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Utilization of metabolic energy under saline conditions: changes in properties of ATP dependent enzymes in plant cells grown under saline conditions

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Abstract

The effect of growth in saline medium on the activity of two ATP utilizing enzymes was studied. Hexokinase in carrot *(Daucus carota* L.) cells grown in suspension culture either in the absence or presence of 150 ml NaCl, and tonoplast H^+-ATPase in tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) cells grown in suspension culture either in the absence of presence of 428 mM NaCl. There was no difference in the pH profiles, NaCl sensitivity and kinetic parameters towards glucose of hexokinase activities from carrot cells grown in the presence or the absence of NaCl, but the activity from cells grown in the presence of NaCl was more resistant to inhibition by N-ethylmaleimide and to inactivation by heat. Two separate apparent Km values toward ATP were delineated in the extract from cells grown in presence of NaCl while extracts from cells grown in the absence of NaCl had only one apparent Km value. The tonoplast H^+-ATPase from NaCl grown tobacco cells showed changed kinetic compared to this activity from cells grown in the absence of NaCl. These data may indicate that growth in NaCl results in the appearance of isozymic activity that enhances the ability of plant cells to utilize metabolic energy more efficiently.

Introduction

The use of plant cells in liquid suspensions cultures to study cellular mechanisms of salt tolerance enable to determine physiological and biochemical changes that occur at the cellular level discretely from adjustments that occur in whole tissue or plant. Additionally these cell lines can accommodate to grow in high salt concentrations (Binzel *et al.* 1988).

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; ADH - alcohol dehydrogenase; BTP - bis tris propane; DCCD - *N,N*-dicyclohexyl-carbodiimide; LDH - lactate dehydrogenase; Mes - 2-[morpholino]ethane sulfonic acid; NEM - *N*-ethylmaleimide.

The accommodation to salinity has physiological, biochemical and genetic components which act together or separately to alleviate the effect of stress. Characterization and subsequent isolation of these components can facilitate the growing understanding of salt tolerance mechanisms. A stable biochemical change, accompanied with enhanced tolerance of the cells to salt may point out a role of that protein in salt tolerance. Recent studies suggest that some glycophyte cells respond to salt stress, if allowed to adapt, in a way similar to halophytes (Reuveni *et al.* 1985, 1990).

Changes in protein pattern and gene expression induced by exposure of plant cells to salt stress have been described previously (Singh *et al.* 1985, Ramagopal 1987). However, the function of these proteins was not identified. On the other hand, there are reports of salt-induced changes in many properties of plant enzymes (Chappell and Hahlbrock 1986, Kalir and Poljakoff-Mayber 1981, Kalir *et al.* 1984, Reuveni *et al.* 1990).

Hexokinase was studied because it occupies a central position in the energy supplying pathways. Hexokinase activity in vertebrate cells was shown to be composed of several isozymes that varied in their Km toward ATP and glucose (Ureta 1982). Hexokinase isozyme profile expression also varied developmentally (Ureta 1982).

The tonoplast H^+-ATPase was studied because it pumps protons into the cell's vacuole and generates the $\Delta\mu H^+$ across the tonoplast that in turn is used to drive solute uptake or efflux via H^+ antiports and symports (Rea and Sanders 1987). There are reports of changes in proton efflux activity when plant cells were exposed to salt stress (Matsumoto and Chung 1988, Reuveni *et al.* 1990). The tonoplast H^+-ATPase is part of a family of proton pumps generally known as the vacuolar ATPases (V-ATPase) because they are located mainly on endomembranes such as the vacuole (Rea and Sanders 1987). V-ATPases are characterized by inhibition by NO_3^-, DCCD and bafilomycin. V-ATPases are composed of as many as 10 subunits with an apparent total relative molecular mass of more than 400 kD (Rea and Sanders 1987). The postulated functions of the various subunits of the V-ATPase are as follows: the large subunit, 67-70 kD, has characteristics consistent with being the catalytic subunit (Rea and Sanders 1987). The 56-60 kD polypeptide has a nucleotide binding site that is apparently different from the catalytic binding site and it may function in regulation of activity (Rea and Sanders 1987). The small 16-18 kD subunit is proteolipid-like and binds *N,N'*-dicyclohexyl-carbodiimide (DCCD) which suggest that this subunit is a component of the proton channel (Rea and Sanders 1987). The function of the other subunits is still unknown, but the 31 kD hydrophilic polypeptide was demonstrated to be an essential subunit of the V-ATPase of yeast (Foury 1990).

Energy maintenance cost did not differ between plant cells grown in saline medium and of cells grown in the absence on NaCl (Schnapp *et al.* 1991). Therefore, it has been hypothesized that ATP utilizing enzymes such as hexokinase in carrot cells and tonoplast H^+-ATPase in tobacco cells exposed to NaCl stress may be more efficient under these growth conditions.

Hexokinase activity from cells grown in the presence of NaCl was more resistant to inhibition by NEM (*N*-ethylmaleimide) and to inactivation by heat. Two separate apparent Km values toward ATP were delineated in the extract from cells grown in

presence of NaCl while only one apparent Km value present in extracts from cells grown in the absence of NaCl. The activity of the tonoplast H^+-ATPase was compared between two cell lines derived from tobacco plant (NaCl sensitive plant), one cell line that was adapted to grow in 428 mM NaCl and the other was grown without NaCl (Binzel *et al.* 1985, 1987). The kinetics of the tonoplast H^+-ATPase proton transport activity was assayed in fractions enriched in tonoplast membrane vesicles and was different between the tobacco cell types.

Material and methods

Growth of cell suspensions: Carrot and tobacco cells in liquid suspension cultures were used as experimental material. The carrot cell (*Daucus carota* L.) suspension was a gift from Dr. D. Aviv of the Weizman Institute, Rehovot, Israel, and was maintained in our laboratory. For a more detailed description of this cell line see Harborne *et al.* (1983) and Reuveni *et al.* (1987).

Carrot cells were grown on B_5 salt medium described by Gamborg *et al.* (1968) with added vitamins and sucrose as described previously (Reuveni *et al.* 1987). The only phytohormone added was 0.2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). For exposure to salinity, 30 ml of 7-d-old cell cultures grown in absence of NaCl, were added to 70 ml of medium containing 220 mM NaCl; thus the cells were exposed to final NaCl salinity of 150 mM. The free spaces of the two cell types were similar (Reuveni *et al.* 1991). Fresh and dry masses were determined according to Binzel *et al.* (1985). Dry mass yields of the both carrot and tobacco cells at beginning of the stationary phase was similar when grown with or without NaCl.

Liquid suspension cultures of tobacco cells (*Nicotiana tabacum* L. cv. Wisconsin 38) were grown in the presence of 428 mM NaCl or the absence of NaCl, as described elsewhere (Reuveni *et al.* 1990). Cell lines were grown in the dark on a MS modified nutrient medium (Binzel *et al.* 1985, 1987) and collected at stationary phase of the growth cycle for membrane vesicle preparation (23 - 26 d for unadapted cells and 36 - 45 d for NaCl adapted cells).

Preparation of membrane vesicles and proton transport assay: Membrane vesicle fractions were prepared by discontinuous sucrose gradient as described (Reuveni *et al.* 1990). The discontinuous gradient was centrifuged at 100 000 *g* for 2 h and the 0/22 % interface was collected. The membrane vesicles were diluted 2 fold in a suspension medium (1 mM Mes-BTP buffer pH 7.4, 250 mM sorbitol) and centrifuged at 150 000 *g* for 30 min. The membrane pellet was suspended in a suspension buffer and proton transport was measured in a *Perkin Elmer* fluorescence spectrometer *LS-5* as described by Reuveni *et al.* (1990).

Hexokinase activity: Hexokinase activity was assayed immediately after extraction in extracts of cells grown in the absence or in the presence of 150 mM NaCl for one growth cycle (8 to 10 d, about 3 generations). Cells were separated from the medium by filtration and immediately frozen in a mortar containing liquid nitrogen. The frozen cells were ground to a fine powder, and extraction buffer (1 % BSA, 0.1 M

imidazole, 4 M glycine and 1 mM EDTA pH 7.2; 1 ml of extraction buffer to 1 g of cells) was added. The resulting slurry was brought to 0 °C and centrifuged at 11 000 *g* (*Sorval RC2*) for 15 min. The pellet was discarded, and the supernatant was used as crude enzyme preparation. Enzyme activity in this extract was stable for a week when stored at 4 °C. Enzymatic activity of extracts prepared the same way, as stated above, but in a buffer lacking BSA was lost within 6 to 8 h at 4 °C. Hexokinase activity was measured according to Bergmeyer *et al.* (1974). The reaction buffer contained 100 mM imidazole, pH 7.75, 0.2 % BSA, 5 mM $MgSO_4$, 0.2 mM NADP, and 2-3 units of glucose-6-phosphate dehydrogenase and various concentrations of glucose and ATP. The glucose-6-phosphate formed by hexokinase activity was oxidized by the dehydrogenase, and the rate of NADPH formation at room temperature (22 °C) was monitored at 340 nm in a *Gilford* recording spectrophotometer. Hexokinase activity is expressed as the change in absorbance at 340 nm per min per g fresh mass of cells. The protein content of the extract was not measured due to the high BSA concentration used to stabilize enzyme activity. In 1 g of fresh mass of cells that were grown in the presence of 150 mM NaCl there was more protein in general than in 1 g of fresh mass of cells that were grown in the absence of NaCl. Therefore, comparison of V_{max} values was not possible.

Apparent Km toward ATP was measured in the above described buffer containing 0.5 mM glucose at various ATP concetrations; apparent Km for glucose was measured in the same buffer containing 1 mM ATP at various glucose concentrations. All experiments were repeated at least twice and representative experiments are shown.

Heat inactivation was carried out by exposing the crude extract either to 46 °C or 55 °C for different lengths of time. Activity was assayed at room temperature (22 °C).

Protein was determined according to Bradford (1976).

Enzymes and chemicals were obtained from *Sigma, U.S.A.*

Results and discussion

Both carrot cells and tobacco cells exhibited growth and cell division in NaCl containing media (Binzel *et al.* 1985, Reuveni *et al.* 1991).

Effect of growth in NaCl on hexokinase activity: The activity of hexokinase was measured in crude extracts of carrot cells grown in the absence or presence of 150 mM NaCl. The average apparent Km values toward glucose of the enzymes from both types of cells were very similar (Fig. 1). Similar apparent Km values for glucose were reported for hexokinase from various sources (Ureta 1982). The apparent V_{max} for hexokinase activity in extracts from NaCl grown cells was greater compared to the activity in extracts from cells grown in the absence of NaCl. The apparent V_{max} values were not compared between the two cell types because they relate to specific activity usually on the basis of protein, and since this basis was not available in this study (see Material and Methods). Measuring the amount of protein in the extract was not possible due to the high concentration of BSA used to stabilize

the activity, therefore protein could not be used as a basis for specific activity calculation. Differences in V_{max} were considered artifactual due to the difference in fresh mass between the two cell types.

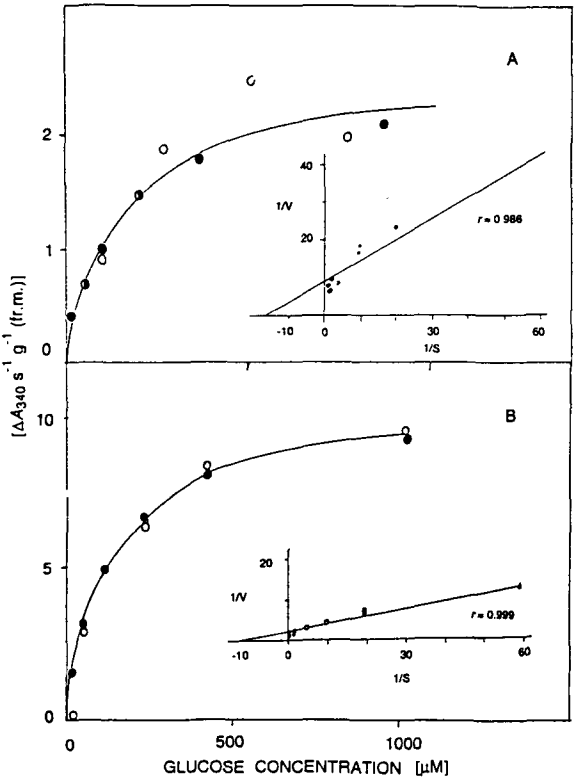

Fig. 1. Kinetics of hexokinase activity toward glucose of extracts prepared from carrot cells in suspension, grown in the presence (*A*) or the absence (*B*) of 150 mM NaCl. Hexokinase activity was assayed either in the presence (*empty symbols*) or the absence (*full symbols*) of 100 mM NaCl. Enzyme activity was tested in extracts from the two cell types in the presence of 1 mM ATP and increasing concentrations of glucose (10 to 800 μM). Apparent Km (*insets*) was estimated using linear transformation of the data according to Dixon and Webb (1979). *r* = regression coeficient.

Compartmentation studies demonstrated that at the stationary growth stage carrot and tobacco cells contain about 100 mM NaCl in the cytosol (Reuveni *et al.* 1991, Binzel *et al.* 1988). Therefore, hexokinase activity was tested at this concentration in order to evaluate the effect of NaCl in the cytosol on hexokinase activity from the two carrot cell types. Addition of 100 mM NaCl (final concentration) to the assay mixture had small non significant effects on the apparent Km towards glucose (Fig. 1) or ATP (data not shown). It was concluded that growth in NaCl did not result in hexokinase activity that was more tolerant to 100 mM NaCl in the assay solution.

Growing the cells in 150 mM NaCl induced a significant change in the apparent Km towards ATP (Fig. 2). Double reciprocal plots were used to deduce the apparent Km of hexokinase activity toward ATP according to Dixon and Webb (1979). The lines were fitted with linear regression with minimal significance level of 95 % or above, according to Johnson (1976), if the points did not fit the 95 % significance level they were fitted with 2 lines. The double reciprocal plot of enzyme activity rate *vs.* ATP concentration for hexokinase activity from NaCl grown cells was best fitted with two distinct, separate lines (Fig. 2). While hexokinase activity in extracts from cells grown in the absence of NaCl was best fitted with one line (Fig. 2). This can be interpreted as indicative of the presence of an additional hexokinase activity in the extracts from cells grown in 150 mM NaCl (Dixon and Webb 1979).

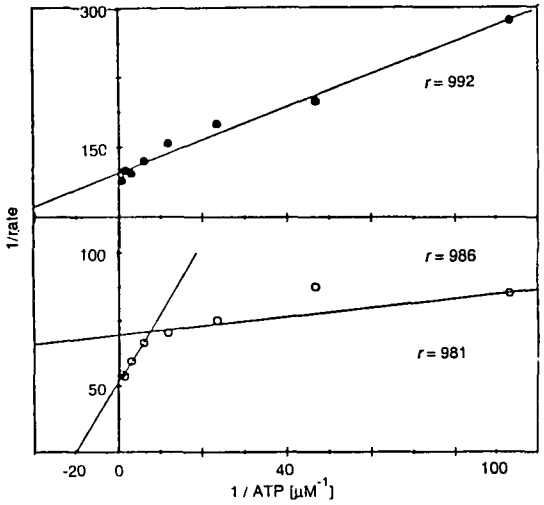

Fig. 2. Lineweaver-Burke plot of hexokinase activity as a function of ATP concentration. Assay conditions as described in Material and methods. ATP concentrations were varied at a constant (0.5 mM) glucose concentration. Extracts from cells grown in absence of NaCl (*full symbols*) or in presence of 150 mM NaCl (*open symbols*) were assayed in the absence of NaCl. Rate was the change in absorbance at 340 nm [ΔA g^{-1}(fr.m.) min^{-1}].

Since a straight line could not be reliably passed through the points that were obtained for hexokinase activity in extracts from NaCl grown cells the alternative was to use two lines that showed a much higher fitness to a linear model (Dixon and Webb 1979). A representative experiment (Fig. 2) demonstrates that in carrot cells exposed to salinity during growth an additional hexokinase activity seems to developed a lower apparent Km value toward ATP (Fig. 2). The value of apparent Km1 of hexokinase activity towards ATP in extracts from NaCl grown cells was practically equal to that of the enzymatic activity in extracts from cells grown in the absence of NaCl (66 ± 30 µM and 65 ± 29 µM, n=5, respectively). The second apparent Km value (apparent Km2 = 6.6 ± 2.3 µM, n=5) toward ATP was about 10

times lower than apparent Km1 and appeared only in cells grown in the presence of NaCl.

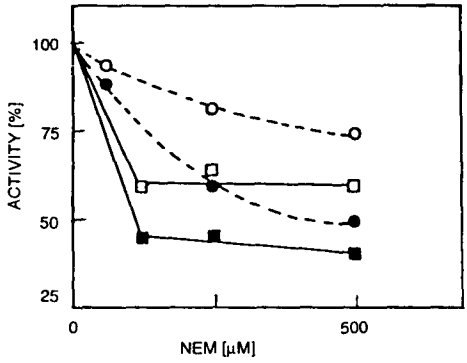

Fig. 3. Effect of *N*-ethylmaleimide (NEM) on hexokinase activity. NEM was added to the assay mixture just before (*squares*) or with (*circles*) the addition of ATP. Activity of enzyme from cells grown in the absence of NaCl (*full symbols*), or in the presence of 150 mM NaCl (*empty symbols*) assayed at pH 7.75. Data expressed as % of activity in absence of NEM.

The pH profiles of hexokinase activity from both cell types were identical, showing a broad peak of maximal activity between pH 8 to pH 9.

NEM (*N*-ethylmaleimide) is an inhibitor known to bind to sulfhydryl group in cysteine and inhibit enzyme activity. Specifically it was found that NEM inhibits ATP requiring enzymes probably due to the presence of a sulfhydryl group at or close to the ATP binding site. Hexokinase activity in extracts from both cell types was inhibited differentially by NEM. The activity in extracts from NaCl grown cells was more resistant to inhibition by NEM (Fig. 3) as well as to heat inactivation (Fig. 4) than the activity in extracts from cells grown in the absence of NaCl.

These results do not by any way show that just one additional isozyme of hexokinase appears after growth in NaCl, the change in kinetic behavior is indicative that some alteration has occurred in the this activity in these cells. These changes in hexokinase activity in cells exposed to salinity stress may be the result of altered gene expression induced by stress, and thus the appearance of additional hexokinase isozyme(s). Another possible explanation of the results is that in cells grown in the presence of NaCl structural changes and/or modifications occurred in the enzyme molecule that affect the kinetic parameters of the enzyme. This different enzyme configuration exhibits increased resistance to NEM, enhanced resistance to inactivation by heat and a lower apparent Km towards ATP. A third possibility is that carrot cell grown in 150 mM NaCl have protecting solutes that maintain the hexokinase activity from inhibition by NEM and heat inactivation and as a by product change the kinetic properties of this enzymatic activity.

Effect of growth in NaCl on tonoplast H^+-ATPase activity: Proton transport by H^+-ATPase into tonoplast membrane vesicle fractions isolated from tobacco cells grown with or without NaCl was MgATP dependent (Fig. 5). The leakage of protons from vesicles from both cell type was similar (Reuveni *et al.* 1990). The dependency of

proton pumping into tonoplast membrane vesicles from NaCl grown tobacco cells, was different from that of tonoplast membrane vesicles isolated from tobacco cells grown without NaCl (Fig. 5). The kinetic of proton pumping into tonoplast membrane vesicles from NaCl grown tobacco cells was sigmoidal whereas that of H^+-ATPase from cells grown without NaCl was hyperbolic. Two dimensional blots of the catalytic subunit did not reveal changes in the number of isozymes of this subunit. The apparent Km value towards MgATP for proton pumping into tonoplast membrane vesicles from cells grown with or without NaCl was calculated to be similar (about 0.5 mM).

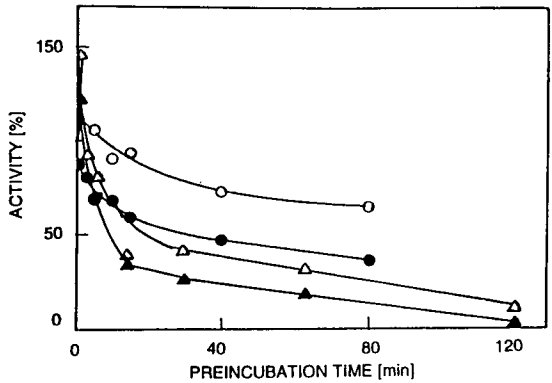

Fig. 4. Effect of preincubation for various periods at 46 °C or 55 °C on hexokinase activity. Extracts from cells grown in the absence (*full symbols*) or in the presence (*empty symbols*) of 150 mM NaCl were pre-incubated for various times at 46 °C (*circles*) or 55 °C (*squares*) and samples were taken and assayed (0.5 mM glucose and 1 mM ATP) at room temperature (22 °C). Data expressed as percentage of activity in without pre-heating.

Both NO_3^- and DCCD are known inhibitors of the tonoplast H^+-ATPase (Rea and Sanders 1988). There was no difference in inhibition of proton pumping activity into tonoplast vesicle from the two tobacco cell types by NO_3^-. A two-fold increase in $I_{1/2}$ of inhibition of proton pumping by DCCD (from 400 to 800 nmol DCCD mg^{-1} membrane protein) was observed in tonoplast vesicles from NaCl grown cells compared to proton pumping into tonoplast vesicles from cells grown without NaCl (Fig. 6). This result indicates that a change in the small subunit (16 kD, *cf.* Rea and Sanders 1988) has occurred after growth of tobacco cells in NaCl. It is not clear how the increased resistance to DCCD is translated to the increased proton pumping capability (Reuveni *et al.* 1990) and altered kinetics that were observed in these tobacco cells after growth in NaCl. Further elucidation of the mechanism of proton transport by this enzyme may explain this observation.

Significance in relation to salt tolerance: Under increased ion fluxes that occur when plant cells are exposed to saline environment maintenance energy costs of plant cells were suggested to increase (Amthor 1984) to accommodate NaCl evacuation from the cytosol to the external and vacuolar media that have been associated with NaCl stress (Läuchli 1984, Watad *et al.* 1986, 1991). The increased use of metabolic energy (ATP) to evacuate ions from the cytosol should reduce the carbon use

efficiency of cells grown in NaCl. This increased demand for energy should increase respiration in cells grown with NaCl compared to cells grown without NaCl. However, after these cells have grown in saline medium their respiration rate equals that of cells grown without NaCl. Furthermore, the carbon use efficiency of NaCl grown cells was greater than that of cells grown without NaCl when both cell types were grown in high sugar levels (Schnapp *et al.* 1991). Reduction in respiration rate of tobacco cells grown with NaCl compared to that of cells grown without NaCl, and the accumulation of organic solutes (Binzel *et al.* 1988), resulted in an apparent higher carbon use efficiency.

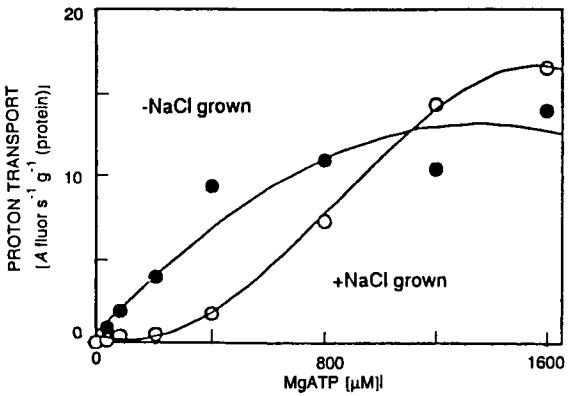

Fig. 5. Effect of MgATP concentration on proton transport activity of H^+-ATPase into tonoplast vesicles. Proton transport rate (measured as the rate on quinacrine quenching) into tonoplast vesicles from tobacco cells that were grown in the absence (*full circles*) or presence (*open circles*) of 428 mM NaCl was measured at various MgATP concentrations. Each point is the average of three separate experiments.

Changes in protein pattern and gene expression induced by exposure of plant cells to salt stress have been described previously (Singh *et al.* 1985, Ramagopal 1987). However, the function of these proteins was not identified. On the other hand, there are reports of salt-induced changes in many properties of enzymes (Chappell and Hahlbrock 1986, Kalir and Poljakoff-Mayber 1981, Kalir *et al.* 1984, Reuveni *et al.* 1990). Examples of changes in enzyme properties that was induced by external stress were reported for LDH and ADH. Different enzymatic activity of these enzymes was reported to appear in response to an aerobic stress (Fagerstedt and Crawford 1986, Hanson and Brown 1985, Roberts *et al.* 1984). These isozymes were assumed to maintain cellular metabolism under conditions of limited supply of energy (Fagerstedt and Crawford 1986, Hanson and Brown 1985, Hoffman *et al.* 1986).

Therefore, in order to survive under these constraints (*i.e.* equal respiration and higher demand for ATP) cells growing in the presence of NaCl seem to express a set of isozymes that exhibit a higher affinity towards ATP. Isozyme(s) of hexokinase exhibiting higher affinity towards ATP in carrot cells grown in the presence of 150 mM NaCl and of the tonoplast H^+-ATPase in tobacco cells grown in 428 mM NaCl were shown to occur. Cells growing in the presence of NaCl seem to have the ability to express new enzyme activity and to control housekeeping metabolic processes,

such as the vacuolar pH maintenance (Reuveni *et al.* 1990) and plasma membrane proton transport (Reuveni *et al.* in preparation), under conditions of increased energy demand and reduced ATP turnover caused by NaCl stress. The expression of these activities enables plant cells to grow in salinized environment. Other mechanisms which protect the sensitive cytosol in cells exposed to NaCl can be envisioned which would include expression of new isozymes which exhibit tolerance to high NaCl concentration in the cytosol, such enzymes have yet to be demonstrated.

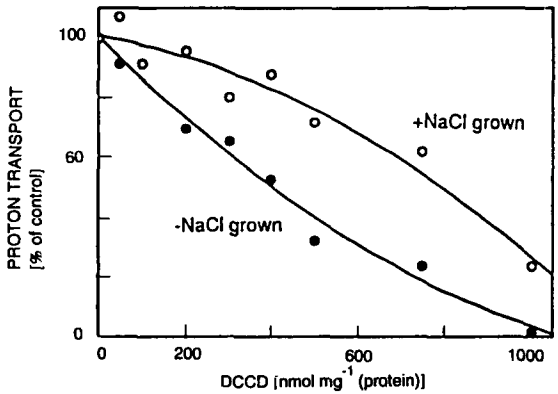

Fig. 6. Inhibition of proton transport by DCCD. Proton transport rate (measured as the rate on quinacrine quenching) into tonoplast vesicles from tobacco cells that were grown in the absence (*full circles*) or presence (*open circles*) of 428 mM NaCl was measured at various DCCD concentrations MgATP concentration was 1.5 mM and the vesicles were pre-incubated with DCCD at least 5 min before MgATP was added. Data expressed as percentage of activity in absence of DCCD.

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