Kinetic Studies on the Mechanism of the Malate Dehydrogenase Reaction*

ELIZABETH HEYDE AND S. AINSWORTH
From the Department of Biochemistry, The University, Sheffield 10, England

SUMMARY
This work is a kinetic investigation of the reaction mechanism of malate dehydrogenase, prepared from washed mince of whole bovine heart by a variation on previous methods.

The forward and reverse reactions catalyzed by this enzyme have been studied at pH 8.0 in the presence and in the absence of one product at a time, with the use of a recording fluorometer to measure changes in the concentration of NADH.

The initial velocity pattern in the absence of products and the product inhibition pattern have been determined. These are consistent with an ordered mechanism which has a kinetically significant ternary complex, and in which the coenzyme substrates combine with the free enzyme.

Values have been determined for all the Michaelis, dissociation, and inhibition constants of the reaction.

The dissociation constants determined for the coenzymes acting as substrates differ from estimates of the same constants obtained by studying the coenzymes as product inhibitors. These effects may be related to substrate inhibition by oxalacetate and substrate activation by malate, which have been observed previously.

The rate constants calculated for the separate reaction steps in the mechanism reveal that the simplest "Ordered Bi Bi" mechanism (Cleland, W. W., Biochim. Biophys. Acta, 67, 104 (1963)) does not apply; they are consistent with an Ordered Bi Bi mechanism in which the enzyme-oxidized coenzyme complex isomerizes. As with other dehydrogenases for which this condition applies, the possibility cannot be excluded that the enzyme-reduced coenzyme complex may also isomerize.

Malate dehydrogenase (l-malate:NAD oxidoreductase, EC 1.1.1.37) appears to have the lowest molecular weight of the known NAD-requiring dehydrogenases. An investigation into the mechanism of the reaction catalyzed by this enzyme might therefore be considered of particular value for gaining insight into common features of dehydrogenase reactions in general.

Whitehead (1) speculated that each of this group of enzymes might consist of one or more units of molecular weight approximately 20,000, because the molecular weights of the individual dehydrogenases tended to be multiples of this value. Malate dehydrogenase from the mitochondrial fraction of bovine heart was reported by Davies and Kun (2) to have a molecular weight of approximately 15,000, and thus appeared to be the only dehydrogenase which might be a monomer. However, Siegel and England (3) prepared the enzyme by a somewhat different method and reported a molecular weight of 62,000. Moreover, their preparation appeared to be homogeneous in the ultracentrifuge, whereas that of Davies and Kun (2) showed a small impurity. Because of the uncertainty concerning the molecular weight of bovine heart mitochondrial malate dehydrogenase, the present work was designed to check on this as well as to study the reaction mechanism of the enzyme. The method of preparation, from acetone-treated whole bovine heart, was similar to that used by Siegel and England (3), but avoided the heat fractionation and gave a higher yield of enzyme with a specific activity intermediate between those reported by Siegel and England (3) and by Davies and Kun (2) or Grimm and Doherty (4). The molecular weight of the enzyme was found to agree with that reported by Siegel and England. Structural studies on the enzyme, together with details of the preparation, will be reported elsewhere.

The present study establishes the complete product inhibition pattern for bovine heart mitochondrial malate dehydrogenase. All experiments were performed at pH 8.0, so that kinetic parameters obtained from studies of the reaction in opposite directions could be compared directly. Moreover, comparisons may be made with the kinetic studies of Raval and Wolfe (5, 6) on pig heart mitochondrial malate dehydrogenase, which were also performed at pH 8.0 in the same buffer. Care was taken to avoid those ranges of concentration of oxalacetate or malate in which substrate inhibition or activation, respectively, occurs (2, 3). The results indicated that the reaction mechanism is of the "Ordered Bi Bi" type (7), although a detailed study of the kinetic and rate constants revealed that the mechanism is more...
Kinetic Studies on Mechanism of Malate Dehydrogenase Reaction

It will be assumed now, and justified later by the experimental results, that the reaction mechanism is of the Ordered Bi Bi type (7), as represented in Scheme 1.

\[
\begin{array}{cccccc}
A & B & P & Q & E \\
E & EA & EAB & EPQ & EQ & E
\end{array}
\]

**SCHEME 1**

Here A, B, P, Q, and E represent NADH, oxalacetate, malate, NAD, and enzyme, respectively. The complete rate equation for this reaction mechanism, in terms of the kinetic constants used by Cleland (7), is

\[
v = \frac{V_f V_r (AB - PQ/K_{eq})}{K_a K_b A + K_c K_d A + K_e K_f A + V_f + \frac{K_g K_f P}{K_a} + \frac{K_h K_d P}{K_b} + \frac{K_i K_c P}{K_d} + \frac{K_j K_b P}{K_e} + \frac{K_k K_a P}{K_f} + \frac{V_f P}{K_p} + \frac{V_r P}{K_q} + \frac{K_a K_b K_c K_d K_e K_f}{V_f V_r K_{eq}}}
\]

This equation may be modified to give all the rate equations for initial velocities in the absence or presence of a product, by setting the appropriate reactant concentrations to zero. \(V_f\) and \(V_r\) represent the maximum velocities of the forward and reverse reactions, respectively. \(K_{ia}\) and \(K_{iq}\) are dissociation constants for the reactions of the free enzyme with A and Q, respectively. The constants \(K_a, K_b, K_c, K_d,\) and \(K_e\) are Michaelis constants for A, B, P, and Q, respectively, and have no obvious significance apart from representing the concentration of each reactant yielding half the maximum velocity when the complementary substrate is at saturating concentration. \(K_{in}\) and \(K_{ip}\) are inhibition constants without obvious physical significance. The equilibrium constant, \(K_{eq}\), is replaced by kinetic constants when required by means of the Haldane relationship

\[
K_{eq} = \frac{V_f V_r K_{eq}}{V_f V_r K_{eq}}
\]

The initial velocity equations in the absence of products are, for the forward reaction

\[
v_f = \frac{V_f}{K_a K_b A + K_c K_d A + K_e K_f A + 1}
\]

and, for the reverse reaction

\[
v_r = \frac{V_r}{PQ + K_a K_b P + K_c K_d P + K_e K_f P + 1}
\]

Thus, whichever substrate is varied, a double reciprocal plot should be a straight line showing both slope and intercept variation with change in concentration of the fixed substrate.

The initial velocity equations in the presence of one product at a time are listed below.

For the forward reaction, with malate (P) as product inhibitor, and (a) NADH (A) varied

\[
1 - \frac{K_a}{V_f} \left(1 + \frac{K_d K_b}{K_a K_b} \left(1 + \frac{K_f K_b}{K_a K_d} \right) \frac{1}{A} \right) + \frac{1}{V_f} \left(1 + \frac{K_b}{B} + K_p \left(\frac{K_a}{K_i} + B \left(1 + \frac{K_i}{K_p}\right)\right)\right)
\]

With NAD (Q) as product inhibitor, and (a) NADH (A) varied

\[
1 - \frac{K_a}{V_f} \left(1 + \frac{K_d K_b}{K_a K_b} \left(1 + \frac{K_f K_b}{K_a K_d} \right) \frac{1}{A} \right) + \frac{1}{V_f} \left(1 + \frac{K_b}{B} + K_p \left(\frac{K_a}{K_i} + \left(1 + \frac{Q}{K_{eq}}\right)\right) \right)
\]

For the reverse reaction, with NADH (A) as product inhibitor, and (b) oxalacetate (B) varied

\[
1 - \frac{K_a}{V_f} \left(1 + \frac{K_d K_b}{K_a K_b} \left(1 + \frac{K_f K_b}{K_a K_d} \right) \frac{1}{A} \right) + \frac{1}{V_f} \left(1 + \frac{K_b}{B} + \left(1 + \frac{Q}{K_{eq}}\right)\right)
\]

and (a) malate (P) varied

\[
1 - \frac{K_a}{V_f} \left(1 + \frac{K_d K_b}{K_a K_b} \left(1 + \frac{A}{K_{ia}}\right) \frac{1}{A} \right) + \frac{1}{V_f} \left(1 + \frac{K_b}{B} + \left(1 + \frac{A}{K_{ia}}\right)\right)
\]

or (b) oxalacetate (B) varied

\[
1 - \frac{K_a}{V_f} \left(1 + \frac{K_d K_b}{K_a K_b} \left(1 + \frac{Q}{K_{eq}}\right) \frac{1}{A} \right) + \frac{1}{V_f} \left(1 + \frac{K_b}{B} + \left(1 + \frac{Q}{K_{eq}}\right)\right)
\]

Thus, the type of inhibition to be expected is competitive when NAD is studied with respect to NADH (and vice versa), but is noncompetitive in all other cases. Each noncompetitive product inhibitor would be expected to affect the slopes and vertical intercepts of a double reciprocal plot to different extents (Equations 3, 4, 6, 7, 9, and 10). In all the product inhibitions, a linear relationship between inhibitor concentration and increase in slope or intercept is predicted. It is clear from the equations that a direct estimate of an inhibition constant can be obtained only from an experiment involving competitive inhibition (Equations 5 and 8). In all other cases the "apparent" inhibition constant determined by analysis of one experiment (see "Methods") is a complex function of the inhibition constant in question with other kinetic constants and sometimes also with the concentration of the fixed substrate. The true inhibition constant must then be obtained by calculation, as indicated in Table II, below.
**Experimental Procedure**

**Materials**

The sodium salts of NAD and NADH were obtained from Sigma (Grade III, 98%). Adenosine 3′-diphosphoribose (Sigma) (0.008 mM) did not inhibit the reaction over the range of concentration of NADH used, and the coenzyme was not further purified (8). Sodium hydrogen malate and oxalacetic acid were products of the British Drug Houses, Ltd., Poole, England (laboratory reagent grade). Tris, obtained from British Drug Houses, was recrystallized from solution in water at 60° by the addition of an equal volume of acetone. Glacial acetic acid and EDTA were also obtained from British Drug Houses (analytical reagent grade). Crystalline bovine plasma albumin was the product of Armour Pharmaceutical Company, Ltd., Eastbourne, England.

**Methods**

Preparation of Malate Dehydrogenase—The method used was essentially that of Straub (9) as modified by Ochoa (10) for the preparation of pig heart malate dehydrogenase, and used by Siegel and Englard (3) to prepare bovine heart mitochondrial malate dehydrogenase. The controlled heating step (3) was not performed, to avoid the possibility of destroying delicate tertiary structures, such as might be involved in an allosteric site. Full details of the preparation will be reported elsewhere. The enzyme was stored at 2° in Tris-acetate buffer (0.5 M acetate, pH 7.5), at concentrations of approximately 10 mg of protein per ml. Under these conditions the specific activity fell approximately 20% in 3 months. All preparations used were homogeneous in the ultracentrifuge, and had specific activities between 300 and 400 μmoles of NADH produced per min per mg of protein under the standard assay conditions (3).

The fluorescence spectrum of the enzyme (10 mg per ml), excited at 340 μm and measured with the use of a Corning 3.73 emission filter on the recording fluorometer, was compared with that of a 10−4 M solution of NADH under the same conditions. The results showed that the amount of endogenous NADH bound per 62,000 μg of enzyme could not be more than 0.025 mole.

**Determination of Protein**—This was carried out by the method of Gornall, Bardawill, and David (11) with crystalline bovine plasma albumin as the standard.

**Fluorometric Measurement of Malate Dehydrogenase Activity**—All assays were carried out in a silica cell at pH 8.0 and 25°, with the use of a continuously recording fluorometer to measure increase or decrease in fluorescence due to change in concentration of NADH. The two-channel fluorometer used for this purpose was designed to minimize fluctuations arising from variations in the intensity of the exciting light. Light from a 500-watt alternating current xenon arc was focused by a Köhler system (with quartz lenses) onto two silica cells, placed one behind the other in holes drilled in a thermostated copper block. The light passed through an ultraviolet filter (Corning 7.60) before entering the first cell, which contained a solution of glycogen to scatter a small percentage of the incident light. Scattered light emitted at right angles to the exciting beam passed a second ultraviolet filter before reaching a 6255 B photomultiplier (E.M.I. Electronics, Ltd., Ruislip, England). The greater part of the exciting light entered the second cell, where it excited the fluorescence of NADH contained in the assay solution. Fluorescent light, again emitted at right angles, passed a complementary filter (Corning 3.72) before reaching a second EMI 6255 B photomultiplier. The outputs of the two photomultipliers were amplified with constant gain, 100-eps tuned amplifiers. After rectification, the two signals were fed to a slightly modified version of the ratio recorder described by Ainsworth and Winter (12). In this recorder, the signal derived from the scattered light was used as the reference voltage, against which the fluorescence signal was balanced. Provision was made to reverse the polarity of the scattered light signal, so that it could also be used as an opposing voltage to offset the larger part of the fluorescence signal obtained when the reaction was studied with high initial concentrations of NADH. With these arrangements, a 20% variation in the exciting light intensity was reflected by a 1% change in the recorded ratio.

Reaction mixtures contained, in a total volume of 3 ml, Tris-acetate buffer (0.1 M in acetate), pH 8.0, with substrates and sometimes a product at the concentrations indicated in the figures. Substrate and product solutions were made up immediately before use in 0.01 M Tris-acetate buffer, pH 8.0, and readjusted to pH 8.0 with 1.0 N NaOH where necessary. After addition of these components the reaction mixtures were kept in ice and then incubated for 5 min at 25° before addition of the enzyme. The amount of enzyme added, in 0.01 ml, corresponded to 0.056 μg of protein for all experiments except those with NADH as a product inhibitor, in which 0.027 μg of protein was added. There was a linear relationship between initial velocity and protein concentration over this range. Immediately after the addition of enzyme the silica cell was inverted three times to initiate the reaction, and the change in fluorescence due to NADH was recorded over the time period during which the initial rate was maintained. This time period was shortest in the reverse reaction, owing to the unfavorable position of the equilibrium of the reaction and to the high affinity of the enzyme for products in this direction.

Because the fluorescence yield is not strictly linear with NADH concentration (13), the initial slope of each reaction record was related to NADH concentration as follows. (a) When NADH was to be oxidized, the reaction mixture was made up to contain 0.010 μmole of NADH less than the initial amount. After temperature equilibration of the reaction mixture, but before the addition of enzyme, 0.05 ml of NADH solution (equivalent to 0.010 μmole of NADH) was added and mixed, and a record was made of the corresponding deflection on the fluorometer. The initial rate of the reaction was then calculated in terms of micromoles of NADH oxidized, by direct comparison with this standard deflection. (b) When NADH was to be produced, a Perspex rod was used as a standard (14); the

**Table I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Michaelis constant</th>
<th>Dissociation constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>$K_m \pm 0.116$</td>
<td>$K_m \pm 0.044$</td>
</tr>
<tr>
<td>Oxalacetate</td>
<td>$K_m \pm 0.0386$</td>
<td>$K_m \pm 0.0443$</td>
</tr>
<tr>
<td>Malate</td>
<td>$K_m \pm 0.073$</td>
<td>$K_m \pm 0.039$</td>
</tr>
<tr>
<td>NADH</td>
<td>$K_m \pm 0.0101$</td>
<td>$K_m \pm 0.039 \pm 0.085$</td>
</tr>
</tbody>
</table>
TABLE II

Kinetic constants derived from product inhibition studies on malate dehydrogenase

The apparent inhibition constants were determined from the data of Figs. 3, 4, 6, 7, 9, and 10 by analysis with the NONCOMP computer program (15). True constants were calculated from the apparent constants by substituting into the relevant formulas for the concentration of the fixed substrate and for the kinetic constants recorded in Table I. Analysis of the data of Figs. 5 and 8 with the COMP computer program (15) yielded true inhibition constants directly.

<table>
<thead>
<tr>
<th>Product inhibitor</th>
<th>Varied substrate</th>
<th>Figure and equation</th>
<th>Apparent $K_i$</th>
<th>Significance of apparent $K_i$</th>
<th>True constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate</td>
<td>NADH</td>
<td>3</td>
<td>$10.8 \pm 2.1$</td>
<td>$3.12 \pm 0.39$</td>
<td>$0.31 \pm 0.22 (K_p)$</td>
</tr>
<tr>
<td></td>
<td>Oxalacetate</td>
<td>4</td>
<td>$3.38 \pm 0.58$</td>
<td>$4.09 \pm 0.41$</td>
<td>$0.56 \pm 0.19 (K_p)$</td>
</tr>
<tr>
<td>NAD</td>
<td>NADH</td>
<td>5</td>
<td>$2.34 \pm 0.57$</td>
<td></td>
<td>$1.1 \pm 0.1 (K_iq)$</td>
</tr>
<tr>
<td></td>
<td>Oxalacetate</td>
<td>6</td>
<td>$1.58 \pm 0.13$</td>
<td></td>
<td>$0.82 \pm 0.26 (K_iq)$</td>
</tr>
<tr>
<td>NADH</td>
<td>Malate</td>
<td>7</td>
<td>$0.00739 \pm 0.00147$</td>
<td></td>
<td>$0.0047 \pm 0.0010 (K_iq)$</td>
</tr>
<tr>
<td></td>
<td>NAD</td>
<td>8</td>
<td>$0.00973 \pm 0.00146$</td>
<td></td>
<td>$0.0073 \pm 0.0033 (K_iq)$</td>
</tr>
<tr>
<td></td>
<td>Oxalacetate</td>
<td>9</td>
<td>$0.00176 \pm 0.00019$</td>
<td>$0.00684 \pm 0.00082$</td>
<td>$0.034 \pm 0.010 (K_s)$</td>
</tr>
<tr>
<td></td>
<td>NAD</td>
<td>10</td>
<td>$0.00914 \pm 0.00021$</td>
<td>$0.00497 \pm 0.00058$</td>
<td>$0.020 \pm 0.016 (K_s)$</td>
</tr>
</tbody>
</table>

The rod was calibrated at frequent intervals against a solution of NADH. However, an additional control was needed when NADH was used as a product inhibitor. Thus 0.01 ml of a solution of NADH (equivalent to 0.0025 µmole of NADH) was added to each reaction mixture, and the deflection produced was later related to that on the record of the corresponding enzyme reaction.

The use of a standard for each assay had the additional advantage of correcting for any small variations in sensitivity of the fluorometer which were not canceled out by the electronic arrangement.

For each experiment, a stock enzyme solution containing 0.066 mg of protein per ml in 0.01 M Tris-acetate buffer, pH 8.0, was prepared, and an intermediate dilution was made after each group of 6 assays. Each experiment of approximately 30 assays was performed at least four times, twice or more in order from the fastest to the slowest reactions and an equal number of times in the reverse order. Mean estimates of the velocities were then taken, thus correcting for any slight decrease in activity of the enzyme over the duration of the experiment, and used in determining kinetic constants.

Analysis of data was done with the computer programs of Cleland (15), modified slightly so that they could be used on the Atlas computer at Harwell. The linearity of individual lines in the double reciprocal plots was checked graphically before the data for a whole experiment were analyzed, according to the type of plot, by means of the SEQUEN, NONCOMP, or COMP program. The estimates of kinetic constants so obtained, together with standard deviations, are recorded in Tables I and II. They were used to draw the lines of the figures, which thus show the agreement between the experimental results and the corresponding velocity equations. The standard deviations of sums, prod-


and quotients were calculated by the following formulas (16), although these are only minimum S.D. values since it is impossible to calculate precise ones.

\[
\text{S.D. } (x + y) = \pm \sqrt{(\text{S.D. } x)^2 + (\text{S.D. } y)^2}
\]

\[
\text{S.D. } (xyz) = \pm \sqrt{x^2(\text{S.D. } y)^2 + y^2(\text{S.D. } x)^2}
\]

\[
\text{S.D. } \left(\frac{x}{y}\right) = \pm \frac{1}{y^2} \sqrt{z^2(\text{S.D. } y)^2 + y^2(\text{S.D. } x)^2}
\]

Weighted mean values and their standard deviations were calculated according to the formulas

\[
\text{Weighted mean of } x \text{ values } = \frac{\sum w_i x_i}{\sum w_i}
\]

where

\[
w = \frac{1}{(\text{S.D. } x_i)^2}
\]

and

\[
\text{S.D. of weighted mean value } = \frac{1}{\sqrt{\sum w_i}}
\]

**RESULTS**

The data of Figs. 1 and 2 show the fit to Equations 1 and 2 of the initial velocities in the absence of products. The kinetic constants determined from these data are recorded in Table I. The maximum velocity for the forward reaction \( (V_f) \), determined with an enzyme preparation of specific activity 388, was 8.49 \( \pm 3.40 \) 

\[
\text{moles of NADH oxidized per min per } \mu\text{g of malate dehydrogenase.}
\]

A different enzyme preparation, specific activity 312, was used in the experiments on the reverse reaction. The maximum velocity in this direction \( (V_r) \), determined from the data of Fig. 2, was 0.109 \( \pm 0.005 \) 

\[
\text{micro moles of NADH produced per min per } \mu\text{g of malate dehydrogenase.}
\]

For comparison with \( V_f \), this value of \( V_r \) has been multiplied by the factor, 388/312, yielding 0.136 \( \pm 0.006 \) 

\[
\text{micro moles of NADH produced per min per } \mu\text{g of malate dehydrogenase.}
\]

If the molecular weight of the enzyme is taken as 63,000, \( V_f \) and \( V_r \) can be expressed as 5.4 \( \times 10^4 \) 

\[
\text{min}^{-1} \text{ and } 8.6 \times 10^3 \text{ min}^{-1}, \text{respectively.}
\]

The product inhibition studies on the forward reaction are illustrated in Figs. 3 to 6. All the inhibitions were of the non-competitive type except for that in Fig. 5, which showed competition between NAD and NADH. These results were in agreement with Equations 3 to 6. The kinetic constants determined directly by analysis of these data are recorded in Table II, together with the true kinetic constants calculated from them.

\[2\] An experiment identical with that illustrated in Fig. 2 was performed with an enzyme preparation of specific activity 240. The maximum velocity determined from this, when multiplied by 312/240, was 0.105 

\[
\text{micro moles of NADH produced per min per } \mu\text{g of malate dehydrogenase, which was in agreement with the value from the data of Fig. 2. All the other kinetic constants were also similar to those from Fig. 2.}
\]
FIG. 3. Inhibition of the forward reaction by malate, with NADH as the variable substrate and with the oxalacetate concentration held constant at 0.020 mM. The concentrations of malate were ▲, 0.0 mM; ◊, 1.5 mM; ●, 3.0 mM; ◐, 4.5 mM; and ■, 6.0 mM. $v$ is expressed as micromoles of NADH oxidized per min per $\mu$g of malate dehydrogenase; each point is the mean from four determinations.

Fig. 4. Inhibition of the forward reaction by malate, with oxalacetate (OAA) as the variable substrate and with the NADH concentration held constant at 0.025 mM. The concentrations of malate were ◊, 0.0 mM; ▲, 0.5 mM; ◐, 1.0 mM; ●, 1.5 mM; and ◥, 2.0 mM. $v$ is expressed as micromoles of NADH oxidized per min per $\mu$g of malate dehydrogenase; each point is the mean from four determinations.
FIG. 5. Inhibition of the forward reaction by NAD, with NADH as the variable substrate and with the oxalacetate concentration held constant at 0.020 mM. The concentrations of NAD were ●, 0.0 mM; ▲, 0.4 mM; □, 0.8 mM; ○, 1.2 mM; and △, 1.6 mM. $v$ is expressed as micromoles of NADH oxidized per min per $\mu$g of malate dehydrogenase; each point is the mean from four determinations.

FIG. 6. Inhibition of the forward reaction by NAD, with oxalacetate (OAA) as the variable substrate and with the NADH concentration held constant at 0.025 mM. The concentrations of NAD were ●, 0.0 mM; ▲, 0.0025 mM; ■, 0.0050 mM; ○, 0.00375 mM; and △, 0.0050 mM. $v$ is expressed as micromoles of NADH produced per min per $\mu$g of malate dehydrogenase; each point is the mean from six determinations.

FIG. 7. Inhibition of the reverse reaction by NADH, with malate as the variable substrate and with the NAD concentration held constant at 0.20 mM. The concentrations of NADH were ●, 0.0 mM; ▲, 0.00125 mM; ■, 0.0025 mM; ○, 0.00375 mM; and △, 0.0050 mM. $v$ is expressed as micromoles of NADH produced per min per $\mu$g of malate dehydrogenase; each point is the mean from six determinations.

Table 1). Possible explanations for these discrepancies will be discussed.

A further test for correlations among the various constants was based on the Haldane relationships. There are two such relationships for an Ordered Bi Bi mechanism, and the values calculated for $K_{eq}$ from these relationships may be compared with the approximate $K_{eq}$ of 10,000 which was determined thermodynamically for this reaction by Raval and Wolfe (5) and which was found to agree with calculated values (17). With the use of kinetic constants obtained from the experiments conducted in the absence of products, the calculated $K_{eq} = V/K_{p}K_{q}/V_{r}K_{a}K_{b}$ was 8,000. This was in agreement with the thermodynamic value of $K_{eq}$, although the minimum standard deviation of the estimate was 6,200. The second Haldane relationship

\[ K_{eq} = \left( \frac{V_{r}}{V_{f}} \right)^{2} \frac{K_{p}K_{q}}{K_{a}K_{b}} \]

yielded a value of 59,000 for $K_{eq}$, with a minimum standard deviation of 44,000. This was significantly different from the previous estimate of $K_{eq}$ from kinetic data and also from the thermodynamic value. However, the increased complexity of the calculation made it a less reliable estimate and, moreover, the difference was not as great as an order of magnitude.

Cleland (7) gives relationships for calculating rate constants for the reaction steps shown in Scheme 2.
Kinetic Studies on Mechanism of Malate Dehydrogenase Reaction

Vol. 243, No. 9

FIG. 8. Inhibition of the reverse reaction by NADH, with NAD as the variable substrate and with the malate concentration held constant at 2 mM. The concentrations of NADH were ○, 0.0 mM; ▲, 0.00125 mM; ●, 0.0025 mM; and △, 0.00375 mM. v is expressed as micromoles of NADH produced per min per µg of malate dehydrogenase; each point is the mean from six determinations.

FIG. 9. Inhibition of the reverse reaction by oxalacetate, with malate as the variable substrate and with the NAD concentration held constant at 0.25 mM. The concentrations of oxalacetate were ●, 0.0 mM; ▲, 0.002 mM; □, 0.004 mM; △, 0.006 mM; and ○, 0.008 mM. v is expressed as micromoles of NADH produced per min per µg of malate dehydrogenase; each point is the mean from six determinations.

FIG. 10. Inhibition of the reverse reaction by oxalacetate, with NAD as the variable substrate and with the malate concentration held constant at 1 mM. The concentrations of oxalacetate were ●, 0.0 mM; ▲, 0.0015 mM; □, 0.0030 mM; △, 0.0045 mM; and △, 0.0060 mM. v is expressed as micromoles of NADH produced per min per µg of malate dehydrogenase; each point is the mean from six determinations.

E \[ \frac{k_{11}}{k_{11}k_{12}} \] \[ \frac{A}{B} \] \[ \frac{P}{Q} \] \[ \frac{E}{EAB} \] \[ \frac{EPQ}{E} \]

SCHEME 2

The relationships, and from them the values for rate constants, are set out in Table III. The rate constants are expressed in the same manner as \( V_f \) and \( V_r \), to facilitate comparison with these kinetic constants. Values could not be calculated for \( k_{13} \) and \( k_{-1} \) because of the negative value obtained for \( k_{13} \). If the mechanism in Scheme 2 fits the results, the maximum velocity in a certain direction of the reaction cannot be greater than any of the individual rate constants in that direction. Thus \( k_{a3} \) and \( k_{-1} \) should be greater than or equal to \( V_f \) (5.4 \times 10^4 min^{-1}), while \( k_{-3} \) and \( k_{-2} \) should be greater than or equal to \( V_r \) (8.6 \times 10^2 min^{-1}). However, \( k_{13} \) and \( k_{14} \) were less than \( V_f \), although \( k_{-1} \) and \( k_{-2} \) were in the expected relationship to \( V_r \). Scheme 2, therefore, does not correctly represent all the individual steps in the reaction sequence. The rate equation for the actual sequence was nevertheless identical with that for Scheme 2 when expressed in terms of kinetic constants rather than rate constants. The significance of this effect will be discussed.

DISCUSSION

Qualitatively, the results were consistent with an Ordered Bi Bi mechanism in which the coenzymes combine with the free
enzyme. The only mechanism not excluded by the results was Iso Theorell Chance; this was, however, rendered unlikely by the evidence for a reaction between NADH and the enzyme.

The quantitative correlations among kinetic constants were in agreement with the predictions for an Ordered Bi Bi mechanism. Thus, the Haldane relationships showed reasonable agreement between the kinetic constants and the equilibrium constant. It is noteworthy that the standard deviation for each calculation of $K_{eq}$ made with the use of these relationships was of the order of the $K_{eq}$ itself. Moreover, these were only minimum estimates of the standard deviations, for precise estimates could not be made. It is therefore clear that the Haldane relationships were not a very sensitive test of the fit of the present results.

The three estimates of each of the constants $K_p$ and $K_q$ were in agreement. On the other hand, the three estimates of each of the constants $K_{iq}$ and $K_{oi}$ obtained from product inhibition data were different from the corresponding estimates with the use of NADH and NAD as substrates. It is noteworthy that when NADH was studied as a substrate the only other reactant present was oxalacetate, whereas when NAD was a product inhibitor present. Oxalacetate has been observed to cause substrate inhibition at higher concentrations than those used in the present work (2, 3), and must therefore be able to combine in two ways with the enzyme. It is plausible that the combination of oxalacetate at a site distinct from the active site reduces the effective combination of NADH and so causes inhibition. Similarly, oxalacetate might be expected to inhibit the combination of NAD, perhaps more strongly than that of NADH, and this effect might well be detectable at lower concentrations of oxalacetate than are required to show substrate inhibition. Oxalacetate may therefore inhibit the combination of NAD with the enzyme as a product inhibitor, causing the value of $K_{iq}$ observed under these conditions to be higher than that observed when NAD functioned as a substrate and oxalacetate was not present.

An analogous explanation can be proposed for the fact that $K_{iq}$ from studies of NAD as a product inhibitor was greater than from experiments in which NAD was a substrate. Under the latter conditions, the only other reactant present was malate, whereas when NAD was a product inhibitor present was malate, whereas when NAD was a product inhibitor present was malate, whereas when NAD was a product inhibitor present was malate. Oxalacetate might be expected to inhibit the combination of NAD, perhaps more strongly than that of NADH, and this effect might well be detectable at lower concentrations of oxalacetate than are required to show substrate inhibition. Oxalacetate may therefore inhibit the combination of NAD with the enzyme as a product inhibitor, causing the value of $K_{iq}$ observed under these conditions to be higher than that observed when NAD functioned as a substrate and oxalacetate was not present.

The lines of the figures illustrating product inhibition by NADH with respect to malate (Fig. 7) and by NAD with respect to oxalacetate (Fig. 6) did not appear to be curved, although nonlinearity would be expected if the above hypotheses were correct. This may be because the changes in $K_{iq}$ and $K_{oi}$ over these concentration ranges of malate and oxalacetate, respectively, were too small to be detected by the experimental technique. If $K_{iq}$ increased 2-fold from 0.0022 mM (Table II) over the range of malate concentration in Fig. 7, the slope of the line with the highest NADH concentration would be approximately 0.7 times as great at the lowest malate concentration as it is at the highest. In Fig. 6, the slope of the line with the highest NAD concentration would be approximately 0.7 times as great at the highest oxalacetate concentration as it is at the lowest, if $K_{iq}$ decreased 2-fold from 1.1 mM (Table II) over this range of oxalacetate concentration. In view of the possibility that effects of this magnitude might be present, the values of $K_{iq}$ and $K_{oi}$ obtained from competitive inhibition data (Figs. 5 and 8), at constant oxalacetate and malate concentrations, respectively, might be more reliable than those from Figs. 6 and 7. This may be reflected in the lower standard deviations of the values from Figs. 5 and 8.

The formation of an inactive $E$-oxalacetate ($EB$) complex could also cause this type of discrepancy between estimates for both $K_{iq}$ and $K_{oi}$. If $EB$ is present, the denominator terms

### Table III

**Rate constants for malate dehydrogenase reaction, calculated on assumption of simple Ordered Bi Bi mechanism (7)**

<table>
<thead>
<tr>
<th>Rate constant</th>
<th>Significance</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{-1}$</td>
<td>$V_f/K_a$</td>
<td>$2.1 \times 10^9$ min$^{-1}$ M$^{-1}$</td>
</tr>
<tr>
<td>$k_{+2}$</td>
<td>$V_f (1 + k_{-2}/k_{+2})/K_a$</td>
<td></td>
</tr>
<tr>
<td>$k_{+3}$</td>
<td>$1/(V_f - 1/K_q)$</td>
<td>Negative</td>
</tr>
<tr>
<td>$k_{+4}$</td>
<td>$V_f/K_{iq}$</td>
<td>$5.1 \times 10^4$ min$^{-1}$</td>
</tr>
<tr>
<td>$k_{-1}$</td>
<td>$V_f/K_{ia}$</td>
<td>$2.8 \times 10^8$ min$^{-1}$</td>
</tr>
<tr>
<td>$k_{-2}$</td>
<td>$1/(1 - 1/K_{ia}/K_{iq})/(V_f - 1/K_{ia})$</td>
<td>$1.2 \times 10^4$ min$^{-1}$</td>
</tr>
<tr>
<td>$k_{-3}$</td>
<td>$V_f/K_p(1+k_{+2}/k_{-2})$</td>
<td></td>
</tr>
<tr>
<td>$k_{-4}$</td>
<td>$V_f/K_q$</td>
<td>$1.4 \times 10^8$ min$^{-1}$ M$^{-1}$</td>
</tr>
</tbody>
</table>

It must be able to combine in two ways with the enzyme. It is reasonable to propose that combination of malate at a site distinct from the active site may enhance the combination of NADH with the enzyme and in this way cause activation. If this were so, malate might also be expected to affect the combination of NADH, since both the coenzymes may be visualized as occupying the same part of the active site because of their structural similarity and the competition observed between them. If malate should enhance the combination of NADH more strongly than that of NAD, the effect would be observed at lower malate concentrations than those required to demonstrate substrate activation, even though the degree of saturation of the second site by malate would be small. It is therefore possible that malate may enhance the combination of NADH as a product inhibitor, causing the value of $K_{iq}$ observed under these conditions to be lower than that observed when NADH functions as a substrate and malate is not present.

An analogous explanation can be proposed for the fact that $K_{iq}$ from studies of NAD as a product inhibitor was greater than from experiments in which NAD was a substrate. Under the latter conditions, the only other reactant present was malate, whereas when NAD was a product inhibitor present was malate. Oxalacetate might be expected to inhibit the combination of NAD, perhaps more strongly than that of NADH, and this effect might well be detectable at lower concentrations of oxalacetate than are required to show substrate inhibition. Oxalacetate may therefore inhibit the combination of NAD with the enzyme as a product inhibitor, causing the value of $K_{iq}$ observed under these conditions to be higher than that observed when NAD functioned as a substrate and oxalacetate was not present.
Kinetic Studies on Mechanism of Malate Dehydrogenase Reaction Vol. 243, No. 9

K_1, K_2, V_m, k_4, k_1, k_2, k_3, K_{cat}, K_m, and K_{cat}/K_m in Equation 1 should each be multiplied by the factor 1 + B/K_{ib}, where K_{ib} is the dissociation constant for the EB complex. Data for all reactions when oxalacetate is present would be affected, and estimates of most constants from these data would therefore be invalid. The value for K_{cat} determined from Fig. 5 (Table II) should then be greater than the value determined in the absence of oxalacetate (Table I) because it would represent K_{cat} (1 + B/K_{ib}). Conversely, the values for K_{cat} determined in the absence of oxalacetate from Figs. 7 and 8 (Table II) should be less than K_{cat} determined from Fig. 1 (Table I). However, this hypothesis leads to the prediction of nonlinear plots in Figs. 1, 4, and 6, as well as a parabolic relationship between line slope and oxalacetate concentration in Fig. 9 and between vertical intercept and oxalacetate concentration in Fig. 10. There did not appear to be experimental support for these effects, although if K_{ib} were large compared with oxalacetate concentration a small effect might not be detected. It is noteworthy that the value of K_{cat} which could be calculated from the data of Fig. 5 (assuming that EB is formed) was approximately 0.010 mM, and hence low enough to cause marked nonlinearity in the figures concerned.

The product inhibition data did not support the existence of either of the inactive ternary complexes, E-NADH-malate or E-NAD-oxalacetate, where malate and oxalacetate as well as the coenzymes are combined at the active site. The present results therefore contrast with those of Zewe and Fromm on lactate dehydrogenase (18) and of Fromm and Nelson on ribitol dehydrogenase (19), in which there appear to be such inactive complexes. While the product inhibition studies on malate dehydrogenase from pig heart (6) were similar to the present work on the bovine heart enzyme in indicating a compulsory order mechanism with a ternary complex (but no inactive ternary complexes), the data in the absence of products indicated, for the pig enzyme only (5), that the ternary complex is not kinetically significant. The ratio V_f/V_r was much higher for the enzyme from bovine heart than for that from pig heart (62 and 3.3, respectively). Comparison of the corresponding Michaelis and dissociation constants for the two enzymes reveals agreement well within an order of magnitude in each case except for the Michaelis constant for NADH. K_a for the bovine enzyme was much higher than that for the pig enzyme, even though the estimates for K_{cat} were similar.

Because K_a was so high in the present work, it was not technically feasible to study the kinetics over a full range of concentration of NADH, up to and beyond K_a (20). Moreover, the range of concentration of oxalacetate which could be used had an upper limit close to the value of K_b, since substrate inhibition was observed at higher concentrations. These limitations probably account for the proportionately large standard deviations recorded for the kinetic constants in this direction of the reaction. It should be noted that the keto form of oxalacetate is the true substrate of the enzyme, and that the concentration of this species under specific experimental conditions is uncertain (21).

When the rate constants for the individual steps of the reaction, calculated on the basis of Scheme 2, were compared with the appropriate maximum velocity, it was seen that k_{cat} and k_{cat} were both less than V_f. This arose because V_f (K_{cat}/K_m) < V_f. In another terminology this can be stated as φ_f = φ_f/K_m < φ_f, which happens to be the reverse of a condition (22) for a simple Ordered Bi Bi reaction mechanism. Cleland (7) has pointed out that kinetic data for glutamate dehydrogenase, lactate dehydrogenase, and yeast alcohol dehydrogenase yield in each case a calculated rate constant for the dissociation of the E-NAD complex which is lower than the maximum velocity in the same direction of the reaction. One explanation for the present results, as well as for those just mentioned, is that the E-NAD complex isomerizes (Reference 7, p. 124); moreover, the E-NADH complex could also isomerize without requiring that the rate constant for dissociation of this complex (k_-) should be less than V_r. Zewe and Fromm (18, 23) reported a similar conclusion on the mechanism of action of rabbit muscle lactate dehydrogenase. However, the rate constants calculated for pig heart malate dehydrogenase on the basis of compulsory order mechanism with a ternary complex (6) were all greater than the corresponding maximum velocities.

A second possible explanation for the present results is that E and NAD form an inactive complex which blocks the reaction (7). If this complex forms, with dissociation constant K_{cat}, the terms K_aV_f/K_{cat}, K_aV_r, and K_aK_{cat}V_r in Equation 1 should each be multiplied by (1 + Q/K_{cat}). The data of Fig. 2 would still give linear plots, but the value obtained for K_{cat} would be lower than the true value. The data of Figs. 5 and 6 also would still yield linear plots, and again the value obtained for K_{cat} would be too small. The value of K_{cat} actually obtained from the product inhibition data was larger than that from Fig. 2, and thus an inactive complex between E and NAD does not appear to be formed.

Although all the experiments in this work were performed in concentration ranges in which substrate inhibition by oxalacetate and substrate activation by malate could not be observed, the possibility remains that effects of this type might not have been completely excluded and might have affected the determination of constants. In connection with the calculations of rate constants (Table III), it can be seen that an approximately 10-fold increase in the value of V_r.K_{cat}/K_m would be needed to make V_f.K_{cat}/K_m equal to V_f (i.e., k_{cat} = k_{cat}) for (or to make k_{cat} decrease on a positive value). The approximate standard deviation of this expression is considerably less than the value of the expression itself, and it would appear unlikely that a slight substrate activation by malate could bring about a change of this magnitude. Alternatively, V_f would need to be in error to the extent of an order of magnitude in order to bring it down to the value of V_r.K_{cat}/K_m, and in spite of the large standard deviation for V_f it appears unlikely that substrate inhibition by oxalacetate could have such a large effect without being observable experimentally. In addition, if the ratio V_f/V_r were in error by an order of magnitude, the observed agreement between the Haldane calculation of K_{cat} and the thermodynamic value would be unlikely.

The very general qualitative and quantitative agreement between the results and predictions for an Ordered Bi Bi reaction mechanism indicates that effects caused by undetected substrate inhibition or activation were slight. Moreover, in the case of the two debatable points which arose, over the K_{cat} and K_{cat} values and the relationship of rate constants to the maximum velocity, the most plausible explanations appear to be those which are not based on the possibility of errors in constants.

It can be concluded that the present kinetic evidence is consistent with a compulsory order reaction mechanism for malate dehydrogenase from acetone powder of whole bovine heart, with the coenzymes reacting first in each direction of the reaction, and a kinetically significant ternary complex. Although the ranges of concentration of both malate and oxalacetate used were lower than those where these reactants cause substrate activation and inhibition, respectively, it appeared likely that, in the concentr-
tions used, oxalacetate affects the combination of NAD and malate affects that of NADH. It also seemed probable that the enzyme-NAD complex isomerizes.

REFERENCES
Kinetic Studies on the Mechanism of the Malate Dehydrogenase Reaction
Elizabeth Heyde and S. Ainsworth


Access the most updated version of this article at http://www.jbc.org/content/243/9/2413

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/243/9/2413.full.html#ref-list-1