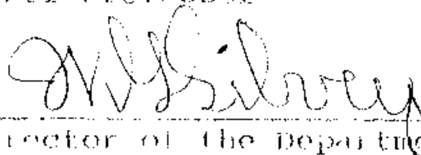
A STUDY OF MALATE DEHYDROGENASE ISOENZYMES IN THE

MIDGE LARVA GLYPTOTENDIPES BARBIPES

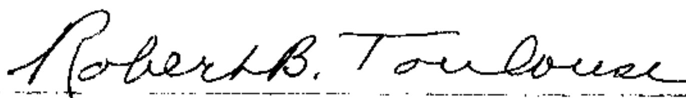
APPROVED:

Major Professor

Minor Professor



Director of the Department of Biology



Dean of the Graduate School

BGN.

Jones, Vicki E., A Study of Malate Dehydrogenase Isoenzymes in the Midge Larva *Glyptotendipes barbipes*. Master of Science (Biology), May, 1972, 56 pp., 3 tables, 16 illustrations, bibliography, 29 titles.

Two isoenzymes of malate dehydrogenase were isolated and partially purified from the midge larva *Glyptotendipes barbipes*. Differential centrifugation followed by cellulose acetate and polyacrylamide gel electrophoresis revealed one isoenzyme associated with the mitochondrial fraction and another form found only in the cytoplasm. Purification procedures that were utilized included ion exchange column chromatography on DEAE-cellulose and Sephadex G-100 column chromatography. The mitochondrial and supernatant forms were purified 16-fold and 56-fold, respectively.

Kinetic studies with the partially purified enzymes revealed significant differences between them. Michaelis constants for malate and oxalacetate were determined for both forms of MDH. The mitochondrial isozyme was found to have apparent Km values of 0.79 mM and 0.015 mM for malate and oxalacetate, respectively. The apparent Km values of the cytoplasmic form were 6.75 mM and 0.1 mM, respectively. The mitochondrial form was found to be inhibited by high concentrations of oxalacetate while the cytoplasmic form was not.

In addition, the two isozymes were found to migrate toward opposite poles during electrophoresis at pH 8.8; and they also exhibited different affinities for DEAE-cellulose at pH 7.5. Both of these observations indicated a difference in charge between the two enzymes.

One similarity in the two isozymes was noted. Neither form was found to be significantly inhibited by the sulfhydryl reagent, N-ethyl maleimide, after 2 hours incubation in a 20 mM solution. This suggests that sulfhydryl groups are not present in the active site of either isozyme.

Observations made on the larvae in the laboratory revealed no evidence that they are able to withstand anoxia for extended periods of time. Furthermore, electron micrographs of mitochondria isolated from midge larvae showed extensive, well-developed cristae which would indicate an aerobic existence.

A STUDY OF MALATE DEHYDROGENASE ISOENZYMES IN THE MIDGE LARVA GLYPTOTENDIPES BARBIPES

THESIS

Presented to the Graduate Council of the

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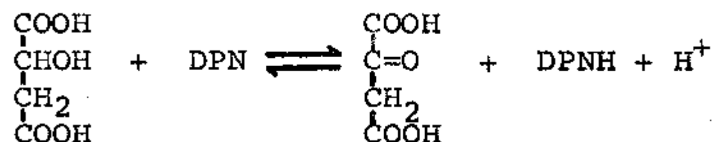
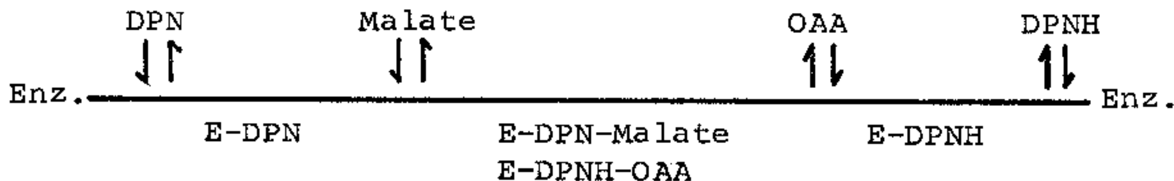
INTRODUCTION

Malate Dehydrogenase

Malate dehydrogenase (L-malate: NAD oxidoreductase, E C 1.1.1.37) catalyzes the reversible reaction:



In most higher plants and animals the enzyme is a dimer of molecular weight 60,000-70,000, although in some bacteria it is a tetramer with molecular weight of about 117,000 (Murphey, 1967). The enzyme has an obligatory binding order which may be illustrated as follows:



The coenzyme must first bond with the enzyme before malate can enter the active site. The transfer of electrons and hydrogen ions then takes place and oxalacetate is the first product to leave, followed by DPNH. For the reverse reaction,

the binding order is the opposite (Harada and Wolf, 1968).

Various isoenzymes of MDH have been found in eucaryotes. Among these the most familiar is the mitochondrial form which functions in the tricarboxylic acid cycle. A cytoplasmic form is also found which is important in the malate-asparate shuttle which transports electrons generated in the cytoplasm into the mitochondrion for oxidation (Christie and Judah, 1953; Thorne, 1960; Kitto and Kaplan, 1966; Zee and Zinkham, 1968). A third form has also been found associated with the glyoxysomes where it takes part in the glyoxylate cycle for the conversion of lipids to carbohydrates (Breidenbach and Beevers, 1967). Another form found in chloroplasts functions in photosynthesis (Rocha, 1968).

Most animals possess only the mitochondrial and cytoplasmic forms; however, within either or both of these subcellular fractions there is often found a variety of electrophoretically separable isozymes (Kitto and Kaplan, 1966; Meizel and Markert, 1968; Davidson and Cortner, 1967; Zee and Zinkham, 1968). There is some disagreement whether these multiple forms of the enzyme within an organelle are conformational isomers of the same protein (conformers), or whether they result from the random association of non-identical subunits. Kitto and Kaplan ascribe to the conformer hypothesis, citing as evidence

the experimental finding that reversible denaturation of the anodal forms produced a species with the same electrophoretic mobility as the cathodal forms. Shows (1970) confirmed this genetically by showing that a single mutation in the homozygous configuration altered the electrophoretic mobility of all bands in the same way. However, Mann and Vestling (1970) have performed fingerprint studies on rat liver mitochondrial isoenzymes, and found that at least three of the forms of MDH were produced from random association of two subunits which differed in composition by only a few peptides.

In most organisms which have been studied, the mitochondrial form of MDH migrates toward the cathode during electrophoresis at pH 8.6, while the cytoplasmic form migrates toward the anode. In the marine snail and some fishes, however, the pattern is reversed (Whitt, 1970).

The mitochondrial and cytosol forms are separate and distinct entities as determined by various physical and chemical tests. McReynolds and Kitto (1970) found that rabbit antisera directed against the mitochondrial form of _Drosophila_ MDH had no effect on the cytoplasmic enzyme from the same organism. Another difference between the two is end product inhibition of the mitochondrial form by DPNH (Sanwal, 1968) and substrate inhibition of the same form by oxalacetate (Kitto and

Kaplan, 1966).

Although the mitochondrion has its own DNA which is capable of coding for some protein, Shows in 1970 demonstrated genetically that both the cytoplasmic and the mitochondrial isozymes of MDH are encoded in nuclear DNA. He further showed that the mitochondrial form is not linked to any other nuclear genes coding for mitochondrial protein, although it is unresolved as to whether the genes coding for mitochondrial and cytoplasmic MDH are linked.

Glyptotendipes barbipes

The organism used in this study was the larval stage of *Glyptotendipes barbipes*, a representative of the family Chironomidae. Larvae of this species live in the benthic zone of bodies of water with a high degree of organic enrichment (Curry, 1962). They reside near the mud-water interface where they build tubes with their own salivary secretions. A net is spun across one end of the tube, and by undulating movements of the larva within the tube, water is drawn through the tube and the net filters out solid materials. The net and its contents are then consumed and another net is spun (Walshe, 1951).

This species, as well as other chironomids, possess a hemoglobin type pigment, erythrocruorin, which has an oxygen

dissociation curve similar to vertebrate myoglobin although the structure is different (Formanek and Amit, 1970). The pigment has a very high oxygen affinity. Fox (1945), using Chironomus plumosus, found that the hemoglobin was one half deoxygenated when the partial pressure of oxygen within the animal reached 0.6 mm. Hg. However, the average oxygen pressure in the water for the complete disappearance of oxy-hemoglobin in the blood was 13 mm. It appears, therefore, that there is a much lower oxygen pressure in the blood than in the water, so a steep gradient of oxygen pressure between water and blood is maintained. This may allow the hemoglobin to be functional when the pO_2 of the water is much higher than the oxygen dissociation curve would indicate.

Walshe (1951) studied the filter feeding of four species of chironomids. Two of the species contained abundant hemo-globin (red species), and two contained very little hemoglobin (pale species). She found that the red species treated with carbon monoxide at low oxygen pressure showed a reduced amount of filter feeding while the pale species maintained normal feeding. This suggested that the hemoglobin is significant in maintaining filter feeding motions under low oxygen con-centrations in the red species. However, the pale species could maintain filter feeding at low oxygen concentrations.

Under total anaerobiosis, the red species lived longer than the pale ones. In addition, cyanide affected the red species more than the pale forms, indicating that the pale species may have an alternative respiratory enzyme system which is not cyanide sensitive and is efficient at low oxygen concentrations.

It has been shown by Augenfeld (1967) that anoxic larvae use more glycogen than their counterparts in an oxygenated environment. Furthermore, the glycogen content is higher in species that are able to tolerate anoxia. However, the glycogen content declines rapidly during this anaerobic incubation.

Chironomid larvae possess an active glycolytic system, as well as succinoxidase and cytochrome oxidase activities. The activity of the latter two enzymes increases during the pupal and adult stages (Augenfeld and Neese, 1961).

Since the habitat of *Glyptotendipes barbipes* larvae is at a location where there would seem to be very little free oxygen, it was of interest to study the capabilities of this organism to live anaerobically. Phosphoenolpyruvate carboxykinase and malic enzyme are two catalysts which are necessary for an essentially anaerobic metabolism in animals (Saz and Lescure, 1969). The presence of these enzymes could not be demonstrated in the chironomid larvae. In addition, lactate

dehydrogenase activity was found to be very low. Malate dehydrogenase activity was present in significant quantities, and since this is an important enzyme in aerobic metabolism, it was decided to study it in greater detail.

MATERIALS AND METHODS

Cellex-D (DEAE cellulose) with an exchange capacity of 0.74 meq per gram was obtained from Bio-Rad Laboratories. Sephadex G-100 was purchased from Pharmacia, Folin-Phenol was obtained from Fisher, and all other chemicals were from Sigma.

Collection of Experimental Organisms

Glyptotendipes barbipes larvae were collected from the stagnation lagoon at Aubrey, Texas, where they were scraped from the floor of the pond along with the slime layer in which they were found to reside. They were brought into the laboratory and placed in aquaria overnight, which were cooled to 0-5°C, then allowed to warm to room temperature. As the water in the aquaria warmed, the larvae would emerge from their tubes in the debris at the bottom and crawl up the sides of the containers. They could then be readily harvested by scraping off the sides with glass plates, after which they were washed several times in distilled water.

Enzyme Assays

Enzyme assays were routinely carried out at 25°C in a

Beckman Model DB-GT Spectrophotometer with recorder. The reaction was run in the direction of malate oxidation, and activity was measured by recording the change in optical density at 340 nm. brought about by the reduction of DPN. Quartz cuvettes with 1 cm light path contained 12 µmoles sodium L-malate titrated to pH 10, 3 µmoles DPN, and enzyme brought to a total volume of 3.0 ml with 0.05 M Glycine buffer, pH 10.1. The reaction was initiated by the addition of enzyme. One unit of activity is defined as the amount of enzyme catalyzing the reduction of 1 µmole of DPN/min.

For the determination of Michaelis constants, L-malate was varied from 0.075 mM to 12 mM with other reagents the same as above, and sufficient enzyme to cause a change in optical density of less than 0.1/min. The Km values for oxalacetate were determined by running the reaction in the opposite direction and measuring the oxidation of DPNH at 340 nm. Freshly prepared OAA titrated to pH 7.8 was varied in concentration from 0.004 mM to 2.4 mM. Three tenths µmoles DPNH and enzyme sufficient to produce a change in optical density from 0.01 to 0.1 in 60 seconds were placed in the cuvette and brought to a total volume of 3.0 ml with 0.05 M Tris, pH 7.8. The recorder was set on an expanded scale to measure an optical density change between 0.01 and 0.1 for

the precise determination of Michaelis constants. Km values were determined with a computer program by the method of Wilkinson (1961), which is an application of a weighted non-linear regression method.

Protein Determination

Protein in the enzyme solutions was determined by a modification of the method of Lowry *et al*. (1951), using bovine serum albumin as the standard. Protein solutions were diluted to a concentration of 20-200 µg in a total volume of 2 ml. Then 2 ml of Solution C, which had been prepared 30 minutes previously, was added to each sample and allowed to stand for 30 minutes at room temperature. Next, 0.2 ml 1 N Folin-Phenol was added to each tube with immediate mixing. After 30 minutes, the optical density was read at 660 nm. Protein concentration of fractions eluted from the columns used in purification was estimated by reading optical density at 280 nm.

Ion Exchange Chromatography and Gel Filtration

DEAE Cellulose was prepared by the method of Peterson and Sober (1962), and equilibrated in 10 mM Tris buffer, pH 7.5. The column was packed under 3 lbs. pressure of nitrogen.

Sephadex G-100 was equilibrated in 10 mM Imidazole buffer pH 7, and columns were packed under 15 cm. hydrostatic pressure.

Electrophoresis

Polyacrylamide gel electrophoresis was performed by the method of Davis (1964) in the Canalco model 200 apparatus. Gels were stained specifically for MDH with an activity stain which contained 100 mg. sodium L-malate titrated to pH 7, 10 mg. DPN, 3 mg. MTT Tetrazolium, and 0.9 mg. phenazine methosulfate in a total volume of 5.3 ml of Tris buffer, pH 7.5.

Cellulose-acetate electrophoresis was performed with Gelman Sepraphore III strips (2.5 x 18 cm.) in a Gelman electrophoresis unit using Gelman high resolution buffer (Tris-barbital sodium-barbital), pH 8.8. Strips were stained with the same specific MDH stain used in gel electrophoresis.

Inhibitor Studies

Tests for inhibition by N-ethyl maleimide were carried out by incubating the enzyme in a 20 mM solution of NEM at room temperature for 2 hours at pH 7.2. Aliquots were removed at intervals for activity tests.

RESULTS

Homogenization and Electrophoresis

Five grams of Glyptotendipes barbipes larvae (fourth instar) were washed in distilled water, weighed, and placed in an ice bath. All subsequent operations were carried out at 0-5°C. They were homogenized in chilled 10 mM Tris buffer, pH 7.5, with 1 mM EDTA (w/v ratio of 1:4) in a Waring blender. The resulting homogenate was centrifuged at 750 g in the Sorvall RC2-B refrigerated centrifuge to remove cell debris. The supernatant was centrifuged at 105,000 g in a Beckman Model L3-40 preparative ultracentrifuge, after which the supernatant was filtered through glass wool. This preparation was designated as the crude extract and was used for preliminary electrophoresis. Cellulose acetate electrophoresis was performed with a constant current of 1.5 mA/strip for 45 min. Two bands of activity were evident as seen in Fig. 1.

Subcellular Localization

In order to determine the subcellular location of the two activities, five grams of larvae were homogenized in chilled 0.25 M sucrose with 1 mM EDTA (w/v ratio 1:4) in a Tenbroek

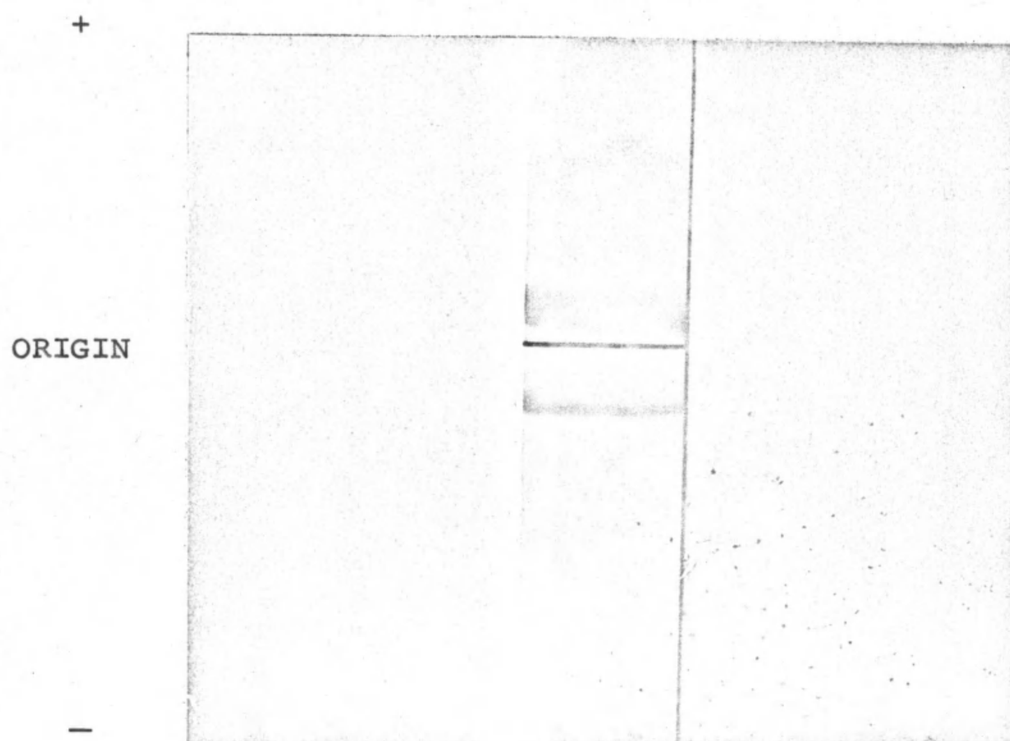


Fig. 1. Cellulose acetate electrophoresis of crude extract from Glyptotendipes barbipes larvae. The strip is stained specifically for MDH as described in "Methods".

hand homogenizer. The homogenate was centrifuged at 700 g to remove nuclei and cell debris; then the supernatant was centrifuged at 10,000 g to sediment mitochondria. The mitochondrial pellet was resuspended in the homogenizing medium and recentrifuged. This washing procedure was repeated two more times. A small portion of the pellet was prepared for electron microscopy (Fig. 2), and the remainder of the mitochondrial pellet was resuspended in about 5 ml of the sucrose solution. The mitochondrial suspension was sonicated for two minutes at 15-second intervals, cooling the mixture in an ice bath between treatments.

Polyacrylamide gel electrophoresis was performed on the mitochondrial fraction, the cytoplasmic fraction and the crude homogenate. Results are pictured in Fig. 3. From the results of this electrophoresis, it was concluded that the band migrating toward the cathode was of mitochondrial origin, while the band migrating toward the anode was cytoplasmic in nature. The weak second band from the cytoplasmic fraction was assumed to be mitochondrial contamination resulting from the rupture of mitochondria during the homogenization procedure.

Enzyme Purification

Seventy-eight grams of *Glyptotendipes barbipes* larvae was

Fig. 2. An electron micrograph of a representative mitochondrion isolated from the midge larvae (X 150,000).

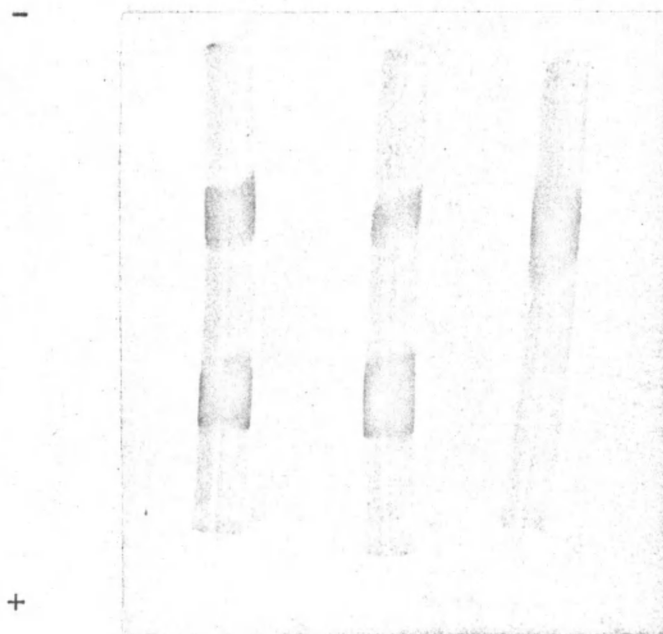


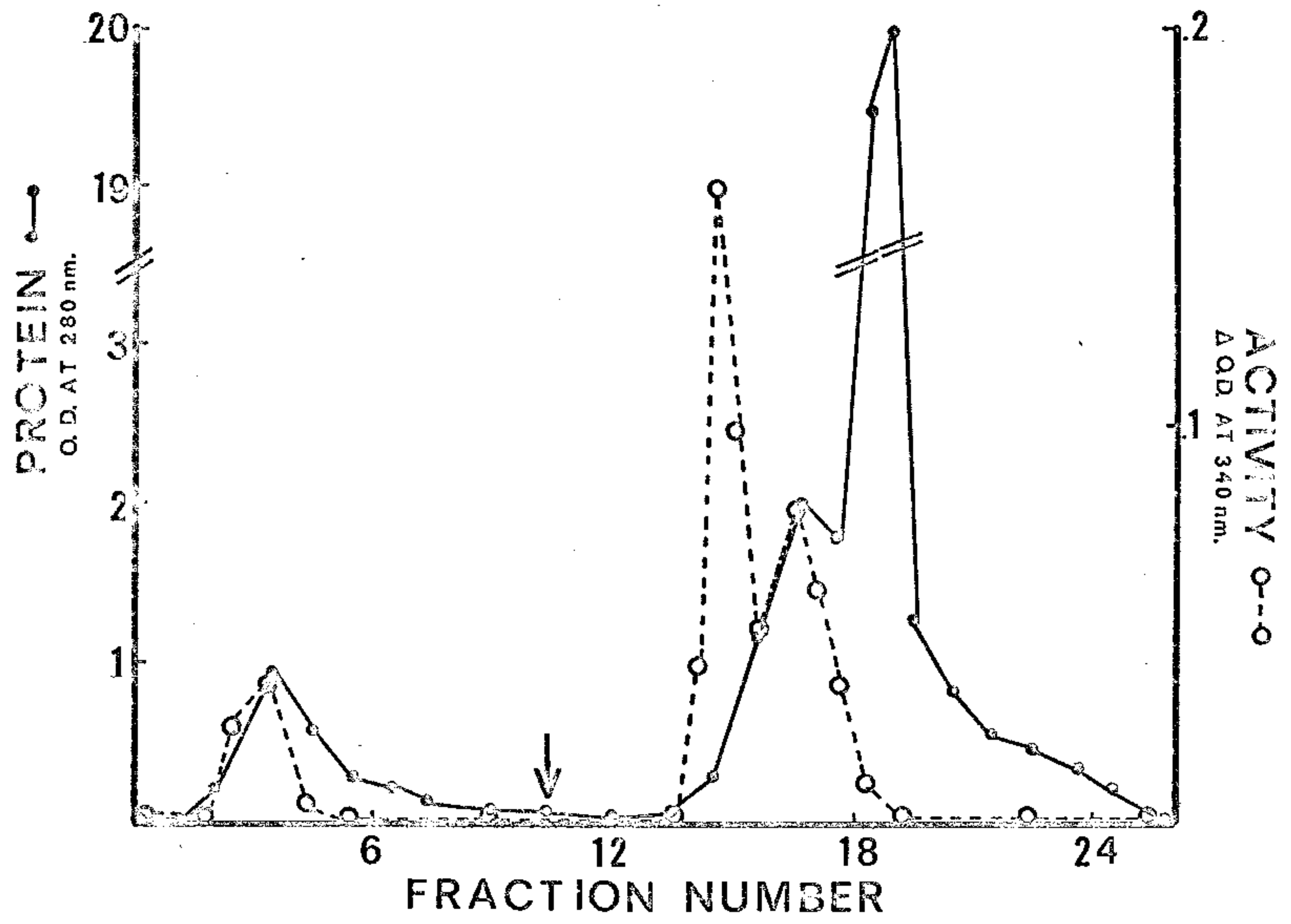
Fig. 3. Polyacrylamide gel disc electrophoresis of MDH. At left is a sample of crude extract, in the center is the supernatant fraction, and at right is a sample of the sonicated mitochondrial fraction.

homogenized in 240 ml of 10 mM Tris buffer, pH 7.5, with 1 mM EDTA in a Waring blender. The homogenate was centrifuged at 700 g for 15 min., after which the pellet was resuspended and rehomogenized in a minimal amount of Tris. The suspension was recentrifuged and the supernatant solutions were combined and centrifuged at 10,000 g for 15 minutes. This supernatant was then centrifuged at 105,000 g for 1 hr. The supernatant, henceforth referred to as the S-105 fraction, was dialyzed overnight against Tris buffer, pH 7.5, containing 1 mM EDTA.

A 2.5 X 45 cm column was packed with washed DEAE cellulose which had been equilibrated in Tris buffer, pH 7.5, containing 1 mM EDTA. Eighty ml of the S-105 fraction was applied to the column and eluted with the above buffer. When no more protein was detectable in the eluant, a linear gradient of 0-1 M KCl was begun. Fractions were collected in 10 ml volumes. The elution pattern of this column is shown in Fig. 4.

The samples containing activity under each peak were pooled and concentrated by dialyzing 3 hr. against saturated ammonium sulfate. They were then dialyzed against 10 mM imidazole buffer, pH 7, containing 1 mM EDTA. A sample from each activity peak was electrophoresed on cellulose acetate strips. Results (Fig. 5 and 6) showed that the activity peak which was eluted with buffer corresponded to the mitochondrial

Fig. 4. Elution pattern of the crude extract from DEAE-cellulose column. Arrow indicates the point where a linear gradient of KCl was begun. The solid line shows protein elution pattern as measured by optical density at 280 nm. Dotted line illustrates MDH activity. See "Methods" for details.


PROTEIN
O.D. AT 280 nm.
20
19
3
2
1
ACTIVITY
Δ O.D. AT 340 nm.
.2
.1
6
12
18
24
FRACTION NUMBER

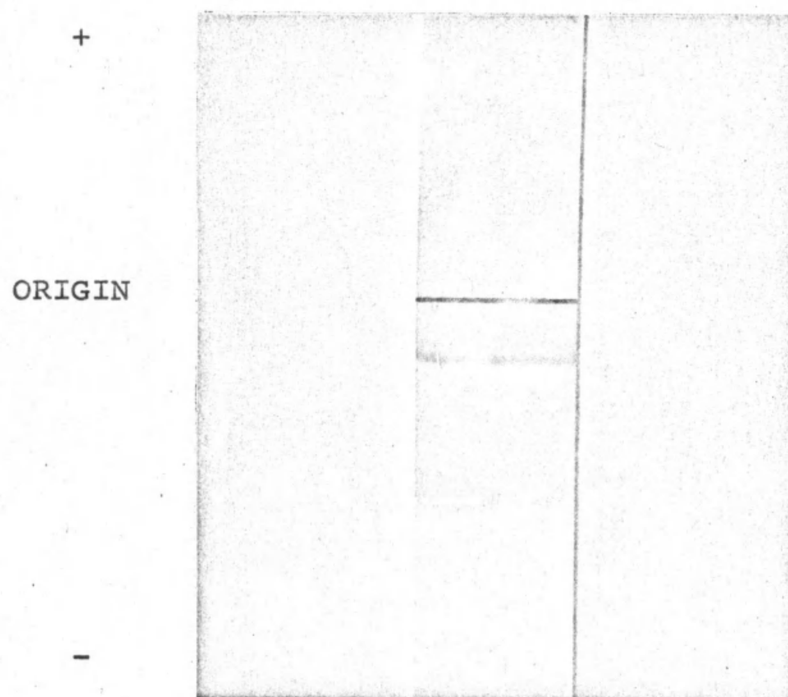


Fig. 5. Cellulose acetate electrophoresis of the MDH activity eluted with buffer from the DEAE cellulose column.

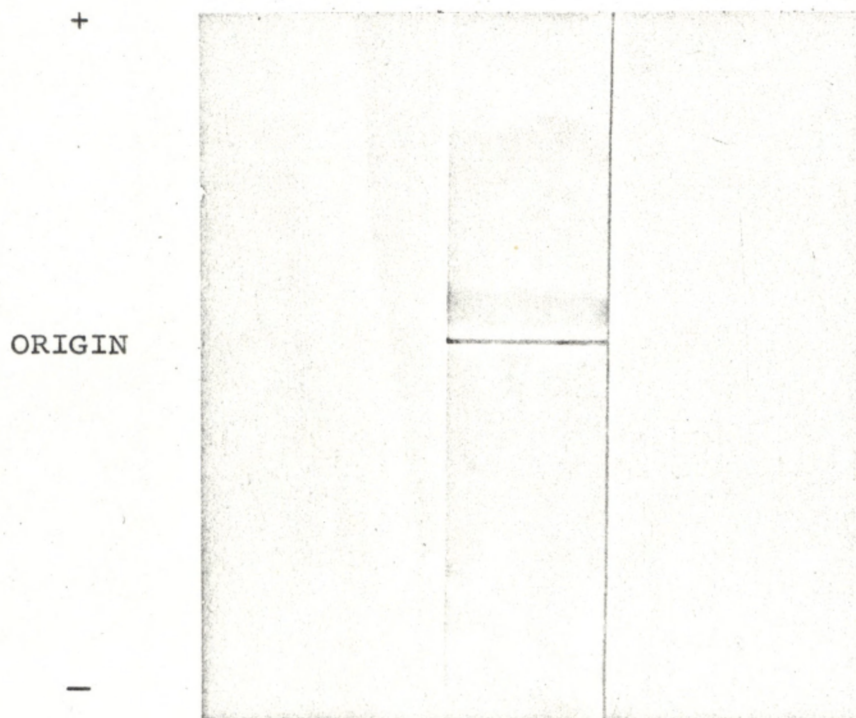


Fig. 6. Cellulose acetate electrophoresis of the MDH activity eluted with salt from the DEAE-cellulose column.

enzyme, while the peak which eluted with the salt gradient migrated identically with the cytoplasmic enzyme. Furthermore, no contamination from the other isozyme was noticeable in either fraction.

Two 1.5 X 30 cm columns were packed with Sephadex G-100. One column was loaded with 10 ml of the eluted mitochondrial activity, and 10 ml of the eluted cytoplasmic activity was applied to the other column. Fractions were collected in 5 ml volumes. Elution patterns from these two columns are shown in Figures 7 and 8. From each column, the fractions containing the highest specific activity were pooled for kinetic studies. Table I shows the degree of purification attained for each isozyme in the purification procedure.

Kinetic Studies

The Michaelis constants for both isozymes were determined by standard methods and the results are depicted in Figures 9-16. The cytoplasmic enzyme is not affected by high concentrations of oxalacetate (Fig. 15), whereas the mitochondrial enzyme is inhibited by this substrate (Fig. 11). The Km values can be seen in Table II.

Inhibition Studies

Anderton (1970) demonstrated that the sulfhydryl reagent

Fig. 7. Elution pattern of mitochondrial MDH isozyme from Sephadex G-100. Solid line illustrates protein elution pattern, dotted line indicates MDH activity. Details are described in "Methods".

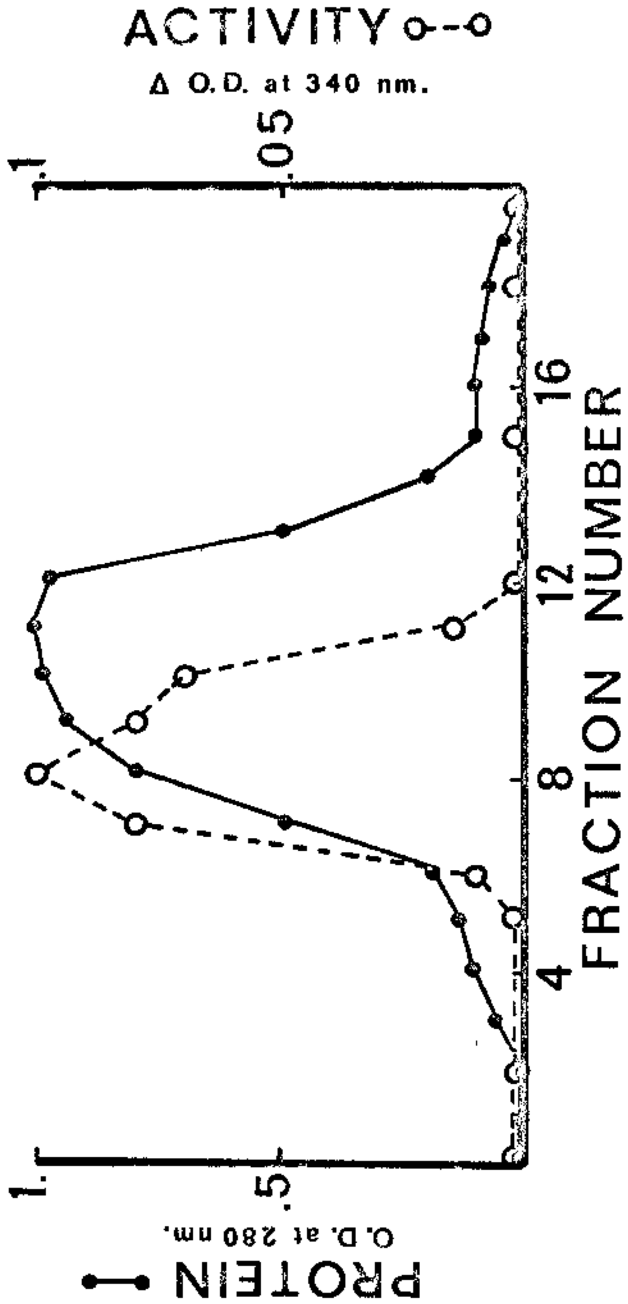

ACTIVITY o--o
Δ O.D. at 340 nm.
.1
.05
FRACTION NUMBER
4
8
12
16
PROTEIN •—•
O.D. at 280 nm.
1.
.5

Fig. 8. Elution pattern of cytoplasmic isozyme from Sephadex G-100 column. Solid line illustrates elution of protein, dotted line indicates elution of MDH activity. See "Methods" for details.

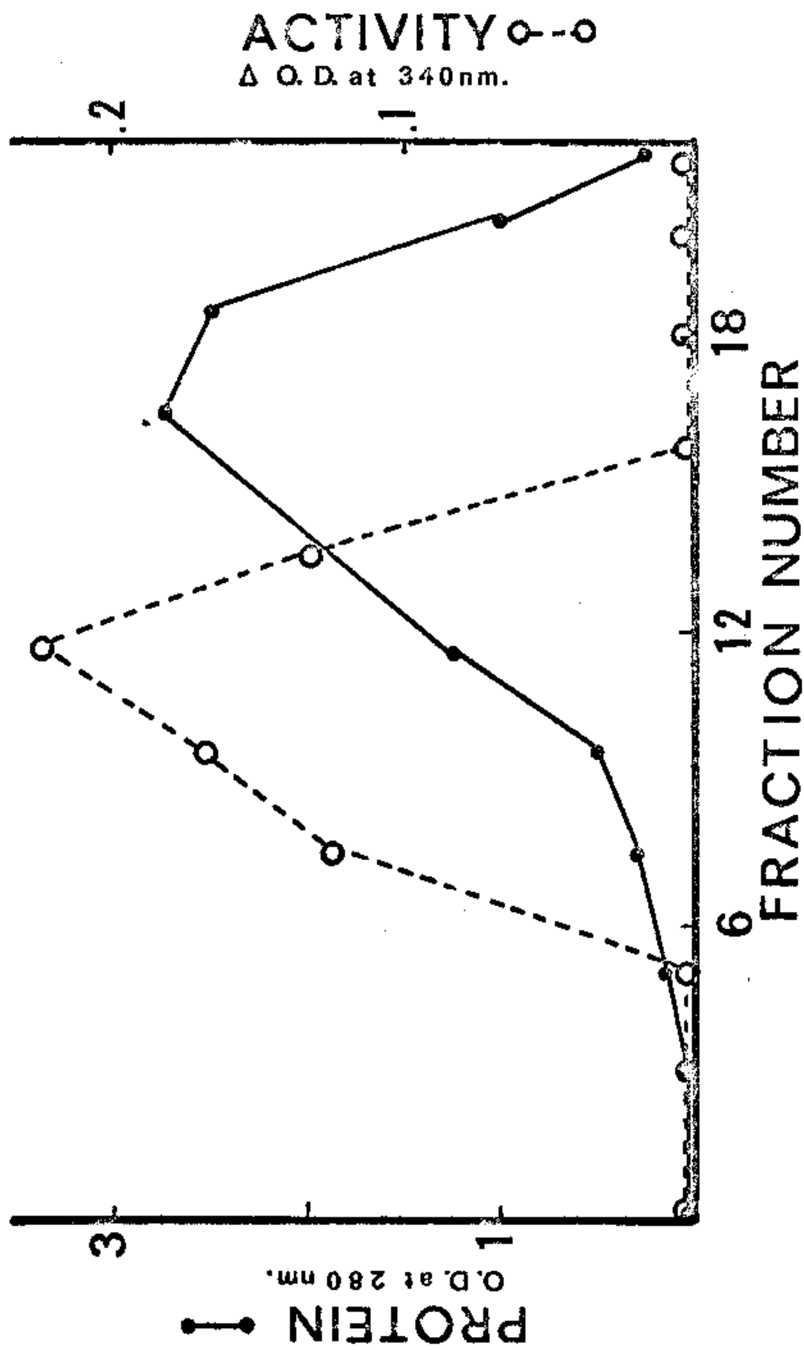

ACTIVITY o--o
Δ O.D. at 340nm.
.2
.1
PROTEIN •—•
O.D. at 280 nm.
3
1
FRACTION NUMBER
6
12
18

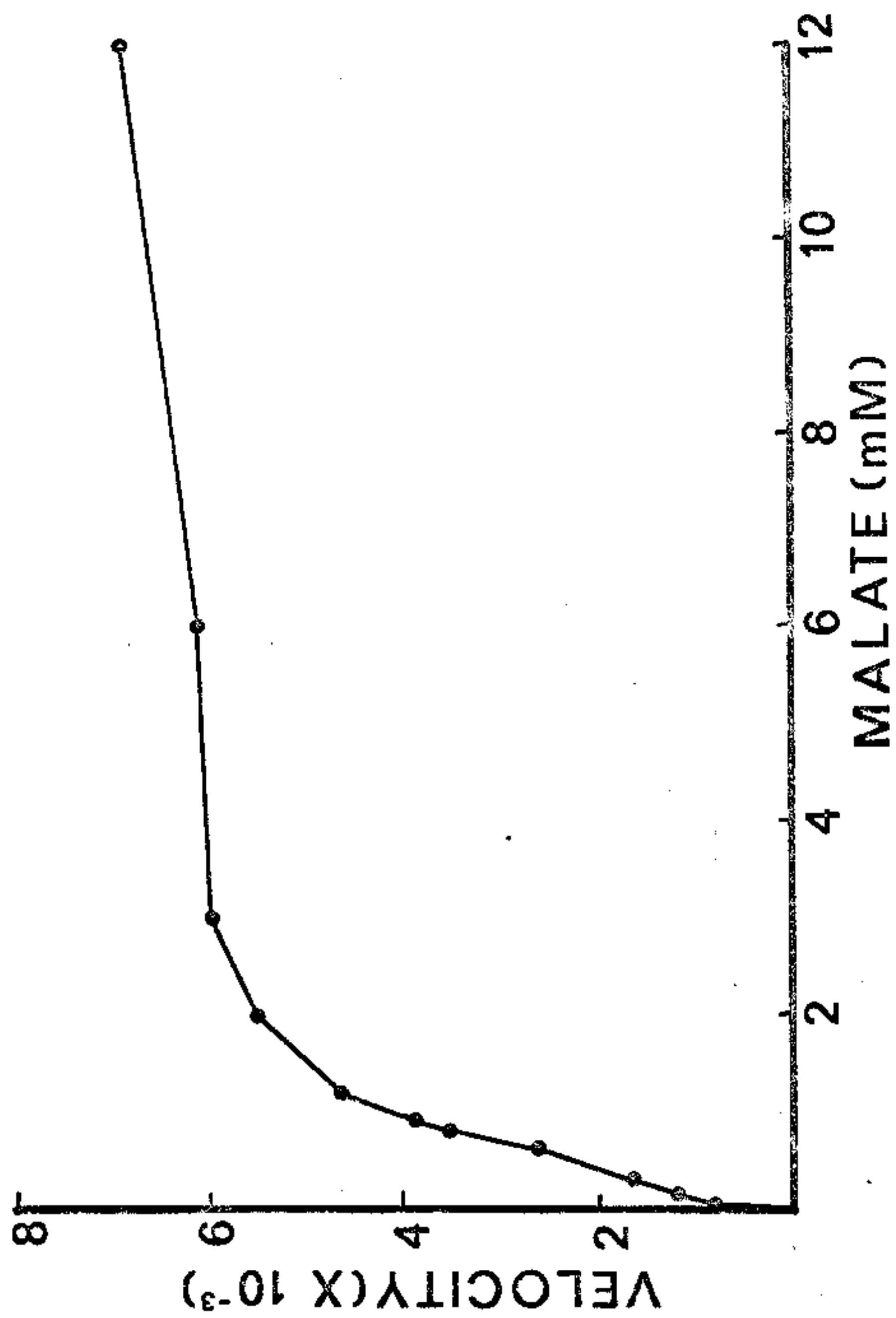
TABLE I

PARTIAL PURIFICATION OF THE CYTOPLASMIC AND MITOCHONDRIAL FORMS OF MALATE DEHYDROGENASE FROM GLYPTOTENDIPES BARBIPES

Treatment	Total Activity*	Specific Activity	Yield (%)	Purification
		Cytoplasmic		
Crude Extract	720	0.0659	—	—
DEAE Cellulose	324	0.603	45	9
Sephadex	223	3.759	31	55.8
		Mitochondrial		
Crude Extract	360	0.0329	—	—
DEAE Cellulose	159	0.3477	45	11
Sephadex	65	0.5237	18.5	15.9

*A total of 1080 units of activity from the crude homogenate were put on the column. From the yield it was calculated that 720 units represented the cytoplasmic enzyme and 360 units were present as mitochondrial enzyme.

Fig. 9. Plot of velocity vs. substrate concentration of the mitochondrial enzyme using malate as the variable substrate. Velocity is given as μmoles DPN reduced per minute. Each point is the average of 4 runs. See "Methods" for explanation of assay conditions.



VELOCITY(X 10^-3)
8
6
4
2
MALATE (mM)
2
4
6
8
10
12

Fig. 10. Double reciprocal plot of initial velocity as a function of malate concentration utilizing the mitochondrial enzyme. Velocity is μmoles DPN reduced per minute.

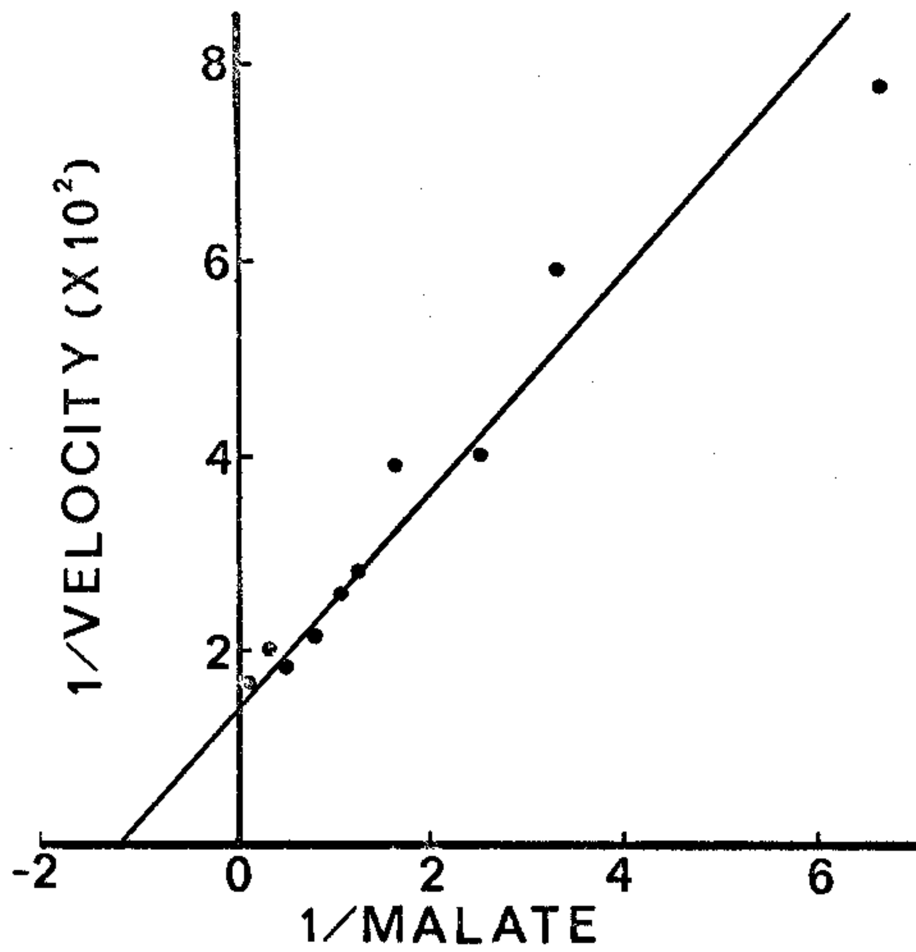
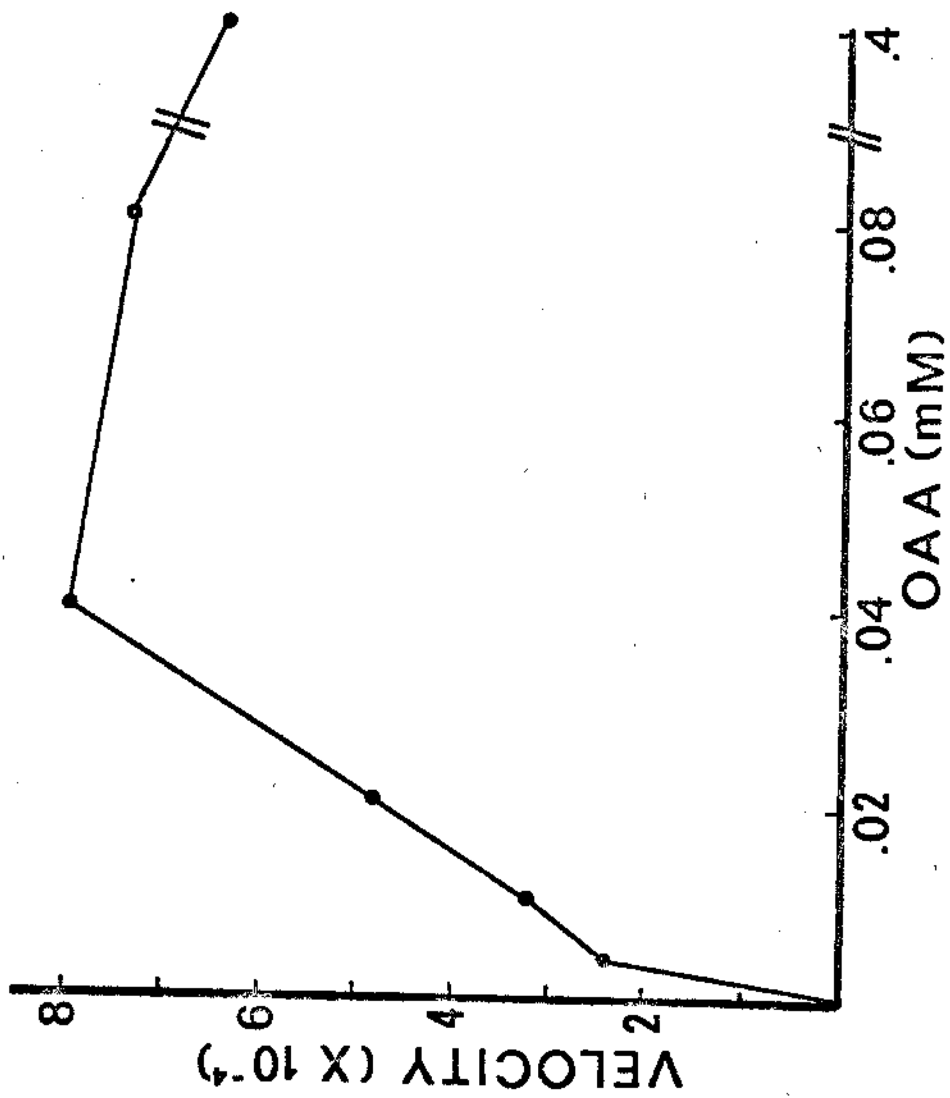

1/VELOCITY (X 10^2)
8
6
4
2
-2
0
2
4
6
1/MALATE

Fig. 11. Plot of velocity vs. substrate concentration of the mitochondrial enzyme using OAA as the variable substrate. Velocity is given as μmoles DPNH oxidized per minute. Each point is the average of 2 runs. See "Methods" for explanation of assay conditions.



VELOCITY (X 10⁻⁴)
8
6
4
2
.02
.04
.06
.08
.4
OAA (mM)

Fig. 12. Double reciprocal plot of initial velocity as a function of OAA concentration utilizing the mitochondrial enzyme. Velocity is µmoles of DPNH oxidized per minute.

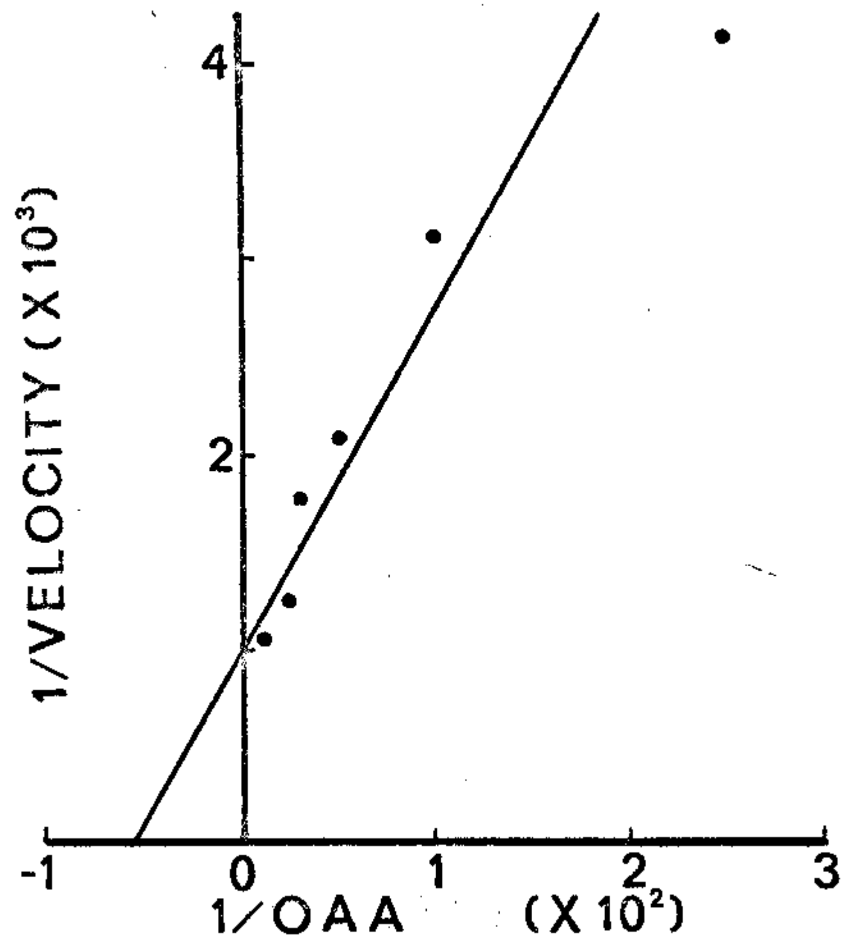

4
2
1/VELOCITY (X 10^3)
-1
0
1
2
3
1/OAA
(X 10^2)

Fig. 13. Plot of velocity vs. substrate concentration of cytoplasmic MDH using malate as the variable substrate. Velocity is given as µmoles DPN reduced per minute. Each point is the average of 4 runs. See "Methods" for explanation of assay conditions.

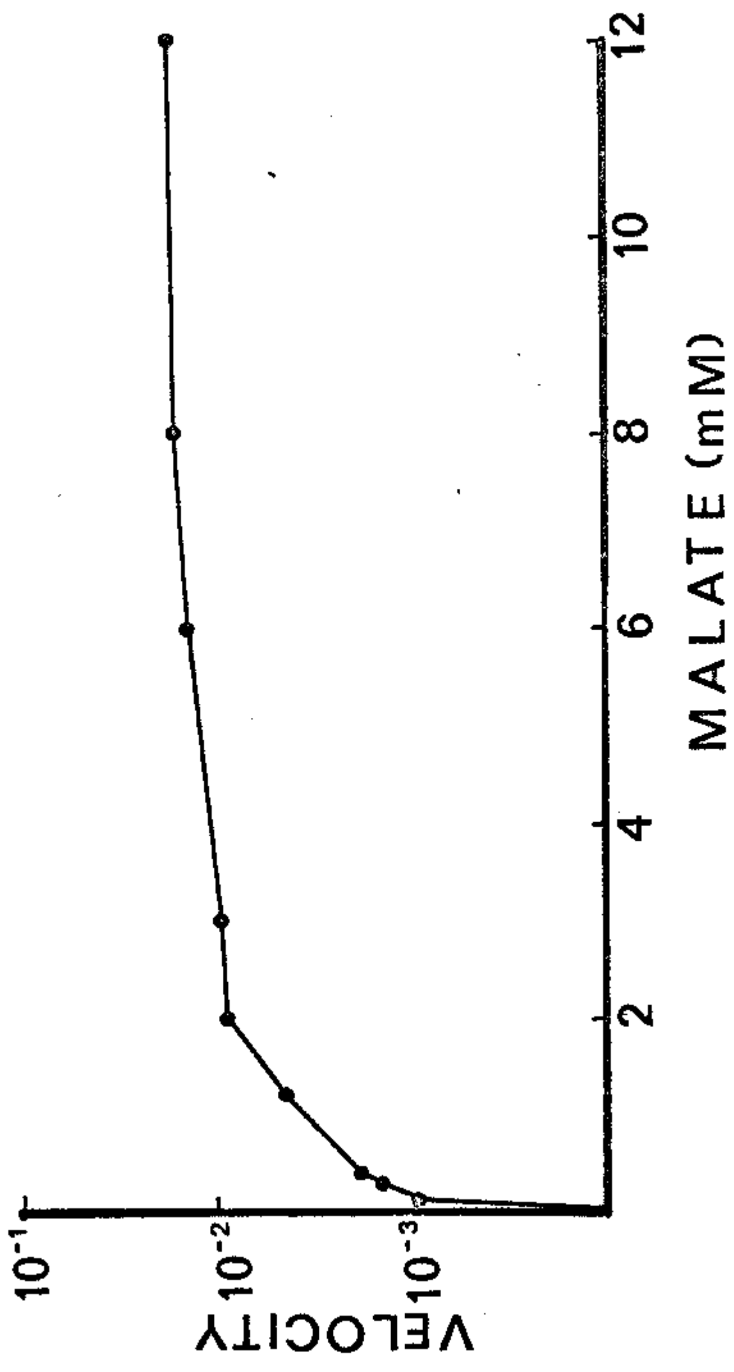
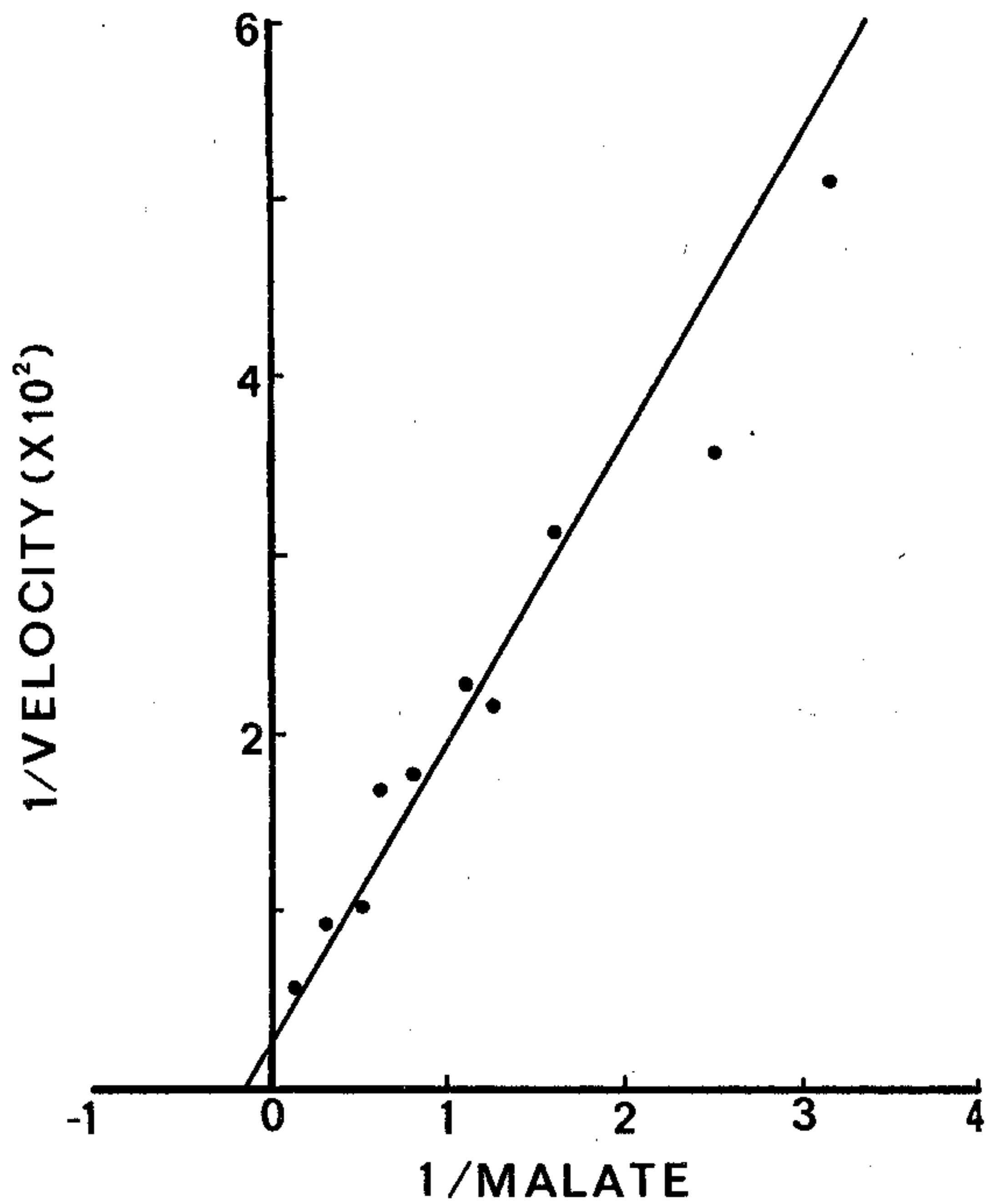

VELOCITY
10^{-1}
10^{-2}
10^{-3}
2
4
6
8
10
12
MALATE (mM)

Fig. 14. Double reciprocal plot of initial velocity as a function of malate concentration using cytoplasmic enzyme. Velocity is μmoles of DPN reduced per minute.



6
4
2
-1
0
1
2
3
4
1/VELOCITY (X10²)
1/MALATE

Fig. 15. Plot of velocity vs. substrate concentration of the cytoplasmic enzyme using OAA as the variable substrate. Velocity is given as μmoles of DPNH oxidized per minute. Each point is the average of 2 runs. See "Methods" for explanation of assay conditions.

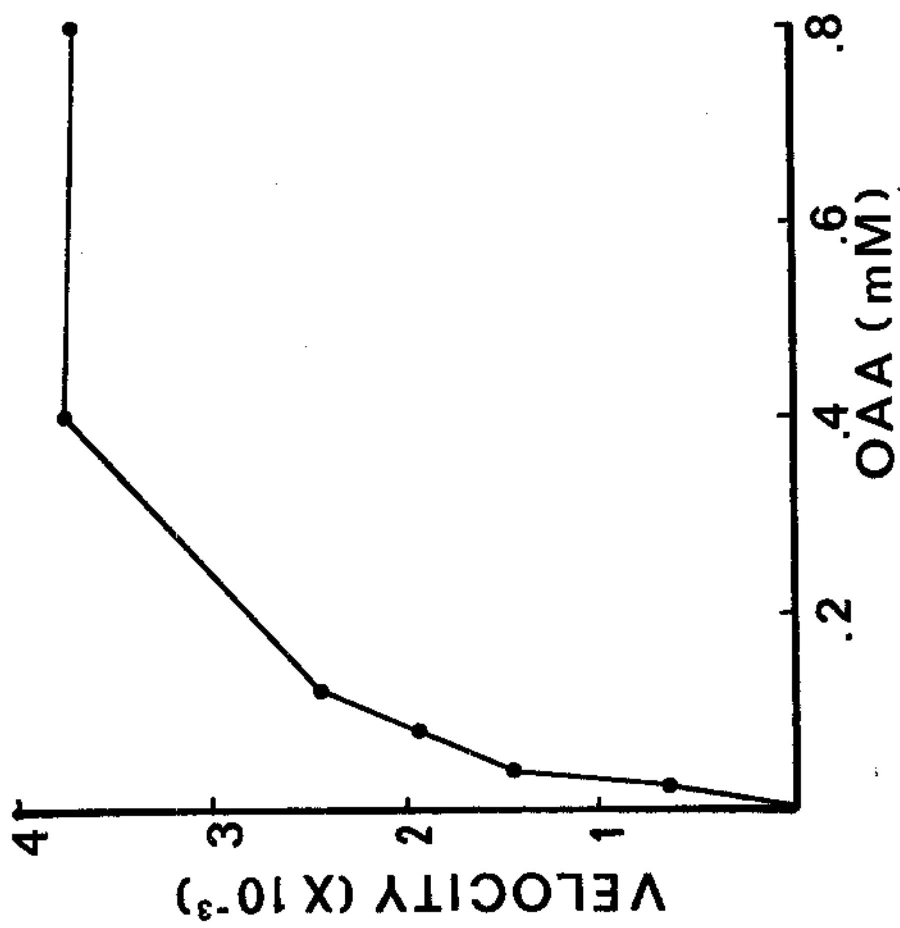
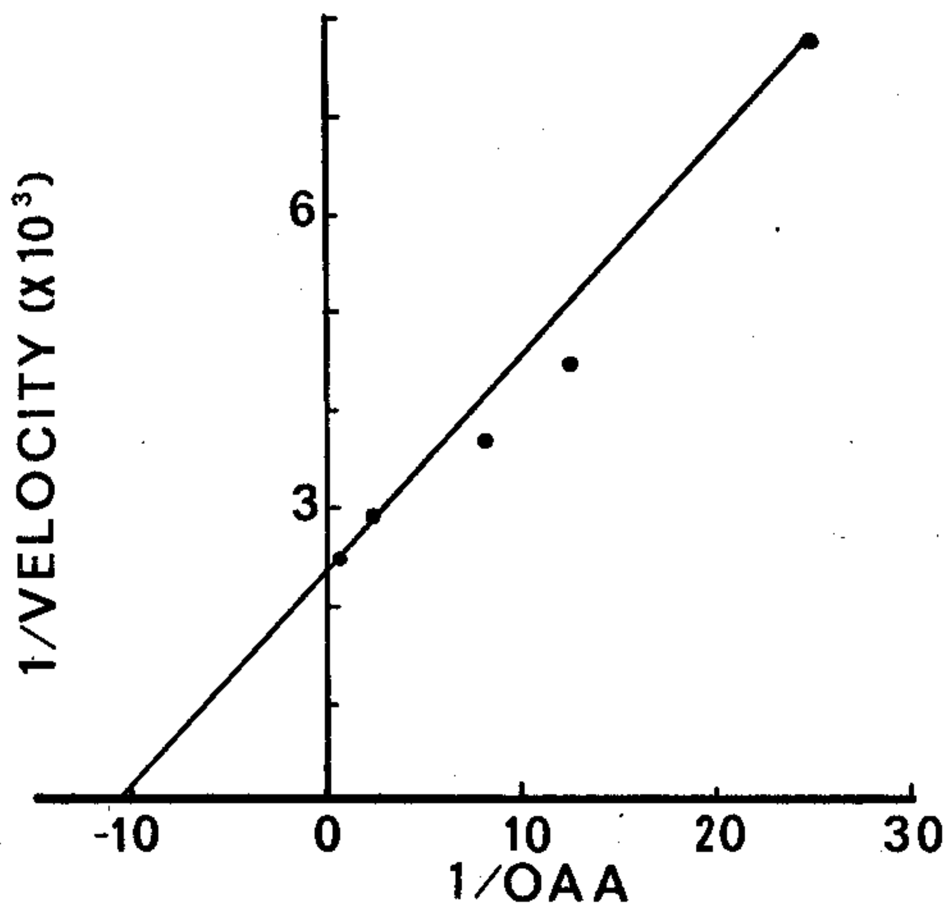

VELOCITY (X 10-3)
OAA (mM)
1
2
3
4
.2
.4
.6
.8

Fig. 16. Double reciprocal plot of initial velocity as a function of OAA concentration using cytoplasmic enzyme. Velocity is listed as µmoles DPNH oxidized per minute.



1/VELOCITY (X 10^3)
6
3
-10
0
10
20
30
1/OAA

TABLE II

COMPARISON OF MICHAELIS CONSTANTS (Km) OF GLYPTOTENDIPES BARBIPES, CHICKEN HEART AND DROSOPHILA

Substrate	Enzyme Source	Km (mM)*		
		G. barbipes	Chicken Heart	*Drosophila*
Malate	Cytoplasmic	6.75 ± 1.00	0.80	8.00
OAA	Cytoplasmic	0.1 ± 0.03	0.05	0.04
Malate	Mitochondrial	0.79 ± 0.097	0.90	20.00
OAA	Mitochondrial	0.015 ± 0.005	0.038	0.047

*The Km values were determined as described in "Methods". The values for chicken heart were taken from Kitto, 1966. The values for *Drosophila* were obtained from McReynolds, 1970.

N-ethylmaleimide inhibited pig heart mitochondrial MDH. Table III shows that neither the cytoplasmic nor the mitochondrial enzyme of the insect larvae were affected by incubation with NEM for two hours.

TABLE III

EFFECTS OF N-ETHYL MALEIMIDE ON MITOCHONDRIAL AND CYTOPLASMIC MALATE DEHYDROGENASES OF GLYPTOTENDIPES BARBIPES

Enzyme	Time	Activity (O.D.340)*
Mitochondrial	0	0.09
	1 hr.	0.1
	2 hr.	0.08
Cytoplasmic	0	0.12
	1 hr.	0.1
	2 hr.	0.09

*See "Methods" for experimental details.

DISCUSSION

Electrophoresis on both polyacrylamide gel and cellulose acetate indicated only two forms of malate dehydrogenase present in *Glyptotendipes barbipes* larvae. This is somewhat unusual since most animals have a variety of electrophoretically separable forms in the cytoplasm, and many also have more than one mitochondrial form (Kitto and Kaplan, 1966; Meizel and Markert, 1968; Davidson and Cortner, 1967; Zee and Zinkham, 1968). Furthermore, the two forms migrated toward opposite poles in electrophoresis, indicating that the charge on the two forms is different at pH 8.8. The migration pattern is typical of many other animals; the cytoplasmic form migrates toward the anode while the mitochondrial form migrates toward the cathode. The difference in electrical charge of the two isozymes is corroborated by the differential affinity for DEAE cellulose exhibited by each. The ctyoplasmic form binds to DEAE cellulose at pH 7.5, while the mitochondrial form does not bind.

Other insects which have been studied up to this time (*Drosophila*, McReynolds and Kitto, 1970; *Corydalus cornutus*; Harris, unpublished observations) appear to possess only two

forms of the enzyme.

In addition, no change of migration pattern was observed at different seasons of the year or when other than fourth instar larvae were utilized. This would indicate no ontogenetic changes in the enzyme with different instars.

In the purification procedure, the activity in the mitochondrial fraction was lower than that found in preliminary work. This is probably because the mitochondria were not sonicated, and homogenization in Tris buffer did not result in rupture of all mitochondria through osmotic shock. The yield of the mitochondrial enzyme was, therefore, low.

In order to calculate the purification factors of the isozymes following the initial DEAE cellulose column, it was necessary to determine the specific activity of the individual isozymes in the crude extract. This activity was estimated by assuming that an equal amount of each isozyme was lost in the column, and that the ratio of mitochondrial to cytoplasmic activity in the crude extract was the same as the ratio of activities eluted from the column. Specific activity is listed for the reaction in the direction of malate oxidation, for which the specific activity of purified enzyme is not extremely high. The specific activities achieved by Stromeyer *et al.* (1971) working with *Chlorella pyrenoidosa* were 33 and 84 for

the particulate and supernatant forms respectively.

The apparent Km values of the cytoplasmic and mitochondrial forms in the midge larvae are significantly different. This points out the distinct uniqueness of the forms and further suggests that the forms are coded for by different genes and, therefore, probably have different functions.

The apparent Michaelis constants that were obtained can best be evaluated by comparing them with work on other animals (Table II). The Km value of the mitochondrial enzyme for malate is comparable with that from chicken heart, while the cytoplasmic Km for malate is closer to that for _Drosophila_. The Km values for malate of both isozymes vary considerably among the three animals; those for oxalacetate are fairly uniform in all three instances. Because of the Dipteran relationship between _Drosophila_ and the chironomids, the enzymes of these two species would be expected to resemble one another. It is interesting therefore, that the mitochondrial form in _Drosophila_ has such a high Km for malate when compared with chironomids and chicken heart.

Inhibition of activity by high concentrations of OAA was observed in the mitochondrial form in _Glyptotendipes barbipes_, but little or none was observed in the supernatant

enzyme. This is in accord with results obtained from other sources (Kun, 1963; McReynolds and Kitto, 1970). No inhibition by high malate concentration was observed in either case.

The inhibition studies with N-ethyl maleimide (Table III) revealed no significant inhibition for either enzyme at pH 7.2. The small loss of activity is not believed to be inhibition by NEM since the control lost some activity also. This is surprising in the case of the mitochondrial form since Anderton (1970) found pig heart m-MDH to be substantially inhibited by NEM, although he concluded that the sulfhydryl groups were not in the active site of the enzyme. It would appear from these data that sulfhydryl groups are not directly involved in catalysis. These data should not be taken as conclusive, however, since Anderton found greater inhibition at higher pH values.

A representative mitochondrion isolated from the chironomid larvae had extensive, well-developed cristae (Fig. 2). This would suggest considerable metabolic activity and would indicate a highly aerobic existence. Observations made in the laboratory corroborated this statement. It was found that the larvae could not survive experimentally induced anaerobiosis for periods of time exceeding 10 hrs. Furthermore,

when larvae were kept in fresh water in aquaria, a constant underwater air supply was necessary. If the air supply was cut off, the larvae would come to the surface, apparently in need of air.

Further studies should be carried out on the isozymes of MDH. Purification to homogeneity would allow more refined studies to be carried out. Techniques such as isoelectric focusing would elucidate the problem of microheterogeneity in the isozymes. Other techniques such as microcomplement fixation could be used to relate the chironomid enzymes with other species. This could give information on the evolutionary adaptations of malate dehydrogenases.

SUMMARY

1. Two distinct forms of malate dehydrogenase were partially purified from Glyptotendipes barbipes larvae utilizing the techniques of ion exchange column chromatography and Sephadex G-100 column chromatography.

2. Subcellular localization of the two forms of the enzyme was carried out by differential centrifugation and zone electrophoresis. The anodal-migrating isozyme was cytoplasmic in origin, whereas the cathodal-migrating enzyme was found only in the mitochondria.

3. The mitochondria isolated had extensive, well-developed cristae which is indicative of an aerobic metabolism.

4. Kinetic studies were performed on the two enzymes in order to determine Michaelis constants for the substrates. For the cytoplasmic enzyme, the apparent Km for malate was 6.75 mM and the value for oxalacetate was 0.1 mM. The apparent Km values of malate and oxalacetate for the mitochondrial enzyme were 0.79 mM and 0.015 mM, respectively.

5. Studies with the sulfhydryl reagent N-ethyl maleimide resulted in no significant inhibition of activity of either

form of MDH. This suggests that sulfhydryl groups do not form an integral part of the active site of either enzyme.

BIBLIOGRAPHY

Anderton, B. H. (1970). The reaction of N-ethyl maleimide with malate dehydrogenase. FEBS Letters 12: 65-68.

Augenfeld, John M. and John C. Neese. (1961). Observations on the respiratory enzymes of various life-stages of Chironomus plumosus, Chironomus staegeri, and Aedes aegypti. Biol. Bull. 120: 129-138.

Augenfeld, John M. (1967). Effects of oxygen deprivation on aquatic midge larvae under natural and laboratory conditions. Physiol. Zool. 40: 149-158.

Breidenbach, R. W. and Harry Beevers. (1967). Association of the glyoxylate cycle enzymes in a novel subcellular particle from castor bean endosperm. Biochem. Biophys. Res. Commun. 27: 462-469.

Christie, G. S. and J. D. Judah. (1953). Intracellular distribution of enzymes. Proc. Roy. Soc. London Ser. B 141: 420-433.

Curry, L. L. (1962). A survey of environmental requirements for the midge (Diptera: Tendipedidae). In Biological Problems in Water Pollution, Third Seminar (Ed. by C. M. Tarzell), U. S. Department of Health, Education, and Welfare, Division of Water Supply and Polution Control, Cincinnati, Ohio. pp. 127-141.

Davidson, R. G. and J. A. Cortner. (1967). Mitochondrial malate dehydrogenase: A new genetic polymorphism in man. Science 157: 1569-1571.

Davis, B. J. (1964). Disc electrophoresis II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121: 404-427.

Formanek, Helmut and Norma Amit. (1970). Die sauerstoff-affinität des Chironomus thummi erythrocruorins. Eur. J. Biochem. 15: 19-21.

Fox, H. Munro. (1945). The oxygen affinities of certain invertebrate haemoglobins. J. Exp. Biol. 21: 161-165.

Harada, Kiyoshi and R. G. Wolfe. (1968). Malic dehydrogenase VII. The catalytic mechanism and possible role of identical protein subunits. J. Biol. Chem. 243: 4131-4137.

Kitto, G. B. and N. O. Kaplan. (1966). Purification and properties of chicken heart mitochondrial and supernatant malic dehydrogenase. Biochemistry 5: 3966-3980.

Kun, E. (1963). In The Enzymes (Ed. by P. D. Boyer, H. Lardy, and K. Myrbach), Vol. 7, Academic Press, N. Y., 149-160.

Lowry, D. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. (1951). Protein measurement with the Folin Phenol reagent. J. Biol. Chem. 193: 265-275.

McReynolds, Maureen Sims and G. Barrie Kitto. (1970). Purification and properties of Drosophila malate dehydrogenases. Biochem. et Biophys. Acta, 198: 165-174.

Meizel, S. and C. L. Markert. (1968). Malate dehydrogenase isozymes of the marine snail, Ilyanassa obsoleta. Arch. Biochem. Biophys. 122: 753-765.

Murphey, William H., G. Barrie Kitto, Johannes Everse, and Nathan O. Kaplan. (1967). Malate dehydrogenases. I. A survey of molecular size measured by gel filtration. Biochemistry 6: 603-609.

Peterson, E. A. and H. A. Sober. (1962). Column chromatography of proteins: substituted cellulose. In Methods of Enzymology. (Ed. by Colowick, S. P. and Kaplan, N. O.), Vol. 5, pp. 3-32, Academic Press, N. Y.

Rocha, Victor, S. K. Mukerji and Irwin P. Ting. (1968). Chloroplast malic dehydrogenase: a new malic dehydrogenase isozyme from spinach. Biochem. Biophys. Res. Commun. 31: 890-894.

Sanwal, B. D. (1969). Regulatory mechanisms involving nicotinamide adenine nucleotides as allosteric effectors I. Control characteristics of malate dehydrogenase. J. Biol. Chem. 244: 1831-1837.

Saz, H. J. and O. L. Lescure. (1969). The functions of phosphoenolpyruvate carboxykinase and malic enzyme in the anaerobic formation of succinate by Ascaris lumbricoides. Comp. Biochem. Physiol. 30: 49-60.

Shows, Thomas B., Verne M. Chapman and Frank H. Ruddle. (1970). Mitochondrial malate dehydrogenase and malic enzyme: Mendelian inherited electrophoretic variants in the mouse. Biochem. Genet. 4: 707-718.

Stromeyer, Cheryl Thomas, Francis E. Cole and Pierre C. Arquembourg. (1971). Purification and properties of malate dehydrogenase from Chlorella pyrenoidosa. Catalytic mechanism of the particulate form. Biochemistry 10: 729-735.

Thorne, C. J. R. (1960). Characterization of two malic dehydrogenases from rat liver. Biochem. Biophys. Acta 42: 175-176.

Walshe, Barbra M. (1951). The function of haemoglobin in relation to filter feeding in leaf-mining chironomid larvae. J. Exp. Biol. 28: 57-61.

Walshe, B. M. (1951). Feeding habits of chironomid larvae. Zool. Soc. (London), Proc. 121 (1): 63-79.

Whitt, G. J. (1970). Genetic variation of supernatant and mitochondrial malate dehydrogenase isozymes in the teleost Fundulus heteroclitus. Experientia 26: 734-736.

Wilkinson, G. N. (1961). Statistical estimations in enzyme kinetics. Biochem. J. 80: 324-332.

Zee, David S. and Wm. H. Zinkham. (1968). Malate dehydrogenase in Ascaris suum: Characterization, ontogeny, and genetic control. Arch. Biochem. Biophys. 126: 574-584.