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Evidence for an ammonium transport system in free-living and symbiotic cyanobacteria

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Abstract. The free-living cyanobacterium *Anabaena variabilis* showed a biphasic pattern of $^{14}CH_3NH_3^+$ uptake. Initial accumulation (up to 60 s) was independent of $CH_3NH_3^+$ metabolism, but long-term uptake was dependent on its metabolism via glutamine synthetase (GS). The $CH_3NH_3^+$ was converted into methylglutamine which was not further metabolised. The addition of L-methionine-DL-sulphoximine (MSX), to inhibit GS, inhibited $CH_3NH_3^+$ metabolism, but did not affect the $CH_3NH_3^+$ transport system.

NH_4^+, when added after the addition of $^{14}CH_3NH_3^+$, caused the efflux of free $CH_3NH_3^+$; when added before $^{14}CH_3NH_3^+$, NH_4^+ inhibited its uptake indicating that both NH_4^+ and $CH_3NH_3^+$ share a common transport system. Carbonylcyanide *m*-chlorophenylhydrazone and triphenylmethylphosphonium both inhibited $CH_3NH_3^+$ accumulation indicating that the transport system was $\Delta\psi$-dependent. At pH 7 and at an external $CH_3NH_3^+$ concentration of 30 µmol dm^{-3}, *A. variabilis* showed a 40-fold intracellular accumulation of $CH_3NH_3^+$ (internal concentration 1.4 mmol dm^{-3}). Packets of the symbiotic cyanobacterium *Anabaena azollae*, directly isolated from the water fern *Azolla caroliniana*, also showed a $\Delta\psi$-dependent NH_4^+ transport system suggesting that the reduced inhibitory effect of NH_4^+ on nitrogenase cannot be attributed to the absence of an NH_4^+ transport system but is probably related to the reduced GS activity of the cyanobiont.

Key words: Ammonium transport – *Anabaena azollae* – *Anabaena variabilis* – Cyanobacteria – Methylammonium transport – Symbiosis

It is well established that in free-living N_2-fixing organisms, nitrogenase activity and synthesis are inhibited when the organisms are exposed to high concentrations of combined nitrogen (see Eady et al. 1982; Stewart 1982). This does not appear to be due to end-product inhibition (Gordon et al. 1981) but to an inhibition of *nif* gene transcription (see Postgate 1982) or to uncoupling by NH_4^+ (Salminen 1981; Murry et al. 1983). There is also evidence that in N_2-fixing cyanobacteria (Stewart and Rowell 1975; Ownby 1977; Thomas et al. 1982) nitrogenase inhibition involves a product of NH_4^+ assimilation via glutamine synthetase. In symbiotic systems nitrogenase inhibition on adding NH_4^+ does not occur or is reduced (Stewart and Rowell 1977; Houwaard 1979; Gadd et al. 1980; Laane et al. 1980; Rai et al. 1980; Peters et al. 1982) and it has been suggested that in bacteroids this is because they lack an NH_4^+ transport system (Laane et al. 1980). Kleiner et al. (1981) hypothesise that the NH_4^+ transport system may also be affected in symbiotic cyanobacteria. We have examined both the free-living heterocystous cyanobacterium *Anabaena variabilis* and the symbiotic packets of *Anabaena azollae* isolated from the water fern *Azolla caroliniana* for an NH_4^+ uptake system using $CH_3NH_3^+$, an analogue of NH_4^+. The findings are reported here.

Materials and methods

1. Organisms and growth conditions

Anabaena variabilis Kütz (ATCC 29413) was grown in continuous culture in BG-11_0 medium (Rippka et al. 1979) at 25° C and at a photon fluence rate of 50 µmol m^{-2} s^{-1}. The culture vessel was bubbled with air at a rate of 1 dm^3 min^{-1}. *Azolla* was grown and packets of *Anabaena azollae* were obtained from it as described by Peters and Mayne (1974).

2. Chlorophyll *a* estimations

Chlorophyll *a* was extracted from the cyanobacteria as before (Mackinney 1941) and absorption was measured at 663 nm using a Pye Unicam SP 1800 spectrophotometer.

3. Measurement of $^{14}CH_3NH_3^+$ uptake

The cyanobacterium, from a continuous culture unless indicated otherwise, was centrifuged, washed and resuspended in 10 mmol dm^{-3} HEPES/NaOH buffer pH 7 and equilibrated for 20 min at 25° C at a photon fluence rate of 50 µmol m^{-2} s^{-1}. ^{14}C-labelled $CH_3NH_3^+$ was then added to the incubation mixture to a final concentration of 30 µmol dm^{-3} (specific activity 10 kBq cm^{-3}), except where stated otherwise, and after 60 s the cells were separated from their bathing medium by microcentrifugation through Dow Corning 550 silicon oil/dinonylphthalate (40:60, v/v) into perchloric acid/water (15:85, v/v) (Scott and Nicholls 1980). In long term experiments, i.e. up to 1000 s, samples were incubated with $^{14}CH_3NH_3^+$ for varying periods of time and

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; GS, glutamine synthetase; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid; MSX, L-methionine-DL-sulphoximine; $\Delta\psi$, membrane potential; ΔpH, transmembrane pH difference; $TPMP^+$, triphenylmethylphosphonium

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then subjected to silicon oil microcentrifugation. Samples of the bathing medium and perchloric acid fractions were withdrawn for estimation of ^{14}C incorporation. ^{14}C-labelling was determined by liquid scintillation spectrometry using a toluene-based scintillant and a Packard Tricarb 2660 scintillation spectrometer, as before (Lawrie et al. 1976).

Uptake studies were also performed using the flow dialysis technique detailed by Ramos et al. (1979). The upper and lower chambers were separated by a dialysis membrane. The upper chamber contained 10 mmol dm^{-3} HEPES/NaOH buffer, pH 7, with or without cells. The same buffer was pumped through the lower chamber at a rate of 500 mm^3 min^{-1}, 1 cm^3 fractions were collected and radioactivity determined as described above. The reaction was started by adding $^{14}CH_3NH_3^+$ to the upper chamber (final concentration 30 µmol dm^{-3}).

4. $CH_3NH_3^+$ metabolism

Measurements of $CH_3NH_3^+$ incorporation into amino acids were carried out by incubating the cells in 10 mmol dm^{-3} HEPES/NaOH buffer, pH 7.0, containing 30 µmol dm^{-3} $^{14}CH_3NH_3^+$ (specific activity 4 kBq cm^{-3}). The cells were extracted in ethanol/water (80:20, v/v) for 6 h at 4° C in darkness and the filtrate evaporated to dryness at 40° C in a rotary evaporator. The residue was then redissolved in 1 cm^3 of 200 mmol dm^{-3} lithium citrate buffer, pH 2.2 and analysed using a LKB-4400 amino acid analyser and a lithium citrate buffer system. 3 cm^3 fractions of the eluent were collected from the amino acid analyser and a 100 mm^3 aliquot from each fraction was counted for radioactivity as above to determine the ^{14}C-labelling of each amino acid.

$CH_3NH_3^+$ incorporation into protein was determined by incubating the cells with $^{14}CH_3NH_3^+$ as above. Samples were then extracted in ethanol for 6 h at 4° C, the ethanol-soluble and ethanol-insoluble (protein containing) fractions separated by filtration and the incorporation of ^{14}C measured.

5. Non-specific binding of $^{14}CH_3NH_3^+$

Non-specific binding of $^{14}CH_3NH_3^+$ was determined by measuring its incorporation in the presence of NaCl (from 2 to 100 mmol dm^{-3}) or into toluene-treated cells of *Anabaena variabilis*. For toluene treatment, cells were harvested by centrifugation and resuspended in 1% (v/v) toluene in 10 mmol dm^{-3} HEPES/NaOH buffer, pH 7.0. After 15 min incubation, the cells were separated from toluene by centrifugation and resuspended in 10 mmol dm^{-3} HEPES buffer, pH 7.0 and $^{14}CH_3NH_3^+$ uptake measured as above.

6. Measurement of intracellular volume

The internal cell volume of the cyanobacterial filaments was estimated using [U-^{14}C]sorbitol and [^3H]water as described by Bakker et al. (1976). Separation of cells and incubation medium was achieved by silicon oil microcentrifugation (Scott and Nicholls 1980).

7. Chemicals

[^{14}C]methylammonium hydrochloride, [^{14}C]sorbitol and [^3H]water were purchased from Amersham International (Amersham, UK). All other chemicals were from Sigma Chemical Company (Poole, Dorset, UK).

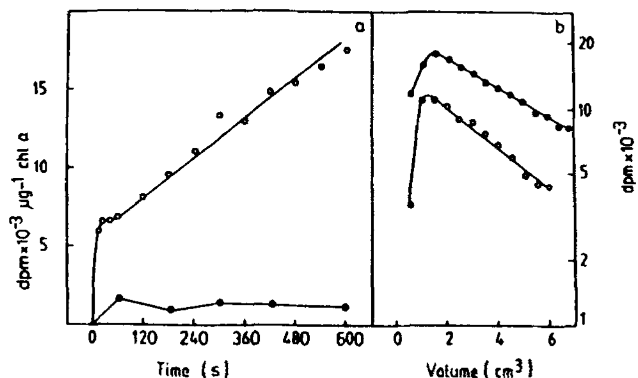

Fig. 1a, b. $CH_3NH_3^+$ uptake by *Anabaena variabilis*. Exponentially growing *A. variabilis* cultures were centrifuged, washed and then resuspended in 10 mmol dm^{-3} HEPES buffer, pH 7. Where required the cells were treated with toluene for 15 min, centrifuged and resuspended in HEPES buffer as above. $^{14}CH_3NH_3^+$ (final concentration of 30 µmol dm^{-3}) was added, at 0 time, and the incorporation of ^{14}C into the cells was determined over the time period shown. **a** Using the silicon oil microcentrifugation technique (○ control; ● toluene-treated filaments); **b** using the flow dialysis technique (○ with *A. variabilis* filaments present, ● without cyanobacterium). In the Tables and Figures presented in this paper each value given is the mean of at least 3 replicates

Results

Uptake and accumulation of $^{14}CH_3NH_3^+$ by Anabaena variabilis

Anabaena variabilis cells, grown in BG-11_0 medium (Rippka et al. 1979), were used to study the NH_4^+-uptake system using the NH_4^+ analogue, $^{14}CH_3NH_3^+$. *A. variabilis* cells incubated in the presence of $^{14}CH_3NH_3^+$ at pH 7.0 showed a rapid initial uptake during the first 60 s and this was followed by a slower secondary uptake which remained linear for the ensuing 600 s (Fig. 1a). Toluene-treated cells showed a small amount of $^{14}CH_3NH_3^+$ incorporation which remained constant over the 600 s experimental period and represented only about 17% of the uptake by untreated cells after 60 s. $^{14}CH_3NH_3^+$ uptake was also studied using the flow dialysis technique (Ramos et al. 1979; Laane et al. 1980). Shortly after adding $^{14}CH_3NH_3^+$ to the upper chamber, radioactivity appeared in the dialysate (lower chamber) and, after an initial rapid increase, decreased slowly (Fig. 1b). When *A. variabilis* cells were present in the upper chamber radioactivity in the dialysate was lower and decreased more rapidly, indicating that $^{14}CH_3NH_3^+$ was taken up and probably metabolised by the cells. The initial internal concentration of $^{14}CH_3NH_3^+$ in the cells was calculated to be 1.25 mmol dm^{-3}, which is 40-fold higher than the external concentration. All further experiments were carried out using the silicon oil microcentrifugation technique because of the difficulties encountered in uniformly illuminating the very dense suspensions of cyanobacteria required in such flow dialysis experiments.

The fate of $^{14}CH_3NH_3^+$ taken up by Anabaena variabilis

The biphasic pattern of $^{14}CH_3NH_3^+$ uptake and the non-parallel decrease of ^{14}C in the dialysate in flow dialysis experiments (Fig. 1) suggested a possible metabolism of $^{14}CH_3NH_3^+$ by *A. variabilis*. This was examined by supplying $^{14}CH_3NH_3^+$ to the cyanobacterium and, at time intervals thereafter, following the incorporation of radioactivity into

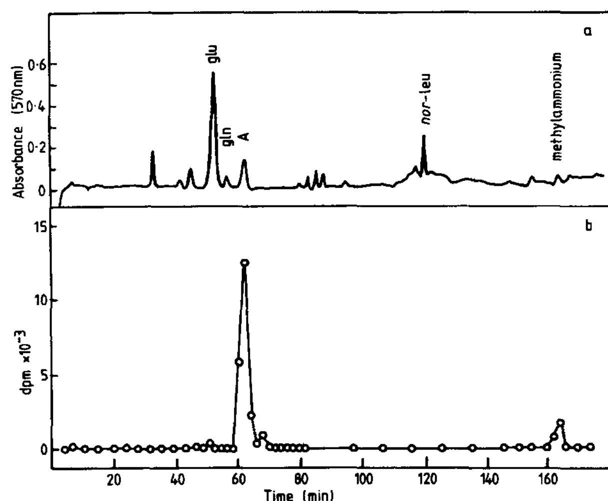

Fig. 2a, b. $CH_3NH_3^+$ metabolism by *Anabaena variabilis*. Exponentially growing *A. variabilis* cultures were harvested and resuspended as in Fig. 1. $^{14}CH_3NH_3^+$ was added to a final concentration of 30 μmol dm^{-3} and after 30 min the cells were harvested, washed and then extracted for amino acid analysis (see materials and methods). **a** Elution profile from the amino acid analyser (note the methylglutamine peak, '*A*'); **b** radioactivity in the corresponding fractions. Nor-leucine served as internal standard

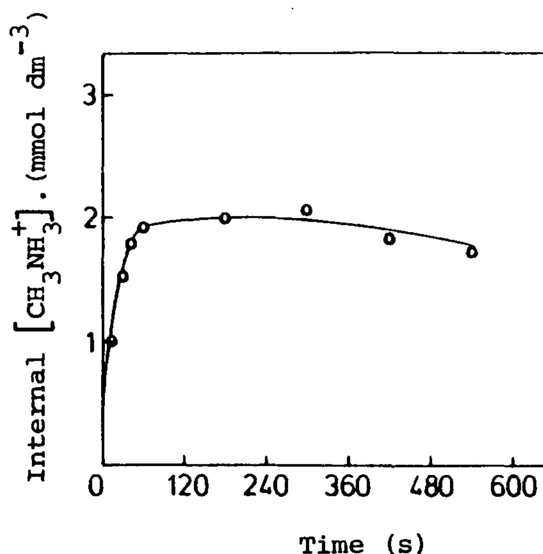

Fig. 3. Effect of L-methionine-DL-sulphoximine on $CH_3NH_3^+$ uptake by *Anabaena variabilis*. Exponentially growing *A. variabilis* cultures were harvested and resuspended as in Fig. 1. MSX was then added to a final concentration of 10 μmol dm^{-3} and after 1 h the cells were centrifuged and resuspended in fresh incubation medium containing MSX. $^{14}CH_3NH_3^+$ was then added to a final concentration of 30 μmol dm^{-3} (0 time) and, at timed intervals thereafter, samples were withdrawn and the cells separated by silicon oil microcentrifugation

the ethanol-soluble and ethanol-insoluble (protein-containing) fractions. Incorporation of radioactivity into the ethanol-insoluble fraction was insignificant and did not increase over a 6 h experimental period (data not shown). However, when the ethanol-soluble fractions were analysed for amino acids and the amino acid fractions measured for ^{14}C accumulation, it was found that in $^{14}CH_3NH_3^+$ fed cells there was, apart from a $CH_3NH_3^+$ peak, a new amino acid peak, eluted just after glutamine, which contained the bulk of the radioactivity (Fig. 2). This fraction was eluted in a similar position to methylglutamine (see Yoch et al. 1983) and when hydrolysed, it yielded equimolar amounts of $CH_3NH_3^+$ and glutamate (data not shown), indicating that the compound was indeed methylglutamine. While the pool of free $CH_3NH_3^+$ remained constant over the 30 min experimental period at an internal concentration of 1.4 mmol dm^{-3} the pool of methylglutamine continued to increase (about 12 mmol dm^{-3} after 30 min).

The role of GS in $^{14}CH_3NH_3^+$ metabolism

It is generally accepted that the route of synthesis of methylglutamine is via GS in prokaryotes. To investigate this in *A. variabilis* we have studied the effect of L-methionine-DL-sulphoximine (MSX), an inhibitor of glutamine synthetase (GS), on $^{14}CH_3NH_3^+$ uptake by *A. variabilis* (Fig. 3). There was a rapid initial accumulation within 60 s but there was no further uptake. Such data suggest that, while an NH_4^+ transport system occurs in *A. variabilis*, sustained uptake is dependent on the further metabolism via GS of the $CH_3NH_3^+$ which enters the cell. The fact that the MSX had no observable effect on $CH_3NH_3^+$ accumulation over the first 60 s indicates that NH_4^+ transport *per se* is not affected by MSX.

The presence and size of a free intracellular $CH_3NH_3^+$ pool was determined by adding NH_4^+ to the cultures at different times during the period of exposure to $^{14}CH_3NH_3^+$ (Fig. 4). The cyanobacterium was then separated from the bathing medium by silicon oil microcentrifugation and the internal $CH_3NH_3^+$ pool examined. It was found that the addition of NH_4^+ rapidly displaced a large fraction of the $^{14}CH_3NH_3^+$ taken up. Addition of NaCl from 2 to 100 mmol dm^{-3}, on the other hand, displaced little $^{14}CH_3NH_3^+$ (<15% of that displaced by NH_4^+). Assuming that the amount of $CH_3NH_3^+$ displaced by NH_4^+ represented the internal pool of free $CH_3NH_3^+$, the internal concentration of $CH_3NH_3^+$ was calculated to be 1.4 mmol dm^{-3} and was constant when measured after 60 s and 600 s. Such data indicate that the internal pool of $CH_3NH_3^+$ had equilibrated within 60 s, with most of the later uptake being due to $CH_3NH_3^+$ metabolism. The $CH_3NH_3^+$ which was not released by subsequent addition of NH_4^+ in 60 s experiments was probably due to non-specific binding; any increase in $^{14}CH_3NH_3^+$ uptake after 60 s mainly reflected its metabolism.

Characterisation of the $CH_3NH_3^+$ transport system in Anabaena variabilis

We have provided evidence above that uptake during the first 60 s of exposure to $^{14}CH_3NH_3^+$ represented transport of the analogue into the cell. Various factors affecting such transport have been investigated. The data in Table 1 show the effect of NH_4^+, the uncoupler CCCP and $TPMP^+$ which, at high concentrations, collapses $\Delta\psi$, on $CH_3NH_3^+$ accumulation over a 60 s period. NH_4^+ inhibited $CH_3NH_3^+$ uptake by 69% indicating that $CH_3NH_3^+$ and NH_4^+ share a common transport system; CCCP inhibited the $CH_3NH_3^+$ uptake to a similar extent (67%) indicating that the uptake was energy

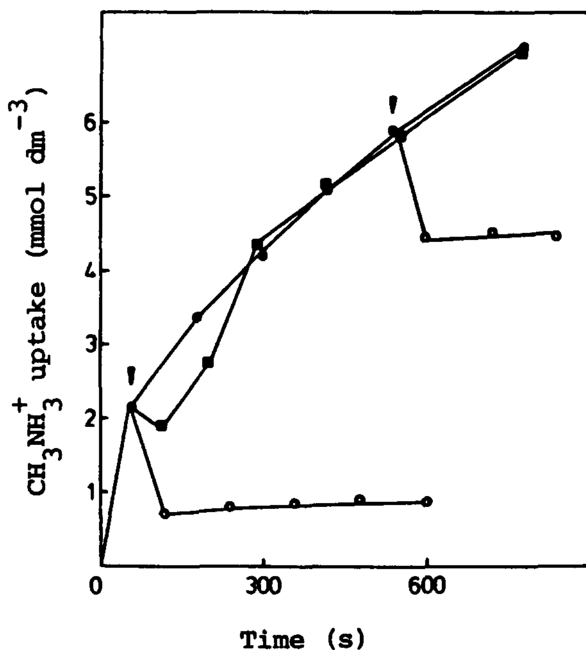

Fig. 4. Effect of NH_4^+ on the internal $CH_3NH_3^+$ pool of *Anabaena variablilis*. Exponentially growing *A. variabilis* cultures were harvested and resuspended as in Fig. 1 and four sets of experimental flasks were then set up. In the first set (●) $^{14}CH_3NH_3^+$ was added at 0 time, samples were withdrawn at various time intervals, starting from 60 s, and radioactivity in the cells measured using the silicon oil microcentrifugation technique. In the second and third sets NH_4Cl (○) or NaCl (■) was added (final concentrations: NH_4Cl, 200 µmol dm^{-3}; NaCl, 5 mmol dm^{-3}) to the incubation medium 60 s after the addition of $^{14}CH_3NH_3^+$ and thereafter samples were withdrawn at the time intervals shown. In the fourth set NH_4Cl was added 600 s after the addition of $^{14}CH_3NH_3^+$ (○)

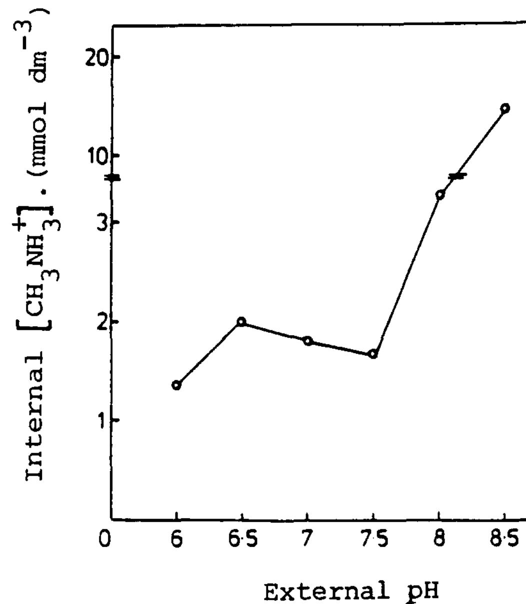

Fig. 5. Effect of external pH on $CH_3NH_3^+$ accumulation by *Anabaena variabilis*. Exponentially growing *A. variabilis* cultures were harvested and resuspended in 10 mmol dm^{-3} HEPES buffer at the desired pH (6.0 to 8.5). $^{14}CH_3NH_3^+$ was added at 0 time and after 60 s the cells were separated by silicon oil microcentrifugation and analysed for ^{14}C incorporation

Table 1. Effect of ammonium, CCCP and $TPMP^+$ on $CH_3NH_3^+$ accumulation by *Anabaena variabilis* Kütz. Exponentially growing *A. variabilis* cells were centrifuged and resuspended in 10 mmol dm^{-3} HEPES buffer, pH 7.0. $^{14}CH_3NH_3^+$ was added at 0 time and after 60 s ^{14}C-incorporation into the cells determined. Ammonium chloride (200 µmol dm^{-3}), when used, was added just before the addition of $^{14}CH_3NH_3^+$. CCCP (10 µmol dm^{-3}) and $TPMP^+$ (100 µmol dm^{-3}) additions were made 30 min prior to the addition of $^{14}CH_3NH_3^+$

Treatments	$CH_3NH_3^+$ accumulation		
	dpm µg^{-1} chl *a*	mmol dm^{-3}	% of control
Control	6300	2.1	100
$+NH_4Cl$	1980	0.66	31
+CCCP	2200	0.70	33
$+TPMP^+$	1825	0.60	28

dependent and $TPMP^+$ inhibited $CH_3NH_3^+$ uptake by 72% which suggested that $CH_3NH_3^+$ was taken up in response to $\Delta\psi$. The remaining 30% of the uptake was probably due to non-specific binding and was similar to the value obtained in Fig. 4. Thus NH_4^+ and $CH_3NH_3^+$ appear to be transported into the cell via a common $\Delta\psi$-dependent transport system.

Figure 5 presents data on the accumulation of $^{14}CH_3NH_3^+$ at different external pH values. $CH_3NH_3^+$ accumulation was similar when measured over a pH range of 6.0 to 7.5. However, $^{14}CH_3NH_3^+$ accumulation at higher pH values increased many fold possibly due to diffusion of uncharged methylamine across the membrane.

When the external concentration of $CH_3NH_3^+$ was increased there was a corresponding increase in the internal pool of free $CH_3NH_3^+$ up to an external concentration of 40 µmol dm^{-3} (Fig. 6) but beyond that concentration the internal pool of free $CH_3NH_3^+$ remained constant. It is not possible to say how this is distributed within the cells or between the cell types (heterocysts and vegetative cells). This is probably the highest concentration of internal free $CH_3NH_3^+$ which the transport system can maintain.

The uptake and accumulation of $CH_3NH_3^+$ by Anabaena azollae packets isolated from the water fern Azolla caroliniana

Having established the presence of an NH_4^+ transport system in the free-living cyanobacterium *A. variabilis* we then examined the symbiotic cyanobacterium present in packets of *Azolla caroliniana* in view of the possibility suggested by work on legume bacteroids (Laane et al. 1980; Kleiner et al. 1981) that such an NH_4^+ transport system may be lacking in symbiotic prokaryotes, thus explaining the failure of NH_4^+ to inhibit nitrogenase in such systems (see e.g. Stewart et al. 1980). The data obtained (Fig. 7) show that, as in the case of the free-living *A. variabilis* (Fig. 1), $CH_3NH_3^+$ uptake by *A. azollae* packets was biphasic. The initial rapid uptake over 60 s was similar to that of *A. variabilis*. However, a second phase of uptake although noted was much slower compared with that for *A. variabilis*. The symbiotic *A. azollae* shows only 25% of the GS activity of free-living cyanobacteria (see Peters et al. 1979; Gadd et al. 1980) and such data, showing a reduced rate of $^{14}CH_3NH_3^+$ uptake, are in accord with our

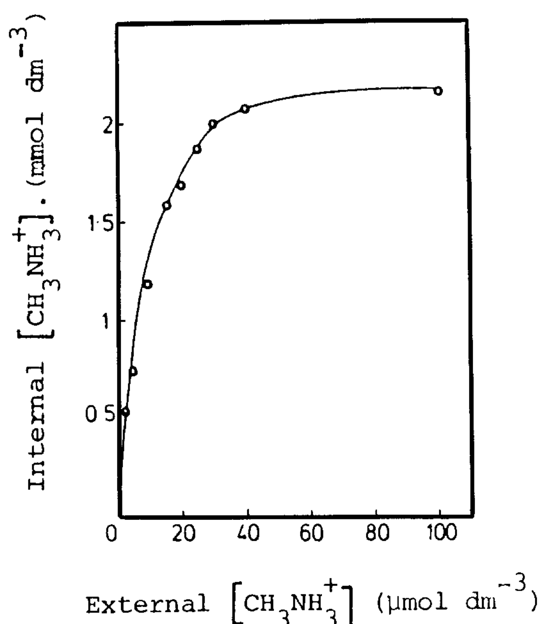

Fig. 6. Effect of external $CH_3NH_3^+$ concentration on $CH_3NH_3^+$ accumulation by *Anabaena variabilis*. Exponentially growing *A. variabilis* cultures were harvested and resuspended as in Fig. 1. $^{14}CH_3NH_3^+$ was added to a desired final concentration (2 to 100 µmol dm^{-3}) at 0 time and after 60 s the cells were separated by silicon oil microcentrifugation and analysed for ^{14}C incorporation

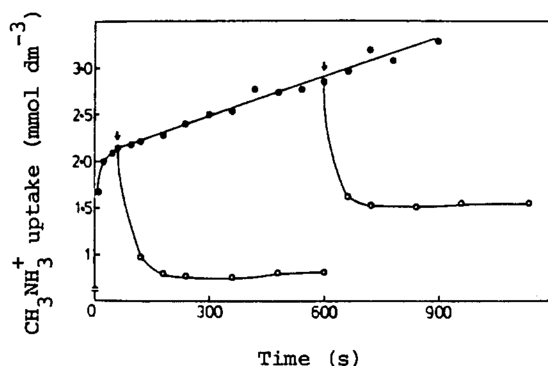

Fig. 7. $CH_3NH_3^+$ uptake (●) in symbiotic *Anabaena azollae* and the effect of NH_4^+, added after 60 s or 600 s (○), on the internal $CH_3NH_3^+$ pool. *A. azollae* cells were isolated from *Azolla caroliniana* and $^{14}CH_3NH_3^+$ uptake measured as in Fig. 4

findings of a requirement for an active GS for sustained $CH_3NH_3^+$ uptake. Addition of NH_4^+ subsequent to $^{14}CH_3NH_3^+$ caused the release of the $^{14}CH_3NH_3^+$. The amount of $CH_3NH_3^+$ released was similar whether NH_4^+ was added 60 s or 600 s after $CH_3NH_3^+$ and the internal pool of free $CH_3NH_3^+$ was calculated to be 1.3 mmol dm^{-3}, which is comparable to our findings above for *A. variabilis*. NH_4^+, CCCP and $TPMP^+$ all inhibited the $CH_3NH_3^+$ uptake by over 60% (Table 2).

These data for the free-living *A. variabilis* and the symbiotic *Anabaena* packets from *Azolla* suggest that in both systems $CH_3NH_3^+$ and NH_4^+ share a common, $\Delta\psi$-dependent transport system, at least within the pH range tested. The symbiotic cyanobacterium *A. azollae* thus appears to be different from the bacteroids of *Rhizobium leguminosarum* where an NH_4^+ transport system is considered to be absent (Laane et al. 1980).

Table 2. Effect of NH_4^+, CCCP and $TPMP^+$ on $CH_3NH_3^+$ accumulation by the symbiotic cyanobacterium *Anabaena azollae*. The experimental details are as shown in the legend to Table 1

Treatments	$CH_3NH_3^+$ accumulation		
	dpm μg^{-1} chl *a*	mmol dm^{-3}	% of control
Control	8218	2.15	100
$+NH_4Cl$	3187	0.80	40
+CCCP	3092	0.78	39
$+TPMP^+$	3080	0.78	39

Discussion

The data presented in this paper provide information on the kinetics of $^{14}CH_3NH_3^+$ uptake by the cyanobacterium *Anabaena variabilis* and demonstrate both the presence of an NH_4^+ transport system and that metabolism via GS is necessary for sustained uptake. Evidence for the presence of a $\Delta\psi$-dependent NH_4^+ transport system comes from the facts that *A. variabilis* accumulated $^{14}CH_3NH_3^+$, that NH_4^+ inhibited $CH_3NH_3^+$ accumulation, that accumulated $CH_3NH_3^+$ was released from the cells by the subsequent addition of NH_4^+ indicative of an internal pool of free $CH_3NH_3^+$ within the organism, and that $CH_3NH_3^+$ accumulation was CCCP- and $TPMP^+$-sensitive. Such findings of an NH_4^+ transport system in a free-living cyanobacterium are in keeping with the findings for the heterotrophic N_2-fixing bacteria *Azotobacter vinelandii* (Laane et al. 1980; Gordon and Moore 1981) and *Rhizobium* sp. (Gober and Kashket 1983) and for the photosynthetic N_2-fixing bacterium *Rhodospirillum rubrum* (Alef and Kleiner 1982; see also Kleiner 1981). The presence of such an NH_4^+ transport system in N_2-fixing cyanobacteria is of importance for the retention of NH_4^+ produced during N_2-fixation and for utilisation of NH_4^+ from the external medium which relieves the highly energy demanding process of N_2-fixation.

We have also demonstrated the presence of a $\Delta\psi$-dependent NH_4^+ transport system in the packets of symbiotic *Anabaena azollae* directly isolated from the water fern *Azolla caroliniana*. Such findings must be interpreted cautiously because the *Azolla* plants used were not axenic and also because the packets used would necessarily contain filaments of the cyanobacterium at different stages in symbiosis. However, as Fig. 7 shows, the activity of the transport system in the symbiotic *A. azollae* was as great as in the free-living *A. variabilis*. Such results differ from those for legume bacteroids which are reported to lack an NH_4^+ transport system (Laane et al. 1980).

It has been reported that NH_4^+ uptake inhibits nitrogenase activity in *Azotobacter vinelandii* by lowering the membrane potential (Haaker et al. 1980; Laane et al. 1980) which is involved in electron donation to nitrogenase in bacteria (see Haaker et al. 1980) and cyanobacteria (Hawkesford et al. 1981, 1982). Laane et al. (1980) have suggested that the lack of an NH_4^+ effect on bacteroid nitrogenase is because of the lack of an NH_4^+ transport system in the bacteroids. The lack of NH_4^+ inhibition of nitrogenase in symbiotic cyanobacteria, however, cannot be explained in this way, in view of our current findings.

Our data also show, that in addition to the presence of an NH_4^+ transport system, NH_4^+ metabolism via GS is required

for sustained uptake. Evidence for this comes from our studies using MSX which inhibits GS, the primary NH_4^+ assimilating enzyme in cyanobacteria (Stewart and Rowell 1975). While MSX had no effect on the initial $CH_3NH_3^+$ uptake (first 60 s) it totally inhibited the subsequent uptake of $CH_3NH_3^+$. That is, MSX affected metabolism-dependent uptake but not the transport system as such. Haaker et al. (1982) have also found that MSX had no effect on the NH_4^+ transport system of *Rhodopseudomonas sphaeroides*. Kleiner and Castorph (1982), however, have reported MSX inhibition of NH_4^+ transport in *Klebsiella pneumoniae*, although it is possible that such results may have been due to an NH_4^+ build-up during MSX treatment inhibiting uptake of $CH_3NH_3^+$ rather than to MSX inhibiting the transport system directly.

Overall, we conclude that there is an NH_4^+ transport system in both the free-living and symbiotic cyanobacteria and our data support the assumption that nitrogenase regulation by NH_4^+ involves a product of NH_4^+ assimilation via GS.

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