BEEF-HEART MALIC DEHYDROGENASES

II. PREPARATION AND PROPERTIES OF CRYSTALLINE SUPERNATANT MALIC DEHYDROGENASE

SASHA ENGLARD AND HILDA H. BREIGER

Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, New York, N.Y. (U.S.A.)

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SUMMARY

Differential extraction of beef-heart muscle revealed the presence of two malic dehydrogenases. One, apparently derived from the supernatant fraction, is easily extractable and exhibits no inhibition of oxidation of DPNH by elevated concentrations of oxaloacetate. The other, presumably mitochondrial in origin, requires more drastic means of extraction and under similar defined conditions of assay is inhibited by elevated concentrations of oxaloacetate.

A method of purifying the supernatant malic dehydrogenase is described. By a combination of ammonium sulfate fractionation, heat inactivation, chromatography on DEAE-cellulose, starch-block electrophoresis, and finally dialysis against ammonium sulfate solutions, a crystalline malic dehydrogenase has been prepared.

Electrophoretic studies over a wide range of pH values revealed that the preparation was essentially homogeneous.

The enzyme was also homogeneous in the ultracentrifuge, and had an $s^0_{20,w}$ of $5.1 \cdot 10^{-13}$ sec and a $D^0_{20,w}$ of $9.1 \cdot 10^{-7}$ cm^2/sec. From these data, assuming a partial specific volume of 0.74 ml/g, a molecular weight of 52000 was calculated.

Some kinetic constants of the enzyme have been determined and its specificity examined.

INTRODUCTION

In formulating a study of the pertinent structural features of enzymes catalyzing stereospecific hydrogen transfer reactions, we thought an enzyme of choice, because of its reportedly low molecular weight (15000–20000) was malic dehydrogenase prepared from acetone-dried powders of ox-heart mitochondria[1]. However, the kind of study considered would require large amounts of enzyme, the preparation of which from isolated mitochondria could only be tedious and perhaps impracticable. On the other hand, it is known that malic dehydrogenase obtained from acetone-dried powders of thoroughly washed minces of whole pig heart[2–4] (molecular weight, 40000 ± 5000 (see ref. 4), 56000 (see refs. 5, 6)) behaves kinetically like the "mitochondrial" enzyme of other tissues[7,8], *i.e.*, it is inhibited by elevated concentrations of oxaloacetate[9].

Similarities between the purified pig-heart enzyme and the malic dehydrogenases of apparent mitochondrial origin in other tissues have also been observed with other kinetic criteria[10,11] and from patterns of chromatographic behavior on various ion exchangers[10]. Encouraged by these findings, it was thought feasible to isolate the corresponding lower molecular weight mitochondrial malic dehydrogenase from acetone-dried powders of whole beef heart without first engaging in the separation of mitochondria.

While engaged in this aspect of the problem, we became aware of the presence of another malic dehydrogenase in freshly prepared minces of beef heart. This enzyme differed from the "mitochondrial" enzyme in its ease of extractability, its kinetic properties and physical characteristics. These findings agree with similar observations reported for other tissues[7,8,10,12–17] concerning the multiplicity of malic dehydrogenases with different properties.

The present paper reports the preparation in crystalline form of beef-heart supernatant malic dehydrogenase. In addition, some kinetic and molecular properties of this enzyme are described. A preliminary communication of this work has appeared[11].

EXPERIMENTAL

Throughout this study only glass-distilled water, deionized before distillation, was used. All other materials were used as previously described[6].

Methods of assay

Throughout purification, malic dehydrogenase activity was measured by the rate of DPNH oxidation at 360 mμ in a model PMQ II Zeiss spectrophotometer. Assays were carried out in cuvettes of 10-mm light path maintained at 30° using a total volume of 3.0 ml containing: 150 μmoles of triethanolamine, pH 7.6; 15 μmoles of EDTA (neutralized to pH 7.6–7.8); 0.440–0.474 μmole of DPNH; and 0.379 μmole of freshly dissolved oxaloacetic acid (unneutralized). Reactions were generally initiated by the addition of 10 μl of the various enzyme fractions diluted appropriately with 0.1 *M* potassium phosphate, pH 7.4; readings of absorbancy were taken at 30-sec intervals. A linear relationship was generally maintained for the first 5–6 min provided the changes in absorbancy at 360 mμ did not exceed 0.035/min. A unit of enzyme activity was defined as the amount of enzyme causing a change in absorption at 360 mμ of 0.010/min under the above specified conditions of assay. Specific activity was defined as the number of units/mg of protein as determined by the method of LOWRY *et al.*[18] using crystalline bovine serum albumin as a standard for protein content.

Kinetic data were obtained with a model 14 Cary recording spectrophotometer, and changes in absorbancy were recorded at 340 mμ. In some of these experiments, in order to increase the sensitivity of the method for measurements of initial rates, cylindrical silica cells of 5-cm light path were used.

Differential extraction of beef-heart muscle

The steps described by DELBRÜCK *et al.*[7] for the fractional extraction of various tissues were applied with several minor modifications to minces of beef-heart muscle. 2.5 g of fresh or 2-week old frozen, ground beef-heart muscle were suspended in 25 ml

of 0.25 *M* sucrose buffered at pH 7.3 with 0.01 *M* triethanolamine, and the mixture stirred mechanically for 15 min. The suspension was centrifuged at 78000 × *g* for 20–25 min in a preparative Spinco ultracentrifuge. The supernate was collected and the residue was re-extracted twice with fresh portions of buffered isotonic sucrose. The residue was then extracted for 2 min with 22.5 ml of the buffered sucrose solution by grinding in a heavy smooth-walled glass tube fitted with a teflon pestle and the suspension centrifuged as before. This procedure was repeated twice. The residue was next ground for 1 min with 12.5 ml of 0.1 *M* potassium phosphate pH 7.4 in a Potter-Elvehjem glass homogenizer; the suspension was diluted with an additional 5 ml of the same buffer and centrifuged in the preparative ultracentrifuge for 15 min at 105000 × *g*. The residue was again subjected to the same treatment except that grinding in the glass homogenizer was for 2 min. The residue was then resuspended in 17.5 ml of 0.1 *M* potassium phosphate, pH 7.4, and blended for 1 min at maximum speed in a Lourdes Multi-mixer high speed homogenizer. The suspension was centrifuged at 105000 × *g* and the residue extracted by the last procedure two more times.

Each extract, obtained sequentially as described, was assayed for malic dehydrogenase activity at two levels of oxaloacetate concentration. The composition of the reaction mixtures for the low oxaloacetate assay was identical with that described for the standard assay procedure (*i.e.*, 0.379 μmoles oxaloacetate, $1.26 \cdot 10^{-4}$ *M*). The high oxaloacetate assay reaction mixtures differed only in that the substrate concentration was increased by a factor of 10 (3.79 μmoles oxaloacetate, $1.26 \cdot 10^{-3}$ *M*). Assays were carried out at room temperature and initial rates of DPNH oxidation recorded with a model 14 Cary spectrophotometer at 360 mμ. Changes in absorption per minute at at 360 mμ, when assays were performed, could then be converted using an experimentally determined ratio to changes in absorbancy per minute at 340 mμ, and enzyme activity expressed as μmoles of reduced pyridine nucleotide oxidized/min was calculated from the extinction coefficient of $6.22 \cdot 10^6$ cm²/mole for DPNH (see ref. 19).

RESULTS

Multiplicity of malic dehydrogenases in beef-heart muscle

In agreement with observations reported for *Locusta migratoria* flight muscle[7] and several rat tissues[8], beef-heart muscle appears to contain two malic dehydrogenases differing in ease of extractability from minced tissue and in sensitivity to inhibition by elevated concentrations of oxaloacetate (Table I). From ratios of enzymic activities measured at low and high oxaloacetate concentrations, it may be seen in this table that "mitochondrial" enzyme, recognized by its strong capacity for inhibition by oxaloacetate, is extracted from tissue minces only when comparatively drastic procedures are used for solubilization. This enzyme is relatively stable to treatment with acetone and ethanol, and has been extracted and purified from acetone-dried powders of thoroughly washed minces of whole beef heart* [5,6]. The supernatant enzyme, obtained in soluble form by merely stirring the ground tissue with isotonic sucrose, does not appear to be significantly affected by higher concentrations of oxaloacetate under the specified conditions of assay. The method for purification of this malic dehydrogenase is outlined below.

* Henceforth in this paper, when reference is made to this enzyme it will be denoted as M-MDH.

Purification of beef-heart supernatant malic dehydrogenase

Unless otherwise indicated all operations were carried out at 3–5°. All additions of solid ammonium sulfate were carried out at 0° with the pH of each solution being maintained between 7.1 and 7.3 by dropwise addition of approx. 2 *N* ammonium hydroxide.

TABLE I

DIFFERENTIAL EXTRACTION OF BEEF-HEART MALIC DEHYDROGENASE

Conditions of sequential extractions	*Number of extractions using indicated conditions*	*Total units of malic dehydrogenase in supernate**	*Specific activity***	*Ratio of low: high oxaloacetate assay*
Mechanical stirring with 0.25 *M* sucrose	1	431	9.5	1.12
+ 0.01 *M* triethanolamine, pH 7.3	2	45	—	1.15
	3	14	—	1.22
Homogenization in teflon homogenizer	1	55	—	2.25
with 0.25 *M* sucrose + 0.01 *M*	2	28	—	2.50
triethanolamine, pH 7.3	3	22	—	2.62
Homogenization in glass homogenizer	1	211	15.8	3.17
with 0.1 *M* potassium phosphate, pH 7.4	2	46	—	2.32
Homogenization in high speed Lourdes	1	115	35.8	2.47
homogenizer with 0.1 *M* potassium	2	46	—	2.44
phosphate, pH 7.4	3	19	—	2.40

* Expressed as μmoles of DPNH oxidized/min/g wet weight of freshly minced tissue as assayed at the low oxaloacetate concentration (*cf.* text). The recovery of malic dehydrogenase activity for the combined fractions was equivalent to that of an independent experiment in which the tissue was exhaustively extracted by the single procedure of blending in a Lourdes high speed homogenizer with 0.1 *M* potassium phosphate, pH 7.4.

** Expressed as μmoles of DPNH oxidized/min/mg of protein as assayed at the low oxaloacetate concentration.

Step 1: Fresh beef hearts, obtained from the slaughter house and transported to the laboratory on ice, were dissected as free as practical of fat, diced and passed through a mechanical meat grinder. One kilogram of mince was suspended in 5 l of ice cold 0.25 *M* sucrose buffered at pH 7.6 with 0.01 *M* triethanolamine and stirred mechanically for 15 min. The suspension was passed through several layers of cheese cloth and the residue squeezed with a Pexton hand press*. The initial extract usually contained a total of 29.96 to 35.64 g of protein with a specific activity of 1230 to 1639 units/mg protein. From the residue, after additional washings with cold distilled water, an acetone-dried powder was prepared from which M-MDH was subsequently extracted and purified[5,6].

Step 2: The pH of the crude extract was raised to 7.2 by addition of dilute ammonium hydroxide, solid ammonium sulfate was added slowly to 40% saturation (28.3 g/100 ml), and the mixture was stirred for 45 min. One gram of Hyflo Supercel (Johns-Manville) was then added for each 100 ml of suspension, and the mixture filtered through Eaton and Dikeman grade No. 192 folded filter paper. Ammonium sulfate (30.4 g/100 ml of initial extract) was added gradually to the clear filtrate to 82% saturation and the mixture stirred for 60 min. The suspension was centrifuged

* Supplied by the Lee Engineering Company, Milwaukee 1, Wisconsin (U.S.A.).

for 40 min at 18000 × *g* or alternatively was passed through a KSB-R Servall 8-tube continuous flow system at a rate of approx. 40 ml/min while employing a rotor speed of 18500 rev./min. The supernate was discarded; the precipitate was dissolved in 0.05 *M* potassium phosphate pH 7.4 in 0.001 *M* EDTA to a total volume equivalent to 40% of the initial weight of the mince (400 ml). Specific activity, 3021–4533 units/mg protein.

Step 3: The solution obtained in step 2 was next brought to approx. 35% saturation with salt by addition of solid ammonium sulfate (24.7 g/100 ml). The mixture was stirred for 45 min, centrifuged for 1 h at 18000 × *g*, and the residue discarded. The clear supernate was rapidly heated to 60° in a boiling water bath (2.25–2.5 min), and maintained between 62 to 64° for 10 more minutes. After rapid cooling in a salt–ice bath at —10°, the heat-treated solution was kept at 0° for 30–45 min and centrifuged as before. Solid ammonium sulfate was added to the supernate to 43% saturation (5.7 g/100 ml), and the solution stirred for 45 min. The suspension was centrifuged and the precipitate discarded. The ammonium sulfate concentration of the supernate was raised to 72% saturation by further addition of salt (20.4 g/100 ml), and the solution stirred for 1 h. The precipitate was collected by centrifugation at 18000 × *g* for 90 min, dissolved in a minimum volume of 0.01 *M* potassium phosphate buffer of pH 6.9 and dialyzed against this buffer for 18–24 h; several changes of dialyzing medium were made. The dialyzed solution was clarified by centrifugation at 18000 × *g* for 75–90 min. Specific activity, 5833–9364 units/mg protein.

Step 4: DEAE-cellulose with an exchange capacity of 0.7 mequiv./g was washed successively with potassium phosphate buffers, pH 6.7 to 6.9, of decreasing concentrations starting with 0.2 *M*. The anion exchange cellulose was finally equilibrated with 0.01 *M* potassium phosphate, pH 6.9. A slurry containing 50 g of equilibrated DEAE was poured into a 4-cm I.D. column and the cellulose permitted to pack by gravity and occasional gentle tapping to liberate trapped air bubbles. Under these conditions a column height of 21.5–22 cm was generally obtained. The dialyzed solution from step 3 was passed through this column and 40 ml of 0.01 *M* potassium phosphate, pH 6.9, were added to the glass column above the DEAE-cellulose bed. A dropping funnel containing the same buffer was then attached to the column and approx. 660 ml of effluent was collected (including the effluent obtained by the initial passage of the dialyzed enzyme solution through the column). This solution, deep red in color, contained 31–40% of the protein and 2.4–6.2% of the total units of malic dehydrogenase initially placed on the column. The enzyme in this fraction was sensitive to elevated concentrations of oxaloacetate, and therefore appeared to be malic dehydrogenase of mitochondrial origin. A similar unretarded behavior of rat liver "mitochondrial" malic dehydrogenase on DEAE-cellulose (at pH 8.2 in Tris buffer) has recently been reported[10]. The column was next connected to a mixing flask containing 400 ml of 0.01 *M* potassium phosphate, pH 6.9, which in turn was connected to a reservoir containing 0.05 *M* potassium phosphate, pH 6.9. A rapidly spinning magnetic bar in the mixing flask insured thorough mixing of the dilute buffer with the more concentrated buffer entering from the reservoir. A uniform and gradual increase in potassium phosphate concentration of solution entering the DEAE-cellulose bed was thus achieved. Eluate was collected at a rate of 30–35 ml/h. Thus an initial 500-ml fraction containing insignificant malic dehydrogenase activity was

collected. Small fractions (15–20 ml) were then collected with an automatic fraction collector. Malic dehydrogenase of increased specific activity emerged from the column in effluent fluid which had a phosphate concentration of 0.028 *M*. Fractions with increased specific activity (5 fold or greater) were pooled, and the combined solution was reduced in volume to 3–5 ml by means of pressure dialysis. The concentrated enzyme solution, moderately red in color, was dialyzed against sodium barbital buffer of 0.038 ionic strength at pH 8.6 for 22–24 h; at least two changes of buffer were made during the dialysis. At this stage of purification, the specific activity ranged from 42698 to 47151 units/mg protein.

Step 5: 500 g of Mallinckrodt potato starch was washed and equilibrated with sodium barbital buffer of 0.038 ionic strength at pH 8.6. After equilibration, a thick paste was made by suspending the starch in 225–250 ml of the same buffer. The paste was poured onto a hollow lucite platform with water at 0° circulating beneath it. Excess buffer was removed and the starch block was trimmed to a size of 25.5 × 15 cm (height 1.0–1.2 cm). A strip of 0.3–0.5 cm width was removed from the center of the block. The dialyzed solution obtained in step 4 was mixed with dry starch and the heavy paste was poured into the prepared channel. Electrophoresis was performed for 18–20 h at a constant potential gradient of 350 V (current initially, 27 to 29 mA). At termination of electrophoresis the block was sliced into 0.5-cm strips starting at the center and moving toward the anode. Each starch sample was washed 7 times with 3-ml portions of 0.1 *M* potassium phosphate, pH 7.4, on a sintered glass filter of medium porosity. The fractions obtained in this way were adjusted to a total volume of 25.0 ml, and assayed for enzymic activity and for protein content. Under normal conditions, malic dehydrogenase with the highest specific activity and in greatest amount was located in a 1.5-cm band approx. 4.5 cm from the origin in the direction of the anode, clearly separated from a slower moving diffuse light red band. Since no loss of enzymic activity was experienced in this step, the fractions of lower specific activity could be combined, concentrated and after dialysis again subjected to electrophoretic separation in order to increase further the total yield of material of high specific activity*.

Step 6: The electrophoretically separated fractions with similar highest specific activities were combined (specific activity ranging from 70572 to 77486 units/mg protein) and reduced in volume by means of pressure dialysis so that a solution containing 25–30 mg protein/ml was obtained. The concentrated enzyme solution was then equilibrated by dialysis with 55% saturated ammonium sulfate in 0.05 *M* potassium phosphate, pH 6.2 (prepared by diluting a neutralized solution of ammonium sulfate saturated at 3–5° appropriately with 0.5 *M* potassium phosphate, pH 6.2, and water). If a turbidity developed at this stage, the solution was clarified by centrifugation. The dialyzing medium was then changed to 58% ammonium sulfate in 0.05 *M* potassium phosphate, pH 6.2, and dialysis continued for at least 24 h. The solution was centrifuged for 1 h at 41000 × *g* to remove a slight amorphous precipitate which had formed. The clear supernate was then dialyzed against 60% ammonium sulfate in 0.05 *M* potassium phosphate, pH 6.2, and slow crystallization

* The more active fractions, after a second electrophoresis, yielded some cuts with specific activities equalling that of the crystalline enzyme obtained subsequently. The total units of activity in these fractions, however, indicated poorer recovery than that obtained by inclusion of step 6 in the purification procedure.

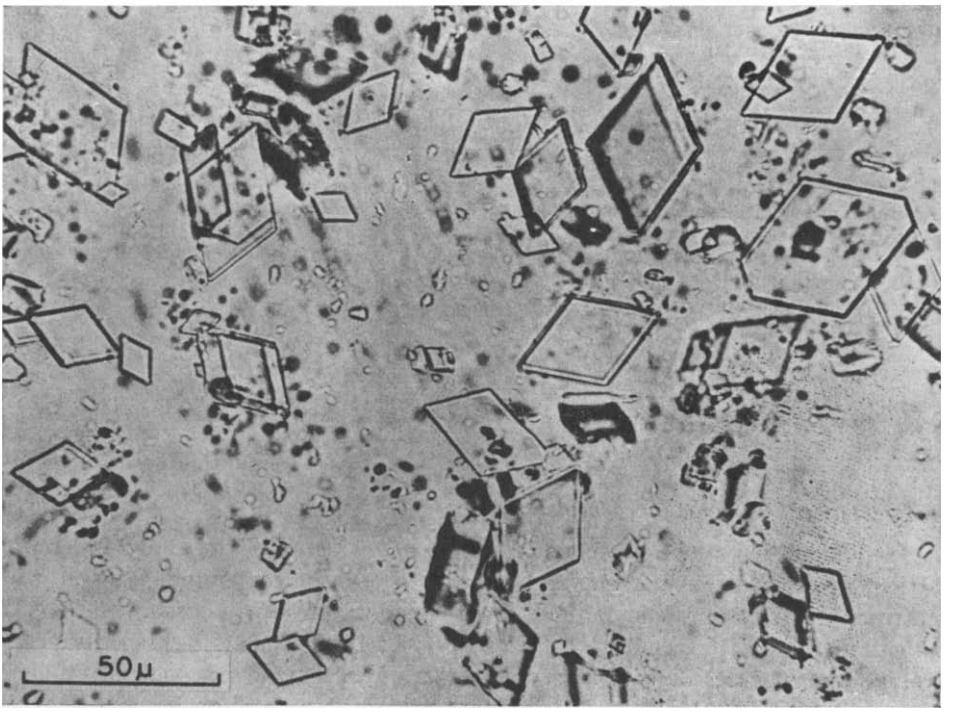

Fig. 1. Crystalline precipitate of beef-heart supernatant malic dehydrogenase.

ensued as evidenced by appearance of a characteristic silky sheen when the dialysis bag was swirled. (Crystallization usually began within 24–36 h.) The crystals settled rapidly, and were maximally precipitated under these conditions within 48–72 h from the time of appearance of the first turbidity*. Crystals were harvested by centrifugation, dissolved in 0.1 *M* potassium phosphate, pH 7.4, and recrystallized between 58 and 60% saturated ammonium sulfate in 0.05 *M* potassium phosphate, pH 6.2, using the procedure just described. The specific activity of the enzyme in the first crystalline crop and after one recrystallization was significantly increased, and repeated recrystallizations did not lead to further significant increases in specific activity. The specific activity of the first mother liquor was substantially lower than that of the first crystalline material, but mother liquors obtained after repeated recrystallizations finally approached the specific activity of the crystals. Fig. 1 shows the diamond-shaped appearance of crystals of supernatant malic dehydrogenase obtained after one recrystallization. In absence of 0.05 *M* potassium phosphate, pH 6.2, the enzyme crystallizes at a slightly higher concentration of ammonium sulfate (between 62 and 65% saturation). The crystals obtained under these conditions, however, consist of large plates with elongated hexagonal faces.

Table II summarizes the pertinent data from the various steps of a typical preparation.

* If crystallization was incomplete, as revealed by the presence of significant levels of malic dehydrogenase activity in the mother liquor, additional quantities of crystalline material could be obtained by slowly increasing the ammonium sulfate concentration of the dialyzing medium up to 62–63% saturation.

TABLE II

PURIFICATION OF BEEF-HEART SUPERNATANT MALIC DEHYDROGENASE

Steps	*Total malic dehydrogenase units* $\times 10^{-3}$	*Total protein (mg)*	*Specific activity (units/mg protein)*	*Yield (percent)*
1. Crude extract	53 295	32 510	1 639	—
2. Ammonium sulfate fractionation	40 000	10 816	3 698	75.1
3. Heat inactivation and ammonium sulfate fractionations	41 920	5 769	7 267	78.7
4. DEAE fractionation	23 608	552.9	42 698	44.3
5. Starch electrophoresis	18 000	232.3	77 486	33.8
6. Crystallization:				
1st crystalline crop	11 464	131.6	87 112	21.5
1st mother liquor	4 138	66.5	62 226	—
1st recrystallization	7 523	72.7	103 445	14.1
Mother liquor of recrystallization	4 403	45.9	95 929	—

Sedimentation and diffusion coefficients

Sedimentation studies were carried out as previously described[6] using 4 concentrations of purified enzyme ranging from 2.15 to 5.70 mg protein/ml. Prior to ultracentrifugation, the protein samples were equilibrated by dialysis with 0.05 *M* potassium phosphate in 0.001 *M* EDTA, pH 7.1. Experiments were performed at average temperatures ranging from 8.5 to 13.2°. The enzyme sedimented as a single component (Fig. 2) and the sedimentation velocity appeared to be essentially independent of protein concentration. $s^0_{20,w}$ was calculated to be $5.1 \cdot 10^{-13}$ sec.

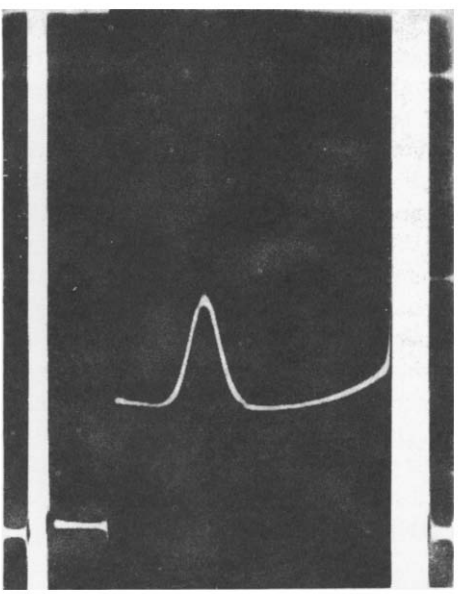

Fig. 2. Sedimentation velocity pattern of supernatant malic dehydrogenase. 4.90 mg protein/ml in 0.05 *M* potassium phosphate and 0.001 *M* EDTA, pH 7.1, at an average temperature of 8.5°. Rotor speed, 59780 rev./min. The exposure was taken 72 min after full speed was attained, using a bar angle of 50°.

Diffusion measurements were performed as previously described[6] using 4 concentrations of the enzyme ranging from 3.54 to 6.41 mg protein/ml. The diffusion of the enzyme was studied at average temperatures of 0.6 to 0.8° in the same buffer as employed in the ultracentrifugation studies. $D^0_{20,w}$ was calculated to be $9.1 \cdot 10^{-7}$ cm²/sec.

Assuming a partial specific volume of 0.74 ml/g, a molecular weight of 52000 was calculated from the sedimentation and diffusion data.

Electrophoretic behavior

Samples of the purified supernatant malic dehydrogenase, at protein concentrations ranging from 2.08 to 5.68 mg/ml, were dialyzed against the following buffers each containing EDTA in a final concentration of 0.001 *M*: 0.035 *M* sodium citrate, pH 3.91; 0.025 *M* sodium citrate, pH 4.73; 0.1 *M* sodium citrate, pH 4.97; 0.02 *M* sodium citrate, pH 5.15; 0.1 *M* sodium acetate, pH 4.55; 0.05 *M* potassium phosphate, pH 6.20; 0.05 *M* potassium phosphate, pH 7.12; and 0.045 *M* sodium barbital, pH 8.75. Following dialysis small samples of the enzyme in each of the buffers studied were removed and assayed for activity. It was thus ascertained that no significant loss in activity occurred in the pH range of 4.55 to 8.75. At pH 3.91, considerable inactivation occurred, approx. 22% of the initial activity remaining after dialysis. Electrophoretic measurements were then made in a model 38A Perkin-Elmer instrument at an average temperature of 1°. Electrophoresis was generally continued until the boundary migrated across the cell. Within limits of the electrophoretic analysis at the pH values studied the protein exhibited a single peak migrating at a uniform rate. A plot of the electrophoretic mobility as a function of pH (Fig. 3) permitted an approximation of the isoelectric point (pH at which no migration occurs), and a value of pH 4.6 to 4.7 was obtained.

Kinetics

At pH 6.7 in 0.5 *M* potassium phosphate, the rate of DPNH oxidation reached a maximum when oxaloacetate concentration was $1.3 \cdot 10^{-4}$ *M* and no inhibitory effects

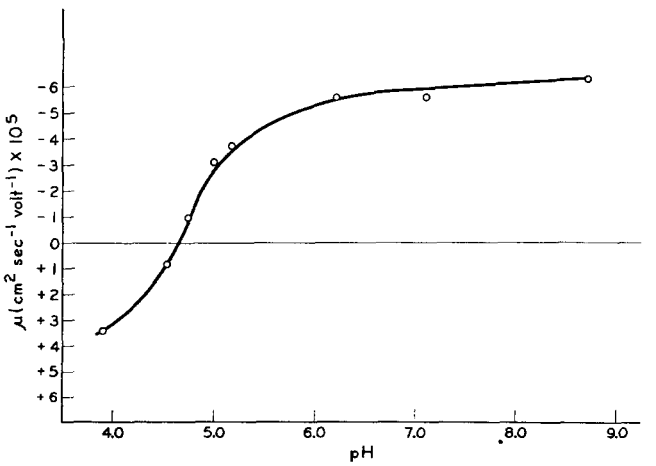

Fig. 3. Electrophoretic mobility as a function of pH for the supernatant malic dehydrogenase. pH as measured with glass electrode at 0°. Buffers and other conditions as described in text.

were observed at substrate concentrations as high as $1.9 \cdot 10^{-3}\,M$. In presence of a constant concentration of DPNH ($1.36 \cdot 10^{-4}\,M$), the apparent K_m value for oxaloacetate was $4.2 \cdot 10^{-5}\,M$ at pH 6.7 (Fig. 4).

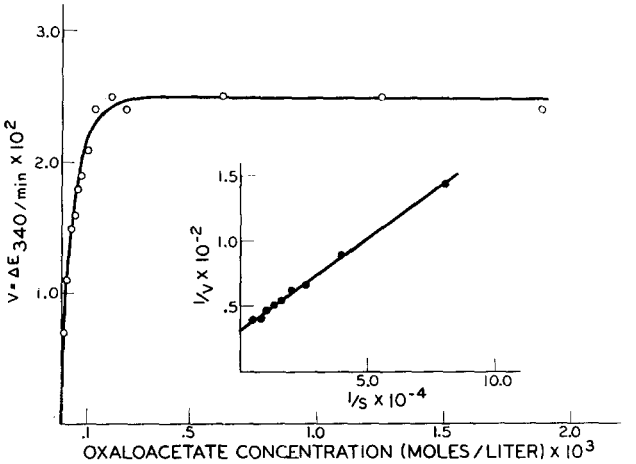

Fig. 4. Relationship between initial velocity of DPNH oxidation and concentration of oxaloacetate. In addition to oxaloacetate, the reaction mixtures contained 1500 μmoles of potassium phosphate, pH 6.7, 6.4 μmoles of EDTA, 0.41 μmole of DPNH and 0.026 μg of enzyme protein in a total volume of 3.02 ml.

The apparent K_m value for DPNH, determined at a constant concentration of oxaloacetate of $2.5 \cdot 10^{-4}$ at pH 6.7 in 0.5 M potassium phosphate, was $2.7 \cdot 10^{-5}\,M$.

As shown in Fig. 5, the rate of DPN reduction at pH 8.4 in 0.1 M Tris reached a maximum at L-malate concentration of $1.6 \cdot 10^{-2}\,M$ and inhibitory effects were already evident at substrate levels of $3.9 \cdot 10^{-2}\,M$. The apparent K_m values determined by extrapolation of the straight line obtained in the double reciprocal plot[20] in the non-inhibitory range of L-malate concentrations, was $4.7 \cdot 10^{-4}\,M$ at pH 8.4 in 0.1 M Tris at a constant DPN concentration of $5.35 \cdot 10^{-4}\,M$ (Fig. 5).

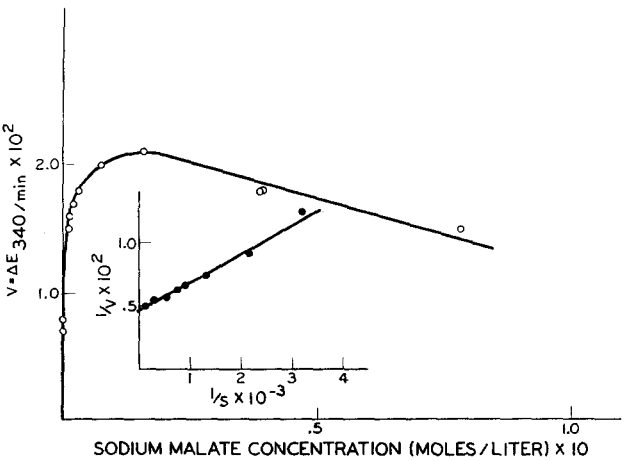

Fig. 5. Relationship between initial velocity of DPN reduction and concentration of L-malate. In addition to L-malate the reaction mixtures contained 1600 μmoles of Tris, pH 8.4, 8.61 μmoles of neutralized DPN and 0.131 μg of enzyme protein in a total volume of 16.1 ml.

Under similar conditions of assay, at a constant optimum concentration of $1.55 \cdot 10^{-2}$ *M* L-malate, an apparent K_m of $9.9 \cdot 10^{-5}$ for DPN was obtained.

Specificity

α-Ketobutyrate and pyruvate were inactive as substrates for supernatant malic dehydrogenase. Although DPNH oxidation and DPN reduction were detectable at high enzyme concentrations in the presence of α-ketoglutarate and tartronate, respectively, rates were extremely slow. With L-malate (0.1 *M*) the rate of DPN reduction was 94 μmoles/min/mg of protein at pH 9.9 in 0.09 *M* glycine–NaOH, compared with corresponding specific activities of 0.32 for mesotartarate (0.16 *M*) and 0.44 for D(−) tartarate (0.16 *M*). The enzyme was completely inactive with L(+) tartarate. At pH 6.7 in 0.5 *M* potassium phosphate, relative specific activities of 487 and 13, in terms of μmoles of DPNH oxidized/min/mg of protein, were obtained with oxaloacetate ($2.5 \cdot 10^{-4}$ *M*), and mesoxalate ($8.3 \cdot 10^{-3}$ *M*), respectively. The rate of oxidation of TPNH by oxaloacetate in 0.025 *M* glycylglycine pH 7.5 was 1.1% of that observed with DPNH.

DISCUSSION

Much of the evidence for the occurrence of multiple forms of an enzyme in a single tissue rests on electrophoretic distributions obtained using either crude homogenates or extracts of various organs and may, in addition to the uncertainties inherent in the methodology[21–22], also be complicated by existence of different cell types in the tissue. However, a similar multiplicity of electrophoretically distinct dehydrogenases of similar catalytic specificities, has also been demonstrated recently in cytologically homogeneous material such as cells grown in tissue culture[14].

At another level, the two forms of malic dehydrogenase so far recognized appear to be connected with different subcellular units. Thus, results obtained by differential centrifugation of a rat liver homogenate indicate that malic dehydrogenase is localized in both supernatant and mitochondrial fractions[23]. A similar pattern of distribution can be deduced from the results obtained by DELBRÜCK *et al.*[7,8] for flight muscle of *Locusta migratoria* and for several rat tissues. The extra- and intramitochondrial malic dehydrogenases in these tissues can also be differentiated from one another by the kinetics with which they catalyze the reduction of oxaloacetate by DPNH[7,8], by their electrophoretic mobilities[13] and by chromatographic behavior on Amberlite IRC-50 and DEAE-cellulose[10]. The results obtained in this study on the distribution, kinetics, and chromatographic behavior of beef-heart malic dehydrogenases are therefore in agreement with similar observations reported for these enzymes in other tissues. Similar results with beef heart have also been reported by GRIMM AND DOHERTY[24].

The enzyme purified from acetone-dried powders of thoroughly washed minces of beef heart shows the same kinetic properties as the enzyme extracted from fresh tissues by more drastic procedures, and can therefore be identified as the malic dehydrogenase of mitochondrial origin. Essentially homogeneous preparations of this enzyme have been obtained and some of its physical and kinetic properties described[5,6]. The purification and crystallization of the supernatant malic dehydrogenase permitted characterization of this enzyme so that comparison could be made with the enzyme of mitochondrial origin. Some physical and kinetic properties of the two beef heart malic

dehydrogenases are compared in Table III. The sedimentation coefficients are significantly different, and the diffusion coefficients show almost a 50% difference. These differences are also reflected in the calculated molecular weights of the two enzymes.

TABLE III

COMPARISON OF PURIFIED BEEF-HEART SUPERNATANT MALIC DEHYDROGENASE WITH THE PURIFIED M-MALIC DEHYDROGENASE ISOLATED FROM ACETONE-DRIED POWDERS OF WHOLE BEEF HEART

	Supernatant malic dehydrogenase	*"Mitochondrial" malic dehydrogenase (M-MDH)**
Molecular properties		
$s^0_{20,w}$ (sec)	$5.1 \cdot 10^{-13}$	$4.3 \cdot 10^{-13}$
$D^0_{20,w}$ (cm²/sec)	$9.1 \cdot 10^{-7}$	$6.5 \cdot 10^{-7}$
Molecular weight	52000	62000
Isoelectric point	4.6–4.7	5.5–5.6
Apparent Michaelis const.**		
Koxaloacetate	$\cdot \cdot 10^{-5}$ M	$3.4 \cdot 10^{-5}$ M
KDPNH	$2.7 \cdot 10^{-5}$ M	$5.2 \cdot 10^{-5}$ M
Kmalate	$4.7 \cdot 10^{-4}$ M	$2.5 \cdot 10^{-4}$ M
KDPN	$9.9 \cdot 10^{-5}$ M	$9.9 \cdot 10^{-5}$ M

* Data from SIEGEL AND ENGLARD[5,6].

** Values for the same substrate determined in the same buffer at the same pH for each of the two enzymes (*cf.* text).

The isoelectric points, as determined by the method of moving electrophoresis, are considerably different. It is to be noted that the maximum mobility values for the supernatant malic dehydrogenase are much greater than those previously reported for the M-MDH[6]. These differences probably account for the observed electrophoretic and chromatographic behavior of the two enzymes in crude extracts or homogenates of various tissues. The stability of the two malic dehydrogenases also differ to a remarkable extent. The supernatant enzyme retains full activity at pH 4.6 whereas the purified M-MDH was completely inactivated at this pH and retained only 51% of its initial activity at pH 5.5 (see ref. 6). The apparent K_m values for the two enzymes, determined under identical conditions of assay, do not appear to be significantly different. However, the kinetics of reduction of oxaloacetate and of oxidation of malate by the two purified beef heart malic dehydrogenases are considerably different. With purified M-MDH, at pH 6.7 in 0.5 M potassium phosphate buffer, the rate of oxaloacetate reduction was maximum at a substrate concentration of $1.3 \cdot 10^{-4}$ M and inhibition already was apparent at an oxaloacetate concentration of $2.6 \cdot 10^{-4}$ M (see refs. 5, 6). Under similar conditions of assay, purified supernatant enzyme achieved optimum rates at approximately the same substrate concentration* but maximum rates were unaffected by oxaloacetate concentrations as high as

* Although the supernatant malic dehydrogenases of both *Locusta migratoria* and rat liver also differ considerably from their corresponding mitochondrial enzymes with respect to concentration of oxaloacetate yielding maximum rates[7,8], THORN[10] has indicated that for each enzyme the optimal substrate concentration depends on both the pH and the nature of the buffer used. It is therefore not surprising that under the arbitrary conditions of assay used in the present study both beef-heart enzymes achieve maximum rates at the same oxaloacetate concentrations.

$1.9 \cdot 10^{-3}$ *M*. On the other hand, although elevated concentrations of L-malate inhibited supernatant malic dehydrogenase at pH 8.4 in 0.1 *M* Tris, M-MDH was unaffected under these conditions of assay. Actually, using other conditions of assay (0.09 *M* glycine–NaOH, pH 9.8), purified M-MDH was activated in an anomalous manner by elevated concentrations of L-malate[5,6]. Such behavior has previously been described for other preparations of malic dehydrogenase of apparent mitochondrial origin[1,4].

The persistent kinetic and molecular differences between essentially homogeneous preparations of M-MDH and supernatant malic dehydrogenase from beef-heart muscle leave little doubt that we are dealing with structurally different protein entities. Comparison of the structural features of these two enzymes, particularly an examination of their active centers, would be a singularly opportune way of studying the mechanism of action of these dehydrogenases.

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