5. AMMONIUM/METHYLAMMONIUM TRANSPORT IN NOSTOC ANTH

5.1. INTRODUCTION:

N₂-fixing cyanobacteria enter into symbiotic associations with plants ranging from algae to angiosperms (see Stewart et al., 1983; Smith and Douglas, 1987). In symbiosis, the cyanobiont undergoes extensive structural and metabolic alterations which lead to a higher rate of N₂-fixation and transfer of fixed-N from cyanobiont to the eukaryotic partner (Stewart et al., 1983; Rai et al., 1989; Rai, 1988; 1989).

Among the bryophyte-cyanobacterial symbiosis, the hornwort Anthoceros-Nostoc symbiosis is better characterized. N₂-fixing Nostoc sp. develops in cavities on the undersurface of the Anthoceros-gametophyte thallus. The cyanobiont shows a high heterocyst frequency, is photosynthetically inactive and liberates nearly 90% of the nitrogenase derived ammonia to the eukaryotic partner (see Stewart et al., 1983; Meeks et al., 1985; Rai et al., 1989). This liberation of ammonia by the cyanobiont has been suggested to be due to low GS activity (Stewart et al., 1983; Meeks et al., 1985).

The cyanobiont of Anthoceros punctatus was isolated and cultured in free-living state. It showed a marked difference in methylammonium metabolism compared to other cyanobacteria (see chapter 3). In the present chapter, characteristics of ATS in the cultured (free-living) cyanobiont of A. punctatus (Nostoc ANTH) was investigated to see if ATS in this cyanobacteria has any
unique features. A study of ATS in the cyanobiont of *A. punctatus*,
directly isolated from the thallus, could not be done because of
the problem of isolating enough clean cells from the thallus.

5.2. MATERIALS AND METHODS:

5.2.1. Organism and growth conditions:

*Nostoc* ANTH, an isolate from *A. punctatus*, was grown in
axenic aerated batch cultures in BG-11 medium (Rippka *et al.*, 1979),
at 28 ± 1°C and at a photon fluence rate of 50 μmol.m⁻².s⁻¹ as described in chapter 2. When required NH₄Cl (1 mmol.dm⁻³),
glutamine (1 mmol.dm⁻³), glucose (50 mmol.dm⁻³) or CH₃NH₂Cl (5 mmol.dm⁻³) was added to the medium and buffered with 10 mmol.dm⁻³
HEPES-NaOH (pH 7.5).

5.2.2. Chlorophyll and protein determinations:

Chl a and protein content of cyanobacteria were measured
according to Mackinney (1941) and Lowry *et al.* (1951),
respectively.

5.2.3. Measurement of ¹⁴CH₃NH⁺⁺ uptake:

¹⁴CH₃NH⁺⁺ uptake was assayed at 28°C and at a photon fluence
rate of 50 μmol.m⁻².s⁻¹., unless otherwise indicated, and non-
specific binding of ¹⁴CH₃NH⁺⁺ by cell membranes was measured by
using toluene treated cells. (Details given in Chapter 2).
5.2.4. Measurement of GS activity:

GS biosynthetic and transferase activities were measured according to Sampaio et al. (1979).

5.2.5. Chemicals:

$^{14}$CH$_3$NH$_2$Cl, $^3$H-dextran and $^3$H-water were purchased from Amersham International plc, Amersham, U.K. Silicon DC 550 and dinonylphthalate were purchased from Fluka AC, Buchs, Switzerland. All other chemicals were obtained from Sigma chemical company, U.S.A.

5.3. RESULTS:

5.3.1. $^{14}$CH$_3$NH$_3^+$ uptake in N$_2$-grown *Nostoc* ANTH cells at pH 7:

5.3.1.1: $^{14}$CH$_3$NH$_3^+$ uptake:

The ammonium transport in *Nostoc* ANTH cells was studied using $^{14}$CH$_3$NH$_3^+$ as probe. N$_2$-grown *Nostoc* ANTH cells, at pH 7 and external $^{14}$CH$_3$NH$_3^+$ concentration of 50 µmol.dm$^{-3}$, showed a biphasic pattern of $^{14}$CH$_3$NH$_3^+$ uptake with an initial rapid phase during first 60 s followed by a slower second phase which remained linear over the 12 min experimental period. The uptake rates during initial rapid and slower second phase were found to be 0.119 and 0.0156 nmol.min$^{-1}$.µg$^{-1}$ Chl a, respectively (Fig 5.1). The toluene treated cells showed a much lower level of $^{14}$CH$_3$NH$_3^+$ incorporation during first 30 s which remained constant over the 12 min experimental period (Fig 5.1). This incorporation was found to be 6.16 kBq.mg$^{-1}$ Chl a representing about 22% of the total uptake by
Fig 5.1 $^{14}$CH$_3$NH$_3^+$ uptake at pH 7 by *Nostoc* ANTH filaments grown on N$_2$-medium. O, control; •, toluene treated cells. (In this and all other experiments the data are means of four replicates obtained from two repeat experiments. The variation range was between 5 - 10% from the average.)
untreated cells after 60 s (first phase). These values were considered to be non-specific adsorption of $^{14}$CH$_3$NH$_3^+$ by cell membranes and subtracted from the respective values in untreated cells, before data were plotted in all other experiments which follow.

5.3.1.2. Effect of NH$_4$Cl:

Simultaneous addition of NH$_4$Cl and $^{14}$CH$_3$NH$_3^+$ resulted in total inhibition of $^{14}$CH$_3$NH$_3^+$ uptake, during both phases, in N$_2$-grown cells, at pH 7, indicating a common transport for NH$_4$Cl and $^{14}$CH$_3$NH$_3^+$ (Fig 5.2). This suggested that, as in the case of Anabaena 7120, $^{14}$CH$_3$NH$_3^+$ can be used as a probe to study the NH$_4^+$ uptake in Nostoc ANTH. When NH$_4$Cl was added subsequent to the addition of $^{14}$CH$_3$NH$_3^+$, it showed two effects: a) a sudden efflux of preaccumulated $^{14}$C-label from the cells into the cell suspension, and b) complete inhibition of further $^{14}$CH$_3$NH$_3^+$ uptake by cells (Fig 5.2). The NH$_4^+$-displaceable $^{14}$C-label remained constant over the experimental period and equaled to the $^{14}$C-label which was transported during initial rapid phase. This indicated existence of a free $^{14}$CH$_3$NH$_3^+$ pool inside the cells which built up during initial phase of $^{14}$CH$_3$NH$_3^+$ uptake. Considering that the NH$_4^+$-displaceable $^{14}$CH$_3$NH$_3^+$ represented the internal free $^{14}$CH$_3$NH$_3^+$ pool, the latter was calculated to be 4.9 mmol.dm$^{-3}$. Thus, at an external $^{14}$CH$_3$NH$_3^+$ concentration of 50 μmol.dm$^{-3}$, Nostoc ANTH cells showed a nearly 100 fold accumulation of $^{14}$CH$_3$NH$_3^+$.

While $^{14}$CH$_3$NH$_3^+$ displaced by NH$_4^+$ remained constant, with time more and more $^{14}$C-label remained inside the cells. This is not surprising since $^{14}$CH$_3$NH$_3^+$ is metabolized in Nostoc ANTH cells by
Fig 5.2 Effect of NH4Cl on 14CH3NH3+ uptake, at pH 7, by N2-grown Nostoc ANTH filaments. NH4Cl was added at times indicated (arrows) to a final concentration of 200 μmol.dm−3. O, control (14CH3NH3+ only); •, NH4Cl and 14CH3NH3+ added simultaneously at zero time; △, NH4Cl added 3 min after 14CH3NH3+ addition; ▲, NH4Cl added 8 min after 14CH3NH3+ addition; □, 14CH3NH3+ uptake in NH4Cl (1 mmol.dm−3)-grown cells.
GS-GOGAT pathway (see chapter 3). Such data indicate that the second phase of $^{14}\text{CH}_3\text{NH}_3^+$ uptake represents metabolism of the transported $^{14}\text{CH}_3\text{NH}_3^+$. 

In NH$_4^+$-grown cells no $^{14}\text{CH}_3\text{NH}_3^+$ accumulation/incorporation was observed (Fig 5.2). In these cells the $^{14}\text{CH}_3\text{NH}_3^+$ uptake pattern was similar to that observed in toluene-treated cells. These data are consistent with earlier findings on Anabaena variabilis (Rai et al., 1986a), Anabaena 7120 (see Rai and Prakasham, 1989), and other bacteria (Kleiner, 1985a) showing repression of ATS in NH$_4^+$-grown cells.

5.3.1.3. Effect of MSX:

In one set of experiments cells were preincubated with MSX (10 µmol.dm$^{-3}$) for 1 hour to inactivate GS and then $^{14}\text{CH}_3\text{NH}_3^+$ uptake was measured subsequently in absence of MSX. In such experiments only the first phase of $^{14}\text{CH}_3\text{NH}_3^+$ uptake was detectable (Fig 5.3); the second phase was absent. This indicated that the first phase was MSX insensitive. The lack of second phase of uptake in such cells may be due to blockage of $^{14}\text{CH}_3\text{NH}_3^+$ metabolism in the cells since GS was inhibited. It was also possible, however, that MSX may have affected $^{14}\text{CH}_3\text{NH}_3^+$ uptake during the second phase at transport level as found in a GS mutant of Anabaena cycadeae (singh et al., 1985b). In an attempt to distinguish the effect of MSX at uptake level from that at the level of GS inhibition, further experiments were done to see the effect of MSX on $^{14}\text{CH}_3\text{NH}_3^+$ uptake in cells where GS remained unaffected during the experimental period. In these experiments no preincubation with MSX was done. MSX (10 µmol.dm$^{-3}$) was added together with $^{14}\text{CH}_3\text{NH}_3^+$ at zero
Fig. 5.3 Effect of MSX on $^{14}$CH$_3$NH$_3^+$ uptake, at pH 7, by N$_2$-grown _Nostoc_ ANTH filaments. MSX was added at times indicated (arrows) to a final concentration of 10 μmol.dm$^{-3}$. ○, control ($^{14}$CH$_3$NH$_3^+$ only); ●, MSX and $^{14}$CH$_3$NH$_3^+$ added simultaneously at zero time; ▲, MSX added 5 min after $^{14}$CH$_3$NH$_3^+$ addition; △, $^{14}$CH$_3$NH$_3^+$ uptake in MSX-preincubated cells (cells were incubated with 10 μmol.dm$^{-3}$ MSX for 1 h, then washed and resuspended in fresh buffer and uptake was studied without MSX being present in the medium.)
time or 5 min after the addition of $^{14}$CH$_3$NH$_3^+$ and $^{14}$CH$_3$NH$_3^+$ uptake measured during the next 12 min in the presence of MSX (during this period GS remained fully active; see Rai and Prakasham, 1989). When MSX and $^{14}$CH$_3$NH$_3^+$ were added simultaneously, $^{14}$CH$_3$NH$_3^+$ uptake occurred at a rate similar to that in the control for the first 30 s after which the uptake continued at a slower rate till the $^{14}$CH$_3$NH$_3^+$ in cell reached a level similar to that at the end of first phase of $^{14}$CH$_3$NH$_3^+$ uptake in control cells (Fig 5.3). Such data indicated that although MSX partially affected the rate of $^{14}$CH$_3$NH$_3^+$ uptake during first phase it did not affect the overall pool size built up during this phase. On the other hand, it totally blocked the second phase of $^{14}$CH$_3$NH$_3^+$ uptake (Fig 5.3). Since GS activity was not affected during this period it was concluded that this effect of MSX was at the level of $^{14}$CH$_3$NH$_3^+$ uptake. Thus, as in Anabaena 7120 (Rai and Prakasham, 1989), Nostoc ANTH also has two uptake systems for NH$_4^+$/CH$_3$NH$_3^+$; one MSX-insensitive and the other MSX-sensitive. These observations are consistent with MSX having two targets of inhibitory action; one at GS level and the other at transport level, as found in other cyanobacteria (see Singh et al., 1985b; Rai and Prakasham, 1989).

Addition of MSX to the cell suspension during the second phase of $^{14}$CH$_3$NH$_3^+$ uptake caused an immediate inhibition of $^{14}$CH$_3$NH$_3^+$ uptake and an efflux (15 Bq.µg$^{-1}$ Chl a) of preaccumulated $^{14}$C-label from the cells (Fig 5.3). The former observation is similar to the effect of MSX found in A. variabilis (Rai et al., 1984), Anabaena 7120 (Rai and Prakasham, 1989) and A. cycadeas (Singh et al., 1985b). However, the efflux of $^{14}$C-label caused by MSX contrasts with the observations in other cyanobacteria (Rai
14C-efflux was caused by MSX. It is probable that MSX caused partial efflux of some intracellular 14CH3NH3+. It should be noted here that MSX did partially affect, transiently, the build up of internal free pool of 14CH3NH3 during the first phase of 14CH3NH3 uptake. Alternatively, the 14C-efflux may have been due to displacement by MSX of an internal pool of some metabolized product of CH3NH3+. (Unlike other cyanobacteria, Nostoc ANTH does metabolize CH3NH3 as N-source; chapter 3).

5.3.1.4. Kinetics of concentration-dependent 14CH3NH3+ uptake via the two ATS:

14CH3NH3+ uptake rates, in Nostoc ANTH, at pH 7, via the two ATS at various external 14CH3NH3+ concentrations (1-500 μmol.dm⁻³) were studied (Fig 5.4a - 5.4c) as in Anabaena 7120 (see chapter 4). First, the 14CH3NH3+ uptake rates via the MSX-insensitive ATS were studied by following 14CH3NH3+ uptake during the first phase (initial 60 s) at 15 s intervals after 14CH3NH3+ addition to the cell suspension. Rates were calculated from the linear portions of the curve. A biphasic pattern of concentration-dependent 14CH3NH3+ uptake rate, similar to that in Anabaena 7120 (see chapter 4), was observed (Fig 5.4a) with Vmax values of 0.125 and 0.225 nmol.min⁻¹.μg⁻¹ Chl a, in the external concentration range of 1 - 15 and 15 - 500 μmol.dm⁻³, respectively (Fig 5.4a; data not shown beyond 150 μmol.dm⁻³). The corresponding Km values (calculated from Lineweaver-Burk plots) were 3 and 45 μmol.dm⁻³, respectively (Fig 5.4b & 5.4C).

Next, 14CH3NH3+ rates, via the second ATS were studied, at
Fig 54a Concentration-dependent $^{14}$CH$_3$NH$_3^+$ uptake rates, at pH 7, during initial MSX-insensitive rapid phase by N$_2$-grown *Nostoc* ANTH filaments showing dual isotherm Michaelis-Menten Kinetics. O, uptake rates at an external concentration range of 1 - 15 μmol.dm$^{-3}$ $^{14}$CH$_3$NH$_3^+$; Δ, uptake rates at an external concentration range of 15 - 150 μmol.dm$^{-3}$ $^{14}$CH$_3$NH$_3^+$. 
Fig 5.4 b & c Lineweaver-Burk plots for $^{14}\text{CH}_3\text{NH}_3^+$ uptake during high affinity mode (1 - 15 $\mu$mol.dm$^{-3}$ external $^{14}\text{CH}_3\text{NH}_3^+$ concentration, O) and low affinity mode (15 - 150 $\mu$mol.dm$^{-3}$ external $^{14}\text{CH}_3\text{NH}_3^+$ concentration, $\Delta$) calculated from Fig 4a as before (Lineweaver and Burk, 1934).
$^{14}\text{CH}_3\text{NH}_3^+$ concentration range and conditions similar to the above. The rates were calculated from linear second phase of $^{14}\text{CH}_3\text{NH}_3^+$ uptake (between 5 and 12 min after $^{14}\text{CH}_3\text{NH}_3^+$ addition to cell suspension). The observed Vmax values were 0.023 and 0.0205 mmol.min$^{-1}$.g$^{-1}$ Chl a, in the external concentration range of 1 - 20 and 50 - 400 µmol.dm$^{-3}$, respectively (Fig 5.5a). The corresponding Km values were 4.6 and 135 µmol.dm$^{-3}$, respectively (Fig 5.5b & 5.5c; calculated from Lineweaver-Burk plots).

Thus, as in Anabaena 7120 (see chapter 4), in Nostoc ANTH also both ATS showed dual affinity mode: a high affinity mode at low substrate concentration and a low affinity mode at high substrate concentration. However, the pattern of change in $^{14}\text{CH}_3\text{NH}_3^+$ uptake rates, via the second ATS, in response to external $^{14}\text{CH}_3\text{NH}_3^+$ concentration, differed in the two organisms (see Fig 4.8a of chapter 4 and Fig 5.5a of this chapter). In Anabaena 7120, the $^{14}\text{CH}_3\text{NH}_3^+$ uptake rate via the second ATS saturated below 10 µmol.dm$^{-3}$. When external $^{14}\text{CH}_3\text{NH}_3^+$ concentration was increased beyond 10 µmol.dm$^{-3}$ a further increase in the rate of uptake occurred which saturated at 150 µmol.dm$^{-3}$. Further increase in external substrate concentration did not result in any increase in uptake rate (see chapter 4 Fig 4.8a). In Nostoc ANTH, however, the uptake rate increased in response to increase in external substrate concentration upto 20 µmol.dm$^{-3}$. Further increase in external $^{14}\text{CH}_3\text{NH}_3^+$ concentration resulted in a transient decrease in $^{14}\text{CH}_3\text{NH}_3^+$ uptake rate. However, when external $^{14}\text{CH}_3\text{NH}_3^+$ concentration was raised beyond 50 µmol.dm$^{-3}$, an increase in uptake rate was observed. This increase continued upto external substrate concentration of 300 µmol.dm$^{-3}$. Further increase in
Fig 5.5a Concentration-dependent $^{14}$CH$_3$NH$_3^+$ uptake rates, at pH 7, during the subsequent MSX-sensitive slower phase by N$_2$-grown Nostoc ANTH filaments showing dual isotherm Michaelis - Menten kinetics. O. uptake rates at an external concentration range of 1 - 400 µmol.dm$^{-3}$ $^{14}$CH$_3$NH$_3^+$. 
Fig 5.5 b & c Lineweaver-Burk plots for $^{14}$CH$_3$NH$_3^+$ uptake during high affinity mode (1 - 25 μmol.dm$^{-3}$ external $^{14}$CH$_3$NH$_3^+$ concentration, △) and low affinity mode (50 - 300 μmol.dm$^{-3}$ external substrate concentration, ▲) calculated from Fig 5a as before (Lineweaver and Burk. 1934).
external $^{14}\text{CH}_3\text{NH}_3^+$ concentration again resulted in a decrease in uptake rate (Fig 5.5a). Furthermore, in *Nostoc ANTH*, the Vmax value decreased during the shift from high to low affinity mode whereas, in *Anabaena 7120* the Vmax value increased (see Fig 5.5a of this chapter and Fig 4.8a of chapter 4).

5.3.1.5. Effect of CCCP and TPMP$^+$:

Ammonium transport in various prokaryotes, so far tested, is found to be active and energy-dependent and driven by transmembrane electrical potential (Kleiner, 1981; 1985a; Rai *et al.*, 1984; 1986a; Singh *et al.*, 1985b; 1987; Rai and Prakasham, 1989). Therefore, the effect of CCCP and TPMP$^+$ on $^{14}\text{CH}_3\text{NH}_3^+$ uptake, in this cyanobacterium, at pH 7, was studied. CCCP caused complete elimination of $^{14}\text{CH}_3\text{NH}_3^+$ uptake during both phases (Fig 5.6) suggesting that $^{14}\text{CH}_3\text{NH}_3^+$ uptake in *Nostoc ANTH* also is an active and energy-dependent process like that in *Anabaena 7120* (Rai and Prakasham, 1989). TPMP$^+$ treated cells showed a markedly lower level of $^{14}\text{CH}_3\text{NH}_3^+$ uptake (Fig 5.6). In TPMP$^+$ treated cells the uptake rate during first and second phase was 0.06 and 0.005 nmol.min$^{-1}$.µg$^{-1}$ Chl a, respectively. This represented 50 and 29.5% of the corresponding values in control cells, respectively. These results indicate that while transmembrane electrical potential is necessary for optimum rates of uptake during both phases of $^{14}\text{CH}_3\text{NH}_3^+$ uptake, $^{14}\text{CH}_3\text{NH}_3^+$ uptake can still occur in absence of transmembrane electrical potential. This contrasts with the results in *Anabaena 7120* (Rai and Prakasham, 1989) and *A. variabilis* (Rai *et al.*, 1984) where TPMP$^+$ treated cells showed total lack of $^{14}\text{CH}_3\text{NH}_3^+$ uptake.
Fig 5.6 $^{14}\text{CH}_3\text{NH}_3^+$ uptake, at pH 7, by N$_2$-grown Nostoc ANTH filaments in the presence (●, △) or absence (○) of CCCP (△) and TPMP$^+$ (●). CCCP (10 μmol.dm$^{-3}$) and TPMP$^+$ (100 μmol.dm$^{-3}$) were added 30 min before $^{14}\text{CH}_3\text{NH}_3^+$ addition.
5.3.1.6. Effect of glutamine and glutamate:

Glutamine and glutamate are the initial products of primary ammonia assimilation in cyanobacteria (Stewart, 1980). Hence, effects of these compounds on ATS, in this cyanobacterium was studied.

$^{14}\text{CH}_3\text{NH}_3^+$ uptake, in glutamine (1 mmol.dm⁻³) grown Nostoc ANTH cells, at pH 7, is shown in fig 5.7a. The uptake pattern was found to be similar in N₂- and glutamine-grown cells. The rate of $^{14}\text{CH}_3\text{NH}_3^+$ uptake during the first phase was similar in glutamine- and N₂-grown cells, during the second phase $^{14}\text{CH}_3\text{NH}_3^+$ uptake was slightly higher in glutamine-grown cells. Such results suggest that glutamine is not a repressor of $^{14}\text{CH}_3\text{NH}_3^+$ uptake in Nostoc ANTH cells. This contrasts with the finding of Singh et al. (1987) who have shown that $^{14}\text{CH}_3\text{NH}_3^+$ uptake was repressed in glutamine grown cells of A. cycadeae.

The effect of various external glutamine concentration on $^{14}\text{CH}_3\text{NH}_3^+$ uptake, at pH 7, in N₂-grown Nostoc ANTH cells is shown in Fig 5.7b. In the presence of 200 μmol.dm⁻³ glutamine, $^{14}\text{CH}_3\text{NH}_3^+$ uptake during the first phase was severely inhibited with little or no effect on the rate of uptake during the second phase. Increasing concentration of glutamine caused progressively more inhibitory effect, however the uptake did resume after 5 min (i.e. the second phase). Thus, glutamine seems to inhibit the MSX-insensitive ATS while having little or no affect on the MSX-sensitive ATS.

*Nostoc* ANTH did not grow in glutamate containing medium. Therefore, for studying effect of glutamate on ATS, experiments were performed in N₂-grown cells only. As shown in fig 5.8,
Fig 5.7a $^{14}\text{CH}_3\text{NH}_3^+$ uptake at pH 7. by N$_2$-grown (O) and glutamine (1 mmol.dm$^{-3}$)-grown (●) *Nostoc* ANTH filaments.
Fig 5.7b Effect of glutamine addition on $^{14}\text{CH}_3\text{NH}_3^+$ uptake, at pH 7, by 
N$_2$-grown *Nostoc* ANTH filaments. Glutamine was added along 
with $^{14}\text{CH}_3\text{NH}_3^+$ at zero time. O, control ($^{14}\text{CH}_3\text{NH}_3^+$ only); ●, 
200 μmol.dm$^{-3}$ glutamine; △, 500 μmol.dm$^{-3}$ glutamine; ▲, 
1000 μmol.dm$^{-3}$ glutamine.
Fig 5.8  Effect of glutamate addition on $^{14}\text{CH}_3\text{NH}_3^+$ uptake, at pH 7, by N$_2$-grown Nostoc ANTH filaments. Glutamate was added along with NH$_4$Cl at zero time. O, control ($^{14}\text{CH}_3\text{NH}_3^+$ only); ●, 200 μmol dm$^{-3}$ glutamate.
presence of 200 \mu mol.dm^{-3} glutamate affected the first phase of $^{14}$CH$_3$NH$_3$\(^+\) uptake but not the second phase. This was similar to the effect of glutamine on $^{14}$CH$_3$NH$_3$\(^+\) uptake in N$_2$-grown cells (Fig 5.7b) and indicated that, like glutamine, glutamate affected only the MSX-insensitive ATS.

5.3.2. $^{14}$CH$_3$NH$_3$\(^+\) uptake in glucose-grown cells:

A comparative study of $^{14}$CH$_3$NH$_3$\(^+\) uptake in autotrophically-grown, photoheterotrophically-grown and heterotrophically-grown H.ostoc ANTH cells is presented in fig 5.9. The $^{14}$CH$_3$NH$_3$\(^+\) uptake pattern in photoheterotrophically-grown cells (cells grown in N$_2$-medium in light + 50 mmol.dm$^{-3}$ glucose) and heterotrophically-grown cells (cells grown in N$_2$-medium in dark + glucose) was similar to that in the control (autotrophically-grown cells; cells grown in N$_2$-medium in light). However, both photoheterotrophically-grown and heterotrophically-grown cells accumulated less $^{14}$CH$_3$NH$_3$\(^+\) than autotrophically-grown cells.

The rate of $^{14}$CH$_3$NH$_3$\(^+\) uptake during the second phase (representing $^{14}$CH$_3$NH$_3$\(^+\) metabolism) in autotrophically-grown and heterotrophically-grown cells was essentially identical. However, the rate in photoheterotrophically-grown cells was 57% higher indicating a higher rate of $^{14}$CH$_3$NH$_3$\(^+\) metabolism. This may reflect the fact that compared to the autotrophically- and heterotrophically-grown cells, there was higher availability of C-skeleton and energy, for $^{14}$CH$_3$NH$_3$\(^+\) metabolism, in photoheterotrophically-grown cells.
Fig 5.9 $^{14}\text{CH}_3\text{NH}_3^+$ uptake, at pH 7, by Nostoc ANTH filaments grown autotrophically, photoheterotrophically, and heterotrophically. O, cells grown in N$_2$-medium in light; •, cells grown in N$_2$-medium in light + 50 mmol.dm$^{-3}$ glucose; Δ, cells grown in N$_2$-medium in dark + 50 mmol.dm$^{-3}$ glucose.
5.3.3. $^{14}$CH$_3$NH$_3^+$ uptake in CH$_3$NH$_3^+$-grown Nostoc ANTH cells:

5.3.3.1. $^{14}$CH$_3$NH$_3^+$ uptake:

_Nostoc_ ANTH has an ability to metabolize CH$_3$NH$_3^+$ as N-source (see chapter 3). Since, in NH$_4^+$-grown _Nostoc_ ANTH cells the methylammonium/ammonium transport system was repressed (Fig 5.2), it would be interesting to know whether such a transport system is operative in CH$_3$NH$_3^+$-grown cells. Therefore, $^{14}$CH$_3$NH$_3^+$ uptake in CH$_3$NH$_3^+$-grown _Nostoc_ ANTH cells was investigated (Fig 5.10). In contrast to the NH$_4^+$-grown cells where no $^{14}$CH$_3$NH$_3^+$ uptake occurred, CH$_3$NH$_3^+$-grown cells showed $^{14}$CH$_3$NH$_3^+$ uptake indicating that despite CH$_3$NH$_3^+$ being used as N-source by _Nostoc_ ANTH, CH$_3$NH$_3^+$ transport system was not repressed by CH$_3$NH$_3^+$.

The uptake pattern in CH$_3$NH$_3^+$-grown cells was different than that in N$_2$-grown cells. Unlike in N$_2$-grown cells, $^{14}$CH$_3$NH$_3^+$ uptake in CH$_3$NH$_3^+$-grown cells did not show as distinct a biphasic pattern as in N$_2$-grown cells.

5.3.3.2. Effect of NH$_4$Cl:

As seen in fig 11, addition of NH$_4$Cl at zero time caused a progressive inhibition of the $^{14}$CH$_3$NH$_3^+$ uptake. However, for the initial 2 - 3 min uptake was similar to that in control. Similar effect was observed when NH$_4$Cl was added subsequent to $^{14}$CH$_3$NH$_3^+$ addition. Furthermore, NH$_4^+$ did not cause any efflux of $^{14}$C-label from cells into the medium (Fig 5.11). This is in contrast to the finding in N$_2$-grown cells where addition of NH$_4^+$ caused immediate and complete inhibition of $^{14}$CH$_3$NH$_3^+$ uptake and NH$_4^+$ addition subsequent to $^{14}$CH$_3$NH$_3^+$ addition caused efflux of preaccumulated $^{14}$CH$_3$NH$_3^+$ (see Fig 5.2). These data thus, indicate that the
Fig 5.10 $^{14}$CH$_3$NH$_3^+$ uptake, at pH 7, by N$_2$-grown (O), CH$_3$NH$_3^+$ (5 mmol.dm$^{-3}$)-grown (●), and NH$_4^+$ (1 mmol.dm$^{-3}$)-grown (△) *Nostoc* ANTH filaments.
Fig 5.11 Effect of NH$_4$Cl on $^{14}$CH$_3$NH$_3^+$ uptake, at pH 7, by CH$_3$NH$_3^+$-grown *Nostoc* ANTH filaments. NH$_4$Cl (200 µmol.dm$^{-3}$) was added at times (arrows) indicated. O, control ($^{14}$CH$_3$NH$_3^+$ only); ⋄, NH$_4$Cl and $^{14}$CH$_3$NH$_3^+$ added simultaneously at zero time; Δ, NH$_4$Cl added 5 min after $^{14}$CH$_3$NH$_3^+$ addition.
carrier of $^{14}$CH$_3$NH$_3^+$ in CH$_3$NH$_3^+$-grown cells is a specific CH$_3$NH$_3^+$ carrier distinct from the NH$_4^+/CH_3$NH$_3^+$ carrier in N$_2$-grown cells.

The inhibition of $^{14}$CH$_3$NH$_3^+$ uptake by NH$_4^+$ after the initial 2 - 3 min may reflect the fact that NH$_4^+$ did not affect the $^{14}$CH$_3$NH$_3^+$ accumulation in CH$_3$NH$_3^+$-grown cells but affected the metabolism of the internal $^{14}$CH$_3$NH$_3^+$ because of it being the natural substrate for GS. This conclusion is also supported by the fact that: a) the $^{14}$CH$_3$NH$_3^+$ uptake in various cyanobacteria after 60 s is dependent on the metabolism of transported species (Rai et al., 1984; Singh et al., 1985b; 1986; 1987; Reglinski et al., 1989), b) CH$_3$NH$_3^+$ and NH$_4^+$ are assimilated by the same enzyme in cyanobacteria (see chapter 3) and c) GS has a higher affinity for NH$_4^+$ than CH$_3$NH$_3^+$ because of the former being the natural substrate (see Kerby et al., 1987). Such overall results suggested existence of a specific transport system for CH$_3$NH$_3^+$ in CH$_3$NH$_3^+$-grown Nostoc ANTH cells. These observations are similar to those observed in the CH$_3$NH$_3^+$ resistant cyanobacterium *A. variabilis* (Reglinski et al., 1989).

5.3.3.3. Effect of MSX:

MSX was found to be an inhibitor of the second phase of $^{14}$CH$_3$NH$_3^+$ uptake in N$_2$-grown Nostoc ANTH (see Fig 5.3) and in *Anabaena* 7120 (see chapter 4). Therefore, the effect of MSX during $^{14}$CH$_3$NH$_3^+$ uptake in CH$_3$NH$_3^+$-grown Nostoc ANTH cells, at pH 7, was also investigated (Fig 5.12). MSX is a known inhibitor of GS (Stewart, 1980). To avoid MSX inhibition of CH$_3$NH$_3^+$ uptake via inhibition of GS and to ensure that the observed effects of MSX on $^{14}$CH$_3$NH$_3^+$ uptake, if any, were at the level of uptake, the experi-
Fig. 5.12 Effect of MSX on $^{14}$CH$_3$NH$_3^+$ uptake at pH 7, by CH$_3$NH$_3^+$-grown *Nostoc* ANTH filaments. MSX (10 μmol.dm$^{-3}$) was added at times (arrows) indicated. O, control ($^{14}$CH$_3$NH$_3^+$ only); Δ, MSX and $^{14}$CH$_3$NH$_3^+$ added simultaneously at zero time; ▲, MSX added 5 min after $^{14}$CH$_3$NH$_3^+$ addition.
ments were conducted only for 12 min (inhibition of GS by MSX at a concentration of 10 μmol.dm⁻³ was undetectable before 30 min).

Addition of 10 μmol.dm⁻³ MSX at zero time did not affect ¹⁴CH₃NH⁺₃ uptake in CH₃NH⁺₃-grown cells. Addition of MSX, 5 min after ¹⁴CH₃NH⁺₃ addition also gave similar results i.e. no inhibition of ¹⁴CH₃NH⁺₃ uptake. This is in contrast to the findings in N₂-grown cells where MSX inhibited the second methylammonium/ammonium transport system.

These results further suggest that the CH₃NH⁺₃ transport system in CH₃NH⁺₃-grown cells is different from the methylammonium/ammonium transport systems in N₂-grown cells.

5.3.3.4. Effect of CCCP and TPMP⁺:

Addition of CCCP caused a severe inhibition of ¹⁴CH₃NH⁺₃ uptake in CH₃NH⁺₃-grown cells (Fig 5.13). This is consistent with the finding in N₂-grown cells and indicate that the process is energy-dependent. TPMP⁺ also caused a similar inhibition indicating that the process is dependent on transmembrane electrical potential.

5.3.4. ¹⁴CH₃NH⁺₃ uptake at pH 9 in N₂-grown Nostoc ANTH cells:

Fig 5.14 show ¹⁴CH₃NH⁺₃ uptake, at pH 9, in N₂-grown Nostoc ANTH. As at pH 7, a biphasic pattern of ¹⁴CH₃NH⁺₃ uptake was also observed at pH 9. However, the ¹⁴CH₃NH⁺₃ accumulation (first phase) was over 2 fold higher at pH 9 (40 Bq.μg⁻¹ Chl a higher than at pH 7; see Fig 5.14 and Fig 5.1). This may be explained by the fact that at pH 9 more methylamine occurs as uncharged species (CH₃NH₂) which can diffuse without need of the transport system.
Fig 5.13 $^{14}$CH$_3$NH$_3^+$ uptake, at pH 7, by CH$_3$NH$_3^+$-grown Nostoc ANTH filaments in the presence (Δ, ▲) or absence (○) of CCCP (▲) and TPMP$^+$ (Δ). CCCP (10 μmol.dm$^{-3}$) and TPMP$^+$ (100 μmol.dm$^{-3}$) were added 30 min before $^{14}$CH$_3$NH$_3^+$ addition.
Fig 5.14 $^{14}\text{CH}_3\text{NH}_3 /^{14}\text{CH}_3\text{NH}_2$ uptake, at pH 9, by N$_2$-grown Nostoc ANTII filaments.
and get trapped in the cell by protonation. The uptake during the second phase, at pH 9, was also higher than that at pH 7. This may reflect a higher level of metabolism of the intracellular \( \text{CH}_3\text{NH}_3^+ \) via GS due to higher level of \( \text{CH}_3\text{NH}_3^+ \) entry into the cells.

Addition of \( \text{NH}_4^+ \) during \( ^{14}\text{CH}_3\text{NH}_3^+ \) uptake at pH 9 caused an efflux of preaccumulated \( ^{14}\text{CH}_3\text{NH}_3^+ \) (Fig 5.15). However, the amount effluxed was far less than the \( ^{14}\text{CH}_3\text{NH}_3^+ \) accumulated during the first phase. This contrasts with the finding at pH 7 where \( \text{NH}_4^+ \) caused total efflux of the preaccumulated \( ^{14}\text{CH}_3\text{NH}_3^+ \) during the first phase (Fig 5.2). When a pH shift, from pH 9 to pH 7, was induced simultaneously with addition of \( \text{NH}_4^+ \) there was a total efflux of preaccumulated \( ^{14}\text{CH}_3\text{NH}_3^+ \) from the cells. Probably, the intracellular \( \text{CH}_3\text{NH}_3^+ \) pool was in two compartments, one of which was displaced by \( \text{NH}_4\text{Cl} \) while the other by the pH shift.

\( \text{NH}_4^+ \) at pH 9, as well as after the shift from pH 9 to 7, apart from causing efflux of \( ^{14}\text{C} \)-label from the cells, also caused an inhibition of further \( ^{14}\text{CH}_3\text{NH}_3^+ \) uptake (Fig 5.14 & 5.15). Inhibition of \( ^{14}\text{CH}_3\text{NH}_3^+ \) uptake in the presence of \( \text{NH}_4^+ \) can be easily explained by the fact that \( \text{NH}_4^+ \) is the natural substrate and therefore preferred over \( \text{CH}_3\text{NH}_3^+ \).

5.3.4.1. Effect of MSX:

Addition of 10 \( \mu \text{mol.dm}^{-3} \) MSX after \( ^{14}\text{CH}_3\text{NH}_3^+ \) addition resulted in a transient efflux of \( ^{14}\text{C} \)-label from the cells followed by continued \( ^{14}\text{CH}_3\text{NH}_3^+ \) uptake at a rate similar to that in control cells (Fig 5.16). This \( ^{14}\text{C} \)-displacement by MSX was similar to that found at pH 7 (Fig 5.3). However, the continued uptake of \( ^{14}\text{CH}_3\text{NH}_3^+ \) at pH 9 in presence of MSX was in contrast to
Fig 5.15 Effect of NH$_4$Cl and NH$_4$Cl + pH shift (from pH 9 to 7) on $^{14}$CH$_3$NH$_3^+$/$^{14}$CH$_3$NH$_2$ uptake, at pH 9, by N$_2$-grown *Nostoc* ANTH filaments. NH$_4$Cl and was added at times indicated (arrows) to a final concentration of 200 µmol dm$^{-3}$. pH shift was achieved by addition of 4.5 mm$^3$ of HCl to 1 cm$^3$ of cell suspension at pH 9 to bring it down to pH 7. O, control ($^{14}$CH$_3$NH$_3$ only); Δ. NH$_4$Cl added 5 min after $^{14}$CH$_3$NH$_3^+$ addition; ▲. NH$_4$Cl added 10 min after $^{14}$CH$_3$NH$_3^+$ addition; ■. NH$_4$Cl + HCl added 5 min after $^{14}$CH$_3$NH$_3^+$ addition; ■. NH$_4$Cl + HCl added 10 min after $^{14}$CH$_3$NH$_3^+$ addition.
Fig 5.16 Effect of MSX on $^{14}$CH$_3$NH$_3^+$/$^{14}$CH$_3$NH$_2$ uptake, at pH 9, by N$_2$-grown *Nostoc* ANTH filaments. MSX (10 μmol.dm$^{-3}$) was added at time (arrows) indicated. O, control ($^{14}$CH$_3$NH$_3^+$ only); Δ, MSX added 5 min after $^{14}$CH$_3$NH$_3^+$ addition. ▲, MSX added 10 min after $^{14}$CH$_3$NH$_3^+$ addition.
the finding at pH 7 where MSX inhibited $^{14}$CH$_3$NH$_3^+$ uptake at the transport level (Fig 5.3). This may again reflect the fact that unlike at pH 7 where the $^{14}$CH$_3$NH$_3^+$ uptake was carrier mediated, at pH 9 much of the uptake occurred via diffusion. The diffused CH$_3$NH$_2$ was metabolized in the cells since MSX, during the short experimental period, did not affect GS.

5.3.4.2. Effect of CCCP and TPMP$^+$:

Addition of CCCP or TPMP$^+$ to N$_2$-grown Nostoc ANTH cells, at pH 9 did not change the $^{14}$CH$_3$NH$_3^+$ uptake pattern and accumulation rates (Fig 5.17). Such results indicate that the $^{14}$CH$_3$NH$_3^+$ uptake at pH 9, in this cyanobacterium is an energy-independent process. These results are similar to those observed in other cyanobacteria (Kerby et al., 1986; Boussiba, 1989; Reglinski et al., 1989; Rai and Prakasham, 1989).

5.4. DISCUSSION:

The $^{14}$CH$_3$NH$_3^+$ uptake studies presented in this chapter show that, as in Anabaena 7120 (see chapter 4; Rai and Prakasham, 1989), N$_2$-grown cells of Nostoc ANTH possess two methylammonium/ammonium transport systems (one MSX-insensitive fast ATS and the other MSX-sensitive slower ATS). The arguments for existence of these two ATS are the same as those discussed in chapter 4. In addition, the differential effects of glutamine, glutamate and mode of C-nutrition (Fig 5.7, 5.8 and 5.9) on the two phases of $^{14}$CH$_3$NH$_3^+$ uptake also suggest that the biphasic $^{14}$CH$_3$NH$_3^+$ uptake pattern observed is a reflection of two distinct methylammonium/
Fig 5.17 $^{14}\text{CH}_3\text{NH}_3^+/^{14}\text{CH}_3\text{NH}_2$ uptake, at pH 9, by N$_2$-grown Nostoc ANTH filaments in the presence ($\Delta, \bullet$) or absence (O) of TPMP$^+$ ($\Delta$) and CCCP ($\triangle$). TPMP$^+$ (100 $\mu$mol.dm$^{-3}$) and CCCP (10 $\mu$mol.dm$^{-3}$) were added 30 min before $^{14}\text{CH}_3\text{NH}_3^+$ addition.
ammonium transport systems. Most characteristics of these ATS are similar to those of Anabaena 7120. However, some differences were also evident. First, unlike the case in Anabaena 7120 where TPMP$^+$ totally blocked $^{14}$CH$_3$NH$_3^+$ transport, in Nostoc ANTH cells only a partial inhibition of $^{14}$CH$_3$NH$_3^+$ uptake was observed in the presence of TPMP$^+$ (Fig 5.6). Thus, while $^{14}$CH$_3$NH$_3^+$ transport in Anabaena 7120 was wholly dependent on transmembrane electrical potential, the uptake in Nostoc ANTH was only partially dependent on transmembrane electrical potential. Second, while MSX did not cause any efflux of $^{14}$C-label from Anabaena 7120 cells, in Nostoc ANTH cells MSX did cause such an efflux either by displacing the intracellular $^{14}$CH$_3$NH$_3^+$ pool or a metabolized product of CH$_3$NH$_3^+$. Third, in Anabaena 7120 the shift to lower affinity mode showed an increase in Vmax values for the second ATS while in Nostoc Vmax decreased.

Another distinctive feature of methylammonium/ammonium transport system in Nostoc ANTH was the fact that glutamine-grown cells still possessed the two methylammonium/ammonium transport systems. That is glutamine was not a repressor for methylammonium/ammonium transport system in Nostoc ANTH. In contrast glutamine has been reported to be a repressor of methylammonium/ammonium transport system in A. cycadeae (Singh et al., 1987).

In Anabaena 7120 existence of two intracellular CH$_3$NH$_3^+$ pools was argued based on the $^{14}$CH$_3$NH$_3^+$ uptake and metabolism studies (see chapter 4; Rai and Prakasham, 1989). The same arguments apply here too. In addition, the results of $^{14}$CH$_3$NH$_3^+$ uptake studies at pH 9 further strengthen the arguments for two intracellular pools of CH$_3$NH$_3^+$ (see Fig 5.15). At pH 9 NH$_4^+$ could only partially
displace the intracellular pool of CH$_3$NH$_3^+$. However, NH$_4^+$ together with a pH shift from 9 to 7 resulted in a total efflux of the intracellular free CH$_3$NH$_3$ (see Fig 5.15 and the relevant result section).

In addition to the methylammonium/ammonium transport systems, a specific methylammonium transport system was found in CH$_3$NH$_3^+$-grown _Nostoc_ ANTH cells. That this methylammonium transport system was specific for CH$_3$NH$_3^+$ and that it did not transport NH$_4^+$ was concluded from the fact that:

1) Unlike the CH$_3$NH$_3^+$ accumulation by methylammonium/ammonium transport system (Fig 5.2), the CH$_3$NH$_3^+$ accumulation by the methylammonium transport system was not inhibited by addition of ammonium (Fig 5.11), and

2) In contrast to the observations in N$_2$-grown cells (Fig 5.2), addition of NH$_4^+$ did not cause efflux of the preaccumulated CH$_3$NH$_3^+$ from CH$_3$NH$_3^+$-grown cells (Fig 5.11).

The methylammonium transport system in CH$_3$NH$_3^+$-grown cells had the following characteristics:

1) CH$_3$NH$_3^+$ transport through methylammonium transport system was an energy-dependent process, driven by transmembrane electrical potential as shown by inhibition of CH$_3$NH$_3^+$ uptake by CCCP (an uncoupler) and TPMP$^+$ (an agent causing collapse of transmembrane electrical potential) (Fig 5.13).

2) Unlike the observation in N$_2$-grown cells where MSX inhibited the Methylammonium/ammonium uptake during the second phase, CH$_3$NH$_3^+$ uptake by methylammonium transport system was unaffected by MSX (Fig 5.12). That is, this methylammonium transport system was MSX-insensitive.
3) While methylammonium/ammonium transport systems are known to be repressed by excess availability of its own substrate: ammonium (Rai et al., 1986b; Rai and Prakasham, 1989; see also Fig 5.2), methylammonium transport system was not repressed in cells grown on CH$_3$NH$_3^+$.

The methylammonium transport system seems to be an inducible system since it developed only in CH$_3$NH$_3^+$-grown cells. This is supported by the fact that:

1) In NH$_4^+$-grown cells no CH$_3$NH$_3^+$ uptake occurred, and

2) In N$_2$-grown cells CH$_3$NH$_3^+$ uptake occurred only through methylammonium/ammonium transport systems. If the specific methylammonium transport system was operative in N$_2$-grown cells then NH$_4^+$ should not have caused total inhibition of CH$_3$NH$_3^+$ uptake and total efflux of pre-accumulated CH$_3$NH$_3^+$ in such cells.

Overall, the data show that like other cyanobacteria, N$_2$-grown cells of Nostoc ANTH possess two energy-dependent methylammonium/ammonium transport systems. Both these methylammonium/ammonium transport systems show affinity modulation in response to external substrate concentration. A specific methylammonium transport system was found to be induced in CH$_3$NH$_3^+$-grown cells of Nostoc ANTH. This is the first report of existence of a specific methylammonium transport system in cyanobacteria so far, except in a CH$_3$NH$_3^+$-resistant mutant strain of A. variabilis (Reglinski et al., 1989).