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MALATE DEHYDROGENASE (DECARBOXYLATING) (NADP) ISOENZYMES OF OPUNTIA STEM TISSUE

MITOCHONDRIAL, CHLOROPLAST, AND SOLUBLE FORMS

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SUMMARY

Three malate dehydrogenase (decarboxylating) (NADP) (L-malate:NADP oxidoreductase (decarboxylating), EC 1.1.1.40) isoenzymes were separated from a partially purified Opuntia extract by anion-exchange column chromatography and starch-gel electrophoresis. One isoenzyme (C-MDH) corresponded chromatographically to a malate dehydrogenase (decarboxylating) (NADP) isolated from non-aqueously prepared chloroplasts while another (M-MDH) corresponded with a form isolated from mitochondria purified by sucrose density-gradient centrifugation. An additional form, not associated with an organelle, was designated as a soluble isoenzyme (S-MDH). Some differences were noted among kinetic and enzymatic properties such as pH optima, heat of activation (ΔH), thermostability, K_m's for L-malate, manganese, and NADP. Differential inhibition by substrate analogs such as D-malate, citraconate, and citramalate was observed. D-Malate was a competitive inhibitor with respect to L-malate for the mitochondrial enzyme while a non-competitive inhibitor for the chloroplast and the supernatant forms. Thus three distinctly different proteins with malic enzyme activity are present in green tissue of Opuntia, *viz.*, a mitochondrial form (M-MDH), a chloroplast form (C-MDH), and a soluble or non-particulate form (S-MDH).

INTRODUCTION

In a previous paper concerned with the intracellular distribution of CO_2 metabolism enzymes in *Opuntia phylloclades*, we reported malate dehydrogenase (decar-

Abbreviations: C-MDH, M-MDH and S-MDH, chloroplast, mitochondrial and soluble malate dehydrogenase (decarboxylating) (NADP) isoenzymes, respectively; MES, 2-(*N*-morpholino) ethane sulfonic acid; TES, *N*-Tris (hydroxymethyl) methyl-2-amino ethane sulfonic acid; PMS, phenazine methosulfate; NBT, nitro blue tetrazolium.

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boxylating) (NADP) (L-malate:NADP oxidoreductase (decarboxylating), EC 1.1.1.40) activity in mitochondria purified by sucrose density gradients and in non-aqueously purified chloroplasts[1]. It was of importance to ascertain whether the enzymic activity in the different subcellular particles represented different proteins, *i.e.*, isoenzymes, and if different, whether or not their kinetic properties were different. Multiple enzymes are well known in plant tissues[2], however, to our knowledge no reports concerning compartmentation of malate dehydrogenase (decarboxylating) (NADP) isoenzymes are published. The question of biological significance of enzymes necessitates a precise knowledge of intracellular distribution.

MATERIALS AND METHODS

Reagents

L-Malic acid, D-malic acid, NADP, 2-(*N*-morpholino) ethane sulfonic acid (MES), *N*-Tris (hydroxymethyl) methyl-2-amino ethane sulfonic acid (TES), DEAE-cellulose, citraconic acid, tartronic acid, and phenazine methosulfate (PMS) were obtained from Calbiochem (Los Angeles, Calif.). Trizma–HCl, Trizma–base, dihydroxymaleic acid, citramalic acid, and nitro blue tetrazolium (NBT) were obtained from Sigma Chemical Corp. (St. Louis, Mo.). Hydrolyzed starch for starch-gel electrophoresis was from Connaught Medical Research Laboratories, Toronto, Canada. All other chemicals were of reagent grade.

Enzyme preparation

Mitochondria, non-aqueous chloroplasts and soluble supernatant fractions were prepared similarly to our earlier report[1] from young stem tissue of *Opuntia ficus-indica* Mill. After homogenation in a Waring blender, the initial extract was prepared by $(NH_4)_2SO_4$ fractionation between 0.2 and 0.9 satn. After 12 h in the cold, the resulting precipitate was centrifuged at 20 000 $\times$ *g* for 30 min. The precipitate was resuspended in 0.01 M Tris (pH 7.4) and dialyzed against three changes of the same buffer. A second $(NH_4)_2SO_4$ precipitation from 0.3 to 0.8 satn. was treated similarly. The final suspension of protein in 0.01 M Tris (pH 7.4), was used for subsequent work.

DEAE-cellulose column chromatography

Three malate dehydrogenase (decarboxylating) (NADP) isoenzymes were separated on a 1 $\times$ 10 (2.5 cm $\times$ 25 cm of 0.61 mequiv/g) DEAE-cellulose column equilibrated with 0.005 M Tris buffer (pH 7.4). The partially purified enzyme preparation (914 mg protein) was placed on the column and eluted with the following buffers (all pH 7.4): 40 ml of 0.005 M Tris, 40 ml of 0.02 M Tris, 80 ml of 0.05 M Tris, 100 ml of 0.1 M Tris, 100 ml of 0.15 M Tris, 130 ml of 0.2 M Tris, 200 ml of 0.18 M Tris *plus* 0.1 M NaCl, 150 ml of 0.15 M Tris *plus* 0.2 M NaCl, and 250 ml of 0.2 M Tris *plus* 0.2 M NaCl. Fractions of 8 ml were collected. The fractions with maximum activity were combined and used for subsequent kinetic and electrophoretic studies.

Assays

Enzymic activity was determined with a dual beam Cary spectrophotometer equipped with an automatic recorder and sample changer by measuring the increase in absorbance of NADP at 340 mμ during reduction. Heat of activation and other

temperature studies were conducted with a temperature control cuvette holder. Assay mixtures contained 0.5 mM L-malate, 0.4 mM $MnCl_2$, 0.1 mM NADP, 0.05 M Tris (pH ranges between 7.2 and 8.5) or 0.05 M MES (pH ranges between 5.0 and 7.0) or 0.05 M TES (pH ranges between 6.5 and 8.5) and enzyme. The total volume was 3.0 ml. Initial rates were used in calculations of activities. Specific activities are expressed as change in absorbance/min per mg protein.

Protein was determined in the purified preparations by the 280–260 mμ method of LAYNE[3]. During the initial stages of purification, the phenol reagent method[4], as described earlier[5], was used.

Temperature stability was estimated by diluting the enzyme preparation with 0.005 M Tris buffer (pH 7.4) to obtain an activity of 0.1 A/min per ml enzyme. Preparations were treated by heating at the appropriate temperatures in the reservoir of a constant-temperature bath. Temperature was controlled to within 0.1°. Samples were

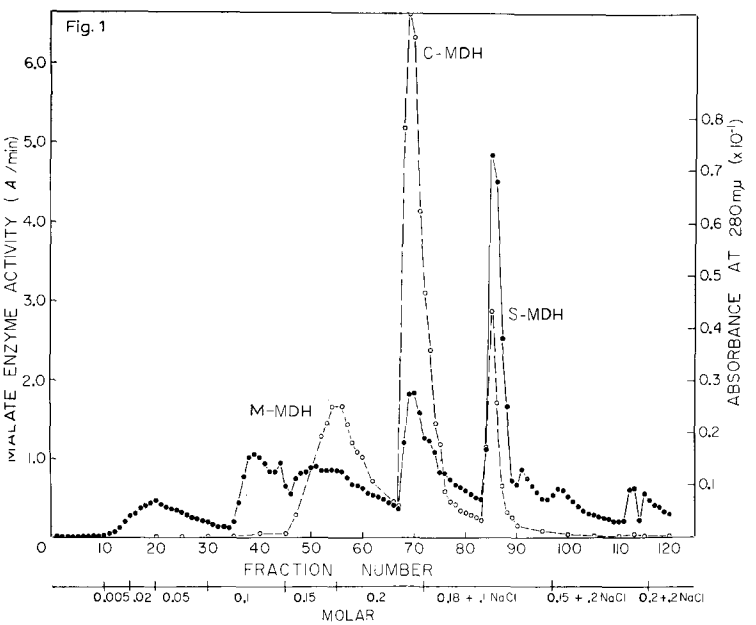


Fig. 1. DEAE-cellulose separation of three proteins with malate dehydrogenase (decarboxylating) (NADP) activity. Column, 2.5 cm × 25 cm of 0.61 mequiv/g (equilibrated with 5 mM Tris (pH 7.4). 914 mg of protein were added and eluted with the given concentrations of salt and buffer. 8-ml fractions were collected. ●, protein profiles; ○, malate dehydrogenase (decarboxylating) (NADP) activity.

withdrawn at various times, chilled immediately in an ice bath, and subsequently assayed for malate dehydrogenase (decarboxylating) (NADP) activity.

Starch-gel electrophoresis

Starch-gel electrophoresis was performed according to FINE AND COSTELLO[6] as described earlier[7] using phosphate–citrate buffer at pH's 7.0 and 8.0.

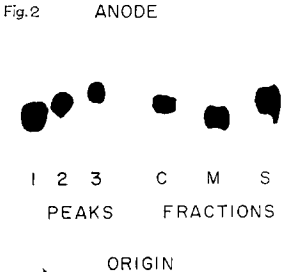
RESULTS

DEAE-cellulose column separation of malate dehydrogenase (decarboxylating) (NADP) isoenzymes

Three peaks with malate dehydrogenase (decarboxylating) (NADP) activity were eluted from a DEAE-cellulose column with a Tris–NaCl buffer (Fig. 1). Peak 1, eluted at approx. 0.15 M Tris, Peak 2 at about 0.2 M Tris, and Peak 3 at 0.1 M NaCl in 0.18 M Tris. Starch-gel electrophoresis indicated that each peak was enzymatically pure with respect to malate dehydrogenase (decarboxylating) (NADP). The combined activities of the three peaks accounted for all of the malate dehydrogenase (decarboxylating) (NADP) activity applied to the column. 60% of the total activity was in Peak 2, 30% in Peak 1, and about 10% in Peak 3. Enrichment of Peaks 1 and 2 was 5- and 10-fold, respectively. No enrichment was obtained with Peak 3.

Starch-gel electrophoresis

Starch-gel electrophoresis of malate dehydrogenase (decarboxylating) (NADP) activity isolated from non-aqueously isolated chloroplasts and aqueously isolated mitochondria suggested that Peak 1 was a mitochondrial enzyme and Peak 2 was a



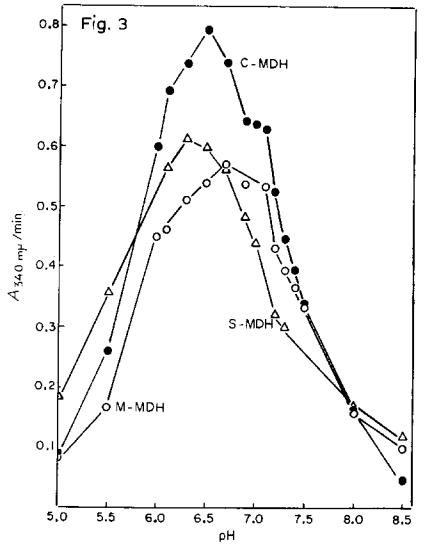


Fig. 2. Starch-gel electrophoresis of malate dehydrogenase (decarboxylating) (NADP) proteins from cactus phylloclades. Left: Peaks 1, 2, 3 from DEAE-cellulose column separation (see Fig. 1). Right: C, fractions isolated from chloroplasts which were prepared and purified by non-aqueous extraction and density-gradient centrifugation; M, fraction isolated from mitochondria prepared and purified by sucrose density-gradient centrifugation; S, supernatant from particles. The electrophoretic mobilities of C and M correspond to Peaks 2 and 1, respectively. The mobility of S suggests contamination with the particulate enzymes as well as an additional band corresponding to Peak 3. The peaks were designated as C-MDH (Peak 2), M-MDH (Peak 1), and S-MDH (Peak 3). Phosphate–citrate buffer at pH 7.0. 150 V at 4° for 18 h.

Fig. 3. pH optima curves for C-MDH, M-MDH, and S-MDH. Reaction mixtures contained 0.5 mM L-malate, 0.4 mM $MnCl_2$, 0.1 mM NADP in 50 mM buffer (MES, pH 5.0–7.0; TES, pH 6.5–8.5). Total vol., 3 ml.

chloroplast enzyme (Fig. 2). The evidence, however, rests solely on their electrophoretic mobilities. Electrophoresis of the supernatant fraction from the particles gave an elongated band with a leading edge extending further toward the anode than either the chloroplast or mitochondrial fractions. Furthermore, the mobility of the leading edge corresponded exactly with the mobility of the Peak 3 protein. These data suggested that the supernatant fraction contained the fastest isoenzyme and perhaps was also contaminated with the other two. Also, since the same pH was used for the cellulose column chromatography and the starch-gel electrophoresis, one would predict that the first isoenzyme to elute from the anion column would be the slowest electrophoretically, and the last to elute would be the fastest electrophoretically. Despite the fact that a mixture of three malate dehydrogenase (decarboxylating) (NADP) proteins could not be separated on starch gels, repeated electrophoresis of them always resulted in the same relative mobilities. Thus Peaks 1, 2, and 3 were designated as mitochondrial malate dehydrogenase (decarboxylating) (NADP) (M-MDH), chloroplast malate dehydrogenase (decarboxylating) (NADP) (C-MDH), and soluble malate dehydrogenase (decarboxylating) (NADP) (S-MDH).

pH optima

At a L-malate concentration of 0.5 mM, the estimated pH optima for S-MDH, M-MDH, and C-MDH were 6.3, 6.7 and 6.5, respectively (Fig. 3). Rates at pH 5.5 were 30, 30 and 60% of the maxima for C-MDH, M-MDH, and S-MDH, respectively

TABLE I

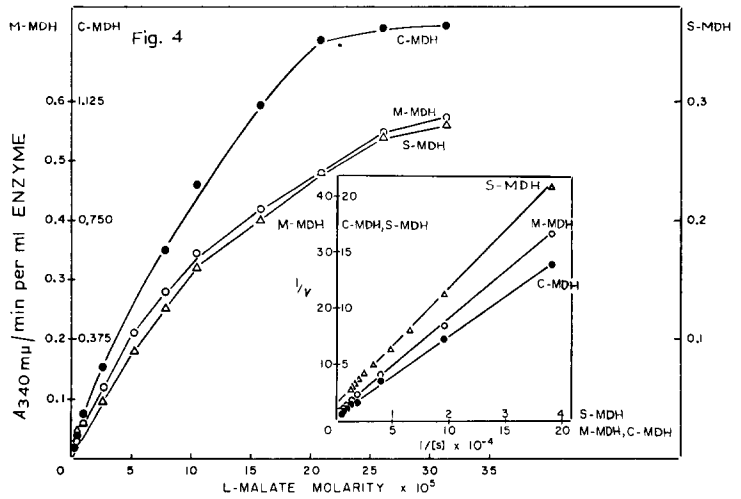
MICHAELIS CONSTANTS FOR MALATE DEHYDROGENASE (DECARBOXYLATING) (NADP) ISOENZYMES

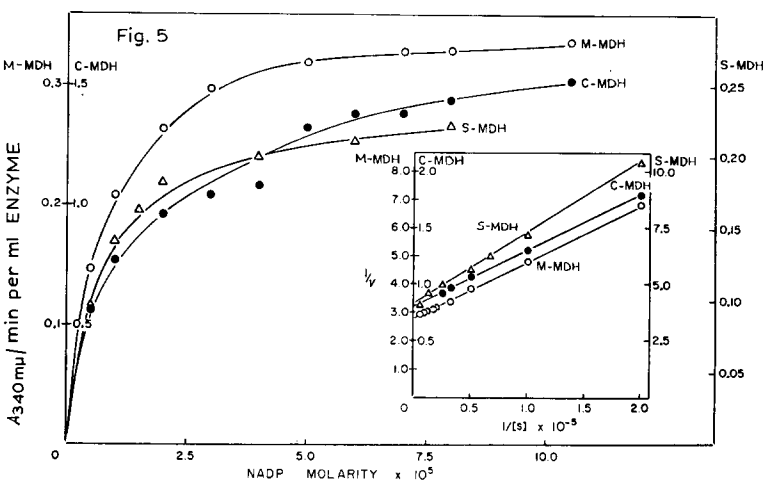
Isoenzyme	*Substrate*	K_m *(M)*	*Number of observations*
M-MDH from	L-Malate	$1.3 \cdot 10^{-4} \pm 0.14 \cdot 10^{-4}$	12
Peak 1 of	NADP	$7.2 \cdot 10^{-6} \pm 0.080 \cdot 10^{-6}$	10
Fig. 1	Mn^{2+}	$1.6 \cdot 10^{-5} \pm 0.17 \cdot 10^{-5}$	9
C-MDH from	L-Malate	$1.5 \cdot 10^{-4} \pm 0.21 \cdot 10^{-4}$	9
Peak 2 of	NADP	$8.4 \cdot 10^{-6} \pm 0.95 \cdot 10^{-6}$	9
Fig. 2	Mn^{2+}	$1.7 \cdot 10^{-5} \pm 0.11 \cdot 10^{-5}$	10
S-MDH from	L-Malate	$3.1 \cdot 10^{-4} \pm 0.17 \cdot 10^{-4}$	10
Peak 3 of	NADP	$7.8 \cdot 10^{-6} \pm 0.34 \cdot 10^{-6}$	8
Fig. 3	Mn^{2+}	$1.6 \cdot 10^{-5} \pm 0.083 \cdot 10^{-5}$	8

while at pH 7.5 rates were 30, 50 and 40% of maxima. These data suggested differences in pH optima.

Kinetic constants

Michaelis constants (K_m) for the three main substrates (L-malate, manganese, and NADP) were calculated from rate *versus* substrate concentration data with an electronic computer using the program of ELMORE, KINGSTON AND SHIELDS[8]. Excellent fits to the expected hyperbola were obtained and hence low errors were computed (Table I and Figs. 4–6). The greatest significant difference was obtained for the





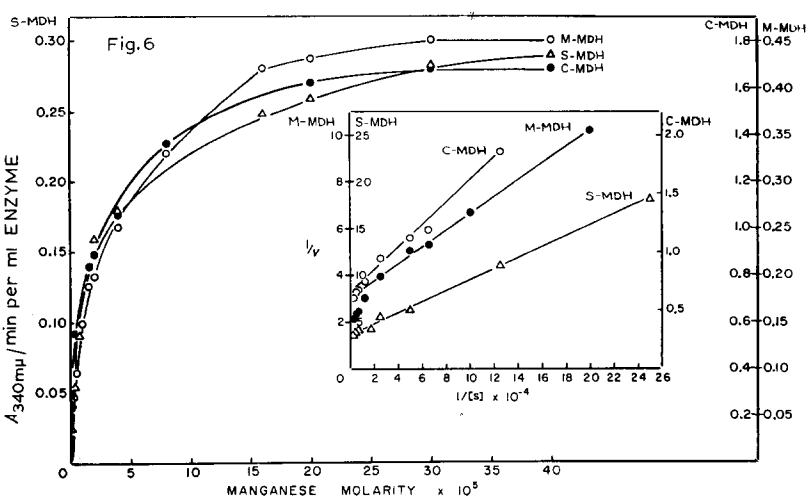
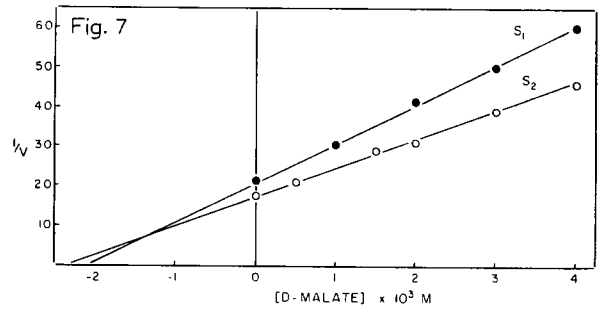
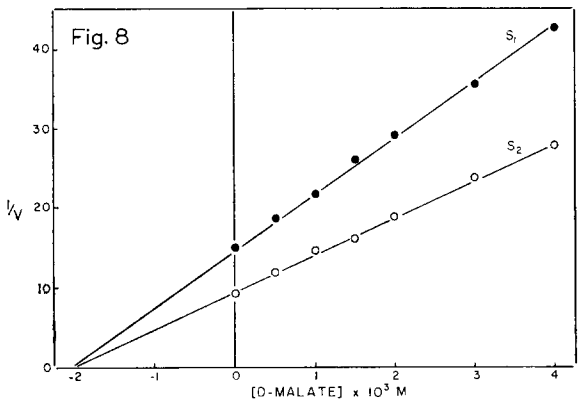


Fig. 4. Rate *versus* malate concentration for M-MDH, C-MDH, and S-MDH, and double reciprocal plots (see Table I for kinetic constants). NADP, 0.1 mM; $MnCl_2$, 0.4 mM. Total vol., 3 ml. Other conditions as given in text.

Fig. 5. Rate *versus* NADP concentration for M-MDH, C-MDH, and S-MDH, and double reciprocal plots (see Table I for kinetic constants). L-Malate, 0.5 mM; $MnCl_2$, 0.4 mM. Vol., 3 ml. Other conditions as given in text.

Fig. 6. Rate *versus* manganese concentration for M-MDH, C-MDH, and S-MDH and double reciprocal plots (see Table I for kinetic constants). L-Malate, 0.5 mM; NADP 0.1 mM. Vol., 3 ml. Other conditions as given in text.





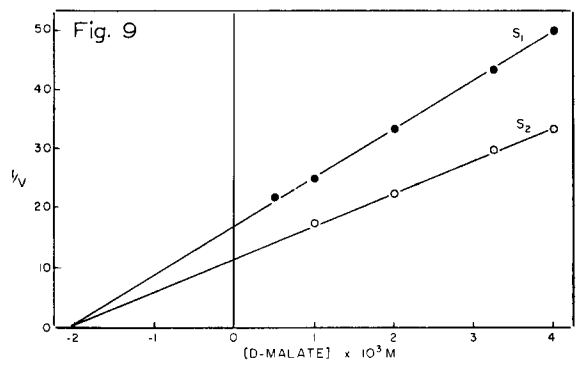


Fig. 7. Effect of several concentrations of D-malate on M-MDH activity. NADP, 0.1 mM; $MnCl_2$, 0.4 mM; L-malate: S_1, 0.1 mM; S_2, 0.2 mM; pH 6.7; 0.05 M MES. v, A/min. Competitive inhibition with respect to L-malate is shown.

Fig. 8. Effect of several concentrations of D-malate on C-MDH activity. NADP, 0.1 mM; $MnCl_2$, 0.4 mM; L-malate: S_1, 0.1 mM; S_2, 0.2 mM; pH 6.5; 0.05 M MES. v, A/min. Non-competitive inhibition with respect to L-malate is shown.

Fig. 9. Effect of several concentrations of D-malate on S-MDH activity. NADP, 0.1 mM; $MnCl_2$, 0.4 mM; L-malate: S_1, 0.1 mM; S_2, 0.2 mM; pH 6.3; 0.05 M MES. v, A/min. Non-competitive inhibition with respect to L-malate is shown.

K_m's for L-malate; the S-MDH K_m being 2-fold greater than either the M-MDH or C-MDH. Differences among others were slight and therefore we would expect that the mechanisms of the three isoenzymes, if not identical, are quite similar. Because each isoenzyme was assayed at the estimated pH optimum (see Fig. 3), differences among constants may reflect pH differences.

Substrate specificity

DIXON-WEBB[9] graphical analyses of the effect of D-malate on the rates of reaction with L-malate as a substrate indicated that for M-MDH, D-malate was a competitive inhibitor (K_i = 1.45 mM) while for both S-MDH and C-MDH D-malate acted as a non-competitive inhibitor (K_i's = 2.05 mM) (Figs. 7–9). The effects of several compounds (1.0 mM) as inhibitors are summarized in Table II. Dihydroxymaleic acid

TABLE II

PERCENTAGE INHIBITION OF MALATE DEHYDROGENASE (DECARBOXYLATING) (NADP) ISOENZYMES BY VARIOUS SUBSTRATE ANALOGS

*Inhibitor**	*M-MDH*	*C-MDH*	*S-MDH*
Dihydroxymaleic acid	100	100	100
Maleic acid	11	17	20
Tartronic acid	91	90	95
Malonic acid	72	71	68
DL-Citramalic acid	17	11	27
Citraconic acid	44	17	19
D-Malic acid	16	30	22

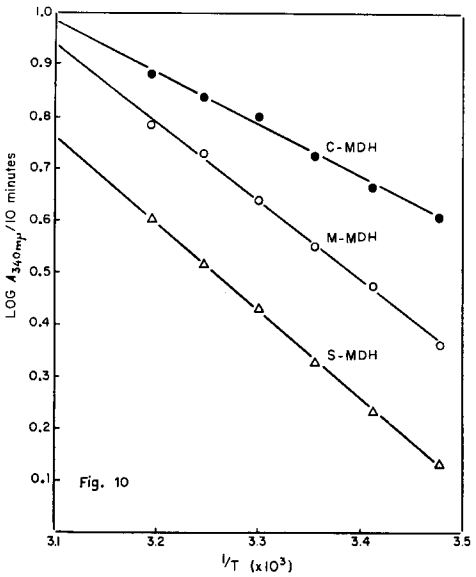
* Inhibitor concn.: 1 mM in 0.05 M MES (pH 6.5); L-malate, 0.5 mM; other assay conditions as given in text.

seemed to be the strongest inhibitor while tartronic acid inhibited all three isoenzymes 90–95%. Citramalic acid inhibited S-MDH more than either M-MDH or C-MDH while citraconic acid inhibited M-MDH to a greater extent. Differences among the three isoenzymes are most pronounced if rates in the presence of the inhibitors are compared. For example, the ratios of the rates of reaction in the presence of citramalic to that in the presence of citraconic were 1.5 for M-MDH, 1.07 for C-MDH, and 0.9 for S-MDH. Thus these inhibitor studies also suggested different kinetic properties for the three enzymes.

Effect of temperature

Energies or heats of activation (ΔH) computed from Arrhenius plots (Fig. 10) were 6923 ± 252, 4581 ± 213, and 7754 ± 634 cal/mole for M-MDH, C-MDH, and S-MDH, respectively.

Rates of heat inactivation at 57° were determined from initial log (rate) *versus* time curves: 0.553, 3.60, and 2.55% per min for M-MDH, C-MDH, and S-MDH, respectively. The curves, however, did not extrapolate to 100% activity at zero heating time, and thus there is a suggestion that the total effect was not solely dependent upon temperature. Linear plots of these data are shown in Fig. 11. The M-MDH seems to be quite stable at 57° for short periods whereas the S-MDH is very



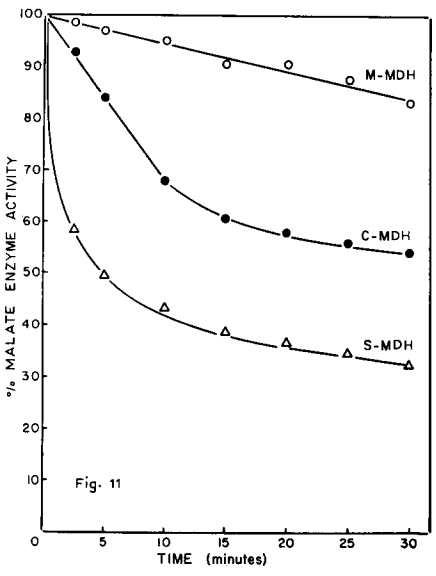


Fig. 10. Arrhenius plots of log (rate) *versus* reciprocals of absolute temperature ($1/T$) for C-MDH, M-MDH, and S-MDH. Reaction conditions: L-malate, 0.5 mM; NADP, 0.1 mM; $MnCl_2$, 0.4 mM; pH 6.5; 0.05 M MES. Total vol., 3 ml. Heats of activation (ΔH) computed from the slopes are: C-MDH, 4581 ± 213; M-MDH, 6923 ± 252; S-MDH, 7754 ± 634 cal/mole.

Fig. 11. Rates of heat inactivation of malate dehydrogenase (decarboxylating) (NADP) isoenzymes at 57°. Percent remaining activity is plotted against time at 57°. Preparations were heated for the times given and then cooled in an ice bath prior to assays. Reaction conditions: L-malate, 0.5 mM; NADP, 0.1 mM; $MnCl_2$, 0.4 mM; pH 6.5; 0.05 M MES. Total vol., 3 ml.

labile. The M-MDH fraction afforded thermostability to both S-MDH and C-MDH. Mixtures of M-MDH with either C-MDH or S-MDH resulted in less inactivation than expected whereas a mixture of C-MDH and S-MDH resulted in the expected inactivation (Table III). In these experiments, the protein concentration was 0.521, 0.316, and 3.43 mg/ml for M-MDH, C-MDH and S-MDH, respectively; thus there was no correlation between protein concentration and heat stability.

DISCUSSION

Cactus stem tissue, *i.e.*, phylloclades, similarly to other succulent tissues, fix CO_2 *via* "dark", non-photosynthetic reactions as well as by photosynthetic reactions. The dominant product, malic acid, tends to fluctuate diurnally such that maximum concentrations are reached after several hours in the dark followed by minimum concentrations toward the end of the subsequent light period[10]. Because of the products of dark CO_2 fixation (malate, aspartate, *etc.*) in cactus, we can speculate that malate dehydrogenase (decarboxylating) (NADP) as well as phosphoenolpyruvate carboxylase, malate dehydrogenase (NAD), and transaminases are most certainly involved. A further point of interest is that malate is a major organic acid product of CO_2 fixation by cacti in light[10]. Experiments conducted to date, however, do not differentiate between "dark" CO_2 fixation in the light and photosynthetic CO_2 fixation.

TABLE III

HEAT STABILITY OF THREE MALATE DEHYDROGENASE (DECARBOXYLATING) (NADP) ISOENZYMES

Preparations were heated for 15 min at 57° then chilled in ice prior to assays. Actual loss of activity is the percentage decrease after heating. The theoretical loss is the average expected loss predicted from the individual experiments. M-MDH fractions seem to protect the S-MDH and C-MDH fractions.

Isoenzyme	*Before heat* $A/min \times 10^2$	*After heat* $A/min \times 10^2$	*Loss of activity (%)*	
			Actual	*Theoretical*
M-MDH	14.6	12.4	15.5	
C-MDH	18.0	12.6	30.0	
S-MDH	14.7	6.3	57.2	
M-MDH + S-MDH	24.3	20.0	17.5	36.3
M-MDH + C-MDH	24.7	25.0	0	22.7
C-MDH + S-MDH	31.0	18.1	41.8	43.6

Presently, we have extracted three proteins (isoenzymes) with malate dehydrogenase (decarboxylating) (NADP) activity from green stem tissue (phylloclades) of Opuntia and separated them by anion-exchange column chromatography and starch-gel electrophoresis. Chromatography of enzyme preparations from subcellular organelles indicated that the three isoenzymes were compartmentalized in (1) the chloroplasts, (2) the mitochondria, and (3) a non-particulate or soluble fraction. Hence the isoenzymes were designated as C-MDH, M-MDH, and S-MDH. Although the experimental methods used tended to suggest kinetic differences, *i.e.*, pH optima, K_m's, *etc.*, apparent physical differences manifested by chromatographic separations were most pronounced. In mouse tissue, mitochondrial and supernatant malate dehydrogenase (decarboxylating) (NADP) isoenzymes have been reported[11].

Reaction rates of all three isoenzymes were adequately described by Michaelis–Menten kinetics. The data obtained over substrate concentration ranges of three orders of magnitude tended to fit the hyperbolic function of Michaelis–Menten with no apparent tendency toward sigmoid or parabolic kinetics. Statistical fits of these data were significant at the 0.001 confidence level in all cases. As can be seen from the plots of the data (see Figs. 4–6), there was a slight, but not significant, tendency toward "substrate activation". It appears to be important that with the single exception of the malate K_m for S-MDH, K_m's for specific substrates were quite similar.

A previously described protein with malate dehydrogenase (decarboxylating) (NADP) activity showed activation with respect to D-malate[12]. Our experiments did not reveal any activating molecules (see Table II) and D-malate was an inhibitor of all three proteins. Interestingly and probably relevant to the mechanism of the reaction, D-malate was a competitive inhibitor with respect to L-malate for M-MDH, but showed non-competitive kinetics with S-MDH and C-MDH. Ratios of activities in the presence of several substrate analogs also suggested kinetic differences.

Our data do not allow conclusions concerning specific metabolic functions for the isoenzymes other than the obvious one that they most certainly are involved in malate metabolism in their respective compartments. It is worth noting that most evidence suggests that *in vivo* the malate dehydrogenase (decarboxylating) (NADP) catalyzes the decarboxylation of malate rather than the carboxylation of pyruvate[13]. In this regard we suspect that these isoenzymes function in malate utilization rather

than malate synthesis. In non-green plant tissues, evidence is available which suggests more than one pool of malate[14,15], and the pattern of CO_2 fixation by cactus roots also suggests compartmentation of products[16]. Hence the compartmentation of enzymes which have malate as a substrate is consistent with these findings.

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