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Salt Tolerance in Suspension Cultures of Sugar Beet[1]

INDUCTION OF Na^+/H^+ ANTIPORT ACTIVITY AT THE TONOPLAST BY GROWTH IN SALT

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ABSTRACT

Cell suspension cultures of sugar beet were grown at various salinities (0–200 millimolar NaCl). Their tolerance to Na^+ was comparable to that of the intact plant. Tonoplast vesicles were prepared by sucrose density gradient centrifugation of microsomal membranes and shown to be highly purified. The vesicles were subjected to a pH jump in the presence of acridine orange and the rate of recovery of fluorescence after addition of Na^+ was used as a measure of Na^+-dependent H^+ efflux. In the presence of K^+ and valinomycin, the Na^+/H^+ antiport showed saturation kinetics. Increasing Na^+ in the growth medium did not change the apparent K_m for Na^+, but increased V_{max} to about twice the control value, suggesting a specific induction of antiport synthesis by salt.

In a previous study (3) we characterized a Na^+/H^+ antiport in tonoplast vesicles isolated from storage tissue of red beet and sugar beet. Since the vacuolar accumulation of sodium is characteristic of salt-tolerant species (7, 8) this tonoplast Na^+/H^+ antiport may be one of the principal physiological factors conferring salt tolerance on the plant. Further work on the expression and regulation of this transport system is therefore needed. A recent report (14) shows that the marked increase in capacity for Na^+ accumulation which develops during washing of sliced storage tissue of red beet (11) is accompanied by a progressive decrease in apparent K_m of the tonoplast Na^+/H^+ antiport.

In the present work, we use sugar beet cell suspensions to investigate the regulation of antiport activity in response to extracellular Na^+, and present evidence for a specific induction of antiport activity by salt.

MATERIALS AND METHODS

Plant Material. Cell suspension cultures of sugar beet (*Beta vulgaris* L.) were initiated from callus obtained from Dr. J. W. Saunders, East Lansing, MI. Cell suspensions were maintained in 500 ml Erlenmeyer flasks containing 250 ml Murashige and Skoog medium (15) in the dark at 25°C on a rotatory shaker and subcultured weekly.

Dry Weight. Cells were collected by filtration on preweighed Whatman No. 1 filter paper and dried at 70°C for 4 h, cooled, and reweighed for dry weight.

Isolation of Tonoplast Vesicles. Cells were collected by vacuum filtration onto Whatman No. 1 filter paper on a Büchner funnel, and washed with an equal volume of distilled H_2O. Cells (50–60 g) were mixed with 150 ml homogenization medium with 0.5 mm diameter glass beads and homogenized with four pulses of 30 s each at 4°C in a Bead Beater Cell Homogenizer (Biospec Products, Bartlesville, OK). The homogenization medium consisted of 5% PVP, 0.5% BSA, 1 mM PMSF,[3] 30 mM Tris, 5 mM DDT, 5 mM EGTA, 5 mM $MgSO_4$, 0.5 mM butylated hydroxytoluene, 0.5 mM dibucaine, and 0.25 M mannitol, adjusted to pH 8.0 with H_2SO_4. The isolation was performed at 4°C throughout. The homogenate was filtered through four layers of cheesecloth and centrifuged for 20 min at 8,000*g* to remove debris and mitochondria. Pellets were discarded and the supernatants were centrifuged for 35 min at 80,000*g* in a Beckman type 35 rotor. The supernatant was aspirated and the microsomal pellet was resuspended with a Teflon pestle homogenizer in a medium containing 1.1 M glycerol, 1 mM Tris-EDTA, 0.5 mM dibucaine, 0.5 mM butylated hydroxytoluene, 3 mM DDT, 10 mM Tris-Mes (pH 7.5), and 0.15 M KI. The membranes were again sedimented at 80,000*g* for 35 min, resuspended in suspension medium without KI, and layered on sucrose gradients. For linear sucrose gradients, the KI-treated membranes were resuspended in 4 ml suspension medium and layered on a 33 ml linear gradient of 10 to 45% (w/w) sucrose in suspension medium. After centrifugation at 80,000*g* for 2 h in a Beckman SW 27.1 rotor, the gradient was divided into 22 to 24 fractions for subsequent assay.

For routine preparation of tonoplast vesicles, the KI-treated microsomal pellet was resuspended in 20 ml suspension medium, and 5 ml was layered onto each of four step gradients consisting of 16 ml 15% (w/w) sucrose layered over 16 ml 23% (w/w) sucrose in suspension medium. After centrifugation at 80,000*g* for 2 h in a Beckman SW 27.1 rotor, membranes at the 0 to 16% sucrose interface were removed with a Pasteur pipette. The membranes were diluted 10- to 20-fold with the desired experimental solution, sedimented at 80,000*g*, and resuspended in 1 ml of the same solution.

Enzyme Assays. Phosphate hydrolyzing activity was measured as the liberation of Pi according to Ames (1). ATPase activity was measured in a 0.5 ml reaction volume containing 30 mM Tris/Mes, 50 mM KCl, 1 mM Tris-ATP, 1 mM $MgSO_4$, 5 μM gramicidin D, 1 mM NaN_3, and 150 μM Na-molybdate. Nitrate-sensitive ATPase activity was measured at pH 7.8 in the presence of 50 mM KNO_3. Vanadate-sensitive ATPase activity was measured at pH 6.5 in the presence of 100 μM Na-vanadate. PPiase activity was measured in a 0.5 ml reaction volume containing 30 mM Tris/Mes (pH 7.8), 50 mM KCl, 0.3 mM PPi-Tris, 0.5 mM $MgSO_4$, 5 μM gramicidin D, and 100 μM Na-molybdate.

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[3] Abbreviations: PMSF, phenylmethyl sulfonyl fluoride; TMA, tetramethylammonium. PPiase, inorganic pyrophosphatase.

UDPase activity was measured in a 0.5 ml reaction volume containing 30 mM Tris/Mes (pH 6.5), 3 mM UDP-Tris, 3 mM $MnSO_4$ in the presence and absence of 0.03% Triton X-100. When Triton X-100 was present, Pi was measured following the precipitation of detergent with TCA and $HClO_4$ (10). Antimycin A-insensitive NADH-Cyt *c* reductase and Cyt *c* oxidase were assayed spectrophotometrically at 25°C from the absorption change at 550 nm (9).

Protein Determination. Protein was measured by a modification of the dye-binding method (6) as previously described (4).

Fluorescence Assays. The fluorescence quenching of acridine orange was used to monitor the dissipation of inside-acid pH gradients across the membranes of the tonoplast vesicles (2, 3). In all experiments, tonoplast vesicles (15 μg protein) loaded with a solution containing 250 mM mannitol, 15 mM K-gluconate, 1 mM DDT, 250 mM glycerol, and 10 mM Tris/Mes (pH 5.8) were added to 2 ml buffer containing 250 mM Mannitol, 15 mM K-gluconate, 1 mM DDT, 250 mM glycerol, 10 mM Tris/Mes (pH 8.0), and 5 μM acridine orange at 25°C. Subsequent changes in the fluorescence were monitored with a Perkin-Elmer spectrofluorimeter model LS-3 at excitation and emission wavelengths of 495 and 540 nm, respectively, and a slit width of 10 nm for both excitation and emission. During the measurement, the samples were continuously stirred. A present ΔpH was obtained by mixing tonoplast vesicles (pH_i 5.8) with a buffer solution at pH_o 8.0 ('pH jump') (3). On addition of vesicles, there was an initial quenching of fluorescence, followed by a progressive recovery of fluorescence indicating the rate of dissipation of the pH gradient (*cf.* 3, 5). The rate of change of quench is proportional to proton flux, at least when comparing initial rates of change of quench starting at the same ΔpH (2). Aliquots from stocks of 0.5 M $(TMA)_2SO_4$ or Na_2SO_4 solutions were injected during continuous fluorescence recording, and the resulting initial change in rate of fluorescence recovery was determined. To prevent pH changes in the outside buffer, all stock solutions of cations were buffered at pH 8.0. The initial rates were determined by drawing the tangents of the recorded traces obtained in the first 15 s following the addition of salts. The initial rates of dissipation of pH gradient by Na^+ were corrected for volume and/or pH changes by subtraction of those obtained by addition of TMA^+ and were expressed as rate of change in fluorescence per min (3).

RESULTS AND DISCUSSION

Effects of NaCl on Cell Growth. We have used cell suspensions derived from sugar beet to study the effects of salt on Na^+/H^+ antiport activity at the tonoplast. Cells were grown in Murashige and Skoog medium with the addition of 0, 50, 100, and 200 mM NaCl. Growth curves (0–10 d after transfer) showed very little effect of NaCl on growth rates. The increase in dry weight of the cells over a period of from 0 to 10 d after transfer to fresh medium is shown in Figure 1. Cells grown in the presence of 50 mM NaCl exhibited a growth rate similar to that of the control cells, while there was a slight increase in growth rate in the presence of 100 mM NaCl, and a slight decrease in the presence of 200 mM NaCl. Thus the tolerance of the suspension cultures to Na^+ is comparable to that of the intact plant. This material may therefore serve as a convenient model for the further investigation of salt tolerance.

Isolation of Tonoplast Vesicles. Figure 2 shows a sucrose gradient profile of the KI-treated microsomal pellet sedimented at 80,000*g*. Note that we have plotted enzyme specific activities, rather than total activities, in order to illustrate the extent of purification of various fractions. The distribution of marker enzymes on the continuous sucrose gradient showed that the two predominant enzyme activities associated with tonoplast (nitrate-sensitive, azide-insensitive ATPase; PPiase) were very well separated from the bulk of membrane protein as well as from vanadate-sensitive ATPase (marker for plasma membrane), Cyt *c* oxidase (mitochondria), latent UDPase (Golgi), and the bulk of the antimycin-insensitive NADH-Cyt *c* reductase (ER). A small fraction of the latter enzyme is thought to belong to the tonoplast (12). In the present work, a 0 to 16% sucrose step was used to isolate tonoplast vesicles, which eliminated essentially every trace of contaminating markers. Comparison of sucrose gradient profiles of marker enzymes after growth in the presence and absence of NaCl showed no significant differences (results not shown).

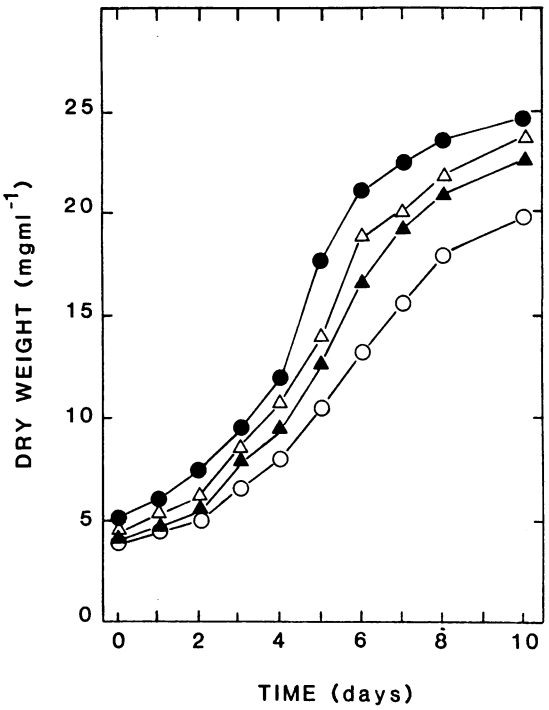

FIG. 1. Growth curves of sugar beet cell suspensions grown in increasing NaCl concentrations. Dry weight was measured as described in "Materials and Methods." (▲) control; (△) 50, (●) 100, and (○) 200 mM NaCl.

The Induction of Na^+/H^+ Antiport Activity by Salt. The effect of Na^+ on the dissipation of a transmembrane pH gradient was tested in tonoplast vesicles basically as described before (3, 5). The addition of tonoplast vesicles equilibrated at pH 5.8 to a pH 8.0 buffer (pH jump) caused quenching of acridine orange fluorescence. To avoid the generation of electrically driven H^+ movements through conductive pathways (*i.e.*a H^+ diffusion driven by a Na^+ diffusion potential), the pH jumps were generated in the presence of equimolar K^+ concentrations (15 mM) across the membrane and valinomycin, which should abolish the electrical membrane potential across the tonoplast membranes (3, 13). The quenched fluorescence was gradually restored after addition of different Na^+ concentrations. Sodium-dependent H^+ fluxes, measured as percent change of fluorescence quench (Q) per min, were recorded as described in "Materials and Methods." In the experimental range, the initial rate of H^+/Na^+ exchange (fluorescence recovery), in the presence of K^+ plus valinomycin displayed saturation kinetics with respect to extravesicular Na^+ concentrations in tonoplast vesicles isolated from sugar beet cell suspensions grown at increasing NaCl concentrations (0–200 mM) (Fig. 3). Figure 4 shows the effect of NaCl in the cell growth medium on the apparent K_m and V_{max} for Na^+/H^+ antiport in isolated tonoplast vesicles. No change was seen in apparent K_m, while the V_{max} increased with increasing Na^+ in the growth medium up to about 100 mM NaCl, reaching a maximum of about twice the

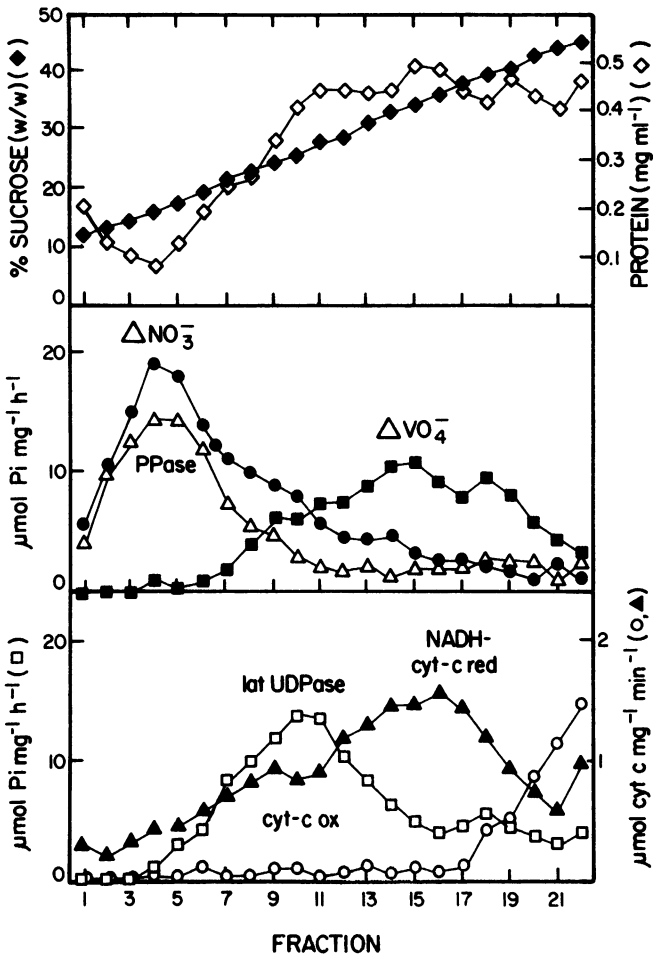

FIG. 2. Distribution of marker enzyme specific activities on a linear sucrose gradient. Marker activities were measured as described in "Materials and Methods." ΔNO^-_3 and ΔVO^-_4 refers to the difference in ATPase activity in the presence and absence of 50 mM KNO_3 and 100 μM Na-vanadate, respectively. Azide, molybdate, and gramicidin D were included throughout. (◆), % sucrose (w/w); ◇, protein; (●), ΔNO^-_3; (△), PPase; (■), ΔVO^-_4; (○), Cyt *c* oxidase; (▲), antimycin A-insensitive NADH-Cyt *c* reductase; (□), latent UDPase.

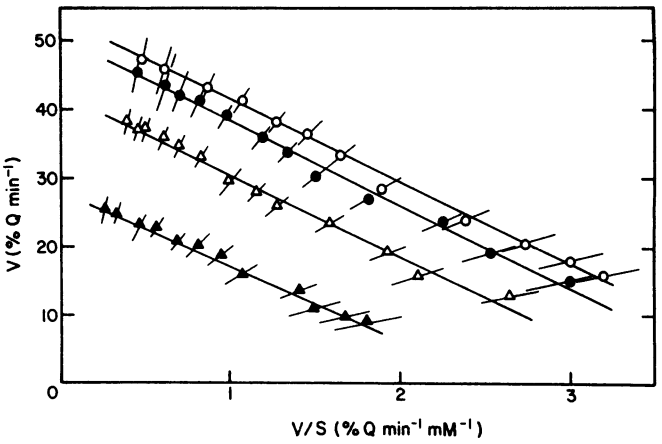

FIG. 3. Eadie-Hofstee plot of the Na^+ dependence of the rate of recovery of acridine orange fluorescence quenching after a pH jump in the presence of equimolar K^+ concentrations (15 mM) across the membrane and 1 μM valinomycin. The rate of recovery of acridine orange fluorescence quenching was measured as described in "Materials and Methods." Values are mean ± SD (n = 5). The line is that of best fit by the least square method. (▲) control cells; (△) cells grown in the presence of 50 mM, (●) 100 mM, and (○) 200 mM NaCl. Q, fluorescence quenching.

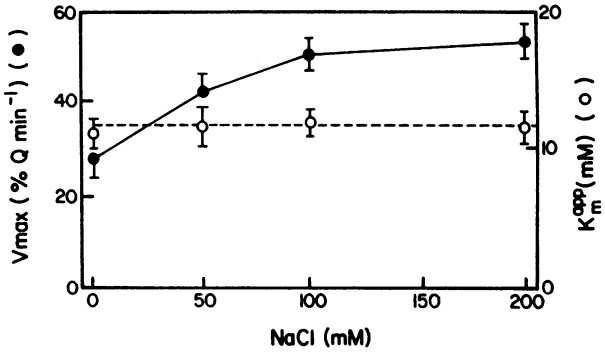

FIG. 4. Effect of NaCl in the growth medium on Na^+-dependent H^+ efflux measured in tonoplast vesicles isolated from sugar beet cell suspension cultures. The apparent K_m for Na^+ and the V_{max} for H^+ efflux were calculated from Figure 3.

control value.

An increase in V_{max} for the antiport with no change in apparent K_m suggests the addition of more antiport molecules to the tonoplast in response to NaCl in the growth medium. Whether there is, in fact, a specific induction of antiport synthesis by NaCl remains to be investigated.

Although most of the data were obtained after a few culture cycles, the effect of Na^+ on the antiport was seen in the first culture cycle, *i.e.* after no more than two cell divisions. Thus, it does not represent a selection of genetic variants. It is not likely to be an expression of changes in growth pattern, since the effects of Na^+ on cell growth are small, and are reversed at 200 mM NaCl. It is not a response to osmotic pressure, since no effect on the Na^+/H^+ antiport activity was observed when the cells were grown in the presence of 200 mM mannitol (results not shown), thus there appears to be a specific induction of antiport activity by NaCl.

In addition to the component of antiport activity dependent on Na^+, there is a component (about half the maximal activity) which remains in cells not exposed to Na^+. Halophytes grown in absence of salt are known to partially retain some characteristics related to salt tolerance, *e.g.* high intracellular ion concentrations and a certain level of synthesis of enzyme-compatible solutes such as betaine (8). The retention of a 'constitutive level' of Na^+/H^+ antiport in absence of salt may be related to this general pattern in halophytes.

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