

Amine Accumulation in Acidic Vacuoles Protects the Halotolerant Alga *Dunaliella salina* Against Alkaline Stress

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ABSTRACT

Amines at alkaline pH induce in cells of the halotolerant alga *Dunaliella* a transient stress that is manifested by a drop in ATP and an increase of cytoplasmic pH. As much as 300 millimolar NH_4^+ are taken up by the cells at pH 9. The uptake is not associated with gross changes in volume and is accompanied by K^+ efflux. Most of the amine is not metabolized, and can be released by external acidification. Recovery of the cells from the amine-induced stress occurs within 30 to 60 minutes and is accompanied by massive swelling of vacuoles and by release of the fluorescent dye atebtrin from these vacuoles, suggesting that amines are compartmentalized into acidic vacuoles. The time course of ammonia uptake into *Dunaliella* cells is biphasic—a rapid influx, associated with cytoplasmic alkalization, followed by a temperature-dependent slow uptake phase, which is correlated with recovery of cellular ATP and cytoplasmic pH. The dependence of amine uptake on external pH indicates that it diffuses into the cells in the free amine form. Studies with lysed cell preparations, in which vacuoles become exposed but retain their capacity to accumulate amines, indicate that the permeability of the vacuolar membrane to amines is much higher than that of the plasma membrane. The results can be rationalized by assuming that the initial amine accumulation, which leads to rapid vacuolar alkalization, activates metabolic reactions that further increase the capacity of the vacuoles to sequester most of the amine from the cytoplasm. The results indicate that acidic vacuoles in *Dunaliella* serve as a high-capacity buffering system for amines, and as a safeguard against cytoplasmic alkalization and uncoupling of photosynthesis.

Ammonia is a common source of nitrogen for plants, algae, and cyanobacteria. It was demonstrated that in most photosynthetic eucaryotes, ammonia is assimilated primarily through the glutamine synthetase/glutamate synthetase in the chloroplast (11). Green algae possess two glutamine synthetase isoenzymes, one in the chloroplast and another in the cytoplasm (4), and assimilate ammonia very efficiently also in the dark (20).

However, ammonia or ammonium ions have several physiological side effects. Excess of ammonia in the chloroplast may lead to uncoupling of photophosphorylation, a drop in ATP production, and inhibition of CO_2 fixation (8, 19). Ammonia may also affect intracellular pH in various ways. The assimilation of ammonia to glutamine involves release of protons and acidification, which necessitates extrusion of excess protons from the cell. The influx of ammonia into the cell may produce rapid cytoplasmic pH changes, whose direc-

tion depends on the mechanism of transport. Diffusion of NH_3 into the cells, which is favored at alkaline pH, will induce cytoplasmic alkalization due to internal protonation of NH_3 , whereas transport of NH_4^+ into the cells may produce acidification due to dissociation of H^+ resulting from assimilation of NH_3 or its diffusion into acidic vacuoles (7). Indeed, several previous works reported cytoplasmic alkalization or acidification resulting from addition of amines to plants and algae (3, 9, 12, 13, 17). There are also good indications for the existence of a specific high affinity uniport mechanism for NH_4^+ uptake in plants and algae as well as for free diffusion of NH_3 into cells at alkaline pH (reviewed in ref. 7). It is not clear how photosynthetic cells regulate intracellular pH and avoid uncoupling of photosynthesis during amine uptake and metabolism; however, there are indications for accumulation of amines in acidic vacuoles from observations of vacuolar pH changes during amine accumulation (3, 12, 13), which may suggest that acidic vacuoles function as amine buffering systems.

In a preliminary recent work, we have reported that influx of ammonia into cells of the halotolerant alga *Dunaliella salina* induces a rapid alkalization of the cytoplasm, followed by recovery of the cytoplasmic pH, which occurs in parallel with massive hydrolysis of polyphosphates (15). However, the mechanism of pH recovery, and the localization of the amine in the cell, have not been elucidated. In the present study, we have focused on the role of acidic vacuoles in the compartmentalization of amines. We utilize the fluorescent amine 6-chloro-9-[4(diethylamino)-1-methylbutylamino]-2-methoxy acridine (atebtrin), which accumulates in acidic vacuoles of *D. salina* (22), as a marker to follow amine uptake into the vacuoles and also compare ammonia (pK 9.25) with two nonmetabolizable amines (benzylamine, pK 9.35; triethylamine, pK 10.9) to ensure that the response of the cells is not specific to ammonia. It is demonstrated that the recovery of the cells results from compartmentation of the amine into acidic vacuoles. In a subsequent work, it will be demonstrated that the capacity of the cells to accumulate amines depends on hydrolysis of polyphosphates in these vacuoles.

MATERIALS AND METHODS

Culture

Dunaliella salina cells were grown in batch cultures in an illuminated New Brunswick Psychotherm Incubator in 0.5 M NaCl medium as previously described (2). For all the experiments, cells were pelleted by centrifugation (2000 rpm \times 10) and resuspended in incubation medium containing 0.5 M

NaCl, 5 mM MgCl₂, 5 mM KCl, and 20 mM Tris-Cl, pH 9. Cell number and cell volume were determined in a Coulter counter.

Preparation of Lysed Cells

Concentrated *D. salina* cells (3×10^7 – 10^8 cells/mL) were lysed by two treatments in a Yeda-press pressure cell at 300 and 100 p.s.i. at 4°C, and assayed within 1 h after lysis.

Atebrin Fluorescence

Cells were preloaded with 3 μ M atebrin for 20 min at 24°C in incubation medium, centrifuged, and resuspended in fresh incubation medium. Atebrin fluorescence was measured in a Perkin-Elmer MPF-44A spectrofluorimeter with the excitation and emission wavelengths set at 359 and 505 nm, respectively (22).

NMR Measurements

Intracellular pH of *D. salina* cells was estimated from the Pi peak resonance position of ³¹P-NMR spectra as described in ref. 16.

NH₄⁺ and K⁺ Content

Uptake of ammonium was measured as follows. Concentrated *D. salina* cells (2×10^8 cells/mL) were preincubated for 20 to 30 min before the addition of ammonia in incubation medium in the dark. After the addition of 20 mM NH₄Cl, samples of 200 μ L were applied to 0.4 mL Eppendorf microfuge tubes containing 100 μ L silicon oil (AP-100, Wacker) and cells were separated by centrifugation for 45" at 12,000g as previously described (5). Ammonium content was determined in 3% TCA extracts with a Nessler reagent (1). K⁺ content was determined on the same samples in a flame photometer, with reference to standard K⁺ solutions.

Electron Microscopic Techniques

For electron microscopy, cell cultures were fixed on ice for 10 h in 0.5 M NaCl medium containing 1.5% glutaraldehyde and trapped in 3.4% agarose. The preparation was postfixed with 1% osmium tetroxide and stained with 2% uranyl acetate. After dehydration, samples were embedded in Epon, sectioned, and stained with 0.4% lead citrate and 2% uranyl acetate. The samples were observed in a Philips 410 transmission electron microscope, operating at 80 kV.

Other Analytical Techniques

ATP concentration was determined in frozen cell samples containing 2 to 4 $\times 10^7$ cells with the luciferase assay in a Lumac 3M Biocounter.

RESULTS

Amine Induced Transient Stress in *Dunaliella*

Amines at alkaline pH (8–10) induce in *D. salina* cells a transient stress that is manifested by reduced motility, inhi-

bition of O₂ evolution, and a decrease in cellular ATP level (Fig. 1B). All three tested amines induce a similar transient drop in ATP. However, within 30 to 60 min, the cells recover their motility and photosynthetic capacity, and their ATP level is restored. In order to eliminate effects of amines that result from inhibition of photosynthesis, all the experiments described below were carried out in the dark.

A possible reason for the amine-induced stress is intracellular alkalinization. To test this possibility, the intracellular pH in *D. salina* cells was measured with ³¹P-NMR by following the shift in the peak resonance position of Pi. For this purpose, cells were immobilized in agarose beads and perfused with a high-oxygen medium, as previously described (2). These measurements were carried out at 10°C in order to slow down intracellular pH changes to enable a more accurate measurement. At this temperature, the level of intracellular ATP is only slightly decreased and is similarly affected by amines except for the slower, or absence of, recovery with ammonia or BzNH₂, respectively (Fig. 1B). The rates of O₂ uptake in the dark and of oxygen evolution in the light are slower by three- to fivefold in comparison with the rates at 23°C. As is demonstrated in Figure 1A, addition of either ammonia, BzNH₂,¹ or TEA induces internal alkalinization. Comparison of Figure 1A and B shows (a) that the extent of

¹ Abbreviations: BzNH₂, benzylamine; TEA, triethylamine.

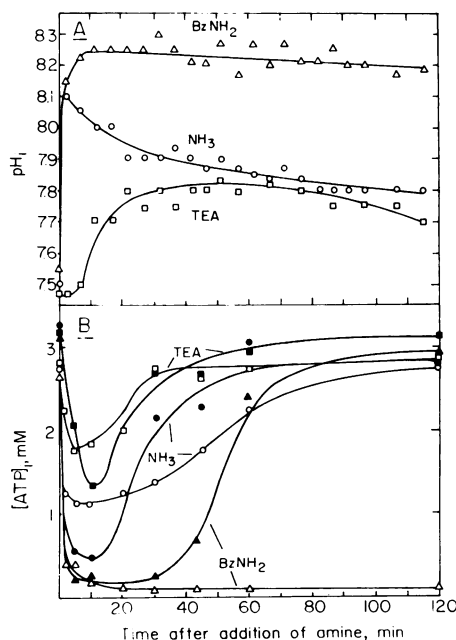


Figure 1. Effect of amines on intracellular pH and ATP in *Dunaliella*. Intracellular pH (A) of *D. salina* cells trapped in agarose beads was measured at 10°C by ³¹P-NMR as described in "Materials and Methods." ATP measurements (B) were carried out at 10°C (open symbols) or at 23°C (solid symbols) following 30 min of preincubation at pH 9 (5×10^8 cells/mL) in the dark. After addition of 20 mM NH₄Cl or 20 mM BzNH₂Cl or 50 mM TEA-Cl, samples of 50 μ L were frozen in liquid nitrogen at the indicated times and analyzed for ATP by the luciferase assay.

pH rise is correlated with the extent of ATP drop ($\text{BzNH}_2 > \text{NH}_3 > \text{TEA}$) and (b) that the kinetics of recovery of pH with ammonia and the absence of recovery with BzNH_2 resemble the changes in cellular ATP. With TEA, the recovery of cellular ATP is not correlated with the recovery of pH. However, it may be noted that, whereas the recovery in ATP level for NH_3 and TEA is complete, pH approaches a new steady-state about 0.3 units higher than the original value. Therefore, although these two parameters are not fully correlated, these results suggest that the transient amine-induced stress results from internal alkalinization. It also suggests that the cells have the capacity to counterbalance internal alkalinization by a process that depends on metabolic energy.

Is Ammonia Assimilation Involved in Recovery from Stress?

The recovery of the cells from the amine induced stress may result either from elimination of the amine, from internal compartmentation, or from metabolic assimilation. Because green algae have the capacity to assimilate ammonia in the dark, it seemed possible that recovery of the cells from stress may be due to assimilation of ammonia. To test this possibility, ammonium content of the cells was determined. As is demonstrated in Figure 2, ammonium uptake at pH 9 is biphasic: a rapid phase, completed within 1 min, followed by a temperature-dependent slower phase. The calculated average intracellular ammonium concentration reaches approximately 300 mM. To test whether ammonia accumulates inside the cells or is assimilated, cells were preloaded for 1 h and the external amine was washed away. As is demonstrated in Figure 3, at pH 9 there is a slow decrease of about 30% of the amine content of the cells. Illumination of loaded cells for 4 h at pH 9 does not lead to a further decrease of ammonia content (not shown). At pH 6, there is a rapid loss of about two-thirds of the amine, which is correlated with its appearance in the external medium. This rapid efflux seems to result from inversion of the pH gradient between the cytoplasm and

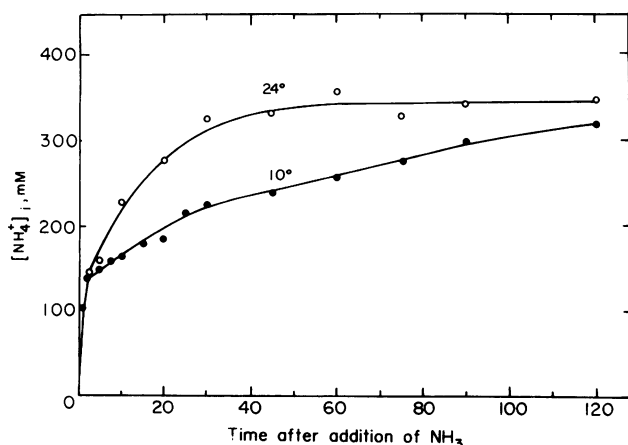


Figure 2. Time course of NH_4^+ uptake. *D. salina* cells were incubated at 24 or 10°C at pH 9 with 20 mM NH_4Cl . At the indicated times, cell samples were separated through silicon oil and their ammonium content was determined as described in "Materials and Methods."

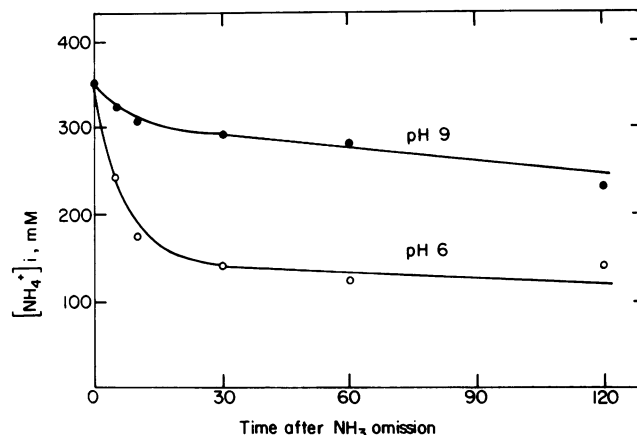


Figure 3. Efflux of NH_4^+ from loaded *D. salina*. *D. salina* cells were preincubated for 1 h with 20 mM NH_4Cl at 24°C at pH 9, centrifuged, and resuspended in fresh incubation medium buffered to pH 9 or pH 6. At the indicated times, samples of cells were analyzed for NH_4^+ content as in Figure 2.

external medium, and establishment of a new equilibrium between the external, cytoplasmic, and vacuolar compartments (see below). The experiment demonstrates that within the time of the experiment most of the ammonia is not metabolized inside the cells. It can be concluded that the recovery of *Dunaliella* from amine-induced stress does not result from elimination from the cell or from metabolic assimilation.

Effects of Amines on Cell Volume and on K^+ Content

In view of the high content of accumulated ammonia, reaching an average concentration of about 300 mM (Fig. 2), it may be expected that the amine accumulation should lead to a significant swelling of the cells. However, volume measurements indicated that within 1 to 2 h, ammonia induces only a minor change in volume (less than 5%, Fig. 4A), whereas TEA and benzylamine lead to a minor swelling of about 10% (not shown). Because the two major intracellular osmotic components in *D. salina* are glycerol and K^+ , it seemed possible that the minor volume change involved in amine uptake may be due to a parallel loss of either glycerol or K^+ . Examination of the glycerol content during amine uptake revealed no losses of glycerol (not shown). However, ammonia, and to a lesser extent TEA and benzylamine, induce a significant K^+ efflux amounting to 30 to 50% of the cellular K^+ content. A parallel analysis of NH_4^+ uptake and K^+ efflux at two external pH values demonstrates a clear kinetic and quantitative correlation between loss of K^+ and NH_4^+ uptake at pH 7, whereas at pH 9, the extent of NH_4^+ uptake is significantly greater than the loss of K^+ , mainly due to a rapid initial phase of NH_4^+ uptake, which is not correlated with K^+ efflux (Fig. 4B, C). These results indicate a possible linkage between NH_4^+ uptake and K^+ efflux.

Involvement of Acidic Vacuoles in Amine Uptake

Electron micrographs of *D. salina* cells before and after incubation with NH_4Cl manifest a major enlargement of

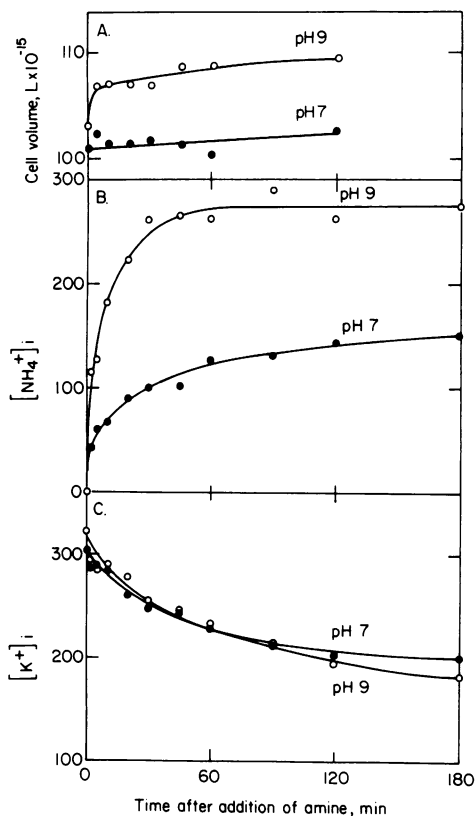


Figure 4. Correlation between NH_4^+ influx, K^+ efflux, and cell volume. *D. salina* cell volumes (A) after addition of 20 mM NH_4Cl , at pH 7 (solid) or pH 9 (open) at 24°C, were measured in a Coulter counter and expressed in fL/cell. Ammonium (B) and K^+ content (C) were determined under the same conditions in cell samples separated through silicon oil.

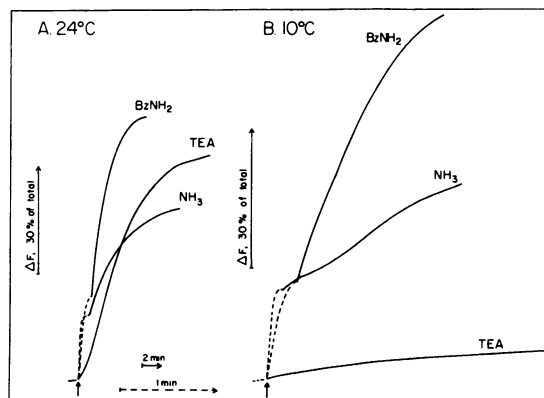


Figure 6. Time course of atebtrin fluorescence changes by three amines. Ammonium Cl (20 mM), BzNH_2Cl (20 mM), or TEA-Cl (50 mM) were added to atebtrin-labeled cells (arrows) and the atebtrin fluorescence changes were recorded as described in "Materials and Methods." Note that broken lines indicate a faster time scale.

vacuolar structures (compare C, D with A, B in Fig. 5). A prolonged adaptation of *D. salina* cells to ammonia at alkaline pH (16 mM NH_4Cl , pH 8.5) also induces a large increase in the number and size of vacuoles, which literally occupy most of the cytoplasm of the cells (Fig. 5E, F). These results indicate that high concentrations of ammonium ions accumulate in the vacuoles and lead to their osmotic swelling.

We have recently demonstrated that the fluorescent amine atebtrin accumulates in acidic vacuoles of *Dunaliella* and can be used as a specific indicator for these organelles (22). We have also demonstrated that amines induce the release of atebtrin from the cells at alkaline pH, suggesting that the amines also accumulate in acidic vacuoles. As is demonstrated in Figure 6 all three amines induce an atebtrin fluorescence enhancement when added to atebtrin-loaded cells, indicative

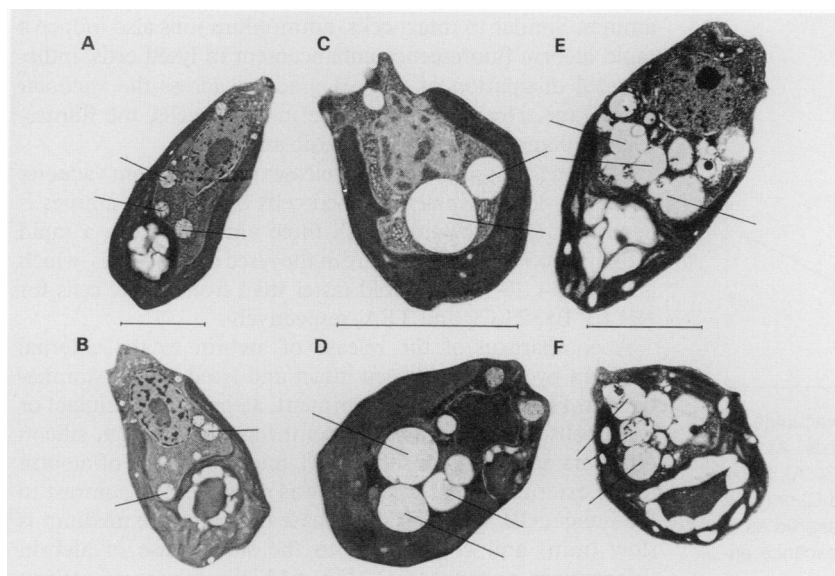


Figure 5. Effect of ammonia on the morphology of *Dunaliella*. Electron micrographs of control cells (A, B), cells loaded for 1 h with 20 mM NH_4Cl (C, D), or cells adapted to 16 mM NH_4Cl for several weeks (E, F) were obtained as described in "Materials and Methods." Representative vacuoles are indicated. Bars = 5 μm .

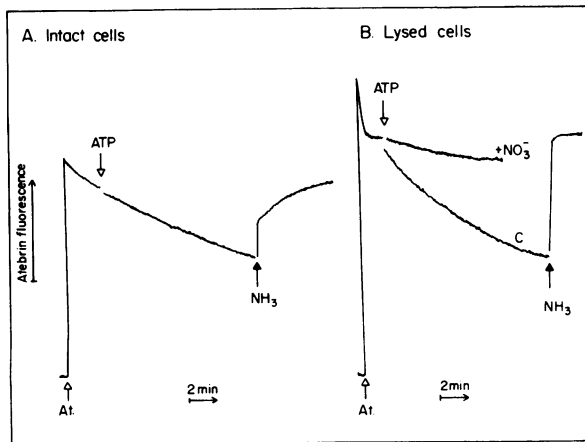


Figure 7. ATP-dependent uptake of atebtrin into acidic vacuoles in *D. salina* lysed cells. Intact (A) or lysed (B) *D. salina* cells were incubated in buffered suspension medium at pH 8. Additions of 3 μ M atebtrin (At.), 0.5 mM ATP, or 20 mM NH_4Cl are indicated by arrows. C, control; $+\text{NO}_3^-$, cells to which 25 mM NaNO_3 was added before ATP.

for release of the dye from the vacuoles. Ammonia and benzylamine induce a biphasic fluorescence enhancement that was already reported in our previous paper. We suggested that the rapid phase reflects release of atebtrin from the vacuoles to the cytoplasm because (a) fluorescence microscopy reveals redistribution of atebtrin within the cell shortly after addition of amines and (b) the initial fluorescence enhancement is not correlated with release of dye from cells (22, see also Fig. 9). The temperature-dependent slower phase presumably reflects release of the dye from the cells because it is kinetically correlated with the appearance of atebtrin in the external medium (22). TEA induces a slow fluorescence enhancement, which is preceded by a lag period and is strongly temperature dependent. These results are well correlated with

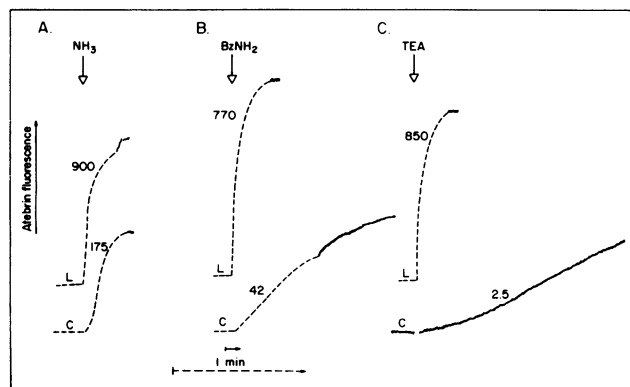


Figure 8. Comparison of the release of atebtrin from vacuoles by different amines between lysed and intact *D. salina* cells. Atebtrin fluorescence changes induced by addition of 5 mM NH_4Cl (A), 5 mM BzNH_3Cl (B), or 20 mM TEA-Cl (C) to intact (trace labeled C) or lysed (trace labeled L) atebtrin-loaded *D. salina* cells were measured as in Figure 7. Numbers indicate initial rates of atebtrin fluorescence enhancement in relative fluorescence units per min.

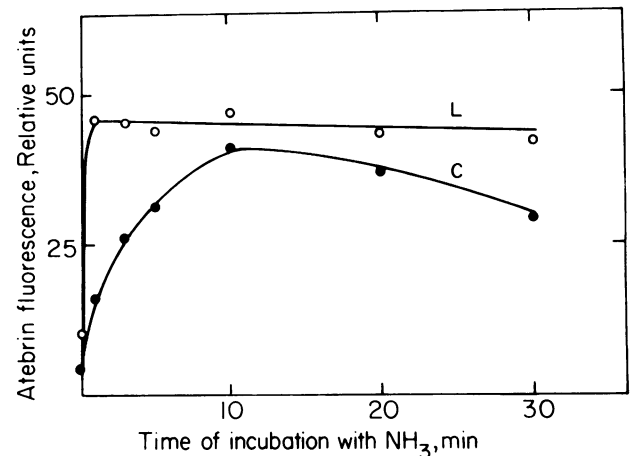


Figure 9. Comparison of atebtrin release to the medium from intact and lysed *D. salina* cells by NH_4Cl . Atebtrin-loaded intact (C) or lysed (L) cells were separated by centrifugation through silicon-oil, after the addition of 20 mM NH_4Cl , and the amount of atebtrin released to the upper medium was determined from atebtrin fluorescence.

the effects of ammonia, BzNH_2 , and TEA on intracellular pH and ATP content (Fig. 1) and indicate that the accumulation of amines in acidic vacuoles is associated with intracellular pH changes.

To understand the complex kinetics of the amine-induced atebtrin fluorescence changes, an attempt was made to lyse the plasma membrane of the cell, and to expose the vacuoles. As is demonstrated in Fig. 7B, treatment of *D. salina* cells with a Yeda press pressure cell impairs the capacity of the cells to accumulate atebtrin, but addition of ATP, which has no effect on intact cells (Fig. 7A), induces atebtrin fluorescence quenching in the lysed cells. The observation that nitrate, an inhibitor of the vacuolar H^+ -ATPase, inhibits atebtrin uptake indicates that the dye is accumulated in acidic vacuoles in response to an ATP-dependent H^+ uptake. These observations indicate that the vacuoles in the lysed cell preparation maintain an acid-inside pH gradient and, therefore, accumulate amines. Similar to intact cells, ammonium ions also induce a rapid atebtrin fluorescence enhancement in lysed cells, indicative of dissipation of the pH gradient across the vacuolar membrane. However, unlike the in intact cells, the fluorescence enhancement is monophasic and rapid.

A kinetic comparison of the release of atebtrin from vacuoles of atebtrin-loaded intact or lysed cells by different amines is demonstrated in Figure 8. All three amines induce a rapid monophasic atebtrin release from the lysed cell vacuoles, which is about 5-, 20-, or 350-fold faster than from intact cells for NH_4Cl , BzNH_3Cl , and TEA, respectively.

A comparison of the release of atebtrin to the external medium by NH_4Cl between intact and lysed cells is summarized in Figure 9. In this experiment, atebtrin-loaded intact or lysed cells were separated by centrifugation through silicon oil layers after addition of NH_4Cl , and the release of atebtrin to the external (upper) medium was measured. In contrast to the intact cells, in which the release of dye to the medium is slow (min) and corresponds to the slow phase of atebtrin fluorescence enhancement (Fig. 6A), the release of atebtrin

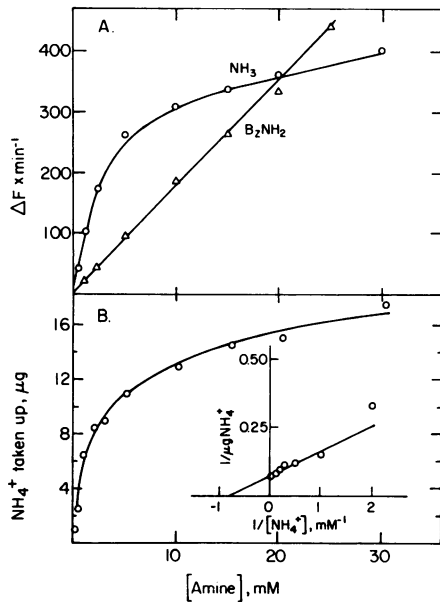


Figure 10. Concentration dependence of ammonia and benzylamine uptake. In A, the initial rate of atebtrin fluorescence enhancement by different NH_4Cl or BzNH_2Cl concentrations are expressed in relative fluorescence units (ΔF). In B, the ammonia taken up within 5 min of incubation with the indicated NH_4Cl concentration was measured. Both experiments were performed at pH 9.

from lysed cells is completed within 1 min. Therefore, this experiment is consistent with the interpretation that the rapid ammonia-induced fluorescence enhancement in intact cells reflects release of atebtrin from the vacuoles to the cytoplasm, whereas in the lysed cells, the trapped dye is released directly to the medium. These results indicate that the vacuolar membrane is much more permeable to amines than the plasma membrane.

Kinetic Analysis of Amine Uptake

The observation that the plasma membrane constitutes the major permeability barrier to amine uptake and that amines induce a rapid release of atebtrin from the vacuoles, which can be followed by fluorescence enhancement, provides a technique to analyze the transport mechanism of amines across the plasma membrane. To get an insight about the mechanism of transport across the plasma membrane, the dependence of the initial rate of atebtrin release on amine concentration and on extracellular pH was determined for several amines.

Analysis of the concentration dependence for ammonia and benzylamine (Fig. 10) reveals saturation kinetics for ammonia and a linear concentration dependence for benzylamine. A similar saturable concentration dependence is obtained for ammonia also from uptake measurements (Fig. 10B). The calculated apparent K_m values for ammonia are 1.4 and 2.8 mM from atebtrin fluorescence changes and uptake experiments, respectively. The difference may result from the different cell concentration in these measurements, from the fact that the ammonia uptake is not a true initial rate (5 min),

or from additional factors that influence atebtrin release. These results indicate a carrier-catalyzed uptake mechanism for ammonia and a diffusion mechanism for benzylamine.

The pH dependence of amine uptake can be very informative for understanding the transport mechanism. If the amine is transported by diffusion, then the rate of uptake should be proportional to the free amine concentration and stimulated at alkaline pH. Conversely, if the protonated amine is the transported species, the uptake should be stimulated below the amine pK at acidic or neutral pH. As is demonstrated in Figure 11, the uptake of all three amines, as reflected by atebtrin fluorescence enhancement, is stimulated at alkaline pH. Most pronounced is the stimulation of atebtrin release by TEA at alkaline pH, which is increased approximately 10-fold per pH unit from pH 7 to pH 10 (Fig. 11C), as expected for a diffusion mechanism.

Analysis of the initial rate of atebtrin release by ammonium chloride (Fig. 12) demonstrates that the rate is proportional to NH_3 concentration in the pH range 6 to 9, consistent with transport of NH_3 , and similar results were obtained for BzNH_2 (not shown). Also consistent with transport of NH_3 and not of NH_4^+ is the observation that the K_m for NH_4Cl uptake decreases at lower pH (from 1.4 mM at pH 9 to 9.5 mM at pH 8). The anomalous biphasic fluorescence enhancement and quenching obtained with NH_3 at pH 6 may reflect reuptake of atebtrin from the cytoplasm into the vacuoles as a result of regeneration of the pH gradient across the vacuolar membrane. These results indicate that all three amines are transported through *Dunaliella* plasma membrane in the free amine form either by diffusion (BzNH_2 , TEA) or via a carrier mechanism (ammonia).

DISCUSSION

The results described herein indicate that the amine-induced stress at alkaline pH results from cytoplasmic alkalization, and that the recovery is achieved by compartmentation of the amine into acidic vacuoles.

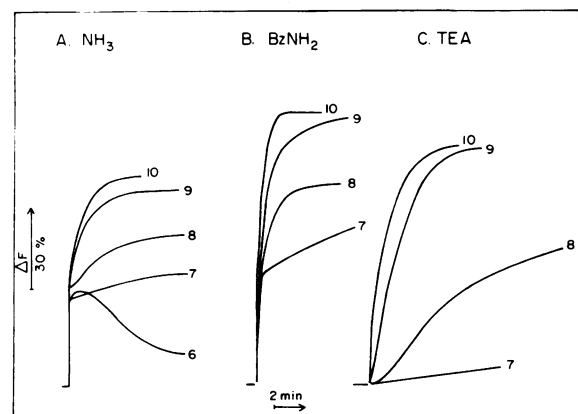


Figure 11. Effect of medium pH on amine-induced atebtrin release from acidic vacuoles. *D. salina* cells were loaded with atebtrin and preequilibrated for 20 to 40 min at the indicated pH values. Atebtrin release was initiated by addition of 20 mM NH_4Cl (A), 20 mM BzNH_2Cl (B), or 50 mM TEA-Cl (C).

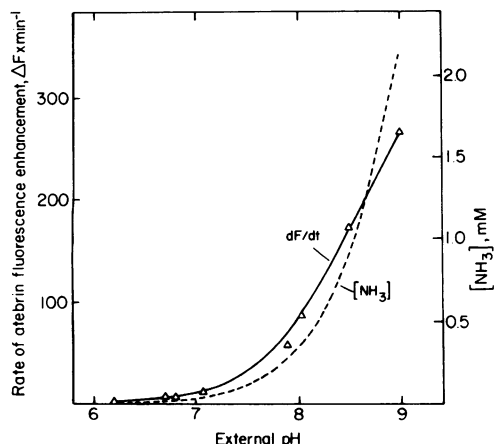


Figure 12. Correlation between rate of atebtrin release from acidic vacuoles and NH_3 concentration at different external pH values. The initial rate of atebtrin fluorescence enhancement (ΔF), induced by 5 mM NH_4Cl in atebtrin-loaded *D. salina* cells, was measured at different external pH media as in Figure 11. The free NH_3 concentration (broken line) was calculated from the pK of ammonia.

That stress is induced by cytoplasmic alkalization is clearly indicated by the correlations between cytoplasmic pH changes, as measured by ^{31}P -NMR, the drop in cellular ATP, and amine influx. Amines that have a lower permeability across the plasma membrane, such as TEA, also induce slower and smaller changes in cytoplasmic pH. Therefore, it can be assumed that the protonation of the amine within the cells leads to cytoplasmic alkalization.

The rapid release of atebtrin from acidic vacuoles by amines and the massive swelling of the vacuoles clearly indicate that amines accumulate in acidic vacuoles in *Dunaliella*. Although it has not been determined which fraction of the intracellular amine is sequestered within the vacuoles, there are two considerations that indicate that most of the amine eventually accumulates inside the vacuoles and very little is left in the cytoplasm.

First, the measured cytoplasmic pH change is far too small to be accounted for by protonation of the amount of amine taken up by the cells. Our measurements show that influx of ammonia, equivalent to intracellular concentrations of hundreds of millimolar, induces cytoplasmic pH changes of less than 1 pH unit. Because the estimated intracellular buffering capacity of whole cells is only 30 mM/pH unit (6), namely 5 to 10% of ammonium in the cells, it has to be concluded that most of the amine should be compartmentalized and buffered in intracellular compartments. Second, the recovery of motility, ATP level, and photosynthetic capacity indicates that most of the amine has to be excluded from the cytoplasm. It may be noted that ATP formation in isolated *D. salina* thylakoid preparations is completely inhibited by 10 mM NH_4Cl at pH 8. Because the calculated averaged intracellular concentration of ammonia is 30 to 50 times higher, this should result in complete inhibition of photosynthesis. Yet, ammonia-treated *Dunaliella* cells recover within 30 min their normal ATP level and 30 to 70% of their photosynthetic capacity (not shown). These considerations

indicate that most of the amine has to be excluded from the chloroplast and the cytoplasm and compartmentalized into the vacuoles.

The observation that acidic vacuoles in *Dunaliella*, which occupy only 3 to 5% of the cell volume (Fig. 5, refs. 10, 22), have the capacity to buffer and accumulate massive amounts of amine is surprising. In view of the high permeability of the vacuolar membrane to amines, it might be expected that rapid accumulation of amine within the vacuoles should lead to rapid intravacuolar alkalization and a decrease of the pH difference between the vacuole and the cytoplasm, which should limit further accumulation of amines. The observation that amine accumulation and atebtrin release continue, while the cells actually recover, indicate that additional processes that further increase the capacity of vacuoles to sequester most of the amine from the cytoplasm have been activated. Because the recovery of the cells, and the correlated slow phase of ammonia uptake, are temperature-dependent, it appears that this process may involve metabolic reactions. In the accompanying paper, we will demonstrate that the recovery is associated with hydrolysis of polyphosphates within the vacuoles (16).

The release of K^+ , which correlates with ammonium accumulation, may also be related to polyphosphate hydrolysis, because a major part of the K^+ in *Dunaliella* appears to be associated with polyphosphates (14). The release of K^+ from vacuoles may provide a means to decrease the intravacuolar osmolarity during amine accumulation to prevent their lysis within the cell.

The strategy of compartmentation of amines into acidic vacuoles may confer several benefits to photosynthetic organisms. First, acidic vacuoles may serve as a high-capacity ammonia-buffering system, controlling the level of cytoplasmic and chloroplastic ammonia according to the needs and the capacity of the ammonia assimilation system. Second, it can serve as a safeguard against uncoupling of photosynthesis, as already discussed. It has been observed that accumulation of ammonia in plants, under conditions that inhibit NH_3 assimilation, does not significantly uncouple photosynthesis, as might be expected (18, 21). It seems possible, therefore, that in plants as well, access of ammonia is compartmentalized into acidic vacuoles. Third, vacuoles may have an important role in pH homeostasis, by preventing amine-induced cytoplasmic alkalization. This may be a quite common stress among aquatic alkaliphilic microorganisms. Rapid compartmentation of the free amine into acidic vacuoles can protect cytoplasmic pH, as appears to be the case in *Dunaliella*.

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