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Malic Dehydrogenase from *Tamarix* Roots

EFFECTS OF SODIUM CHLORIDE *IN VIVO* AND *IN VITRO*[1]

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ABSTRACT

Soluble and mitochondrial malic dehydrogenases (MDH) were isolated from root tips of the halophyte *Tamarix tetragyna* L. grown in the presence and absence of NaCl. The activity of the enzymes isolated from root tips grown in the presence of NaCl was lower than that of the enzymes isolated from roots grown in absence of NaCl. The mitochondrial MDH was much more sensitive to salinity than the soluble MDH. The soluble enzyme from roots grown in NaCl had a higher Km for malate and lower Km for NAD than enzyme from the control roots. Addition of NaCl *in vitro* at 72 mM significantly stimulated the reductive activity of soluble MDH, while higher NaCl concentrations (240 mM and above) depressed enzyme activity. The inhibition of enzyme activity by various salts was found to be in the order $MgCl_2 > NaCl = KCl > Na_2SO_4$. Mannitol at equiosmotic concentrations had no effect. Substrate inhibition, typical for oxaloacetate oxidation, was not observed at high NaCl concentrations *in vitro* and high substrate concentrations neutralized the inhibitory effect of NaCl. Increased coenzyme concentrations had no effect. *In vitro* NaCl increased the Km for malate and oxaloacetate already at relatively low concentrations. At the same time NaCl decreased the Km for NAD and NADH. The inhibitory effect of NaCl on enzyme activity seems not to be due to the effect on the Km alone. Soluble and mitochondrial MDH had different responses to pH changes, mitochondrial MDH being more sensitive. Mitochondrial MDH released from the particles had a similar response to that of the entire particles. Changes of pH modified the effect of NaCl on enzyme activity. It was postulated that NaCl apparently induces conformational changes in the enzyme.

Many plants are capable of growing in saline substrate. This adaptation is usually accompanied by decreased internal osmotic potential, in order to maintain a gradient for water uptake. The mechanism for this osmotic adaptation involves increased ion uptake and changes in the balance between the inorganic and organic ions in the cell sap (3, 4). However, the increased ionic concentration and the lower osmotic potential may affect the structure and function of enzyme proteins (5, 16).

Halophytes are plants that are naturally adapted to grow in saline substrate. This group apparently consist of plants with diverse mechanisms of adaptation, the common denominator being the ability to endure the adverse conditions of a saline habitat. In many cases, the halophytes show typical morphological and anatomical features; many of them have very low osmotic potentials in their cell sap; some are able to regulate the internal salts concentration, either by salt excretion or by accumulation of excessive water in their cells, *i.e.* succulence. However, they usually show considerable tolerance to high internal osmotic concentrations. The whole problem was recently reviewed by Waisel (29). He concluded that no satisfactory explanation exists for the mechanism of this tolerance.

Another group of organisms showing a very high tolerance to substrate salinity is the group of the halophilic bacteria. The enzymes of these organisms are apparently adapted to function in the presence of high ionic concentrations and are activated by them (1, 2, 6, 13–16, 21, 22). Holmes and Halvorson (13) studied the effect of NaCl on malic dehydrogenase from obligatory halophilic bacteria, facultative halophilic bacteria, and animal tissue. The enyzme from the animal tissue showed maximal activity in the absence of NaCl and the activity decreased with increasing NaCl concentration. The enzyme isolated from obligatory halophilic bacteria, on the other hand, showed the highest activity at the high NaCl concentrations (up to 4 M) and its activity decreased with decreasing NaCl concentrations. Hubbard and Miller (14, 15) showed similar to halophilic bacteria, or whether the enzyme was simi- It was of interest, therefore, to examine whether an enzyme of a halophytic plant showed salt requirement for its activity, similar to halophilic bacteria, or whether the enzyme was similar to that of the glycophytes. Malic dehydrogenase (L-malate-NAD oxidoreductase EC 1.1.1.37) isolated from *Tamarix* roots was examined.

MATERIALS AND METHODS

Cuttings of *Tamarix tetragyna L.* were collected from a tree from Mediterranean coastal salt marshes in the Naaman area and were rooted and grown in vermiculite moistened either with half strength Hoagland's solution (12) or with Hoagland's solution containing 0.12 M NaCl (osmotic potential $-\pi = -5$ atm). When the cuttings began to root, salinity was stepped up to final salinities of 0.12 M, 0.24 M, 0.36 M, and sometimes 0.48 M NaCl (corresponding to $\pi = -5, -10, -15$, and -20 atm, respectively) and higher. Loss of water was compensated daily.

Isolation of Mitochondria and Preparation of Enzyme. Root tips, 2 cm long, were collected and blotted with filter paper. Two g of root were ground with sand and 0.5 g of Polyclar AT in 0.1 M tris-HCl buffer (pH 7.4) containing 0.5 M sucrose, 5 mM EDTA, and 1 mM dithioerythritol (buffer I in Fig. 1) and treated as outlined in Figure 1.

For kinetic studies, the soluble and the solubilized enzymes were partially purified by precipitation with solid $(NH_4)_2SO_4$.

[1] This paper is taken from the Ph.D. thesis of A. K.

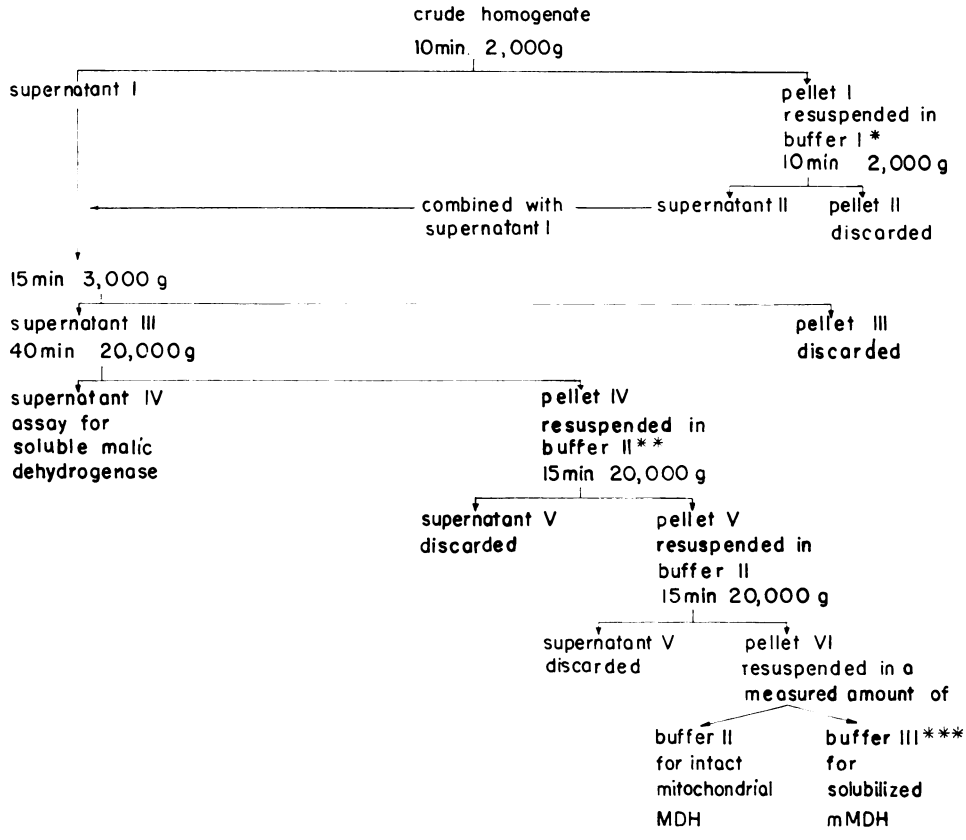

FIG. 1. Isolation procedure of the crude mitochondrial and soluble malic dehydrogenase. Buffer I*: see text; buffer II** was the same as buffer I with 1 mg/ml BSA; buffer III*** was the same as buffer I without sucrose and containing 0.5% Triton X-100. The solubilized mitochondria and the supernatant IV were passed through a column of Sephadex G-25 and served as a source of sMDH.

Table I. *Malic Dehydrogenase Activity in Mitochondrial and Soluble Fractions Isolated from Tamarix Roots Grown at Different Levels of Salinity*

The reaction followed NADH oxidation in the presence of OAA at 340 nm. Reaction mixture contained in 3 ml: 1 μmole of OAA, 0.2 μmole of NADH, 3 μmole of EDTA, 1 μmole of cysteine·HCl, 300 μmole of tris-HCl, pH 7.5. The results are means of at least three replicates. In part A, no NaCl was present in the assay mixture. In part B, NaCl was present in the assay medium at the same concentration as in growth medium.

	NaCl in Growth Medium		mMDH			sMDH		
			Specific activity[1]	Control	Total activity[2]	Specific activity[2]	Control	Total activity[2]
	atm	*mM*		%	× 10^2		%	× 10^2
A. No NaCl in assay medium	−0.5	0	0.78	100	8.4	0.43	100	99.4
	−5.5	120	0.73	94	7.7	0.38	88	83.4
	−10.5	240	0.49	63	5.5	0.30	70	78.0
	−15.5	360	0.31	40	3.5	0.28	65	59.6
B. NaCl present in assay medium	−0.5	0	0.78	100	8.4	0.43	100	94.4
	−5.5	120	0.72	92	7.6	0.44	102	91.0
	−10.5	240	0.38	48	4.3	0.24	43	61.0
	−15.5	360	0.19	24	2.0	0.16	37	34.6

[1] Change in absorbance/mg protein·min.
[2] Change in absorbance/g fresh roots·min.

The fraction precipitating between 55 and 75% saturation was collected. The fraction was dissolved in 50 mM tris-HCl buffer (pH 8.0) and passed through a Sephadex G-75 column. More than 70% of the total activity of the crude preparation was located in this fraction. The activity of malic dehydrogenase was followed spectrophotometrically at 340 nm in both directions—oxidation of malate and reduction of OAA[2].

All the preparatory stages were absolutely necessary, otherwise appreciable activity was not achieved. Substitution of Polyclar by Na-ascorbate was not successful.

Protein was estimated according to Lowry *et al.* (18).

RESULTS

sMDH and mMDH enzymes were found in the roots of *Tamarix*. The activity of both isoenzymes was studied in roots grown at different levels of salinity in the presence or absence of NaCl in the assay mixture. The results are given in Table I.

Growing the roots in saline media resulted in decreased malic dehydrogenase activity, specific and total, mitochondrial and soluble. The inhibition was significant only at concentrations higher than 120 mM (π = approximately −5 atm), and the higher the salinity the stronger was the effect. This effect was more pronounced on the mitochondrial than on the soluble enzyme. At the two higher levels of salinity, inclusion of NaCl, at equivalent concentrations, in the assay mixture made the inhibition even higher.

Salinity may affect the enzyme in two ways. (*a*) NaCl in growth medium may depress or repress the protein synthesis, thus less enzyme will be synthesized. This will be a strictly *in*

[2] Abbreviations: OAA: oxaloacetate; sMDH: soluble malic dehydrogenase; mMDH: mitochondrial malic dehydrogenase; DTT dithiothreitol.

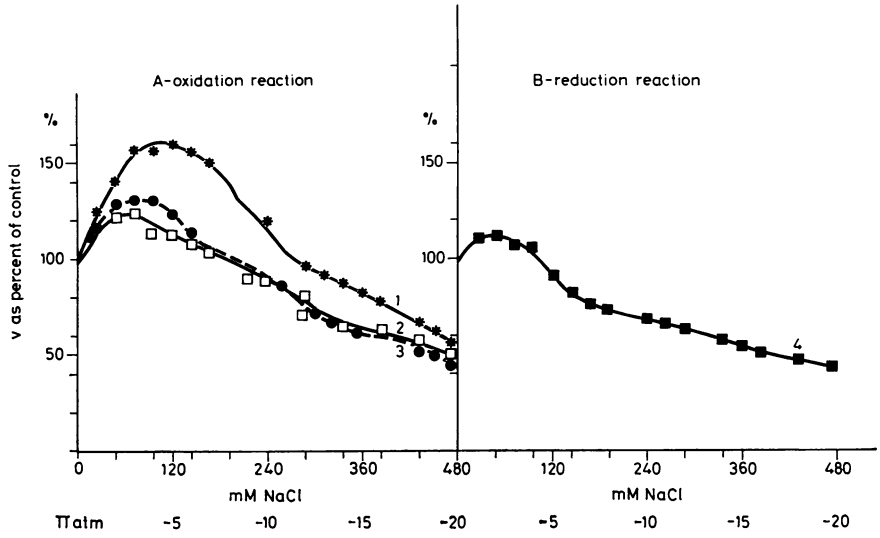

FIG. 2. Effect of NaCl in the reaction mixture on the specific activity of malic dehydrogenase. Results are given as percentage of activity in absence of NaCl. All points on graph are means of three replicates. For malate oxidation, the reaction mixture contained in mM: malate, 10; NAD, 1; glycine·KOH, 100; pH 9.5. For OAA reduction reaction, the mixture contained in mM: OAA, 0.33; NADH, 0.1; tris·HCl, 100; pH 7.5. A: oxidation reaction; 1: soluble enzyme from control plants (✳——✳); 2: mitochondrial enzyme of control plants (□——□); 3: soluble enzyme from roots grown at 360 mM NaCl (●- -●). B: reduction reaction; 4: soluble enzyme from control plants (■——■). The mitochondrial enzyme was solubilized after isolation of the particles.

vivo effect. (*b*) High salt concentration in the cell may affect the structure of the enzyme; conformational changes may occur and thus affect the catalytic activity and the ability to bind the substrate or the coenzyme or both. This type of effect could operate *in vivo* and *in vitro*. It was expected to differentiate between these two types of effects mainly with aid of a kinetic analysis.

Kinetic studies were carried out on the soluble enzymes derived from control roots and roots grown at 360 mM NaCl. The reaction studied was that of malate oxidation. No NaCl was present in the reaction mixture. Apparent K*m* values were calculated for malate and NAD. The K*m* for L-malate was 11.7 mM for the enzyme from salt-treated plants as against 7.6 mM for the control. The parallel K*m* values for NAD were 1.4 for the salt-treated and 2.4 for the controls. These data and other data not reported here on the response of the enzyme to salinity suggested the possibility of conformational changes in the enzyme molecule, which may have been induced by the continuous presence of NaCl in the cell. If this is the case, it should be possible to duplicate the effect *in vitro*.

The effect of NaCl *in vitro* on the malic dehydrogenase was therefore studied. NaCl was added to the assay mixture for the mMDH and sMDH, isolated from control roots and roots exposed to salinity (Fig. 2). The assay was run in a rather high buffer concentration but preliminary experiments showed that in the absence of NaCl in the reaction medium the activity of the enzyme was the same at 0.1, 0.05, or 0.025 M buffer. The addition of NaCl (or other salts) to any of these, changed the activity.

As shown in Figure 2, enzyme isolated from plants grown in nonsaline medium was stimulated *in vitro* by relatively low NaCl concentrations. The most effective concentration being 80 to 100 mM. The stimulation was most pronounced with the soluble enzyme in the oxidation reaction (line 1), and least apparent in the soluble enzyme in the reduction reaction where the effect was practically nonsignificant (line 4). The reduction reaction by the mitochondrial enzyme from roots grown in the absence of NaCl (line 2, Fig. 2) was less sensitive to NaCl *in vitro* than the soluble enzyme. These results are similar to those reported by Hiatt and Evans (11) and by Greenway and

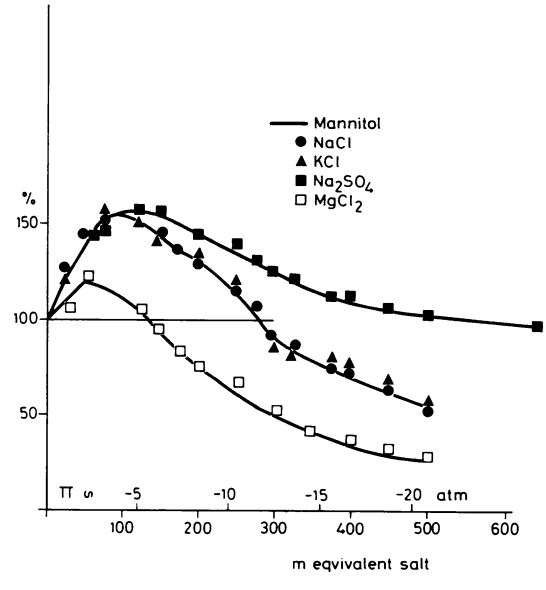

FIG. 3. Effect of various salts *in vitro* as compared with the purely osmotic effect of mannitol on the oxidative activity of sMDH from roots grown in the absence of NaCl. The reaction mixture contained in μmoles: sodium malate, 26; NAD, 2.7; EDTA, 3; DTT, 0.6; glycine·KOH, 300, pH 9.5. Results are expressed as percentage of activity in reaction mixture without salt.

Osmond (10). Weimberg (30) has reported stimulation of sMDH from imbibed pea seeds by 10 and 25 mM NaCl, higher concentrations had an inhibitory effect. In our experiments, the *in vitro* stimulatory effect was usually larger for the soluble enzyme from control roots (Fig. 2, line 1) than for that isolated from roots exposed to 360 mM NaCl during growth (line 3), but the difference was not always reproducible.

The effect of sodium and chloride ions on the enzyme activity was studied also in combination with other ions. Effects of NaCl, KCl, Na_2SO_4, and $MgCl_2$ added *in vitro* to the reaction mixture are shown in Figure 3. The effect of isoosmotic

Table II. *Effect in Vitro of Various Salts on Malic Dehydrogenase Activity of Entire Mitochondria from Control Roots and Roots Grown in NaCl*

Results are given as percentage of the activity (OAA reduction) in reaction mixture without salt. The activity in the mitochondria from the NaCl-treated roots was 40% lower than that of the mitochondria from control roots.

Salts in Reaction Mixture	Mitochondria from Control Roots				Mitochondria from Roots Grown in 360 mM NaCl			
	NaCl	KCl	Na_2SO_4	$MgCl_2$	NaCl	KCl	Na_2SO_4	$MgCl_2$
meq								
0	100	100	100	100	100	100	100	100
48	95	90	96	86	105	103	106	82
120	89	85	92	46	90	90	100	53
192	81	81	88	35	86	85	98	42
240	74	76	86	29	78	80	95	35
288	66	70	84	28	73	70	94	27
360	60	65	80	25	66	65	92	20
432	52	56	72	20	59	55	84	18
480	48	51	66	17	53	50	73	16

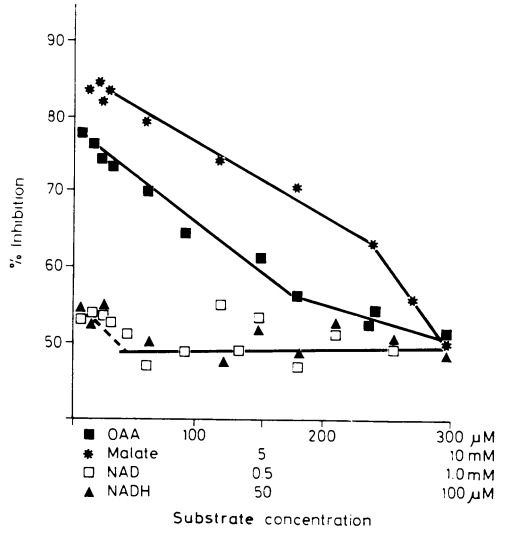

FIG. 4. Effect of substrate concentration on the inhibition of mMDH activity by 480 mM NaCl. The results are expressed as percentage inhibition compared with reaction mixture without NaCl.

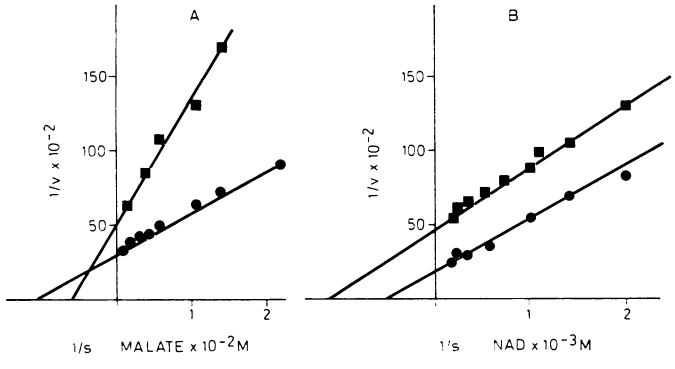

FIG. 5. Double reciprocal plot for the activity of sMDH with changing malate concentration (A) or NAD concentration (B). NAD concentration at A was 1 mM and malate concentration at B was 10 mM. Reaction mixture without NaCl (●); 480 mM NaCl in reaction mixture (■). Plants were grown without NaCl.

concentrations of mannitol, in absence of salts, was studied in parallel.

The effects of NaCl and KCl on the oxidation reaction with the soluble enzyme were practically identical—both stimulated the reaction at low concentrations (80–100 mM) and inhibited at high concentrations (250 mM and higher). Sodium sulfate was less inhibitory in higher concentration and the stimulatory effect could be observed at a wider range of concentrations. Magnesium chloride, however, had practically no stimulatory effect at low concentrations and was much more damaging at high concentrations. Mannitol had no effect. Similar effects of NaCl and Na_2SO_4 were also observed when the malic dehydrogenase activity was followed during OAA reduction.

In the experiment depicted in Figure 2, the mMDH was released from the particles by solubilization. The possibility exists that inside the intact mitochondrion, the enzyme may react to salinity in a different way than the liberated enzyme. It was suggested that the malic dehydrogenase is located in the external membrane of the mitochondrion (7, 17); if this is so, permeability should not be a limiting factor. However, respiratory control mechanisms, functioning in the intact particle, may cause a different response to the effect of NaCl and other salts than in free enzymes. The results in Table II show that, to some extent, this was the case. No stimulation of malic dehydrogenase activity was caused by low salt concentration in mitochondria isolated from control roots, or in mitochondria isolated from roots exposed during growth to 360 mM NaCl. Otherwise the effects of the different salts were similar to their effects on the solubilized enzyme.

Increase in substrate concentration, either malate or OAA, markedly decreased the inhibitory effect of 480 mM NaCl on the mitochondrial enzyme at the optimal pH (Fig. 4). Change of the coenzyme concentrations had almost no effect. Similar results were obtained with the soluble enzyme. A double reciprocal plot of the results for the soluble enzyme is shown in Figure 5. Similar plots were obtained for the mitochondrial enzyme. It follows that NaCl added to the reaction mixture decreased the affinity of the enzyme (soluble and mitochondrial) for the substrate malate, but increased the affinity for the coenzyme (NAD).

If the reduction of OAA is the reaction studied, a clear effect of substrate inhibition is evident, and this effect is abolished by addition of NaCl to the reaction mixture (Fig. 6), although the reaction as a whole is inhibited. The magnitude of the inhibition of the reaction by NaCl depends on the substrate concentration: in supraoptimal concentrations, inhibition decreased with increasing substrate concentrations.

The interaction between substrate and NaCl concentration in their effect on the activity of sMDH was studied at 72 mM, the stimulating concentration, and at 480 mM, the inhibitory concentration. Roots grown in absence of NaCl (Fig. 7A) and roots exposed to salinity of 360 mM during growth (Fig. 7B) were used. In the presence of the stimulatory concentrations of NaCl in the reaction mixture, much higher substrate concentrations are necessary to demonstrate substrate inhibition than in the absence of NaCl (Fig. 7A). Moreover, NaCl stimulation *in vitro*, is evident only at relatively high substrate concentration (only above 0.05 mM OAA). In the presence of the inhibitory NaCl concentrations, no inhibitory effect of substrate was found even at the concentration of OAA of 3.2 mM. When the plants were exposed to salinity during growth, no inhibitory substrate concentration was found (in the range tested) for any of the NaCl *in vitro* treatments (Fig. 7B). The stimulatory and inhibitory effects of NaCl *in vitro* were evident as in enzyme derived from control plants. The double reciprocal plots of the data from Figures 6 and 7 are given in Figure 8. These plots show clearly that if NaCl is

added *in vitro* the apparent K*m* and the V_{max} for both the substrate (OAA) and the coenzyme (NADH) are affected (Fig. 8, A and B). A similar effect could be observed also for the soluble enzyme. Figure 8, C and D, shows that any addition of NaCl, whether in stimulatory or inhibitory concentration, increases the K*m*, but the effect on V_{max} differs with the two NaCl concentrations. Results reported by Weimberg (30) for sMDH from imbibed pea seeds show that salt increased the apparent maximal velocity of the reaction but decreased the affinity for OAA. He used much lower salt concentration than were used by us.

The effect of NaCl on the activity of the enzyme at different pH values was studied. Siegel and Englard (25, 26) and Sulebele and Silverstein (27) have shown that mMDH and

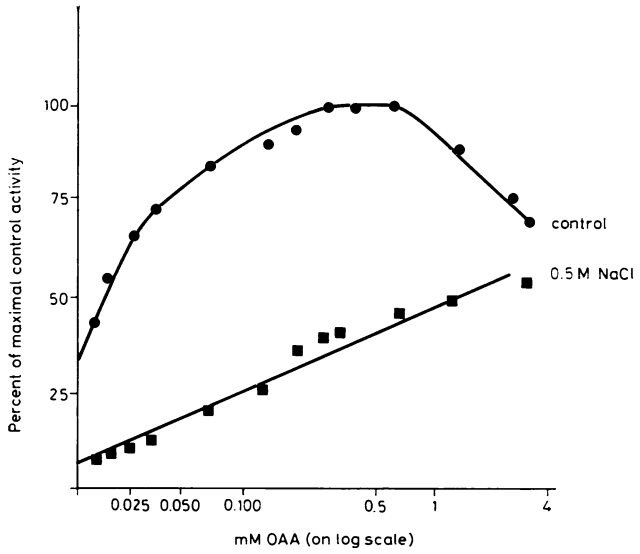

FIG. 6. Effect of NaCl at changing substrate concentrations on the reductive activity of mMDH. Plants were grown in nonsaline medium. NaCl (0.5 M) was added into the reaction mixture. Composition of the reaction mixture in μmoles: OAA, 1; NADH, 0.2; tris-HCL, 300; EDTA, 3; DTT, 0.6. Results are expressed as percentage of maximal activity of the control.

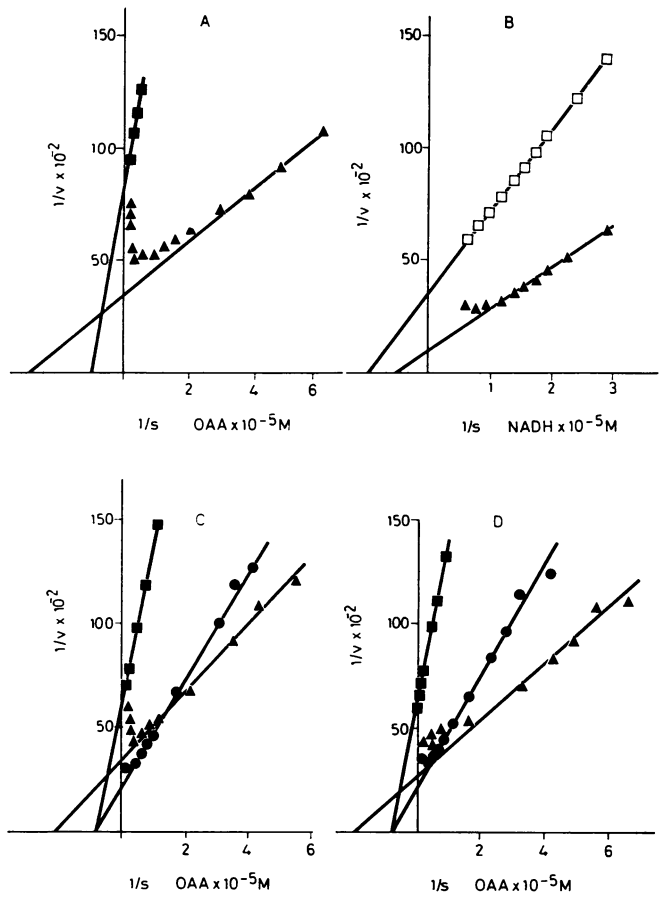

FIG. 8. Double reciprocal plots for data of Figs. 6 and 7 and additional data for changing concentrations of NADH with mitochondrial enzyme. A and B: Mitochondrial enzyme from plants grown in absence of NaCl; C: soluble enzyme from plants grown in absence of NaCl. D: soluble enzyme from plants exposed during growth to 360 mM NaCl and then 480 mM or 72 mM NaCl were added to the reaction mixture. The reaction mixture contained 600 mM NaCl (□); 480 mM NaCl (■); 72 mM NaCl (●); no NaCl (▲).

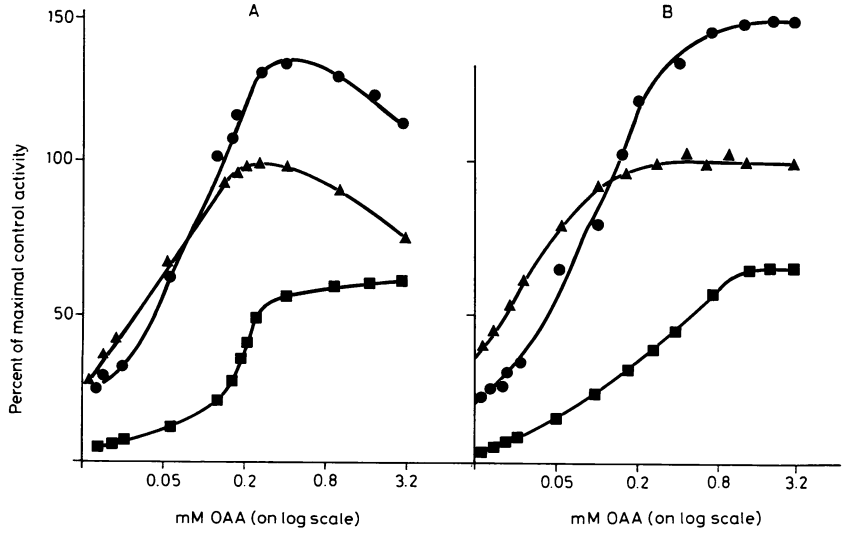

FIG. 7. Effect of NaCl and various substrate concentrations on the reductive activity of sMDH. Reaction in absence of NaCl (▲); 72 mM NaCl (●); 480 mM NaCl (■). A: Plants grown in absence of NaCl; B: plants grown in 360 mM NaCl. Reaction mixture the same as in Fig. 6. Maximal control specific activity was 4.37.

sMDH have different pH requirements. Figure 9 shows that, indeed, this is the case for the reaction of OAA reduction. In the soluble enzyme, the rate of reduction decreased with increasing pH from 7.5 to 10 (Fig. 9B). For the mitochondrial enzyme when inside the particle, a broad peak between pH 5.7 and 8.5 was found, but if the reaction was studied with solubilized mitochondrial enzyme, the pH optimum was at 8.7 and the peak was more defined (Fig. 9A).

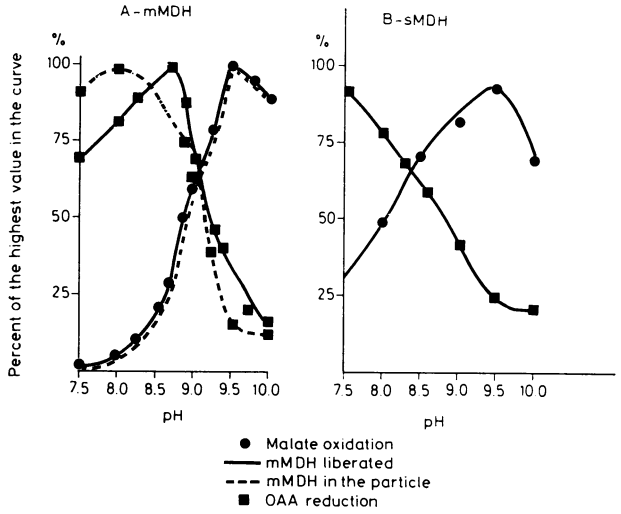

FIG. 9. Effect of pH on the activity of malic dehydrogenase from control roots. The reaction mixture contained in mM: glycine·KOH, 100; EDTA, 2; NAD, 1; malate, 10, or NADH 0.2; OAA, 0.3; DTT, 0.1. Results are given in arbitrary units calculated on the basis of maximal activity for each substrate. A: Mitochondrial enzyme: liberated enzyme (——); enzyme inside the particles (– – –). B: Soluble enzyme: malate oxidation (●); OAA reduction (■).

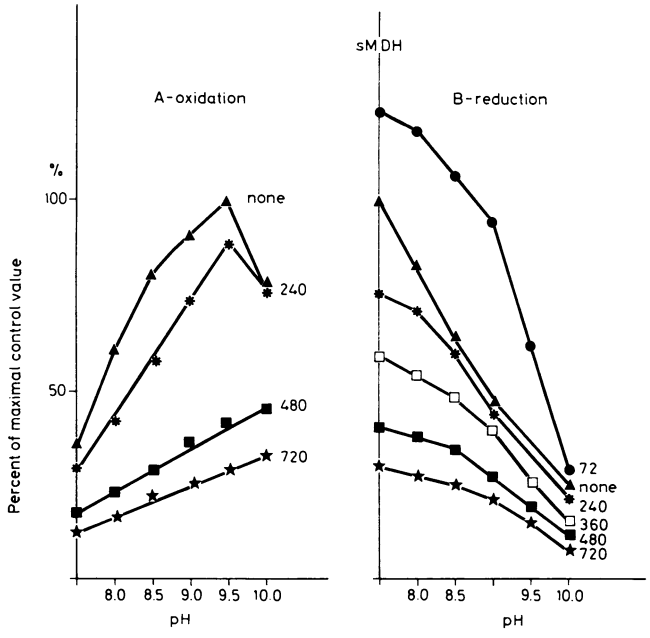

FIG. 10. Effect of NaCl on the specific activity of sMDH at different pH value. Results are given as percentage of the maximal control value. A: Oxidation of malate; B: reduction of OAA. Reaction mixtures are the same as in legend to Fig. 5. Numbers on the right-hand side (A and B) indicate mM concentration of NaCl added into the reaction mixture. Plants were grown in absence of NaCl.

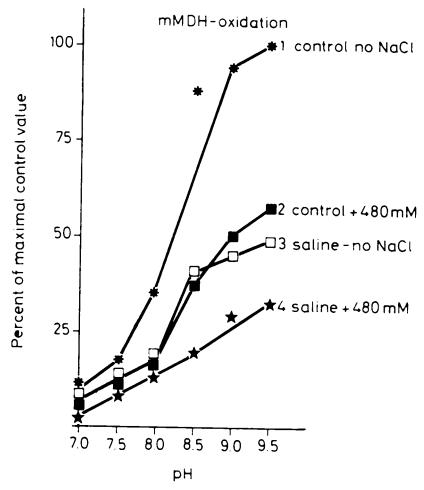

FIG. 11. Effect of NaCl on the activity of mMDH at different pH values. Results are given as percentage of the maximal control value. Grown and reaction in absence of NaCl-control (✳); grown in absence of NaCl, reaction in presence of 480 mM NaCl (■); grown in presence of 360 mM NaCl reaction without NaCl (□); 360 mM NaCl in growth medium and 480 mM NaCl in reaction medium (★).

The mitochondrial enzyme shows almost no oxidative activity (either in the particle or when solubilized) at pH 8.0 and lower. The activity increases rather sharply with increasing pH to an optimum at 9.5 (Fig. 9A). The optimal pH for the oxidative activity of the soluble enzyme was also 9.5 (Fig. 9B), but 50% of the activity was still detected at pH 8.

If NaCl was added into the reaction mixture of the soluble enzyme, at concentrations higher than 240 mM, the oxidation reaction (Fig. 10A) was severely depressed, the depression being stronger at the optimal than in the other pH values, thus abolishing the optimum peak. The reduction reaction was also increasingly inhibited by increasing concentrations of NaCl, the inhibition being stronger at the lower pH range (7–8.5) than in the higher, similar to the findings of Weimberg (30). The stimulatory effect of low NaCl concentration was evident almost throughout the whole pH range tested (Fig. 10B), also similar to the findings of Weimberg (30) for sMDH from pea seeds.

The mitochondrial enzyme from plants grown in the absence of NaCl was much less sensitive to the effect of NaCl *in vitro* than the soluble enzyme; this was especially evident at the pH range of 7.5 to 8.5 (Fig. 11, lines 1 and 2, as compared to Fig. 10A).

If the plants were exposed to 360 mM NaCl during growth, the mitochondrial enzyme isolated from their roots reacted to different pH conditions almost in an identical way to the control enzyme with 480 mM NaCl added *in vitro* (Fig. 11, lines 2 and 3). If NaCl was added *in vitro* to enzyme isolated from plants exposed to NaCl also during growth, an even stronger inhibition of activity occurred at pH values of 8.5 to 9.5 (Fig. 11, line 4).

DISCUSSION

The data show that malic dehydrogenase isolated from roots of the halophyte *Tamarix tetragyna* L. had no obligatory requirement for salt. Although low NaCl concentrations did stimulate enzyme activity, concentrations above 100 mM had a strong inhibitory effect. This is similar to the effect of NaCl on the enzymes isolated from glycophytes. However, obligatory

requirements for high salt concentrations (to 4 M) are well known for enzymes of halophilic bacteria (13).

The data in Figure 3 and in Table II support the notion that the effect of salinity on the malic dehydrogenase is not a simple effect of ionic strength. There certainly are specific effects of the different ions. The order of the salts for the effect was

$$MgCl_2 > KCl = NaCl > Na_2SO_4$$

This order is different from that reported for halophilic bacteria, where considerable differences were found between the effects of NaCl and KCL (1).

An attempt was made to analyze the effect of NaCl on the kinetics of the malic dehydrogenase. Duporque and Kun (8) found that increase in phosphate concentration from 50 to 100 mM increased 8-fold, the apparent K*m* value for OAA. They concluded that phosphate behaved as a competitive inhibitor of OAA. The effect of chloride was considered to be similar to that of phosphate. Also Weimberg (30) suggested that NaCl at inhibitory concentrations had a competitive effect. Hiatt and Evans (11) found that in the presence of 67 mM NaCl the K*m* for OAA and for NADH increased 2-fold, but they concluded that the enzyme was activated at this NaCl concentration. These findings suggested that the inhibition is not strictly competitive. Kinetic analysis of our experiments (Figs. 5 and 8) show that NaCl *in vitro* caused an increase in the K*m* for the substrate (either malate or OAA) and a decrease in the K*m* for the co-enzyme; the latter is contrary to the findings of Hiatt and Evans (11) for the enzyme from spinach leaves.

The kinetic behavior of malic dehydrogenase is often explained by formation of a ternary complex. First there is a binding of the enzyme and the co-enzyme, then the binding of the substrate to the enzyme occurs. Raval and Wolfe (23, 24) suggest four steps in the reaction:

$$MDH + NAD \underset{K2}{\overset{K1}{\rightleftharpoons}} MDH\cdot NAD \quad (1)$$

$$MDH\cdot NAD + Malate \underset{K4}{\overset{K3}{\rightleftharpoons}} \quad (2)$$

MDHXY (activated ternary complex)

$$MDHXY \underset{K6}{\overset{K5}{\rightleftharpoons}} MDH\cdot NADH + OAA \quad (3)$$

$$MDH\cdot NADH \underset{K8}{\overset{K7}{\rightleftharpoons}} MDH + NADH \quad (4)$$

The fourth step seems to be the rate-limiting one—the dissociation of the complex MDH·NADH (K7). The increased affinity for the coenzyme induced by salinity, as reported in this paper, will cause the increase in rate of step 1, but it will also stabilize the complex of "enzyme-reduced coenzyme" and thus will reduce considerably the rate of step 4. Increase of the K*m* for the substrate, in this course of events, will have an inhibitory effect. The rate of the over-all reaction will depend on the compulsory sequence of the above four steps.

The graphs of 1/v *versus* 1/s for malate oxidation (Fig. 5) and for OAA reduction (Fig. 8) indicate the possibility that NaCl exerted partially mixed noncompetitive inhibition and possibly inhibited the dissociation of the reaction products from the enzyme. At the same time, an increased K*m* indicated the possibility of disturbances in binding the substrate and competition with the substrate. On the other hand, the curves for the coenzyme (Figs. 5B and 8B) suggested coupling or uncompetitive inhibition, *i.e.* NaCl increased the affinity for the coenzyme but at the same time inhibited the rate of reaction as suggested above.

Table III. *Kinetic Data for Effect of NaCl on Activity of Malic Dehydrogenase*

K*m* and V_{max} are data in absence of NaCl in reaction mixture; K′*m* V'_{max} are data in presence of NaCl in reaction mixture. α indicates change in affinity of the enzyme to the substrate.

Enzyme	NaCl Concn in Growth Medium	Substrate	K*m*	NaCl Concn in Reaction Mixture	V_{max}/V'_{max}	K′*m*	K*i*	α
	mM		μM	mM	ratio	μM		
sMDH	0	Malate[1]	7,450	480	1.65	13,300	0.37	3.0
	0	NAD	1,820	480	2.67	910	0.48	
	0	OAA[2]	52	480	3.02	134	0.06	4.8
	360	OAA	53	480	2.96	139	0.08	4.2
mMDH	0	Malate	11,600	600	2.18	23,100	0.39	3.0
	0	OAA	33	480	2.50	106	0.15	4.8
	360	OAA	37	480	1.90	74	0.31	3.1
	360	NADH	25	480	3.50	11,600	0.42	

[1] pH 9.5: When malate concentration was changed, NAD concentration was 0.9 mM. When NAD concentration was changed, malate concentration was 9 mM.
[2] pH 7.5: When OAA concentration was changed, NADH concentration was 67 μM. When NADH concentration was changed, OAA concentration was 0.33 mM.

Table III shows the apparent kinetic data for the effect of NaCl on malic dehydrogenase. Apart from the changes in K*m* already mentioned above, K*i* and α for malate, for both isoenzymes sMDH and mMDH, were practically identical. This was not the case for the K*i* for OAA of the two isoenzymes. A great similarity was found between the data (OAA reduction) for the soluble enzyme from control roots and roots grown in presence of NaCl (K*m*, K′*m*, K*i*, α). The similarity was less apparent between the mitochondrial enzymes from the two sources.

An interaction was found between substrate concentration and salinity level. Salinity counteracted substrate inhibition but an increasing substrate concentration neutralized the inhibitory effect of NaCl (Figs. 4 and 6).

In vitro NaCl seems to have three types of effects on the soluble enzyme (Fig. 8, C and D). (*a*) Change in affinity to the substrate that is manifested by changes in the K*m*: this required comparatively low NaCl concentrations, as seen for the soluble enzyme (Fig. 8, C and D) where the lines for both NaCl concentrations intercept the axis at the same place. (*b*) Effect on rate of reaction: low NaCl concentrations increased V_{max} of the soluble enzyme, high NaCl concentrations decreased V_{max}, thus V_{max}/V'_{max} was > 1. V_{max} is apparently not directly dependent on K*m* and is not affected by the effect of NaCl on the affinity between substrate and enzyme only. (*c*) Counteracting substrate inhibition: this may be caused by NaCl either *in vivo* (Fig. 7B) or *in vitro* (Fig. 7A). *In vitro* NaCl did not affect the K*m* for OAA (Table III), but it did affect the K*m* for malate and NAD.

It was shown that the NaCl inhibition of the enzyme activity depends on substrate concentration; increased substrate concentration counteracted the damaging salinity effect and thus may have a protective function under saline conditions. It would appear that the internal pH conditions may have a similar effect. Ting (28) and Zschoche and Ting (31) showed that OAA substrate inhibition was affected by pH. The soluble and mitochondrial enzymes showed different pH requirements (Fig. 9). Similar findings were reported previously by Siegel and Englard (25, 26) for enzymes isolated from beef hearts: the soluble enzyme was less sensitive to pH changes than the

mitochondrial enzyme. At pH 8.0 the mitochondrial enzyme had practically no oxidative activity (Fig. 9A), while the reductive activity showed half of its maximal activity at this pH (Fig. 9B). The reductive activity showed a reversed trend: the optimal pH for the mitochondrial enzyme was at pH 8.5, while the soluble enzyme had its optimal activity around pH 7 to 7.5. There was very little difference between the mitochondrial enzyme inside the particles and when solubilized.

Addition of NaCl *in vitro* to the soluble enzyme resulted in decreased sensitivity to pH changes (Fig. 10). The effect was less pronounced for the mitochondrial enzyme (Fig. 11). The similarity of the effect of the same NaCl concentration *in vivo* and *in vitro* suggests that also inside the cell, under saline conditions, the mitochondrial enzyme is exposed to the effect of NaCl.

Flowers (9) postulates that the tolerance of the halophytes to salinity is due to their ability to accumulate the ions inside the vacuole and counteract their osmotic effect by accumulation of sugars in the cytoplasm. It was shown that in *Tamarix* roots the mitochondrial enzyme is somehow exposed to the effect of NaCl in the cell as an *in vivo* effect was demonstrated; therefore, the compartmentalization cannot be complete.

The results suggest that the halophyte can counteract the harmful effect of salinity by several mechanisms, among them possible changes of pH and, mainly, changes in metabolic pathways resulting in accumulation of substrate. This may have an osmotic balancing effect as postulated by Flowers (9), but also may result in reduction of the inhibition of enzyme activity caused by salinity.

Our data would indicate that NaCl has very similar effects on the enzyme *in vivo* and *in vitro*. No evidence was presented in this paper that NaCl depresses enzyme synthesis, but the evidence suggests very strongly that conformational changes occur in the enzyme due to salinity. This view is supported by the findings showing the effect of NaCl on K*m* of substrate and coenzyme, and by the finding that the effect of NaCl on enzyme activity is not due solely to the effect on the K*m*. Similar views that the effect of salinity on enzyme is through conformational changes were expressed by Duporque and Kun (8), Datta (6), and by Lieberman and Lanyi (16), but it has not yet been proven beyond doubt.

LITERATURE CITED

1. Aitken, D. M., A. J. Brown, and A. D. Brown. 1970. Properties of a halophil nicotinamide adenine dinucleotide phosphate-specific isocitrate dehydrogenase. Preliminary studies of the salt relations and kinetics of the crude enzyme. Biochem. J. 116: 125–134.
2. Baxter, R. M. 1959. An interpretation of the effect of salts on the lactic dehydrogenase of *Holobacterium salinarum*. Can. J. Microbiol. 5: 47–55.
3. Bernstein, L. 1961. Osmotic adjustment of plants to saline media. I. Steady state. Amer. J. Bot. 48: 909–918.
4. Bernstein, L. 1963. Osmotic adjustment of plants to saline media. II. Dynamic phase. Amer. J. Bot. 50: 360–370.
5. Cohen, P. and M. A. Rosenmayer. 1969. Human glucose 6-phosphate dehydrogenase. Purification of the erythrocyte enzyme and the influence of ions on its activity. Eur. J. Biochem. 8: 1–7.
6. Datta, P. 1971. Homoserinedehydrogenase of *Rhodospirillum rubrum*. Conformational changes in the presence of substrates and modifiers. Biochemistry 10: 402–408.
7. Davies, D. D. and E. Kun. 1957. Isolation and properties of malic dehydrogenase from ox heart mitochondria. Biochem. J. 66: 307–316.
8. Duporque, D. and E. Kun. 1969. Malate dehydrogenase of ox kidney. 2. Two substrate kinetics and inhibition analyses. Eur. J. Biochem. 7: 242–252.
9. Flowers, T. J. 1972. The effect of sodium chloride on enzyme activities from four halophyte species of Chenopodiaceae. Phytochemistry 11: 1881–1886.
10. Greenway, H. and C. B. Osmond. 1972. Salt responses of enzymes from species differing in salt tolerance. Plant Physiol. 49: 256–259.
11. Hiatt, A. J. and H. J. Evans. 1960. Influence of salts on activity of malate dehydrogenase from spinach leaves. Plant Physiol. 35: 622–627.
12. Hoagland, D. R. and D. I. Arnon. 1950. The water culture method for growing plants without soil. Calif. Agric. Exp. Stat. Cir. 347: 32p.
13. Holmes, P. K. and H. O. Halvorson. 1965. Purification of a salt-requiring enzyme from an obligatory halophilic bacterium. J. Bact. 90: 312–315.
14. Hubbard, J. S. and A. B. Miller. 1969. Purification and reversible inactivation of the isocitrate dehydrogenase from an obligate halophile. J. Bact. 99: 161–168.
15. Hubbard, J. S. and A. B. Miller. 1970. Nature of the inactivation of the isocitrate dehydrogenase from an obligate halophile. J. Bact. 102: 677–681.
16. Leiberman, M. N. and J. K. Lanyi. 1972. Threonine deaminase from extremely halophilic bacteria. Cooperative substrate kinetics and salt dependence. Biochemistry 11: 211–221.
17. Livne, A. and N. Levine. 1967. Respiration and oxidative phosphorylation of NaCl-treated leaves. Plant Physiol. 42: 107–113.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
19. Macrae, A. R. 1971. Malic enzyme activity in plant mitochondria. Phytochemistry 10: 2343–2347.
20. Marquez, E. D. and A. F. Brodie. 1970. Electron transport in halophilic bacteria. Involvement of a menbaquinone in the reduced nicotinamide adenine dinucleotide oxidative pathway. J. Bacteriol. 103: 261–263.
21. Rafaeli-Eshkol, D. 1968. Studies on halotolerance in a moderately halophilic bacterium. Effect of growth conditions on salt resistance of the respiratory system. Biochem. J. 109: 675–685.
22. Rafaeli-Eshkol, D. and Y. Avi-Dor. 1968. Studies on halotolerance in a moderately halophilic bacterium. Effect of betaine on salt resistance of the respiratory system. Biochem. J. 109: 687–691.
23. Raval, D. N. and R. S. Wolfe. 1962a. Malic dehydrogenase. III. Kinetic studies of the reaction mechanism by product inhibition. Biochemistry 1: 1112–1117.
24. Raval, D. N. and R. S. Wolfe. 1962b. Malic dehydrogenase. IV. pH dependence of the kinetic parameters. Biochemistry 1: 1118–1123.
25. Siegel, L. and S. Englard. 1961. Beef heart malic dehydrogenases. I. Properties of the enzyme purified from extracts of acetone dried powder. Biochim. Biophys. Acta 54: 67–76.
26. Siegel, L. and S. Englard. 1962. Comparative studies of some properties of mitochondrial malic dehydrogenase and soluble malic dehydrogenase. Biochim. Biophys. Acta 64: 101–110.
27. Sulebele, G. and E. Silverstein. 1969. Malate dehydrogenase and aspartate aminotransferase of *Phycomyces blakesleeanus*. Arch. Biochem. Biophys. 133: 425–435.
28. Ting, J. P. 1968. Malic dehydrogenases in corn root tips. Arch. Biochem. Biophys. 129: 1–7.
29. Waisel, Y. 1972. Biology of Halophytes. Academic Press, New York.
30. Weimberg, R. 1967. Effects of sodium chloride on the activity of a soluble malate dehydrogenase from pea seeds. J. Biol. Chem. 242: 3000–3006.
31. Zschoche, W. C. and J. P. Ting. 1973. Purification and properties of malate dehydrogenase from *Spinacea oleracea* leaf tissue. Arch. Biochem. Biophys. 159: 767–776.