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Salt tolerance in the halophyte *Suaeda maritima*: some properties of malate dehydrogenase

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Malate dehydrogenase activity in a high speed supernatant fraction prepared from homogenates of the shoots of the halophyte *Suaeda maritima* was separated into two fractions by gel filtration. Sodium chloride stimulated the activity of the larger molecular mass form of the enzyme to a greater extent than that of the smaller molecular mass form: the latter constituted the bulk of the activity. The degree of stimulation by sodium chloride increased with increasing substrate concentrations. Differences between the properties of the enzymes isolated from plants grown with and without sodium chloride are described.

The results obtained with the halophyte enzyme were very similar to those obtained with the glycophyte *Pisum sativum*. Maximum activation of enzyme activity was obtained with a salt concentration of 50 mol m^{-3} in both species and at higher concentrations activity was inhibited. The results are discussed in relation to the mechanism of salt tolerance in halophytes.

Introduction

A high salt concentration is an important factor contributing to the loss of agricultural production, particularly in the arid and semi-arid regions of the world. Such high salt concentrations may arise in soils as a result of geological action or due to the influence of saline groundwaters. Anions such as carbonate (or bicarbonate), sulphate and chloride and cations such as calcium, magnesium, potassium and sodium are prevalent at high concentrations in many terrestrial groundwaters (Craig 1970). Sodium and chloride ions also form the bulk (86% by mass) of the ions present in sea water (Harvey 1966), a source of salinity which may often affect the land. Finally, man's influence through irrigation may also directly lead to the accumulation of salt in the soil profile.

The total area of salt affected land is hard to evaluate precisely, although it is generally recognized to be high. For example, in 1962 Bernstein estimated that there were some 75×10^6 ha (hectares) of salt-affected land in the U.S.S.R., while Strogonov (1964) put the figure at 10% of the surface or about 200×10^6 ha. In the Punjab region of India some 7.4×10^6 ha of the 31×10^6 ha of agricultural land were estimated to be saline and land was going out of agricultural production at the rate of 0.25×10^6 ha per year due to increasing salinity brought about by mans use of irrigation (Raheja 1966).

The importance of high salinity to agriculture is that the growth of most crop plants is very sensitive to salt concentrations much above 100 mol m^{-3} (Bernstein & Hayward 1958; Bernstein 1962; Nieman 1962; Greenway 1973). During osmotic adjustment these salt sensitive crop plants absorb ions which bring about the lowering of the plant water potential necessary for the maintenance of a positive water balance. While there is no evidence to suggest that it is the low water potential *per se* which brings about the growth inhibition (Flowers & Hanson 1969:

Hsiao 1973), there are a number of lines of evidence which suggest that the growth reductions result from direct toxic effects of ions on metabolism.

Many metabolic processes in salt sensitive plants are inhibited by high concentrations of ions when measured *in vitro*; namely, enzyme activity (see Evans & Sorger 1966), protein synthesis (see Hall & Flowers 1973) and the activity of mitochondria (see Flowers 1974) and chloroplasts (Ben-Amotz & Avron 1972). Furthermore, while the presence of ions in these plants is correlated with growth inhibition, salt tolerant varieties of normally sensitive crops such as soybean, barley and rice exclude sodium and chloride ions from their tissues (see Greenway 1973).

Enigmatically, halophytes not only tolerate high salt concentrations but also accumulate ions to high levels within the cells (see Flowers 1975). In contrast to the situation occurring in glycophytes, the growth of halophytes is apparently stimulated by the high concentrations of ions present in the growth medium. Both fresh and dry mass of plants growing in 150–300 mol m^{-3} sodium chloride are commonly some two to three times greater than those of plants growing in the absence of added sodium chloride. However, the metabolism of these plants has recently been shown not to differ qualitatively in terms of salt tolerance, from that of glycophytes. Enzyme activity in halophytes is, *in vitro*, as sensitive to high salt concentrations as that in glycophytes (Johnson *et al.* 1968; Flowers 1972*a*, *b*; Greenway & Osmond 1972; Heimer 1973; Austenfeld 1974), although recent work on malate dehydrogenase (decarboxylating) indicates that the sensitivity of enzymes to salts may be modulated by the substrate concentration (Greenway & Sims 1974). It is not known, however, if this property is unique to the enzymes of halophytes or if it applies equally to the enzymes of glycophytes. However, even if desensitization of the enzyme to salts were a particular property of halophytes, the work on malate dehydrogenase (decarboxylating) indicates that it alone could not account for the ability of halophyte metabolism to function at the tissue salt concentrations calculable by expressing the salt content on the basis of the total water content (500–1000 mol m^{-3}). Subcellular compartmentation has been invoked to explain the enigma (see Hall & Flowers 1973; Flowers 1975).

Although much has been made of the sensitivity of halophyte enzymes to high salt concentrations and of the contrast with the halophilic bacteria where enzymes require high salt concentrations for maximal activity (Larsen 1967), little work has been carried out on the activation of halophyte enzymes by ions. This paper reports the effects of sodium chloride in particular on the soluble malate dehydrogenase activity of a halophilic member of the Chenopodiaceae, *Suaeda maritima* and a very salt sensitive glycophyte, *Pisum sativum*. Malate dehydrogenase was chosen because certain forms exist in the 'soluble' phase of the cell and so should be subject to the general cytoplasmic ion levels. A knowledge of the sensitivity of the enzyme to ions will contribute to the estimation of the concentration of ions in the cytoplasm: earlier work (Flowers 1972*a*) needs re-evaluating in the light of the more recent work of Greenway & Sims (1974). Furthermore, this enzyme is intimately concerned with the presence of a pool of malate which exists outside the mitochondria in at least some species (see Oaks & Bidwell 1970). Ion accumulation has been shown to be related to the synthesis of organic acid in grasses (see Torii & Laties 1966), to oxalate in some members of the Chenopodiaceae (Williams 1960; Osmond 1967), and specifically to malate in beet (Osmond & Laties 1969). Malate also appears to play a central role in carbon fixation by certain halophilic members of the Aizoaceae (Winter & von Willert, 1972; Winter 1973*c*).

Methods

Abbreviations: The following abbreviations are used in the text: TES, *N*-tris(hydroxymethyl) methyl-2-aminoethane sulphonic acid; tris, tris(hydroxymethyl)aminomethane; MTT, 2,5-diphenyl-3-(4,5-dimethylthiazol-2)-tetrazolium bromide; PMS, methylphenazonium methosulphate; NAD^+ and $NADP^+$, nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate respectively.

Plants of *Suaeda maritima* L. Dum. were grown for 45–50 days (from seed collected from Cuckmere Haven, Sussex – Grid reference, TV 519978) in sand irrigated either with a culture solution alone or with the addition of 2 g/100 ml sodium chloride (340 mol m^{-3}) as described by Flowers (1972*a*): the latter are referred to in the text as salt grown plants.

Peas (*Pisum sativum* L. cv. Alaska) were grown over water for 10–14 days.

The plant tops (10g) were homogenized in sucrose (30 ml, 400 mol m^{-3}) containing TES (20 mol m^{-3}, pH adjusted to 7.0 with sodium hydroxide) using a pestle and mortar and centrifuged at a g_{max} of 190000 for 30 min in an MSE 65 centrifuge. The supernatant was used as a source of malate dehydrogenase activity.

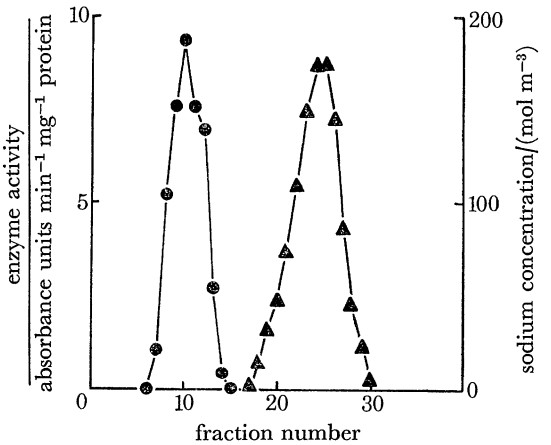

Figure 1. Gel filtration of malate dehydrogenase activity from salt grown *Suaeda maritima* plants. Enzyme activity (●) in the supernatant fraction was separated from sodium ions (▲) by filtration on Sephadex G25.

Since the *Suaeda* plants contain large quantities of ions (mainly sodium and chloride in salt plants and potassium and nitrate in plants grown on culture solution alone (Yeo 1974)), the supernatants from homogenates of these plants and from the peas were desalted by gel filtration. The supernatant fraction (25 ml) was applied to a column (400 mm long × 20 mm diameter) of Sephadex G25 (coarse) and eluted with a dilute buffer TES, (5 mol m^{-3} pH adjusted with tris to 7.0 and designated TES-tris, see below) into separate 5 ml fractions by using a Buchler Fractomat 3-4200 fraction collector at a flow rate of 0.9 ml/min. Malate dehydrogenase activity was confined to fractions 7–14 (40 ml) and separated from dissolved salts (figure 1).

The fractions containing the enzyme activity were either lyophilized or subjected to ammonium sulphate precipitation. The majority of the malate dehydrogenase activity was precipitated by ammonium sulphate at between 30 and 90% saturation, although some activity was present in the 0–30% ammonium sulphate fraction (2–3% of the fractionated activity) and at saturations greater than 90% (4–14%). The lyophilization procedure was

adopted to avoid the loss of specific fractions of the total malate dehydrogenase activity. The protein precipitated by ammonium sulphate (30–90% saturation) or the lyophilized effluent from the Sephadex G25 was resuspended in sucrose (2 ml of 15 g/100 ml sucrose containing 5 mol m^{-3} TES-tris, pH 7.0) and applied to a column containing Sephadex G200 (700–750 mm × 15 mm diameter). The column was eluted with dilute buffer (5 mol m^{-3} TES-tris, pH 7) at a flow rate of 0.1 ml min^{-1} and enzyme activity determined in various fractions. The whole procedure was carried out at 2–5 °C and enzyme activity was normally assayed 24 h after the application to the G200. In certain circumstances enzyme which had been stored at −10 °C was used for subsequent assays and this is indicated in the text.

After gel filtration, enzyme activity was normally measured by determining the oxidation of NADH at 25 °C by using a Pye Unicam SP800 recording spectrophotometer fitted with a scale expansion unit. The assay medium contained NADH (50 mmol m^{-3}), oxalacetate (25–250 mmol m^{-3}), sodium or potassium chlorides (0–200 mol m^{-3}) and buffer (normally TES-tris, 6.7 mol m^{-3}, but see also results) in a total volume of 3 ml. In the absence of added sodium chloride the assay medium contained less than 3 mol m^{-3} of sodium. The assay medium used for determining activity in the fractions contained 50 mol m^{-3} sodium chloride.

Enzyme activity was also determined by assaying the reduction of NAD^+ in the presence of sodium malate. The assay medium contained sodium malate (4 mol m^{-3}), NAD^+ (0.4 mol m^{-3}) and TES-tris (6.7 mol m^{-3}, at a final pH of 8.6) together with sodium chloride (0–200 mol m^{-3}).

All assays were duplicated and the experiments repeated at least once.

In the initial trial of the effect of buffer type on activity of malate dehydrogenase a variety of buffers were used. In each case the concentration of the first mentioned component or components of the buffer system was at a defined concentration while the pH was adjusted with the second component; e.g. TES-tris (5 mol m^{-3}) is TES at a concentration of 5 mol m^{-3} adjusted to the required pH with tris.

Malate dehydrogenase activity was also fractionated by polyacrylamide gel electrophoresis. Desalted, lyophilized supernatant fractions were applied in sucrose (10 g/100 ml) to a stacking gel of 2.5% acrylamide containing tris-phosphate (59 mol m^{-3}, pH 6.7) and separated on 7.5% acrylamide containing tris-Cl (375 mol m^{-3}, pH 8.9). The compartment buffer was tris-glycine (49.5 mol m^{-3}, pH 8.4) (Maurer 1971). Electrophoresis was carried out at 4 °C by using 3 mA per tube. Gels were stained by incubation in a medium containing sodium malate (100 mol m^{-3}), NAD^+ (0.5 mol m^{-3}), MTT (0.5 mol m^{-3}) and PMS (50 mmol m^{-3}) in tris-Cl (500 mol m^{-3}, pH 8.1) as described by Wilkinson (1970).

Protein was determined either by the method of Lowry, Rosebrough, Farr & Randall (1951) or by calculation from the absorbance measured at 260 and 280 nm (protein in mg ml^{-1} $= 1.45A_{280} - 0.74A_{260}$; see Leggett Bailey 1967). Protein estimations made by the two procedures on the same samples were in good agreement.

Results and discussion

Conditions for the assay of malate dehydrogenase activity

The nature of the buffer system used for enzyme assays can, in itself, have marked effects on the activity of the enzyme under investigation. Consequently, the response of malate dehydrogenase activity to sodium was determined in a variety of buffers. The rationale behind the choice of buffer for further experiments was to combine maximum sodium activation with a

low activity in the buffer alone (i.e. as little as possible activation by the buffer alone but with the absence of inhibitory effects as evidenced by the ionic activation).

An enzyme fraction was prepared from salt grown *Suaeda* by ammonium sulphate fractionation and gel filtration. Enzyme activity was determined in a series of buffers immediately after fractionation and following a period of storage at −10 °C (table 1): the pH and sodium content of the assay media were also determined. Maximum activation occurred in TES buffers whose pH was adjusted either by sodium hydroxide or tris. These buffers also evinced the lowest rates of enzyme activity in the absence of added sodium chloride, indicating minimum activation by the buffer alone. Neither the differences between buffers nor the activation by sodium was altered by storage at low temperature. Since the sodium content of the TES-NaOH varies at different pH values, the use of TES-tris was preferred. Furthermore, the concentration of the TES buffers had no effect on the reaction rate over the range 4–10 mol m^{-3}, whereas tris + maleate-KOH or tris + maleate-NaOH buffers had a profound effect on the reaction rate over the same concentration range.

TABLE 1. THE EFFECT OF VARIOUS BUFFERS ON THE ACTIVATION OF MALATE DEHYDROGENASE FROM *SUAEDA MARITIMA* GROWN IN THE PRESENCE OF 2% SODIUM CHLORIDE.

(The oxalacetate concentration was 250 mmol m^{-3} and the buffers were at a concentration of 6.7 mol m^{-3})

buffer	sodium (mol m^{-3})	pH	enzyme activity (absorbance units min^{-1} mg^{-1} protein)	%
TES-NaOH	4.8	6.8	5.9	100
	41.0	6.7	11.7	197
TES-tris	3.5	6.8	5.9	100
	42.5	6.8	11.1	188
Tris-maleate	9.8	6.9	8.7	147
	45.5	6.8	13.2	224
Tris-Cl	3.5	6.7	7.4	125
	39.5	6.6	12.6	214
Tris-bicine	3.0	7.2	9.8	166

Enzyme fractionation

Lyophilized enzyme prepared from extracts of salt grown *Suaeda* and applied to polyacrylamide gels yielded six distinct bands of activity with R_f values (with respect to bromophenol blue) of 0.7, 0.9, 3.4, 4.6, 5.7 and 6.7 (figure 2). Plants grown in the absence of sodium chloride had 3 additional bands (R_f values of 2.0, 4.2 and 7.1), one of which (R_f = 2.0) stained a redder colour than any of the other bands. The difference in the growth conditions therefore appeared to influence the complement of material staining for malate dehydrogenase activity, a point which is reiterated later. Enzyme prepared from *Pisum* showed 5 bands of activity (R_f values of 0.7, 2.7, 3.2, 5.5 and 6.6) although there was an additional area of diffuse staining covering the region from the origin to an R_f of 5.5. Electrophoresis of a soluble fraction from pea seeds on starch gels showed a similar distribution of activity (Weimberg 1967).

Elution of the same enzyme fractions from Sephadex G200 with the dilute TES buffer indicated only two clear peaks of activity (figure 3) which were present in both *Suaeda* grown with (figure 3*a*) and without (figure 3*b*) sodium chloride and in preparations from *Pisum*

(figure 8). Ammonium sulphate precipitated enzyme from *Suaeda* was also fractionated into two peaks of activity on elution from G200.

Neither unfractionated supernatants nor fraction I or II from either species oxidized NADH in the absence of oxalacetate. Furthermore, the rate of the reverse reaction (i.e. malate + NAD^+ → oxalacetate + NADH + H^+) was only a small percentage of that for the oxidation of NADH and was markedly inhibited by oxalacetate. Malate dehydrogenase (decarboxylating) activity could be demonstrated, but required magnesium ions and was specific to $NADP^+$. The rate of reaction was of the same order of magnitude as for the reduction of NAD^+.

The discrepancy between the number of bands separating on gel electrophoresis and in gel filtration has been noted previously (O'Sullivan & Wedding 1972) and is related to the existence of both proteins of differing molecular mass and of similar molecular mass, but

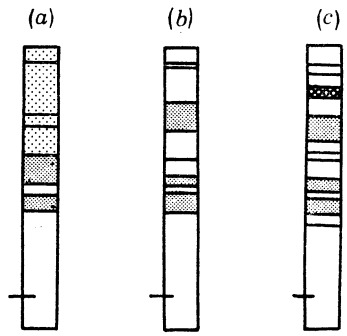

FIGURE 2. A diagram of polyacrylamide gels stained for malate dehydrogenase activity. *a*, *Pisum*; *b*, *Suaeda* grown in sodium chloride (2 %) *c*, *Suaeda* grown in culture solution alone. Gel *a* contained an area of diffuse staining (light stippling) and gel *b* a red band (heavy stippling). The bar indicates the position of a bromophenol blue band.

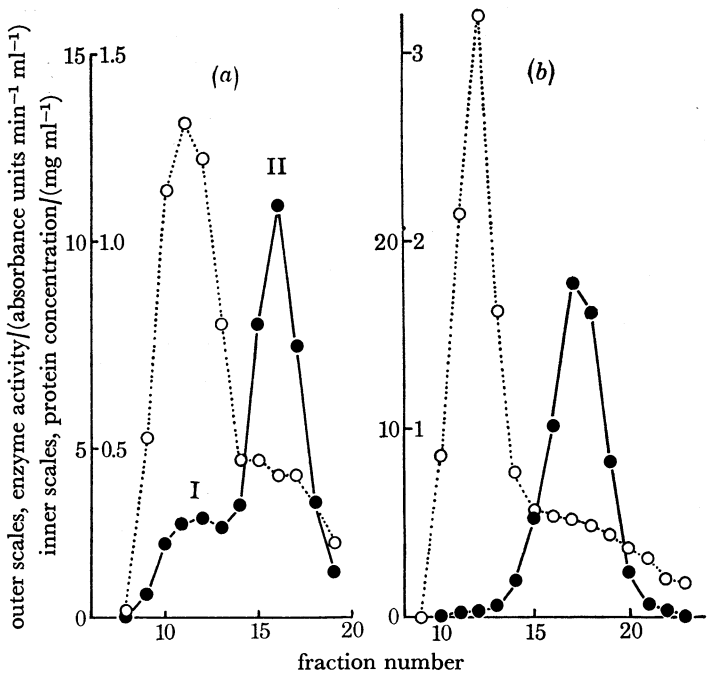

FIGURE 3. Gel filtration (G200) of malate dehydrogenase activity (●) from *Suaeda maritima*. (*a*) salt grown plants; (*b*) plants grown in the absence of sodium chloride. Total protein is represented by the broken line (○).

different charge. The status of all these forms as distinct isoenzymes is questionable, since modification of the molecular size can be brought about by treatment with divalent and monovalent cations, NADH and dithiothreitol (Jefferies, Laycock, Stewart & Sims 1969; Benveniste & Munkres 1973; Ziegler 1974) or by the incubation in glycine buffer (O'Sullivan & Wedding 1972).

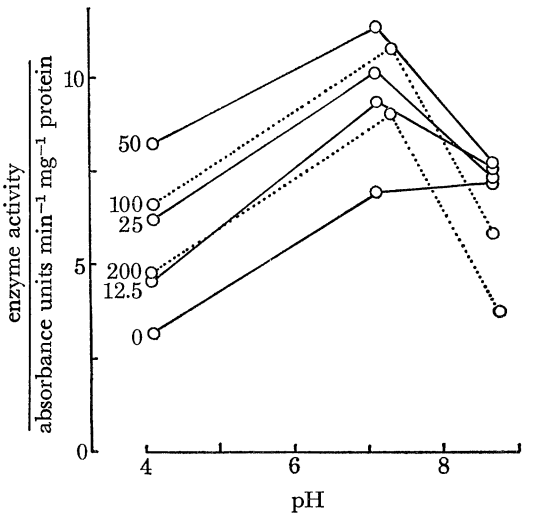

FIGURE 4. The effect of pH on the activity of malate dehydrogenase activity (fraction II – see text) from salt grown *Suaeda maritima* plants. Enzyme activity was assayed at the sodium chloride concentrations indicated on the figure; the effect of pH at above optimal sodium chloride concentrations is represented by broken lines.

Effects of sodium chloride on enzyme activity

Malate dehydrogenase activity in crude supernatant preparations from *Suaeda* was previously shown to be optimal at pH values of 7–8 regardless of whether the plants were grown in the presence or absence of sodium chloride. Assays were conducted in 50 mmol m^{-3} NADH and 100 mmol m^{-3} oxalacetate in tris + maleate-NaOH (25 mol m^{-3}) containing some 20 mol m^{-3} of sodium.

Enzyme activity in fraction II (the major part of the enzyme activity; figure 3) assayed in the presence of 250 mmol m^{-3} oxalacetate and 50 mmol m^{-3} NADH also showed more activity at pH 7 than at pH values of 4 or 9 (figure 4) provided that sodium chloride was added to the buffer. Interestingly, in buffer alone (6.7 mol m^{-3} with some 3.5 mol m^{-3} of sodium) there was a much less marked pH optimum when the enzyme was prepared from salt grown plants (figure 4). This difference in pH optimum was not apparent in preparations from plants grown in the absence of additional sodium chloride (results not shown).

The data for fraction II (figure 4) also indicate that sodium stimulation in salt grown plants was very much pH dependent. Maximum activation (to 250% of the rate in buffer alone) was brought about by sodium chloride at a concentration of 50 mol m^{-3} and occurred at a pH of 4, while there was little activation (to 107%) at pH 8.5 (figure 5*a*). However, activity was always greater at pH 7 than at 4, the rates in 50 mol m^{-3} sodium chloride being 11.4 absorbance units min^{-1} mg^{-1} protein (165% of the rate in buffer alone) and 8.3 absorbance units min^{-1} mg^{-1} protein (256%) respectively.

There was a noticeable difference between the response of fractions I and II to sodium chloride. Under the same conditions (oxalacetate at 250 mmol m^{-3}, NADH at 50 mmol m^{-3},

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pH 7) enzyme activity in fraction I was very much more highly stimulated (to 460 % of the rate in buffer alone for enzyme from salt grown plants) and by higher (100 mol m^{-3} as opposed to 50 mol m^{-3}) concentrations of sodium chloride than was fraction II (figure 5*a*). A similar difference in the behaviour was apparent for enzyme prepared by ammonium sulphate fractionation.

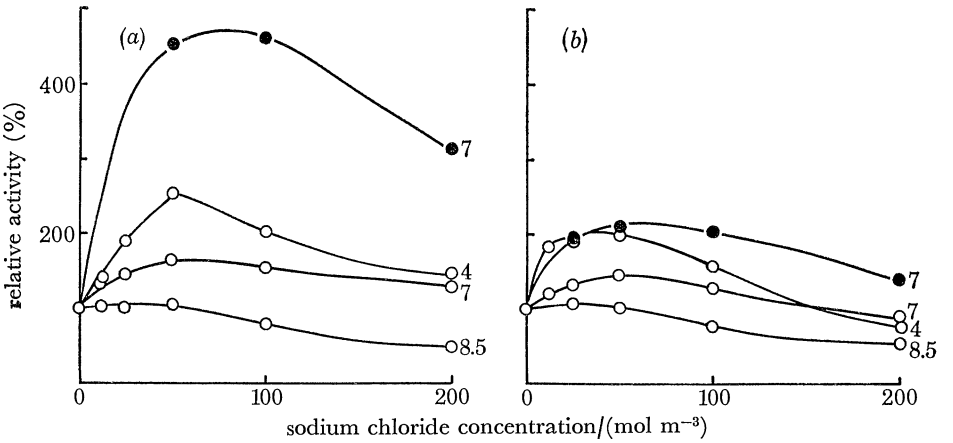

FIGURE 5. The effect of sodium chloride concentration on malate dehydrogenase activity prepared (*a*) from salt grown *Suaeda maritima* and (*b*) non-salt grown plants. Activity is expressed relative to the rate in buffer alone and at the indicated pH values for fraction I (●) and II (○) enzyme.

TABLE 2. THE DEGREE OF ACTIVATION AND THE PROPORTION OF FRACTIONS I AND II IN MALATE DEHYDROGENASE ACTIVITY FROM *SUAEDA MARITIMA* GROWN UNDER DIFFERENT CONDITIONS

(The table presents the rates of enzyme activity in the presence of sodium chloride (50 mol m^{-3}) as percentages of the rates in the absence of added sodium chloride. The proportion of activity in the two fractions was calculated from data such as figure 1 (as areas).)

growth conditions	expt.	enzyme activation (%) fraction I	enzyme activation (%) fraction II	proportion of activity as fraction I
+NaCl	*a*	455	165	0.39
	b	350	150	0.25
−NaCl	*c*	211	149	0.02
	d	266	196	0.11

Where plants were grown in the culture solution alone, activation of fraction II was again greater at a pH of 4 (to 198 %) than at 7 (to 149 %) or 8.5 (108 %) – figure 5*b*. Interestingly, fraction I enzyme prepared from such plants was also more highly activated than enzyme of fraction II (to 211 % of the rate in buffer alone as opposed to 149 % for fraction II in 50 mol m^{-3} sodium chloride, pH 7) although the degree of activation did appear to be lower than for the equivalent fraction from plants grown in the presence of sodium chloride (figure 5*b*; table 2). Furthermore, the proportion of the total activity which was present as fraction I was much lower in plants grown in the absence of salt than in salt grown plants (table 2). However, since the overall activity was dominated by fraction II there was no significant difference in the response to sodium chloride of the total enzyme activity from plants grown under the different salt regimes (figure 10).

It is also noteworthy that under the same conditions of pH and substrate concentration, addition of 200 mol m^{-3} sodium chloride to fraction II from salt grown plants reduced the

enzyme activity close to that in the absence of added sodium chloride, while activity of fraction I was still highly stimulated, i.e. fraction I was both more highly activated by sodium chloride and more tolerant of high sodium chloride conditions. This was also true for preparations made from *Suaeda* plants grown on culture solution alone.

It is perhaps worth while reiterating at this point the difference that the assay conditions can make to the results obtained. Sodium chloride at a concentration of 200 mol m^{-3} activates malate dehydrogenase of fraction I to some 330% of the rate in the buffer alone, which is

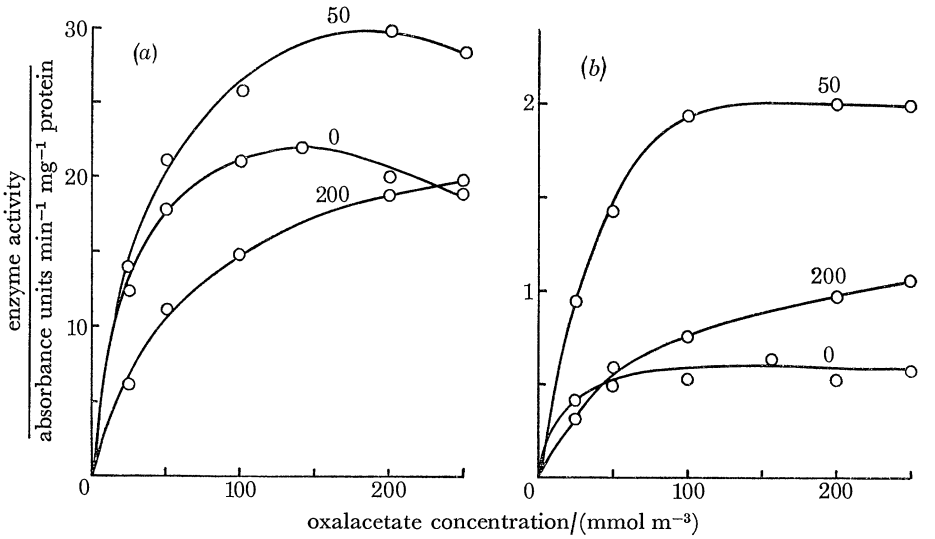

FIGURE 6. The effect of oxalacetate concentration on the activity of malate dehydrogenase of salt grown *Suaeda maritima* plants. (*a*) fraction II; (*b*) fraction I. The concentration of sodium chloride in the assay medium (mol m^{-3}) is indicated on the figure.

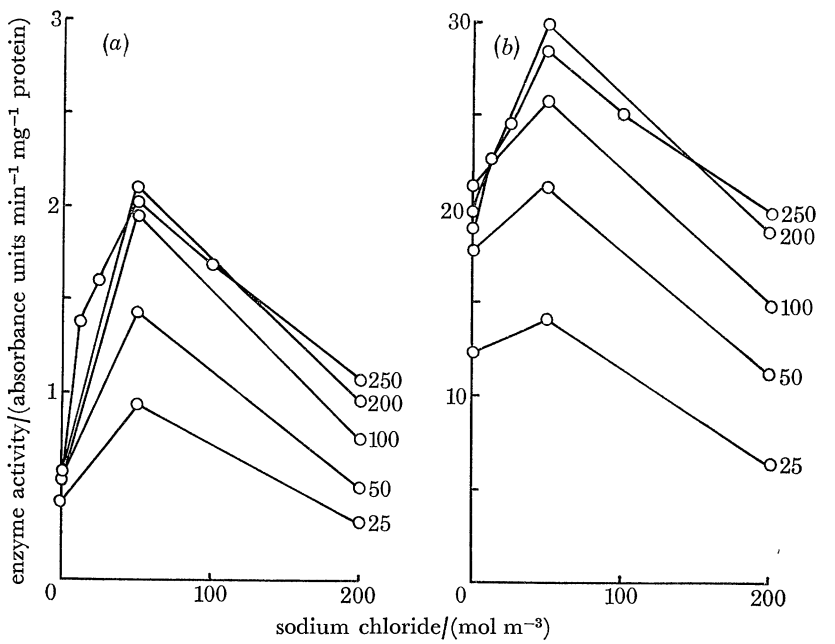

FIGURE 7. The effect of sodium chloride on malate dehydrogenase activity from salt grown *Suaeda maritima* at different oxalacetate concentrations (mmol m^{-3} as indicated on the figure). (*a*) fraction I; (*b*) fraction II.

used as the reference point. If the reference point were a buffer system containing sodium of 25 mol m^{-3}, then the rate in sodium chloride at an added concentration of 200 mol m^{-3} would only be some 87% of the reference value.

Two features were particularly noticeable in the interaction between sodium chloride and oxalacetate concentration. For preparations made from *Suaeda* plants, the inhibition of fraction II activity by high oxalacetate concentrations (200 to 250 mmol m^{-3}) was overcome by increasing sodium chloride levels (figure 6*a*): fraction I, however, was not subject to substrate inhibition at these concentrations (figure 6*b*). Secondly, inhibition of activity by sodium chloride was much more apparent at low substrate concentrations than at high oxalacetate concentrations; this was apparent both for plants grown with and without sodium chloride and for fractions I and II (figure 7*a* and *b* show results for fractions I and II from salt grown plants). However, this effect was more closely related to a decrease in stimulation rather than an increase in inhibition since as far as the inhibitory effect is concerned, the decrease in enzyme activity per unit change in salt concentration (negative gradients of figure 7) are approximately independent of the substrate concentration. For the decarboxylating malate dehydrogenase from *Triglochin striata*, potassium chloride at a concentration of 200 mol m^{-3} was less inhibitory (40%) in the presence of high malate concentrations (4 mol m^{-3}) than in the presence of low (0.1 mol m^{-3}) substrate concentrations, wherein the same salt concentration brought about an 80% inhibition of enzyme activity (Greenway & Sims 1974). The enzyme was apparently not activated by potassium chloride at any of the concentrations used, although control assays contained some 20 mol m^{-3} potassium.

There are some differences between the enzyme activity of *Suaeda* plants grown with and without sodium chloride, these being:

(1) a difference in the effect of pH on the activity of the fraction II enzymes for plants grown in the presence and absence of sodium chloride,

(2) a lower degree of activation of fraction I by sodium for plants grown in the absence of sodium than for plants grown in its presence (table 1),

(3) a difference in the proportions of fractions I and II in plants grown in the different conditions (table 1),

(4) a smaller number of bands staining for malate dehydrogenase on polyacrylamide gels for plants grown with additional sodium chloride (figure 2).

All these factors might be taken to be indicative of real differences between the state of the enzymes in the plants grown in the presence and absence of sodium chloride. Similar differences in the level of activation of malate dehydrogenase from *Lemna minor* but by calcium ions were reported by Jefferies *et al.* (1969). Plants growing on high calcium concentrations contained an enzyme which was activated to a greater extent by somewhat higher calcium concentrations than enzyme prepared from plants growing on lower calcium concentrations. This difference was attributed to a change in the number of sub-units in the functional enzyme within the tissue. Stewart (1968) also reported a correlation between enzyme activation by sodium chloride and the natural salinity of the habitat of a number of different species.

The effect of monovalent cations on the sub-unit structure of a number of different malate dehydrogenases has been investigated. Ziegler (1974) reported that the molecular mass of the native enzyme from spinach (which is also salt stimulated – Hiatt & Evans 1960) is some 64000 as determined by gel filtration. A form of the enzyme with a molecular mass of 127000 was also demonstrated and this was stabilized by magnesium ions, while potassium ions

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at a concentration of 100 mol m^{-3} in tris (50 mol m^{-3}) produced enzyme forms with molecular masses of 186000, 127000, 91000 and 64000 of which the latter predominated. For malate dehydrogenase from *Neurospora*, the variety of molecular masses is reduced to one of 65000 by treatment with potassium chloride at a concentration of 100 mol m^{-3} (Benveniste & Munkres 1973). If the malate dehydrogenase of *Suaeda* is an oligomeric protein and it appears that this is so for a range of malate dehydrogenases (e.g. also beef heart, Siegel 1967; rat liver, Mann & Vestling 1969) then it may be inferred that the differences in the properties of the enzyme from plants grown with and without sodium chloride are related to their ability to form the two molecular sizes, since the preparations applied to the G200 were previously desalted.

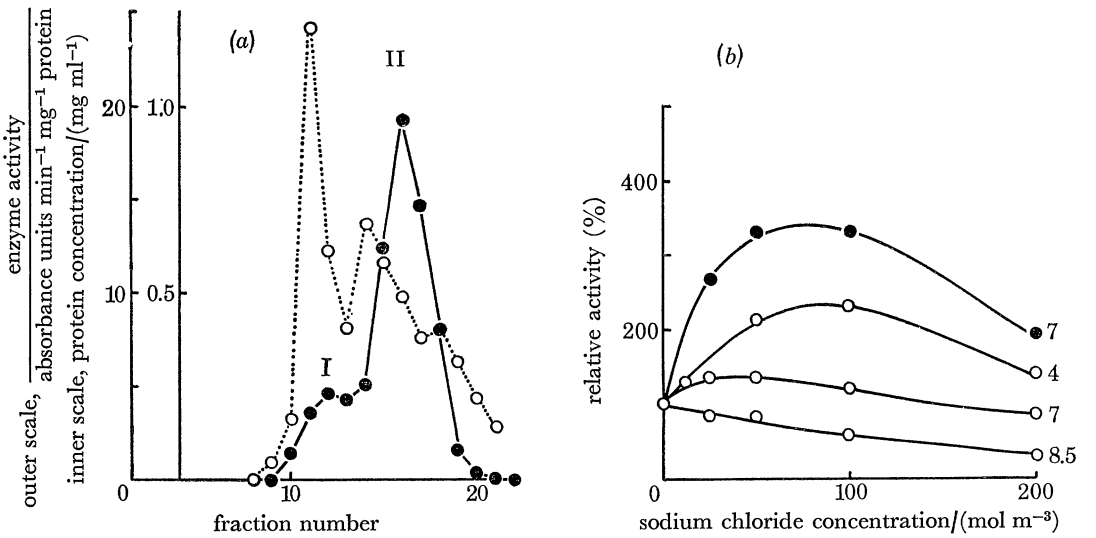

FIGURE 8 (*a*) The elution profile of malate dehydrogenase activity (●) and total protein (○) from lyophilized supernatants of *Pisum sativum*. (*b*) The effect of sodium chloride concentration on the activation of malate dehydrogenase activity from *Pisum*. Activity is expressed relative to the rate in buffer alone and at the indicated pH values for fractions I (●) and II (○).

The question arises as to whether the response of the two fractions from *Suaeda* is unique to halophytes or also occurs in salt sensitive species such as *Pisum*. Preparations of malate dehydrogenase activity from shoots of *Pisum* yielded two separable fractions (figure 8*a*). For fraction II, optimal activity was again at pH 7, except that in the absence of added sodium chloride when activity at pH 7 was approximately the same as that at pH 9. Activation by sodium chloride at a concentration of 50 mol m^{-3} was greater at pH 4 (to 213%) than at pH 7 (to 134%) although in contrast to the situation in *Suaeda* activity at pH 8.5 was inhibited (to 80% of the control value – figure 8*b*). However, there was no great difference from *Suaeda* in the overall degree of activation and fraction I was again highly activated by sodium chloride (to 334% by 50 mol m^{-3}). Activation by potassium chloride was marginally (10%) more effective than by sodium chloride for fraction I. Thus the presence of a highly activated enzyme fraction was not restricted to the halophyte, but was also present in the glycophyte (figure 10).

Substrate inhibition at high oxalacetate concentrations was again alleviated by increasing sodium concentrations (figure 9). These results are in essential agreement with those of Weimberg (1967). Although he did not note the rather higher pH optimum in the absence of added

sodium chloride, he did report greater activation of an enzyme prepared by cellulose chromatography at low pH values. This activation was substantial and similar to that reported for a spinach malate dehydrogenase (Hiatt & Evans 1960). Maximum activation occurred at a sodium chloride concentration of 20 mol m^{-3} and at higher sodium chloride concentrations, activity was inhibited. In the experiments reported herein, inhibition of fraction II activity was brought about by sodium chloride concentrations greater than 50 mol m^{-3} and for fraction I by concentrations greater than 100 mol m^{-3}.

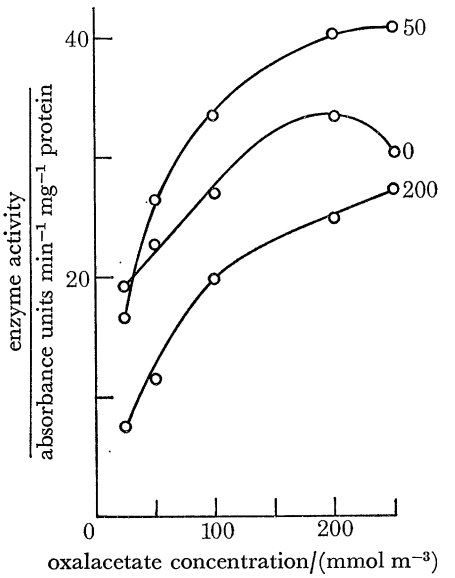

FIGURE 9. The effect of oxalacetate concentration on the activity of malate dehydrogenase of *Pisum sativum*. The concentration of sodium chloride added to the assay medium is indicated on the figure.

TABLE 3. THE EFFECT OF SODIUM CHLORIDE CONCENTRATIONS ON THE K_M VALUES (mmol m^{-3}) FOR MALATE DEHYDROGENASE ACTIVITY IN FRACTIONS I AND II OF *SUAEDA* DETERMINED FOR VARIATIONS OF THE OXALACETATE CONCENTRATION

	K_M/(mmol m^{-3}) for plants grown			
	−NaCl		+NaCl	
sodium chloride in assay / mol m^{-3}	fraction I	fraction II	fraction I	fraction II
0	24	8	15	31
50	40	26	59	37
200	72	62	87	74

The mechanism of enzyme inhibition by inorganic ions is poorly understood, but is thought to be related to effects induced by ion binding on enzyme conformation (see von Hippel & Schleich 1969). It has often been noted that the inhibitory concentrations of ions exhibit the characteristics of competitive kinetics (see, for example, Weimberg 1967, Susz, Haber & Roberts 1966). The double reciprocal plots obtained with enzyme from *Suaeda* in which the oxalacetate concentration was varied indicate that increasing the sodium chloride concentration

from 50 to 200 mol m^{-3} increased the value of the K_M (table 3) by about twofold although the actual increase was a function of both the enzyme fraction and the conditions during growth. The effect of sodium chloride with NADH as the variable substrate are also required to distinguish the site of inhibition. The lowering of the affinity of an enzyme for its substrate by high salt concentrations has been used by Greenway & Sims (1974) in their interpretation of experiments on malate dehydrogenase (decarboxylating) of *Triglochin maritima* and *T. striata*. They postulated a rise in the levels of intermediates which would reduce the inhibition by salts.

The activation of enzymes by monovalent cations has been noted on many occasions and considerable data collected by Evans & Sorger (1966). They drew a number of conclusions about the activation of enzymes by monovalent cations of which the most pertinent is that the majority of enzymes were activated by potassium and that these enzymes were usually also activated by rubidium and ammonium ions, but not by lithium. Enzymes which were activated by sodium were mostly from halophilic bacteria and were then also activated by lithium ions,

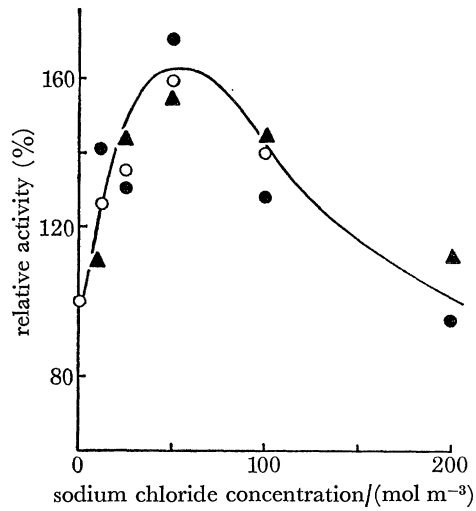

FIGURE 10. The effect of sodium chloride concentration on the activity of malate dehydrogenase from *Pisum* (▲), and *Suaeda* either grown with (●) or without (○) added sodium chloride. The assays were carried out at pH 7 in the presence of oxalacetate at a concentration of 250 mmol m^{-3}.

but to a much lesser extent by potassium, rubidium and ammonium ions. Of the fifty or so enzymes or enzyme systems (excluding those of the halophilic bacteria) in which mention was made of the effects of both sodium and potassium in only about 20% of these was sodium required or replaced potassium to any significant degree. Some 10% of these cases were the sodium/potassium activated ATPases of animal cells. Thus activation of an enzyme to a similar extent by both sodium and potassium is not a common occurrence. The effect of potassium on the malate dehydrogenase activity of *Suaeda* was essentially the same as that of potassium. For example, activation of fraction II prepared from *Suaeda* grown in the absence of sodium chloride by 40 mol m^{-3} potassium and sodium chlorides was to 146 and 143% of the rate in the absence of added ions, respectively, while for *Pisum* activation by potassium was only marginally greater than for sodium. The nature of the activation, as with the inhibition, is not well understood although the cations appear to interact with the enzyme rather than the substrate, to generally reduce the K_M for the substrate and to have their effect by altering the

conformation of the enzyme (Suelter 1974). As far as malate dehydrogenase activity from *Suaeda* and *Pisum* is concerned, in both cases the K_M was increased by activating concentrations of sodium chloride.

Conclusions

The effect of sodium chloride on malate dehydrogenase activity has a bearing on a number of aspects of halophyte metabolism. The similarity in behaviour of the enzyme fractions prepared from the halophyte and the glycophyte (figure 10) point to the fundamental similarity in the two types of plant, a point which has been made on a number of previous occasions (Jennings 1968; Flowers 1972*a*, *b*; Greenway & Osmond 1972; Hall & Flowers 1973). The optimum sodium chloride concentration for activation is similar to that for maximum activation of malate dehydrogenase activity from peas and of other glycophytes (Evans & Sorger 1966). As far as *Suaeda* is concerned, the lack of inhibition of activity from fraction I by a sodium chloride concentration of 200 mol m^{-3} may be of some significance, since if the enzyme existed in this form within the cell its activity would be relatively unimpaired by the salt concentrations estimated to be present in the cytoplasm by Yeo (1974). For salt grown plants the leaf cytoplasmic sodium concentration was estimated to be approximately 165 mol m^{-3} by analysis of the efflux of radioisotope. Plants grown on culture solution alone, although having a lower ion content overall contained a similar level of potassium (140 mol m^{-3}) within the cytoplasmic phase of the cell. Thus although the overall ion content may be doubled (2.9×10^{-3} mol g^{-1} dry tissue for plants growing on culture solution alone and 7.2 mol g^{-1} dry tissue for plants growing in culture solution containing 2% sodium chloride) by including 2% sodium chloride in the culture solution, cytoplasmic ion levels may be similar in plants growing under the different salinity regimes. The functional significance of the different levels of activation and in the proportions of the two fractions of the enzyme activity from plants under the different growth regimes is difficult to evaluate. Furthermore, it is not possible to estimate the proportion of the two enzymes which occurs *in vivo*. There is clearly room for a close control of the activity by monovalent anions or cations and since the higher molecular mass form of the enzyme appears to be stabilized at least *in vitro* by divalent cations, the possible variations in molecular mass and form make this enzyme at one and the same time both intriguing and difficult to study.

The influence of ions in the control of the metabolism has been discussed on a number of occasions (e.g. by Wyatt 1964; Bygrave 1967): there is little specific information as far as halophytes are concerned, however. The most extensive work has been on the facultative halophyte *Mesembryanthemum crystallinum* (Winter & von Willert 1972; Winter 1973*a*, *b*, 1974*a*, *b*, *c*). This plant, which is a member of the Aiozaceae, shows a marked stimulation in dark fixation of carbon dioxide and of malate accumulation characteristic of the so-called crassulacean acid metabolism (c.a.m.) in the presence of sodium chloride in the growth medium. Since this change can be brought about by high light intensities and low relative humidities, Winter (1974*a*) believes that the change in the mode of carbon fixation which follows transfer to saline conditions may be hormonally controlled. Water deficits have in fact been shown to alter the levels of abscissic acid in the leaves (see Hsiao 1973). The change to the utilization of c.a.m. in *Mesembryanthemum* must presumably involve a large increase in the activity of phosphoenolpyruvate carboxylase activity although this has not been determined in this species. However, Shomer-Ilan & Waisel (1973) have recently reported a large increase in the activity in this

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enzyme in the salt-tolerant grass *Aeluropus litoralis* when the plant is cultivated in the presence of sodium chloride. Earlier work (Joshi, Dolan, Gee & Saltman 1962) on dark fixation in the marine angiosperm *Zostera marina* showed a relatively large proportion of the total carbon fixed appeared as aspartate while Webb & Burley (1965) reported a larger proportion of $^{14}CO_2$ fixed by salt marsh plants was present as amino acids rather than organic acids. Furthermore, incorporation of $^{14}CO_2$ in the dark into leaf homogenates of spinach was increased by the presence of sodium chloride in the incubation medium (Joshi *et al.* 1962). These observations are complementary rather than contradictory, since malate arises by reduction of oxalacetate (by malate dehydrogenase) produced by the carboxylation of phosphoenolpyruvate, while aspartate may be formed by the transamination of oxalacetate from the same source, with glutamate. The fact that the change in the pattern of dark fixation in spinach can occur in leaf homogenates, suggests that the change in pathway is not wholly, if at all, hormonally controlled.

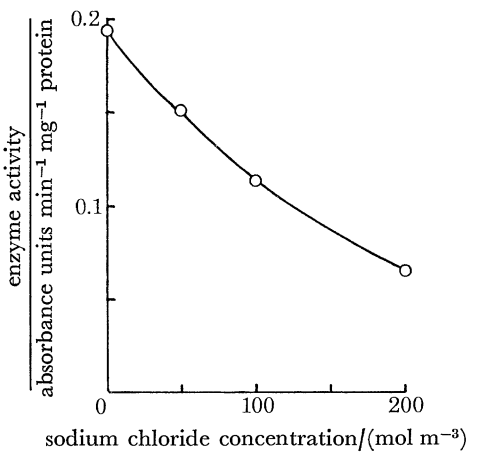

FIGURE 11. The effect of sodium chloride on malate dehydrogenase (fraction II) from salt grown *Suaeda*. Activity was assayed for the reverse reaction from that generally used (see Methods).

The role of malate itself in ion accumulation is also unclear. The formation of malate from oxalacetate would clearly be stimulated in the presence of sodium (or potassium) and chloride ions. Interestingly, the reverse reaction, from malate to oxalacetate is not stimulated by ions (figure 11 and Weimberg 1967; Hason-Porath & Poljakoff-Mayber 1969) and consequently both the equilibrium and the rate of reaction would favour the formation of malate. Malate does appear to exist in two separate pools within plant cells (Lips & Beevers 1966) and the pool which is labelled by dark fixation is relatively large and may be vacuolar in distribution (see Oaks & Bidwell 1970). If this is the case then evidence for a connection with ion accumulation is stronger albeit only circumstantial. A more direct connection between ion transport and malate accumulation has been presented for beet disks (Osmond & Laties 1969) while organic acids were implicated in a general way in ion transport in maize (Torii & Laties 1966) and oxalate has been shown to balance excess cations in a number of species mostly from deserts and members of the *Chenopodiaceae* (Williams 1960; Osmond 1967). However, the level of malate dehydrogenase activity in different species is not uniquely related to their ability to accumulate ions (namely, *Pisum* and *Suaeda* where the specific activity of enzymes from pea is apparently higher than for *Suaeda* – Flowers 1972). Interestingly, Hason-Porath & Poljakoff-Mayber (1969) found that low sodium salinities increased the specific activity of the soluble malate

dehydrogenase of pea roots while the level of mitochondrial malate dehydrogenase was suppressed (although the level of NADP dependent malate dehydrogenase increased). Such an increase in activity could be related to an increase in ion transport, although the significance of this finding is not entirely clear since results of this nature were not reported by Weimberg (1970) in similar experiments also using peas: he did, however, find a small increase in the specific activity of malate dehydrogenase activity in the leaf tissue. The lower specific activity of malate dehydrogenase in *Suaeda* growing under saline as opposed to non saline conditions is also difficult to explain. It may be, however, that the rate of ion transport in plants growing in media dominated by potassium ions is of necessity greater than in conditions in which sodium is accumulated owing to the greater permeability of the tonoplast to potassium which allows loss of accumulated ions back into the cytoplasm (Yeo 1974). Thus, although the role of organic acids in ion accumulation has not yet been fully elucidated, malate seems to play an important part in salt tolerance in some species, while malate dehydrogenase is an enzyme whose activity is clearly subject to ionic regulation of its activity.

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