Phytochemistry, 1976, Vol. 15, pp. 1231–1234. Pergamon Press. Printed in England.

SALT TOLERANCE IN THE HALOPHYTE *SUAEDA MARITIMA*. FURTHER PROPERTIES OF THE ENZYME MALATE DEHYDROGENASE

T. J. FLOWERS, J. L. HALL and MARGARET E. WARD
School of Biological Sciences, University of Sussex, Falmer, Brighton, Sussex, England

(*Revised received* 5 *February* 1976)

Key Word Index—*Suaeda maritima*; Chenopodiaceae; effect of salts; malate dehydrogenase; halophyte.

Abstract—Malate dehydrogenase activity in supernatant fractions prepared from the halophyte *Suaeda maritima* was modified by added NaCl with an optimal concentration for activation of about 50 mM. At this ionic strength of 0.05 the chlorides of sodium, potassium, ammonium, rubidium, calcium and magnesium all produced a similar degree of stimulation, while the nitrates of potassium and sodium were somewhat less effective. A similar result was obtained whether the plants were grown in the presence or absence of NaCl. Furthermore, malate dehydrogenase activity in preparations from the glycophyte *Pisum sativum* behaved in a similar manner. The enzyme activity from both *Suaeda* and *Pisum* was separable into two fractions (I and II) by gel filtration on Sephadex G200. The MW of fraction II from *Suaeda* was estimated to be 165000 and that from *Pisum* approximately 282000: fraction I from both species eluted at the void volume of Sephadex G200. Storage of lyophilised supernatant resulted in the loss of enzyme activity from fraction I and a decrease in the overall stimulation by NaCl. Treatment of the lyophilised enzyme with NaCl at a concentration of 100 mM also resulted in the loss of enzyme activity from fraction I.

INTRODUCTION

The mechanism of salt tolerance in higher plants is still poorly understood, although recently it has been established that this tolerance does not appear to parallel that in halophilic bacteria. The proteins of such bacteria are highly specialised and not only tolerate high (a few molar) salt concentrations but require these salt levels for optimal enzyme activity [1]; the enzymes of halophytes, on the other hand, are not, *in vitro*, particularly more tolerant of high salt concentrations than those of glycophytes [2–14]. Furthermore, the salt concentrations required for optimal enzyme activity are similar to those required by enzymes from glycophytes [13–15].

Malate dehydrogenase activity from *Suaeda maritima* showed maximum activation by NaCl concentrations of about 50 mM when assayed in a low ionic strength buffer at its pH optimum of 7 [15], and activity was separable, by gel filtration, into two fractions differing in the degree of this activation. Enzyme activity in fraction I (which eluted earlier than fraction II from Sephadex G200) was stimulated to some 400% of the rate in the buffer alone by NaCl, while fraction II (which constituted the bulk of the enzyme activity) was stimulated to only 150% by a similar NaCl concentration. It was not clear, however, whether the activation was specific to particular ions, nor whether the effects of the salts were associated with changes in enzyme conformation or of the quaternary structure. This paper reports the effects of a variety of ions on the activation of malate dehydrogenase prepared from *Suaeda maritima* and the effect of salt on the molecular weights of the enzyme, together with comparable studies on *Pisum sativum*.

RESULTS

Malate dehydrogenase activity in a desalted extract of salt grown *Suaeda* was stimulated optimally by NaCl at a concentration of about 50 mM (Fig. 1). Maximum rates were some 160% of the rate in the absence of added NaCl although there was some variability between experiments (see below). The response of supernatant enzyme prepared from *Pisum* was indistinguishable from that of *Suaeda* (Fig. 1). In the presence of NaCl at a concentration of 500 mM (the approximate overall sodium concentration of the leaf cells of *Suaeda* [3], the rate was reduced to 48% of the rate in the absence of added NaCl

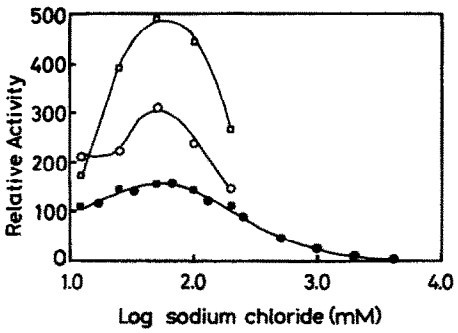

Fig. 1. The effect of NaCl on malate dehydrogenase activity in mitochondrial and supernatant preparations from *Pisum* and *Suaeda* grown in the presence of NaCl. Activity is expressed relative to that in the buffer alone: *Suaeda* supernatant (●) and mitochondria (○); *Pisum* supernatant (■) and mitochondria (□).

Table 1. The effect of lyophilising and storage on malate dehydrogenase activity from a single homogenate of *Suaeda maritima* grown in the presence of NaCl

	Enzyme activity in the absence of NaCl (OD min^{-1} mg^{-1} protein)	Rate in the presence of NaCl (50 mM) (% control)
Freshly prepared enzyme	2.4	225
Lyophilised, stored 5d	3.1	190
Lyophilised, stored 40d	3.2	150

Table 2. The effect of storage on the stimulation of malate dehydrogenase fractions from Sephadex G200 by NaCl (50 mM)

Days of storage	Relative activity [(+Na)/(−Na)%] in fractions I	II
0	455	157
4	350	150
7	142	133
42	136	—

or 31% of the maximum activity: some enzyme activity (5%) was detectable even in 4 M NaCl. Interestingly, enzyme activity in sonicated mitochondrial fractions from both *Suaeda* and *Pisum* showed a greater stimulation by sodium than was apparent for the supernatant enzyme (Fig. 1). For preparations from *Suaeda* the maximum rate for the mitochondrial enzyme was some 300% of the rate in buffer alone, while the enzyme from *Pisum* was stimulated to 500% of the rate in the absence of added NaCl.

The effect of storage on supernatant enzyme activity

Neither lyophilisation of the enzyme from *Suaeda* nor its subsequent storage at −10° over periods of up to 40 days caused any significant loss of activity assayed in the absence of added ions. However, storage of the enzyme as a frozen solution at −10° resulted in a loss of more than half of this activity within five days, while storage at 2 to 4° resulted in a similar loss of activity, but over a 24 h period. Although storage of the lyophilised material did not result in a decrease of the activity at low ionic strength, there was a decline in the degree of salt stimulation. Storage of one preparation of lyophilised enzyme over a 40-day period brought about a decrease of the stimulation by 50 mM NaCl from 225 to 150% of the rate in the buffer alone (Table 1).

When lyophilised extracts were fractionated on Sephadex G200 immediately after lyophilising two peaks of enzyme activity were apparent (Fig. 2a, solid line). However, storage of the lyophilised material resulted in a progressive decrease in the activity of fraction I until it was almost non-existent (42d—Fig. 2a, broken line). This loss of fraction I was accentuated by a decrease in the degree of salt stimulation of fraction I relative to fraction II (Table 2), since the elution profiles were assayed in the presence of the optimal NaCl concentration (50 mM): fraction I from freshly lyophilised material is more highly salt stimulated than fraction II (Table 2). Results with enzyme stored for a period of up to seven days indicated that the optimum chloride concentration remained at 50 mM.

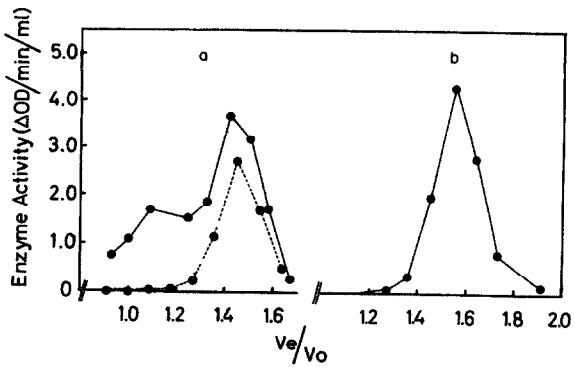

Fig. 2. Elution profiles of *Suaeda* supernatant malate dehydrogenase activity from Sephadex G200. Enzyme activity was assayed at neutral pH in the presence of NaCl (50 mM). A: immediately after lyophilising (solid line) and after storage for 42 d at −10° (broken line). B: salt elution; *Ve*, elution volume; *Vo*, void volume.

The effect of various anions and cations on malate dehydrogenase activity

The effect of different anions and cations on malate dehydrogenase activity was investigated in order to ascertain whether the effects were specific to NaCl. With freshly prepared enzyme (desalted, but not lyophilised) from salt grown plants the chlorides of sodium, potassium and ammonium at an ionic strength of 0.05 were all very similar in effect but more effective than $NaNO_3$ at the same concentration (Table 3). A similar picture emerged with preparations from *Pisum*: the monovalent chlorides were more effective than the monovalent nitrates. The chlorides of magnesium and calcium (also at an ionic strength of 0.05) possibly produced marginally higher rates of activity than those of the monovalent cations (Table 3).

This result was not altered when the preparations were lyophilised, rehydrated and kept for one day at 2° before assaying (Table 3,c), there being again little difference between the effect of the various monovalent chlorides all of which were more stimulatory than the nitrates. Calcium and magnesium chlorides at an ionic strength of 0.05 were again marginally more stimulatory than the

Table 3. The effect of various ions on the malate dehydrogenase activity from *Suaeda* and *Pisum*. The conditions were as described in the Experimental and the ions were all added at an ionic strength of 0.05. The rates of enzyme activity are expressed relative to those in the presence of NH_4Cl

Salt	a	b	c	d	e
NH_4Cl	100	100	100	100	100
NaCl	98	94	94	91	112
KCl	98	99	92	94	97
RbCl	—	96	90	91	92
$NaNO_3$	88	84	75	74	84
KNO_3	—	85	81	81	85
$CaCl_2$	—	102	100	106	107
$MgCl_2$	—	102	97	118	102
LSD ($p = 0.05$)	14	4	7	9	12

a: Salt grown *Suaeda* (not lyophilised); b: *Pisum* (not lyophilised); c: non-salt grown *Suaeda*: lyophilised extract stored 47d; d: fraction I from G200 elution of salt grown *Suaeda*; e: fraction II from G200 elution of salt grown *Suaeda*.

monovalent chlorides. The results were similar with preparations both from plants grown in the presence and absence of NaCl (Table 3 shows the results for plants grown in the absence of NaCl) and for fractions I and II following gel filtration on Sephadex G200 (Table 3, d & e).

Molecular weights

Gel filtration of malate dehydrogenase activity of freshly lyophilised enzyme preparations in the presence of NaCl at a concentration of 100 mM (a concentration which is stimulatory, but slightly above the optimal concentration) resulted in a single peak of enzyme activity (Fig. 2b). This occurred both with preparations made from plants of *Suaeda* grown in the presence of NaCl and with preparations from *Pisum* (results not shown). Under these conditions, the MWs of the malate dehydrogenase from *Suaeda* estimated following calibration of the Sephadex G200 column with proteins of known MW but in the absence of NaCl (Fig. 3) was some 107000. In the absence of sodium chloride estimates of the molecular weight for fraction II from both salt and non-salt grown *Suaeda* ranged from 132000 to 186000 with a mean of 165000 ± 10000 (*Ve/Vo* ranging from 1.42 to 1.50, Fig. 3). For *Pisum* the elution volume of fraction II lay outside the calibrated range. However, since the relationship is likely to remain linear to a MW of 300000 [30] it is possible to extrapolate and calculate a mean value of 280000 for this fraction. Ratios of *Ve/Vo* for fraction I from both *Suaeda* and *Pisum* approached one indicating MWs of 500000–800000 or more [30].

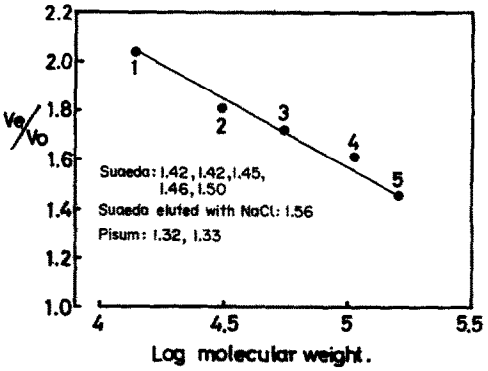

Fig. 3. Calibration of Sephadex G200. The co-ordinates mark the relative elution volumes of 1, RNAase; 2, carbonic anhydrase; 3, hexokinase monomer; 4, hexokinase dimer; 5, aldolase. The table inset shows the relative elution volumes for separate elutions of malate dehydrogenase activity from *Suaeda* and *Pisum* at low ionic strength and from *Suaeda* in the presence of 100 mM NaCl.

DISCUSSION

Malate dehydrogenase appears to be an oligomeric protein in all those cases investigated [16–24]. The range of MWs reported is, however, very large. For malate dehydrogenase isolated from animal cells, the native enzyme commonly has a MW of 60 to 70000, see e.g. [19–21] and consists of four sub-units e.g. [19] as does the enzyme from *Neurospora* [17]. Electrophoresis of plant extracts indicate numerous protein bands with malate dehydrogenase activity [22, 25, 26]; these are, however, both charge isomers and aggregates which are interconvertible [22 and below]. Thus MWs ranging from 17000 to 700000 have been reported in the literature for plant malate dehydrogenase.

For *Suaeda* two active forms of the enzyme were separated by gel filtration. The malate dehydrogenase activity was eluted from the G200 at low ionic strength and this may have contributed to the high values obtained for the MW, since the ionic environment (as well as the pH) clearly affects the degree of aggregation. Benveniste and Munkres [23] reported an increase in the number of MW species from one to five when the concentration of the Tris-buffer was lowered from 100 to 5 mM. Three of these species were of higher MW than those seen in the 100 mM buffer.

NaCl stimulated malate dehydrogenase activity as previously described [15]. The difference in the effect of NaCl (or ionic strength) on the supernatant and mitochondrial malate dehydrogenases provides the possibility of a diagnostic test for these two dehydrogenases which can be separated by the level of salt stimulation. The difference in the effect of ionic strength on mitochondrial and supernatant malate dehydrogenase is not unique and has been reported for rat kidney [27] although Kalir and Poljakoff-Mayber [13] found with preparations from the root of *Tamarix* that the mitochondrial enzyme was activated to a lesser extent than the supernatant enzyme. However, they utilised a high Tris–Cl buffer concentration. This may also have contributed to the low level of salt stimulation of oxalacetate reduction reported by these workers.

Results reported here confirm those previously reported [15] that enzyme activity in fraction I was activated to a greater degree by NaCl than that in fraction II. The loss of stimulation on storage of lyophilised preparations is thus consistent with a decrease in the proportion of fraction I (Fig. 2a) since the optimum chloride concentration remained at 50 mM. Moreover, since NaCl itself brings about a decrease in the MW with a loss of fraction I, activation of the enzyme appears to be associated with a change in the quaternary structure. Since the preparation of the enzyme took place by homogenising in the presence of NaCl (10 g fresh weight of *Suaeda* grown in the presence of NaCl contains some 6 mmole of NaCl producing a concentration of approximately 200 mM in the homogenate) followed by desalting, the effect of ions on the quaternary structure must be reversible. Furthermore, it has been shown previously [15] that preparations from salt-grown plants contained a higher proportion of fraction I than preparations from non-salt plants. Thus it may be inferred that the enzyme from plants grown in the presence of NaCl differs from the low-salt enzyme in its ability to reform the high MW fraction. Other differences between the enzyme from saline and non-saline grown plants include a difference in the effect of pH on enzyme activity at low ionic strength, a lesser degree of activation of fraction I by chloride for plants grown in the absence rather than the presence of NaCl, and a smaller number of bands staining for malate dehydrogenase on polyacrylamide gels for plants grown with additional NaCl [15].

Malate dehydrogenases from different sources all appear to be influenced by their ionic environment although in rather different ways. While KCl increases the number of MW forms in spinach [24] it decreases the number both in *Neurospora* [23], *Suaeda* (Fig. 2) and *Pisum*. Calcium causes dispersion of the aggregates

in *Neurospora* [23] but, when added to the growth medium, an increase in the MW of malate dehydrogenase from *Lemna* [17].

The activation of malate dehydrogenase from both *Suaeda* and *Pisum* by ions appears to be relatively non-specific, there being little difference in the effect of divalent and monovalent chlorides at the same ionic strength: the chlorides were marginally more effective than the nitrates, however. This is unusual, since enzymes activated by potassium ions are usually also activated by ammonium and by rubidium ions but not by sodium ions [28, 29]. Since the chlorides of calcium and magnesium also activate the enzyme to the same degree at the same ionic strength while the nitrates of potassium and sodium were less effective, it appears that any specificity may be conferred by the anion. This is consistent with the conclusions drawn by Hiatt and Evans [31] on the activation of the spinach malate dehydrogenase by salts. They found the chlorides of sodium, potassium, lithium and ammonium to be of equivalent effect while Na_2SO_4 activated to a lesser degree than NaCl. Malate oxidation by malate dehydrogenase prepared from *Tamarix* roots was activated to the same degree by NaCl, KCl and Na_2SO_4 although $MgCl_2$ was much less effective [13]. The authors concluded that these were specific effects and not simply due to ionic strength.

The mode of action of ions on enzymes is not well understood [29], although it is thought to be related to the effects of the ions on enzyme conformation. For malate dehydrogenase it is also apparent that the activation of the enzyme is related to changes in the quaternary structure of the enzyme. Finally, it is important to emphasise that the effects of ions on the halophyte enzyme are clearly mirrored in the effects on the glycophyte enzyme: there are no obvious differences between halophyte and glycophyte as is the case with the halophilic and salt-sensitive bacteria.

EXPERIMENTAL

Plants of *Suaeda maritima* (L.) Dum. were grown in sand culture as previously described [3] either in the presence of 340 mM NaCl (salt plants) or in its absence. Peas (*Pisum sativum* L.) were grown for 10 to 14 days over H_2O. Plant tops were homogenised with a pestle and mortar in a soln containing sucrose (400 mM) and TES (20 mM, pH adjusted to 7.2 with Tris) at a ratio of 3 ml/g fr. wt and centrifuged at a g_{max} of 190000 for 30 min. Supernatant was desalted on Sephadex G25 (coarse) being eluted with a dil. buffer (5 mM TES pH adjusted to 7.0 with Tris) as previously described [15] and either used directly as a source of enzyme activity or lyophilised overnight. Lyophilised extracts were either used on the following day or stored desiccated at −10°. For gel filtration the lyophilised material was resuspended in 2 ml 15% w/v sucrose containing 5 mM TES–Tris pH 7.0 and applied to a column containing Sephadex G200 (700–750 mm × 15 mm diameter). The column was eluted with the 5 mM TES–Tris buffer at a flow rate of 0.1 ml min^{-1} and enzyme activity determined in the various fractions [15]. In a number of cases NaCl at a concentration of 100 mM was added to the elution buffer and this is referred to in the text as salt elution. Calibration of the Sephadex G200 columns was achieved using RNAase, carbonic anhydrase, hexokinase and aldolase eluted in 5 mM TES–Tris pH 7.0. The void volume was determined at the elution volume of blue dextran.

Mitochondrial malate dehydrogenase was produced by sonication of mitochondrial pellets prepared as described by Flowers [8]. Enzyme activity (E.C.1.1.1.37) was determined in duplicate samples by following the oxidation of NADH (50 μM) in the presence of oxalacetate (250 μM) and TES–Tris buffer (6.7 mM, pH 7.2). Protein was determined both by the method of Lowry *et al.* [32] and by calculation from the formula, protein (mg ml^{-1}) = $1.45A_{280} - 0.74A_{260}$ [33].

Acknowledgement—We gratefully acknowledge the support of this work by the Nuffield Foundation.

REFERENCES

1. Larsen, H. (1967) *Advances in Microbial Physiology* (Rose, A. H. and Wilkinson, J. F., eds.), Vol. I pp. 97–132. Academic Press, New York.
2. Johnson, M. K., Johnson, E. J., MacElroy, P. D., Speer, H. L. and Bruff, B. S. (1968) *J. Bacteriol.* **95**, 1461.
3. Flowers, T. J. (1972) *J. Exp. Botany* **23**, 310.
4. Flowers, T. J. (1972) *Phytochemistry* **11**, 1881.
5. Greenway, H. and Osmond, C. B. (1972) *Plant Physiol.* **49**, 256.
6. Hall, J. L. and Flowers, T. J. (1973) *Planta* **110**, 361.
7. Heimer, Y. M. (1973) *Planta* **113**, 279.
8. Flowers, T. J. (1974) *J. Exp. Botany* **25**, 101.
9. Austenfeld, F-A. (1974) *Z. Pflanzenphysiol.* **71**, 288.
10. Greenway, H. and Sims, A. P. (1974) *Australian J. Plant Physiol.* **1**, 15.
11. Stewart, G. R. and Lee, J. A. (1974) *Planta* **120**, 279.
12. Treichel, S. P., Kirst, G. O. and Von Willert, D. J. (1974) *Z. Pflanzenphysiol.* **71**, 437.
13. Kalir, A. and Poljakoff-Mayber, A. (1975) *Plant Physiol.* **55**, 155.
14. Von Willert, D. J. (1974) *Oecologia* **14**, 127.
15. Flowers, T. J., Ward, M. E. and Hall, J. L. (1976) *Phil. Trans. Roy. Soc. Lond. B.* **273**, 523.
16. Stewart, G. R. (1968) The properties of malate dehydrogenase in relation to ionic adaptation. Ph.D. Thesis, University of Bristol.
17. Jefferies, R. L., Laycock, D., Stewart, G. R. and Sims, A. P. (1969) *Ecological Aspects of Mineral Nutrition of Plants* (Rorison, I. H., ed.), pp. 281–307. Blackwell, Oxford.
18. Munkres, K. D. (1965) *Biochemistry* **4**, 2186.
19. Siegel, L. (1967) *Biochemistry* **6**, 2261.
20. Mann, K. G. and Vestling, C. S. (1969) *Biochemistry* **8**, 1105.
21. Lowenstein, J. M. (1969) *Methods in Enzymology*, pp. 99–106. Academic Press, New York.
22. O'Sullivan, S. A. and Wedding, R. T. (1972) *Plant Physiol.* **49**, 117.
23. Benveniste, K. B. P. and Munkres, K. D. (1973) *Biochem. Biophys. Res. Commun.* **50**, 711.
24. Ziegler, I. (1974) *Phytochemistry* **13**, 2403.
25. Weimberg, R. (1968) *Plant Physiol.* **43**, 622.
26. Cammaerts, D. and Jacobs, M. (1975) *Plant Sci. Letters* **4**, 249.
27. Kun, E., Eanes, R. Z. and Volfin, P. (1967) *Nature* **214**, 328.
28. Evans, H. J. and Sorger, G. J. (1966) *Ann. Rev. Plant Physiol.* **17**, 47.
29. Suelter, C. H. (1974) *Metal Ions in Biological Systems* (Siegel, H., ed.), pp. 201–251. Marcel Dekker, New York.
30. Andrews, P. (1965) *Biochem. J.* **96**, 595.
31. Hiatt, A. J. and Evans, H. J. (1960) *Plant Physiol.* **35**, 662.
32. Lowry, O. H., Rosebrough, N. J., Farr, A. C. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
33. Leggett Bailey, J. (ed.) (1967) *Techniques in Protein Chemistry* pp. 346. Elsevier, Amsterdam.