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THE EFFECT OF SALTS ON MALATE DEHYDROGENASE FROM LEAVES OF *ZEA MAYS*

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Key Word Index—*Zea mays*; Gramineae; maize; malate dehydrogenase; salts; salinity.

Abstract—The effect of sodium and potassium chlorides and of sodium sulphate on malic dehydrogenase (EC 1.1.1.37) from *Zea mays* chloroplasts and cytosol has been investigated. Optimal NaCl concentration (100 mM) doubled the rate of oxaloacetate reduction by the cytosolic enzyme with NADH but caused an increase of some 10-fold with NADPH. Oxidation of malate was also stimulated using NADP but the optimal concentration was less than 20 mM. The chloroplast enzyme activity [with NAD(H)] was not significantly activated by increasing ionic strength.

INTRODUCTION

Under saline conditons, all plants appear to respond to the elevated external salt concentration by increasing their content of those ions predominating in their environment. However, while halophytes are able to regulate the accumulation of substantial quantities of ions in their shoots[1], for the majority of plants, resistance is conferred by excluding as high a proportion of ions as possible from the tops[2]. In all species, however, it appears essential that the cytoplasmic ion concentration be maintained within similar limits if the metabolism is not to be disrupted. It may be assumed that the salt-sensitive species are less successful at achieving this end, under saline conditions, than the more tolerant species, although we have no detailed information on the sequence of events leading to the death of a plant from salinity except that its salt content normally rises to excessive levels.

Salts present in leaf cells under saline conditions may be accumulated in various compartments, for example, cytosol, mitochondria, chloroplasts and vacuoles, and may be expected to affect the plant at least in part through specific effects on particular enzymes. In this paper we report some preliminary findings on the effects of salts on one of our most salt-sensitive crop species, *Zea mays*.

Maize is considered to be the "type species" in which malate is the primary vehicle of carbon flow between mesophyll and bundle sheath chloroplasts. This species also typifies that group of plants in which this is achieved through the activity of an NADP-specific malate dehydrogenase (see [3]). This enzyme, which is more active than the NAD-specific malate dehydrogenase in partially purified extracts from such species [4] is activated by light and dithiothreitol (DTT)[5, 6]: activation is regulated by protein factors [3, 7, 8] which can be separated from the enzyme by chromatography on Sephadex G200. The enzyme probably operates preferably in the direction of malate formation, since its activity in this reaction is three times higher than in the opposite direction[4].

Most studies of NADP-malate dehydrogenase have concentrated on the effects of light and sulphydryl reagents on the activity of the enzyme and have not been concerned with other possible activators. Since the activity of many enzymes is affected by the concentration of inorganic ions, which may be accumulated to particularly high levels in cells and chloroplasts in plants growing under saline conditions, we have investigated the *in vitro* effects of such ions on the activity of NADP- and NAD-malate dehydrogenase. In these preliminary experiments we have observed that after partial purification and desalting with Sephadex, the NADP-specific enzyme activity was dramatically increased by the addition of inorganic ions. A comparison is made between the effects of inorganic ions on the activity of isoenzymes from cytosol and chloroplasts.

RESULTS

Maize plants were grown for 3 weeks in Hoagland's solution and normally contained some 80 mmol of potassium and 2 mmol of sodium/g fr. wt. When sodium chloride was added (at concentrations of either 50 or 100 mM), the sodium concentration increased, although the potassium content remained relatively constant (Table 1). The sodium content of chloroplasts isolated from these leaves also increased with increasing external salinity although the K/Na ratio was much less affected than the overall K/Na ratio by the change in the external conditions (Table 1).

Cytosolic NAD-specific malate dehydrogenase

The activity of malate dehydrogenase was measured in preparations made both from homogenates of whole leaves (but with the main vein

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Table 1. Sodium and potassium contents of leaves and chloroplast fractions from maize plants grown at different salinities

NaCl concn (mM)	Leaf contents (mmol/g fr. wt)			Chloroplast contents (μmol/mg chlorophyll)		
	K	Na	K/Na	K	Na	K/Na
0	81	1.9	43	3.8	1.2	3.3
50	74	44	1.7	3.4	2.7	1.2
100	79	137	0.57	4.1	5.4	0.69

Plants, which were 3 weeks old on harvesting, were grown as described in the Experimental.

removed)—designated cytosolic—, and from mesophyll chloroplasts, using both NAD and NADP as the coenzyme. The purified specific enzyme from the cytosol had an activity of some 30 units/mg protein (17 units/g fr. wt) with apparent K_m values for oxaloacetate and NADH of 88 and 12 μM respectively (pH 7.5; with NADH and oxaloacetate concentrations of 57 and 167 μM respectively). When assayed in the reverse direction the apparent K_m values for L-malate and NAD were 4.6 and 0.36 μM (with NAD and L-malate concentrations of 0.5 and 4.95 mM respectively; pH 9.2)—see also Table 4.

The addition of sodium chloride to the assay medium stimulated the rate of oxaloacetate reduction with an optimum at a concentration of 100 mM (Fig. 1). Sodium chloride was always inhibitory to the oxidation of malate (Fig. 1). The effects of potassium chloride and of sodium sulphate were in general very similar to those of sodium chloride on oxaloacetate reduction, although the degree of stimulation was somewhat greater with the potassium salt (Table 2).

Cytosolic NADP-specific malate dehydrogenase

Malate dehydrogenase activity with NADP as the coenzyme was only 5–10% of that attained with NAD as the coenzyme, when estimated in terms of oxaloacetate reduction. However, the NADP-specific activity was increased by up to about an order of magnitude when the salt concentration was increased from 0 to 100 mM (Fig. 1, Table 3). The oxidation of L-malate was also activated by the addition of sodium chloride to the assay medium, but to a much lesser extent than the reduction of oxaloacetate (Fig. 1). Activation of oxaloacetate reduction linked to NADP was not confined to sodium and chloride ions and was also effected by potassium chloride and sodium sulphate (Table 3).

The increase in the ionic strength of the assay medium also increased the apparent K_m values for both substrates. At an oxaloacetate concentration of 167 μM, increasing the sodium chloride concentration from 33 to 200 mM increased the K_m from 39 to 101 μM (Table 4). Increasing sodium chloride concentrations had a smilar effect on the K_m for NADPH (Table 4).

Chloroplast malate dehydrogenase activity

Malate dehydrogenase activity in preparations from isolated chloroplasts was some 4% of the activity in the cytosolic fraction with NAD as the enzyme but 5–10% with NADP; however, activity with NADP as the coenzyme was only 0.5% of that found with NAD and inconsequence too small to be readily measured. The NAD-specific activity was in general 1.2 units/mg protein. The K_m values determined under the same conditions as used for the cytosolic preparation indicated that the chloroplast enzyme had higher affinities for malate and oxaloacetate than the whole-leaf enzyme, but a lower affinity for NADH; the K_m

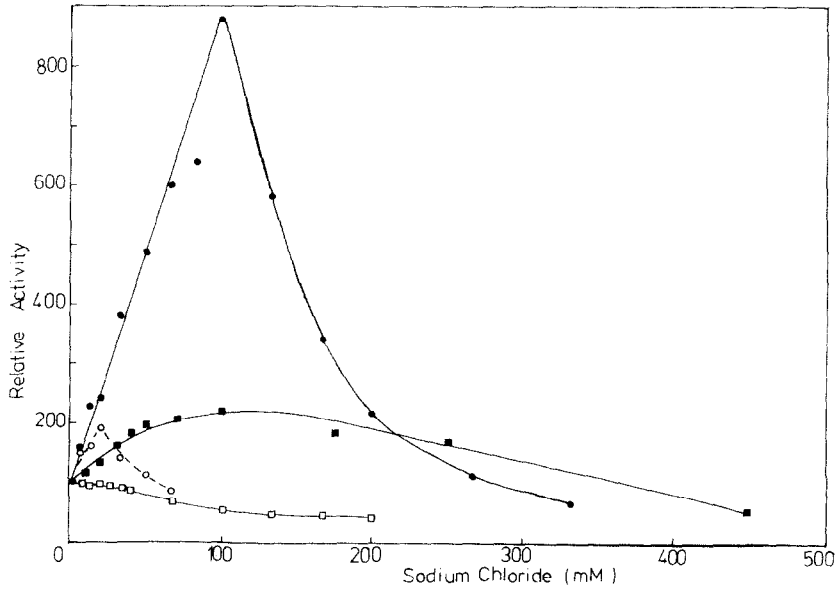

Fig. 1. The effect of sodium chloride on the activity of cytosolic malate dehydrogenase linked to the oxidation or reduction of NAD (square) or NADP (circles). The closed symbols show the effect on the reduction of oxaloacetate and the open symbols on the oxidation of malate.

Table 2. The effect of various salts on the activity of cytosolic NAD-malate dehydrogenase, expressed relative to the activity in the absence of added salts (= 100)

Sodium/potassium concn (mmol/l.)	NaCl	Enzyme activity in KCl (relative units)	Na_2SO_4
10		141	130
20	145	155	141
30		198	145
40		203	164
50	209	232	163
75		307	193
100	227	307	223

Table 3. The effect of various salts on the activity of NADP-malate dehydrogenase, expressed relative to the activity in the absence of added salts

Sodium/potassium concn (mmol/l.)	Enzyme activity in NaCl (relative units)	KCl	Na_2SO_4
17	232	265	245
33	333	333	330
67	453	600	433
83	610	658	466
100	668	705	508
150	520	488	345
200	398	398	395

100 relative units corresponds to 0.17 units/mg protein of malate dehydrogenase.

for NAD was similar in both preparations (Table 4). The chloroplast enzyme was not significantly activated by sodium or potassium chlorides or sodium sulphate at concentrations above 17 mM (Table 5) and shows a marked contrast in this respect to the cytosolic enzyme.

DISCUSSION

The effect of ionic strength on the activity of malate dehydrogenase isolated from maize leaves appears to be a function of both the source of the enzyme (chloroplast or cytosol) and the particular coenzyme (NAD or NADP) utilized. For preparations from the cytosol, the NADH-linked oxidation of oxaloacetate showed maximal activation in the presence of 100 mM NaCl (or KCl) (Table 2, Fig. 1): this is a similar concentration to that previously reported as bringing about maximum stimulation in similar preparations

Table 4. Summary of the apparent K_m values for malate dehydrogenase from maize leaves

Added NaCl (mM)	Fixed substrate (μM)	Apparent K_m(μM) OAA	Malate	NAD(H)	NADP(H)	pH
(A) Cytosolic enzymic						
0	NAD 500	—	4560	—	—	9.2
0	Malate 4950	—	—	360	—	9.2
0	NADH 57	88	—	—	—	7.5
0	OAA 167	—	—	12	—	7.5
(B) Chloroplast–NAD–specific enzyme						
0	NAD 500	—	2970	—	—	9.2
0	Malate 4950	—	—	340	—	9.2
0	NADH 57	33	—	—	—	7.5
0	OAA 167	—	—	360	—	7.5
(C) NADP–specific enzyme						
17	OAA 167	—	—	—	14	7.5
100	OAA 167	—	—	—	117	7.5
200	OAA 167	—	—	—	143	7.5
33	NADPH 57	39	—	—	—	7.5
100	NADPH 57	79	—	—	—	7.5
200	NADPH 57	101	—	—	—	7.5

Table 5. The effect of various salts on the activity of chloroplast NAD-malate dehydrogenase, expressed relative to the activity in the absence of added salts (1.2 units NAD-MDH = 100 units)

Sodium/potassium concn	Enzyme activity in NaCl	KCl	Na_2SO_4
17	99	100	92
33	87	90	90
50	74	70	73
67	58	62	66
100	43	45	50
133	31	37	46
150	29	30	45
200	23	21	34

from *Pisum* and *Suaeda* [9] and maize and barley [10]. There was, however, a dramatic difference with the NADP-specific enzyme when maximum activation approached 10-fold at the optimum salt concentration. With this coenzyme, even the reduction of malate was activated to some extent by NaCl, an occurrence which is not generally apparent with the NAD-linked activity (Fig. 1) [9, 11]. Significant activation of NADP-malate dehydrogenase by monovalent cations has recently been reported by Mackay and Marsh [12] in a preparation from spinach. The activation reported herein for the NADP-linked enzyme from maize was of a greater degree, although this may reflect details of preparation. We have found extensive purification to lessen the activation shown by NAD-malate dehydrogenase to salts and earlier results with *Pisum* and *Suaeda* showed that activation was lost on storage [13].

NADP-linked malate dehydrogenase was too low to be meaasured from chloroplast fractions; the low level of NADP-linked activity was attributed to the effect of Triton X-100 during extraction. There was, however, sufficient NAD-linked activity to enable the effects of changes in ionic strength to be readily determined. Yet another pattern emerged, with activation, if it occurred, being confined to concentrations of less than 17 mM. All the assays were carried out in relatively low concentrations of Tris-acetate buffer to avoid activation of the enzyme activity by the buffer system alone. Differences in the effects of ions on isoenzymes with different subcellular origins have been previously reported for *Pisum*, *Suaeda* [9] and rat kidney [14].

The K_m values here are very similar to those previously reported for malate dehydrogenase activity (e.g. [9]), and invariably increased with salt concentrations that activated and inhibited enzyme activity. The question arises as to whether NaCl-activation is mediated through a regulatory protein, or by some other mechanism, since it has been shown that activation by dithiothreitol or inactivation by O_2 are dependent on such protein(s) [5]. The involvement of regulatory protein(s) may explain why different purification procedures result in different degrees of activation. Both inhibitory and activatory ionic strengths for NADP-malic dehydrogenase affected the enzymic affinity for its substrate and coenzyme in a similar way by decreasing it. Although it is well established that ionic strength and specific ions affect enzyme stability and activity [9, 10, 15–17] two different effects of salt, on one hand activation (100 mM NaCl) and then inhibition at higher concentration (200 mM NaCl) with decreasing enzymic affinity for its substrates as the salt concentration rises, may be better explained as mediated by a regulatory protien, than by suggesting regulation by enzyme–substrate complex formation. Inhibition by higher concentration of salts, which characterizes both enzymes, NAD and NADP-malic dehydrogenase, may be due to conformational changes: regulatory proteins have been identified as being important in the operation of a further enzyme of the C_4 pathway, phosphate dikinase [3], but the effect of ionic strength on its activity is not known.

As far as salinity is concerned, maize, like other glycophytes, belongs to that category of plants which have been labelled "excluders". In essence this means that the greater the resistance of a variety or species, the greater its ability to exclude ions from its shoots [1]. In maize an important mechanism appears to be the reabsorption of sodium and chloride ions by the xylem parenchyma [18]. Ions which do reach the tops are presumed to be sequestered in the vacuoles while the cytoplasmic contents are maintained at lower levels: when this compartmentation cannot be maintained, the death of the cell presumably follows. It appears from the estimated contents of the chloroplasts that this organelle is buffered against large-scale changes in sodium content occurring in the shoot in general. While under saline conditions there is almost two orders of magnitude change in the overall leaf-sodium content the chloroplast content only changed by 4–5 times (Table 1). We recognize that the absolute contents measured in the chloroplast may have been subject to large losses of sodium and potassium during isolation, but such changes should be similar for both ions. The buffering of maize chloroplasts against large changes in their monovalent cation content may partially explain the great sensitivity of NADP-malate dehydrogenase, presumably a chloroplast enzyme [19], to ionic strength.

EXPERIMENTAL

Plants of *Zea mays* (cv LG 11) were grown in aerated Hoagland's soln [20] for between 15 and 25 days in growth cabinet at 22° and *ca* 70% r.h. with a 16-hr light and 8-hr dark period. Where salinity treatments were imposed this was achieved by adding NaCl to the soln to produce final concns of 50 or 100 mM.

Cytosolic malate dehydrogenase. After removal of the main vein, the leaves were cut into pieces and blended for 90 sec in a Tris–acetate buffer (50 mM Tris, pH 8) containing EDTA (2 mM) and DTT (5 mM) and polyvinyl polypyrrolidone (Polyclar AT, 0.2%). The homogenate was filtered through muslin and centrifuged at 40 000 *g* for 15 min. The pellet was discarded and the supernatant used as the source of what we designate as cytosolic malate dehydrogenase in order to distinguish it from the malate dehydrogenase prepared from the chlroroplast fraction.

Protein precipitating between 50 and 75% saturated $(NH_4)_2SO_4$ was resuspended in a minimum of the isolation medium and applied to a DEAE cellulose column prein-

cubated with Tris–acetate (25 mM, pH 8) containing DTT (5 mM) and eluted with 0.4 M KCl dissolved in the same buffer. Fractions rich in malate dehydrogenase were collected and combined, and the protein again precipitated with $(NH_4)_2SO_4$. The ppt was resuspended in the 25 mM Tris–acetate buffer PH 7.5 and applied to a column of Sephadex G50. Fractions rich in malate dehydrogenase activity were combined and utilised for the assays of the NADP-specific malate dehydrogenase activity. The NAD-specific activity was also assayed after a further cycle of $(NH_4)_2SO_4$ precipitation and chromatography on Sephacryl S-200.

Chloroplast malate dehydrogenase. Chloroplasts were isolated by a procedure based on that of ref. [21]: after removing the main vein, the leaf material was homogenized for 25 sec in a soln containing sucrose (0.4 M) Tris–MES (50 mM Tris, pH 7.5), EDTA (2 mM), BSA (0.1%), PVP-40 (0.05%) and DTT (5 mM), to which Polyclar (Sigma) (0.1%) and a few drops of Antifoam A emulsion (Sigma) were added just before use. The homogenate was filtered through muslin and the fraction which sedimented between 200 *g* for 1.5 min and 1500 *g* for 10 min collected. This pellet was washed by resuspension in the chloroplast isolation medium and the final chloroplast fraction sedimented at 6000 *g* for 10 min. Finally, the chloroplast fraction was incubated in a soln of Triton X-100 (2%, w/v) containing Tris–acetate (25 mM Tris, pH 7.5), and DTT (5 mM) for a period of 2 hr at 20° in order to solubilize this enzyme. After centrifugation at 36 000 *g* for 10 min, the supernatant was fractionated with $(NH_4)_2SO_4$ between 50 and 70% satn, the ppt was resuspended in Tris–acetate buffer pH 7.8 25 mM and further purified by chromatography on a DEAE cellulose column equilibrated with the same buffer. The enzymic activity was eluted by 0.4 M NaCl in the above buffer, and the active fractions were reprecipitated by 75% saturated $(NH_4)_2SO_4$. Resuspended ppts were pooled together and repurified on Sephadex G-100 for assay.

Enzyme assays. Malate dehydrogenase activity was assayed using both NAD and NADP (L-malate:NAD(P) oxidoreductase EC1.1.1.37) as the coenzyme as soon as practicable after isolation and purification. The change in the $A_{340\,nm}$ was measured in one of the following assay media: for the reduction of oxaloacetate the medium (3 ml) contained Tris–MES (2.5 μmol Tris, pH 7.5), NAD(P)H 151 nmol, oxaloacetate (500 nmol) and DTT (0.5 μmol). Malate oxidation was assayed in the following medium: glycine–KOH (25 μmol glycine, pH 8.2 in the presence of NAD and pH 8.5 for NADP), NAD(P) (1.5 μmol) and L-malate (15 μmol)—the pH of the malate was adjusted to the required value prior to addition to the assay medium. Sp. act. is defined as a change of 6.2 *A* units/min/mg protein.

Other measurements. Protein concns were determined from $A_{280\,nm}$ and $A_{260\,nm}$ and chlorophyll was estimated according to the method of ref. [27]. Ion concns were determined by flame photometry of acetic acid extracts of leaves and chloroplast fractions.

REFERENCES

1. Flowers, T. J., Troke, P. F. and Yeo, A. R. (1977) *Annu. Rev. Plant Physiol.* **28**, 89.
2. Greenway, H. and Munns, R. (1980) *Annu. Rev. Plant Physiol.* **31**, 149.
3. Kagawa, T. and Hatch, M. D. (1977) *Arch. Biochem. Biophys.* **184**, 290.
4. Hatch, M. D. and Slack, C. R. (1970) *Progr. Phytochem.* **2**, 35.
5. Johnson, H. S. and Hatch, M. D. (1970) *Biochem. J.* **113**, 273.
6. Scheiber, R. and Beck, E. (1979) *Plant Physiol.* **64**, 744.
7. Jacquot, J. P., Vidal, Y. and Gadal, P. (1976) *FEBS Letters* **71**, 222.
8. Vidal, J., Jacquot, J. P. and Membre, H. (1978) *Plant Sci. Letters* **11**, 305.
9. Flowers, T. J., Ward, M. E. and Hall, J. L. (1976) *Phil. Trans. Soc. B.*, **273**, 523.
10. Blackwood, G. C. and Miflin, B. J. (1976) *Plant Sci. Letters* **7**, 435.
11. Weimberg, R. (1967) *J. Biol. Chem.* **242**, 3000.
12. Mackay, A. B. and Marsho, T. V. (1980) *Plant Physiol.* **66**, 754.
13. Flowers, T. J., Hall, J. L. and Ward, M. E. (1976) *Phytochemistry* **15**, 1231.
14. Kun, E., Eanes, R. Z. and Volfin, P. (1967) *Nature, Lond.* **214**, 328.
15. Franks, F. and Eagland, D. (1975) *C. R. C. Crit. Rev. Biochem.* **3**, 165.
16. von Hippel, P. H. and Schleich, T. (1969) in *Structure and Stability of Biological Macromolecules* (Timosheff, S. N. and Fasman, G. P., eds), p. 417. Marcel Dekker, New York.
17. Pollard, A. and Wyn Jones, R. G. (1979) *Planta* **144**, 291.
18. Yeo, A. R., Kramer, P., Läuchli, A. and Gullasch, J. (1977) *J. Exp. Botany* **28**, 17.
19. Marsho, F. V., Sokolove, P. M. and Hackay, A. B. (1980) *Plant Physiol.* **65**, 103.
20. Hoagland, P. R. and Arnon, D. J. (1950) *Calif. Agric. Exp. Stn. Circ.* **347**, 32.
21. Walker, S. H. and Tzawa, S. (1979) *Plant Physiol.* **63**, 133.
22. Arnon, D. I. (1949) *Plant Physiol.* **24**, 1.