TRANSACTIONS, ILL. STATE ACAD. SCI.
Vol. 67, No. 1 (1974)

EFFECT OF LOW WATER POTENTIAL ON THE ACTIVITY OF MITOCHONDRIAL, CHLOROPLAST AND SUPERNATANT MALIC DEHYDROGENASE FROM THE HALOPHYTE, SALICORNIA PACIFICA.

J. H. Yopp
Department of Botany, Southern Illinois University, Carbondale 62901

ABSTRACT. Salicornia spp. are among the most salt tolerant of higher plants with tissue water potentials (ψ) falling to -80 bars. The effect of low ψ on in vitro malic dehydrogenase (MDH) isozyme activity from young (low-salt) and old (high-salt) tissue was examined. Sucrose and NaCl (0.5, 0.75, an 1.0 M) were employed as assay osmotica. Activities of MDH isozymes were found to decrease (60%) but relative proportions of each fraction remained constant with increasing internal salt. A retardation of substrate penetration into intact mitochondria and a concomitant parallel inhibition of solubilized enzyme were observed with decreasing ψ (50% in 0.5 M NaCl or 1.0 M sucrose). Sucrose was more inhibitory than NaCl. Supernatant was more tolerant of low ψ than mitochondrial MDH. Activity of MDH in 1 M NaCl could be restored to 80% of the control with increased (nine-fold) substrate. These data indicate subcellular enzymatic integrity in this halophile at low water potential (-20.7 bars) and possible adaptation through increased in vivo substrate levels.

The great majority of plants are glycophytes or plants adapted to relatively low levels of salinity. Growth of glycophytes on increasingly saline soil results in progressive stunting and finally death. Numerous studies have been conducted to distinguish between effects due to a lowered water potential (ψ) and those due to the specific solute used to obtain it (Bernstei and Hayward, 1958). Many of the recent studies have attempted to ellucidate th mechanisms of inhibition through analysis of solute effects on enzymatic systems. The results of these studies on glycophytes have provided evidence for at least four possible mechanisms. The first concerns the osmotic component of ψ and its effects on enzymes compartmentalized within organelles. As the mitochondrion is subjected to increasing osmotic stress it contracts and component enzymes are inhibited. This inhibition may be due to decreased accessibility of substrate (Johnson and Lardy, 1958; Atmonson and Davis, 1967) or to concentration of endogenous substrates to the point of inhibition (Flowers and Hanson, 1969). The second entails inhibition of the enzyme per se due to the specific chemical nature of the solute. The third involves the change in tertiary structure of the enzyme due to effects of low water activity on protein hydration (Weimberg, 1967). The fourth possibility involves the suppression of enzyme synthesis as the tissue accumulates salt (Porath and Poljakoff-Mayber, 1964 and 1968).

Another view of the mechanisms of enzymatic response to low water activity might be obtained by utilizing a plant adapted to high salinity (i.e., a halophyte). The data presented in this paper are derived from experimentation utilizing the halophyte Salicornia pacifica. The species of the genus Salicornia are among the most salt tolerant of all higher plants, characteristically accumulating very high tissue salt (NaCl) during the growing season (Chapman, 1960). The perennial nature of S. pacifica enables one to study young, low-salt tissue concomitantly with older, high-salt tissue.

Malic dehydrogenase was chosen as the enzyme to be studied since it: 1, has representative isozymes both within the organelles and the cytoplasm; 2, has been extensively studied in glycophytes subjected to saline conditions; and 3, can be measured with a high degree of reproducibility.

MATERIALS AND METHODS

Young, green stems and the older, red stems were obtained from *Salicornia pacifica* collected from the salt marsh adjacent to the marina at Palo Alto, California. The chemical composition of the bay water in which they were growing was given by Gibor (1956). Osmotic values were calculated from freezing point depression values of their expressed sap measured by a Beckman differential thermometer. Protein content of the two types of tissue was determined by means of the method of Lowry, *et al.* (1951) following its precipitation by perchloric acid (7%).

Mitochondrial, chloroplast, and supernatant (cytoplasmic) malic dehydrogenase (MDH) were isolated from the stems by a modification of the method of Brandon (1967) devised for enzymatic studies on succulent tissues. The procedure consisted of grinding 10 g of freshly harvested stems in 50 ml of a medium that was 0.35 M mannitol, 0.1 M Tris-HCl, 0.01 M cysteine, and 2% (w/v) bovine serum albumen (pH 7.6) in a chilled mortar. The homogenate was squeezed through four layers of cheesecloth and centrifuged at 100 x g for 5 minutes. Chloroplasts were sedimented from the supernatant by centrifugation of 1000 x g for 10 minutes. The supernatant from this step was centrifuged for 30 minutes at 15,000 x g to obtain a mitochondrial fraction. The mitochondrial and chloroplast pellets were each suspended in 5 ml of 0.35 M mannitol. The last supernatant was retained as the source of supernatant MDH.

Malic dehydrogenase was assayed by following the oxidation of NADH at 340 nm in a Zeiss PM QII spectrophotometer. The components of the assay medium were: Tris, 200 μmoles, pH 7.4; $MgCl_2$, 10 μmoles; oxaloacetic acid (freshly prepared and neutralized before use), 2 μmoles; NADH, 0.2 μmoles; malic dehydrogenase fraction, 50 μl; and distilled water or the appropriate solute solution to 3.0 ml total volume. Variations in substrate and solute concentration are presented in the results. Both NaCl and sucrose were employed as assay osmotica. Mitochondria and chloroplasts were solubilized where noted by including 50 μl of Tween-20 in the assay medium.

Results are expressed as units (ΔO.D. of 0.01/min) per g fresh weight.

RESULTS

The osmotic values obtained from the young, low-salt tissue and older, high-salt tissue used throughout the experiments were found to average (three determinations) -5.8 and -20.7 bars, respectively.

Protein content (mg/g fresh weight) was found to average (three determinations) 15.4 and 9.6 for the low- and high-salt tissues, respectively.

Mitochondrial MDH from green, low-salt tissue was found to be stimulated by NaCl in concentrations of up to 0.2 M (Table 1). Above 0.2 M, however,

TABLE 1. MDH Activity of Intact and Solubilized Mitochondria in the Presence of Varying Concentrations of NaCl.

Molarity of NaCl in Assay Medium	Units of MDH from Intact Mitochondria Per Gram Fresh Weight of Tissue	% Inhibition (I) or Stimulation (S) over Control	Units of MDH from Solubilized Mitochondria	% Inhibitio (I) or Stim lation (S) over Contro
0 (Control)	70	0	250	0
0.2	105	33.3 (S)	345	38 (S
0.4	70	0	210	15 (I
1.0	30	57 (I)	85	66 (I
1.5	30	57 (I)	75	70 (I

increasing NaCl concentration progressively reduced MDH activity. Solubilization of the MDH brought about approximately a three-fold stimulation of activity in the control and at all concentrations of NaCl up to 1.5 M. Although the MDH activity was always higher following solubilization, the response to increases in NaCl closely paralleled that of the enzyme in the intact mitochondrion.

The salt effect on mitochondrial MDH from low-salt tissue was no different from that of MDH isolated from high-salt tissue (Table 2). The total units of mitochondrial MDH were, however, always about 50% less in the high-salt tissue.

TABLE 2. Effect of Salt on MDH Activity of Intact and Solubilized Mitochondr from Low- and High-Salt Tissue.

Molarity of NaCl in Assay Medium	Units of MDH from Intact Mitochondria Per Gram Fresh Weight of Tissue	% Inhibition	Units of MDH from Solubilized Mitochondria	% Inhibiti
		Green, Low-Salt Tissue		
0.00	75.0	0	300	0
0.75	28.5	62	130	56
		Red, High-Salt Tissue		
0.00	43.0	0	160	0
0.75	17.0	60	75	53

Increasing tissue salt concentration did not affect the relative distribution of the MDH among the subcellular fractions (Table 3). While lower values of MDH (∿50%) were observed in the fractions from high-salt tissue, t relative distribution of MDH was found to be about the same.

TABLE 3. Total Units and Their Distribution in the Various Fractions from *Salicornia pacifica* of Varying Internal Salt Content.

Fraction	Total Units Per Gram Fresh Weight	Percentage of Total in Homogenate
	Green, Low-Salt Tissue	
Homogenate	1400	100.0
Chloroplast	300	21.0
Mitochondrial	260	18.5
Supernatant	720	53.5
	Red, High-Salt Tissue	
Homogenate	660	100.0
Chloroplast	150	22.0
Mitochondrial	160	24.0
Supernatant	360	54.0

When each of these sources of solubilized isozymes were tested for their response to NaCl, the order of increasing resistance was found to be chloroplast, mitochondrial, and finally supernatant MDH (Table 4). WHen these sources of MDH were compared on the basis of origin from low- and high-salt tissue, chloroplast and mitochondrial isozymes display no differences in resistance to high assay NaCl (Table 4). However, the supernatant MDH from high-salt tissue appears to be much more inhibited by NaCl than that from low-salt tissue.

To determine whether the effect of high solute concentration on mitochondrial MDH was osmotic or due to the specific nature of the solute, both sucrose and NaCl were used as assay osmotica. In order to evaluate the potential osmotic effect both intact and solubilized mitochondria from low-salt tissue were employed. Sucrose proved to be most inhibitory to MDH within the intact mitochondrion even though 1 M NaCl provides almost twice as many solute particles as 1 M sucrose (Table 5). However, unlike the parallel inhibition of intact and solubilized mitochondrial MDH exerted by NaCl (Table 1), there appears to be a much greater restoration of sucrose-inhibited, mitochondrial MDH by solubilization (Table 5). It is also evident that once solubilized, the MDH is about equally sensitive to the two osmotic agents.

MDH inhibition by high concentrations of its substrate was observed for the mitochondrial isozyme from *Salicornia*. However, the response of the mitochondrial enzyme from low-salt tissue to increasing substrate concentrations was just the opposite in high concentrations of assay NaCl (Figure 1). In fact, the loss of activity in the MDH of the intact mitochondrion due to the presence of 1 M NaCl could be completely restored by a four-fold increase in substrate.

TABLE 4. Effect of Increasing Amounts of NaCl on Activity of Solubilized and Supernatant MDH from Tissue of Low and High Salt Content.

Fraction	Units Per Gram Fresh Weight	Molarity of NaCl in Assay Medium	% Inhibition
	Green, Low-Salt Tissue		
Chloroplast	300	0.00 (Control)	0
	220	0.50	27
	130	0.75	55
Mitochondrial	300	0.00	0
	160	0.50	45
	130	0.75	56
Supernatant	780	0.00	0
	630	0.50	20
	480	0.75	38
	450	1.00	42
	Red, High-Salt Tissue		
Chloroplast	150	0.00	0
	80	0.50	43
	70	0.75	50
Mitochondrial	160	0.00	0
	100	0.50	34
	70	0.75	53
Supernatant	360	0.00	0
	360	0.50	0
	300	0.75	17
	270	1.00	25

TABLE 5. Effect of the Nature of the Solute on MDH of Intact and Solubilized Mitochondria from Low Salt Tissue.

Solute in Assay Medium	Units of MDH from Intact Mitochondria	% Inhibition	Units of MDH from Solubilized Mitochondria	% Inhibiti
None (Control)	78	0	265	0
NaCl (0.75 M)	32	59	126	51
NaCl (1 M)	30	62	80	70
Sucrose (1 M)	0	100	159	40
Sucrose (1.5 M)	0	100	106	60

DISCUSSION

The MDH isozymes of the halophyte Salicornia pacifica show the rise in activity (in vivo) with increasing salt concentration as has been previously reported (Hiatt and Evans, 1961; Weimberg, 1970). However, important differences in magnitude exist. While the soluble MDH from peas shows maximal activity at 0.02 M NaCl and inhibition to totality at 0.3 M (Weimberg, 1967), that from Salicornia is maximally stimulated at 0.2 M NaCl. Moreover, considerable activity of the Salicornia MDH isozymes may be observed at 1.5 M NaCl. This much greater activity in high NaCl concentration may reflect some structural adaptation in the MDH of Salicornia.

A progressive inhibition of the MDH in the intact mitochondrion due to increasing assay solute concentration was observed. However, the nature of this inhibition is not solely due to an osmotically-induced restriction of substrate penetration as suggested by Atmonson and Davis (1967). There is instead, a parallel decrease in activity under similar solute conditions in the solubilized enzyme. Flowers and Hanson (1969) found a similar response for the MDH of soybean mitochondria. In addition, Atmonson and Davis (1967) found no difference in inhibition exerted by NaCl and sucrose. The MDH of Salicornia whether solubilized or within the mitochondrion, is inhibited to a much greater degree by sucrose. While Flowers and Hanson (1969) observed a difference between sucrose- and KCl-induced inhibition, the KCl was reported to be more inhibitory. The opposite was seen in the present study, where the salt (NaCl) was less inhibitory than sucrose. These findings may indicate an osmotically-induced inhibition due to a non-penetratable sucrose and a more tolerant enzyme to the penetrating NaCl. The similar magnitude of inhibition of the solubilized MDH by sucrose and NaCl indicates that there exists a true inhibitory effect from lowered ψ per se. This may result from an altered hydration as postulated by Flowers and Hanson (1969). It is unlikely that there was inhibition due to increased substrate levels accompanying volume decrease in the osmotically-contracted mitochondrion. The MDH activity suppressed by 1 M NaCl could instead be completely restored by increasing the substrate concentration.

The constancy in distribution of MDH among the subcellular fractions as the tissue rose in internal salt content is probably another manifestation of adaptation in Salicornia (Table 3). The lower activities of MDH in the high-salt tissue are more apparent than real. This results from the expression of activity as units per gram fresh weight. As noted in the Results section, due to increased succulence in the high-salt tissue, the protein values per gram fresh weight declined. Specific activities of the malic dehydrogenases were then not greatly different.

There appeared to be no significant difference in degree of salt inhibition between that exerted on mitochondrial MDH from low- or high-salt tissue. This indicates that there is no change in mitochondrial or MDH enzyme structure as the tissue ages and rises in salt content. The MDH of the supernatant from high-salt tissue does seem to be more salt tolerant to high NaCl concentration in the assay medium (Table 4). The significance of this observation may lie in the fact that this latter isozyme is not compartmentalized and must function in the low water activity that results from increasing tissue salt.

Another mechanism of adaptation to high internal salt may lie in the interpretation of the data in Figure 1. There is little doubt that increasin substrate concentration can bring about greater activity in salt-inhibited MD Such increasing substrate concentration would be very inhibitory to the enzyme were it not in the presence of high salt. The hypothetical characteristics of this adaptation would be two-fold. First there must be some shift to high endogenous substrate levels as internal salt increases. Webb and Burley (1965) and Joshi, *et al.* (1962) have shown that in a facultative halophyte there is a shift towards the formation of more amino acids and mali acid with increasing salinity. This may reflect some mechanism of increasin substrate levels for salt-sensitive enzymes. Secondly, an overcoming of an effect of high NaCl with increased substrate suggests some type of competiti inhibition exerted by the salt. Weimberg (1967) has provided kinetic eviden that there does exist such a competition between NaCl and oxaloacetic acid f the active site of MDH. The data presented here (Table 5) suggests that thi apparent competitive effect might be due to a less available active site resulting from decreased hydration of the enzyme. In this case increasing substrate would increase activity according to the principle of mass action. Of all the possibilities for both the basis of salt inhibition of activity a the mechanism of adaptation to it, this latter hypothesis seems best support by the data of the present study.

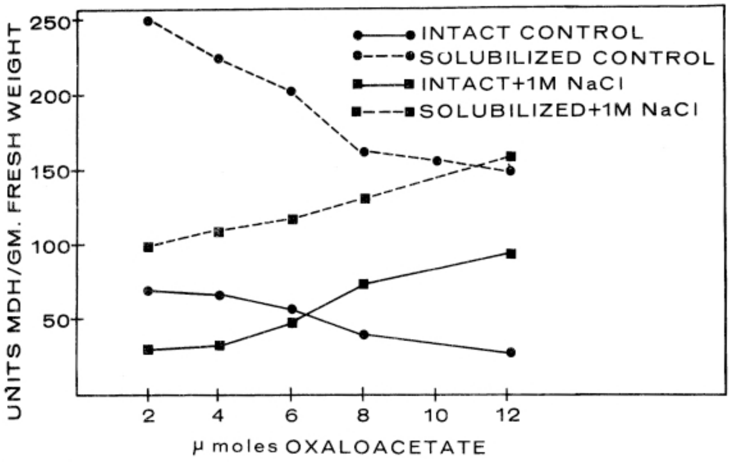

FIGURE 1. EFFECT OF SUBSTRATE CONCENTRATION ON INTACT AND SOLUBILIZED MDH ACTIVITY.

ACKNOWLEDGMENTS

This research was made possible by the award to the author of a Postdoctoral Resident Research Associateship by the National Research Counci The author also wishes to express his appreciation to Ames Research Center (NASA), Moffett Field, California, where this research was conducted.

LITERATURE CITED

ATMONSON, A. and R. P. DAVIS. 1967. Mitochondrial Respiration Under Conditions of Varying Osmolarity. Biochem. Biophys. Acta. 131:221-33.

BERNSTEIN, L. and H.E. HAYWARD. 1958. Physiology of Salt Tolerance. Ann. Rev. Plant Physiol. 9:25-46.

BRANDON, P. C. 1967. Temperature Features of Enzymes Affecting Crassulacean Acid Metabolism. Plant Physiol. 42:977-84.

CHAPMAN, V. J. 1960. Salt Marshes and Salt Deserts of the World. Plant Science Monographs, Interscience Publishing Company, New York. 392 pp.

FLOWERS, T. J. and J. B. HANSON. 1969. The Effect of Reduced Water Potential on Soybean Mitochondria. Plant Physiol. 44:939-45.

GIBOR, A. 1956. The Culture of Brine Algae. Biol. Bull. 111:223-29.

HIATT, A. J. and H. J. EVANS. 1961. Influence of Salts on Activity of Malic Dehydrogenase from Spinach Leaves. Plant Physiol. 35:662-75.

JOHNSON, D. and H. LARDY. 1958. Substrate Selective Inhibition of Mitochondrial Oxidation by Enhanced Tonicity. Nature 181:701-02.

JOSHI, G., T. DOLAN, R. GEE, and P. SALTMAN. 1962. Sodium Chloride Effect on Dark Fixation of CO_2 by Marine and Terrestrial Plants. Plant Physiol. 37:446-49.

LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193:265-75.

PORATH, E. and POLJAKOFF-MAYBER. 1964. Effect of Salinity on Metabolic Pathways in Pea Root Tips. Israel J. Bot. 13:115-21.

PORATH, E. and POLJAKOFF-MAYBER. 1968. The Effect of Salinity in the Growth Medium on Carbohydrate Metabolism in Pea Roots. Plant Cell Physiol. 9:195-203.

WEBB, K. L. and J. W. A. BURLEY. 1965. Dark Fixation of CO_2 by Obligate and Facultative Salt Marsh Halophytes. Can. J. Bot. 43:281-85.

WEIMBERG, R. 1967. Effect of Sodium Chloride on the Activity of a Soluble Malate Dehydrogenase from Pea Seeds. J. Biol. Chem. 242:3000-06.

WEIMBERG, R. 1970. Enzyme Levels in Pea Seedlings Grown on Highly Salinized Media. Plant Physiol. 46:466-470.