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Modulation of Na^+/H^+ Antiporter Activity by Extreme pH and Salt in the Halotolerant Alga *Dunaliella salina*

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ABSTRACT

The effect of different growth conditions on the activity of the Na^+/H^+ antiporter in *Dunaliella salina* has been investigated. Adaptation of *D. salina* cells to ammonia at alkaline pH or to high NaCl concentrations is associated with a pronounced increase in the plasma membrane Na^+/H^+ exchange activity. The enhanced activity is manifested both in vivo, by stimulation of Na^+ influx into intact cells in response to internal acidification, and in vitro, by a larger ^{22}Na accumulation in plasma membrane vesicles in response to an induced pH gradient. Kinetic analysis shows that the stimulation does not result from a change of the K_m for Na^+ but from an increase in the V_{max}. In contrast, adaptation of cells to a high LiCl concentration (0.8 M) depresses the activity of the Na^+/H^+ antiporter. Adaptation to ammonia is also associated with a large increase of three polypeptide bands in purified plasma membrane preparations, indicating that they may compose the antiporter polypeptides. These results suggest that adaptation to ammonia or to high salinity induces overproduction of the plasma membrane Na^+/H^+ antiporter in *Dunaliella*.

The halotolerant green alga *Dunaliella salina* has an exceptional capacity to adapt to extreme pH and salt concentrations while maintaining constant intracellular pH and [Na^+]. Therefore, this alga may be expected to possess transport systems for Na^+ exclusion and internal pH regulation.

An Na^+/H^+ antiporter was previously characterized in plasma membrane preparations of *D. salina* (12, 13). The antiporter is highly specific for Na^+ and is competitively inhibited by micromolar concentrations of Li^+ and amiloride (12, 13). In vivo experiments show that the Na^+/H^+ antiporter is activated by a transient cytoplasmic acidification, indicating that it may be involved in pH homeostasis (11).

In mammalian cells, Na^+/H^+ antiporters have been extensively characterized and their role in pH homeostasis, volume regulation, and osmotic changes has been clearly established (8). In bacteria, antiporters constitute the main Na^+-extruding system (17, 20). A gene that encodes for an Na^+/H^+ antiporter in *Escherichia coli* was found to be indispensable for adaptation to high Na^+ and growth at alkaline pH (19, 26). *E. coli* mutants that overexpress an Na^+/H^+ antiporter were selected by culturing mutagenized cells in the presence of high levels of LiCl (18).

In higher plants, a plasma membrane Na^+/H^+ antiporter was described in roots of the halophyte *Atriplex numularia* (5), but it has not been well characterized. However, a vacuolar Na^+/H^+ antiporter was found and characterized in several plant species, and it was reported that an increase in salt concentration can induce an increased antiporter activity (1, 4, 7).

Under normal growth conditions, the activity of the antiporter in *D. salina* is relatively low. Because there are indications that the Na^+/H^+ antiporter in *Dunaliella* is involved in pH and internal Na^+ regulation, it seems plausible that extreme pH and ionic conditions may affect its synthesis. We attempted to adapt *Dunaliella* cells to the following extreme growth conditions: (a) Prolonged pH stress was induced by culturing cells in media containing a high NH_4Cl concentration at alkaline pH. (b) Cells were grown with LiCl to inhibit the Na^+/H^+ antiporter activity. (c) The activity of the Na^+/H^+ antiporter was examined in cells adapted to increasing NaCl concentrations.

It is demonstrated that the activity of the plasma membrane Na^+/H^+ antiporter is overproduced in cells adapted to ammonia or to high NaCl and depressed by adaptation to LiCl.

MATERIALS AND METHODS

Growth Conditions

Control Cells

Dunaliella salina were grown in media containing 1 M NaCl and 25 mM $NaHCO_3$ as previously described (10). Cultures were grown under continuous illumination at 26°C with slow continuous shaking. The pH was maintained at 7.0 by addition of gaseous CO_2 through a pH-controlled unit.

NH_3-Adapted Cells

Adaptation to NH_3 was achieved by stepwise transfers of cells to increasing concentrations of NH_4Cl (1–18 mM) at pH 8.2 (maintained with a pH stat). Otherwise, the growth medium was identical with control conditions (see above) except for omission of NO_3^-, for which NH_4^+ was substituted. Complete adaptation to 18 mM NH_4Cl took about 3 weeks.

Li^+-Adapted Cells

Control cells were gradually adapted to LiCl by a stepwise increase in the ratio of Li^+ to Na^+ in the medium. Control cells grown in 1 M NaCl were transferred to medium containing the same osmolarity but increasing concentration of LiCl

[1] Professor Avron, the motivator and inspirer of this research, died on March 29, 1991.

(starting from 0.1 M LiCl/0.9 M NaCl to 0.8 M LiCl/0.2 M NaCl). The pH was maintained at 7.0.

Salt-Adapted Cells

Dunaliella cells were adapted to media containing from 0.5 to 3 M NaCl (3, 21) for several months before the experiments were carried out.

Preparation of Plasma Membrane Vesicles

Plasma membrane vesicles were prepared from algae that had been adapted to the different growth conditions by osmotic lysis of the cells followed by differential centrifugation, as previously described (12).

Measurement of Na^+/H^+ Exchange Activity

In Plasma Membrane Vesicles

The Na^+/H^+ antiporter activity was measured by imposition of a pH gradient (interior acid) across the plasma membrane and monitoring either the Na^+-stimulated dissipation of the pH gradient with acridine orange (12) or the uptake of ^{22}Na by separation of the vesicles on Dowex-Tris columns (13).

In Whole Cells

Activation of the Na^+/H^+ exchange was induced by internal acidification using a permeable weak acid as previously described (11). Control cell cultures, as well as NH_3- and Li^+-adapted cells, were washed with 1 M NaCl medium before the experiment. Salt-adapted cells were washed in salt media identical in concentration with the growth medium. The acid load was performed at pH 5.5 with 45 mM acetate. Sodium content was measured in samples of cells that were passed through Dowex-Tris columns with a flame photometer.

Assays

Cell number and volume was measured in a model ZM Coulter Counter. Determination of ion content was according to the method of Pick et al. (21). O_2 evolution was measured with a Rank Brothers electrode. The polypeptide composition of plasma membranes was determined by SDS-PAGE using the method of Laemmli (16) with a 7 to 15% polyacrylamide gradient. Protein concentration was measured by a modified Lowry method (2).

All experiments were performed at least three times. Each experimental point represents the mean value of duplicates.

RESULTS

Growth Adaptation

Adaptation to NH_3

An attempt to induce a prolonged pH stress in *Dunaliella* was made by adaptation of cells to gradually increasing concentrations of NH_4Cl at pH 8.2. At alkaline pH, NH_3 is expected to diffuse into the cells and change the internal pH (14). Indeed, in previous work, cytoplasmic alkalinization was observed after addition of NH_4Cl to *Dunaliella* cells (22). Figure 1 shows the growth rate of *Dunaliella* cells that were adapted stepwise to 18 mM NH_4Cl. Control cells, when transferred directly to this medium, stopped growing after 1 d. When NH_3-adapted cells were transferred back to the control growth conditions for several generations, they lost their ability to grow in 18 mM NH_4Cl (data not shown), implying that the adaptation does not reflect a selection of genetic variants. The volume of NH_3-adapted cells was found to be higher than the volume of control cells, but the ion content was identical (Table I).

The growth of NH_3-adapted cells was critically dependent on the external pH, having an upper limit of pH 8.2 (Fig. 2). The strong pH dependence of the growth of NH_3-adapted cells is consistent with an alkaline stress induced by diffusion of NH_3 into the cells. Above pH 8, a substantial proportion of the amine exists as a free base (pK 9.2), and its rate of diffusion into the cells probably exceeds their capacity to cope with the internal alkalinization.

Adaptation to Li^+

The inhibition of the Na^+/H^+ antiporter activity in *Dunaliella* cells growing in media containing high LiCl concentrations is expected to perturb the regulation of the internal pH or $[Na^+]$. In view of the report that *E. coli* mutants, selected for resistance to high LiCl concentration, overexpress the

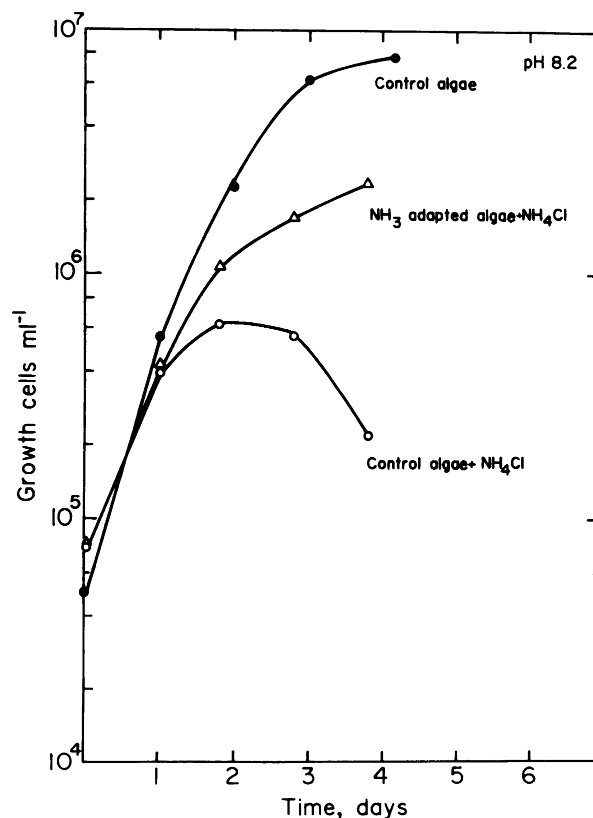

Figure 1. Growth rate of NH_3-adapted and control cells. ●, Control medium; ○ and △, 18 mM NH_4Cl medium.

Table I. *Volume and Ion Content of Control NH_3-Adapted and Li^+-Adapted Algae*

Algae	Volume	$[Na^+]_{in}$	$[Li^+]_{in}$
	fL	*mM*	*mM*
Control	95	20	
NH_3 adapted	140	25	
Li^+ adapted	100	8	35

Na^+/H^+ antiporter (18), we expected that *Dunaliella* cells grown on high LiCl would be enriched with Na^+/H^+ antiporter.

Previous reports showed that *Dunaliella* cells were unable to grow in an Na^+-depleted medium composed exclusively of LiCl (6, 9). Because Li^+ is a competitive inhibitor of the Na^+/H^+ antiporter, we had to increase the concentration of Li^+ to greater than the concentration of Na^+ in the growth medium. As the Li^+/Na^+ concentration ratio was gradually increased, *Dunaliella* cells adapted to grow in a medium containing 0.8 M LiCl and 0.2 M NaCl (Fig. 3). Unadapted cells were unable to grow in this medium. The cell volume of Li^+-adapted and control algae are similar (Table I). Li^+-adapted cells contain low Na^+ (8 mM) and a substantial amount of Li^+ (35 mM), with an Li^+/Na^+ ratio similar to that in the extracellular medium.

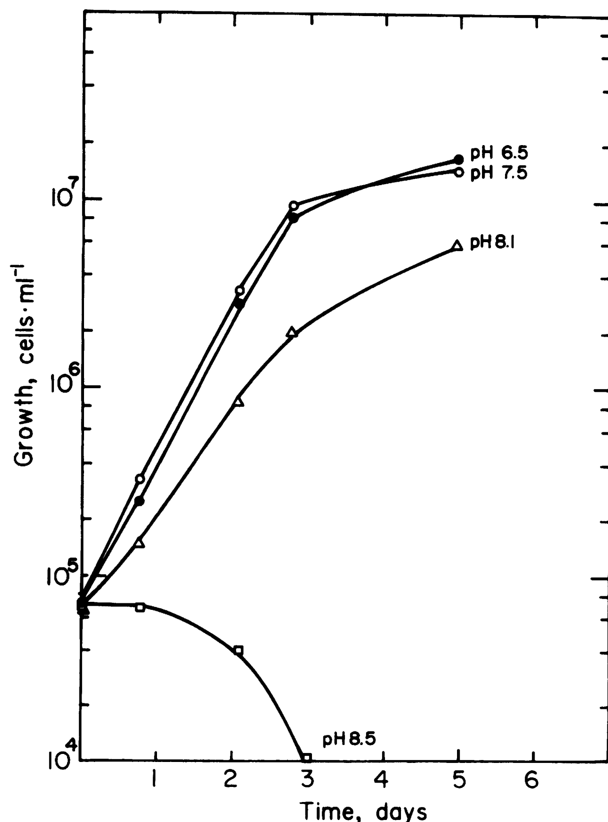

Figure 2. Growth rate of NH_3-adapted cells as a function of the external pH in medium containing 18 mM NH_4Cl.

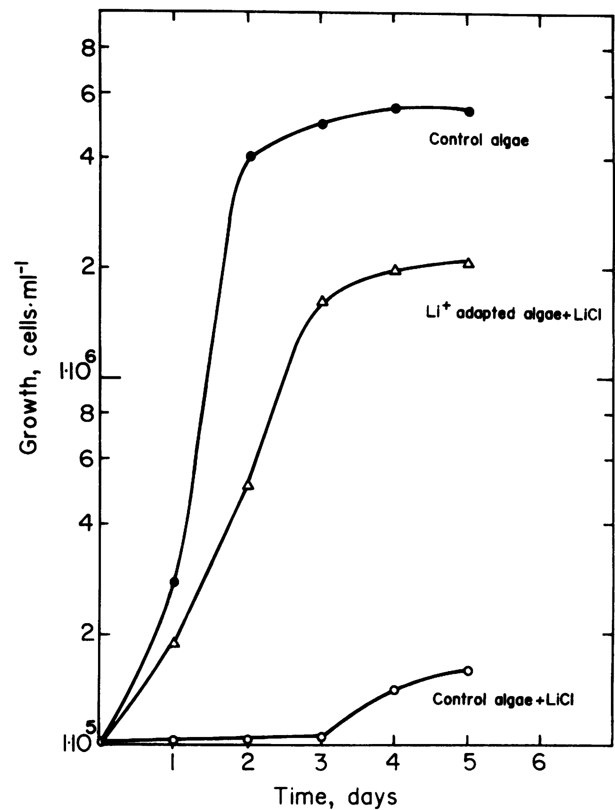

Figure 3. Growth rate of Li^+-adapted cells and control cells. ●, Control medium; ○ and △, 0.8 M LiCl + 0.2 M NaCl medium.

Salt Adaptation

Previously, it was shown that *Dunaliella* cells could be adapted to a wide range of NaCl concentrations (0.1–5.5 M) without significant effects on the growth rate, the internal Na^+ concentration, or cell volume (3, 21).

Na^+/H^+ Antiporter Activity in Intact Cells

We recently demonstrated that it is possible to measure the Na^+/H^+ antiporter activity in vivo by artificially inducing internal acidification using a weak acid, acetate, at pH 5.5 (11). The internal increase in Na^+ content was taken as a measure of the activity of the Na^+/H^+ antiporter (11). Figure 4A shows that both the rate and the extent of Na^+ influx in the NH_3-adapted cells is much higher than in control cells. The increase in Na^+ content is completely dependent upon internal acidification and can be inhibited by Li^+, indicating that it reflects Na^+/H^+ antiporter activity. Conversely, the activity of the Na^+/H^+ antiporter in Li^+-adapted cells is lower than in control cells (Fig. 4B). Figure 5 shows the activity of the antiporter in whole cells adapted to different NaCl concentrations. The antiporter activity increases proportionally with the increase in the NaCl concentration in the medium.

Na^+/H^+ Antiporter Activity in Plasma Membrane Vesicles

Figure 6 shows the activity of the Na^+/H^+ antiporter in plasma membrane vesicles prepared from the different

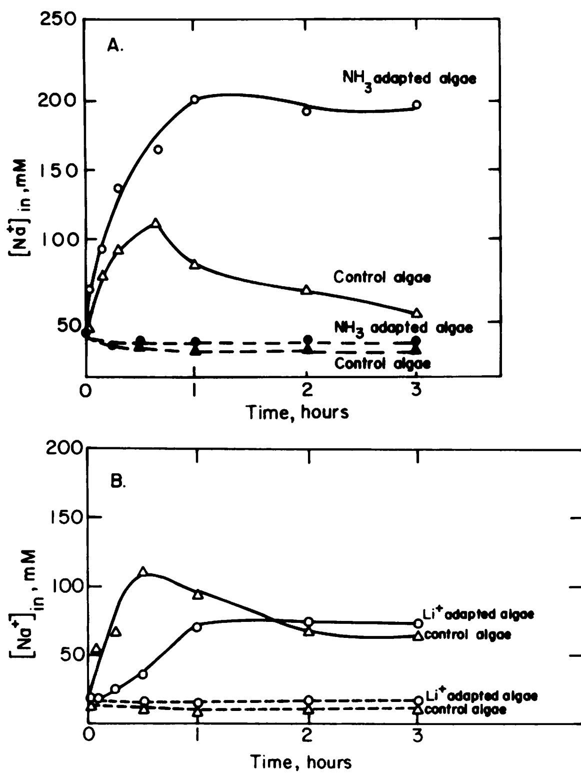

Figure 4. Na^+/H^+ antiporter activity in whole cells. A, NH_3-adapted cells; B, Li^+-adapted cells. Internal Na^+ concentration is measured as function of time from the acidification by acetate (see "Materials and Methods"). ——, + Acetate; - - - - - , − acetate. The cells were washed and resuspended in 1 M NaCl medium before the acidification.

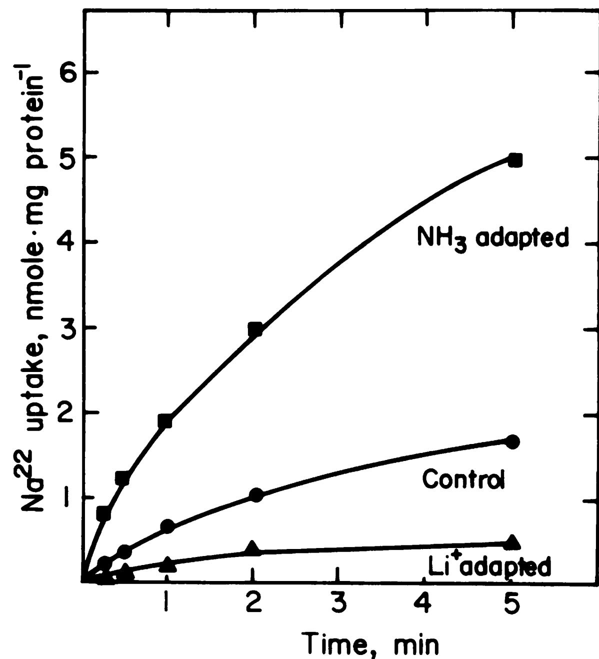

Figure 6. Na^+/H^+ antiporter activity in plasma membrane vesicles. Plasma membrane vesicles prepared from cells: ■, NH_3 adapted; ●, control; ▲, Li^+ adapted. Na^+ uptake was measured by accumulation of ^{22}Na in response to an artificially induced pH gradient as described in "Materials and Methods."

adapted cells. The activity in NH_3-adapted vesicles is about 5 times higher than in the control vesicles. Li^+-adapted vesicles show a decrease in the activity of the antiporter with respect to control vesicles. The activity of the Na^+/H^+ antiporter in the salt-adapted plasma membrane is shown in Figure 7. The activity in vesicles isolated from cells grown in

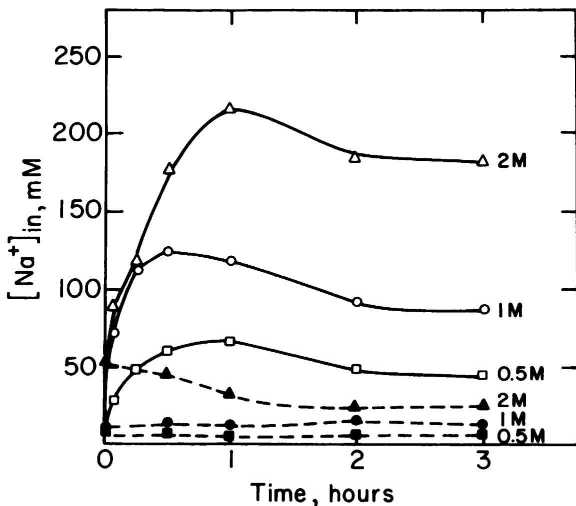

Figure 5. Na^+/H^+ antiporter activity in intact cells adapted to 0.5, 1, or 2 M NaCl. The acidification was performed in media containing the same NaCl as in the growth media. ——, + Acetate; - - - - - , − acetate.

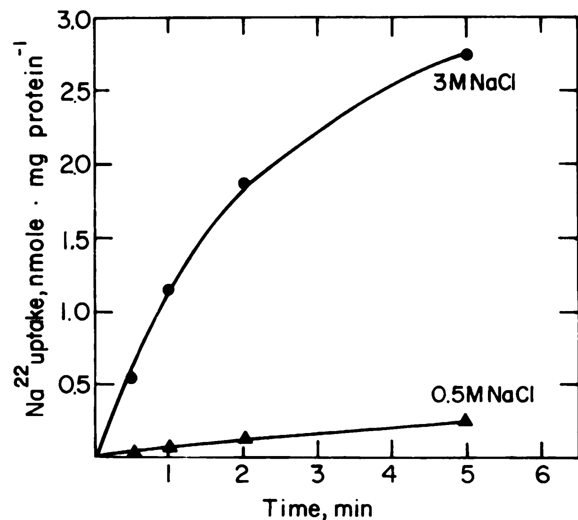

Figure 7. Na^+/H^+ antiporter activity in vesicles prepared from cells adapted to 0.5 M NaCl (▲) and 3 M NaCl (●).

3 M NaCl is 10 times higher than in vesicles isolated from cells grown in 0.5 M NaCl.

To examine whether the changes in the antiporter activity are due to changes in affinity for Na^+ or to changes in the amount of the antiporter, we performed a kinetic analysis of Na^+/H^+ activity as a function of Na^+ concentration (Fig. 8).

The apparent K_m for Na^+ (15 mM) does not change in the NH_3- or Li^+-adapted vesicles, in comparison with control vesicles. However, there is an increase of the V_{max} in the NH_3-adapted vesicles and a decrease of V_{max} in the Li^+-adapted vesicles. This indicates an increase or decrease in the amount of the Na^+/H^+ antiporter in NH_3- or Li^+-adapted cells, respectively. Similarly, the change in the activity of the Na^+/H^+ antiporter in the salt-adapted vesicles is due to a change in the V_{max} and not in the K_m (Fig. 8).

The conclusion that the different adaptations affect only the level and not the properties of the antiporter is further supported by inhibition studies with amiloride and Li^+. We found that $^{22}Na^+$ uptake is inhibited by Li^+ or amiloride and that the K_i values (30 μM) for inhibition are similar to the values in control vesicles (data not shown).

It was important to show that the low activity of Na^+/H^+ antiporter in the Li^+-adapted cells is not due to an Li^+/H^+ exchange. However, we found that internal acidification of Li^+-adapted cells in an Li^+-containing medium does not result in Li^+ uptake, indicating that the antiporter does not transport Li^+ (data not shown).

The polypeptide analysis of the different plasma membrane preparations is illustrated in Figure 9. Three polypeptide bands increase markedly in the NH_3-adapted vesicles (lane 3, arrows) in comparison with the control vesicles (lane 1). Several other polypeptides are either increased or depressed in the Li^+-adapted plasma membrane vesicles.

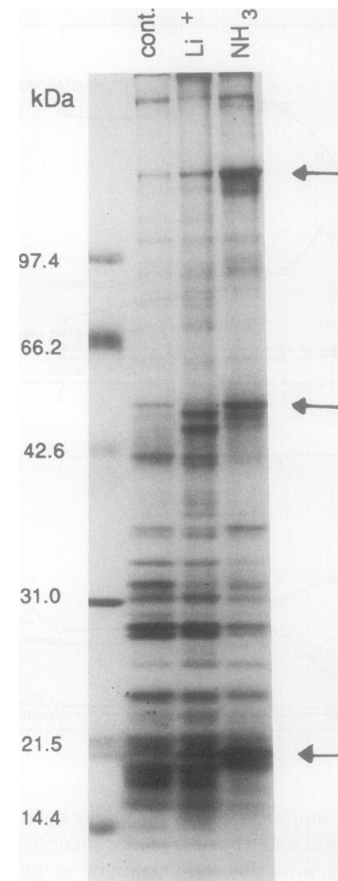

Figure 9. SDS-PAGE analysis of polypeptide composition of plasma membrane vesicles stained with Coomassie blue. Each lane contains 40 mg of protein. Lane 1, Control vesicles; lane 2, Li^+-adapted vesicles; lane 3, NH_3-adapted vesicles.

DISCUSSION

NH_3-adapted cells show an increased activity of the Na^+/H^+ antiporter in whole cells and in plasma membrane vesicles. The kinetic evidence indicates an increase in the maximal transport velocity with no change in apparent K_m. This is consistent with either increased turnover of individual transporters or an elevated number of antiporters. The prolonged adaptation period that was required and the observation that it is associated with an increase of certain plasma membrane proteins suggest that the increase in exchange activity results from overproduction of Na^+/H^+ antiporter.

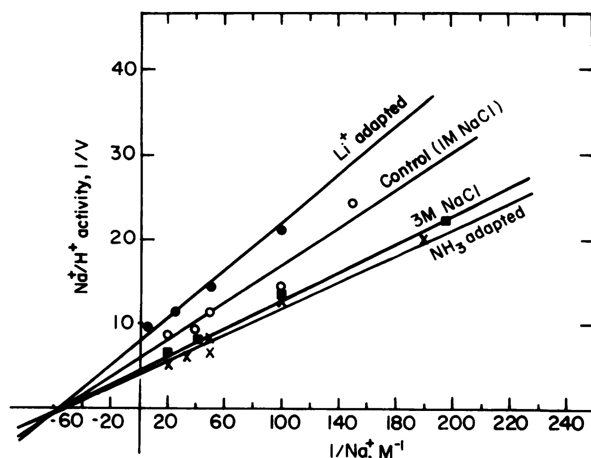

Figure 8. Kinetic analysis of the Na^+/H^+ antiporter activity in vesicles as a function of Na^+ concentration. The data are plotted by the method of Lineweaver and Burk. Na^+/H^+ antiporter activity was measured by the Na^+-stimulated ΔpH decay with acridine orange expressed in relative units as described earlier (see "Materials and Methods" and ref. 12).

The conclusion that NH_3, which induces a pH stress, leads to overproduction of the plasma membrane Na^+/H^+ antiporter in *Dunaliella* is consistent with previous observations in bacteria (15, 19, 26). It was demonstrated that disruption of the Na^+/H^+ antiporter gene in bacteria leads to failure of growth at alkaline pH, particularly in high NaCl media (15, 19, 26). It is surprising that Li^+ adaptation, which was expected to induce an increase in the Na^+/H^+ antiporter activity, resulted in a decrease. In variance with the bacterial system, in *Dunaliella*, Li^+ is an efficient competitive inhibitor of the Na^+/H^+ antiporter and not a substrate, (18). Therefore, excess of toxic cytoplasmic Li^+ cannot be eliminated by the antiporter in *Dunaliella*, which may explain the absence of overproduction of the antiporter in this alga.

It may be noted that, although Li^+-adapted algae have

normal growth rates and photosynthetic and respiratory activities, they are unable to grow at external alkaline pH (8.0–9.5). This defect could be due to reduced activity of the Na^+/H^+ antiporter, consistent with the predicted role in pH homeostasis.

The finding that adaptation to high salinity is associated with an increased Na^+/H^+ antiporter activity, observed both in intact cells and in vesicles, suggests that the expression of the Na^+/H^+ antiporter is also regulated by salt. Whether this is a response to an increase in the extracellular Na^+ or to salt-induced secondary changes is not known at present. It has been reported that exposure of a plant to salt induces an increase in the synthesis of a 170-kD polypeptide in correlation with elevated activity of vacuolar Na^+/H^+ antiport (1), which is probably associated with compartmentation of Na^+ into the vacuole. However, the rationale for this analogy is not clear, because in *Dunaliella* acidic vacuoles have a limited capacity for Na^+ (24), and Na^+ ions that enter the cells are eliminated through the plasma membrane.

The polypeptide composition of the Na^+/H^+ antiporter is still unknown. Three plasma membrane polypeptides of 20, 50, and 150 kD are highly elevated in the NH_3-adapted vesicles in comparison with control vesicles, and it is conceivable that they may be associated with Na^+/H^+ exchange. A similar difference in the polypeptide pattern of plasma membrane from salt-adapted *Dunaliella* cells was shown recently (23). A 150-kD membrane protein in *Dunaliella* was found to be induced by high salt and correlated with adaptation of cells to growth in saline media (23). However, because plasma membranes from Li^+-adapted cells, which have reduced Na^+/H^+ antiport activity, also show a slight increase in this band, its direct correlation with Na^+/H^+ antiport is still unclear. There are two other indications of a possible correlation between the 20- and 50-kD polypeptides and Na^+/H^+ antiport: (a) Partially purified Na^+/H^+ antiporter preparations, extracted by Triton X-100 from *D. salina* vesicles, were found to be enriched in two polypeptides of similar molecular mass (13); (b) in preliminary studies, we found that two similar polypeptides in *D. salina* vesicles are labeled with a photoreactive amiloride analog. It is likely, therefore, that the 20- and 50-kD polypeptides are part of the Na^+/H^+ antiporter. It is hoped that the possibility of inducing overproduction of the Na^+/H^+ antiporter in *Dunaliella* plasma membrane will enable its purification and characterization.

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