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STUDIES ON ENZYMES FROM PARASITIC HELMINTHS

I. PURIFICATION AND PHYSICAL PROPERTIES OF MALIC ENZYME FROM THE MUSCLE TISSUE OF *ASCARIS SUUM*

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SUMMARY

1. A procedure has been developed for the isolation of the mitochondrial malic enzyme (L-malate: NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.39) from muscle tissue of the roundworm *Ascaris suum*.

2. The enzyme is adjudged to be homogeneous by the criteria of analytical ultracentrifugation, zone electrophoresis, isoelectric focusing and rechromatography.

3. Concomitant with the homogeneity studies the following physical parameters were established: $s^0_{20,w} = 8.9 \cdot 10^{-13}$ s; $D_{20,w} = 3.6 \cdot 10^{-7}$ cm$^2 \cdot$s^{-1}; apparent isoelectric pH = 6.63.

4. Sedimentation velocity, sedimentation equilibrium ultracentrifugation, as well as analytical gel filtration, yield a molecular weight of 250 000 for the native enzyme. The enzyme can be dissociated into four identical monomers of molecular weight 64 000.

5. The isolated enzyme utilizes NAD^+ more effectively than $NADP^+$ in the decarboxylation of malate. Oxaloacetate is not decarboxylated in the presence of either coenzyme.

6. The enzyme exhibits an absolute requirement for Mn^{2+} or Mg^{2+} and is inhibited by $(NH_4)_2SO_4$. The inhibition is partially reversed by Mn^{2+}.

INTRODUCTION

An oxidative decarboxylation of malate to pyruvate and carbon dioxide was observed by Moulder *et al.*[1], Lwoff and Cailleau[2], Lwoff *et al.*[3] and Ochoa *et al.*[4]. The enzyme which catalyzed this reaction was termed "malic enzyme"[4]. Rutter and Lardy[5] attempted to isolate this enzyme from the cytosol fraction of pigeon liver, and eventually Hsu and Lardy[6] obtained the enzyme in homogeneous form. Malic enzyme has been studied from a variety of sources including *Escherichia coli*[7–9], group D *Streptococcus*[10], lactic acid bacteria[10–12], rat liver[13,14] and brain[15], bovine adrenal cortex[16], heart[17] and lens[18], cauliflower[19], spinach and barley[20], mice erythrocytes[21],

insects[22], wheat germ[23], protozoa[24], and chicken liver[25]. It has also been studied in the parasitic helminths *Moniliformis dubius*[26], *Hymenolepis diminuta*[27,28], *Fasciola hepatica*[29] and *Ascaris suum*[30].

The primary differences in each of the malic enzymes investigated thus far lie in the specificity for the coenzymes NAD^+ or $NADP^+$ and whether or not the enzyme will decarboxylate oxaloacetate. Three different enzyme classifications of malic enzyme have thus been proposed. The crystalline pigeon liver malic enzyme (L-malate: $NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.40) utilizes $NADP^+$ and will also decarboxylate oxaloacetate, whereas the malic enzyme from the group D *Streptococcus*, cauliflower, and *Ascaris* mitochondria, (L-malate: NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.39) preferentially uses NAD^+ and will not decarboxylate oxaloacetate. The third type of malic enzyme (L-malate: NAD^+ oxidoreductase (decarboxylating), EC 1.1.1.38) from *Lactobacillus arabinosus* was shown by Korkes *et al.*[31] to utilize NAD^+ and to decarboxylate oxaloacetate. Subsequently, Nathan[50] demonstrated that the catalytic activities of this enzyme (EC 1.1.1.38) resided in two separate and distinct proteins.

Hsu[32] demonstrated that the pigeon liver malic enzyme would catalyze three additional reactions: the reduction of pyruvate and oxaloacetate and the decarboxylation of malate to lactate. However, these three reactions constitute less than 15% of the total activity of the enzyme.

The biochemical and physiological functions of the malic enzymes have been investigated by several research groups[16,32–34]. Malic enzyme (EC 1.1.1.40) appears to function as a generator of reducing power for the synthesis of lipids and/or the hydroxylation of steroids[32,16]. Malic enzyme (EC 1.1.1.39) has been implicated in the carbohydrate metabolism of the parasitic helminths as the source of reducing power for an anaerobic phosphorylation within the mitochondria[34]. Recently, Li *et al.*[28] isolated a malic enzyme from the tapeworm, *Hymenolepis diminuta*, and demonstrated that it required $NADP^+$ and could decarboxylate oxaloacetate. On the other hand, Saz and Hubbard[30] found that partially purified malic enzyme from the roundworm, *Ascaris suum*, utilized NAD^+ preferentially and could not decarboxylate oxaloacetate. These two parasites reside in the same environment, the essentially anaerobic small intestine, and have been shown to possess similar carbohydrate metabolic patterns[27,35] It was thus of interest to purify the malic enzyme from the roundworm, *Ascaris suum*, and to study its chemical, physical and catalytic properties.

MATERIALS AND METHODS

Materials

Ion exchangers; diethylaminoethyl cellulose (DEAE, Cellex-D, 0.74 mequiv·g^{-1} Calbiochem) and Cellulose phosphate (0.91 mequiv·g^{-1}, Sigma Chemical Co.) were cleaned and equilibrated according to Peterson and Sober[36]. The sodium salts of NAD^+ NADH, $NADP^+$, NADPH, L-malate; and oxaloacetic acid, pyruvic acid, 2-mercaptoethanol, $(NH_4)_2SO_4$ (enzyme grade), crystalline bovine serum albumin, triethanolamine (enzyme grade), imidazole, ethylenediaminetetraacetic acid, rabbit muscle lactic dehydrogenase (spec. act., 600 units·mg^{-1}), phenazine methosulfate, and MTT tetrazolium (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) were purchased from Sigma Chemical Company. Tris and polyethyleneglycol (20 000)

were from Fisher Scientific Company; while Sephadex G-200, aldolase, chymotrypsin, ovalbumin, and ribonuclease were obtained from Pharmacia Fine Chemicals. Guanidinium chloride and sodium dodecyl sulfate were both "Sequanal grade" and were obtained from Pierce Chemical Company. All other chemicals were reagent grade and all solutions were prepared in double distilled, deionized water.

Dialysis tubing was boiled in $NaHCO_3$ for 45 min, rinsed in distilled water and stored in 95% ethanol. The tubing was rinsed free of ethanol in several changes of double distilled, deionized water before each use.

Protein determinations

Protein concentrations were established by the method of Lowry *et al.*[37] using crystalline bovine serum albumin as the standard. The protein concentration in the effluent from column chromatography was monitored from its absorption at 280 nm. All fractions in which the specific activity was calculated were based on protein concentrations determined by the Lowry method.

Enzyme assays

L-Malic enzyme activity was followed in the forward direction (the decarboxylation of L-malate) according to a modification of the method of Hsu and Lardy[6]. The assay mixture contained, in a final volume of 3.0 ml, 30 μmoles of 2-mercaptoethanol; 30 μmoles of L-malate; 12.0 μmoles $MnCl_2$; and 0.75 μmole of NAD^+. The assay was initiated by the addition of the enzyme and the reduction of NAD^+ was followed at 340 nm in a Beckman Kintrac VII spectrophotometer with a thermostated sample chamber at 25 °C. Each sample was stirred magnetically during the assay. One unit of enzyme activity is defined as the amount of enzyme catalyzing the reaction of one micromole of substrate per min under initial velocity conditions at 25 °C. The specific activity is expressed as units of enzyme per mg of protein. Oxaloacetate decarboxylase activity was monitored according to the method of Hsu and Lardy[6].

Electrophoresis

Cellulose acetate electrophoresis was performed on 2.5 cm × 18 cm Sepraphore III strips in a variety of buffers over a pH range of 6.0–8.5. The ionic strength of the buffers was maintained at 0.05 by the addition of appropriate amounts of NaCl. Protein was visualized by staining the strips with 0.5% (w/v) Ponceau S in 7.5% (w/v) trichloroacetic acid; after staining, the strips were washed in 5.0% (v/v) acetic acid.

Polyacrylamide gel electrophoresis was carried out according to Davis[38] utilizing the Canalco model 200 apparatus. Gels were prepared with a 7.5% monomer concentration in 25 mM Tris–glycine buffer, pH 8.8, and stained with 0.25% (w/v) Coomassie Brilliant Blue[39].

Sodium dodecyl sulfate gel electrophoresis was performed according to the method of Weber and Osborn[39]. The mobilities of the standard proteins were plotted against the log of their subunit molecular weights.

Isoelectric focusing was conducted in a sucrose density gradient according to Rozacky *et al.*[40] and the LKB manual with narrow range ampholines (LKB Productor), pH 5.0–8.0 in a LKB model 8100 apparatus. The enzyme solution was elec-

trofocused for 60 h at 600 V, and the pH of the 1.0 ml fractions was measured immediately after elution at 5 °C.

Chromatography

DEAE-cellulose and cellulose phosphate were equilibrated[36] and packed into columns at 0 to 5 °C with a back pressure of approx. 10 lb/inch^2 maintained by a peristaltic pump. When a concentration gradient of KCl was employed to elute protein from the ion-exchangers the Cl^- concentration of the eluant was monitored according to the method of Schales and Schales[41].

Sephadex G-200 was packed into a 1.5 cm × 110 cm column with a hydrostatic head pressure of 10 cm. The column was calibrated with blue dextran, 3-*O*-methyl-[^{14}C]glucose, and proteins of known physical parameters. The elution volumes were correlated with the molecular weights[42] or with the Stokes' radii[43].

Ultracentrifugation

Ultracentrifugation experiments were conducted in a Beckman-Spinco Model E analytical ultracentrifuge equipped with RTIC temperature control and electronic speed control. The schlieren patterns from the sedimentation velocity experiments were utilized to estimate values for the diffusion coefficient by analyzing the boundary spreading according to Schachman[44]. Sedimentation equilibrium ultracentrifugation experiments were conducted by the meniscus depletion method[45]. Densities and viscosities of all buffers were determined as described previously[40]. The details of the experiments are given in Results or the legends to the figures.

RESULTS

Isolation of Ascaris muscle malic enzyme

All purification steps were performed at 0 to 5 °C unless otherwise specified. A typical purification procedure is given below.

Fractions I and II: Supernatant fraction. Adult female *Ascaris suum* were obtained from a local abattoir and transported to the laboratory[46] where the muscle was dissected free of other tissues. After rinsing the muscle tissue (350 g) in cold distilled water it was homogenized with a Waring blender with 3 vol. of 10 mM Tris buffer, pH 7.0, 7.0 mM glycerol, 10 mM 2-mercaptoethanol, 1.0 mM ethylenediaminetetraacetic acid, and sufficient NaCl to adjust the final ionic strength to 0.20. The homogenates were centrifuged at 12 000 × *g* for 30 min in a refrigerated centrifuge and the supernatant solutions were collected. The pellets were resuspended in 2 vol. of homogenizing buffer, rehomogenized and centrifuged as before for 30 min (Fraction I, 1200 ml). The supernatant solutions were then spun at 105 000 × *g* for 1 h, pooled (Fraction II, 1050 ml) and dialyzed for 24 h against two changes, 20 l each, of 10 mM imidazole buffer, pH 6.5, 10 mM 2-mercaptoethanol, 1.0 mM EDTA, and 7.0 mM glycerol.

Fraction III: First cellulose phosphate chromatography with a linear salt gradient elution. Fraction II was applied to a cellulose phosphate column, 2.5 cm × 95 cm, previously equilibrated with 10 mM imidazole buffer, pH 6.5, 10 mM 2-mercaptoethanol, 1.0 mM EDTA, and 7.0 mM glycerol. The flow rate was maintained at 0.5 ml per min, and the column was washed with the same buffer until the effluent was

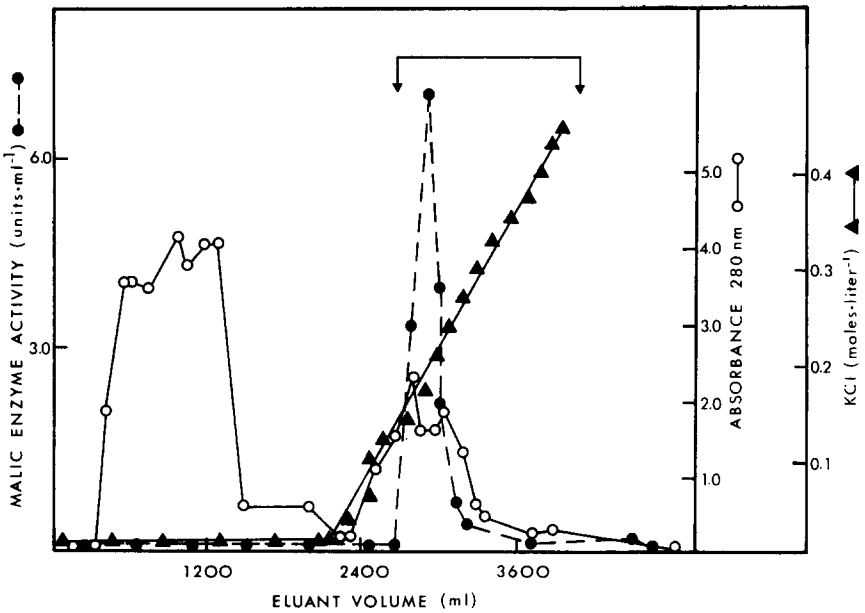

Fig. 1. Column chromatography of malic enzyme on cellulose phosphate. Fraction II (1050 ml, 5.25 mg protein per ml) was applied to a cellulose phosphate column (2.5 cm × 95 cm). The same buffer was pumped through the column at a flow rate of 0.5 ml per min, and 20 ml fractions were collected. After the 1st large peak of protein (○——○) had washed through the column, remaining protein was eluted with a linear salt gradient as described in Results. Fractions containing malic enzyme activity (●——●) were collected and pooled (bar with arrows). The Cl^- concentration (▲——▲) of fractions was determined as described in Methods.

protein free; thereafter, a linear salt gradient was employed to elute the protein. The mixing chamber contained 1.5 l of the same buffer while the reservoir chamber contained an identical quantity of the same buffer with the addition of 0.5 M KCl. Fractions (20 ml) were collected and assayed for malic enzyme activity (Fig. 1). Those fractions containing activity were pooled (1600 ml), concentrated to a volume of 410 ml with polyethyleneglycol, and dialyzed overnight against 20 l of 10 mM Tris buffer, pH 7.5, 10 mM 2-mercaptoethanol, 1.0 mM EDTA and 7.0 mM glycerol (Fraction III).

Fraction IV: DEAE-cellulose chromatography with a linear salt gradient elution. Fraction III was applied to a DEAE-cellulose column, 2.5 cm × 40 cm, previously equilibrated against the above buffer. Buffer was pumped through the column until the eluant was protein free, and a linear salt gradient was applied (Fig. 2). The mixing chamber contained 500 ml of the Fraction III buffer, and the reservoir (500 ml) contained in addition 0.5 M KCl. The fractions (20 ml) were collected at a flow rate of 1.0 ml per min and those containing enzyme activity (380 ml) were concentrated to a final volume of 180 ml with polyethyleneglycol. Subsequently, Fraction IV was dialyzed overnight with 20 vol. of the buffer of Fraction II.

Fraction V: Second cellulose phosphate chromatography with a linear salt gradient elution. Fraction IV was applied to a cellulose phosphate column, 1.5 cm × 25 cm, equilibrated with the above buffer. The flow rate was adjusted to 1.0 ml per min and buffer was pumped through the column until it was free of protein. A linear salt gra-

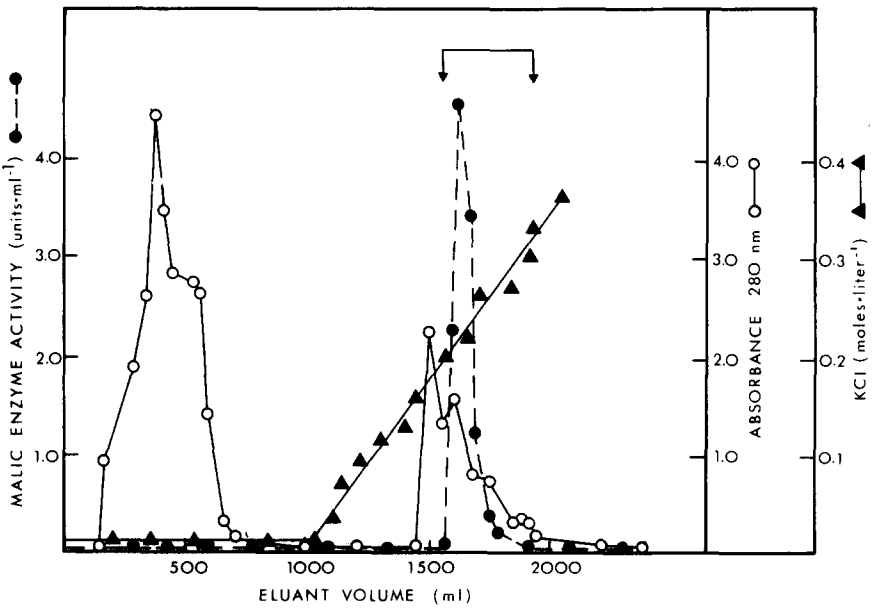

Fig. 2. Column chromatography of malic enzyme on DEAE-cellulose. Fraction III (410 ml, 4.87 mg protein per ml) was applied to a DEAE-cellulose column (2.5 cm × 40 cm). The same buffer was pumped through the column at 1.0 ml per min, and 20-ml fractions were collected. After the initial peak of protein (○——○) was eluted from the column, a linear salt gradient (KCl) (▲——▲) was used to elute the malic enzyme activity (●——●). The indicated fractions (bar with arrows) were pooled.

dient was applied which was composed of 500 ml of the above buffer in the mixing chamber, and 500 ml of this buffer containing 0.5 M KCl in the reservoir. Fractions (20 ml) which contained malic enzyme activity were collected and pooled (Fig. 3). At this stage the enzyme is adjudged to be homogeneous by the criteria presented below.

Table I shows the results of a typical fractionation of *Ascaris* muscle malic enzyme. The recovery (46%) is greater than those obtained with other purification procedures. The purified malic enzyme exhibits a specific activity of approx. 15 units per mg protein which is lower than that of the enzymes from pigeon liver (27 units per mg[6]), rat liver (27 units per mg[13]; 55 units per mg[14]) and *E. coli* (56 units per mg[7]). *Ascaris* muscle appears to be an excellent source of malic enzyme since it contains approx. 550 μg of the enzyme per g of muscle tissue.

Physical studies

Sedimentation velocity ultracentrifugation. Malic enzyme was subjected to sedimentation velocity ultracentrifugation at 60 000 rev./min at four protein concentrations, and the Schlieren patterns were photographed. A single boundary was observed in all cases throughout the runs. The $s^0_{20,w}$ calculated from the Schlieren patterns was $8.9 \cdot 10^{-13}$ s. Boundary spreading analysis was performed[44] and the results are shown in Fig. 4. An apparent diffusion coefficient of $3.58 \cdot 10^{-7}$ cm$^2 \cdot$s^{-1} was obtained, and the linear horizontal plot is indicative of a monodisperse system. When both of

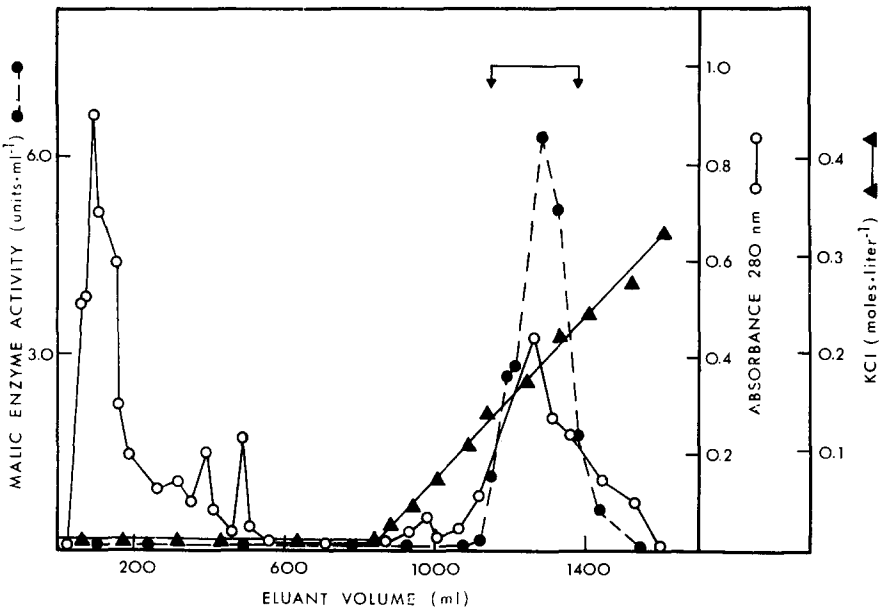

Fig. 3. Column chromatography of malic enzyme on cellulose phosphate. Fraction IV (180 ml, 1.7 mg protein per ml) was applied to a cellulose phosphate column (1.5 cm × 25 cm) previously equilibrated against the same buffer. Buffer was pumped through the column at a flow rate of 60 ml/h, and fractions of 10 ml were collected. After the protein (○——○) had washed through the column, the remaining malic enzyme activity (●——●) was eluted with the use of a linear KCl gradient (▲——▲). Those fractions containing malic enzyme activity were collected and pooled (bar with arrows).

these values are substituted into the Svedberg equation with a partial specific volume assumed to be 0.74 ml·g^{-1} (ref. 6), an apparent molecular weight of 235 000 is obtained (Table II).

Sedimentation equilibrium ultracentrifugation. The linearity of the plot of ln C *versus* x^2 obtained from high-speed equilibrium centrifugation (Fig. 5) is also good evidence for a single protein species. The calculations were made by determining the vertical deflections of a single fringe along the entire length of the cell, and yielded a weight-average molecular weight for the native enzyme of 248 000.

Treatment of the native enzyme with 6.0 M guanidinium chloride resulted in dissociation of the enzyme into its subunits. High speed equilibrium ultracentrifugation yielded a subunit weight-average molecular weight of 66 400. "Aged" enzyme (stored for two months in 0.20 ionic strength buffer at 5 °C) was subjected to sedimentation equilibrium ultracentrifugation and yielded a weight-average molecular weight of 61 000. In both experiments linearity of the plots of ln C *versus* x^2 suggests identical or near identical subunits (Table II).

Sodium dodecyl sulfate gel electrophoresis. The protein standards and malic enzyme sample were dissolved in 1.0 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 1% (w/v) sodium dodecyl sulfate, and 1% (v/v) 2-mercaptoethanol and incubated at 35 °C for 5 h[47]. Electrophoresis was carried out as indicated in Methods and the results (Fig. 6) yield a value of 65 000 for the subunit molecular weight. The subunit molecular weight obtained by this method is in good agreement with the

TABLE I

FRACTIONATION OF L-MALIC ENZYME FROM *Ascaris suum*

Fraction	*Volume (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units·mg⁻¹)*	*Purification*		*Recovery*	
					Over preceding step	*Overall*	*Over preceding step (%)*	*Overall (%)*
I Crude homogenate	1200	15000	2621	0.16	—	—	100	100
II Supernatant 105 000 × *g*	1050	5512	2578	0.46	2.8	2.8	98	98
III Eluant from 1st phosphocellulose column	410	2000	2164	1.1	2.5	6.7	84	83
IV Eluant from 1st DEAE-cellulose column	180	304	1670	5.4	5.0	33.6	77	64
V Eluant from 2nd phosphocellulose column	450	90	1212	13.5	2.5	84.0	73	46

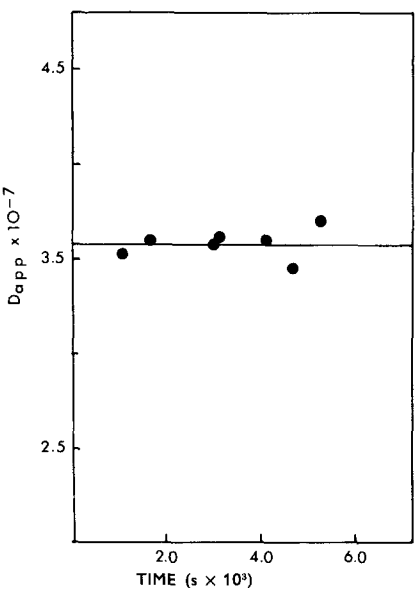

Fig. 4. Analysis of boundary spreading during sedimentation velocity centrifugation of *Ascaris* malic enzyme. The apparent diffusion coefficient (D_{app}) was evaluated as a function of time, as described under Methods, from Schlieren photography. Purified malic enzyme (spec. act. 14.2 units·mg^{-1}, 4.0 mg·ml^{-1}) was dialyzed against 50 mM Tris (Cl^-), pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol and 0.1 M NaCl. The centrifugation was carried out at 20 °C in a 12-mm double sector cell at 60 000 rev./min.

TABLE II

PHYSICAL PROPERTIES OF *Ascaris* MALIC ENZYME

Physical parameter	*Method*	*Value*
Sedimentation coefficient ($s^\circ_{20,w}$)	Sedimentation velocity ultracentrifugation	$8.9 \cdot 10^{-13}$ s
Diffusion coefficient ($D_{20,w}$)	Boundary spreading–velocity sedimentation[44]	$3.58 \cdot 10^{-7}$ cm$^2 \cdot$s^{-1}
	Gel filtration[43]	$3.54 \cdot 10^{-7}$ cm$^2 \cdot$s^{-1}
Molecular weight ($M_{s,D}$)	$s^\circ_{20,w}$; $D_{20,w}$ from boundary spreading	235 000
	$s^\circ_{20,w}$; $D_{20,w}$ from gel filtration	236 000
Molecular weight (M_{eq})	Equilibrium sedimentation[45]	248 000
Molecular weight (M_{gel})	Gel filtration[42]	262 000
Stokes' radius	Gel filtration[43]	60.0 Å
Apparent isoelectric point (pI_{app})	Isoelectric focusing electrophoresis	6.63
Subunit molecular weight (M_{eq})	Equilibrium sedimentation in 6.0 M guanidine	66 400
	Equilibrium sedimentation "aged enzyme"	61 000
Subunit molecular weight (M_{gel})	Sodium dodecyl sulfate disc gel electrophoresis[39]	65 000

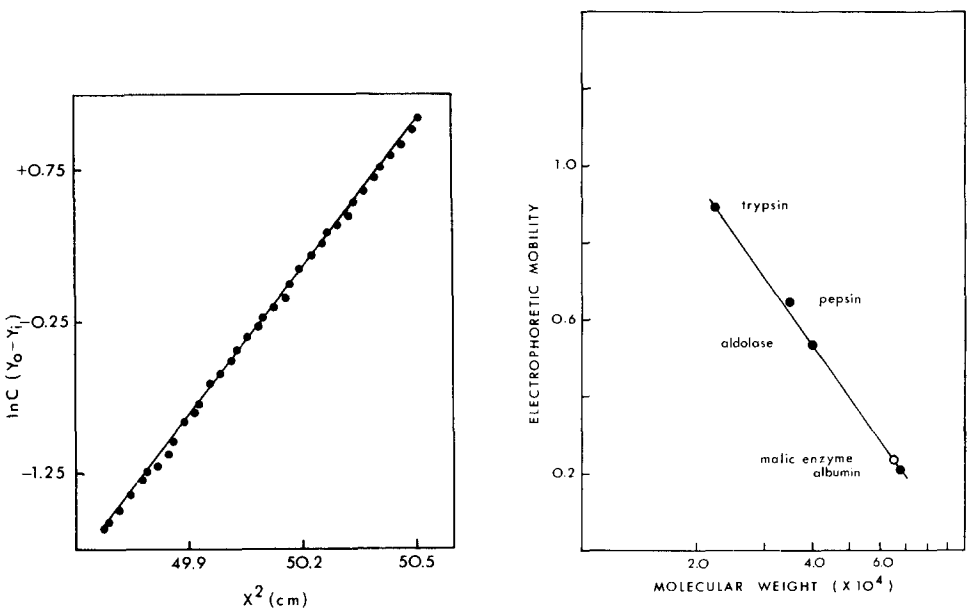

Fig. 5. Equilibrium sedimentation of *Ascaris* malic enzyme. Malic enzyme (spec. act. 15.1 units$\cdot$mg^{-1}) was subjected to meniscus depletion ultracentrifugation at 15 000 rev./min at 18.3 °C for 24 h in the An-D rotor in a 12-mm double sector cell. The sample sector contained 120 μl of enzyme (0.5 mg$\cdot$ml^{-1} in 0.1 M sodium phosphate buffer, pH 7.0). The other sector contained 120 μl of the same buffer.

Fig. 6. Sodium dodecyl sulfate gel electrophoresis of *Ascaris* malic enzyme. Fraction V malic enzyme (0.75 mg, spec. act. 13.5 units$\cdot$mg^{-1}) and standard proteins were prepared as described in Methods and subjected to electrophoresis on separated gels (10% monomer concentration) (8 mA, 5 h). The mobility of each protein was plotted against the log of its molecular weight[38].

values of 66 400 and 61 000 obtained by sedimentation equilibrium ultracentrifugation of guanidine dissociated and aged enzyme, respectively. Also in agreement with the ultracentrifugation studies, a single protein species was observed with dissociated malic enzyme (Fig. 6).

Molecular sieve chromatography. The method of Andrews[42] was used to estimate the molecular weight of the malic enzyme, and the correlation of the elution volume of malic enzyme with the elution volumes of standard proteins gave a molecular weight of 262 000. When the data were analyzed according to the method of Ackers[43] a value of 60.0 Å was obtained for the Stokes' radius of native malic enzyme. Substitution of this value into the Stokes–Einstein equation yields a diffusion coefficient of $3.54 \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$ (Table II) which is in agreement with the value obtained from boundary spreading analysis.

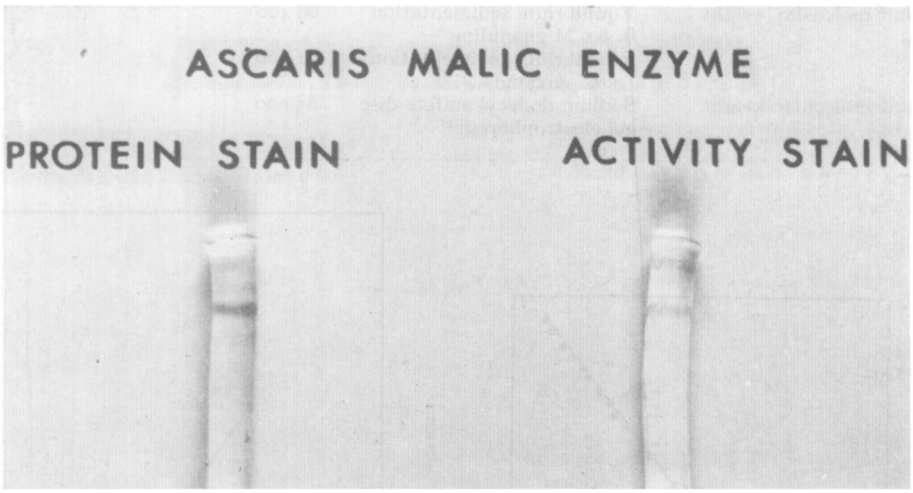

Fig. 7. Polyacrylamide gel electrophoresis of *Ascaris* malic enzyme. Malic enzyme (50 μg with spec. act. 13.5 units·mg^{-1}) was applied to a 7.5% standard 0.6 cm × 7 cm polyacrylamide gel. Electrophoresis was carried out at 5 °C in Tris–glycine buffer pH 8.8. The gel shown on the left was stained for protein as indicated in Methods while the gel on the right was developed in a stain developed for malic enzyme activity[47].

Electrophoresis. Cellulose acetate electrophoresis was performed at a variety of pH values. The results of these experiments yielded a single protein over a pH range of 6.0–8.5.

Polyacrylamide gel electrophoresis was performed according to the method of Davis[38] and the results are shown in Fig. 7. A single band of protein was obtained which showed identical migration as compared to that of malic enzyme when the gel was stained for catalytic activity[48].

Isoelectric focusing was performed as described in Methods, and the results (Fig. 8) indicate a single protein species with an apparent isoelectric point of 6.63.

Enzyme specificity. Both NAD^+ and $NADP^+$ at saturating concentrations were used to measure the decarboxylation of malate. The decarboxylation of malate was more efficient in the presence of NAD^+ than $NADP^+$ and the decarboxylation of

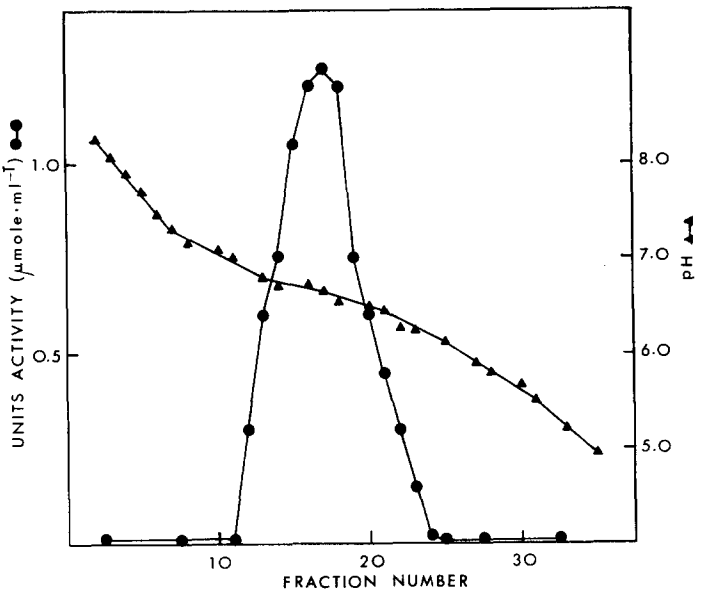

Fig. 8. Isoelectric focusing of *Ascaris* malic enzyme. Fraction V (1 mg, spec. act. 13.5 units·mg^{-1}) was focused in a sucrose density gradient in a 110-ml Ampholine column as indicated in Methods. Narrow range (pH 5–8) carrier ampholines at a concentration of 1% were used and the focusing was carried out at 600 V for 60 h at 3 °C. Fractions of 1.0 ml each were collected and assayed for malic enzyme activity (●——●) and pH (▲——▲) immediately.

TABLE III

SUBSTRATE SPECIFICITY OF *Ascaris* MALIC ENZYME

Substrate	*Coenzyme*	*Metal*	*Activity ($\Delta A_{340\ nm}$·min^{-1})*
L-Malate*	NAD^+	Mn^{2+}	0.049
	$NADP^+$	Mn^{2+}	0.019
	NAD^+	Mg^{2+}	0.025
	$NADP^+$	Mg^{2+}	0.010
Oxaloacetate**			*(μmoles pyruvate formed)*
−enzymes	NAD^+	Mn^{2+}	5.9
−enzymes	$NADP^+$	Mn^{2+}	5.8
+enzymes	NAD^+	Mn^{2+}	5.8
+enzymes	$NADP^+$	Mn^{2+}	5.8

* Malic enzyme (spec. act. 15.1 units·mg^{-1}) was assayed as described in Methods in the presence of 10 mM L-malate, 0.33 mM NAD^+ or $NADP^+$, and 10 mM $MgCl_2$ or $MnCl_2$.

** Malic enzyme (spec. act. 15.1 units·mg^{-1}) was assayed in 10 mM sodium acetate buffer (pH 4.5) in 30 mM oxaloacetate, 0.07 mM NAD^+ or $NADP^+$, 10 mM $MnCl_2$. After incubation for 15 min at 25 °C, aliquots were withdrawn and assayed for pyruvate by coupling to lactate dehydrogenase (spec. act. 600 units·mg^{-1}) and saturating quantities of NADH. Controls were run by incubating oxaloacetate under identical conditions but in the absence of malic enzyme to correct for nonenzymatic decarboxylation.

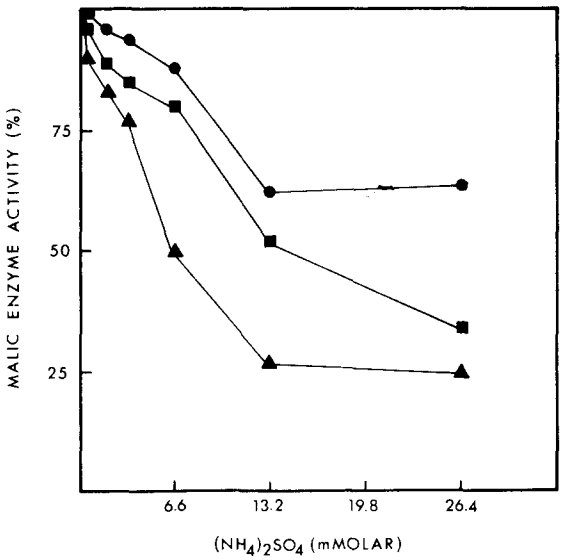

Fig. 9. Inhibition of *Ascaris* malic enzyme activity by $(NH_4)_2SO_4$. The percentage of activity remaining is plotted against the concentration of $(NH_4)_2SO_4$ in the reaction mixture. The $MnCl_2$ was varied in three experiments: ●——●, 10 mM; ■——■, 4.16 mM; and ▲——▲, 2.08 mM. The assay for malic enzyme activity is as described previously in Methods.

oxaloacetate was not detected in the presence of NAD^+ or $NADP^+$ (Table III). Therefore, the EC 1.1.1.39 classification is indeed correct.

Inhibition by $(NH_4)_2SO_4$. During the purification procedure attempts to fractionate or concentrate the malic enzyme solution with ammonium sulfate resulted in loss of malic enzyme activity. Fig. 9 shows that the inhibition by $(NH_4)_2SO_4$ is decreased by increasing the concentrations of $MnCl_2$.

DISCUSSION

Procedures have been described for the purification of malic enzyme from pigeon liver[6], *E. coli*[7], group D *Streptococcus*[10], and rat liver[13,14] and chicken liver[24]. In order to isolate the enzyme from *Ascaris suum* it was necessary to develop an alternate procedure since conditions (*e.g.* heat treatment, organic solvent and $(NH_4)_2SO_4$ fractionation[6]) utilized to isolate the enzyme from other sources resulted in denaturation of the roundworm enzyme. The present isolation procedure utilizing ion exchange chromatography also results in higher overall recovery of the enzyme (46%) than previous methods: 14%[7], 14%[13], 16.5%[14], 29%[10], 28%[24] and 42%[6]. Thus, by the procedure reported here it is possible to obtain relatively large quantities of homogeneous malic enzyme (90–100 mg) from a small amount of aschelminth muscle tissue (350 g). The concentration of roundworm malic enzyme (550–600 μg per g muscle) is larger than in pigeon liver (450 μg per g tissue)[6] or in rat liver (29 μg per g tissue)[13].

A variety of physical methods including analytical ultracentrifugation, gel filtration, and gel electrophoresis yield a molecular weight of approx. 250 000 for the native enzyme. These data are in agreement with the molecular weight of malic en-

zyme from rat liver (250 000)[13] and pigeon liver (250 000–280 000)[6,13], but differ markedly from the *E. coli* malic enzyme (550 000)[7]. It is noteworthy that Li *et al.*[28] recently reported the molecular weight of malic enzyme from the tapeworm, *Hymenolepis diminuta*, to exhibit a value approx. (120 000–130 000) one-half that of the ascarid malic enzyme. Their observations were based upon sedimentation equilibrium and gel filtration studies.

Ascaris malic enzyme appears to be composed of four identical subunits of 64 000 ± 2700. These data are in good agreement with the values obtained from *E. coli*[7] and pigeon liver[49]. All malic enzymes reported thus far seem to be composed of similar sized polypeptide chains, the only differences being the degree of aggregation (*e.g.* tetramer in pigeon liver[49], rat liver[13], octomer in *E. coli*[7], and possibly a dimer in *Hymenolepis diminuta*[28]).

The ascarid malic enzyme was shown to have an apparent isoelectric point of 6.63 by isoelectric focusing and the presence of only one activity was detected under a variety of electrophoretic conditions. Other research groups[15–17,20,21] have demonstrated that malic enzyme from several tissues including brain, heart, liver, adrenal cortex and erythrocytes may exist in isozymic forms. The three electrophoretic studies in this report yielded only one malic enzyme in *Ascaris* muscle.

London *et al.*[10] demonstrated that the group D *Streptococcus* malic enzyme activity was stimulated by ammonium salts at concentrations below 10^{-3} M, but inhibited at higher concentrations. Similarly, Sanwal and Smando[8] observed activation by NH_4^+ in the *E. coli* malic enzyme. In contrast, the ascarid malic enzyme exhibited no stimulation by NH_4^+ under any conditions. $(NH_4)_2SO_4$ inhibits the roundworm enzyme, and the extent of inhibition is dependent upon the concentration of the $MnCl_2$ present in the reaction mixture. London *et al.*[10] were able to establish a requirement for a metal ion only after extensive dialysis against $1.0 \cdot 10^{-3}$ M EDTA. Li *et al.*[28] have demonstrated that the tapeworm malic enzyme is active even in the absence of exogenous metal cofactor. In contrast to the above examples, dilution or dialysis of the roundworm malic enzyme is sufficient to demonstrate a metal requirement and addition of the metal to the assay mixture is an absolute requirement for activity. It would appear that the affinity of the various malic enzymes for the metal cofactors varies considerably. In the case of the aschelminth malic enzyme, it definitely should be considered as a metal requiring enzyme rather than as a metalloenzyme as appears to be the case of *Hymenolepis diminuta*[28].

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