STUDIES ON MALIC ENZYME FROM HYMENOLEPIS DIMINUTA

APPROVED:

[Signatures]

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Malic enzyme from the rat tapeworm, *Hymenolepis diminuta*, has been purified 320-fold to a final specific activity of 29.4. The purification procedure included heat treatment, followed by column chromatography with Sephadex G-20, two phosphocellulose columns, and Sephadex G-200, respectively. The final purified enzyme appeared to be homogeneous on disc gel electrophoresis and G-200 gel filtration.

The molecular weights of malic enzyme from *H. diminuta* as determined by Sephadex G-200 and high speed sedimentation equilibrium ultracentrifugation were $1.2 \times 10^5$ and $1.0 \times 10^5$, respectively. This is the lowest molecular weight ever found for a malic enzyme. This value is about one-half that of pigeon liver malic enzyme. Based on this approximation, a dimer form has been proposed for the enzyme from the parasite.

Oxalacetate decarboxylase activity was demonstrated for the *H. diminuta* malic enzyme at pH 7.5. The very low activity recorded is thought to be due to pH. This activity confirms the classification of this enzyme (EC 1.1.1.40) for this organism.
The apparent Michaelis constants determined for TPN, malate, TPNH and pyruvate were 4.3 μM, 0.25 mM, 23 μM and 15.5 mM respectively. Due to the higher affinities of the enzyme for TPN and malate, it is apparent that the catalysis is primarily in the direction of malate decarboxylation. The TPNH formed by the enzyme may serve a purpose in lipid synthesis or it may reduce fumarate directly or indirectly with concomitant ATP formation. However, under optimal substrate concentrations, the reverse reaction noted here accounted for 36% that of the forward reaction. It is doubtful that the reverse reaction is of any physiological significance.

_H. diminuta_ malic enzyme did not show a loss of activity during the purification, whether or not external metal ions were used in the assay procedure. The activity was lost completely by EDTA treatment without metal ions in the assay system. Manganese (ous) ion was the most effective metal for restoration of the enzyme activity after EDTA treatment. This indicates that there is a metal ion associated with the malic enzyme. However, further studies are required to definitely show that it is a metalloenzyme.

Both N-ethy maleimide and p-chloromercuribenzoic acid inhibited the enzyme activity. Aged malic enzyme could be
reactivated by dithiothreitol. Thus, the tapeworm malic enzyme is believed to be a SH-enzyme.
STUDIES ON MALIC ENZYME FROM HYMENOLEPIS DIMINUTA

THESIS

Presented to the Graduate Council of the North Texas State University in Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

Tung Li, B. S.
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Malic enzyme (malate: TPN$^+$ oxidoreductase (decarboxylating), EC 1.1.1.40) was first characterized in the cell free extract of pigeon liver by Ochoa et al. (1947b, 1948). It catalyzes the following major reaction:

$$\begin{array}{c}
\text{COO}^- \\
\text{CHOH} \\
\text{CH}_2 \\
\text{COO}^-
\end{array} + \text{TPN} \rightleftharpoons \text{Mg}^{++} \text{ or Mn}^{++} \rightleftharpoons \begin{array}{c}
\text{CO}_2 \\
\text{CH}_2 \\
\text{COO}^-
\end{array} + \begin{array}{c}
\text{COO}^- \\
\text{C=0} \\
\text{CH}_2 \\
\text{COO}^-
\end{array} + \text{TPNH} \ (1)$$

1-Malate \hspace{3cm} \text{Pyruvate}

The enzyme could also decarboxylate oxalacetate (OAA) to pyruvate and CO$_2$ with equal efficiency (Ochoa et al. , 1947a):

$$\begin{array}{c}
\text{C=0} \\
\text{CH}_2 \\
\text{COO}^-
\end{array} \xrightarrow{\text{Mg}^{++} \text{ or Mn}^{++}, \text{H}^+} \begin{array}{c}
\text{CO}_2 \\
\text{CH}_3 \\
\text{COO}^-
\end{array} \hspace{3cm} \begin{array}{c}
\text{C=0} \\
\text{CH}_2 \\
\text{COO}^-
\end{array} \ (2)$$

OAA \hspace{3cm} \text{Pyruvate}

This reaction was confirmed later by Hsu and Lardy (1967a) using crystalline malic enzyme isolated from pigeon liver.
In addition to the above reactions, crystalline pigeon liver enzyme has been shown to catalyze the reduction of pyruvate (Hsu and Lardy, 1967b), reduction of OAA, and conversion of L-malate to L-lactate (Hsu, 1970). These activities account for less than 15% of the major reactions (1) and (2) of the native enzyme:

(3) \[ \text{Pyruvate} \rightarrow \text{L-Lactate} \]

(4) \[ \text{OAA} \rightarrow \text{L-Malate} \]

(5) \[ \text{L-Malate} \rightarrow \text{L-Lactate} \]
Malic enzyme has been studied in a variety of organisms. A divalent cation, either Mn$^{++}$ or Mg$^{++}$, is generally required for maximum catalysis. The intracellular localization of the enzyme differs in the various mammalian tissues studied (Table I). Distinctive electrophoretic patterns of cytosol and mitochondrial malic enzyme have also been demonstrated in the mouse kidney (Shows et al., 1970) and bovine heart (Frenkel, 1971).

Malic enzyme has been purified from both pigeon liver cytosol and Escherichia coli (Hsu and Lardy, 1967a; Spina et al., 1970). Table II is a comparison of some of the physical properties of malic enzyme isolated from both sources.

An ordered reaction mechanism was postulated for both pigeon liver (Hsu et al., 1967 and E. coli (Sanwal and Smando, 1969) malic enzyme. Based on reaction (1), an ordered Bi-Ter mechanism has been theorized:

\[
\begin{array}{ccccc}
TPN & \text{Malate} & CO_2 & \text{Pyruvate} & TPNH \\
\uparrow & \uparrow & \uparrow & \uparrow & \uparrow
\end{array}
\]

In regard to the physiological role, it was first thought that malic enzyme participated in gluconeogenesis by functioning in the direction of CO$_2$ fixation through a TPN-linked dismutation with the glucose-6-phosphate
<table>
<thead>
<tr>
<th>Source</th>
<th>Cellular Localization</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosol</td>
<td>Mitochondrion</td>
</tr>
<tr>
<td>Pigeon liver&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Rat brain&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bovine adrenal cortex&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bovine heart&lt;sup&gt;d&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mouse kidney&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

e. Shows <i>et al.</i>, 1970.

dehydrogenase system (Ochoa <i>et al.</i>, 1948, 1950). The malate formed is oxidized to OAA, the precusor of phosphoenolpyruvate (PEP). Thus, through the reverse of Embden-Meyerhof pathway, gluconeogenesis was made possible (Ochoa <i>et al.</i>, 1948, 1950; Utter and Kurahashi, 1959):
However, subsequent evidence strongly suggested that malic enzyme might function to provide TPNH as well as pyruvate in lipogenesis:

1) Lipogenic tissues (liver, adipose tissue, adrenal, mammary gland) are rich in malic enzyme (Utter, 1959; Rees and Huggins, 1960; Wise et al., 1964).

2) Malic enzyme activity in rat liver was found to be inadequate to account for the rate of pyruvate conversion to carbohydrate (Shrago et al., 1963).

3) The hexose monophosphate shunt did not provide enough TPNH for fatty acid synthesis (Flatt and Ball, 1964).

4) Malic enzyme in both rat adipose tissue and liver was altered in such a way by experimental conditions (e.g., change in dietary regime, hormonal treatments) which were known to affect lipogenesis (Fitch and Chaikoff, 1960; Vaughan and Winders, 1964; Wise et al., 1964; Young et al., 1964; Lardy
et al., 1964).

TABLE II

COMPARISON OF SOME PHYSICAL PROPERTIES OF PIGEON LIVER AND E. COLI MALIC ENZYME

<table>
<thead>
<tr>
<th>Physical Parameter</th>
<th>Pigeon Liver\textsuperscript{a}</th>
<th>E. coli\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight (Mw)</td>
<td>$2.8 \times 10^5$</td>
<td>$5.5 \times 10^5$</td>
</tr>
<tr>
<td>Number of subunits</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Subunit Mw</td>
<td>$7.6 \times 10^4$</td>
<td>$6.7 \times 10^4$</td>
</tr>
<tr>
<td>Specific activity</td>
<td>27.3</td>
<td>56.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Hsu and Lardy, 1967a, 1967b.

\textsuperscript{b}Spina et al., 1970.

TPN-dependent malic enzyme has been demonstrated in the rat tapeworm *Hymenolepis diminuta* by Prescott and Campbell (1965). This parasitic helminth resides in an anaerobic environment in the small intestine of rats. A complete scheme of carbohydrate metabolism is still not clear in this organism. However, the carbohydrate metabolism of the parasitic nematode *Ascaris lumbricoides suum*, which resides in an anaerobic environment in the small intestine of pigs, has been studied in some detail. Saz and Lescure (1966) have proposed a pathway for the utilization of carbohydrates in the ascarid
According to the above scheme, PEP formed via the Embden-Meyerhof pathway in the cytoplasm is carboxylated to form OAA via PEP carboxykinase. OAA is then reduced to malate via malate dehydrogenase by using the DPNH produced during the Embden-Meyerhof pathway. The malate then enters the mitochondrion where it can participate in one of following two reactions:

1) via DPN-dependent malic enzyme (EC 1.1.1.39*), malate is oxidized and decarboxylated to pyruvate. The latter is converted to acetate subsequently.

2) via fumarase, fumarate is formed from malate.

*EC 1.1.1.39 is applied to the DPN-dependent malic enzyme which does not decarboxylate OAA. If the enzyme is DPN-dependent and capable of decarboxylating OAA, the EC number then is 1.1.1.38.
Fumarate is then reduced to succinate. This reduction is flavin-linked with a resultant ATP generation (Kmetec and Bueding, 1961; Seidman and Entner, 1961; Saz and Lescure, 1969).

It is proposed that there is a balance between DPN and DPNH inside the mitochondrion. The DPNH formed via malic enzyme is oxidized when fumarate is reduced to succinate. Since malic enzyme is DPN-dependent in the ascarid and it is proposed to provide the reducing equivalent for the reduction of fumarate, the lipogenic function of malic enzyme as applied to the mammalian tissues does not seem to fit in the ascarid. However, it had been shown that DPNH appears to work just as well as TPNH in the fatty acid synthesis in the *Ascaris lumbricoides suum* (Beames et al., 1967). Thus, the possible lipogenic purpose of malic enzyme in the nematode should not be ignored.

In view of the facts that *Hymenolepis diminuta* 1) forms succinate as its major fermentation product (Fairbairn et al., 1961; Scheibel and Saz, 1966), 2) possesses PEP carboxy-kinase and malic enzyme activities (Prescott and Campbell, 1965), 3) appears to be devoid of cytochrome oxidase activity, but does catalyze a mitochondrial $^{32P}$-ATP exchange (Scheibel et al., 1968), and 4) lacks a complete TCA cycle (Scheibel
and Saz, 1966; Ward and Fairbairn, 1970), it would appear likely that a similar carbohydrate metabolism may occur in this tapeworm (Scheibel and Saz, 1966; Saz and Bueding, 1966; Saz and Lescure, 1969). It is also worth noting that despite the high content of lipid, studies with *H. diminuta* showed that this worm did not appear to be capable of carrying out a complete β-oxidation of fatty acids. This is due to the absence of two of the enzymes in that process (Ward and Fairbairn, 1970). In addition, *H. diminuta* seems to have lost its capacity for *de novo* synthesis of fatty acids but may increase the chain length of some higher fatty acids (Jacobson and Fairbairn, 1967). This chain lengthening process would probably still require TPNH. The presence of TPN-dependent malic enzyme in the tapeworm would probably serve a similar purpose as in the mammalian tissues.

Due to its subcellular localization, the physiological role it plays, and its molecular variations, malic enzyme appears to be an interesting enzyme. So far, very few enzyme studies have been done in depth in the parasitic helminths. The enzymology in these parasites is of significance from a comparative point of view. *Hymenolepis diminuta* belongs to the phylum Platyhelminthes which appeared much earlier than the chordates in animal phylogeny. Although
Ascaris suum belongs to a different phylum, Aschelminthes, both the Platyhelminthes and the Aschelminthes are believed to arise from a common acoeloid ancestor. In addition, these two animals reside in a similar anaerobic environment. The presence of TPN-dependent malic enzyme in the tapeworm while the enzyme is DPN-dependent in the ascarid is an interesting question to probe. Whether these two enzymes are evolved divergently or convergently, the physiological importance of this enzyme in both organisms, as well as other studies including catalytic properties and enzyme regulation studies, etc., could yield valuable information on the evolution of these parasites. Comparison is also interesting between the parasitic malic enzyme and the enzyme from other sources. The intent of the present investigation was to purify the malic enzyme from the tapeworm Hymenolepis diminuta with high yield.
MATERIALS AND METHODS

Materials

Maintenance of Worms

The tapeworm (Hymenolepis diminuta) used in this study was maintained in the laboratory essentially as described by Read et al. (1963).

Substrates and Enzymes

Triphosphopyridine nucleotide (TPN), L-malic acid, pyruvate (sodium salt, dimer-free), TPNH (tetrasodium salt), the disodium salt of the reduced diphosphopyridine nucleotide (DPNH), and cis-oxalacetic acid (grade I), and lactic dehydrogenase (EC 1.1.1.27, specific activity 600 units per mg) were obtained from Sigma. Manganese (ous) chloride was the product of Fisher Chemical Company. Sodium bicarbonate was purchased from J. T. Baker Chemical Co.

Materials for Column Chromatography

Cellulose phosphate (coarse, exchange capacity, 0.86 meq/mg) was obtained from Sigma. Chromatography columns, Sephadex G-25 and G-200, and Calibration Kit were supplied
Buffers

Tris (hydroxymethyl) aminomethane was from Fisher. Triethanolamine and imidazole were obtained from Sigma.

Others

Ethylene diamine tetraacetate (EDTA) was the product of J. T. Baker Chemical Co. Polyethylene glycol 20,000 was purchased from Fisher. N-Ethyl-maleimide, p-chloromercuribenzoic acid, and 2-mecaptoethanol were obtained from Sigma. All other chemicals were of analytical grade.

Methods

Enzyme Assays and Definition of Units of Activity

Malic enzyme was assayed according to reaction (1) spectrophotometrically at 340 nm following the reduction of TPN. Curvets of 1.0-cm light path were used. Tris -Cl, 0.1 M, pH 7.5, was used as a buffer unless otherwise stated. The routine reaction mixture consisted of 0.1 mM TPN, 10 mM malate, 4 mM MnCl₂, and 0.1 M Tris, in a final volume of 2.5 ml. Enzyme was added to initiate the reaction. Initial velocities were measured and the average of at least two measurements was reported in the results. The assays were
carried out at 25° with a Beckman Model DB-GT Spectrophotometer attached to a Beckman Ten-Inch Laboratory Potentiometric Recorder. In the case of kinetic studies, the full scale of the recorder was set to equal 0.1 optic density unit. A chart speed of 10 inches per min was used for more precise initial velocity measurements.

Units of malic enzyme activity are defined as micromoles of TPN reduced per min at 25° under the assay conditions described. Specific activity is expressed as units per mg protein.

Oxalacetate decarboxylase activity (reaction (2)) was assayed by incubating at 25° a reaction mixture containing 0.1 M triethanolamine (TEA) buffer at pH 7.5, 10 mM OAA, 0.1 mM TPN, 0.2 mM DPNH, 10 micrograms of lactic dehydrogenase, and malic enzyme. The final volume was 2.5 ml. Malic enzyme was added to start the reaction. Spontaneous decarboxylation of OAA was corrected for by the use of a control without malic enzyme. Changes of OD at 340 nm was the indication of OAA decarboxylase activity.

Protein Determination

Protein was determined by the method of Lowry et al. (1951). Bovine serum albumin was used as the protein
standard. Estimation of protein in fractions during column chromatography was done by measuring the absorbance at 280 nm. All specific activities were calculated from Lowry's protein determination.

**Ion Exchange and Gel Filtration Chromatography**

Phosphocellulose was washed repeatedly with acid and base according to the procedure suggested by Peterson et al. (1962). It was equilibrated with the desired buffer prior to use. After the column was packed, it was washed with buffer in a cold room kept at 0-4°C. The eluant was tested to ensure the desired pH had been attained before the sample was applied. Fractions were collected at the same temperature. Constant flow rate was achieved with a piston minipump from Milton Roy Company.

Sephadex was equilibrated with the desired buffer as suggested by Pharmacia (1970). A 2.5 x 45 cm column was packed with G-25 and a 1.6 x 120 cm column with G-200. Both of the columns were washed with the buffer in the cold room for more than 100 hours till the bed length had been stabilized and a constant flow rate had been achieved. In the case of G-25, the sample was allowed to soak into the column bed, and elution was initiated with the buffer. For G-200 chro-
matography, sucrose (20% w/v) was added to all samples, so that the eluting buffer could be layered above the column without mixing the sample. Calibration of the G-200 column was carried out with blue dextran, aldolase, ovalbumin, chymotrypsinogen A, and ribonuclease A obtained from Pharmacia. The volume collected in each fraction was determined by weighting. Elution volumes were correlated with the molecular weight (Andrews, 1965).

**Polyacrylamide Gel Electrophoresis**

The procedure followed was that of Davis (1964). Protein was stained with 1% napthol blue black in 5% acetic acid. The enzyme activity stain was based on the method of Shows et al. (1970).

**Sedimentation Equilibrium Ultracentrifugation**

High speed sedimentation equilibrium ultracentrifugation was carried out as suggested by Yphantis (1964) and Van Holde (1967). Analysis of fringes was performed by enlarging the photograph with a microprojector. Molecular weight was calculated by the equation:

\[
M_w = \frac{2RT}{(1-\bar{v}p)w^2} \frac{dlnc}{dx^2}
\]
where \( c \) is the concentration expressed in Rayleigh fringes, \( x \) is the distance of the fringe from the center of rotation, \( R \) is the gas constant, \( 8.314 \times 10^7 \) ergs/mole/degree, \( T \) is the absolute temperature, \( \bar{V} \) is the partial specific volume of the solute, \( p \) is the density of the solution, and \( w \) is the angular velocity of the centrifuge rotor in radians per second, (rpm) \((2\pi)/60 \). The value of 0.73 was used for \( \bar{V} \) as an approximation.

**Kinetic Studies**

Michaelis constants (\( K_m \)) for TPN and malate in the forward direction of reaction (1) and TPNH and pyruvate in reverse reaction were evaluated by a computer program (Cleland, 1963). An expanded scale on the recorder was used in the studies on the forward reaction to obtain a precise measurement down to \( 10^{-4} \) OD units. Due to the interference of high concentrations of TPNH and pyruvate at 340 nm, the measurements of the reverse reactions were carried out without expanding the recorder scale. A much larger amount of enzyme was used in the latter case and sodium bicarbonate was added in saturated quantities for the reverse reactions. All the substrates used in kinetic studies were made up fresh and all the reaction measurements were carried out at pH 7.5 and 25°.
**Metal Ion Effects**

In this experiment, the malic enzyme sample was either dialyzed against 10 mM imidazole buffer at pH 7.0 containing 0.1 M EDTA for 24 hours, or incubated with 10 mM EDTA before analysis.

**Identification as a SH-enzyme and Reactivation of Aged Enzyme**

Both of the potent SH-group inhibitors, p-chloromercuribenzoate (PCMB) and N-ethyl maleimide (NEM), were incubated with the enzyme sample in presence or in absence of dithiothreitol (DTT) for 40 min. Enzyme activity was tested every 10 min. Reactivation of aged malic enzyme was achieved with DTT.
RESULTS

Purification Procedure

Step I - Crude Extract

The tapeworms (74 grams), aged from 15 to 30 days, were collected from the small intestine of infected rats and rinsed free of fecal materials with Krebs-Ringer solution (Umbriet et al., 1964), pH 7.4, with 0.05 Tris-HCl. After blotting on filter paper, the worms were suspended in 3 volumes of 10 mM imidazole buffer, pH 6.5, containing 1 mM EDTA, and cut into 2-mm pieces with scissors. All the subsequent steps were carried out at 0-4° in the same buffer unless otherwise noted. The first homogenization was made in a stainless steel semi-micro-Waring blender. The homogenate was centrifuged for 20 min at 12,000 g, and the supernatant solution filtered through glass wool. Rehomogenization of the pellet was made with a motor-operated teflon homogenizer in another 3 volumes of buffer. After centrifugation, the supernatant solution was again filtered and pooled and the pellet discarded. A total of 300 ml was obtained as the crude extract.
**Step II - Heat Treatment**

This procedure was based on Kaufman *et al.* (1951). Magnesium acetate was added to the crude extract to give a concentration of 10 mM. The mixture was divided into 15-ml aliquots in 20 test tubes, and heated with constant stirring for 5 min in a water bath maintained at 58°. The aliquots were then rapidly cooled, combined, and centrifuged for 20 min at 12,000 g. The resulting precipitate was discarded. The supernatant solutions were collected, giving a total volume of 267 ml.

**Step III - Sephadex G-25 Chromatography**

In order to desalt and equilibrate with the buffer before applying to ion-exchange column chromatography, the heat-treated enzyme solution was divided into 5 fractions. Each fraction was then passed through a Sephadex G-25 column (2.5 x 45 cm) pre-equilibrated with the buffer. A pump was used to maintain a constant flow rate of 18 ml/hour. Fractions of 5 ml were collected by an automatic fraction collector. The enzyme activity followed a large protein peak. A typical elution pattern is presented in Fig. 1. Appropriate fractions were pooled and the enzyme solutions from all the five runs were combined.
Fig. 1. Sephadex G-25 chromatography of malic enzyme from Hymenolepis diminuta. Heat treated extract from Step II was separated into 5 fractions. Each fraction was passed through a G-25 column (2.5 x 45 cm, bed length 35 cm) which had been washed with 10 mM imidazole buffer, pH 6.5, containing 1 mM EDTA, extensively. After the sample had soaked into the column bed, the buffer was pumped through the column at a flow rate of 18 ml per hour. Fractions of 7 ml were collected. This is a typical elution pattern of the five runs. The solid bar indicates fractions pooled. ○, malic enzyme activity. ●, protein.
Step IV - First Phosphocellulose Column Chromatography

A column (2.5 x 45 cm) was prepared and packed with phosphocellulose (coarse) with a bed length of 37 cm. The 220-ml enzyme solution from Step III was pumped into the column bed and then the column was washed with the buffer until the first protein peak had emerged. Thereafter, a linear NaCl gradient from 0 to 1.0 M was carried out in a total of two liters of buffer. A constant elution rate of 45 ml/hr was maintained with a pump. Fractions of 7 ml were collected and assayed for both absorbance at 280 nm and malic enzyme activity. Enzyme activity was recovered in the latter half of the third protein peak after the salt gradient had been applied (Fig. 2). Fractions with enzyme activity above 1.0 unit ml⁻¹ were pooled and dialyzed.

Step V - Second Phosphocellulose Column Chromatography

This step was performed in a manner identical with the previous step, with the exception of column size and eluant volume. A 1.5 x 30 cm column was used (bed length 25 cm), and a salt gradient was applied immediately after the sample had been soaked into the column bed. The elution of protein and enzyme activity is shown in Fig. 3. Fractions of 5-ml were
Fig. 2. First phosphocellulose column chromatography of *Hymenolepis diminuta* malic enzyme. Fractions pooled from the Sephadex G-25 column was pumped into a phosphocellulose column (2.5 x 45 cm, bed length 37 cm) (equilibrated with 10 mM imidazole buffer, pH 6.5, containing 1 mM EDTA). The column was washed with the same buffer after the sample had been applied. A constant flow rate of 45 ml per hour was maintained by a pump and 7-ml fractions were collected. After the first protein peak had passed through, a linear NaCl gradient from 0 to 1.0 M was established in 2 liters of buffer. The arrow indicates the starting of the salt gradient and the solid bar fractions pooled. ○, malic enzyme activity. ●, protein.
Fig. 3. Second phosphocellulose column chromatography of *Hymenolepis diminuta*. This step was similar to the first phosphocellulose column chromatography except a small column (1.5 x 30 cm, bed length 25 cm) was used. Dialyzed enzyme solution (with the same buffer used before) from Step IV was pumped into the column bed. A linear NaCl gradient from 0 to 1 M in 1000 ml buffer was applied immediately when the sample had soaked into the column bed. Flow rate was maintained at 36 ml per hour and fractions of 5 ml were collected. Pooled fractions were indicated by the solid bar, •, malic enzyme activity. •, protein.
collected and those with enzyme activity 2 units ml\(^{-1}\) or higher were pooled. Salt was removed by dialysis overnight against 4 liters of 10-mM imidazole buffer at pH 6.5 containing 1 mM EDTA.

**Step VI - Concentration by Sephadex G-200**

The pooled fractions from the above step were put into dialysis tubing. Dry Sephadex G-200 was then poured over the bag. The dextran draws the fluid out of the tubing and a volume of 3 ml was obtained after 40 hours. However, 16% of the total activity was lost in this step.

**Step VII - Sephadex G-200 Column Chromatography**

The concentrated enzyme solution was applied to a Sephadex G-200 column (1.6 x 120 cm, bed length 101 ml). A constant hydrostatic pressure of 15 cm was set up to maintain a flow rate of 6 ml per hour. Fractions of 3 ml were collected. Malic enzyme was found coinciding with the first protein peak (Fig. 4). Appropriate fractions were pooled.

The 320-fold purification of malic enzyme from the tapeworm *Hymenolepis diminuta* is summarized in Table III. Most of the loss, except during the concentrating of the enzyme solution (Step VI), was accounted for by fractions.
Fig. 4. Gel filtration of *Hymenolepis diminuta* on Sephadex G-200. Concentrated enzyme solution from Step VI was added to a Sephadex G-200 column (1.6 x 120 cm, bed length 101 cm) and eluted with 10 mM imidazole buffer, pH 6.5, containing 1 mM EDTA. Appropriate hydrostatic pressure was set up to maintain a constant flow rate of 6 ml per hour. Fractions of 3 ml each were collected and assayed for both malic enzyme activity (○) and protein concentration (■). Solid bar indicates fractions pooled.
<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Total Activity (units)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (units/mg prot)</th>
<th>Purification Over Previous Step</th>
<th>Over all</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Crude extract</td>
<td>300</td>
<td>297.0</td>
<td>3,300.0</td>
<td>0.09</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>II. Heat treatment</td>
<td>267</td>
<td>302.8</td>
<td>1,121.4</td>
<td>0.27</td>
<td>3.0</td>
<td>3.0</td>
<td>(102)</td>
</tr>
<tr>
<td>III. Sephadex G-25 chromatography</td>
<td>220</td>
<td>239.4</td>
<td>374.0</td>
<td>0.64</td>
<td>2.4</td>
<td>7.1</td>
<td>81</td>
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<tr>
<td>IV. First phosphocellulose column chromatography</td>
<td>75</td>
<td>196.9</td>
<td>24.4</td>
<td>8.07</td>
<td>12.6</td>
<td>89.7</td>
<td>66</td>
</tr>
<tr>
<td>V. Second phosphocellulose column chromatography</td>
<td>34</td>
<td>148.9</td>
<td>8.3</td>
<td>18.0</td>
<td>2.2</td>
<td>200.0</td>
<td>50</td>
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<tr>
<td>VI. Concentration by Sephadex G-200</td>
<td>3</td>
<td>101.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>34</td>
</tr>
<tr>
<td>VII. Sephadex G-200 column chromatography</td>
<td>20</td>
<td>80.0</td>
<td>2.7</td>
<td>29.4</td>
<td>1.6</td>
<td>326.6</td>
<td>27</td>
</tr>
</tbody>
</table>
on the leading and trailing edges of column peaks, which were discarded.

Properties of Tapeworm Malic Enzyme

Homogeneity Measurements

Sephadex G-200 gel filtration.--The elution pattern of malic enzyme through the G-200 column in step VII of the purification procedure is symmetrical, with enzyme activity and protein concentration overlapping each other (Fig. 4).

Polyacrylamide gel electrophoresis.--Two bands of protein were found corresponding to the zones which exhibited enzyme activity (Fig. 5).

Molecular Weight Determination

Sephadex G-200 gel filtration.--The same column used in purification was calibrated (Fig. 6). From the position on the calibration curve, the molecular weight of malic enzyme from *H. diminuta* was estimated to be 120,000 g mole$^{-1}$.

Sedimentation equilibrium ultracentrifugation.--A preliminary high-speed sedimentation equilibrium ultracentrifugation was performed. The purified enzyme solution (Preparation III) was concentrated as in Step VI in
Fig. 5. Polyacrylamide gel electrophoresis patterns of *Hymenolepis diminuta* malic enzyme. Disc electrophoresis on polyacrylamide gel was performed at pH 8.6 in Tris-glycine buffer for two hours at a current of 5 mA per gel. Concentrated solution of the 320-fold purified malic enzyme (0.8 mg/ml) was applied in a volume of 50 ul. Protein stain was in napthol blue black and cleared in 5% TCA. Malic enzyme activity stain was cleared in 5% acetic acid.
Fig. 6. Calibration curve and estimation of molecular weight of *Hymenolepis diminuta* malic enzyme. A Sephadex G-200 column (1.6 x 110 cm) was calibrated with (a) ribonuclease A, Mw 13,700, (2) chymotrypsinogen A, Mw 25,000, (3) ovalbumin, Mw 45,000, and (4) aldolase, Mw 158,000. Elution was carried out with 10 mM imidazole buffer, pH 6.5, containing 1 mM EDTA at a flow rate of 6 ml per hour. Fractions of 3 ml were collected. A semi-log scale was used with molecular weight on the log scale. $V_e$ = elution volume for the proteins, $V_o$ = elution volume determined for blue dextran 2000 (void volume). The circle indicates the malic enzyme.
the purification procedure with the exception that polyethylene glycol replaced the Sephadex. The concentrated enzyme solution was dialyzed for 48 hours against a buffer containing 10 mM Tris, pH 7.5, 1 mM EDTA, 0.05% (v/v) 2-mercaptopethanol, and 0.1 M NaCl. The density of the buffer was 1.1011 at 20°. Dilution was made with the dialyzing buffer to obtain a protein concentration of 0.3 mg/ml prior to ultracentrifugation. The photograph of the Rayleigh interference pattern, taken after equilibrium had been established (24 hours), was enlarged with a microprojector. Calculations were made by determining the vertical deflection of a single fringe across the entire length of the liquid column. The slope of ln c versus x² plot (Fig. 7) was substituted to the equation and a molecular weight of 10 x 10⁴ was obtained.

**Determination of OAA Decarboxylase Activity**

Different enzyme quantities were to demonstrate this activity. Enzyme from Preparation III was used in this study. The results are presented in Table IV.

**Kinetic Studies**

The Lineweaver-Burk plots of 1/v against 1/s for TPN and malate in the forward reaction (1), and TPNH and pyruvate in the reverse reaction are presented in Fig. 8 and Fig. 9.
Fig. 7. Equilibrium sedimentation of *Hymenolepis diminuta* malic enzyme. The concentrated 320-fold purified malic enzyme was dialyzed against 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 0.05% (v/v) 2-mercaptoethanol, 0.1 M NaCl. \( p(\text{density}) = 1.1011 \). Prior to the run, the enzyme was diluted with the dialyzing buffer to a concentration of 0.3 mg per ml. The photograph of the Rayleigh interference pattern was measured with a microprojector by determining the vertical deflection of a single fringe across the entire length of the liquid column. \( C \) is the concentration expressed in Rayleigh fringes, and \( X \) is the distance of the fringe from the center of rotation.
respectively. The Michaelis constants (Km) determined are

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Km</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPN</td>
<td>$4.3 \times 10^{-6}$ M</td>
</tr>
<tr>
<td>Malate</td>
<td>$2.5 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>TPNH</td>
<td>$2.3 \times 10^{-5}$ M</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>$15.5 \times 10^{-3}$ M</td>
</tr>
</tbody>
</table>

Using the same amount of enzyme from the same preparation (Preparation II), the maximum velocity of the reverse reaction (Vr) is found to be 36% that of the forward.

**TABLE IV**

**OAA DECARBOXYLASE ACTIVITY OF MALIC ENZYME FROM HYMENOLEPIS DIMINUTA**

<table>
<thead>
<tr>
<th>Experimental Conditions*</th>
<th>0D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>0</td>
</tr>
<tr>
<td>- malic enzyme</td>
<td>0</td>
</tr>
<tr>
<td>- lactic dehydrogenase</td>
<td>0</td>
</tr>
<tr>
<td>Complete with malic enzyme:</td>
<td></td>
</tr>
<tr>
<td>8 ug</td>
<td>0.001</td>
</tr>
<tr>
<td>16 ug</td>
<td>0.0019</td>
</tr>
<tr>
<td>20 ug</td>
<td>0.0025</td>
</tr>
</tbody>
</table>

*Conditions of the complete assays are described under "Methods". An expanded scale on the recorder was used to measure 0 to 0.1 OD units.
Fig. 8. Double reciprocal plots of the activity of *Hymenolepis diminuta* as a function of (a) TPN and (b) malate. The assay mixture was described in MATERIALS AND METHODS. The recorder scale was adjusted to measure 0-0.1 OD units and a chart of 10 inches per min was used. The values for the two interceptions (1/Vmax and 1/Km) were determined by a computer program. Purified enzyme from Preparation II was used.
(a) \[ \frac{1}{V} \]

(b) \[ \frac{1}{V} \]
Fig. 9. Double reciprocal plots of the reverse reaction of *Hymenolepis diminuta* malic enzyme as a function of (a) TPNH and (b) pyruvate. Due to the interference of TPNH and pyruvate at 340 nm, the recorder scale was not expanded. Chart speed was 10 inches per min. Purified enzyme was from Preparation II.
reaction \( \left( V_f \right) \). In carrying out these reactions, the following quantities of the substrates were used:

<table>
<thead>
<tr>
<th>Forward reaction</th>
<th>Reverse reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPN</td>
<td>TPNH</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>0.4 mM</td>
</tr>
<tr>
<td>Malate</td>
<td>Pyruvate</td>
</tr>
<tr>
<td>10.0 mM</td>
<td>30.0 mM</td>
</tr>
<tr>
<td>( \text{NaHCO}_3 )</td>
<td>40.0 mM</td>
</tr>
</tbody>
</table>

\( V_r = 36\% \ V_f \) at pH 7.5, 25°

**Metal Ion Effects**

The enzyme activity from Preparation II was not affected by \( \text{MnCl}_2 \) in the assay medium. However, after dialysis against 0.01 M imidazole buffer at pH 7.0 containing 0.1 M EDTA over night, a metal ion cofactor was necessary for enzyme activity. Manganese chloride (\( \text{MnCl}_2 \)) gave an activity higher than that before dialysis. Magnesium ion (\( \text{Mg}^{++} \)) was about 60% as effective as \( \text{Mn}^{++} \) at a concentration of 2 mM. A variety of divalent cations were tested for their effects on malic enzyme activities (Table V).

The effect of EDTA on malic enzyme was tested in another manner. Enzyme solution from Preparation II was incubated with 10 mM EDTA. After 10 min, enzyme activity was lost completely without \( \text{MnCl}_2 \) added to the assay medium. However,
### Table V

**Metal Ion Effects on Malic Enzyme Activity**

<table>
<thead>
<tr>
<th>Experimental Conditions*</th>
<th>Relative Activity**</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td>Before dialysis</td>
<td>90</td>
</tr>
<tr>
<td>+MnCl₂ 4.0 mM</td>
<td>90</td>
</tr>
<tr>
<td>After dialysis</td>
<td>0</td>
</tr>
<tr>
<td>+MnCl₂ 0.5 mM</td>
<td>100</td>
</tr>
<tr>
<td>+MnCl₂ 4.0 mM</td>
<td>100</td>
</tr>
<tr>
<td>+MgCl₂ 2.0 mM</td>
<td>60</td>
</tr>
<tr>
<td>+MgSO₄ 2.0 mM</td>
<td>63</td>
</tr>
<tr>
<td>+Mg(acetate)₂ 2.0 mM</td>
<td>61</td>
</tr>
<tr>
<td>+CoCl₂ 2.0 mM</td>
<td>53</td>
</tr>
<tr>
<td>+ZnCl₂ 2.0 mM</td>
<td>43</td>
</tr>
<tr>
<td>+CaCl₂ 2.0 mM</td>
<td>13</td>
</tr>
<tr>
<td>+CuSO₄ 2.0 mM</td>
<td>10</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
</tr>
<tr>
<td>Before EDTA added</td>
<td>90</td>
</tr>
<tr>
<td>+MnCl₂ 4.0 mM</td>
<td>90</td>
</tr>
<tr>
<td>After EDTA added</td>
<td>0</td>
</tr>
<tr>
<td>+MnCl₂ 4.0 mM</td>
<td>100</td>
</tr>
</tbody>
</table>

*The assay conditions are described in "Methods" with the exception that metal ions were not added. The added metals are indicated with the concentration used. In experiment 1 the enzyme solution was dialyzed against 0.01 M imidazole buffer containing 0.1 M EDTA at pH 7. The enzyme solution was incubated with 10 mM EDTA in the experiment 2.

**Enzyme activity after EDTA treatment in both the experiments was designated 100. All the activities measured under other conditions were relative values."
a higher activity was again found with the complete assay condition (Table VII).

Identification as a SH-Enzyme and Reactivation of Aged Enzyme

Some partially purified malic enzyme (specific activity of 8.2) was used in this study. The enzyme had been stored at 2°C for one month. A concentration of 0.133 mM PCMB inhibited the enzyme completely within 10 min of incubation while NEM gave only a partial inhibition of malic enzyme in 40 min. However, in the presence of 0.667 mM DTT, the effects of both PCMB and NEM were abolished. In addition, a 30% increase of activity was found with DTT despite the presence of the inhibitors. The reactivation effect of DTT was demonstrated by incubating the enzyme with DTT for 40 min at 25°C, again a 30% increase in enzyme activity was observed. The results are summarized in Fig. 10.
Fig. 10. Effects of N-ethyl-maleimide (NEM) and p-chloromercuribenzoic acid (PCMB) on Hymenolepis diminuta malic enzyme. Partially purified malic enzyme stored at 2° for one month with a specific activity of 8.2 was used in this study. The enzyme was incubated with (a) control, (b) 0.133 mM NEM, (c) 0.133 mM NEM and 0.667 mM DTT, (d) 0.133 mM PCMB, and (e) 0.133 mM PCMB and 0.667 mM DTT at 25°. Enzyme activity was tested every 10 min. The 0-time malic enzyme activity in the control was designated as 1 as the basis of calculating the relative activities.
DISCUSSION

In order to study an enzyme in detail, it is necessary that the enzyme be in as homogenous a state as possible. The present work dealt primarily in the purification of malic enzyme from *Hymenolipis diminuta*.

The specific activity of malic enzyme in the crude extract from the tapeworm (0.09-0.1) compares with 0.21 from pigeon liver (Hsu and Lardy, 1967a), 0.13 from malate-grown *E. coli* (Spina et al., 1970) and 0.03 from rat liver. Due to the limited supply of worms (generally 3 to 4 grams of worms were obtained from a well-infected rat) a purification procedure had to be developed in order to obtain a good final yield of the purified enzyme.

From preliminary experiments, ammonium sulfate and organic solvent (ethanol and acetone) fractionations resulted in at least 20% loss of enzyme activity. So, these methods were not used in purifying the *H. diminuta* malic enzyme. Heat treatment provided an efficient step in the purification process. This procedure had been applied to the purification of malic enzyme from a variety of sources (Hsu and Lardy, 1967a; Spina et al., 1970; Saito et al., 1971).
Although a purification factor of only 2-4 was gained, the yield in this step was always complete. As a matter of fact, a slight increase in activity was frequently observed on this step. A similar increase with pigeon liver malic enzyme has been reported (Hsu and Lardy, 1967a). Saito et al. (1971) found a 20% increase in malic enzyme activity upon heat treatment in the rat liver. The reason for the increase of activity by heat treatment is not clear. It could be due to the removal of inhibitors by heat.

Dialysis always resulted in a partial loss of enzyme activity. Sephadex G-25 gel filtration was thus employed for the purpose of desalting and equilibration. A 2.4-fold purification was also obtained through the Sephadex G-25 gel filtration. In all the column experiments, including G-25, G-200 and phosphocellulose, malic enzyme appeared in a single peak. Almost all the enzyme activity was recovered by calculation of the fractions containing malic enzyme activity. After appropriate fractions were pooled, an average of 80% of the enzyme was recovered through all the individual columns. The 50% yield of the 200-fold purified enzyme at the end of the second phosphocellulose column (Step V, Table III) was still appreciable. However, 16% of the starting enzyme activity was lost during the
concentrating step (Step VI). Concentrating was always a problem in the purification of the enzyme. Ammonium sulfate precipitation was not utilized due to the small amount of protein (8.3 mg in 34 ml). If a good concentrating method could be developed with little loss of enzyme activity, the final yield could be more than 40% after Sephadex G-200 chromatography. Thus, starting with 100 gm of worm tissue, approximately 6 mg of 320-fold purified enzyme would be obtained through the purification procedure described.

On polyacrylamide gel electrophoresis, the corresponding bands of both activity and protein stains afford good evidence for the homogeneity of the 320-fold purified enzyme. The crude extract or partially purified malic enzyme of the tapeworm repeatedly showed only one band of activity on cellulose acetate electrophoresis (unreported data). Further, the enzyme appeared in a single peak through all the columns. Therefore it is not likely that the two bands necessarily indicate the presence of isozymes. As indicated by Saito et al. (1971), these is the possibility that malic enzyme is modified during purification steps. However, the possibility of the presence of closely related isozymes should not be ignored. The G-200 gel filtration data in which the enzyme activity and protein curves match
gives further evidence of the homogeneity of the preparation.

The molecular weight, $1.2 \times 10^5$, of *H. diminuta* malic enzyme, determined by Sephadex G-200 gel filtration is about one-half the molecular weight of pigeon liver malic enzyme (Hsu and Lardy, 1967a). Since a tetramer has been proposed by Hsu and Lardy (1967a) on pigeon liver malic enzyme, the cestode enzyme could possibly exist as a dimer. If this is the case, the variation in molecular weight and subunit content of malic enzyme among *H. diminuta*, pigeon, and *E. coli* (molecular weight $5.5 \times 10^5$, octomer) (Spina et al., 1970) is interesting in terms of possible physiological and evolutionary significance. The encoding of a basic subunit and then aggregation of these monomers into dimers, tetramers or octomers according to the needs of the organisms would be, teleologically speaking, a conservative mechanism. In this regard, it is interesting to note that the octomeric *E. coli* enzyme can be allosterically effected by various compounds (Sanwal et al., 1968; Sanwal and Smando, 1969a; 1969b; 1969c). This phenomenon of regulation has not been demonstrated with the smaller tetrameric (Hsu and Lardy, 1967a) or dimeric forms. However, whether the molecular weight of *H. diminuta* malic enzyme represents the native enzyme as well as the exact submit content requires further investigation. The molecular
weight of $1.0 \times 10^5$ determined by the preliminary high-speed sedimentation equilibrium ultracentrifugation may not be reliable. More runs are necessary for more precise molecular weight determination. The exact value of the partial specific volume ($V$) should also be determined instead of using an approximation. However, the molecular weight of $1.0 \times 10^5$ does show that the malic enzyme is not as large as that from either pigeon liver or \textit{E. coli}. It is also worth noting that the specific activity of the 320-fold purified enzyme (29.4) is comparable to the value of 28 for the crystalline pigeon liver enzyme (Hsu and Lardy, 1967a).

During the development of the purification procedure, it was found that \textit{H. diminuta} malic enzyme did not bind readily to the DEAE-cellulose (cation exchanger) up to pH 8.0. Instead the enzyme bound to the phosphocellulose (anion exchanger) very well at pH 6.5. DEAE-cellulose has been utilized in the purification of malic enzyme from \textit{E. coli} (Spina \textit{et al.}, 1970), pigeon liver (Hsu and Lardy, 1967a), and the mammalian tissues, rat liver (Saito \textit{et al.}, 1971), bovine heart (Frenkal, 1971), and bovine adrenal cortex mitochondria (Simpson and Estabrook, 1969). The ability of a protein to bind to an ion exchanger depends on the charge distribution over the protein molecule at a
certain pH. A difference in the binding capacity of *H. diminuta* malic enzyme toward DEAE-cellulose suggests an amino acid composition that might be quite different from the malic enzymes studied thus far.

The *H. diminuta* malic enzyme did not require an external metal cofactor through the purification. However, EDTA treatment caused a complete loss of malic enzyme activity. This activity could be recovered by adding a metal ion to the assay medium. Manganous ion (Mn$^{++}$) gave a 10% increase in activity after EDTA treatment but magnesium ion (Mg$^{++}$) restored only 60% of the malic enzyme activity at the same concentration. All the other divalent metal ions tried were less effective at a concentration of 2 mM (Table V). It is very likely that the native enzyme is associated with a metal ion. The EDTA chelates and removes the metal and the enzyme thus loses its activity in the absence of an external source of metal ions. The question of a metal as a possible constituent of *H. diminuta* malic enzyme (metalloenzyme) is interesting since no malic enzymes have been reported to be a metalloenzyme as yet. However, the following prerequisites must be fulfilled by further investigation for the identification of a metalloenzyme as suggested by Vallee (1955) and Gracy and Noltmann (1968):
1) the enzyme must be established to be a homogeneous protein;

2) the pure enzyme must be isolated with its full metal complement and with full activity;

3) the ratio of gram atoms of the metal ion to moles of protein should be a small, integral number;

4) metal analysis performed on most fractions of the isolation procedure must show an increase in the "intrinsic metal" to protein ratio with a concomitant decrease in the "extrinsic metal" to protein ratio;

5) other metals must be shown to be present only in stoichiometrically insignificant concentration;

6) the metal content of enzyme preparations purified to different extents should be correlated with their specific activities;

7) enzyme activity is inhibited by metal binding agents;

8) the inhibition can be prevented or reversed by the addition of metals

The purified malic enzyme exhibited oxalacetate de-carboxylase activity. The activity under the assay condition was proportional to the amount of enzyme added (Table IV), although the activity was low. Hsu and Lardy (1967a) reported that the purified enzyme from pigeon liver catalyzes
the OAA decarboxylation reaction at pH 4.5 at a rate comparable to the decarboxylation of malate at neutral pH. The very low OAA decarboxylase activity catalyzed by *H. diminuta* malic enzyme is probably due to the pH (pH 7.5). This experiment was carried out for a qualitative purpose only. The positive result confirms the classification of the *H. diminuta* malic enzyme (L-malate:TPN oxidoreductase (decarboxylating), EC 1.1.1.40). The malic enzyme from *Ascaris suum* does not decarboxylate OAA (Saz and Hubbard, 1957) thus a different EC number is used to distinguish it from the enzyme with OAA decarboxylase activity (See footnote on page 6).

Hsu et al. (1967) proposed a mechanism for the malic enzyme, which was revised by Hsu (1970). Based on this postulation, there is an isomerization in the central enzyme-substrate complex:

\[
E-Mn^{++} + TPN + L-Malate \xrightarrow{\text{E-Mn}^{++}} M^{n+} + TPNH + H^+ + OAA \xrightarrow{\text{E-Mn}^{++}} M^{n+} + CO_2 + Pyruvate + TPNH
\]
Since the OAA decarboxylase activity requires TPN, two conformational states of the enzyme are also postulated (Hsu et al., 1967). Binding of TPN to the enzyme induces a conformational change which then favors the binding of OAA.

Due to the inhibition by PCMB and NEM, and the reactivation of the aged enzyme by DTT, it is very likely that the SH-group (s) on the protein are somehow involved in catalysis. Inhibition by PCMB of malic enzyme from both rat and pigeon liver has also been reported (Saito et al., 1971). Reversible inactivation upon storage and reactivation with DTT have been observed on purified pigeon liver enzyme (Hsu and Lardy, 1967a). The manner in which the SH-groups contribute to the enzyme activity is not clear. The partial reactivation of malic enzyme during storage as postulated by Hsu and Lardy, (1967a) is due to the oxidation of sulfhydryl groups on the protein. Dithiothreitol reduces these groups and thus restores the enzyme activity.

In regard to the kinetic studies, the Michaelis constants (Km) of TPN, malate, TPNH and pyruvate at pH 7.5 from H. diminuta are higher than those from pigeon liver at pH 7.0 (Hsu et al., 1967). However, these values from the tapeworm are comparable to or lower than those values observed at the
the same pH of either pigeon liver (Rutter and Lardy, 1958; Stickland, 1959; and Neubert and Coper, 1965), rat liver (Saito et al., 1971), or bovine adrenal cortex (Simpson and Estabrook, 1969). As pointed out by Rutter and Lardy (1958) and Hsu et al. (1967), the Michaelis constants of malic enzyme for the different substrates may increase with an increase in pH.

For *H. diminuta* malic enzyme, the Michaelis constants of TPNH and pyruvate are about 5 and 60 times higher respectively than the constants of TPN and malate. Therefore, it is probable that the tapeworm enzyme catalyzes the reaction primarily in the direction of malate decarboxylation. Due to the subcellular localization (Prescott and Campbell, 1965), KM's, and the low capacity for lipogenesis in the tapeworm, it can be postulated that the physiological significance of malic enzyme is found in energy metabolism. The TPNH formed by malic enzyme may or may not be expected to participate directly in the reduction of fumarate. Scheibel et al. (1968) have demonstrated a mitochondrial $^{32}$P$_{i}$-ATP exchange in *H. diminuta* via an electron transport system (flavoproteins) with the fumarate acting as the ultimate electron acceptor. In *Ascaris suum*, the reducing equivalent of DPNH is supplied by the DPN-dependent malic enzyme, but the source
of the reducing equivalent in the tapeworm mitochondria is unclear. If a transhydrogenase was present which oxidizes TPNH at the same time reduces DPN, then TPNH might reduce fumarate in *H. diminuta*. However, mechanisms to generate DPNH from DPN by the oxidation of TPNH in the tapeworm mitochondria have not been reported. About 10% activity has been observed of the tapeworm malic enzyme when DPN was used to substitute for TPN (unreported data). Thus, the function of malic enzyme in the *H. diminuta* mitochondria is obscure and further investigation is required.

Under optimal substrate concentrations at pH 7.5 and 25°, the rate of the reverse reaction was 36% the rate of the forward reaction. This result is consistent with Prescott and Campbell's report in 1965. They observed that the rate of decarboxylation of malate by the tapeworm malic enzyme was three times the rate of synthesis of malate from pyruvate and CO2. Carbon dioxide is a requirement for growth and development of the worm, and CO2 fixation is a necessary pre-requisite for the formation of the terminal electron acceptor, fumarate (Scheibel and Saz, 1966; and Scheibel et al., 1968). Studies on CO2 fixation strongly suggested that phosphoenolpyruvate carboxykinase is the most active CO2 fixing
enzyme, while malic enzyme did not seem to contribute a significant part in this respect (Prescott and Campbell, 1965). Whether the possible reverse reaction of malic enzyme may serve any purpose again requires further study.
REFERENCES


