BBA 66048

PURIFICATION AND PROPERTIES OF DROSOPHILA MALATE DEHYDROGENASES

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(Received October 7th, 1969)

SUMMARY

The supernatant and mitochondrial forms of malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37) have been purified from *Drosophila virilis* Texmelucan (1801.1). The mitochondrial enzyme was judged homogeneous by ultracentrifugal and electrophoretic criteria and has an $s_{20,w}$ of 4.0 S. The molecular weights of both enzymes was estimated to be 68 000 by gel filtration on calibrated columns. The mitochondrial and supernatant forms of malate dehydrogenases could readily be differentiated with respect to inhibition by oxaloacetate, thermolability and reactivity with coenzyme analogs. A rabbit antiserum directed against *Drosophila virilis* Texmelucan mitochondrial malate dehydrogenase reacted strongly with this enzyme as judged by inhibition of enzyme activity and by double diffusion in agar. No cross-reaction was observed with the supernatant malate dehydrogenase. A comparison of the supernatant and mitochondrial malate dehydrogenases of *Drosophila virilis* Texmelucan with the corresponding enzymes from *Drosophila melanogaster* Oregon K revealed differences in immunological properties, in inhibition by substrate and in thermolability.

INTRODUCTION

The existence of two distinct forms of malate dehydrogenase, one in the mitochondrial subcellular fraction and the other in the cytoplasm, has been reported from a variety of organisms[1–8] and a large body of information on the physical and kinetic properties of the enzymes is available. Comparisons by gel electrophoresis of malate dehydrogenase from several species of Drosophila have revealed that electrophoretic differences between species are common while variation within a species is rare[9–11]. Considerable evolutionary and genetic information is available for the genus Drosophila; therefore, it should be a useful organism for studies in biochemical evolution. The present work was undertaken to obtain more detailed biochemical information

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on malate dehydrogenase in this group before commencing the taxonomic and evolutionary studies.

MATERIALS AND METHODS

Materials

NADH, NAD^+ and other co-factor analogs used in this study were obtained from PL Laboratories. Oxaloacetate and Cellex-CM were purchased from CalBiochem and L-malic acid from Sigma; Sephadex G-100 from Pharmacia Fine Chemicals; hydrolyzed starch from Connaught Laboratories, Co.; phenazine methosulfate and nitro-blue tetrazolium from Mann Research Laboratories. Other chemicals were reagent grade.

The Drosophila strains were reared on cornmeal–agar medium in pint milk bottles. Adults of *Drosophila virilis* Texmelucan (1801.1) and *Drosophila melanogaster* Oregon K were collected up to 2 days old and stored frozen.

Enzyme assays

Routine assays during the purification of the enzymes were made in a Beckman D.U. spectrophotometer and kinetic studies in a Cary 15 or a Zeiss PMQ II spectrophotometer. Malate dehydrogenase assays were performed as described in KITTO AND LEWIS[8] except that the concentration of L-malate was 67 mM. A unit of malate dehydrogenase activity is defined as the amount of enzyme required to oxidize or reduce 1 μmole of coenzyme per min under the conditions described.

Temperature stability was determined by diluting the enzyme to 1.0 enzyme unit/ml in 0.1 M phosphate buffer, pH 7.5. The sample was heated at constant temperature and aliquots were removed at different times, chilled immediately and assayed for malate dehydrogenase activity.

Determination of protein content

During the purification of the enzymes, protein content was determined by the method of WARBURG AND CHRISTIAN[12]. The protein concentration of purified enzyme solutions was determined by the method of LOWRY *et al.*[13] using bovine serum albumin as a standard.

Electrophoresis

Starch-gel electrophoresis was performed as described by FINE AND COSTELLO[14] using phosphate–citrate buffer at pH 7.0. Regions of enzymatic activity were located on the gels with a tetrazolium staining mixture specific for malate dehydrogenase[15].

Cellulose acetate strips (Gelman Sepraphore III) were prepared and run according to manufacturer's specifications using high-resolution veronal buffer, pH 8.6. The strips were stained for enzyme activity using the same medium as for starch gels and for protein with 10% Amido Black in methanol–acetic acid solution.

Fluorescence studies

The fluorescence spectrum of *D. virilis* Texmelucan mitochondrial malate dehydrogenase was determined on a Farrand Mark I spectrofluorometer with exci-

tation at 280 mμ. Ribonuclease, chicken heart mitochondrial malate dehydrogenase[7] and tyrosine were used as standards.

Ultracentrifugal analysis

Ultracentrifugal analysis of the *D. virilis* mitochondrial malate dehydrogenase was carried out in a Beckman–Spinco Model E using schlieren optics. The sedimentation constant was determined at a rotor speed of 59 870 rev./min and at a temperature of 20°.

Molecular weight determination

The molecular weights of Drosophila mitochondrial and supernatant malate dehydrogenases were estimated by chromatography on Sephadex G-100 columns calibrated for molecular weight determinations, as described by ANDREWS[16]. The proteins used as standards were bovine serum albumin, ovalbumin, soybean trypsin inhibitor, bovine trypsin, cytochrome *c*, chicken heart malate dehydrogenase and γ-globulin. Dextran blue 2000 was used to determine the void volume.

Isolation of mitochondria

The Drosophila mitochondrial and supernatant fractions used in kinetic studies were prepared from freshly collected flies by the method of SACTOR AND COCHRAN[17]. The purity of the mitochondrial and supernatant preparations was checked by starch-gel electrophoresis. As shown in Fig. 1, the mitochondrial malate dehydrogenase and supernatant malate dehydrogenase are well separated by this method.

Immunological procedures

Rabbit antisera against *D. virilis* mitochondrial malate dehydrogenase were

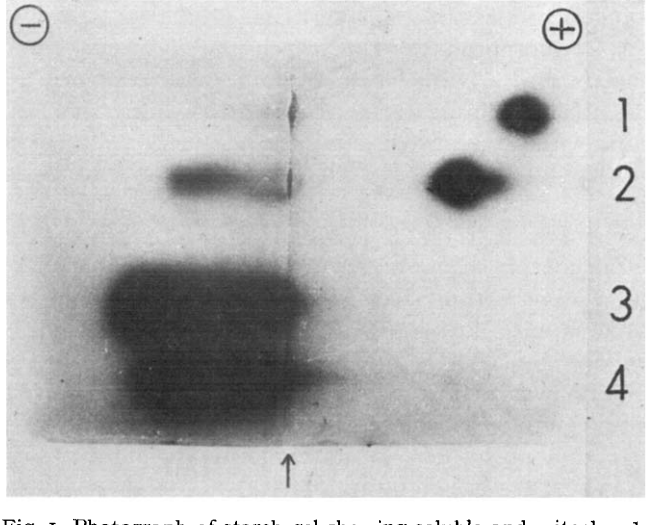


Fig. 1. Photograph of starch gel showing soluble and mitochondrial fractions stained for malate dehydrogenase (see text for details). Samples are: 1, *D. virilis* supernatant fraction; 2, *D. melonagaster* supernatant fraction (contains a small amount of mitochondrial malate dehydrogenase from lysed mitochondria); 3, *D. virilis* mitochondrial fraction; 4, *D. melanogaster* mitochondrial fraction.

obtained in the following manner. A solution of purified mitochondrial malate dehydrogenase containing 100 μg of protein mixed with 0.1 ml Freund's adjuvant and 0.2 ml 0.15 M NaCl was injected into the rabbit's toe pads. This procedure was repeated once a week for 4 weeks. First course sera were obtained after 3 weeks. Rabbits were bled every 2 weeks for 6 weeks and then bled every 3rd week.

Double diffusion tests were performed by the OUCHTERLONY[18] method to characterize the antisera. The antibody well contained about 10 μl of undiluted antiserum. One antigen well contained 10 μl of crude Drosophila tissue extract, one contained 10 μl of crude soluble cellular fraction and a third well contained crud mitochondrial extract. The diffusion was allowed to continue at least 3 days at 4°. The rabbit antisera prepared against *D. virilis* mitochondrial malate dehydrogenase gave a single precipitin band against both the crude tissue extract and the crude mitochondrial fraction. No precipitin band was observed against the soluble cellular fraction.

After the precipitin bands had formed, the agar plates were washed with buffer and placed in the malate dehydrogenase staining mixture used for starch gels. The malate dehydrogenase activity is not completely inhibited by reaction with antibody and the residual activity stained at the site of the precipitin band indicating that the antibody was indeed directed against this enzyme.

Inhibition of malate dehydrogenase activity by antisera was measured in the Tris complement fixation buffer of WASSERMAN AND LEVINE[19]. Equal portions of various dilutions of antibody were mixed with an enzyme solution containing 0.2 enzyme unit/ml. After incubation for 1 h at room temperature, the malate dehydrogenase activity remaining was measured.

RESULTS

Because flies were collected over a considerable period of time, the possibility existed for the introduction of polymorphism. For this reason, the malate dehydrogenase of approx. 50 individual *D. virilis* Texmelucan strain flies was examined by gel electrophoresis at various intervals during these studies. At no time was any electrophoretic variability found within this strain.

Enzyme purification

(1) Extraction of tissues. 100 g of frozen *D. virilis* Texmelucan strain were ground in 500 ml of 0.1 M potassium phosphate buffer (pH 7.5) with 1 mM EDTA, 0.1 mM dithiothreitol. This brei was centrifuged at 37 000 $\times g$ for 20 min, the precipitate discarded, and the supernatant solution recentrifuged at 48 200 $\times g$ for 1 h.

(2) $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ was added to the extract to obtain 50% saturation. The solution was kept on ice for 30 min and then centrifuged for 20 min at 37 000 $\times g$. The precipitate was discarded and more solid $(NH_4)_2SO_4$ was added to bring the supernatant up to 80% saturation. After 30 min the suspension was spun 20 min at 37 000 $\times g$. The resulting precipitate was dissolved in about 50 ml of the phosphate buffer and dialyzed overnight.

(3) Sephadex G-100 filtration. A 5 cm $\times$ 90 cm Pharmacia G-100 column was prepared with 5 mM potassium phosphate buffer (pH 7.5) with 1 mM EDTA, 0.1 mM dithiothreitol and had a void volume of 300 ml. The dialyzed enzyme was placed on

the column and the major peak of malate dehydrogenase activity was eluted at about 650 ml. Fractions containing enzyme activity were combined and the volume reduced at 4° under N_2 through a Diaflo ultra-filter with a UM-1 membrane (exclusion limit of molecules above 10 000 molecular weight). Starch-gel electrophoresis showed that both the supernatant and mitochondrial malate dehydrogenases were present in the concentrate.

(4) Cellex-CM cation exchange. The enzyme solution was placed on a 2.5 cm × 50 cm Cellex-CM column previously equilibrated with the 5 mM potassium phosphate buffer, pH 7.5. The column was eluted with the above buffer and a peak of malate dehydrogenase activity was recovered. The column was then eluted with a linear salt gradient to 0.5 M NaCl until a second peak of malate dehydrogenase activity had been eluted. Starch-gel electrophoresis followed by staining for malate dehydrogenase showed that the first peak contained the supernatant form of the enzyme with a small amount of the mitochondrial form and the second peak contained only the mitochondrial enzyme. The fractions were pooled separately and concentrated as in Step 3.

(5) Further purification of Drosophila mitochondrial malate dehydrogenase. Cellulose acetate electrophoresis revealed that the mitochondrial malate dehydrogenase preparation from Step 4 contained one other protein with no malate dehydrogenase activity. The enzyme solution was placed on a Sephadex G-100 column (18 mm × 50 cm) and two peaks of protein were eluted at 50 and at 80 ml. Malate dehydrogenase activity was coincident with the second peak. This fraction was checked

TABLE I

PURIFICATION OF DROSOPHILA MITOCHONDRIAL MALATE DEHYDROGENASE

Steps	*Total malate dehydrogenase (units)*	*Total protein (mg)*	*Specific* activity (units/mg protein)*	*Yield (%)*	*Purification factor*
1. Crude extract	163 170	39 690	4.1	100	
2. $(NH_4)_2SO_4$, 50–80%	131 760	2 836	46.5	81	11.3
3. Sephadex G-100	30 800	210	147	18.9	35.8
4. Cellex-CM fractionation	5 228	9.6	544	3.20	132.7
5. Sephadex G-100	5 025	3.0	1670	3.1	418

* As measured by oxaloacetate reduction.

on cellulose acetate and only one band of protein was observed which coincided with the site of malate dehydrogenase activity. The purification procedure is summarized in Table I. The specific activity of the purified Drosophila mitochondrial malate dehydrogenase is comparable to that reported for the enzymes from beef heart[5], pig heart[20], ox heart[21] and ox kidney[22]. The enzyme sedimented as a single symmetrical peak in the analytical ultracentrifuge with an $s_{20,w}$ of $4.03 \cdot 10^{-13}$ cm·sec^{-1}. This sedimentation constant is in good agreement with those reported for pig, horse, beef, chicken and tuna mitochondrial malate dehydrogenase[4,7,8,23].

The fluorescence emission spectrum of the purified mitochondrial malate dehydrogenase (activation at 280 mμ) was similar to that reported for chicken[7],

tuna[8], and pig[26] mitochondrial malate dehydrogenases, with a maximum in the vicinity of 307 mμ indicating that the Drosophila enzyme has a low tryptophan content.

(6) Further purification of Drosophila supernatant malate dehydrogenase. Cellulose acetate electrophoresis revealed that the first malate dehydrogenase peak from Step 4 contained several bands of protein and a small amount of mitochondrial malate dehydrogenase as well as supernatant malate dehydrogenase. The sample was applied to an 18 mm × 50 cm Sephadex G-100 column as described above. Fractions with malate dehydrogenase activity were pooled, concentrated and applied to a second Cellex-CM column as described in Step 4. When the malate dehydrogenase activity which was eluted with the starting buffer was examined by cellulose acetate electrophoresis and stained for malate dehydrogenase, only supernatant malate dehydrogenase activity was observed; however, when cellulose acetate strips were stained for protein, four minor bands of protein impurity were found in addition to the major component, which was coincident with supernatant malate dehydrogenase activity. These impurities were estimated to account for less than 10% of the total protein. Attempts at further purification led to drastic losses in enzymatic activity and the experiments reported here were conducted with supernatant malate dehydrogenase purified to this stage.

Starch-gel electrophoresis

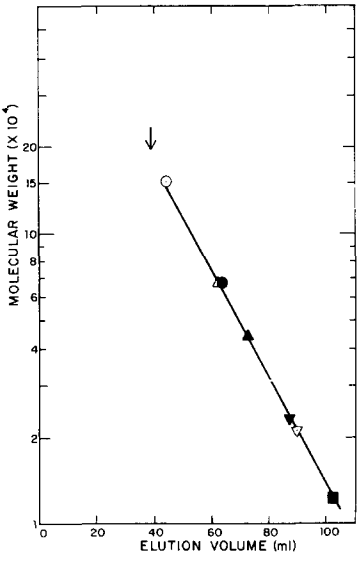
When crude extracts of Drosophila were examined by starch-gel electrophoresis at pH 7.0 in phosphate–citrate buffer, two zones of malate dehydrogenase activity were observed. By cellular fractionation studies the more anodal band was identified as the supernatant malate dehydrogenase (Fig. 1). The mitochondrial enzyme stained on the cathodal side. *D. virilis* supernatant malate dehydrogenase migrated a greater distance than *D. melanogaster* supernatant malate dehydrogenase (Fig. 1). In the *D. melanogaster* supernatant preparation shown in Fig. 1, a minor band of malate dehydrogenase activity is present on the cathodal side of the gel. This band was absent in other subcellular fractionations of *D. melanogaster* and presumably arises from lysis of some of the mitochondria during the fractionation. The two mitochondrial malate dehydrogenases moved about the same distance from the origin toward the cathode, but the *D. melanogaster* mitochondrial enzyme was somewhat slower. Multiple electrophoretic forms of mitochondrial malate dehydrogenase, similar to those described in other organisms[7,15], were observed with extracts from both species of Drosophila.

Molecular weight determination

The molecular weight of *D. virilis* and *D. melanogaster* supernatant and mitochondrial malate dehydrogenases was determined by gel filtration on a Sephadex G-100 column which had been calibrated for molecular weight determination with proteins of known molecular weight. All Drosophila malate dehydrogenases had elution volumes which corresponded to a molecular weight of about 68 000. (Fig. 2) Chicken heart malate dehydrogenases, for which molecular weights of 67 000 have been reported using ultracentrifugal techniques[24], gave identical elution volumes.

Immunological properties

Rabbit antisera prepared against *D. virilis* mitochondrial malate dehydrogenase



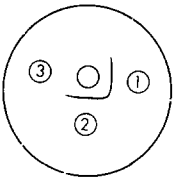


Fig. 2. Gel filtration on Sephadex G-100 of Drosophila malate dehydrogenases. The 1.8 cm × 50 cm column was equilibrated and eluted with 0.1 M potassium phosphate buffer, pH 7.5. The standards used were: ■, cytochrome *c*; △, soybean trypsin inhibitor; ▼, bovine pancreatic trypsin; ▲, ovalbumin; △, bovine serum albumin; ○, γ-globulin. Dextran blue 2000 (↓) was used to determine the void volume. Identical elution volumes (●) were obtained with chicken heart, *D. virilis* and *D. melanogaster* supernatant and mitochondrial malate dehydrogenases.

Fig. 3. A tracing of a double diffusion in agar using an antiserum against *D. virilis* Texmelucan mitochondrial malate dehydrogenase. Center well contained anti-*D. virilis* mitochondrial malate dehydrogenase antibody. Well No. 1 contained crude *D. virilis* extract, well No. 2 mitochondrial subcellular fraction, well No. 3 soluble fraction. (See text for further details.)

reacted strongly on double diffusion agar plates. There was no cross-reaction with the supernatant malate dehydrogenase (Fig. 3). The anti-*D. virilis*-mitochondrial-malate-dehydrogenase was found to inhibit the enzymatic activity of the mitochondrial enzyme, but was without effect on the *D. virilis* supernatant malate dehydrogenase.

TABLE II

INHIBITION OF DROSOPHILA MALATE DEHYDROGENASE BY ANTI-*D. virilis* MITOCHONDRIAL MALATE DEHYDROGENASE ANTIBODY*

Antibody dilution	*Percentage activity remaining*	
	D. virilis mitochondrial malate dehydrogenase	*D. melanogaster mitochondrial malate dehydrogenase*
1:10	22.3	31.5
1:50	50.0	74.0
1:100	75.0	100

* The enzymes were incubated at 25° for 1 h with antibody diluted in Tris complement fixation buffer[17] prior to the assay.

The anti-*D. virilis* mitochondrial malate dehydrogenase antibodies also inhibited the reaction catalyzed by *D. melanogaster* mitochondrial malate dehydrogenase but not as strongly as *D. virilis* mitochondrial malate dehydrogenase (Table II).

Catalytic properties

Substrate inhibition. As is the case with other malate dehydrogenases[5-8] the Drosophila mitochondrial malate dehydrogenase is more susceptible to inhibition by high concentrations of oxaloacetate than the Drosophila supernatant malate dehydrogenase (Fig. 4). Differences were also observed between the two species. *D. melanogaster* mitochondrial malate dehydrogenase is more strongly inhibited and the supernatant malate dehydrogenase of this species less strongly than the corresponding *D. virilis* enzymes.

Using L-malate as substrate, inhibition of the Drosophila enzymes was observed only at high concentrations of L-malate. A similar case had been reported for tuna malate dehydrogenases[8]. Inhibition of Drosophila supernatant malate dehydrogenase is slightly greater than inhibition of the mitochondrial enzyme in this reaction, representative values being 45 and 55% inhibition, respectively, at an L-malate concentration of 0.5 M.

The Michaelis constants of the *D. virilis* enzymes for oxaloacetate are similar to those reported for other malate dehydrogenases but those for L-malate are much higher than those reported for other organisms[4-7,15] (Table III).

TABLE III

MICHAELIS CONSTANTS FOR *D. virilis* MITOCHONDRIAL AND SUPERNATANT MALATE DEHYDROGENASES

Activity was measured using 0.1 M potassium phosphate buffer (pH 7.5) for oxaloacetate and 0.1 M pyrophosphate buffer (pH 8.9) for malate with substrate concentrations in the non-inhibitory range.

	Supernatant malate dehydrogenase (mM)	*Mitochondrial malate dehydrogenase (mM)*
K_m (malate)	8	20
K_m (oxaloacetate)	0.04	0.047

Co-factor analogs. The Drosophila mitochondrial and supernatant malate dehydrogenases can be differentiated on the basis of their ability to use analogs of NAD^+ (Table IV). No significant differences were observed between the two Drosophila species.

Heat inactivation. The results of heat inactivation are shown in Fig. 5. Unlike the situation observed with other malate dehydrogenases[7], the supernatant malate dehydrogenase of Drosophila was more thermolabile than the mitochondrial malate dehydrogenase. The enzymes from the two species also differed in their thermal properties. The *D. melanogaster* mitochondrial malate dehydrogenase is thermally more stable and the *D. melanogaster* soluble malate dehydrogenase is less stable than the corresponding *D. virilis* malate dehydrogenases.

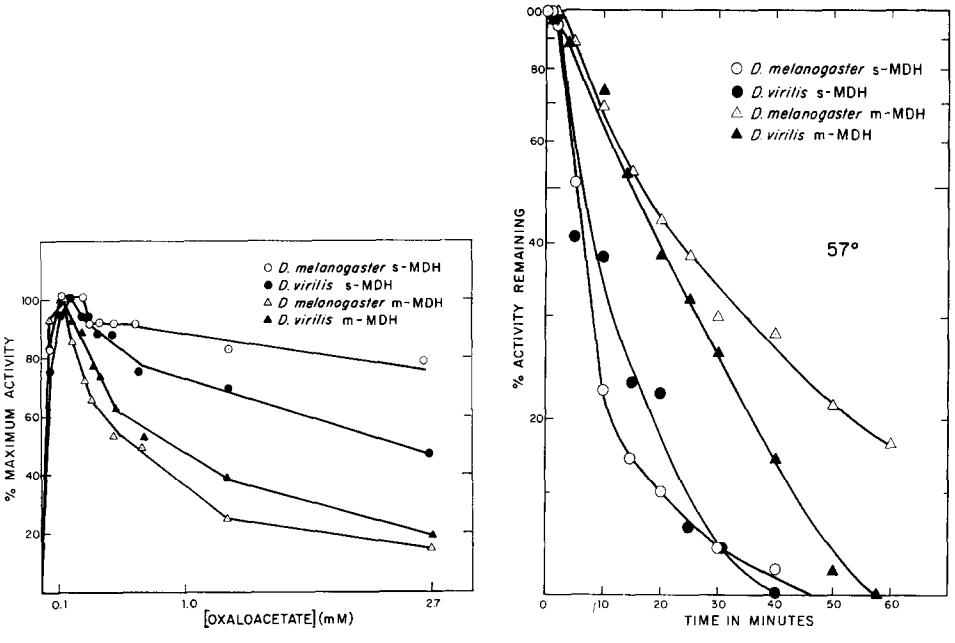


Fig. 4. The effect of oxaloacetate concentration on the activity of *D. virilis* and *D. melanogaster* malate dehydrogenases. s-MDH, supernatant malate dehydrogenase; m-MDH, mitochondrial malate dehydrogenase.

Fig. 5. Rates of thermal inactivation of *D. virilis* and *D. melanogaster* mitochondrial and supernatant malate dehydrogenases at 57°. See text for details. For abbreviations see legend to Fig. 4.

pH optima. The pH optimum of the *D. virilis* and *D. melanogaster* mitochondrial and soluble malate dehydrogenases was around pH 8.5 for oxaloacetate reduction in 0.1 M glycine–NaOH buffer. The pH optimum for malate oxidation was pH 9.0 for both soluble enzymes, but pH 9.5 for the mitochondrial forms.

The pH optimum for oxaloacetate reduction by Drosophila malate dehydrogenases is slightly higher than that reported for chicken malate dehydrogenases and

TABLE IV

RELATIVE RATES OF DROSOPHILA MALATE DEHYDROGENASES WITH ANALOGS OF NAD^+

Assay mixtures contained 67 mM malate, 0.1 M sodium pyrophosphate buffer (pH 8.9) and a coenzyme concentration of 0.3 mg/ml. The rate of reduction of NAD^+ was measured at 340 mμ and reduction of acetylpyridine–adenine dinucleotide and acetylpyridine–hypoxanthine dinucleotide at 365 mμ.

	Supernatant malate dehydrogenase		*Mitochondrial malate dehydrogenase*	
	D. virilis	*D. melanogaster*	*D. virilis*	*D. melanogaster*
Acetylpyridine–adenine dinucleotide/NAD^+	4.5	4.2	12.0	12.9
Acetylpyridine–hypoxanthine dinucleotide/NAD^+	1.65	1.5	0.85	0.9

somewhat lower for L-malate oxidation[7] but similar to that of the beef heart enzyme[4] for both oxaloacetate reduction and L-malate oxidation.

DISCUSSION

One of the first reports of catalytic differences between supernatant and mitochondrial malate dehydrogenases utilized an insect, *Locusta migratoria*[1]. Although malate dehydrogenase has been subsequently purified and studied in detail from a variety of organisms[2–8], we believe that the present report is the most detailed examination of the properties of an insect malate dehydrogenase.

There is a great degree of evolutionary divergence separating Drosophila from most organisms previously studied. It is therefore interesting that the malate dehydrogenases of Drosophila resemble those from vertebrate sources in a number of ways. Inhibition of mitochondrial malate dehydrogenase by oxaloacetate, which was first noted by DELBRÜCK *et al.*[1] for the locust, has also been observed for Drosophila, chicken[7], tuna[8], beef[5,6], and pig[3] mitochondrial malate dehydrogenases. Molecular weights of both the supernatant and mitochondrial enzymes seem to be the same in all higher organisms[24].

The most striking difference between the Drosophila enzymes and previously examined malate dehydrogenases seems to be that the mitochondrial enzyme is more heat stable than the supernatant form. The opposite condition has been reported for mitochondrial malate dehydrogenase from chicken[7] and tuna[8].

The mitochondrial malate dehydrogenases from the two Drosophila species are catalytically more similar to each other than to the supernatant enzyme from the same species, and *vice versa*. However, the malate dehydrogenases from these two species differ from each other in such properties as substrate inhibition, electrophoretic mobility and heat stability. Evidence of appreciable genetic similarity is the ability of anti-*D. virilis* mitochondrial malate dehydrogenase antibodies to partially inhibit *D. melanogaster* mitochondrial malate dehydrogenase activity. The antibody inhibited the enzyme of *D. melanogaster* about 70% as much as the enzyme of *D. virilis* at each dilution. This is similar to the value observed by DUKE AND GLASSMAN[25] for xanthine dehydrogenase in these two species.

D. virilis and *D. melanogaster* belong to different subgenera of the genus Drosophila. Using the complement fixation technique of WASSERMAN AND LEVINE[19], we plan to make quantitative comparisons of the mitochondrial malate dehydrogenase of several other species of Drosophila and other insects. Preliminary results using double diffusion OUCHTERLONY[18] plates suggest that there is a sizable amount of variation in levels of antigen–antibody interaction within the genus Drosophila. Further studies should produce data of considerable evolutionary interest.

ACKNOWLEDGMENTS

We should like to express our appreciation for the interest and support of the late Professor Wilson Stone. The authors are grateful to Mrs. Rebecca Mead and Mr. David Tobey for their technical assistance, and to Dr. Hugh Forrest for making a spectrofluorometer available for these studies.

This work was supported in part by research grants from the Public Health

Service (5 F2-GM-19965-04) and from the National Institutes of Health AI 07853 and GM 11609 (awarded to Dr. W. S. Stone)).

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