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PURIFICATION AND CHARACTERIZATION OF THE NADP-LINKED MALATE DEHYDROGENASE (DECARBOXYLATING) FROM *MANGIFERA INDICA*

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The NADP-linked malate dehydrogenase (decarboxylating) (L-malate : $NADP^+$ oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40), known as 'malic' enzyme, has been isolated and purified to apparent homogeneity from the mango fruit, *Mangifera indica*, by means of extraction, gel chromatography with Sephadex G-200 and anion-exchange chromatography with DEAE-Sephacel. A 16-fold purification with a 49% yield was obtained. The enzyme was physically characterized and its homogeneity determined by polyacrylamide gel electrophoresis, isoelectric focusing, sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and analytical chromatography. An average molecular weight of 258 000 was obtained for the enzyme as well as a Stokes radius of $54.0 \cdot 10^{-8}$ cm and a diffusion coefficient of $3.96 \cdot 10^{-7}$ $cm^2 \cdot s^{-1}$. A frictional ratio, f/f_0 of 1.28 indicated the globular character of the enzyme. The native enzyme consists of four subunits with an average molecular weight of 64 900. The enzyme thus appears to be an oligomeric protein with an apparently homogeneous quarternary structure. The pH optimum for the decarboxylation reaction varied between 6.8 and 7.5, depending on the type of buffer and increased with increasing malate concentration. No cooperativity could be detected between the malate binding sites in the presence of Mn^{2+} as cofactor. It would seem as if Mn^{2+} elicits a positive allosteric effect on the enzyme. Increasing Mn^{2+} concentrations lead to an increase in the K_m value for L(-)malate from 666 μM at 1.0 mM Mn^{2+} to 1.08 mM at 5.0 mM Mn^{2+}. The K_m value determined at pH 7.1 for Mn^{2+} was 14.3 μM. An approximate K_m value of 16 μM was found for $NADP^+$ with some indication of cooperativity between the nucleotide binding sites. The enzyme activity was much more sensitive to regulation when Mg^{2+} served as cation. The allosteric activator, succinate, removed the sigmoidicity observed in the velocity-malate saturation curve and lowered the Hill coefficient from 1.5 to 1.0 Differences were found in the response of the enzyme to certain metabolites depending on whether Mg^{2+} or Mn^{2+} served as cofactor. It appears as if Mg^{2+} and Mn^{2+} stabilize two structurally distinct forms of the enzyme which vary in catalytic and regulatory properties.

Introduction

Malic enzyme (L-malate : $NADP^+$ oxidoreductase (oxalacetate-decarboxylating), EC 1.1.1.40) has been obtained from a variety of different sources including higher animals [1–3], higher plants [4–6] and micro-organisms [7,8]. These enzymes have similar physical and chemical characteristics, although they do differ in certain respects.

The subcellular distribution of malic enzyme in animal tissue appears to be both species and tissue-specific and distinct isozymes are found in the mitochondria and cytosol [9]. Malic enzyme activity has been described in the mitochondria, chloroplast and the cytosol of plant [10,11] and a distinction has been made between the enzyme of C_4-plants, C_3-plants and CAM-plants [12].

According to the literature little is known about

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the malic enzymes from fruits, although the de novo synthesis of malic enzyme during the climacteric phase of ripening has been well established [13], and considerable evidence indicates that the enzyme participates in the ripening process of climacteric fruits. [14–16].

The objective of this investigation was to study the molecular structure and enzymatic properties of the malic enzyme from *Mangifera indica* so as to obtain more information on the physiological role of the enzyme against the background of maturation and senescence of fruit tissue.

Materials and Methods

Materials. Mature, fully ripe fruits, *M. indica* (cv. Kent) were obtained from orchards in the Tzaneen area and stored at −30°C until used.

DEAE-Sephacel and Sephadex G-200 Fine and Superfine were obtained from Pharmacia Uppsala, Sweden. $NADP^+$ and calibration proteins for analytical gel chromatography and SDS electrophoresis were obtained from Boehringer-Mannheim, F.R.G. Polyclar AT, 2-(*N*-morpholino)ethanesulphonic acid (MES), *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (BES) and *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid (TES) were obtained from British Drug Houses, London and Ultrazym 100 from Ciba-Geigy, Switzerland. Ampholine carrier ampholytes were from LKB, Sweden. All other reagents used were from E. Merck, Darmstadt, F.R.G. and were of analytical grade quality.

Assay of malic enzyme activity. The standard assay for the oxidative decarboxylating activity of malic enzyme was carried out spectrophotometrically using a PYE Unicam Sp 1804 spectrophotometer equipped with a recorder and scale expander. The reaction mixture contained the following components: 100 mM Tris-HCl pH 7.1/1.0 mM $MnSO_4$/5.0 mM L-malate/0.5 mM $NADP^+$/enzyme solution in a total volume of 3.0 ml. The reaction was carried out 25°C and was initiated by the addition of enzyme and followed by observing the reduction of $NADP^+$ at 340 nm. Under these conditions the relationship between the reaction rate and enzyme concentration was linear. One unit (I.U.) of enzyme activity was defined as the amount of enzyme that catalyses the conversion on 1 μmol L-malate to pyruvate and CO_2/min.

Protein concentrations were routinely determined by the method of Ohnishi and Barr [17] based on the method of Lowry et al. [18], using bovine γ-globulin as a standard.

Analytical polyacrylamide gel electrophoresis and isoelectric focusing. Analytical polyacrylamide gel electrophoresis was used, inter alia, to determine the homogeneity of purified malic enzyme preparations [19]. A Shandon instrument was used in these experiments which were performed at 5°C. Gels were prepared according to a standard procedure described by Gabriel [19]. Usually 7% polyacrylamide gels were prepared and electrophoresis was performed using the pH 8.3/8.9 Tris-glycine buffer system and a current of 8 mA/gel.

SDS-polyacrylamide gel electrophoresis was conducted according to the method described by Laemmli [20], while the dissociation and reduction of the purified malic enzyme as well as the molecular weight calculations were carried out as described by Weber et al. [21]. The following standard proteins were included for the determination of the molecular weight of the malic enzyme subunits: bovine serum albumin (M_r = 68 000) and the α, β and β′ subunits of the RNA-polymerase from *Escherichia coli* (M_r = 39 000, 155 000 and 165 000, respectively).

Polyacrylamide gel isoelectric focusing was also used to determine the homogeneity of purified malic enzyme preparations [22]. The technique as described by Wrigley [22] was used. Usually 7% polyacrylamide gels were prepared through chemical polymerization. The purified enzyme solutions were incorporated into the gel mixture. Fractionation was performed at 5°C and a current of 1 mA/tube was maintained for 1 h.

Coomassie Brilliant blue G250 was used as staining reagent [23] for the detection of protein bands following electrophoresis or isoelectric focusing, whilst malic enzyme activity was located by immersing the gel in the standard reaction mixture for assaying malic enzyme containing 0.08% nitroblue tetrazolium chloride and 0.014% phenazinemethosulphate [24].

Analytical gel chromatography. The molecular weight, Stokes radius and diffusion coefficient of malic enzyme were determined by analytical gel

chromatography. The experiments were performed as described by Andrews [25] with a column (2.6 × 55 cm) of Sephadex G-200 Fine, equilibrated with 50 mM Tris-HCl buffer, pH 7.1/100 mM KCl. The following standard proteins were used for the calibration of the column: cytochrome *c* (M_r = 12 500), chymotrypsinogen A (M_r = 25 000), ovalbumin (M_r = 45 000), bovine serum albumin (M_r = 67 000), aldolase (M_r = 158 000), catalase (M_r = 240 000), xanthine oxidase (M_r = 275 000) and apoferritin (M_r = 450 000).

Isolation and purification of malic enzyme. The flesh of mango fruit, *M. indica* was used as the source of malic enzyme. All isolation procedures were carried out at 0–5°C. Enzyme activity as well as protein concentration was determined after each purification step.

Extraction (Step 1). The frozen fruit was peeled and sliced into 1.5-cm cubes of which 100 g were transferred to a pre-cooled Waring blendor together with 200 ml 50 mM Tris-HCl buffer, pH 7.1/5 mM cystein/100 μM diethyldithiocarbamate. 1.0 g Polyclar AT (an insoluble poly(vinylpirrolidone)) and 1.0 g Ultrazym 100 (a pectinase) were added and the fruit tissue was homogenized for 3 min.

The pH of the homogenate was adapted to pH 4.5 for optimal activity of the pectinase. The suspension was kept at 7°C with constant stirring for 10 min to effect the degradation of the pectin. The homogenate was adapted to pH 7.1 and was then centrifuged at 15 000 × *g* for 20 min in a precooled Beckman J-21B centrifuge.

The supernatant was lyophilized to concentrate the diluted extract for further purification steps (300 ml concentrated to 30 g). Subsequent subcellular fractionation studies [26], performed on fresh fruit at different maturation stages, located the enzyme in the cytosolic fraction of the fruit cell.

Sephadex G-200 Fine gel chromatography (Step 2). A column of Sephadex G-200 Fine, 70 × 2.6 cm was prepared and equilibrated with 50 mM Tris-HCl buffer, pH 7.1/5.0 mM cystein. 4.0 g of the lyophilized extract (equivalent to 13.0 g mango tissue or 40 ml of the crude extract), obtained in Step 1, were dissolved in this buffer to a final volume of 6.0 ml and applied to the column. Elution was carried out with the same buffer. The flow rate was 16 ml/h (3.01 ml/cm² per h) and fractions of 3.0-ml were collected.

DEAE-Sephacel chromatography (Step 3). The combined fractions containing malic enzyme obtained from Step 2 were applied to a DEAE-Sephacel column (1.6 × 20 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.3/5 mM cystein. The column was eluted with the same buffer. The flow rate was 28 ml/h (13.0 ml/cm² per h) and 3.0-ml fractions were collected. After about 130 ml buffer had passed through, a linear concentration gradient of 0–60 mM NaCl in the starting buffer was effected over a total volume of 180 ml. The peak activity was eluted at approx. 27 mM NaCl. The column was then eluted with a 6.0 M NaCl solution to elute the remaining absorbed protein. Fractions containing malic enzyme activity were combined as indicated in the chromatographic profile shown in Fig. 1.

Sephadex G-200 Superfine gel chromatography (Step 4). The combined fractions of the previous step were concentrated to 5.0 ml by Amicon Ultrafiltration cells, using a 10 000 molecular weight cut-off membrane. This concentrated fraction was applied to a Sephadex G-200 Superfine column (2.6 × 26 cm), prepared and equilibrated with 300 mM Tris-HCl buffer, pH 7.1/5.0 mM cystein. The column was eluted with this buffer. The flow speed was 12 ml/h (2.3 ml/cm² per h) and 2.5-ml fractions were collected. Active fractions were then pooled and used for the physical and chemical characterization of the enzyme.

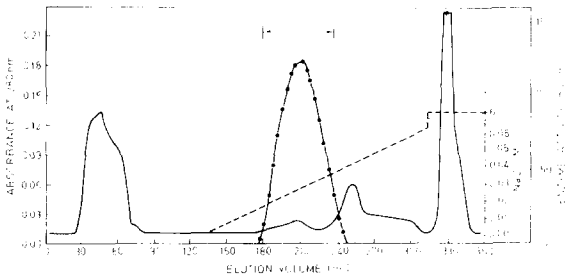


Fig. 1. Purification of malic enzyme on DEAE-Sephacel. 45 ml (4.75 mg protein, 11 761 mU) were applied to the column (1.6 × 20 cm) at a flow rate of 28 ml/h. The column was developed with 50 mM Tris-HCl buffer, pH 7.3/5 mM cystein for 130 ml and then with a linear gradient of 0–60 mM NaCl in the starting buffer, effected over 180 ml; before final elution with 6 M NaCl. 3.0-ml fractions were collected. Protein concentration (————) was determined by absorption at 280 mm. Enzyme activity (•————•) was expressed in mU/ml (nmol substrate converted/min per ml). Fractions containing enzyme activity were combined as indicated.

Results and Discussion

Purification. The results of the various purification steps are summarized in Table I. A final 16-fold purification factor and a specific activity of 7910 mU/mg protein were obtained. The total amount of enzyme isolated from 13.0 g mango tissue was 0.76 mg at a final yield of 49%. The homogeneity of the preparation obtained was confirmed by analytical polyacrylamide gel electrophoresis, polyacrylamide gel isoelectric focusing and SDS-polyacrylamide gel electrophoresis (Fig. 2).

Physical properties. The molecular weight, Stokes radius and diffusion coefficient of the enzyme were determined by means of analytical gel chromatography. An average molecular weight of 258 000 was obtained for malic enzyme with a Stokes radius of $54.0 \cdot 10^{-8}$ cm and a diffusion coefficient of $3.96 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1}. By employing a value of 0.741 ml $\cdot$ g^{-1} as partial specific volume [27] a frictional ratio f/f_0, of 1.28 could be calculated which indicates the globular character of the enzyme.

These results are in good agreement with the physical characteristics of the malic enzymes purified from pigeon liver and other animal tissues [9,27,28].

Not much is known about the physical parameters

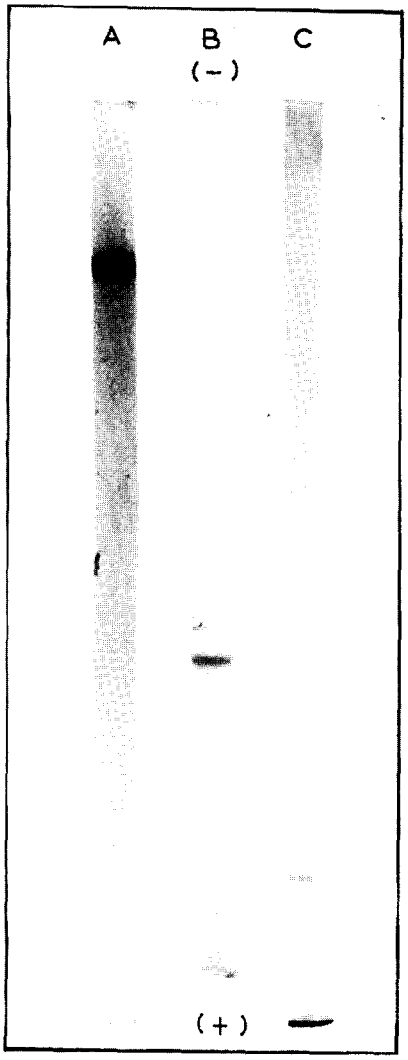


Fig. 2. Analytical polyacrylamide gel electrophoresis of the purified malic enzyme from *Mangifera indica* (A), the SDS-treated enzyme (B) and polyacrylamide gel isoelectric focussing of the enzyme (C). Gels were stained for protein using Coomassie brilliant blue G250. Experimental conditions are as described in the text.

TABLE I

PURIFICATION OF MALIC ENZYME (L-MALATE: NADP$^+$ OXIDOREDUCTASE (OXALACETATE-DECARBOXYLATING), EC 1.1.1.40) FROM *MANGIFERA INDICA*

Step	Volume (ml)	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Purification (-fold)	Yield (%)
1. Crude extract	6.0	25.0	12 379.7	495.2	1	100
2. Sephadex G-200 Fine chromatography	45.0	4.75	11 761.2	2476.0	5	95
3. DEAE-Sephacel chromatography	60.0	0.95	6 595.2	6 942.3	14	53
4. Sephadex G-200 Superfine chromatography	15.0	0.76	6 011.9	7 910.4	16	49

of malic enzymes from plant and especially fruit tissues. The obtained molecular weight value of 258 000 of the enzyme from *M. indica* is comparable to those from *Pyrus communis*, 224 000 [29] and *Zea mays*, 280 000 [11].

Subunit structure of malic enzyme. Following dissociation and reduction [21] of the purified malic enzyme, a single band was obtained with SDS-polyacrylamide gel electrophoresis (Fig. 2). An average molecular weight of 64 900 for the malic enzyme subunits was estimated from its relative mobility by interpolation of a plot of the electrophoretic mobility vs. log molecular weight of the various protein standards.

Similar subunit molecular weights of 63 000 and 65 800 were reported for the enzymes from *Zea mays* and pigeon liver [4,28]. The native form of malic enzyme has been suggested to exist as even-numbered multiples of a monomer unit in nature [28].

The overall results demonstrate that the native form of malic enzyme from *M. indica* is a tetramer comprising four subunits of identical or very similar size. However, it cannot be assumed that the subunits are identical to all other aspects and that only one kind of subunit is present [30].

Effect of pH on the enzyme action. The effect of pH variation on the catalytic action of malic enzyme was determined using four different buffer systems. The pH optimum depended on the type of buffer: in MES-NaOH a value of pH 6.8 was obtained, in BES-NaOH, pH 7.0, in Tris-HCl, pH 7.1 and in TES-NaOH, pH 7.5. The pH-activity curves of the enzyme in the different buffer systems are not shown. The pH optimum in Tris-HCl buffer shifted towards higher pH values with increasing malate concentrations, a typical effect of some malic enzymes [11, 13]. With malate concentrations of 0.1, 1.0 and 10.0 mM, the respective optima were pH 6.95, 7.05 and 7.15. The observed effect of the enzyme from *M. indica* was not so pronounced as for the pigeon liver malic enzyme [31] and resembled more the effect on the enzyme from *Bryophyllum tubiflorum* [5].

Kinetic parameters. The initial velocity of the

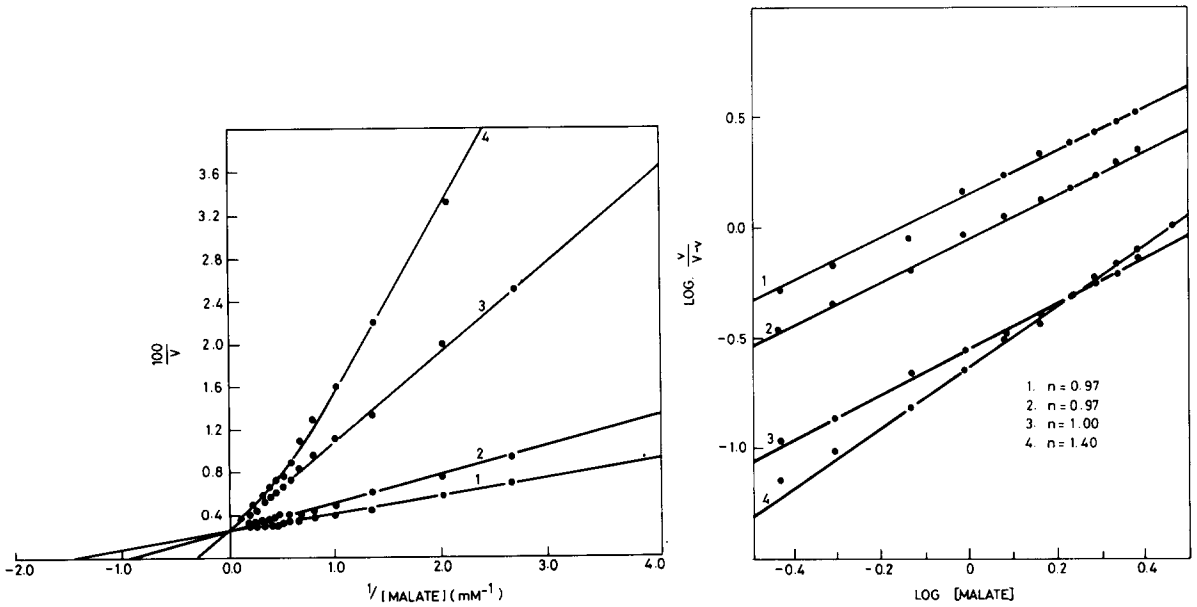


Fig. 3. The effect of different combinations of pH and cofactor concentration on the double-reciprocal plots and Hill plots of L-malate as the variable substrate. Basic reaction medium: 100 mM Tris-HCl buffer/0.5 mM $NADP^+$ 0–50 mM L-malate with combinations of cofactors and pH as indicated: (1) 1 mM Mn^{2+}, pH 7.1; (2) 5 mM Mn^{2+} pH 7.1; (3) 5 mM Mn^{2+}, pH 7.6; (4) 5 mM Mg^{2+}, pH 7.1. Total volume 3.0 ml. (v = reaction velocity, V = reaction velocity at saturating concentrations of substrate, n = Hill coefficient).

malate decarboxylating reaction was studied as a function of malate concentration in the presence of saturating concentrations of $NADP^+$ (0.5 mM) and divalent metal cofactor. To determine the effect of the metal ion on the activity of the enzyme, a series of experiments were conducted, using the following combinations of cofactor concentrations and pH: 5.0 mM Mg^{2+}, pH 7.1; 5.0 mM Mn^{2+}, pH 7.1; 1.0 mM Mn^{2+}, pH 7.1 and 5.0 mM Mn^{2+}, pH 7.6. The Lineweaver-Burk double-reciprocal plots are shown in Fig. 3a.

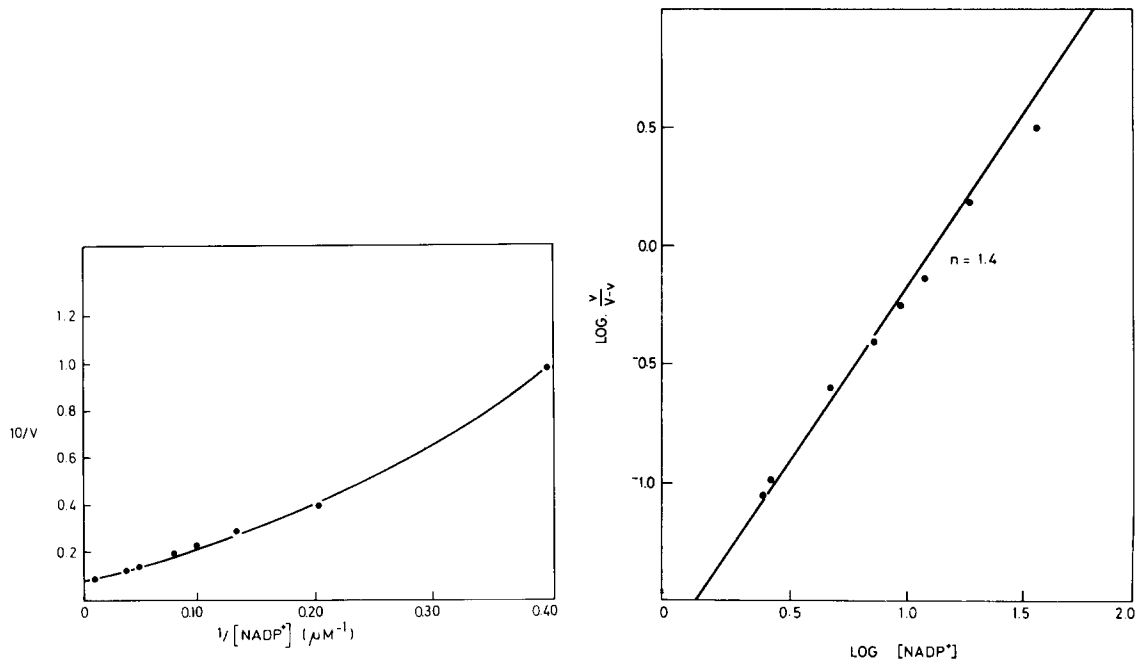
With Mg^{2+} as cofactor, the enzyme followed sigmoid kinetics and the double-reciprocal plot was concave upwards (Fig. 3a). A Hill coefficient of 1.4 was obtained from the Hill plot (Fig. 3b).

In comparison with the results of Mg^{2+} as cofactor, Mn^{2+} the more effective cofactor, appears to elicit a positive allosteric effect on the enzyme and in the presence thereof, normal Michaelis-Menten kinetics were observed with linear double-reciprocal plots (Fig. 3a) and a Hill coefficient of 0.97 (Fig. 3b).

Allosteric behaviour has been reported for some malic enzymes at pH values higher than the optimal pH [32,33], as well as a masking effect of optimal pH and high malate and $NADP^+$ concentrations of cooperativity [6,32]. No similar increase in cooperativity was observed when the pH of the reaction medium was increased to pH 7.6, but the K_m value increased from 1.08 mM at pH 7.1 to 3.57 mM at pH 7.6 with a 5 mM concentration of Mn^{2+} (Fig. 3a).

Increasing the Mn^{2+} concentration in the reaction mixture from 1.0 to 5.0 mM led to an increase in the K_m value for L(-)malate from 666 μM to 1.08 mM. It would seem that Mn^{2+} might have the ability to regulate the activity of the enzyme towards malate. This aspect was further investigated using Mn^{2+} concentrations ranging from 0.5 to 1.0 mM. Increasing Mn^{2+} concentration in the reaction medium had a progressive inhibitory effect on the enzyme activity. Similar results were reported for the pigeon liver enzyme [34] and for the enzymes from *B. diagremontianum* and *P. communis* with Mg^{2+} as cofactor [6,33].

The velocity saturation curves and double-reci-



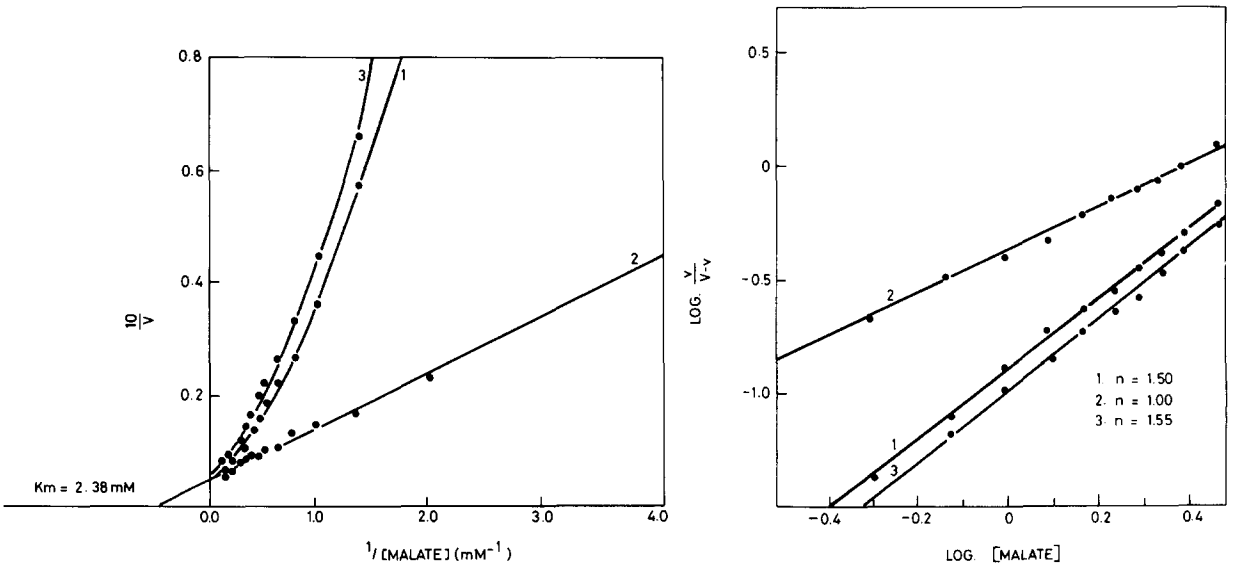
Figs. 4. Double-reciprocal plot and Hill plot for $NADP^+$ as variable substrate in the presence of saturating concentrations of L-malate (5.0 mM) and Mn^{2+} (1.0 mM) in 100 mM Tris-HCl buffer, pH 7.1. The kinetic assays were performed according to the standard reaction conditions as described in the text. (v = reaction velocity, V = reaction velocity at saturating concentrations of substrate, n = Hill coefficient).

procal plot for Mn^{2+} as variable substrate in the presence of saturating concentrations of malate (5.0 mM) and $NADP^+$ (0.5 mM) showed normal Michaelis-Menten kinetics with a K_m value of 14.3 μM. No cooperativity could be detected under the experimental conditions between the Mn^{2+} binding sites. Similar K_m values were reported for malic enzymes from other species: 16 μM for *P. communis* [33] and 13 μM for *E. coli* [35].

The velocity saturation curve for $NADP^+$ as variable substrate in the presence of saturating concentrations of malate (5.0 mM) and Mn^{2+} (1.0 mM) appeared to exhibit a simple hyperbolic response with an approximate K_m of 16 μM. This value compares well with K_m values from other malic enzymes: *B. tubiflorum* (14 μM) [5] and *P. communis* (16 μM) [16]. However, the Lineweaver-Burk double-reciprocal plot of the data indicates some cooperativity between the $NADP^+$ binding sites with a Hill coefficient of 1.4 (Figs. 4a and 4b).

To our knowledge definite cooperative behaviour between the nucleotide binding sites has not yet been reported for malic enzymes from higher plant and animal tissue, although Davies found that the addition of ADP led to the introduction of sigmoidicity into the initial velocity plot of NADP as variable substrate for the enzyme from *Solanum tuberosum* [32]. The importance of nucleotides as regulatory entities has already been shown for the malic enzymes from *E. coli* [7] and *Trypanosoma cruzi* [36].

Regulatory properties. When the velocity of the reaction was studied as a function of malate concentrations in the presence of saturating $NADP^+$ concentrations (0.5 mM) and with Mg^{2+} as divalent cofactor (5.0 mM), a sigmoid response was obtained (Fig. 5a). The apparent Hill coefficient, calculated from the Hill plot (Fig. 5b) was 1.5. The positive cooperativity between the malic binding sites was abolished by the presence of 1.0 mM succinate in the reaction medium, leading to a linear double-reciprocal plot with a K_m value of 2.38 mM for malate (Fig. 5a) and a Hill coefficient of 1.00 (Fig. 5b). In the presence of 1.0 mM citrate in the reaction medium, the sigmoidicity of the initial velocity pattern seems more pronounced (Fig. 5a) leading to an increase in the Hill coefficient of 1.55 (Fig. 5b).



Figs. 5. The effect of succinate and citrate on the oxidative decorboxylation of malate catalysed by the malic enzyme from *Mangifera indica* as shown in the double-reciprocal plots and Hill plots of L-malate as variable substrate with Mg^{2+} as divalent metal cofactor. The kinetic assays were performed in a basic reaction medium containing 5.0 mM Mg^{2+} and 0.5 mM $NADP^+$ in 100 mM Tris-HCl buffer, pH 7.1, and at the indicated malate concentrations (1). The effector concentrations used were 1 mM succinate (2) and 1 mM citrate (3). See text for kinetic constants. (v = reaction velocity, V = reaction velocity at saturating concentrations of substrate, n = Hill coefficient).

Allosteric behaviour was reported for malic enzymes from bovine heart mitochondria [9], *Solanum tuberosum* [32], *T. cruzi* [36] and *E. coli* [7]. Succinate seems to be a positive effector in most cases [9,36] but a number of dicarboxylic acids were reported as activators of the malic enzyme from *S. tuberosum* [32], especially when the malate concentration was low and the pH alkaline. At high malate concentrations the dicarboxylic acids behaved as inhibitors [32]. Comparable results were reported for the enzyme from *P. communis* [33].

Some dicarboxylic acids of metabolic importance were tested for possible regulation of the enzyme. The test substance was present in a 3.33 mM concentration in the reaction mixture containing malate (5.0 mM)/$NADP^+$ (0.5 mM)/Mg^{2+} (5.0 mM). Citrate, isocitrate and α-ketoglutarate inhibited the enzyme respectively by −14, −18 and −10%. Fumarate, succinate and L(+)tartrate activated the enzyme by +14, +6 and +10%, respectively. The results obtained support those by Drouet and Hartman in the case of the malic enzyme from *P. communis* [33], in that the products of malate in the Krebs cycle inhibit the enzyme and that the precursors of malate activate the enzyme at low concentrations.

A different response was obtained with the above mentioned dicarboxylic acids when a 5.0 mM concentration of Mn^{2+} replaced the Mg^{2+}. Citrate, isocitrate and α-ketoglutarate had a small activating effect on the enzyme (+5%) while fumarate, succinate and L(+)-tartrate inhibited the enzyme by −6, −10 and −8%, respectively.

It is obvious from the results presented in this study that the catalytic and regulatory properties of the enzyme are significantly altered by the choice of metal cofactor. Mg^{2+} and Mn^{2+} appear to be important regulatory entities and might stabilize two structurally distinct forms of the enzyme which lead to the variation in catalytic and regulatory properties.

Any postulate on the function and metabolic regulation of the enzyme in the fruit tissue of *M. indica* should take into account the differential effect of Mn^{2+} and Mg^{2+} on the cooperative behaviour as well as the concentration dependance of the activation or inhibition by different effectors at varying concentrations of cofactor and coenzyme and different pH values. A knowledge about the subcellular structures of the fruit cell and the changes that occur in them throughout the phase of ripening and senescence, is furthermore needed as a key to the understanding of metabolic control mechanisms [37]. The physiological significance of the cooperative behaviour of malic enzyme remains uncertain and requires further investigation.

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