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# Permeability of Ammonia, Methylamine and Ethylamine in the Cyanobacterium, *Synechococcus* R-2 (*Anacystis nidulans*) PCC 7942

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**Summary.** Permeabilities of ammonia ($NH_3$), methylamine ($CH_3NH_2$) and ethylamine ($CH_3CH_2NH_2$) in the cyanobacterium (cyanophyte) *Synechococcus* R-2 (*Anacystis nidulans*) have been measured. Based on net uptake rates of DCMU (dichlorophenyldimethylurea) treated cells, the permeability of ammonia was 6.44 ± 1.22 $\mu$m sec$^{-1}$ ($n$ = 13). The permeabilities of methylamine and ethylamine, based on steady-state $^{14}C$ labeling were more than ten times that of ammonia ($P_{methylamine}$ = 84.6 ± 9.47 $\mu$m sec$^{-1}$ (76), $P_{ethylamine}$ = 109 ± 11 $\mu$m sec$^{-1}$ (55)). The apparent permeabilities based on net uptake rates of methylamine and ethylamine uptake were significantly lower, but this effect was partially reversible by ammonia, suggesting that net amine fluxes are rate limited by proton fluxes to an upper limit of about 700 nmol m$^{-2}$ sec$^{-1}$. Increasing concentrations of amines in alkaline conditions partially dissipated the pH gradient across the cell membrane, and this property could be used to calculate the relative permeabilities of different amines. The ratio of ethylamine to methylamine permeabilities was not significantly different from that calculated from the direct measurements of permeabilities; ammonia was much less effective in dissipating the pH gradient across the cell membrane than methylamine or ethylamine. An apparent permeability of ammonia of 5.7 ± 0.9 $\mu$m sec$^{-1}$ could be calculated from the permeability ratio of ammonia to methylamine and the experimentally measured permeability of methylamine. The permeability properties of ammonia and methylamine are very different; this poses problems in the interpretation of experiments where $^{14}C$-methylamine is used as an ammonia analogue.

**Key Words** *Synechococcus* · permeability · ammonia · methylamine · ammonium transport

## Introduction

Ammonia is the preferred source of fixed nitrogen for many plants, fungi and bacteria (Kleiner, 1981). A major difficulty in studies of nitrogen uptake and metabolism is that a convenient nitrogen radioisotope is not readily available. $^{14}C$-methylamine may be used as an ammonia analogue for transport studies in several organisms including *Synechococcus* (Hackette et al., 1970; Boussiba, Dilling & Gibson, 1984*a*; Boussiba & Gibson, 1985; for reviews *see* Kleiner 1981, 1985*b* and Gibson, 1984).

The weakly basic amines (R-$NH_2$), ammonia, methylamine and ethylamine have p$K_a$'s of 9.25, 10.65 and 10.75, respectively, at 25°C (Segel, 1976). The permeability ($P_j$) of a chemical species ($j$) is a measure of its ability to pass through the membrane. Biological membranes are generally very impermeable to ions unless pores or specific carriers are present, but are readily permeable to small uncharged molecules such as water and oxygen (Collander, 1954; Nobel, 1983). Thus uncharged $NH_3$, $CH_3NH_2$ and $CH_3CH_2NH_2$ should pass readily through the cytoplasmic membrane of a cell. Many procaryotes have a transport mechanism for ammonium cation which is repressed when passive diffusion of $NH_3$ is rapid enough to supply metabolic needs (*see* Kleiner, 1981; 1985*b*). Because other nitrogen sources such as nitrate, nitrite, urea and molecular nitrogen, in the case of diazotrophs (nitrogen fixers), are all reduced to $NH_3$ before final fixation into amino acids (Kleiner, 1981; Kleiner, 1985*b*), the permeability of $NH_3$ is important to the efficiency of assimilation of all inorganic nitrogen sources.

The relative importance of uptake of $NH_4^+$ and the passive diffusion of $NH_3$ cannot be assessed unless the permeability of $NH_3$ ($P_A$) is known. Kleiner (1981) points out that although it is generally agreed that $NH_3$ is highly permeable, only a few actual measurements have been made. The permeability of $CH_3NH_2$ ($P_{MA}$) in *Klebsiella* was found to be about 15 $\mu$m sec$^{-1}$ and $P_A$ was inferred to be about 18 $\mu$m sec$^{-1}$ (Kleiner, 1985*a*,*b*).

Accurate direct measurement of $P_A$ is difficult because a chemical assay must be employed. However, for many investigations it is sufficient to know the ratio of the permeability of ammonia ($P_A$) to that of methylamine ($P_{MA}$) or ethylamine ($P_{EA}$).

Ammonia and other amines act as indirect uncoupling agents of chloroplasts, and the degree of uncoupling is a function of the concentration of the uncharged amine (Hind & Whittingham, 1963; Crofts, 1966*a,b*, 1967; Gaensslen & McCarty, 1971). The effectiveness of amines as inhibitors of photophosphorylation increases with the length of the hydrophobic hydrocarbon chain (McCarty & Coleman, 1970; Gaensslen & McCarty, 1971).

In the present paper we show that the permeabilities of amines (including $NH_3$) can be measured in the unicellular cyanobacterium *Synechococcus sp*. A distinction needs to be made between permeabilities based on net uptake or loss of amines by cells and permeabilities measured by exchange fluxes (*i.e.*, exchange of $^{14}C$-amine for unlabeled amine) in the steady state. Theoretical aspects of the calculation of permeabilities and the effect of amines on the pH gradient across the cell membrane are dealt with in the Appendix.

## Materials and Methods

### Chemicals and Radiochemicals

Methylamine · HCl and Fluorinert® (specific gravity 1.85), were purchased from Sigma Biochemicals, St. Louis, MO, and ethylamine · HCl from Eastman Kodak, Rochester, NY. Polycarbonate membrane filters (pore sizes, 0.8 and 1.0 $\mu$m) were purchased from Nucleopore, Pleasanton, CA. The silicone oils, SF96(50)® and Versilube® F-50, were gifts from General Electric (Silicone Products Division, Waterford, NY). Radiochemicals were purchased from New England Nuclear, Boston, MA. DCMU (dichlorophenyldimethylurea) was a gift from R.E. McCarty.

### Experimental Organism and Growth Conditions

*Synechococcus sp* R-2 (*Anacystis nidulans*) (PCC 7942) was maintained as axenic stock cultures (Ihlenfeldt & Gibson, 1975). Cells for experiments were grown in ammonia-free BG-11 medium (Allen, 1968) but with 0.245 mM $CaCl_2$ and without sodium silicate. During growth the pH was between 7.5 to 8.0. Cultures were grown in 1-liter Roux bottles in an illuminated (2 cool-white 40 W fluorescent lights) constant temperature bath at 30°C and were continuously bubbled with approximately 5% $CO_2$ in air. Growth was followed by measuring absorbance at 750 nm ($A_{750}$) using a Zeiss PMQII spectrophotometer. Cell numbers were determined using a Petroff-Hausser counting chamber and phase contrast microscopy.

Cultures had a doubling time of about 12 hr and were grown semi-continuously by daily dilution; cells were used for experiments when $A_{750}$ of a culture was between 0.5 and 1.3, equivalent to a protein content of about 40 to 100 $\mu$g ml$^{-1}$. Cell numbers and protein were found to be directly proportional to absorbance within the above range; the protein content of cultures was 81.6 $\pm$ 6.17 $\mu$g ml$^{-1}$ $A_{750}^{-1}$ ($n$ = 77). All error-bars are $\pm$95% confidence limits. Cytoplasmic membrane surface areas and intracellular volumes are expressed on a protein basis. Cell sizes were measured on phase contrast photomicrographs; for the calculation of cytoplasmic membrane surface areas, *Synechococcus* cells were taken to have the shape of round-ended cylinders.

### Preparation of Cells for Experiments

Cultures of *Synechococcus* were harvested by centrifugation and suspended in a buffered BG-11 medium containing 10 mM $Na_2CO_3$ buffer or 10 mM CAPS (3-[cyclohexylamino]-1-propanesulphonic acid); the cells were thus provided with nitrate (17.7 mM) as a nitrogen source during the course of experiments. The cells were washed several times (>4) in the experimental buffer to be used in an experiment. For ammonia uptake experiments, only carbonate buffer was used, and the pH adjusted to between 9.5 and 10.0. The cell suspensions (protein content about 0.2 to 1.0 mg ml$^{-1}$) were incubated in BG-11 buffer for about 30 min prior to an experiment. For methylamine and ethylamine experiments either carbonate or CAPS was used and the pH adjusted to 9.8 to 10.1.

In steady-state isotopic labeling experiments, the buffer in which the cells were washed and incubated contained the concentration of methylamine or ethylamine to be used in the labeling experiment. $NH_4Cl$ (200 $\mu$M) was added to prevent the metabolism of methylamine; the presence or absence of ammonia had no effect on the uptake of $^{14}C$-ethylamine. Under these conditions metabolism of methylamine and ethylamine was negligible (Boussiba et al., 1984*a*; Boussiba & Gibson, 1985). For net uptake experiments the cells were equilibrated to an ammonia-free BG-11; in some $^{14}C$-methylamine and $^{14}C$-ethylamine experiments 200 $\mu$M ammonia was added to test if the presence of a permeant amine affected the net flux rate of methylamine or ethylamine, respectively.

### Protein Determinations

Protein determinations were made on cell suspensions with absorbances ranging from $A_{750}$ = 0.15 to 1.3. Four replicate samples of cells were suspended in 5% wt/vol trichloroacetic acid for 5 to 10 min, in an ice bath, then centrifuged, and the supernatant was discarded. The pellets were suspended in 0.1 M NaOH and were boiled or autoclaved for 10 min, and then centrifuged. Protein determinations were made on the supernatant using the Coomassie blue dye binding method and bovine serum albumin in 0.1 M NaOH as the standard (BioRad Handbook; Bradford, 1976).

### Silicone Oil Centrifugation

Centrifugation through silicone oil was used to determine intracellular volumes and pH. A 5 : 1 mixture of Versilube® F-50 and SF96 (50)® had a nominal specific gravity of 1.036. Twenty $\mu$l of Fluorinert® was placed in the bottom of 400 $\mu$l microcentrifuge tubes, followed by 80 to 100 $\mu$l of silicone oil mixture, cell suspension (200 $\mu$l containing about 0.2 mg protein) was placed above the oil. After centrifugation the tubes were carefully cut above and below the pellet which had passed through the silicone oil layer. This pellet was then resuspended in 200 $\mu$l of BG-11 medium in a scintillation vial before adding the scintillation fluid. Equal volumes of cell suspension were added to the standards to correct for quenching.

Intracellular volumes were measured by subtracting the apparent $^{14}$C-sorbitol volume (1.2 mM; sp act approximately 30 GBq mol$^{-1}$) from the $^{3}H_2O$ volume (sp act approximately 1 MBq mol$^{-1}$) of the cell pellets. The intracellular volume for *Synechococcus* was 4.09 ($\pm$ 0.396) $\times$ 10$^{-6}$ m$^3$ g$^{-1}$ protein ($n$ = 60).

Rapid uptake measurements of ammonia, $^{14}$C-methylamine, $^{14}$C-ethylamine were performed using an Eppendorf 5414 microcentrifuge. Silicone oil (80-100 $\mu$l; s.g. 1.036) and 1.2 ml of concentrated cell suspension (protein content about 1 mg ml$^{-1}$) were placed in a 1.5 ml microcentrifuge tube and a 10 to 20 $\mu$l known volume of $^{14}$C-labeled amine (sp. act. approximately 10 to 100 GBq mol$^{-1}$) or ammonia standard solution, as appropriate, were placed on the inside of the cap. The closed tube was then shaken and quickly put into the centrifuge. A loading time as short as 2 sec could be achieved (Howitz & McCarty, 1985). After centrifuging, aliquots of the supernatant were taken for counting or for ammonia assay, as appropriate.

## Membrane Filtration Techniques

For filtration assays, the sintered glass surface of a Millipore filtration apparatus was lightly coated with silicone oil (SF-96) to prevent the media from soaking through the membrane filter until suction was applied. In steady-state uptake experiments, 100 $\mu$l of cell suspension (about 0.1 mg protein) containing a known concentration of unlabeled amine was first placed on a membrane filter, then 100 $\mu$l of the same concentration of $^{14}$C-amine (sp act about 10-100 GBq mol$^{-1}$) was added. The cells were incubated either for about 5 sec (short label) or for 30 or 60 sec (long label), then filtered and counted. The counts taken up by the cells were corrected for the $^{14}$C-amine adsorption by the membrane filter. The correction was no more than 5% of the total count of the labeled cells. Conversely, the rate of loss of $^{14}$C-methylamine by *Synechococcus* cells was measured by first equilibrating the cells to a known concentration of $^{14}$C-amine, then washing the filtered cells with media containing the same concentration of unlabeled amine for a measured time. Equation (A4) (*see* Appendix) was used to calculate the permeability of the $^{14}$C-amine in uptake experiments and Eq. (A6) used to calculate the permeability of $^{14}$C-methylamine by efflux.

Net uptake experiments followed a similar protocol. The cell suspension in methylamine and ethylamine-free buffer media (100 $\mu$l) was first added to a filter disk, then 100 $\mu$l of a known concentration of $^{14}$C-labeled amine in buffered medium was added and thoroughly mixed. Incubations were timed and counted as for the steady-state experiments. The final equilibrium concentration of amine in the bulk electrolyte was corrected for the net uptake of amine by the cells.

## Ammonia Assay

The ammonia content of experimental media and cell extracts was assayed using the indophenol color reaction (Solorzano, 1969) adapted for a sample volume of 1 or 2 ml. Standard curves were linear up to about 100 $\mu$M ammonia, and the detection limit was about 1 $\mu$M. Phosphate and borate buffers (10 mM) or high concentrations of phenol (50 mM) did not interfere, although phenol slowed the rate of color development; carbonate buffers lowered absorbances by about 10% but did not affect the linearity of standard curves. Triethanolamine, Tris (tris-hydroxymethylaminomethane) and Good buffers as MES (2[N-morpholino] ethanesulphonic acid) and Tricine (N-tris(hydroxymethyl) methylglycine) and CAPS could not be used.

## Intracellular Ammonia Pools

Several different extractants were compared, including perchloric acid (Boussiba, Resch & Gibson, 1984*b*), 10% (vol/vol) butanol, 10% (vol/vol) toluene and 50 mM phenol. It was found that it was important to kill the cells rapidly, since dying cells rapidly evolved ammonia, yielding apparent intracellular ammonia pools as high as 13 mM.

The efficiencies of 10% toluene and 50 mM phenol as extractants for amines were compared by measuring removal of $^{14}$C methylamine from cells equilibrated in 100 $\mu$M ($^{14}$C-MA; sp act about 10 GBq mol$^{-1}$) and then exposed to phenol or toluene for 2 min before centrifugation. Phenol reproducibly removed in excess of 95% of the methylamine from the cells, but toluene yielded erratic results varying from 20 to 90% efficiency.

## Corrections for Metabolism of Ammonia

Measurements of the ammonia uptake rates of control cells were corrected for the metabolism of ammonia using compartmental analysis techniques (Atkins, 1969; Walker & Pitman, 1976). The apparent permeability of ammonia was also measured in cells treated with the specific photosynthetic inhibitor, DCMU (10 $\mu$M) to inhibit the metabolism of ammonia (Boussiba et al., 1984*b*). The apparent permeability of $NH_3$ in control cells was significantly higher than that found in DCMU-treated cells, indicating that the assumption of a constant rate of ammonia metabolism which had to be made in the compartmental analysis was invalid.

## Measurement of Intracellular pH

The intracellular pH ($pH_i$) can be calculated from the equilibrium accumulation of the weak base amines and the pH of the bulk electrolyte using the Henderson-Hasselbalch equation; it was assumed that the $pK_a$ of the weak base was similar in the bulk medium and within the cell (Butler, Waddell & Poole, 1967; Gaensslen & McCarty, 1971; Heldt et al., 1973).

## Effect of Amines on the pH Gradient

The effects of amines on $\Delta pH_{i,o}$ was measured by adding known amounts of buffered amine solutions to 1 ml volumes of concentrated cell suspensions (about 1 mg ml$^{-1}$ protein) equilibrated to 100 $\mu$M $^{14}$C-ethylamine (sp act about 100 Gbq mol$^{-1}$) in BG-11 medium (pH 10). The pH gradient was calculated from the accumulation ratio of the $^{14}$C-ethylamine. The cells were equilibrated with the added amine for 1 min and then 300 $\mu$l aliquots filtered through Nucleopore filters for counting. Unfiltered 300-$\mu$l aliquots were used as standards. To minimize error, the effects of two different amines on the pH gradient were measured on the same cell suspension. The equilibrium concentration of amine in the bulk electrolyte was corrected for accumulation by the cells using the known volumes of the bulk electrolyte ($v_o$), the intracellular volume ($v_i$) and the measured accumulation ratio of $^{14}$C-ethylamine.

**Table 1.** Stereological data on *Synechococcus*

| | |
|---|---|
| Cell length ($\mu$m) | 3.142 ± 0.0620 (500) |
| Cell width ($\mu$m) | 0.939 ± 0.058 (127) |
| Cytoplasmic surface area/cell volume ratio ($\times 10^6$ $m^{-1}$) | 4.731 ± 0.359 |
| Cytoplasmic membrane surface area ($\times 10^{-12}$ $m^2$ $cell^{-1}$) | 9.269 ± 0.610 |
| ($\times$ $m^2$ $g^{-1}$ protein) | 34.22 ± 3.72 |
| Intracellular volume ($\times 10^{-6}$ $m^3$ $g^{-1}$ protein) | 4.092 ± 0.396 (60) |
| Protein per cell ($\times 10^{-15}$ g $cell^{-1}$) | 271 ± 23.7 (77) |

Error bars are ±95% confidence limits with the number of replicates in brackets.

**Table 2.** Permeabilities of methylamine and ethylamine for *Synechococcus* based on steady-state exchange experiments

| | Permeabilities on cytoplasmic membrane surface area basis ($\mu$m $sec^{-1}$) | | |
|---|---|---|---|
| | Membrane filtration | Centrifugation | Overall mean |
| $CH_3NH_2$ | 88 ± 9.3 (51) | 77.5 ± 22.6 (25) | 84.6 ± 9.47 (76) |
| $CH_3CH_2NH_2$ | 113 ± 16 (20) | 107 ± 15 (35) | 109 ± 11 (55) |

Error bars are ±95% confidence limits with the number of replicates in brackets. Steady-state $CH_3NH_2$ and $CH_3CH_2NH_2$ permeabilities were measured at 10 to 200 $\mu$M (total amine, R-$NH_2$ + R-$NH_3^+$) and an external pH of about 10.

## Results

### Stereological Data on Synechococcus

Table 1 shows data on the size, cytoplasmic membrane surface area, intracellular volumes and numerical densities of *Synechococcus* grown under standard conditions. Cytoplasmic membrane surface areas and intracellular volumes are expressed on a protein basis. The intracellular volume of *Synechococcus* is comparable to previously published values (Boussiba et al., 1984*b*).

### Intracellular Ammonia Pools

The extracellular ammonia ($NH_3$ + $NH_4^+$) found when *Synechococcus* cells were incubated in "ammonia-free" BG-11/buffer at pH 10, remained at less than 2 $\mu$M. The intracellular $NH_3/NH_4^+$ pool in control cells was 351 ± 70 $\mu$M (24) and 467 ± 94 $\mu$M (20) in DCMU poisoned cells, based on the release of ammonia by the cells when poisoned with 50 mM phenol. This "background" intracellular pool of ammonia was taken into account in the calculation of the apparent intracellular pH from the accumulation of ammonia and the $NH_3$ flux.

### Exchange Fluxes of Methylamine and Ethylamine

The permeability of $CH_3NH_2$ ($P_{MA}$) in *Synechococcus* was measured in uptake and efflux experiments using filtration methods; the results were not significantly different and so have been combined to yield mean values (Table 2). $P_{MA}$ and $P_{EA}$ were measured at concentrations from 10 to 200 $\mu$M; as no systematic effect of concentration was found, overall means were calculated. $P_{MA}$ and $P_{EA}$ were also measured using the silicone oil centrifugation technique. Table 2 shows they were not significantly different from those measured using the membrane filter method and so overall means ±95% confidence limits could be calculated. $P_{MA}$ and $P_{EA}$ are of the order of 100 $\mu$m $sec^{-1}$ across the cytoplasmic membrane of *Synechococcus*; $CH_3NH_2$ is less permeable ($P_{MA}$ about 90 $\mu$m $sec^{-1}$) than $CH_3CH_2NH_2$ ($P_{EA}$ about 120 $\mu$M $sec^{-1}$).

### Net Fluxes of Amines

Only net uptake or loss of ammonia by the cells can be conveniently measured. For $^{14}$C-methylamine and $^{14}$C-ethylamine both net fluxes and exchange

**Table 3.** Apparent permeabilities of amines for *Synechococcus* based on net uptake fluxes

| Amine | Conditions | Apparent permeability ($\mu$m sec$^{-1}$) |
|---|---|---|
| $NH_3$ | Control | 9.4 ± 1.2 (22)[a] |
| | DCMU poisoned cells | 6.44 ± 1.25 (13) |
| $CH_3NH_2$ | Control, no ammonia | 38.3 ± 4.15 (16) |
| | 200 $\mu$M ammonia | 49 ± 6.6 (22) |
| $CH_3CH_2NH_2$ | 10 $\mu$M, no ammonia | 73 ± 6.5 (24) |
| | 100 $\mu$M, no ammonia | 43 ± 14 (12) |
| | 100 $\mu$M, 2 $\mu$M valinomycin, no ammonia | 33 ± 6.6 (12) |
| | 100 $\mu$M, 200 $\mu$M ammonia | 73 ± 12 (12) |

Error bars are ±95% confidence limits with the number of replicates in brackets. Apparent $NH_3$ permeabilities were measured at $[NH_3 + NH_4^+]$ of 100 to 120 $\mu$M. The apparent $CH_3NH_2$ permeabilities in net uptake experiments were measured at total methylamine concentrations of 100 $\mu$M; the apparent permeability of $CH_3CH_2NH_2$ was measured in 10 and 100 $\mu$M total ethylamine. The ammonia experiments were conducted at an external pH of 9.5 to 10 and the methylamine and ethylamine experiments at pH 9.8 to 10.1.

[a] Note that the calculated permeability of $NH_3$ in control cells is an overestimate (*see* text).

fluxes can be determined. The net flux of uncharged amine may have been limited by the proton flux and/or fluxes of other ions involved in intracellular charge balance. Hence apparent permeabilities based on net fluxes may be less than the true permeability of the species.

Most $NH_3$ entering a *Synechococcus* cell will be converted to $NH_4^+$ because the pH of the cytoplasm is between 7 and 8. Thus net uptake of $NH_3$ should lead to an alkalinization of the cytoplasm which may be compensated for by a net uptake of protons (Padan, Zilberstein & Rottenberg, 1976; Masamoto & Nishimura, 1978; Gibson, 1981). Thus if the intracellular pH is maintained, one +ve charge must move across the cell membrane for each uncharged amine molecule taken up; the charge displacement involved in this maintenance of charge balance can be large. For example, an apparent permeability of 50 $\mu$m sec$^{-1}$ of an uncharged amine at a concentration of 10 $\mu$M of uncharged amine in the bulk medium is equivalent to a flux of 500 nmol m$^{-2}$ sec$^{-1}$. Barr, Koh and Ryan (1974) used net efflux of ammonia across the plasmalemma of *Nitella* as a minimum estimate of proton efflux.

$NH_3$ permeability ($P_A$) was measured using the silicone oil/centrifugation method. The ammonia concentration at $t = 0$, was determined by adding 10 $\mu$l of $NH_4Cl$ standard solution to a 1-ml aliquot of the supernatant of a previously centrifuged cell suspension. The intracellular ammonia pool of cells in the "ammonia-free" BG-11 buffer was usually measured on the same batch of cells.

The $P_A$ in control and DCMU poisoned cells are compared in Table 3. Cells were treated with 10 $\mu$M DCMU for 5 min before an experiment. The $t$ test shows that the apparent $NH_3$ permeability of control cells were significantly different from that of DCMU-treated cells. Apparent permeabilities based on net fluxes of $CH_3NH_2$ and $CH_3CH_2NH_2$ are also shown in Table 3. Thus $NH_3$ is much less permeable than either methylamine or ethylamine in this organism.

The apparent permeabilities of methylamine and ethylamine based on net fluxes ($P_{MA}$ = 38.3 ± 4.15 $\mu$M sec$^{-1}$, $P_{EA}$ = 43 ± 14 $\mu$m sec$^{-1}$; Table 3) are considerably below those based on exchange fluxes ($P_{MA}$ = 84.6 ± 9.47, $P_{EA}$ = 109 ± 11 $\mu$m sec$^{-1}$; Table 2). The apparent permeability of ethylamine is significantly higher in 1.51 $\mu$M than in 15.1 $\mu$M $CH_3CH_2NH_2$. The net fluxes of methylamine and ethylamine were thus rate limited. The provision of a permeant amine (ammonia) to exchange $^{14}$C-amine significantly increased the apparent permeability of both methylamine and ethylamine. The $K^+$ specific ionophore, valinomycin, did not increase the apparent permeability of $CH_3CH_2NH_2$ (Table 3). The net uptake of ethylamine was therefore not limited by charge balance because this would have been compensated by a net flux of $K^+$.

## Intracellular pH Measurements

Table 4 shows the calculated intracellular pH values for *Synechococcus* in media at pH 9.5 to 10.1 when the accumulated amines have reached equilibrium. The values derived from both methylamine and ethylamine in exchange flux and net flux experiments are not significantly different (pH 7.3), but the apparent intracellular pH based on the uptake of ammonia is significantly lower (pH 7.1).

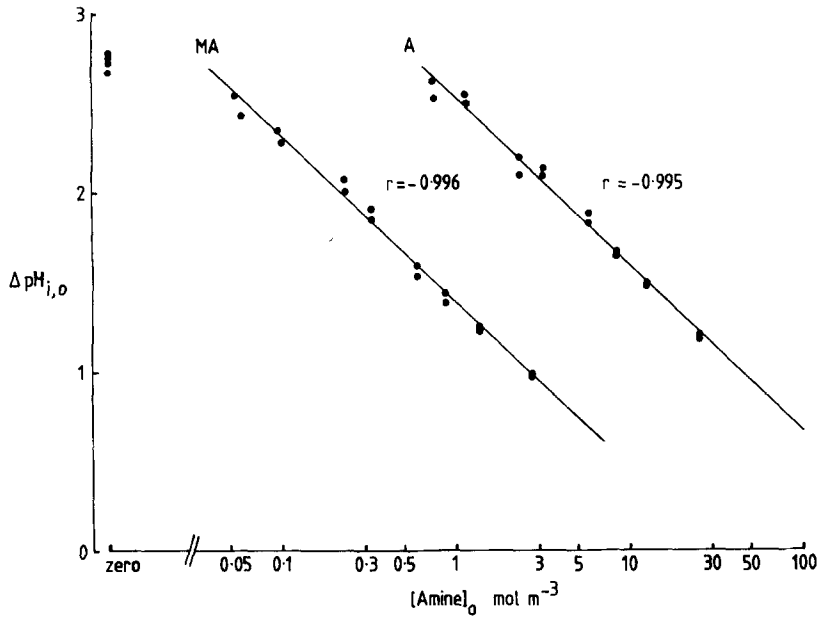


**Fig.** pH gradient across the cell membrane *vs.* log [$NH_3$] and log [$CH_3NH_2$]. The pH gradient across the cell membrane *vs.* the logarithm of the ammonia concentration (*A*) and of the methylamine concentration (*MA*) was measured on the same suspension of cells in BG-11 and 10 mM carbonate buffer at pH 10.01 as described in the text. The pH gradient was calculated from the accumulation ratio of 100 μM $^{14}$C-ethylamine. The zero controls are shown on the left. The lines were fitted by linear regression; the correlation coefficients are shown and the slopes and constants of the regressions are tabulated in Table 5

**Table 4.** Apparent intracellular pH of *Synechococcus*

| Amine | Apparent intracellular pH |
|---|---|
| Exchange flux experiments | |
| Methylamine | 7.35 ± 0.03 (76) |
| Ethylamine | 7.32 ± 0.03 (55) |
| Net flux experiments | |
| Ammonia | 7.11 ± 0.04 (39) |
| Methylamine | 7.33 ± 0.04 (22) |
| Ethylamine | 7.33 ± 0.03 (60) |

Apparent intracellular pH of *Synechococcus* using uptake of ammonia, methylamine or ethylamine. The data is derived from the experiments shown in Tables 2 and 3. Error bars are ±95% confidence limits with the number of replicates in brackets.

## Relative Permeabilities of Amines

The relative permeabilities of amines was determined from the effect of high concentrations (0.1 to 25 mM) of amines at alkaline pH (pH 10) on the pH gradient across the cytoplasmic membrane calculated from the accumulation ratio of $^{14}$C-ethylamine. Preliminary experiments showed that ammonia, methylamine and ethylamine had no significant effect on the apparent $\Delta pH_{i,o}$ until a threshold concentration was reached; thus uncharged $NH_3$ had no effect when below 1.0 mM.

The Figure shows the pH gradient across the cell membrane of *Synechococcus* as functions of log [$NH_3$] (A) and log [$CH_3NH_2$] (MA) in the bathing electrolyte when the external pH was 10.01. Lines were fitted by linear regression [Eq. (A9a)] and have correlation coefficients of 0.996 and 0.997, respectively; the slopes ($m$ ± 95% conf. limits) and intercepts of the regression lines ($b$ ± 95% conf. limits) are shown in Table 5. Methylamine is a much more potent dissipator of the pH gradient, being effective at concentrations of about 1/15th that of $NH_3$. The ammonia and methylamine curves are parallel.

The regression data for the effect of uncharged amine concentrations on $\Delta pH_{i,o}$ in separate experiments are summarized in Table 5. Part A compares $CH_3NH_2$ and $CH_3CH_2NH_2$ and Part B that of $NH_3$ and $CH_3NH_2$ (from the Figure). The slopes of the regression lines (the exponent $m$) are about −0.9; the slopes of the ΔpH *vs.* concentration of uncharged amine curves were parallel when measured on the same batch of cells. The value of the constant ($b$) varies depending on the amine; the ratio of these constants should be governed by the relative permeabilities of the amine species.

The constant ($b$) of $CH_3NH_2$ is only slightly greater than that of $CH_3CH_2NH_2$; yielding a permeability ratio of about 1.23. This apparent permeability ratio compares very well to a value of 1.29 ± 0.19 calculated from the ratio of the experimentally determined permeabilities of $CH_3NH_2$ and $CH_3CH_2NH_2$ shown in Table 2. The permeability ratio of $NH_3$ to $CH_3NH_2$, on the other hand, is very low, about 1 : 15 (6.7 ± 0.8%); taking the mean $P_{MA}$ shown in Table 2, this predicts a permeability of $NH_3$ of 5.7 ± 0.9 μM sec$^{-1}$, which is not significantly different from that measured in DCMU-treated cells (Table 3).

**Table 5.** Effects of amines on ΔpH and permeability ratios of amines

| A. $\Delta pH_{i,o}$ vs. log [ethylamine] and log [methylamine] | Correlation ($r$) | Slope ($m$) | Intercept log ($b$) |
|---|---|---|---|
| Ethylamine ($n$ = 14) | −0.997 | −0.8522 ± 0.04248 | 1.2706 ± 0.03149 |
| Methylamine ($n$ = 14) | −0.998 | −0.8581 ± 0.03028 | 1.3603 ± 0.02015 |
| **B. $\Delta pH_{i,o}$ vs. log [ammonia] and log [methylamine]** | **Correlation ($r$)** | **Slope ($m$)** | **Intercept log ($b$)** |
| Ammonia ($n$ = 16) | −0.995 | −0.9260 ± 0.05498 | 2.5229 ± 0.04370 |
| Methylamine ($n$ = 16) | −0.996 | −0.9189 ± 0.04921 | 1.3843 ± 0.03254 |
| Permeability ratios | | $P_{EA}/P_{MA}$ = 1.23 $^{+0.10}_{-0.096}$ | $P_A/P_{MA}$ = 0.0669 $^{+0.008}_{-0.007}$ |

ΔpH gradient across the cell membrane of *Synechococcus vs.* the log of the concentration of unchanged amine. Lines of best fit were calculated by linear regression and the error bars of the slopes ($m$) and constant (log $b$) were calculated as described by Zar (1974).

## Discussion

Measurements of the permeability of uncharged amines can demonstrate whether a steady-state uptake rate and/or the size of the steady-state intracellular pool observed under specific experimental conditions may be accounted for by passive diffusion of the uncharged species. This is a straightforward application of Fick's law [Appendix: Eq. (A1)] as the null hypothesis, but some practical difficulties arise. It is necessary to establish that the permeability value used was measured under conditions where the amine was passively distributing across the cytoplasmic membrane. In ammonia experiments it is not valid to assume that the intracellular concentration of the unchanged species is zero, but the intracellular ammonia pool may be difficult to measure.

Values for the intracellular pH, calculated on the basis of the equilibrium accumulation of methylamine and ethylamine, are similar (Table 4). This is good evidence that the uptake of methylamine and ethylamine was by passive diffusion driven by the pH gradient rather than a result of cation transport or metabolism of these amines. The apparent intracellular pH calculated using the ammonia data is about 0.2 pH units lower than that calculated using methylamine or ethylamine, suggesting that some transport of $NH_4^+$ might have been occurring. An apparent intracellular pH of about 7.3 is comparable to published values for *Synechococcus* species (Kallas & Dahlquist, 1981; Gibson, 1981; Miller, Turpin & Canvin, 1984) and other prokaryotes (Padan et al., 1976; Masamoto & Nishimura, 1978; Reed, Rowell & Stewart, 1980). *Synechococcus* can maintain a ΔpH of nearly +3 units across the cell membrane; Miller et al. (1984) also noted that *Synechococcus leopoliensis* had a very efficient pH-stat mechanism in alkaline media (pH 9.6).

The intracellular ammonia pools ($NH_3 + NH_4^+$) found in the present study (about 0.4 mM) are considerably below those previously reported for cells incubated at lower pH (Boussiba et al., 1984*a*; Kleiner, 1985*a*,*b*). However, the size of the intracellular $NH_3 + NH_4^+$ pools of *Synechococcus* in alkaline ammonia-free media still indicates that a transport system for $NH_4^+$ is present in these cells. If the intracellular pH of *Synechococcus* was taken to be about 7.3 (Table 4) and the total intracellular $NH_3 + NH_4^+$ pool was 351 ± 70 μM, then [$NH_3$] within the cells would be 3.9 ± 0.77 μM. The bulk electrolyte at pH 10 contained less than 2 μM total ammonia species and so could contain no more than 2 μM $NH_3$. Thus the cells were maintaining a concentration gradient of at least 2 μM $NH_3$.

The permeabilities of $CH_3NH_2$ found in the present study for *Synechococcus* (about 80 μm $sec^{-1}$) are much higher than those in *Klebsiella* (15 μm $sec^{-1}$) (Kleiner, 1985*a*). Although a permeability of about 2 μm $sec^{-1}$ (Smith & Walker, 1978; Nobel, 1983) has often been used as a standard value for the permeability of ammonia and methylamine in eukaryotic cells, more recent measurements of $P_{MA}$ by Walker, Smith and Beilby (1979),

Beilby, Smith, and Walker (1980) and Ritchie (1987) on the giant-celled alga, *Chara*, have yielded considerably higher values ranging from 6 to 18 $\mu$m $sec^{-1}$. The permeability of ammonia in charophytes is about 6-7 $\mu$m $sec^{-1}$ (*Nitella*: Barr et al., 1974; *Chara*: Ritchie, 1987). Kleiner (1981) was correct in suspecting that $P_A$ and $P_{MA}$ were frequently underestimated.

Walter and Gutknecht (1986) have recently reported that artificial phosphatidylcholine membranes have even higher amine permeabilities of about 1000 $\mu$m $sec^{-1}$ but found that amine solubility in organic solvents was highly variable. The permeabilities of amines across artificial membranes would vary widely depending on the phospholipid used. No natural membrane has been reported to have such a high amine permeability.

In *Synechococcus* the apparent permeability of ammonia obtained by direct measurement is much lower than that of methylamine and ethylamine (Tables 2 and 3); this is confirmed by the experiments in which the effect of amines on $\Delta$pH were investigated (Table 5 and the Figure). Thus two separate lines of evidence show that methylamine is more permeable than ammonia in *Synechococcus*. The apparent permeabilities of methylamine and ethylamine vary according to the way in which they are measured. Permeabilities based on exchange fluxes (i.e., steady state) are higher than those based on the net uptake of these amines (Table 2 *vs.* Table 3), indicating that net uptake fluxes were rate limited, and consequently the amine permeabilities were underestimated. The maximum net uptake flux of these amines at pH 10 in 100 $\mu$M total amine is approximately 700 nmol $m^{-2}$ $sec^{-1}$ ($\varnothing_{MA}$ (max) = 700 $\pm$ 76 nmol $m^{-2}$ $sec^{-1}$; $\varnothing_{EA}$ (max) = 650 $\pm$ 210 nmol $m^{-2}$ $sec^{-1}$). The apparent permeabilities of methylamine and ethylamine in net uptake experiments ($P_{MA,net}$ and $P_{EA,net}$) increased in the presence of ammonia (Table 3) and $P_{EA,net}$ was significantly higher when measured in 1.51 $\mu$M than in 15.1 $\mu$M $CH_3CH_2NH_2$. The apparent $P_{EA,net}$ measured at lower concentrations would more closely approach that measured in exchange flux experiments where the net flux if zero. $P_{EA,net}$ was also unaffected by the presence of the potassium ionophore, valinomycin (Table 3). Net uptake of methylamine and ethylamine were thus rate limited by the net movement of protons required to maintain the intracellular pH while net uptake of an amine was taking place, rather than by charge imbalances.

Only net uptake of ammonia could be measured directly in the present study (Table 3), and it is not possible experimentally to eliminate $NH_4^+$ transport completely. The lower value for the intracellular pH using ammonia as a pH probe (Table 4) indicates that there was about 60% more ammonia inside the cells than could be accounted for by the pH gradient (Table 4). In control experiments, the apparent $P_A$ was 9.4 $\pm$ 1.2 (22) $\mu$m $sec^{-1}$, based on experiments at about pH 10 and 100 $\mu$M total ammonia; at this pH the extracellular concentration of $NH_3$ would be about 85 $\mu$M, yielding a net uptake flux of 800 $\pm$ 100 nmol $m^{-2}$ $sec^{-1}$, which is close to the rate-limited net uptake fluxes of methylamine and ethylamine. The apparent permeability of $NH_3$ was decreased by DCMU (Table 3), which blocks incorporation of ammonia into glutamine, presumably by depleting the ATP supply (Boussiba et al., 1984*b*). DCMU should have had no direct effect on the permeability of $NH_3$ but would have inhibited the metabolism of ammonia. The DCMU experiments (Table 3) and the effect of amines on the pH gradient (Table 5) yield estimates of the ammonia permeability of 6.44 $\pm$ 1.25 (13) and 5.7 $\pm$ 0.9 (32) $\mu$m $sec^{-1}$, respectively, and these results are not significantly different.

In studies where $^{14}$C-methylamine uptake has been used to identify $NH_4^+$ transport systems it has usually been implicitly assumed that the passive fluxes of $NH_3$ and $CH_3NH_2$ were similar. However, our measurements demonstrate that passive $CH_3NH_2$ fluxes can be an order of magnitude greater than previously suspected. This means that the case for specific $NH_4^+$ carriers, based on experiments using $^{14}$C-methylamine as an analogue, may be less clear cut than usually thought. For example, Raven and Farquhar (1981) calculated an apparent $P_{MA}$ of about 17 $\mu$m $sec^{-1}$ across the plasmalemma of *Phaseolus vulgaris* (bean) leaf slices and, considering this value too high, concluded that methylammonium transport must be the major uptake mechanism and that bean leaf cells had a $NH_4^+$ transporter. An apparent $P_{MA}$ value of 17 $\mu$m $sec^{-1}$ is not really high enough to exclude passive diffusion of $CH_3NH_2$.

In several studies of putative $NH_4^+$ transport systems in bacteria, the accumulation ratio of $NH_4^+$ ($[NH_4^+]_i/[NH_4^+]_o$) is greater than that observed for methylamine ($[MA^+]_i/[MA^+]_o$) (*see* Kleiner, 1985*b*). Transport of $NH_4^+$ is usually thought necessary to at least balance the "leak" of $NH_3$ which is governed by Fick's Law (Kleiner, 1985*a,b*). If the permeability of methylamine is substantially greater than that of ammonia, as found in the present study, then these observations are readily accounted for by the greater passive "leak" of $CH_3NH_2$. Furthermore, the large difference in the passive leak of $NH_3$ and $CH_3NH_2$ would lead to a slower apparent uptake flux of $CH_3NH_3^+$ compared to $NH_4^+$. Such discrepancies have usually been attributed to discrimination properties of the amine cation transport

system (Gordon & Moore, 1981; Kleiner, 1985*b*; O'Hara et al., 1985).

The permeability values of $NH_3$ can be used to deduce some properties of the $NH_4^+$ transport system (facilitated diffusion and/or active transport) acting to balance an equal but opposite efflux or "leak" of $NH_3$ (Kleiner, 1985*b*). For *Synechococcus* in "ammonia-free" BG-11 using nitrate as the nitrogen source, $P_{NH_3} = 6.44 \pm 1.25$ $\mu$m sec$^{-1}$ and an intracellular $NH_3 + NH_4^+$ pool of $0.35 \pm 0.07$ mM (i.e., $[NH_3]_i = 3.9 \pm 0.77$ $\mu$M), a constant uphill flux of $25 \pm 7$ nmol m$^{-2}$ sec$^{-1}$ implies. Such a flux is not large compared to the rates of transport of other ions across the cytoplasmic membranes of either prokaryotic or eukaryotic cells (Slayman, 1970; Hope & Walker, 1975; Smith & Raven, 1976; Reed, Rowell & Stewart, 1981*a*,*b*; Spanswick, 1981).

Gordon and Moore (1981) proposed that phosphate bond energy was required for $NH_4^+$ transport in *Azotobacter*. Suppose *Synechococcus* cells grown on nitrate used an ATP-driven pump to recycle $NH_4^+$ back into the cells to counterbalance the "leak" of the $NH_3$ synthesized intracellularly from the nitrate ions taken up. The electrochemical potential of an $[NH_4^+]_i/[NH_4^+]_o$ gradient of about 200 is equivalent to 13 kJ mol$^{-1}$; the hydrolysis of ATP (50 kJ mol$^{-1}$) would theoretically provide enough energy to transport two $NH_4^+$ per ATP hydrolyzed. The power requirements needed for transporting $NH_4^+$ at a rate of 25 nmol m$^{-2}$ sec$^{-1}$ would amount to 0.6 mW m$^{-2}$ (Reid & Walker, 1983; Ritchie, 1985). The measured rate of ammonia metabolism in *Synechococcus* is $6.4 \pm 0.67$ nmol m$^{-2}$ sec$^{-1}$ ($n = 36$); and if glutamine synthetase uses one ATP per ammonia (Meister, 1974), this is equivalent to a power consumption rate of $0.32 \pm 0.034$ mW m$^{-2}$. Thus the maintenance of the intracellular ammonium pool of *Synechococcus* is about twice as costly to the cell as the fixation step (ammonia + glutamate into glutamine). These results are similar to those of Kleiner (1985*a*) who estimated that about 4 ATPs were used in retaining ammonia within *Klebsiella* cells per ATP used in the synthesis of glutamine.

Work on *Klebsiella* (Kleiner, 1985*a*,*b*) and on the eukaryotic *Chara* cell (Walker et al., 1979; Beilby et al., 1980) suggest that $NH_4^+$ is taken up by a facilitated diffusion mechanism driven by the membrane potential. Preliminary data, based on the uptake of $^{86}Rb^+$ in the presence of 5 $\mu$M valinomycin (Mitchell & Moyle, 1969; Mewes & Rafael, 1981) show that the membrane potential of *Synechococcus* is about $-125 \pm 5$ mV (20) at pH 10 (R.J. Ritchie and J. Gibson, *unpublished*). For cells growing on nitrate in "ammonia-free" media (< 1 $\mu$M $NH_4^+$) the equilibrium potential of $NH_4^+$ ($\Delta\mu_{NH_4^+}$) would be more than $-150$ mV, and so it is difficult at present to distinguish between an amine uniporter driven by the membrane potential and an ATP-driven pump.

An $NH_4^+/H^+$ antiport system driven by the proton motive force ($pmf_{i,o}$) (Mitchell, 1966; West, 1980; Nicholls, 1982; Ritchie, 1985) cannot account for the size of the intracellular ammonia pools ($NH_3 + NH_4^+$) of cells in alkaline "ammonia-free" media because the membrane potential and pH gradient tend to cancel each other out if the interior of the cell is maintained near neutrality (Krulwich, 1986). The $\Delta$pH gradient is equivalent to about +160 mV and so the $pmf_{i,o}$ available is only about +30 mV, whereas the electrochemical potential of $NH_4^+$ is more than $-150$ mV. The $pmf_{i,o}$ is too low and of the wrong sign.

We have shown that it is possible to measure amine permeabilities. The very large difference between the permeabilities of ammonia and higher amines in *Synechococcus* may or may not be a general feature of prokaryotic cells (*cf*: Kleiner, 1985*a*); however, the permeability of $NH_3$ is also about an order of magnitude lower than that of methylamine and ethylamine in *Rhodobacter sphaeroides* (R.J. Ritchie and J. Gibson, *unpublished*). In the eukaryotic *Chara* cell, in contrast, they are very similar (Ritchie, 1987). Estimates of $NH_4^+$ transport derived from $^{14}C$-methylamine experiments may be very misleading. The distinction between permeabilities based on exchange and net fluxes has not been pointed out before. Permeability values provide a means to investigate quantitatively the problem of $NH_3$ diffusion *vs.* $NH_4^+$ transport.

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## References

Allen, M.M. 1968. Simple conditions for growth of unicellular blue-green algae. *J. Phycol.* **4**:1–3

Atkins, G.L. 1969. Multicompartment Models in Biological Systems. Methuen, London

Barr, C.E., Koh, M.S., Ryan, T.E. 1974. $NH_3$ efflux as a means for measuring $H^+$ extrusion in *Nitella*. *In:* Membrane Transport in Plants. U. Zimmermann and J. Dainty, editors. pp. 180–185. Springer-Verlag, New York–Heidlberg–Berlin

Beilby, M.J., Smith, F.A., Walker, N.A. 1980. The processes transporting $CH_3NH_2$ and $CH_3NH_3^+$ across the *Chara* plasmalemma. *In:* Plant Membrane Transport: Current Conceptual Issues. R.M. Spanswick, W.J. Lucas, and J. Dainty, editors. pp. 601–602. Elsevier/North Holland Biomedical, Amsterdam

Boussiba, S., Dilling, W., Gibson, J. 1984*a*. Methylammonium transport in *Anacystis nidulans* R-2. *J. Bacteriol.* **160**:204–210

Boussiba, S., Gibson, J. 1985. The role of glutamine synthetase activity in ammonium and methylammonium transport in *Anacystis nidulans*. *FEBS Lett.* **180:**13–16

Boussiba, S., Resch, C.M., Gibson, J. 1984*b*. Ammonia uptake and retention in some cyanobacteria. *Arch. Microbiol.* **138:**287–292

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye-binding. *Anal. Biochem.* **72:**248–254

Butler, T.C., Waddell, W.J., Poole, D.T. 1967. Intracellular pH based on the distribution of weak electrolytes. *Fed. Proc.* **26:**1327–1332

Collander, R. 1954. The permeability of *Nitella* cells to non-electrolytes. *Physiol. Plant.* **7:**420–445

Crofts, A.R. 1966*a*. Uptake of ammonium ion by chloroplasts, and the mechanism of amine uncoupling. *Biochem. Biophys. Res. Commun.* **24:**127–134

Crofts, A.R. 1966*b*. Uptake of ammonium by chloroplasts and its relation to photophosphorylation. *Biochem. Biophys. Res. Commun.* **24:**725–731

Crofts, A.R. 1967. Amine uncoupling of energy transfer in chloroplasts. *J. Biol. Chem.* **242:**3352–3359

Gaensslen, R.E., McCarty, R.E. 1971. Amine uptake in chloroplasts. *Arch. Biochem. Biophys.* **147:**55–65

Gibson, J. 1981. Movement of acetate across the cytoplasmic membrane of the unicellular cyanobacteria *Synechococcus* and *Aphanocapsa*. *Arch. Microbiol.* **130:**175–179

Gibson, J. 1984. Nutrient transport by anoxygenic and oxygenic photosynthetic bacteria. *Annu. Rev. Microbiol.* **38:**135–59

Gordon, J.K., Moore, R.A. 1981. Ammonium and methylammonium transport by the nitrogen-fixing bacterium *Azotobacter vinelandii*. *J. Bacteriol.* **148:**435–442

Hackette, S.L., Skye, G.E., Burton, C.U., Segel, H.I. 1970. Characterisation of an ammonium transport system in filamentous fungi with methylammonium-$^{14}C$ as substrate. *J. Biol. Chem.* **245:**4241–4250

Heldt, H.W., Werdan, K., Milovancev, M., Geller, G. 1973. Alkalization of the chloroplast stroma caused by light dependent proton flux into the thylacoid space. *Biochim. Biophys. Acta* **314:**224–241

Hind, G., Whittingham, C.P. 1963. Reduction of ferricyanide by choloroplasts in the presence of nitrogenous bases. *Biochim. Biophys. Acta* **75:**194–202

Hope, A.B., Walker, N.A. 1975. The Physiology of Giant Algal Cells. Cambridge University Press, Cambridge

Howitz, K.T., McCarty, R.E. 1985. Kinetic characteristics of the chloroplast envelope glycolate transporter. *Biochemistry* **24:**2645–2652

Ihlenfeldt, M.J.A., Gibson, J. 1975. $CO_2$ fixation and its regulation in *Anacystis nidulans* (Synechococcus). *Arch Microbiol.* **102:**13–21

Kallas, T., Dahlquist, F.W. 1981. Phosphorous-31 nuclear magnetic resonance analysis of internal pH during photosynthesis in the cyanobacterium *Synechococcus*. *Biochemistry* **20:**5900–5907

Kleiner, D. 1981. The transport of $NH_3$ and $NH_4^+$ across biological membranes. *Biochim. Biophys. Acta* **639:**41–52

Kleiner, D. 1985*a*. Energy expenditure for cyclic retention of $NH_3/NH_4^+$ during $N_2$ fixation by *Klebsiella pneumoniae*. *FEBS Lett.* **187:**237–239

Kleiner, D. 1985*b*. Bacterial ammonium transport. *FEMS Microbiol. Rev.* **32:**87–100

Krulwich, T.A. 1986. Bioenergetics of alkalophilic bacteria. *J. Membrane Biol.* **89:**113–125

Masamoto, K., Nishimura, M. 1978. Estimation of the internal pH in cells of blue-green algae in dark and under illumination. *J. Biochem.* **82:**483–487

McCarty, R.E., Coleman, C.H. 1970. Effect of hydrocarbon chain length on the uncoupling of photophosphorylation by amines. *Arch. Biochem. Biophys.* **141:**198–206

Meister, A. 1974. Glutamine synthetase of mammals. *In:* The Enzymes. Vol. 10 (3rd Ed.) pp. 699–754. P.D. Boyer, editor. Academic, New York–London

Mewes, H.-H.W., Rafael, J. 1981. The 2-(dimethylaminostyryl)-1-methylpyridinium cation as indicator of the mitochondrial membrane potential. *FEBS Lett.* **131:**7–10

Miller, A.G., Turpin, D.H., Canvin, D.T. 1984. $Na^+$ requirement for growth, photosynthesis, and pH regulation in the alkalotolerant cyanobacterium *Synechococcus leopoliensis*. *J. Bacteriol.* **159:**100–106

Mitchell, P. 1966. Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation. Glynn Research Laboratories, Bodmin, Cornwall, England

Mitchell, P., Moyle, J. 1969. Estimation of membrane potential and pH difference across the cristae of rat liver mitochondria. *Eur. J. Biochem.* **7:**471–484

Nicholls, D.G. 1982. Bioenergetics: An Introduction to the Chemiosmotic Theory. Academic, London

Nobel, P.S. 1983. Introduction to Biophysical Plant Physiology, Freeman, San Francisco

O'Hara, G.W., Riley, I.T., Glenn, A.R., Dilworth, M.J. 1985. The ammonium permease of *Rhizobium leguminosarum* MNF 3841. *J. Gen. Microbiol.* **131:**757–764

Padan, E., Zilberstein, D., Rottenberg, H. 1976. The proton electrochemical gradient in *Escherichia coli* cells. *Eur. J. Biochem.* **63:**533–541

Raven, J.A., Farquhar, G.D. 1981. Methylammonium transport in *Phaseolus vulgaris* leaf slices. *Plant Physiol.* **67:**859–863

Reed, R.H., Rowell, P., Stewart, W.D.P. 1980. Components of the proton electrochemical potential gradient in *Anabaena varibilis*. *Biochem. Soc. Trans.* **8:**707–708

Reed, R.H., Rowell, P., Stewart, W.D.P. 1981*a*. Uptake of potassium and rubidium ions by the cyanobacterium *Anabaena variabilis*. *FEMS Microbiol. Lett.* **11:**233–236

Reed, R.H., Rowell, P., Stewart, W.D.P. 1981*b*. Characterisation of the transport of potassium ions in the cyanobacterium *Anabaena variabilis* Kutz. *Eur. J. Biochem.* **116:**323–330

Reid, R.J., Walker, N.A. 1983. Adenylate concentrations in *Chara:* Variability, effects of inhibitors and the relationship to protoplasmic streaming. *Aust. J. Plant Physiol.* **10:**373–383

Ritchie, R.J. 1985. Energetic considerations of ion transport in *Enteromorpha intestinalis* (L.) Link. *New Phytol.* **100:**5–24

Ritchie, R.J. 1987. The permeability of ammonia, methylamine and ethylamine in the charophyte, *Chara corallina* (C. australis). *J. Exp. Bot. (in press)*

Segel, I.H. 1976. Biochemical Calculations. John Wiley & Sons, New York

Slayman, C.L. 1970. Movement of ions and electrogenesis in microrganisms. *Am. Zool.* **10:**377–392

Smith, F.A., Raven, J.A. 1976. $H^+$ transport and regulation of cell pH. *In:* Encyclopedia of Plant Physiology. (New Series) Vol. 2, part A: Cells. pp. 317–346. U. Luttge and M.G. Pitman, editors. Springer-Verlag, Berlin

Smith, F.A., Walker, N.A. 1978. Entry of methylammonium ions into *Chara* internodal cells. *J. Exp. Bot.* **29:**107–120

Solorzano, L. 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. *Limnol. Oceanogr.* **14:**799–801

Spanswick, R.M. 1981. Electrogenic ion pumps. *Annu. Rev. Plant Physiol.* **32**:267–289

Walker, N.A., Pitman, M.G. 1976. Measurements of fluxes across membranes. *In:* Encyclopedia of Plant Physiology. (New Series) Vol. 2, Part A: Cells. pp. 93–126. (U. Luttge and M.G. Pitman, editors.) Springer-Verlag, Berlin

Walker, N.A., Smith, F.A., Beilby, M.J. 1979. Amine uniport at the plasmalemma of charophyte cells. II. Ratio of matter to charge transported and permeability of free base. *J. Membrane Biol.* **49**:283–296

Walter, A., Gutknecht, J. 1986. Permeability of small nonelectrolytes through lipid bilayer membranes. *J. Membrane Biol.* **90**:207–217

West, I.A. 1980. Energy coupling in secondary active transport. *Biochim. Biophys. Acta* **604**:91–126

Zar, J.H. 1974. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, N.J.

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## Appendix

### Measurement of Permeability

Fick's first law states that the flux of an ideal uncharged species ($j$) across a diffusion barrier, such as a biological membrane, between two compartments, compartment $o$ (the bulk medium) and compartment $i$ (the inside of a cell), is proportional to the concentration gradient across the membrane (Nobel, 1983). The permeability ($P$) includes terms for the diffusion coefficient ($D$), the partition coefficient ($K$) of the species ($j$) between the aqueous and membrane phases and the width of the membrane. The permeability of a species is usually given in the dimensions m · $sec^{-1}$. Thus,

$$P = \frac{\varnothing_{o,i}}{\Delta[C]_{o,i}} \tag{A1}$$

where,

$\varnothing_{o,i}$ is the flux (usually in mol $m^{-2}$ $sec^{-1}$) of species ($j$) from compartment $o$ to compartment $i$, $\Delta\,[C]_{o,i}$ is the concentration gradient of species ($j$) between compartments $o$ and $i$ ($[C]_o - [C]_i$) in mol $m^{-3}$,

The time course of equilibration of an unchanged species, between two compartments $o$ and $i$, can be described by simple exponential functions. For uptake into compartment $i$,

$$a_{i,t} = a_{i,\infty}\,(1 - e^{-kt}) \tag{A2}$$

where $a_{i,t}$ is the uptake of the diffusable species in compartment ($j$) at time ($t$) in mol $m^{-2}$; and $a_{i,\infty}$ is the total uptake at equilibrium (infinite time).

Differentiating Eq. (A2), eliminating $k$, and substituting into Eq. (A1) yields

$$P = \frac{a_{i,\infty} \cdot \ln\{a_{i,\infty}/(a_{i,\infty} - a_{i,t})\}}{t\Delta[C]_{o,i}}. \tag{A3}$$

In experiments where species ($j$) is initially absent from the intracellular phase, Eq. (A3) simplifies to

$$P = \frac{a_{i,\infty}.\ln\{a_{i,\infty}/(a_{i,\infty} - a_{i,t})\}}{t[C]_{o,t(0)}} \tag{A4}$$

where $[C]_{o,t(0)}$ is the concentration of the diffusible species in the external medium (bulk electrolyte), at zero time.

Equation (A4) can be used to calculate apparent permeabilities based on the net uptake of a species and is also appropriate for the measurement of exchange fluxes, *i.e.*, radioactive tracer experiments in which the unlabeled species ($j$) is in the steady state between the intracellular and bulk electrolyte phases, the labeled species is added to the bulk electrolyte, and its uptake into the cells followed. Equation (A4) can also be used to calculate apparent permeabilities based on the net uptake of a species.

If the loss of a species from the bulk medium (compartment $o$) can be followed more easily, then taking $a_{i,\infty}$ as the total uptake by the cells at equilibrium ($a_{i,\infty} = a_{o,t(0)} - a_{o,\infty}$), and following a similar procedure as outlined above [Eqs. (A1) to (A4), then

$$P = \frac{a_{i,\infty} \cdot \ln\{a_{i,\infty}/(a_{o,t} - a_{i,\infty})\}}{t\Delta[C]_{o,i}} \tag{A5}$$

and if the initial intracellular concentration is zero this simplifies to

$$P = \frac{a_{i,\infty} \cdot \ln\{a_{i,\infty}/(a_{o,t} - a_{i,\infty})\}}{t[C]_{o,t(0)}}. \tag{A6}$$

Thus the permeability of a species which is not metabolized can be calculated if the net loss or uptake of the species by either compartment $i$ or $o$ can be measured at equilibrium and at an intermediate time $t$ before equilibrium has been achieved. For uncharged species, such as oxygen and water, at equilibrium $[C]_i = [C]_o$, and so Eqs. (A3)-(A6) can be simplified to forms in which only the volume-to-surface area ratio of the cells need to be known. Thus Eq. (A4) reduces to

$$P = \frac{V}{A} \cdot \frac{\ln\{a_{i,\infty}/(a_{i,\infty} - c_{i,t})\}}{t}. \tag{A7}$$

Equation (A7) is often quoted in studies of permeability (Collander, 1954; Nobel, 1983) but it is not applicable to weak electrolytes such as the $CO_2/HCO_3^-$ and $NH_3/NH_4^+$ systems. In Eqs. (A4) and (A6), the equilibrium uptake of species ($j$) by the cell ($a_{i,\infty}$) refers to the total net uptake of both charged and uncharged species of a weak electrolyte system, whereas $[C]_{o,t(0)}$ refers only to the concentration of the uncharged species in the bulk electrolyte.

Equations (A3)-(A6) can be used to calculate permeabilities of the uncharged forms of weak acids and bases provided that the pH of the bulk electrolyte and the $pK_a$ of the weak electrolyte are known; the pH needs to be close enough to the $pK_a$ so that the relative contribution of an amine cation transport system to the total flux is small. Furthermore, if the intracellular pH is known, the Henderson-Hasselbalch equation can be used to simplify Eqs. (A4) and (A6) into forms where only the surface area-to-volume ratio of the cells need to be known. For example Eq. (4) simplifies to

$$P = \frac{V}{A} \cdot \frac{\ln\{a_{i,\infty}/(a_{i,\infty} - c_{i,t})\}}{t} \cdot (1 + 10^{pK_a - pH_i}). \tag{A8}$$

## Effects of Amines on the pH Gradient

Very low concentrations of amines have little effect on the pH gradient, but beyond a threshold concentration the pH gradient across a biological membrane ($\Delta pH_{i,o}$) will be logarithmically related to the external amine concentration (Hind & Whittingham, 1963). Thus if the external pH ($pH_o$) is held constant over a range of external amine concentrations ($[Amine]_o$)

$$\Delta pH_{i,o} = m \log[amine]_o + \log(b) \quad \text{(A9a)}$$

or

$$[H^+]_i = b \cdot [amine]^m. \quad \text{(A9b)}$$

For an ideal system the exponent $m$ would be $-1$. The constant ($b$) is directly proportional to the permeability of the amine and the ratio of the permeability of amines can be determined from the $y$ intercept of a graph of the form $y = mx + b$.

The permeabilities of methylamine and ethylamine can easily be measured experimentally and the calculated relative permeabilities compared to those derived from measurements of the effects of these amines on the pH gradient.