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On ammonia futile cycling in a marine unicellular alga

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Abstract

Futile cycling of ammonia, involving passive release of NH_3 and active transport of NH_4^+, is potentially a major energetic cost to a unicellular organism. Nitrogen-starved cells of the marine diatom *Phaeodactylum tricornutum* possess a sodium-dependent transport system for the ammonium (NH_4^+) analogue methylammonium ($CH_3NH_3^+$). In nitrogen-replete cells and nitrogen-starved cells incubated in the absence of sodium the rate of methylamine uptake was low and increased as a linear function of increasing methylamine concentration. Cells incubated with 21 μM ammonia or 500 μM methylamine (which give the same concentration of uncharged base), had similar rates of uptake in both nitrogen-replete and nitrogen-starved cells. In nitrogen-replete cells there was no inhibition of ammonia or methylamine uptake in the absence of sodium, but there was a marked inhibition for both with nitrogen-starved cells. However, despite the abolition of active $CH_3NH_3^+$ and NH_4^+ uptake by nitrogen-starved cells in the absence of sodium, these cells did not release ammonia. Moreover, neither urea or low pH (which decreased the rate of ammonia uptake) induced release of ammonia in the absence of sodium. In contrast, nitrogen-replete cells released ammonia in darkness, with greater release occurring in the absence of sodium. Absence of sodium was as effective as the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) in inhibiting methylamine uptake by nitrogen-starved cells. However, release of preaccumulated methylamine by nitrogen-starved cells only occurred in the presence of CCCP; there was little release in the absence of sodium. These results suggest that futile cycling of ammonia across the plasma membrane of *P. tricornutum* is not quantitatively significant.

Keywords: Futile cycle; Ammonia; Methylammonium uptake; Compartmentation; Marine unicellular alga; (*P. tricornutum*)

1. Introduction

Futile cycling may be defined as a cyclical pathway which results in the net consumption of ATP or the dissipation of an electrochemical gradient. An excellent example of a futile cycle would be active transport of NH_4^+ and passive release of NH_3 across cell membranes, a process which has been implicated as a major energetic cost in microorganisms [1–6]. Consequently, one possible role for the NH_4^+ transport system is to counteract the passive release of NH_3. However, an additional function of the transport system in microbes, including microalgae, is to accumulate ammonia inside the cell at a sufficiently high concentration to enable the ammonia assimilatory enzyme(s) to function at reasonable rates. This role would be of considerable importance to microbes in nitrogen-deficient environments [7–11]. Clearly, both functions could operate simultaneously. Indeed, futile cycling of ammonia is more likely to occur during accumulation of NH_4^+.

Research on ammonia futile cycling has concentrated on its role in prokaryotes [1–6]. However, despite the bioenergetic consequences of ammonia futile cycling in bacteria, little is known of its importance in eukaryotic organisms. One group of organisms in which ammonia futile cycling is potentially of great importance is marine unicellular algae. Ammonia concentrations in seawater are frequently at or below the analytical detection limit. Despite the alkaline pH of seawater, it is probable that the NH_3 gradient would be directed out of the cell and therefore ammonia futile cycling could be an important energetic cost to these organisms [12–14].

The NH_4^+ transport system has been widely studied by the use of the analogue methylammonium ($CH_3NH_3^+$) [15]. Nitrogen-starved cells of the marine unicellular alga *Phaeodactylum tricornutum* possess a sodium-dependent $CH_3NH_3^+$ transport system [16]. In this paper, evidence is presented that inhibition of this transport system in nitrogen-starved cells does not result in release of ammonia or

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preaccumulated methylamine, suggesting that the energetic cost of ammonia futile cycling across the plasma membrane in *Phaeodactylum* is minimal.

2. Materials and methods

The marine diatom *Phaeodactylum tricornutum* Bohlin (N.E.R.C. Culture Collection of Algae and Protozoa strain No. 1052/6) was grown axenically in ASP2 medium [17] (pH 8.0), with the following modifications; NaCl 240 mM, KCl 10 mM, $NaNO_3$ 10 mM, K_2HPO_4 115 μM, Na_2EDTA 95 μM and vitamin B_{12} 3.7 nM. Cultures were grown for 3 d (nitrogen-replete cells) in 500 mL Dreschel bottles at 20 ± 0.5°C in light (150 μmol photons m^{-2} s^{-1}, photosynthetically active radiation) and aerated with sterile, moistened air. To obtain nitrogen-starved cells, nitrogen-replete cultures were harvested aseptically by centrifugation at 1500 × *g* for 7 min, washed three times, resuspended in nitrogen-free medium and maintained as described above for 24 h before use.

Prior to each experiment, cultures were harvested by centrifugation at 1500 × *g* for 7 min, washed three times and resuspended in nitrogen-free medium in the presence or absence of sodium to give, unless stated otherwise, a cell density of $5 \cdot 10^7$ cells mL^{-1}. In experimental media Tris buffer was replaced by 8.25 mM Hepes-KOH (pH 8.0), because Tris interferes with the method used for determining ammonia. Sodium-deficient medium contained 470 mM mannitol instead of NaCl and 95 μM K_2EDTA instead of Na_2EDTA. In experiments where the effect of pH on ammonia uptake was determined the buffer was either 8.25 mM Hepes or Mes. It should be noted that Mes inhibits colour development [18] if the Solorzano [19] method for determining ammonia is used. However, I found no inhibition of colour development using Sigma reagents as described below. The pH was adjusted with a known volume of 1 M KOH and the total K^+ concentration was maintained at 10 mM by titration with 1 M KCl.

For methylamine uptake experiments, cell suspensions were incubated at 20°C in light (150 μmol photons m^{-2} s^{-1}) and the assay started by the addition of [^{14}C]methylammonium chloride to give 3.7 kBq and 100 nmol mL^{-1} cell suspension. In experiments in which methylamine uptake at different external methylamine concentrations was followed, the specific activity was kept constant at 37 Bq $nmol^{-1}$. Samples (1 mL) were taken at intervals, filtered through 2.5 cm diameter Whatman GF/C filter discs and the cells washed with 25 mL unlabelled medium. The filter discs were transferred to vials containing 5 mL scintillation fluid (1:1 toluene/methanol containing 5 g L^{-1} diphenyloxazole) and counted in a Beckman LS 3801 scintillation counter. All samples were corrected for non-specific binding of [^{14}C]methylamine to the filter discs. In experiments with carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) the protonophore was added as an ethanol solution to give a final concentration of 100 μM; controls received the same amount of ethanol.

For ammonia uptake experiments cell suspensions were incubated as described above and the assay started by the addition of 21 or 400 μM ammonium chloride. Samples were taken immediately after the addition of ammonia and at intervals thereafter. The cells were removed by vacuum filtration (Millipore sampling manifold) through Whatman GF/C filter discs and ammonia concentrations in the filtrates were determined. Ammonia release experiments were done as described [20]. Ammonia concentrations were determined by the alkaline hypochlorite/phenol nitroprusside method (Sigma Technical Bulletin No. 640) after the addition of 87 mM trisodium citrate to prevent precipitation of magnesium and calcium salts [19].

All experiments were done at least three times.

3. Results

Nitrogen-replete cells of *Phaeodactylum* took up methylamine slowly, with little difference between cells incubated in the presence or absence of sodium (Fig. 1a). In contrast, there was appreciable uptake of methylamine by nitrogen-starved cells and this uptake was markedly inhibited in the absence of sodium (Fig. 1b). The latter data confirm those of Wright and Syrett [16]. However, it is not clear from their data whether active transport in the absence of sodium is completely abolished. Rates of methylamine uptake by nitrogen-replete cells in both the presence and absence of sodium and by nitrogen-starved cells in the absence of sodium were a linear function of increasing methylamine concentration (Fig. 2), suggesting that the rates represented passive fluxes of the uncharged base (CH_3NH_2). The K_m for methylamine uptake by nitrogen-starved *Phaeodactylum* in the presence of sodium is 35 μM [16].

Despite considerable evidence which suggests that both NH_4^+ and $CH_3NH_3^+$ are taken up by the same transport system in microalgae [9,21], including *Phaeodactylum* [16], there is compelling evidence that some bacteria possess a transport system which takes up NH_4^+ only, in addition to one which takes up both NH_4^+ and $CH_3NH_3^+$ [22–24]. Of particular importance is that in *Anacystis nidulans* the two transport systems can be distinguished by the insensitivity of the NH_4^+-specific transport system to the absence of sodium [22]. To investigate the possibility of such a system existing in *Phaeodactylum*, the rates of uptake of ammonia and methylamine by both nitrogen-replete and nitrogen-starved cells in the presence and absence of sodium were determined (Table 1). In these experiments the concentration of uncharged base was kept constant at about 1.1 μM. Based on pK_a values of 9.25 for ammonia and 10.65 for methylamine [11] (and assuming that the effects of temperature and ionic composition of the medium on the pK_a values are proportional) the

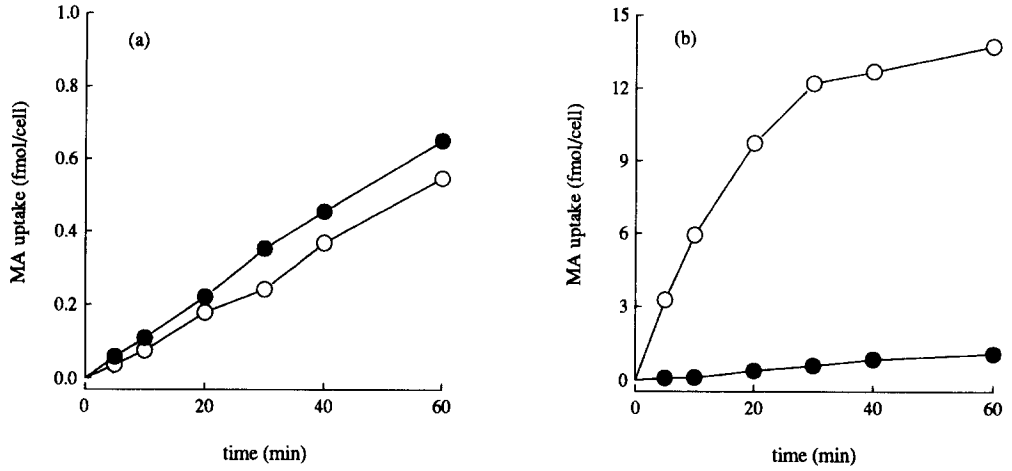

Fig. 1. Methylamine (MA) uptake by (a) nitrogen-replete and (b) nitrogen-starved *Phaeodactylum* in the presence (○) or absence (●) of sodium. The cell density in (b) was $5 \cdot 10^6$ cells mL^{-1}.

initial concentrations were set at 21 μM for ammonia and 500 μM for methylamine. Nitrogen-replete cells took up ammonia and methylamine at similar rates (Table 1). Though there was no effect of the absence of sodium on either ammonia or methylamine uptake in the presence of bicarbonate, ammonia uptake was inhibited in the absence of both sodium and bicarbonate. This effect was probably due to the sodium requirement for photosynthesis at low inorganic carbon concentrations in air-grown *Phaeodactylum* [25]. These results, together with the linearity of the relationship between the rate of methylamine uptake and methylamine concentration (Fig. 2) suggest that nitrogen-replete cells do not possess a transport system for NH_4^+ or $CH_3NH_3^+$. In nitrogen-starved cells in the presence of sodium the rates of ammonia and methylamine were similar, particularly in the presence of bicarbonate (Table 1).

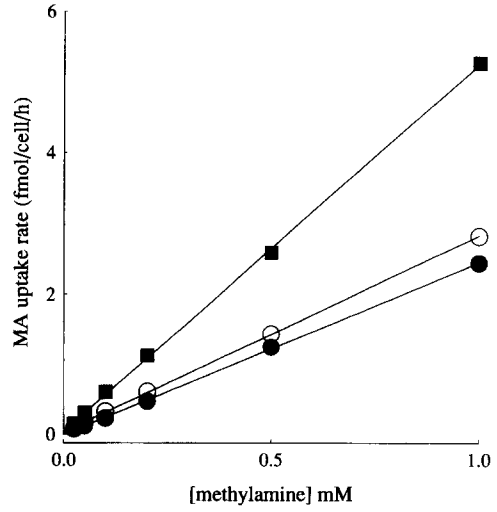

Fig. 2. Effect of methylamine concentration on the rate of methylamine (MA) uptake by nitrogen-replete cells in the presence (○) or absence (●) of sodium and nitrogen-starved *Phaeodactylum* in the absence of sodium (■). Rates were determined after 30 min incubation in [^{14}C]methylamine.

The rates of uptake of both ammonia and methylamine were markedly inhibited in the absence of sodium. In the absence of sodium, rates of ammonia uptake were slightly, but not consistently higher than those for methylamine uptake. However, assuming that, under these conditions, methylamine uptake was purely passive (Fig. 2) and that the difference was due to an additional NH_4^+-specific transport system [22], then the capacity of this system was less than the passive component. Moreover, in determining these low rates, small errors would have a disproportionally large effect on the calculated rates of uptake. For example, if the rates of methylamine uptake are not cor-

Table 1
Effect of bicarbonate on ammonia and methylamine uptake by nitrogen-replete and nitrogen-starved *Phaeodactylum* in the presence or absence of sodium

	Uptake (fmol $cell^{-1}$ h^{-1})	
	ammonia	methylamine
Absence of bicarbonate		
N-replete cells		
$+Na^+$	1.2±0.2	1.7±0.1
$-Na^+$	0.5±0.1	1.8±0.3
N-starved cells		
$+Na^+$	45.7±1.2	56.8±1.5
$-Na^+$	6.0±1.0	4.2±0.3
Presence of bicarbonate		
N-replete cells		
$+Na^+$	1.2±0.2	2.0±0.1
$-Na^+$	1.0±0.2	2.2±0.3
N-starved cells		
$+Na^+$	50.7±4.4	56.2±2.1
$-Na^+$	6.4±0.3	3.5±0.1

Cell suspensions ($5 \cdot 10^6$ cells mL^{-1}) were incubated in the presence or absence of 5 mM $KHCO_3$. Rates were determined for 21 μM ammonium chloride or 500 μM [^{14}C]methylammonium chloride as described in Materials and Methods, with incubation times of 30 min for nitrogen-replete cells and either 1 min ($+Na^+$) or 5 min ($-Na^+$) for nitrogen-starved cells. Values are the means±S.E. for three or four determinations.

rected for non-specific binding of [^{14}C]methylamine to the filters, the rates are equal to (+bicarbonate) or slightly in excess of (−bicarbonate) those for ammonia. Moreover, the differences in the effect of sodium on the rates of ammonia and methylamine uptake were minimal compared with those for *Anacystis nidulans* [22].

A further consideration in comparing ammonia and methylamine uptake is that the former includes a component due to assimilation by glutamine synthetase (GS)/glutamate synthase (GOGAT), whereas methylamine is not metabolized by *Phaeodactylum* [16]. This distinction was explored further by comparing the effects of presence and absence of sodium on ammonia uptake in the presence of bicarbonate by nitrogen-replete and nitrogen-starved cells where the GS activity per mL was kept constant (Fig. 3). In the absence of sodium the initial rate per mL was 3.3-fold higher in nitrogen-starved cells than in nitrogen-replete cells (Fig. 3). This compares with a 6.4-fold increase in the rate per cell (Table 1). These results suggest that about half of the increase per cell (Table 1) in nitrogen-starved cells was due to a higher GS activity per cell and that, at least some, of the difference between ammonia uptake and methylamine uptake, in the absence of sodium, may have been due to GS maintaining a low intracellular concentration of ammonia.

If a major role of the NH_4^+ transport system is to counteract the leakage of NH_3, then release of ammonia should occur in nitrogen-starved cells incubated in the absence of sodium, but should not occur in nitrogen-replete cells. Nitrogen-starved cells did not release ammonia in darkness in the absence of sodium (Fig. 4), whereas nitrogen-replete cells released ammonia in darkness, with

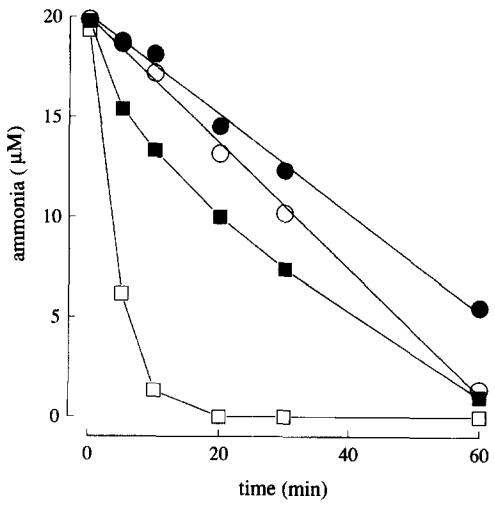

Fig. 3. Ammonia uptake by nitrogen-replete (circles) and nitrogen-starved (squares) *Phaeodactylum* in the presence (open symbols) or absence (closed symbols) of sodium. The cell density was adjusted to give the same glutamine synthetase activity in each treatment (about 500 nmol mL^{-1} h^{-1} at 20°C (Rees, T.A.V., Larson, T.R., Heldens, J. and Huning, F., unpublished data)); $16 \cdot 10^6$ cells mL^{-1} for nitrogen-replete cells and $6.5 \cdot 10^6$ cells mL^{-1} for nitrogen-starved cells. All cell suspensions were incubated in the presence of 5 mM $KHCO_3$.

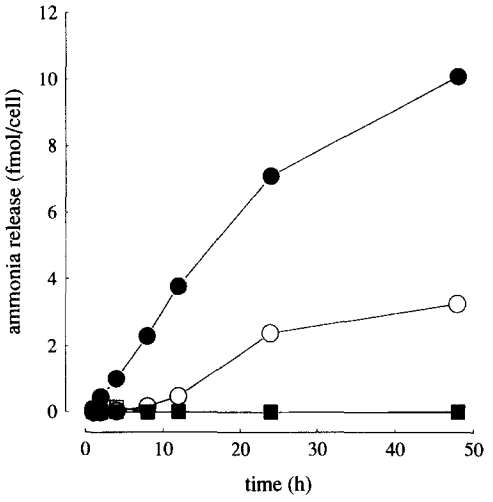

Fig. 4. Ammonia release by *Phaeodactylum* in darkness. Nitrogen-replete (circles) and nitrogen-starved (squares) cells were incubated in the presence (open symbols) or absence (closed symbols) of sodium.

greater release occurring in the absence of sodium (Fig. 4). The difference in the ammonia release characteristics between nitrogen-replete cells incubated in the presence and absence of sodium may be due, in part, to low levels of inorganic carbon in the medium (Table 1; Rees, T.A.V., unpublished data). There was no release of ammonia in light by either nitrogen-replete or nitrogen-starved cells (data not shown).

An alternative explanation for the lack of ammonia release is that nitrogen-starved cells possess low intracellular concentrations of ammonia. Moreover, at the medium pH of 8 it is probable that the intracellular pH is lower and, therefore, the likelihood of NH_3 diffusion out of the cell is decreased. These possibilities were tested in two ways. The effect of increasing the intracellular concentration of ammonia was tested by following the release of ammonia when nitrogen-starved cells were incubated with 0.5 mM urea in darkness in the absence of sodium. Despite the absence of active urea uptake under these conditions [26], uptake can occur by passive diffusion [27]. No ammonia release was detected (data not shown). The only conditions under which ammonia release was observed with nitrogen-starved cells was in the presence of CCCP (0.016 fmol $cell^{-1}$ h^{-1}) and CCCP + urea (0.03 fmol $cell^{-1}$ h^{-1}). However, these rates were substantially less than those obtained with nitrogen-replete cells (Fig. 4) The second possibility was investigated by comparing the effects of pH on rates of ammonia uptake by nitrogen-starved cells in the presence and absence of sodium (Fig. 5). At pH 8 and an initial ammonia concentration of 400 μM, rates of ammonia uptake were the same in the presence or absence of sodium. At lower pH values, the rate of ammonia uptake decreased, with the decrease being greater in the absence of sodium. However, the decrease in the rate of ammonia uptake in the absence of sodium was less than would be expected. One possible explanation for this is

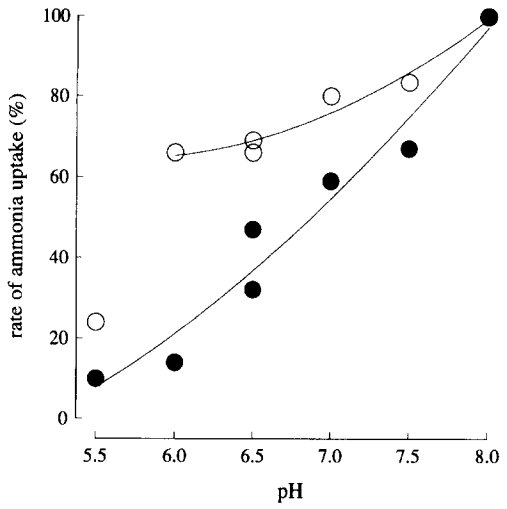

Fig. 5. Effect of pH on dark ammonia uptake by nitrogen-starved *Phaeodactylum* in the presence or absence of sodium. Nitrogen-starved cells were incubated in darkness with 400 μM ammonium chloride in the presence (open circles) or absence (closed circles) of sodium. Samples were taken after 0 and 1 min for ammonia determinations, as described in Materials and Methods. Rates are expressed as % of the rate at pH 8 ($+Na^+ = 70 \pm 4$ fmol cell^{-1} h^{-1}; $-Na^+ = 75 \pm 2$ fmol cell^{-1} h^{-1}).

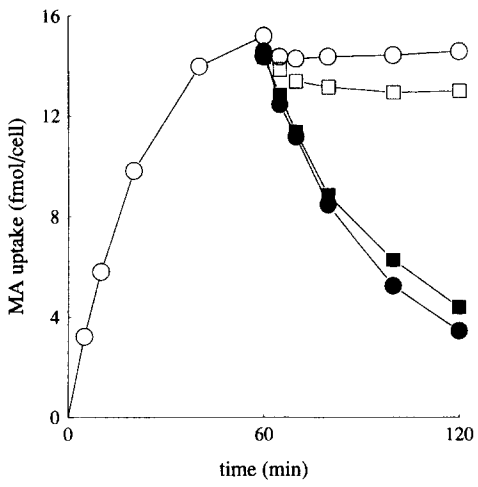

Fig. 6. Effect of the absence of sodium and CCCP on release of preaccumulated methylamine (MA) by nitrogen-starved *Phaeodactylum*. Nitrogen-starved cells ($5 \cdot 10^6$ cells mL^{-1}) were incubated with [^{14}C]methylamine in the presence of sodium (open circles). After 60 min incubation, the cell suspension was divided into four portions. Two portions each were harvested, washed three times and resuspended in methylamine-free, control medium (circles) or minus sodium (squares) medium. To one of the control and minus sodium cell suspensions CCCP was added as an ethanol solution to give 100 μM (closed symbols); the other cell suspensions received the same amount of ethanol (open symbols). The amount of [^{14}C]methylamine per cell was determined as described in Materials and Methods.

that intracellular pH homeostasis was compromised in the absence of sodium. There was no release of ammonia by nitrogen-starved cells maintained in continuous darkness in the absence of sodium at pH 5.5–8 (data not shown).

These results suggest that counteracting the leakage of NH_3 is not a physiological role of the NH_4^+ transport system. However, it is more likely that leakage would occur during transport of NH_4^+ (or $CH_3NH_3^+$), because of the substantial, outwardly-directed gradient of NH_3 (or CH_3NH_2) that would be generated. To test this hypothesis, nitrogen-starved cells of *Phaeodactylum* were allowed to accumulate methylamine, before being transferred to methylamine-free control or minus sodium medium in the presence or absence of CCCP. Methylamine uptake was inhibited markedly in minus sodium medium and in the presence of CCCP, with the inhibition by CCCP being slightly (and consistently) greater (Table 2). Very little efflux occurred in control or minus sodium medium, but substantial efflux occurred in the presence of CCCP in both media (Fig. 6).

Table 2
Effect of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on rates of methylamine uptake by nitrogen-starved *Phaeodactylum* in the presence or absence of sodium

	Rate of methylamine uptake (fmol cell^{-1} h^{-1})
$+Na^+$	43.4 ± 5.3
$+Na^+ +$CCCP	0.8 ± 0.5
$-Na^+$	1.3 ± 0.4
$-Na^+ +$CCCP	0.2 ± 0.1

Cell suspensions ($5 \cdot 10^6$ cells mL^{-1}) were preincubated in light for 10 min with or without CCCP before the addition of [^{14}C]methylamine. CCCP was added as an ethanol solution to give a final concentration of 100 μM; controls received the same amount of ethanol. Rates were determined after 1 min incubation in [^{14}C]methylamine as described in Materials and methods. Values are means $\pm$ S.E. for four determinations

4. Discussion

In the absence of sodium, the ability of nitrogen-starved *Phaeodactylum* to take up methylamine was markedly decreased (Fig. 1b) and rates of uptake were a linear function of increasing methylamine concentration (Fig. 2). Methylamine uptake rates in nitrogen-replete cells were low, irrespective of the presence or absence of sodium (Fig. 1a), and increased linearly with increasing methylamine concentration (Fig. 2). Moreover, rates of ammonia and methylamine uptake in the absence of sodium were similar in both nitrogen-replete and nitrogen-starved cells and in nitrogen-replete cells there was no inhibition of either ammonia or methylamine uptake in the absence of sodium (Table 1). These data are consistent with the absence of active NH_4^+ or $CH_3NH_3^+$ uptake in nitrogen-replete cells and in nitrogen-starved cells which are incubated in the absence of extracellular sodium, with the low rates of uptake being due to passive diffusion of the uncharged base. Assuming $CH_3NH_3^+$ uptake is a true reflection of the capacity of the cells to transport NH_4^+ and that a major function of the transport system is to counteract leakage of NH_3, it would be reasonable to assume that

biosynthesis of the transport system during nitrogen-starvation is in response to a condition which favours release of NH_3. However, ammonia release only occurred in nitrogen-replete cells (Fig. 4), which apparently possess little or no ability to transport $CH_3NH_3^+$ or NH_4^+ (Table 1; Fig. 2), and no release occurred when transport activity was abolished in nitrogen-starved cells by incubating them in the absence of sodium (Fig. 4). Moreover, there was little release of pre-accumulated methylamine in nitrogen-starved cells which were transferred to sodium-deficient medium (Fig. 6), despite the marked inhibition of active uptake under these conditions (Table 2). There appears to be no convincing evidence for futile cycling of ammonia across the plasma membrane in *Phaeodactylum*.

The foregoing assumes that $CH_3NH_3^+$ uptake is a valid measure of total NH_4^+ uptake. Though the available evidence for *Phaeodactylum* (Table 1; Fig. 2) is, in general, consistent with this, the difference between the rates of uptake of ammonia and methylamine by nitrogen-starved cells in the absence of sodium (Table 1) suggests the possibility of a separate transport system for NH_4^+ alone [22–24]. Moreover, if a major role of this transport system is to recover leaked NH_3, then the rates in Table 1 would represent net uptake by the cells and not the true capacity of the transport system. However, the rates of ammonia uptake in the absence of sodium were markedly less than in its presence, in contrast to *Anacystis nidulans*, where there is no sodium-effect [22]. In addition, as there was negligible release of ammonia by these cells in the presence of CCCP (see above) this would represent the only known transport system in *Phaeodactylum* which is insensitive to both CCCP and the absence of sodium [25].

In studies where rates of ammonia and methylamine uptake are compared it is important that consideration is given to the differences in pK_a values for the two molecules (Table 1). If there were no active transport system for either molecule, then in the presence of identical external concentrations the rate of ammonia uptake would be about 25 times greater than that for methylamine. Consequently, comparisons of rates of uptake of the two molecules are of limited use unless the difference in pK_a values is taken into account.

Futile cycling of ammonia is potentially a considerable energetic cost to any single-celled organism. Kleiner [2] calculated that futile cycling of ammonia during nitrogen-fixation by *Klebsiella pneumoniae* would cost 4 ATP per NH_3 molecule. However, experimental investigations of ammonia metabolism during nitrogen-fixation by free-living *Azorhizobium caulinodans* provided no evidence for NH_3 release [28].

Transport of NH_4^+ occurs via the low-affinity K^+ transport system in *Rhodobacter capsulatus* [4] and the high-affinity K^+ transport system (Kdp) in *E. coli* [6]. A combination of this transport and NH_3 leakage results in a marked decrease in the protonmotive force [4] and enhanced O_2 consumption [6]. However, though NH_4^+ causes a large membrane ionic current in *R. capsulatus*, which is not additive with K^+- or Rb^+-dependent ionic currents [4], it either stimulates or has no effect on Rb^+ uptake [29]. To account for these data, Golby et al. [29] suggest that NH_4^+ increases the affinity of the low-affinity K^+ transport system, either by a direct effect of NH_4^+ on the protein or as a consequence of passive NH_3 uptake and alkalinisation of the cytosol. However, it should be noted that *R. capsulatus* possesses two transport systems for NH_4^+ [23].

A mutant of *Klebsiella pneumoniae*, which lacks NH_4^+ transport activity, releases ammonia [1]. However, the mutant also possesses decreased glutamine synthetase and glutamate synthase activities [1]. In contrast, mutants of *Anabaena variabilis* [30] and *Chlamydomonas reinhardtii* [31] lacking the ability to take up NH_4^+ or $CH_3NH_3^+$, but possessing wild-type levels of glutamine synthetase [30,31] and glutamate synthase [31] do not release ammonia. Moreover, $^{15}N/^{14}N$ experiments with *Chlamydomonas* indicate that no NH_3 release occurs during uptake of ammonia [32]. With the exception of futile cycling via the Kdp transport system in *E. coli* and possibly in *R. capsulatus*, there appears to be little compelling evidence for widespread occurrence of ammonia futile cycling across microbial plasma membranes.

There are a number of sources of intracellular ammonia in microalgae. These include transported NH_4^+ and ammonia derived from nitrate reduction, amino acid deamination and photorespiration. Clearly the potential for NH_3 release exists unless ammonia is efficiently reassimilated by glutamine synthetase and glutamate synthase. If these enzymes are inhibited, either directly by methionine sulphoximine [33–35] or indirectly by limiting the supply of 2-oxoglutarate [20,36], algal cells will release ammonia. Moreover, nitrogen-replete *Phaeodactylum* did not release ammonia in light but released substantial amounts of ammonia if the cells were incubated in light in the presence of the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), with this release being greater than that which occurred in darkness (Rees, T.A.V., unpublished data). However, methylamine is not metabolised [16] and either accumulates in the cytosol or is stored in an intracellular compartment, with a likely possibility being the vacuole. Methylamine would accumulate in the acidic vacuole by "acid-trapping", with diffusion of the uncharged base across the tonoplast and protonation within the vacuole. Though involving passive diffusion of the uncharged base the process would, in effect, be active because energy would be required to maintain the protonmotive force across the tonoplast. In maize roots, the majority of tissue ammonia is located in the vacuole [37,38]. Such compartmentation would explain why only 10% ($\pm$ 1.6% S.E., $n = 3$) of preaccumulated methylamine (possibly representing the cytosolic pool) was released in the absence of sodium, but substantial release occurred if the protonophore CCCP, which abolishes the pH gradient across the tonoplast [39], was added to these cells (Fig. 6).

These results are particularly striking given that the absence of sodium was nearly as effective as CCCP in preventing methylamine uptake into the cells (Table 2). Moreover, it suggests that the inhibitory effect of the absence of sodium on uptake is restricted to the plasma membrane.

The foregoing suggests that, for microalgae at least, futile cycling of ammonia across the plasma membrane is unlikely to be quantitatively significant. However, there is a considerable experimental evidence that microalgae produce ammonia intracellularly with release occurring if its reassimilation is prevented [20,33–36] (Fig. 4). The major ammonia assimilatory enzyme in these organisms is glutamine synthetase, which catalyzes an ATP-dependent reaction. Most of the reactions which generate ammonia intracellularly do not involve ATP. Therefore, this represents a futile cycle [40]. Whether this futile cycle is an unavoidable consequence of cellular metabolism or has regulatory significance [40] remains to be resolved.

In conclusion, it is suggested that the NH_4^+ transport system does not have a role in counteracting NH_3 release. In contrast, the assimilatory enzymes glutamine synthetase and glutamate synthase, appear to play a central role in preventing loss of intracellular ammonia in microalgae.

Acknowledgements

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