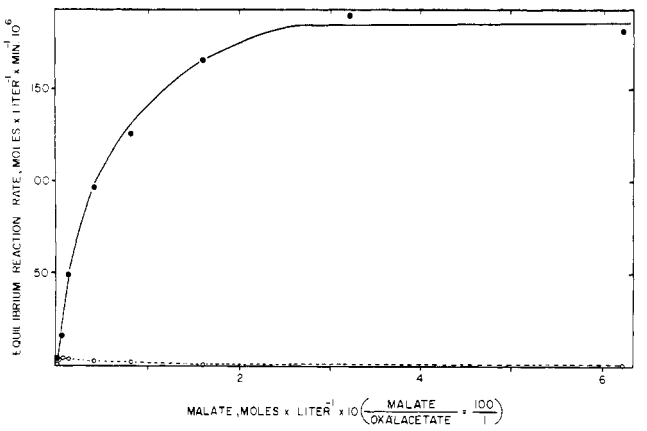

FIGURE 4: Effect of malate/oxalacetate concentration on oxalacetate ⇄ malate and NAD ⇄ NADH reaction rates at equilibrium catalyzed by pig heart mitochondrial malate dehydrogenase. Reaction mixtures contained 48.9 mM NAD, 491 μM NADH, 1.84 μg/ml of enzyme, oxalacetate, and malate as indicated in 70 mM Tris-NO_3 at pH 8.0[2] and 20°.

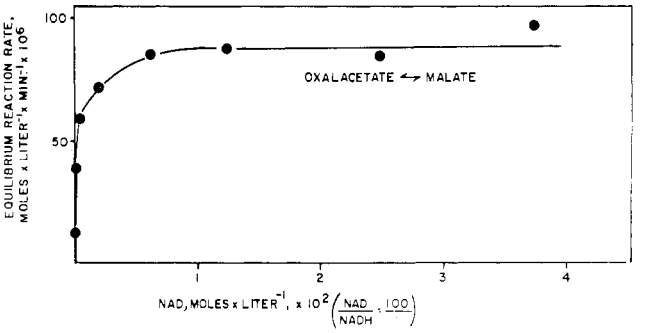

FIGURE 5: Effect of NAD/NADH concentration on the oxalacetate ⇄ malate reaction rate at equilibrium catalyzed by pig heart mitochondrial malate dehydrogenase. Reaction mixtures contained 3.31 mM oxalacetate, 331 mM malate, 7.4 μg/ml of enzyme, and NAD and NADH as shown in 70 mM Tris-NO_3 at pH 8.0[2] and 1°.

Results

Effect of Enzyme Concentration and Time of Reaction. The equilibrium reaction velocities oxalacetate ⇄ malate and NAD ⇄ NADH were found to be directly proportional to enzyme concentration as are initial net velocities, and thus are a valid measurement of enzyme concentration (Figure 2A). The extent of reaction was directly proportional with time, indicating constancy of rate during the intervals measured (Figure 2B). This relationship was utilized in measuring greatly disparate rates by use of different time periods of reaction.

Effect of Substrate Concentration on Equilibrium Reaction Rates. Increasing oxalacetate/malate[4] concentration to saturating levels at constant NAD/NADH concentration at pH 8.0[2] resulted in up to 8-fold reduction from maximum value in the NAD ⇄ NADH rate without any reduction in the oxalacetate ⇄ malate rate (Figure 3). The marked disparity between the NAD ⇄ NADH and oxalacetate ⇄ malate rates increased from about 3-fold at low substrate concentration and maximum NAD ⇄ NADH to about 75-fold at highest substrate concentration. The initial rate of oxalacetate reduction is greater than both equilibrium rates, while the initial rate of malate oxidation was about half the oxalacetate ⇄ malate rate, but eight times the NAD ⇄ NADH rate (Table II).

Equilibrium reaction rates at constant NAD/NADH concentration and pH 8.0 at 10° and 20° (Figure 4) were similar to those obtained at 1°. This finding gives confidence that the response of the enzyme-catalyzed equilibrium reaction rates to substrate concentration at 1° is qualitatively similar to that at temperatures closer to physiologic range. The ratios of maximum oxalacetate ⇄ malate/NAD ⇄ NADH at 20° (52.0) and 10° (11.4) are greater than at 1° (9.1). The initial rate at 10° was 1.71 times greater than the maximum oxalacetate ⇄ malate rate and 19.7 times greater than the maximum NAD ⇄ NADH rate.

Increasing NAD and NADH concentration to more than tenfold k_m with oxalacetate and malate constant at pH 8.0 resulted in an increase in the oxalacetate ⇄ malate rate to a plateau (Figure 5). This finding argues against the presence of a compulsory binding order with substrate binding prior to coenzyme.

Considerably less disparity in rate and depression of NAD ⇄ NADH rate at elevated oxalacetate/malate concentration was found at pH 9.0[2] than at pH 8.0 (Figure 6). A phenomenon similar to that previously observed with bovine heart lactate dehydrogenase at pH 9.7 but not at pH 7.9 was a significant depression in the oxalacetate ⇄ malate rate to a constant value above about 100 mM malate. Half-maximal NAD ⇄ NADH rates occurred at higher malate ⇄ oxalacetate concentration at pH 9.0 than at pH 8.0 suggesting larger coenzyme dissociation constants. The initial rate of oxalacetate reduction was 1.62 times the maximum oxalacetate ⇄ malate rate and 9.76 times the maximum NAD ⇄ NADH rate.

The nonreactive substrate pairs oxalacetate and NAD and malate and NADH were increased while maintaining equilibrium to test for the possible formation of abortive or nonreactive complexes by binding of nonreactive substrate pairs at active sites. Increasing oxalacetate/NAD concentration resulted in marked inhibition in the NAD ⇄ NADH rate with no inhibition in the oxalacetate ⇄ malate rate (Figure 7). If active sites were bound with nonreactive complexes one would expect both oxalacetate ⇄ malate and NAD ⇄ NADH rates to fall. On the other hand, this result strongly supports a compulsory binding order. The initial rate from the oxalacetate–NADH side was 1.15 times the maximum oxalacetate ⇄ malate rate and 210 times the maximum NAD–NADH rate. On the other hand, increasing levels of malate/NADH resulted in increasing moderate depression in both the oxalacetate ⇄ malate and NAD ⇄ NADH rates suggesting the formation of the abortive complex E–NADH–malate (Figure 8). The initial rate was 4.53 times the maximum oxalacetate ⇄ malate rate and 9.26 times the maximum NAD ⇄ NADH rate.

Minimum values for dissociation constants may be obtained from the relationship between equilibrium reaction rate and substrate concentration. For example, when only oxalacetate concentration is rate limiting, from the rate equation for oxacaletate ⇄ malate it can be shown that the ratio of slope to intercept of a plot of 1/oxalacetate ⇄ malate *vs.* 1/(oxalacetate) yields $K_3/(1 + (k'/k))$ (Figure 9) (Boyer and Silverstein, 1963; Silverstein and Boyer, 1964a). The minimum

[4] Oxalacetate/malate concentration indicates oxalacetate and malate concentration at constant ratio. Similar designations are used for other reactant concentrations.

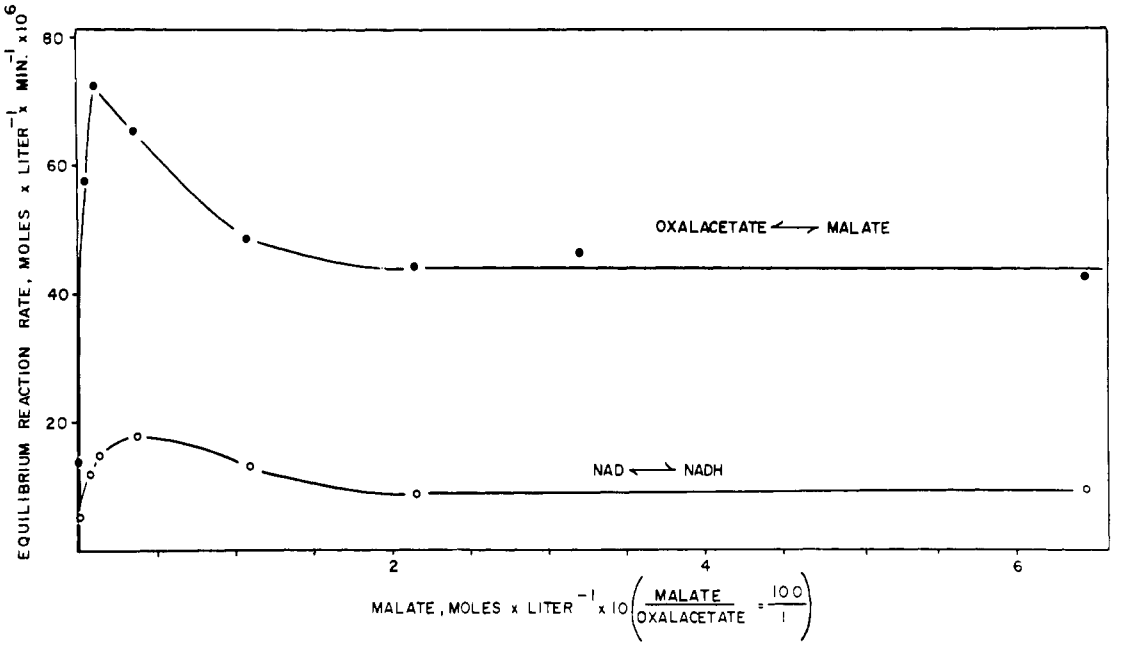

FIGURE 6: Effect of malate/oxalacetate concentration on oxalacetate ⇄ malate and NAD ⇄ NADH reaction rates at equilibrium catalyzed by pig heart mitochondrial malate dehydrogenase. Reaction mixtures contained 7.18 mM NAD, 721 μM NADH, 2.96 μg/ml of enzyme and oxalacetate and malate as indicated in 213 mM Tris-NO_3 at pH 9.0[2] and 1°.

TABLE II: Comparison of Initial and Equilibrium Rates.[a]

Substrate (mol × l.$^{-1}$)	Rate (mol × l.$^{-1}$ × min^{-1} × 10^6)			Initial Rate/Equilibrium Rate	
		Equilibrium			
	Initial	Oxalacetate ⇄ Malate	NAD ⇄ NADH	Oxalacetate ⇄ Malate	NAD ⇄ NADH
Oxalacetate[b]	Oxalacetate malate				
6.08×10^{-5}	44.0	11.3	3.27	3.89	13.4
6.08×10^{-4}	70.2	19.9	1.28	3.53	54.8
6.08×10^{-3}	27.5	16.9	0.36	1.63	76.5
Malate	Malate oxalacetate				
6.08×10^{-2}	10.2	19.9	1.28	0.512	7.96

[a] Initial rate was assayed in 200 mM Tris-NO_3 at pH 8.0[2] and 1° in a volume of 1.645 ml. For oxalacetate reduction 137.2 μM NADH was used, while malate was oxidized in the presence of 10.23 mM NAD. Enzyme concentration was 1.84 μg/ml for equilibrium rate and 0.316 μg/ml for initial rate determinations. Conditions for equilibrium rate measurements are given in Figure 6. Initial and equilibrium rates are normalized with respect to enzyme concentration. [b] Malate concentration was always 100 times higher than oxalacetate for the equilibrium measurements.

estimates of dissociation constants for the reactant in low and likely rate limiting concentration approximate values derived from net reaction velocity studies and are generally lower (Table III).

Discussion

If the malate dehydrogenase reaction has a compulsory order as indicated by the solid arrows in Figure 9, such that the binary complexes E–NAD and E–NADH exist, but E–oxalacetate and E–malate do not, increasingly elevated levels of oxalacetate and malate with maintenance of equilibrium would tend to depress the NAD ⇄ NADH but not the oxalacetate ⇄ malate rate since dissociation of E–NAD and E–NADH would increasingly be prevented by binding of substrate to re-form the ternary complexes from which NAD and NADH cannot dissociate and exchange with the preponderantly non-enzyme bound coenzyme pool. Thus, while the chemical con-

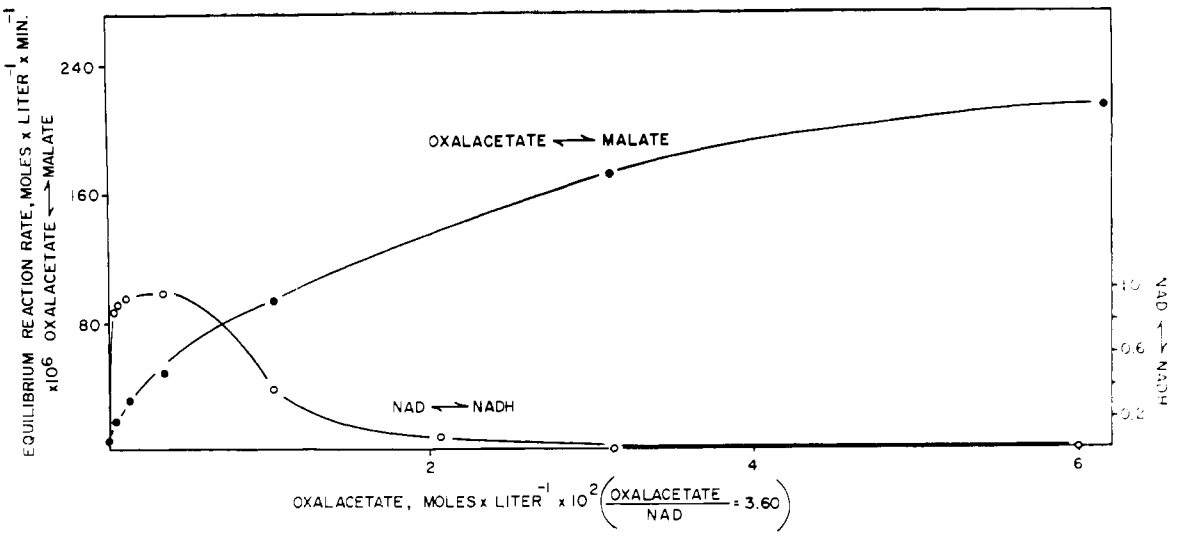

FIGURE 7: Effect of oxalacetate/NAD concentration on oxalacetate ⇄ malate and NAD ⇄ NADH reaction rates at equilibrium catalyzed by pig heart mitochondrial malate dehydrogenase. Reaction mixtures contained 258 mM malate, 7.23 μM NADH, 5.92 μg/ml of enzyme, NAD and oxalacetate as indicated in figure, 70 mM Tris-NO_3 at pH 8.0[2] and 1°.

TABLE III: Estimates of Minimum Values for Dissociation Constants from $1/R'^{a}$ *vs.* $1/S$ Plots.

Substrate	Condition	Dissociation Constant
	Net Reaction Kinetics	
Oxalacetate	pH 8.0, 50 mM Tris-acetate, 25° (Raval and Wolfe, 1962)	4.0×10^{-5} (K_m)
	pH 9.0, Tris-acetate, 0.05 μ, 25° (Raval and Wolfe, 1963)	1.0×10^{-4} (K_m)
NADH	pH 8.0, 50 mM Tris-acetate, 25° (Raval and Wolfe, 1962)	5.1×10^{-6}
NAD	pH 8.0, 50 mM Tris-acetate, 25° (Raval and Wolfe, 1962)	7×10^{-4}
	Equilibrium Kinetics	
Oxalacetate	pH 8.0,[2] variable oxalacetate/malate, 1°	3.0×10^{-5}
	pH 8.0, variable oxalacetate/malate, 10°	3.1×10^{-5}
	pH 8.0, variable oxalacetate/malate, 20°	5.6×10^{-4}
	pH 9.0, variable oxalacetate/malate, 1°	2.5×10^{-5}
NADH	pH 8.0, variable malate/NADH, 1°	4.2×10^{-6}
	pH 8.0, variable NAD/NADH, 1°	3.2×10^{-6}
NAD	pH 8.0, variable oxalacetate/NAD, 1°	4.2×10^{-4}

[a] R' refers to the oxalacetate ⇄ malate reaction rate at equilibrium.

version of substrate and coenzyme occurs stoichiometrically at equal rate on the enzyme, rate inequality of reactant interchange may occur if dissociation steps are rate limiting (Boyer, 1959; Boyer and Silverstein, 1963; Silverstein and Boyer, 1964a,b). These results thus support such a compulsory reaction pathway in the mechanism of pig heart mitochondrial malate dehydrogenase at pH 8.0. This finding is in accord with previous evidence obtained by means of initial rate kinetics (Raval and Wolfe, 1962). A compulsory reaction pathway has been previously demonstrated by equilibrium kinetics for lactate dehydrogenase from mammalian heart and skeletal muscle (Silverstein and Boyer, 1964a) but not for the alcohol dehydrogenases from yeast and horse liver (Silverstein and Boyer, 1964b) and is thus not a mechanism common to all dehydrogenases as has been suggested (Raval and Wolfe, 1962).

Increasing concentration of a nonreactive pair (NAD and oxalacetate and NADH and malate) will result in decrease in all equilibrium reaction rates if abortive complexes form, removing enzyme active sites from catalysis (Boyer and Silverstein, 1963) (Figure 9). These findings thus support the formation of the abortive ternary complex E–NADH–malate. This suggests that the presence of enzyme bound NAD specifies the binding of malate to the substrate site, whereas E–NADH may bind oxalacetate or malate. The enzyme would thus appear to be more perfectly designed to ensure catalysis in the more physiologic direction of oxidation than reduction. E–NADH–malate was not detected previously by fluorescence techniques (Thorne, 1960), although the system used differs from the present one. It should be noted that the malate/NADH effect could conceivably be due to decrease in rate-

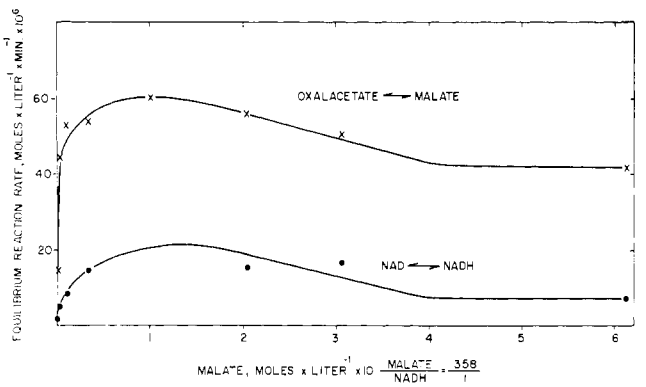

FIGURE 8: Effect of malate/NADH concentration on oxalacetate ⇄ malate and NAD ⇄ NADH reaction rates at equilibrium catalyzed by pig heart mitochondrial malate dehydrogenase. Reaction mixtures contained 258 μM oxalacetate, 7.19 mM NAD, 5.92 μg/ml of enzyme, malate, and NADH as indicated, and 70 mM Tris-NO_3 at pH 8.0[2] and 1°.

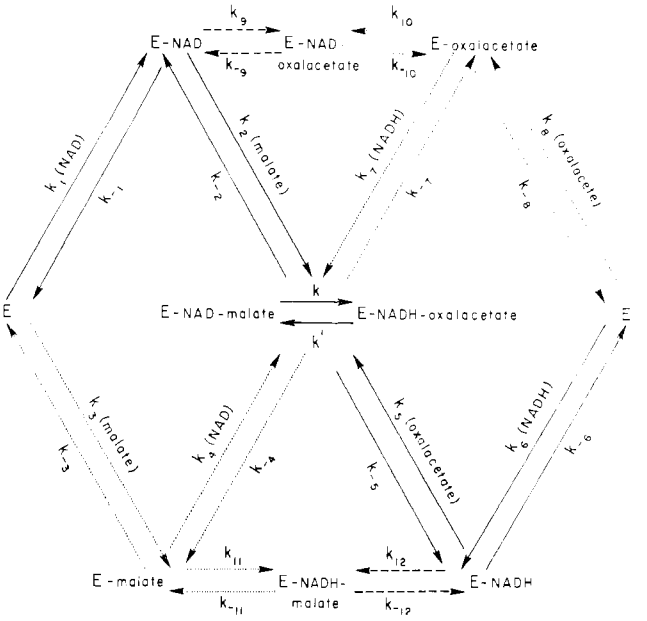

FIGURE 9: Kinetic scheme for the reaction malate + NAD ⇄ oxalacetate + NADH with two reactive and two unreactive ternary complexes. The compulsory reaction pathway is indicated by solid arrows, abortive ternary complexes by dashed arrows and other pathways by dotted arrows.

limiting steps in both interchanges, perhaps by binding of malate, NADH or both at sites other than the catalytic sites. Since this possibility would involve simultaneous influence of very similar degree on two dissociation steps of differing rate, it would seem far less likely than the model proposed.

The NAD ⇄ NADH interchange at pH 9.0 is much less inhibited by elevated oxalacetate and malate concentration than at pH 8.0. Such a result is compatible with a partially compulsory pathway at this pH in which dissociation of coenzyme may occur from ternary complex but at a slower rate than from enzyme–coenzyme complex (Boyer and Silverstein, 1963).

At pH 9.0 the oxalacetate ⇄ malate rate decreased to plateau levels with increasing concentration of malate and oxalacetate. Such behavior could result from binding of malate, oxalacetate or both at a noncatalytic modifier site having a lesser binding affinity than the chemical transformation site, resulting in a decrease in a rate-limiting constant, perhaps mediated through a change in enzyme conformation (Atkinson, 1966). Binding of pyruvate at a noncatalytic site has analogously been suggested to explain pyruvate inhibition of initial reaction velocity (Schwert and Winer, 1963). A similar phenomenon may affect coenzyme interchange and result in substrate inhibition. The suggestion that oxalacetate may bind at the NADH site thereby causing inhibition of initial reaction velocity seen at elevated oxalacetate concentration (Vestling *et al.*, 1960) would appear to be incompatible with the finding at equilibrium that the oxalacetate ⇄ malate rate is not also inhibited.

The marked inequality of the equilibrium rates above initial low levels of oxalacetate and malate indicates that chemical transformation is not rate limiting. The considerably lesser inequality of rate which occurs at pH 9.0 may be indicative of a closer approximation to rate limitation of chemical transformation than at pH 8.0. The greater rate disparity found at 20° than at 10 or 1° suggests that chemical transformation greatly exceeds the slowest step at this temperature, but it is not possible from the data at hand to differentiate between dissociation or chemical transformation steps as rate limiting in the oxalacetate ⇄ malate rate (Boyer and Silverstein, 1963).

Since the initial velocity of oxalacetate reduction was always greater than the equilibrium velocity (Table II), it can be shown from the respective rate equations that $1/k' + 1/k_{-5} + k'/kk_{-5}$ must be greater than $1/k_{-1}$ (Silverstein and Boyer, 1964a). This relationship would hold if k' were larger than the other rate constants listed, which is quite possible from the nature of the equilibrium.

The difference in net initial rate of oxalacetate and malate reaction relative to their interconversion rate at equilibrium (Table II) is likely due to a slower rate-limiting step (E–NADH dissociation) for malate oxidation than for oxalacetate reduction (E–NAD dissociation). The decrease in the ratio of initial to equilibrium velocity for the conversion of oxalacetate into malate with increasing oxalacetate concentration may reflect substrate inhibition of rate-limiting coenzyme dissociation steps which have no effect on the oxalacetate ⇄ malate rate but govern the initial velocity. The greater inhibition of NAD ⇄ NADH than net initial velocity of oxalacetate reduction with increasing oxalacetate concentration may reflect the additional presence of malate in the equilibrium system.

Recently Harada and Wolfe (1968a,b) have proposed a reciprocating compulsory order mechanism for pig heart mitochondrial malate dehydrogenase on the basis of kinetic data with the unnatural substrate ketomalonate and its reduced analog, hydroxy malonate. Surprisingly, hydroxy malonate acted only as an inhibitor and not as a substrate. This interesting mechanism differs from the compulsory order mechanism depicted in Figure 9 in allowing for chemical transformation at one of two catalytic sites only when the second is not occupied by reactants, in providing for ordered dissociation of product from the first site only after the second site is bound in an ordered manner by reactants, and in the absence of free enzyme and binary enzyme–reactant complexes.

Such a mechanism would predict that highly saturating

levels of malate and oxalacetate at equilibrium would result in decrease in the oxalacetate $\rightleftarrows$ malate rate since the concentration of the pentenary complex

$$\begin{matrix}\text{malate} & & \text{NADH} \\ & >\text{E}< & \\ \text{NAD} & & \text{oxalacetate}\end{matrix}$$

in which chemical transformation cannot take place, would be maximized, while

$$>\text{E}<_{\text{oxalacetate}}^{\text{NADH}} \quad \text{and} \quad >\text{E}<_{\text{NAD}}^{\text{malate}}$$

in which chemical transformation may take place, would be minimized. For similar reasons increasing the reactant pairs NAD and NADH or NAD and oxalacetate would also tend to diminish the concentration of enzyme species in which chemical transformation may take place and would therefore be expected to decrease the oxalacetate $\rightleftarrows$ malate rate. These experiments (Figures 3–5 and 7), done at a pH similar to that used by Harada and Wolfe (1968a,b), fail to show the inhibition in the oxalacetate $\rightleftarrows$ malate rate expected for the reciprocating mechanism. The reciprocating compulsory order mechanism thus appears to be incompatible with the equilibrium exchange experiments here reported. This conclusion is strengthened by the observation that the reciprocating compulsory mechanism has the uneconomical feature of apparently allowing for half the catalytic centers to be functioning in substrate turnover at any one time. It would thus require twice as many enzyme molecules to attain the same reaction rate as with simultaneously functioning catalytic centers, and would therefore seem wasteful of the organism's economy and disadvantageous to its evolutionary survival.

Two explanations for decreased maximum initial velocity of reduction of ketomalonate as compared with oxalacetate other than the reciprocating compulsory order mechanism seem plausible. First, the chemical transformation step or hydroxy malonate dissociation step for ketomalonate reduction may be rate limiting, unlike the case in oxalacetate reduction where NAD dissociation is rate limiting. Second, enzyme conformation change, which was hypothesized to occur in a quantitatively different manner for binding of ketomalonate as compared with oxalacetate, could conceivably be slower than dissociation of NAD from enzyme, and thus account for an effect of substrate binding on the coenzyme binding site after substrate has already dissociated, as alluded to by Harada and Wolfe (1968b) as a memory effect. That ketomalonate reduction may not be entirely similar to oxalacetate reduction is suggested by our observations, which will be reported more fully elsewhere, that *p*-hydroxymercuribenzoate activation of oxalacetate reduction is not seen with ketomalonate as substrate, and by the lack of back-reaction with hydroxy malonate, which may perhaps indicate a reaction more complex than the apparent interconversion of ketomalonate and hydroxy malonate. It should also be noted that the formation of the abortive complex, E–NAD–oxalacetate, as an explanation of substrate inhibition (Harada and Wolfe, 1968b) is not supported by equilibrium experiments reported herein.

It is concluded that the compulsory order mechanism depicted in Figure 9 for one catalytic center apparently obtains for all catalytic centers on the enzyme, and is most compatible with the present information. Apparently all catalytic centers may catalyze the reaction simultaneously, although there may certainly be influence of catalytic centers on each other through reactant binding and protein conformational change.

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