# ENZYME SYSTEMS IN THE MYCOBACTERIA

## II. The Malic Dehydrogenase

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As part of a study of the oxidative metabolism of the Mycobacteria a number of enzymes have been isolated from cell-free extracts of this bacillus. A previous communication from this laboratory (Goldman, 1956) described the purification and properties of an isocitric dehydrogenase isolated from the H37Ra strain of *Mycobacterium tuberculosis* var. *hominis*. The present communication concerns the non-decarboxylating malic dehydrogenase of this organism.[1]

Non-decarboxylating malic dehydrogenases are widely distributed in animal, plant, and bacterial cells (Green, 1936; Adler and Michaelis, 1936; Lynen and Franke, 1941; Booth and Green, 1938; Gale and Stephenson, 1939). This enzyme catalyzes the reversible oxidation of L-malate to oxalacetate and requires DPN[2] as its coenzyme (reaction 1).

(1) L-malate + $DPN^+ \rightleftharpoons$ oxalacetate + DPNH + $H^+$

The malic dehydrogenase of heart muscle has been extensively purified by Straub (1942).

A cell-free enzyme system which oxidizes L-malate and uses molecular oxygen as the terminal electron acceptor has been isolated from *M. tuberculosis* strain H37Ra (Millman and Youmans, 1954 and 1955). This oxidation was stimulated by the addition of cofactors. The product of oxidation of L-malate by this system was not identified.

[1] A preliminary account of this work was presented at the 46th annual meeting of the American Society of Biological Chemists, San Francisco, April 1955 (Goldman, 1955).

[2] The following abbreviations are used: diphosphopyridine nucleotide, oxidized and reduced, DPN and DPNH; triphosphopyridine nucleotide, TPN; tris(hydroxymethyl)aminomethane, Tris; 2-amino-2-methyl-1, 3-propanediol, Diol.

### MATERIALS AND METHODS

*Materials.* DPN (purity 0.95 or greater) was obtained from the Pabst Laboratories and Sigma Chemical Company; L-malic acid from Nutritional Biochemicals Corporation. Cell-free extracts of *M. tuberculosis* strain H37Ra were prepared as described previously (Goldman, 1956).

*Methods.* Protein concentration was measured by the biuret reaction (Gornall *et al.*, 1949). Pyruvic acid was determined colorimetrically as the 2,4-dinitrophenylhydrazone (Friedemann and Haugen, 1943). Oxalacetate was determined either colorimetrically as the 2,4-dinitrophenylhydrazone (Friedemann and Haugen, 1943) or by decarboxylation in the presence of either aniline citrate (Greville, 1939) or $Al^{+++}$ (Krebs, 1942).

*Enzyme assay.* The assay system for the malic dehydrogenase of H37Ra contains the following: glycine buffer of pH 10.0 (60 μmoles), L-malate (20 μmoles), enzyme (0.3 to 15 μg) and DPN (1 μmole). The final volume was 1.0 ml. The reaction is started by the addition of the enzyme and is carried out at 21 C. The change in optical density at 340 mμ is followed in the Beckman DU spectrophotometer. Four readings are taken at 30-sec intervals. Under these conditions the rate of reaction is linear for changes in optical density of 0.010 to 0.120 per min. A unit of enzyme activity is defined as that amount of enzyme causing the reduction of 1.0 μmole of DPN per min. Specific activity is defined as units of enzymatic activity per mg protein. The molecular extinction coefficient of DPN is taken as $6.22 \times 10^6$ cm$^2$ $\times$ mole$^{-1}$ (Horecker and Kornberg, 1948).

*Enzyme purification.* All procedures were carried out at 1 to 3 C unless otherwise specified.

*First ammonium sulfate.* The crude cell-free

TABLE 1

*Purification of the malic dehydrogenase of H37Ra*

| Fraction | Volume | Protein | | | Activity | | |
|---|---|---|---|---|---|---|---|
| | | Mg per ml | Total | Per cent recovered | Specific activity | Total units | Per cent recovered |
| | *ml* | | *mg* | | | | |
| Crude extract | 1040 | 13.3 | 13,800 | 100 | 1.07 | 14,800 | 100 |
| AS-1 | 134 | 42.5 | 5,700 | 41 | 2.27 | 13,000 | 88 |
| AS-2 | 255 | 1.95 | 497 | 3.6 | 8.20 | 4,100 | 27 |
| Alc-1 | 18.8 | 5.26 | 99.5 | 0.72 | 15.9 | 1,590 | 11 |

extract is brought to 0.3 saturation by the addition of 20 g of $(NH_4)_2SO_4$ per 100 ml of extract. The resulting suspension is centrifuged at 20,000 ×G for 1 hr, the residue being discarded. The clear supernatant solution is next saturated with $(NH_4)_2SO_4$ by the addition of about 50 g of the salt per original 100 ml of crude cell-free extract. The suspension is filtered overnight. The residue (AS-1) is removed from the filter paper and the filtrate is discarded.

*Second ammonium sulfate.* AS-1 is dissolved in 0.02 M $KHCO_3$ and a neutral saturated solution of ammonium sulfate is added to a final salt concentration of 0.65 saturation. The resulting precipitate is removed by centrifugation and discarded. The supernatant solution is next saturated with ammonium sulfate and allowed to stand several hours with gentle stirring. The precipitate (AS-2) is removed by filtration, recovered from the paper, dissolved in and dialyzed against 0.02 M Tris buffer of pH 7.5.

*Alcohol fractionation.* To the dialyzed solution of AS-2 is added sufficient 0.10 M zinc acetate solution to reach a final $Zn^{++}$ concentration of 0.0125 M. The precipitate is removed by centrifugation and discarded. Sufficient cold (−15 C) ethanol is added to the supernatant solution to reach a final ethanol concentration of 12 per cent by volume. During the addition of the alcohol the tube containing the protein solution is immersed in a cold bath (−15 C) and the temperature of the protein solution is never allowed to rise above 0 C. The mixture is stirred for 30 min, during which time the temperature is allowed to fall to about −5 C. The precipitate (Alc-1) is removed by centrifugation (−5 C) at 20,000 × G for 15 min. Alc-1 is dissolved in 0.1 M citrate buffer of pH 7.5 and dialyzed against 0.01 M Tris buffer of pH 7.5.

Solutions of the malic dehydrogenase at this level of purity show a slight loss of activity after 3 months' storage at −15 C. A typical fractionation is shown in table 1.

The highest specific activity obtained by the procedure described above was 32; specific activities of 15 to 20 are routinely obtained.

## RESULTS

*Characteristics of the assay system.* The dependence of the velocity of reduction of DPN on the concentrations of L-malate and DPN are shown in figure 1. The pH dependence of the malic dehydrogenase is shown in figure 2. Figure 3 shows the linearity of the system with respect to time and enzyme concentration.

*Formation of oxalacetate.* The oxidation product of L-malate by the malic dehydrogenase of H37Ra is oxalacetate. The 2,4-dinitrophenylhydrazone of the oxidation product has an absorption spectrum identical with that of known oxalacetate-2,4-dinitrophenylhydrazone. The decomposition points of both the unknown and reference 2,4-dinitrophenylhydrazones are identical

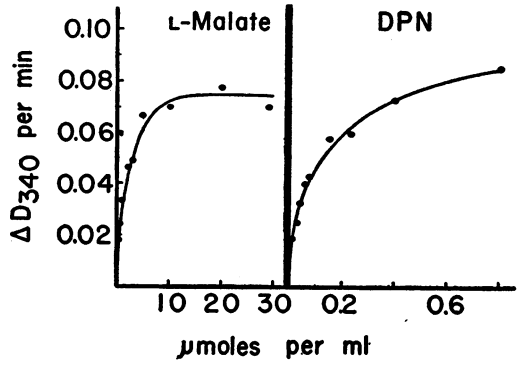


*Figure 1.* Activity of the malic dehydrogenase assay system as a function of the concentrations of L-malate and DPN. Conditions as described in the text.

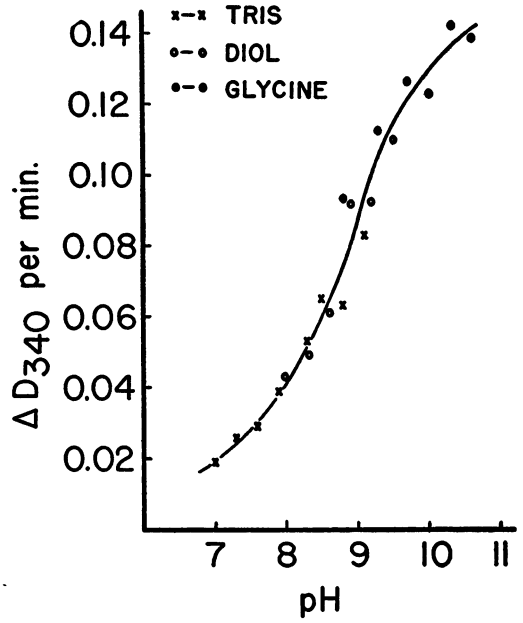


*Figure 2.* Activity of the malic dehydrogenase assay system as a function of the pH of the reaction mixture. Conditions as described in the text except that 60 μmoles of the indicated buffers were used. Abscissa represents final reaction mixture pH.

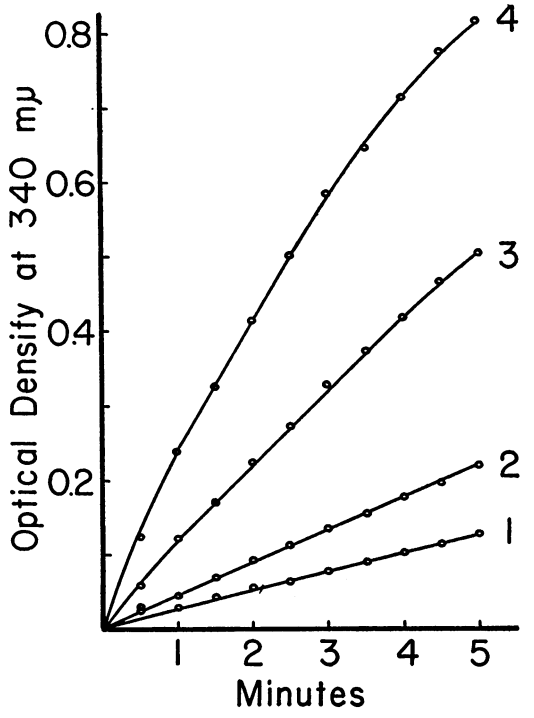


*Figure 3.* Activity of the malic dehydrogenase assay system as a function of time and enzyme concentration. Ordinate represents total change in optical density at 340 mμ corrected for a no-enzyme blank. Enzyme concentrations: curve 1, 0.122 μg; curve 2, 0.244 μg; curve 3, 0.610 μg; curve 4, 1.22 μg.

(214 C). Chromatrography of the dinitrophenylhydrazone of the oxidation product on alumina columns (LePage, 1950) shows the presence of only one component and this has mobilities in different solvents which are identical with those of known oxalacetate-2,4-dinitrophenylhydrazone. Decarboxylation of the oxidation product by aniline citrate or $Al^{+++}$ yields approximately 1 μmole of $CO_2$ for each mole DPNH formed. These results are shown in table 2.

Decarboxylation of the oxidation product in the presence of $Al^{+++}$ yields a keto-acid which has been identified as pyruvic acid. The 2,4-dinitrophenylhydrazones of both the unknown and pyruvic acid have identical spectra in alkaline solution and have the same melting point (217 C, uncorrected) which is not depressed in a mixed melting point determination.

*Reversibility of the oxidation of* L-*malate.* The product of oxidation of L-malate by the malic dehydrogenase of H37Ra is reduced by the dehydrogenase at neutral pH in the presence of DPNH. The oxidation of L-malate is competitively inhibited by oxalacetate (table 3). In

TABLE 2

*Formation of oxalacetate from* L-*malate*

| Experiment No. | μmoles DPNH Formed | μmoles Oxalacetate Formed |
|---|---|---|
| 2:183 | 1.23 | 1.25* |
| 3:21 | 2.80 | 2.12† |
| 3:78 | 1.80 | 1.60† |
| 3:78 | 1.80 | 1.20† |

* Determined colorimetrically.

† Determined manometrically.

Each ml of reaction mixture contained: L-malate (250 μmoles), glycine buffer of pH 10.0 (60 μmoles), DPN (10 μmoles) and enzyme (1.0 mg). The reaction was allowed to proceed for 5 min at 20 C; an aliquot was removed for DPNH assay. After deproteinization ($HClO_4$ for manometric procedure, meta-phosphoric acid for colorimetric procedure), oxalacetate was determined on another aliquot.

the latter experiments the pH was held at 9.1; results at pH 10 are similar.

*Coenzyme and substrate specificity.* The malic dehydrogenase of H37Ra is DPN-specific; no detectable reaction obtains when 1.0 μmole of TPN is substituted in the assay for the 1.0 μmole DPN normally present. D-malate is not oxidized by the malic dehydrogenase and is not an inhibitor of the enzyme.

*$K_s$ determinations.* $K_s$ values (Michaelis and Menten, 1913) for DPN and L-malate were found to be $9.0 \times 10^{-5}$ and $9.5 \times 10^{-4}$ moles $\times$ $L^{-1}$, respectively.

*Equilibrium constant.* The apparent equilibrium constants, $K'_{eq}$,

$$K'_{eq} = \frac{(DPNH)(oxalacetate)}{(DPN^+)(L\text{-}malate)} \quad (2)$$

for the oxidation of L-malate by the malic dehydrogenase are shown in table 4. These values are in good agreement with those of Burton and Wilson (1953).

TABLE 3

*Inhibition of L-malate oxidation by oxalacetate*

| Exp. No. | Concentration L-Malate | Concentration Oxalacetate | DPNH Formed | Per Cent Inhibited |
|---|---|---|---|---|
| | *μmoles/ml* | *μmoles/ml* | *μmoles/min* | |
| 1 | 20 | 0 | 0.0137 | 0 |
| | 12 | 0 | 0.0130 | 0 |
| | 8 | 0 | 0.0113 | 0 |
| | 3 | 0 | 0.0119 | 0 |
| 2 | 20 | 0.06 | 0.0101 | 26 |
| | 12 | 0.06 | 0.0085 | 35 |
| | 8 | 0.06 | 0.0072 | 36 |
| | 3 | 0.06 | 0.0043 | 64 |
| 3 | 20 | 0.15 | 0.0048 | 65 |
| | 12 | 0.15 | 0.0046 | 64 |
| | 8 | 0.15 | 0.0037 | 67 |
| | 3 | 0.15 | 0.0018 | 85 |

Each microcuvette contained the following: glycine buffer of pH 9.1 (60 μmoles), DPN (1.0 μmole), L-malate and oxalacetate as indicated, enzyme of specific activity 6 (3.7 μg). Final volume was 1.0 ml.

## DISCUSSION

The malic dehydrogenase of the H37Ra strain of *Mycobacterium tuberculosis* is very similar to that of animal tissues. Certain differences between the animal and bacterial enzymes were noted. The pH optimum of the bacterial enzyme is about 10.4 while that of the soluble, dissociated apoenzyme of kidney or liver is 9.0 (Huennekens, 1951). The Michaelis constant for L-malate in the animal tissue malic dehydrogenase system has been reported to be $2.5 \times 10^{-2}$ moles $\times$ $L^{-1}$ (Huennekens, 1951). An earlier determination by Green (1936) reported the pH optimum as 8 and the L-malate Michaelis constant as $1 \times 10^{-2}$ moles $\times$ $L^{-1}$. The bacterial enzyme described above has a Michaelis constant of $9.5 \times 10^{-4}$ moles $\times$ $L^{-1}$. These differences, however, are probably not of significance and may reflect certain structural differences in the various apoenzymes. The fact that the coenzyme, substrate, and product specificities of the bacterial enzyme are identical with those of the animal enzyme

TABLE 4

*Equilibrium constant of the malic dehydrogenase of H37Ra*

| pH | Initial Concentration (M/L) | | Final Concentration (M/L) | | | $K'_{eq}$* |
|---|---|---|---|---|---|---|
| | L-malate | DPN | L-malate | DPN | DPNH | |
| 7.6 | $2.0 \times 10^{-2}$ | $0.50 \times 10^{-2}$ | $2.0 \times 10^{-2}$ | $0.50 \times 10^{-2}$ | $0.56 \times 10^{-5}$ | $3.1 \times 10^{-5}$ |
| 8.3 | 2.0 | 0.50 | 2.0 | 0.50 | 1.16 | $1.4 \times 10^{-4}$ |
| 8.9 | 2.0 | 0.50 | 2.0 | 0.48 | 2.1 | $4.6 \times 10^{-4}$ |
| 9.4† | 2.0 | 0.40 | 1.97 | 0.37 | 3.1 | $1.3 \times 10^{-3}$ |
| 10.0 | 2.0 | 0.40 | 1.93 | 0.34 | 6.5 | $6.6 \times 10^{-3}$ |

* Each value recorded is an average of at least 3 experiments.

† These determinations were carried out using 9.6 μg of an enzyme of specific activity 22.

Each microcuvette contained the following: L-malate and DPN as indicated, buffer (60 μmoles), enzyme of specific activity 7 (23.7 μg). Final volume was 1.0 ml. Buffers used were Tris, pH 7.6; Diol, pH 8.3 and 8.9; glycine, pH 9.4 and 10.0. Reactions carried out at 20 C.

indicates that the enzymes are basically the same.

In earlier work with this enzyme, certain difficulties were encountered in demonstrating that the the keto acid formed on the oxidation of L-malate was oxalacetate. In these experiments no decarboxylatable product could be demonstrated. Enzymatic decarboxylation of oxalacetate or oxidative decarboxylation of L-malate could be ruled out by the lack of formation of $CO_2$ during the oxidative reaction. These results have not been confirmed. The present data leave no doubt as to the identity of the product. No explanation can be advanced at this time for these earlier results.

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SUMMARY

A soluble, DPN-specific malic dehydrogenase has been isolated from cell-free extracts of *Mycobacterium tuberculosis* strain H37Ra. The enzyme has been purified about 15-fold through salt and ethanol fractionations. The enzyme is specific for L-malate; oxalacetate is the product of oxidation. Minor differences have been noted between this enzyme and the malic dehydrogenase of animal tissues with respect to Michaelis constants and pH optimum.

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