

Methylammonium/ammonium transport and metabolism in the heterocystous cyanobacteria : *Anabaena* 7120 and *Nostoc* ANTH

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ABSTRACT

Methylammonium (CH_3NH_3^+) transport and metabolism was studied in *Anabaena* 7120, a free-living cyanobacterium, and in *Nostoc* ANTH, a free-living isolate of *Anthoceros punctatus*. CH_3NH_3^+ was assimilated as methylglutamine in *Anabaena* 7120, whereas, it was further metabolised as N-source in *Nostoc* ANTH. Both cyanobacteria possess two ammonium transport systems (ATS): one is responsible for accumulation of CH_3NH_3^+ as internal free pool and is insensitive to methionine sulfoximine (MSX); the other is dependent on the assimilation of transported species and sensitive to MSX. These two systems showed dual affinity modulation in *Anabaena* 7120.

INTRODUCTION

A better understanding of nitrogen metabolism is a prerequisite for enhancing the potential use of diazotrophic cyanobacteria as biofertilizer. Ammonium transport mechanism is one of the crucial aspects which needs a detailed study. This is because ammonium is the first product of N_2 -metabolism and it exerts regulatory effect on various processes of N-metabolism in diazotrophic cyanobacteria. For example, ammonium acts as repressor of nitrogenase synthesis, heterocyst differentiation and uptake and utilization of other nitrogenous compounds (Whitton and Carr, 1982; Gibson, 1984; Bagchi *et al.*, 1985; 1985b; Mackerras and Smith, 1986). Occurrence of ammonium transport system (ATS) is well known in various prokaryotes (Kleiner, 1985). Characterisation of this system in cyanobacteria was started in 1984 by Rai *et al.* and considerable work has been done since then in this regard (Boussiba *et al.*, 1984; Kashyap and Johar, 1984; Singh *et al.*, 1985, 1986, 1987; Kerby *et al.*, 1986; Rai *et al.* 1986; Rai and Prakasham, 1989).

Using radioactive analogue of ammonium, $^{14}\text{CH}_3\text{NH}_3^+$ as probe, we have characterized the ATS in the two strains of diazotrophic cyanobacteria *Anabaena* 7120 (a free-living form) and *Nostoc* ANTH (an isolate from *Anthoceros punctatus*).

CH_3NH_3^+ is a growth inhibitor of various organisms (Singh *et al.*, 1983). However, it can be utilized as carbon and/or nitrogen source by some prokaryotes

depending on the growth conditions (Holtel and Kleiner, 1985). Therefore, before using it to characterize ATS in cyanobacteria we also studied the effect of CH_3NH_3^+ on growth and the fate of intracellular CH_3NH_3^+ in these two strains.

MATERIALS AND METHODS

Axenic cultures of *Anabaena* 7120 and *Nostoc* ANTH were grown continuously in BG-11₀ medium (Rippka *et al.*, 1979) at 25 °C and at a photon fluence rate of 50 $\mu\text{mol. m}^{-2}. \text{s}^{-1}$. The growth medium was supplemented with CH_3NH_3^+ (5mM) and/or dichlorophenyl dimethylurea (DCMU; 10 μM) as and when required and the medium was buffered with hydroxymethylpiperazine ethane sulfonic acid (HEPES) pH 7.5 (10mM) before autoclaving.

The protein and chlorophyll *a* content was estimated according to Lowry *et al.* (1951) and Mackinney (1941), respectively. The glutamine synthetase (GS) and nitrogenase activities were measured using methods of Sampaio *et al.* (1979) and Stewart *et al.* (1967), respectively.

$^{14}\text{CH}_3\text{NH}_3^+$ uptake studies were done as described by Rai and Prakasham, (1989) using a final concentration of 50 $\mu\text{mol. dm}^{-3}$ $^{14}\text{CH}_3\text{NH}_3^+$ (specific activity 9.25 KBq. dm^{-3}). Nonspecific adsorption of $^{14}\text{CH}_3\text{NH}_3^+$ was measured by following ^{14}C -incorporation in toluene-treated cells, and these values were subtracted before plotting the data.

Extraction and estimation of amino acid pool were performed by resuspending the incubated cyanobacterial cells in ethanol/water (70/30;v/v) mixture and incubated for 6 hours at 4°C. Then the filtrate was subjected to rotary evaporation at 35°C. The residue was dissolved in 2 cm^3 of sodium citrate buffer (pH 2.2) and amino acids were detected by using HPLC (Shimadzu LC, 4A). ^{14}C -incorporation in amino acids was measured by Liquid Scintillation Spectrophotometer LS 1801 (Beckman).

$^{14}\text{CH}_3\text{NH}_3^+$ was obtained from Amersham, U.K. All other chemicals used were purchased from Sigma Chemical Co., U.S.A.

RESULTS AND DISCUSSION

Table 1 shows the growth, heterocyst frequency and nitrogenase activity in *Anabaena* 7120 and *Nostoc* ANTH grown in the presence and absence of CH_3NH_3^+ . Both organisms grew well in N_2 -medium. The observed specific growth rates were 1.065 and 0.62 per day for *Anabaena* 7120 and *Nostoc* ANTH, respectively. These data indicate that both cyanobacteria are able to utilize N_2 as nitrogen source. This conclusion was also supported by the observation that both these organisms showed

heterocyst differentiation and nitrogenase activity in air-grown cultures. The observed heterocyst frequency was 5.4% in *Anabaena* 7120 and 6.5% in *Nostoc* ANTH and nitrogenase activity was 4.25 nmol C₂H₂ reduced. h⁻¹ μg⁻¹ Chl *a* in *Anabaena* 7120 and 5.25 nmol C₂H₂ reduced.h⁻¹ μg⁻¹ Chl *a* in *Nostoc* ANTH (table 1).

Table 1. Growth (specific growth rate, day⁻¹), heterocyst frequency (%) and nitrogenase activity (nmol C₂H₂ reduced. h⁻¹. μg⁻¹ Chal *a*) in absence and presence of CH₃NH₃⁺ and/or DCMU in *Nostoc* ANTH and *Anabaena* 7120.

Organism	CH ₃ NH ₃ ⁺	Growth		Heterocyst frequency	Nitrogenase activity
		+DCMU	-DCMU		
<i>Nostoc</i> ANTH	+	0.00	0.76	0.5 ± 0.02	ND*
	-	0.00	0.62	6.5 ± 0.3	5.25 ± 0.025
<i>Anabaena</i> 7120	+	0.00	0.00	0.00	0.00
	-	0.00	1.065	5.4 ± 0.02	4.25 ± 0.50

* = Not detectable

The growth pattern, however, in CH₃NH₃⁺ supplemented medium was different in the two cyanobacteria. This medium did not support the growth of *Anabaena* 7120, while it was found to be growth supportive in *Nostoc* ANTH (table 1). The specific growth rate of *Nostoc* ANTH, in CH₃NH₃⁺ supplemented medium, was 0.76 per day although heterocyst differentiation and nitrogenase activity were severely inhibited (table 1). These results suggested that CH₃NH₃⁺ can be utilized as N-source by this cyanobacterium. The growth of *Nostoc* ANTH, was inhibited when CH₃NH₃⁺ medium was supplemented with DCMU indicating that CH₃NH₃⁺ is metabolised as N-source but not as C-source.

Further CH₃NH₃⁺ metabolism was studied by incubating the cells with ¹⁴CH₃NH₃⁺ and at time intervals thereafter, following the ¹⁴C-incorporation in ethanol soluble fractions. These fractions were analyzed for amino acids and then measured for ¹⁴C-incorporation. When *Anabaena* 7120 cells were analyzed after incubation with ¹⁴CH₃NH₃⁺, radioactivity was detected in an amino acid peak, eluted near glutamine peak, and in methylamine peak (fig 1a). The amino acid, when analyzed for its composition after hydrolysis, yielded ¹⁴CH₃NH₃⁺ (Fig 1b) and glutamate (data not shown) suggesting that the compound was methylglutamine. Such results are consistent with the results observed in *Anabaena variabilis* (Rai *et al.*, 1984; Kerby *et al.*, 1986, 1987). In case of *Nostoc* ANTH, however, ¹⁴C-incorporation was found in three peaks (after 10 min incubation with ¹⁴CH₃NH₃⁺) corresponding to aspartic

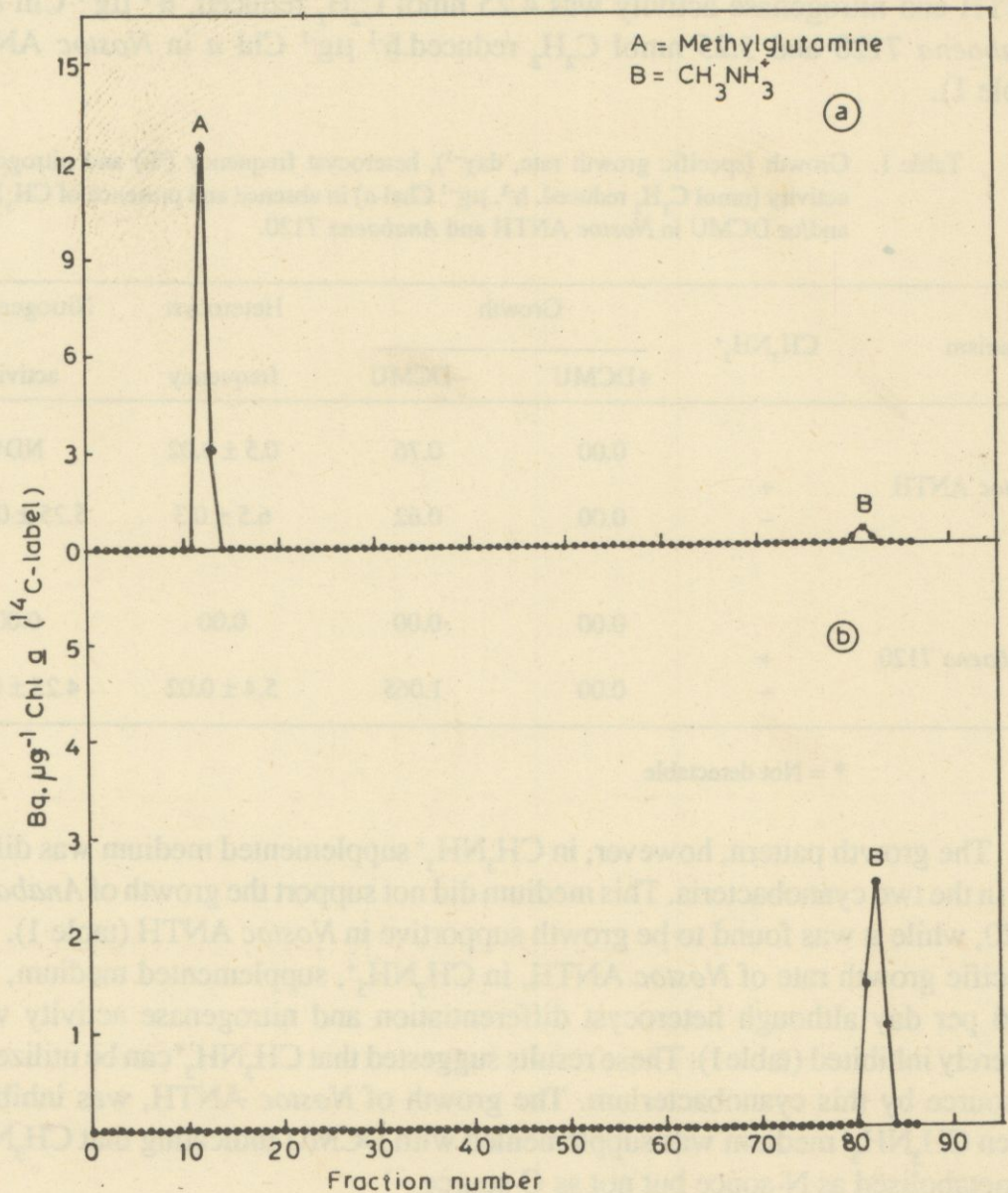


Fig 1. $^{14}\text{CH}_3\text{NH}_3^+$ metabolism by *Anabaena* 7120. Exponentially growing filaments (in this and next two experiments) were harvested and resuspended in HEPES pH 7.0 (10mM) buffer. $^{14}\text{CH}_3\text{NH}_3^+$ was then added to a final concentration of $50 \mu\text{M}$ and after specified incubation times, the cells were harvested, washed and then extracted for amino acid analysis (see materials and methods). a) Elution profile of ^{14}C -amino acids in 60 min $^{14}\text{CH}_3\text{NH}_3^+$ incubated *Anabaena* 7120 cells detected by measuring the radioactivity in fractions (fractions collected at every min during amino acid separation by HPLC). b) Hydrolyzed ^{14}C -amino acid peak of Fig 1a.

acid, glutamate and alanine (fig 2a). The ^{14}C -incorporation in these peaks increased with increase in time (fig 2b, 2c, & 2d) indicating that $^{14}\text{CH}_3\text{NH}_3^+$ is metabolised as N-source by this cyanobacterium. In contrast, longer incubations of *Anabaena* 7120 cells with $^{14}\text{CH}_3\text{NH}_3^+$ resulted in higher accumulation of methylglutamine only

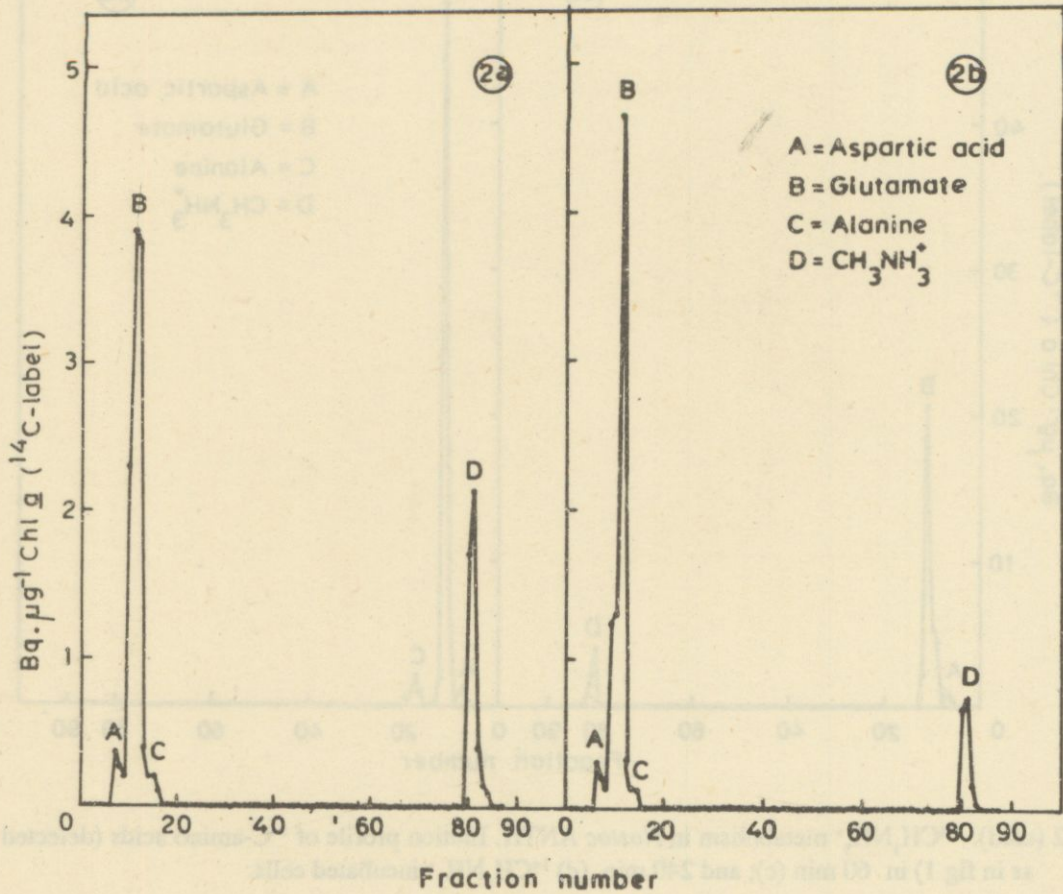


Fig. 2(a & b). $^{14}\text{CH}_3\text{NH}_3^+$ metabolism in *Nostoc* ANTH. Elution profile of ^{14}C -amino acids (detected as in fig 1) in 10 min, (a); and 30 min, (b) $^{14}\text{CH}_3\text{NH}_3^+$ incubated cells.

(data not shown). Azaserine pretreated *Nostoc* ANTH cells showed ^{14}C -incorporation only in methylglutamine (fig 3) similar to that of *Anabaena* 7120. These results suggest that CH_3NH_3^+ is assimilated, in *Nostoc* ANTH, via primary ammonia assimilating pathway (glutamine synthetase-glutamate synthase) while in *Anabaena* 7120 CH_3NH_3^+ is metabolized only upto methylglutamine.

Thus, the *Nostoc* ANTH, a cultured isolate, seems quite different from *Anabaena* 7120, a free-living cyanobacterium with regard to CH_3NH_3^+ metabolism. In subsequent studies we characterized the $^{14}\text{CH}_3\text{NH}_3^+$ uptake in N_2 -grown cultures of the two cyanobacteria.

A biphasic $^{14}\text{CH}_3\text{NH}_3^+$ uptake pattern was observed in both cyanobacteria, with an initial rapid phase followed by a slower second phase (fig 4). The uptake rates, however, differed in both organisms. In *Anabaena* 7120, these were observed to be 0.111 and 0.0216 $\text{nmol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1} \text{Chl } a$ (fig 4a) while in *Nostoc* ANTH, they were 0.119 and 0.0156 $\text{nmol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1} \text{Chl } a$ (fig 4b), during initial and second phases, respectively.

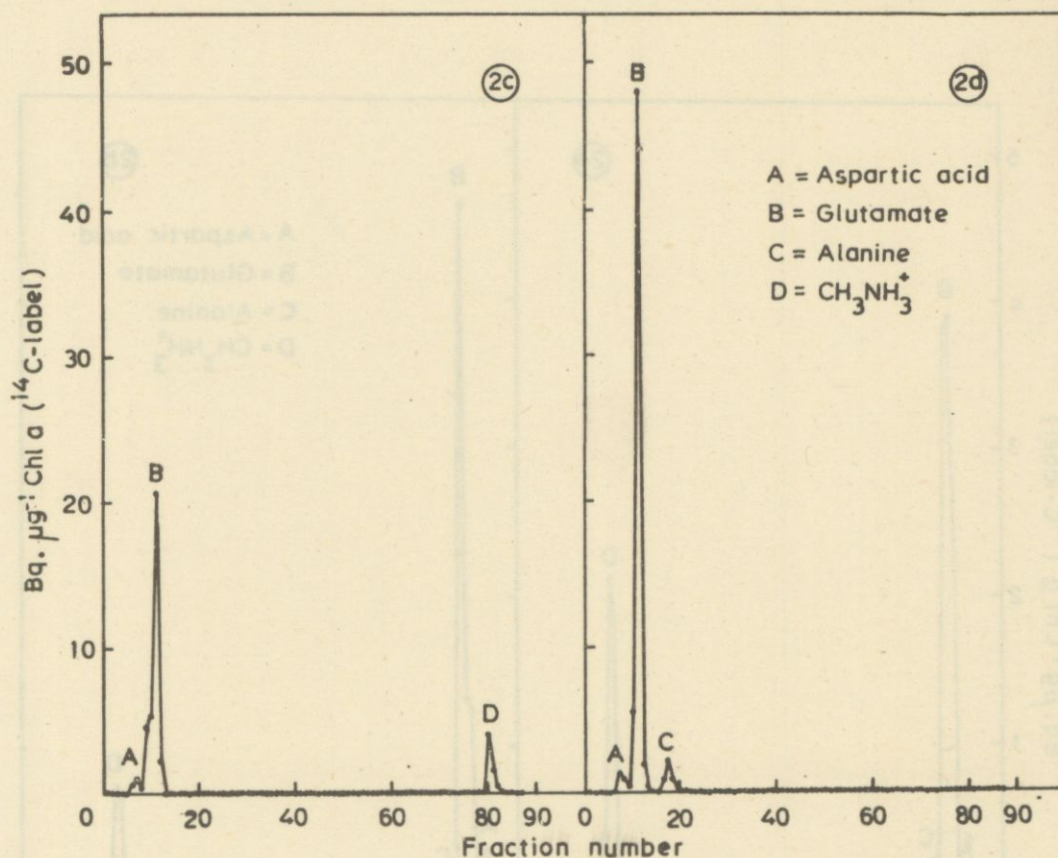


Fig. 2 (c&d). $^{14}\text{CH}_3\text{NH}_3^+$ metabolism in *Nostoc* ANTH. Elution profile of ^{14}C -amino acids (detected as in fig 1) in 60 min (c); and 240 min, (d) $^{14}\text{CH}_3\text{NH}_3^+$ incubated cells.

Simultaneous addition of NH_4Cl and $^{14}\text{CH}_3\text{NH}_3^+$ caused total inhibition of $^{14}\text{CH}_3\text{NH}_3^+$ uptake in both cyanobacteria (fig 5). When NH_4Cl was added subsequent to $^{14}\text{CH}_3\text{NH}_3^+$ addition, it showed an immediate inhibition of $^{14}\text{CH}_3\text{NH}_3^+$ uptake and an immediate efflux of preaccumulated ^{14}C -label from cells (fig 5). These results suggested the occurrence of a common transport system for both NH_4Cl and $^{14}\text{CH}_3\text{NH}_3^+$ in these cyanobacteria. These findings and interpretations are consistent with observations in other cyanobacteria (Rai *et al.*, 1984; Kleiner, 1985; Singh *et al.*, 1985, 1986, 1987, Kerby *et al.*, 1986; 1987, Reglinski *et al.*, 1989).

The NH_4 -displaceable ^{14}C -label remained constant over the experimental period (Fig 5). The amount of ^{14}C -label displaced by NH_4Cl was 20 and 21 $\text{Bq. } \mu\text{g}^{-1}$ Chl *a* in *Anabaena* 7120 and *Nostoc* ANTH, respectively. This amount of ^{14}C -label was similar to the $^{14}\text{CH}_3\text{NH}_3^+$ transported into the cells during initial rapid phase. Such data indicate the presence of free $^{14}\text{CH}_3\text{NH}_3^+$ pool inside the cells and that such a pool seems to be built up during the initial phase. These results also suggest that the first rapid phase is responsible for accumulation of $^{14}\text{CH}_3\text{NH}_3^+$ as free pool inside the cells.

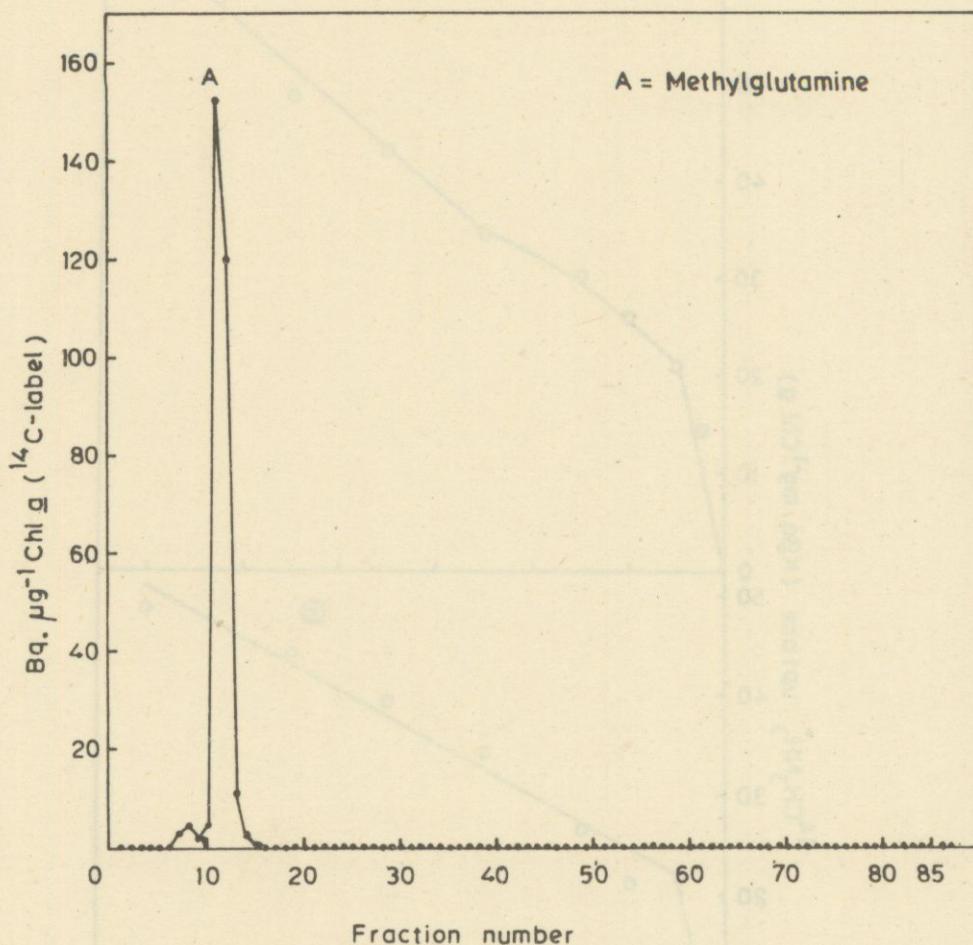


Fig 3. $^{14}\text{CH}_3\text{NH}_3^+$ metabolism in 60 min azaserine ($100\mu\text{M}$) pretreated *Nostoc* ANTH cells. The $^{14}\text{CH}_3\text{NH}_3^+$ incubation time was 60 min.

The NH_4 -undisplaceable ^{14}C -label inside the cells, however, increased with increase in time (fig 5). This increase corresponded to the linear $^{14}\text{CH}_3\text{NH}_3^+$ uptake during the second phase in control cells suggesting that NH_4 -undisplaceable ^{14}C label is the assimilated product of $^{14}\text{CH}_3\text{NH}_3^+$, which is not effluxed by NH_4Cl addition. These findings indicated that the second $^{14}\text{CH}_3\text{NH}_3^+$ uptake phase is dependent on the assimilation of transported species. Indeed our HPLC data confirm this by showing increased ^{14}C -methylglutamine accumulation, in *Anabaena* 7120, with increase in time of $^{14}\text{CH}_3\text{NH}_3^+$ incorporation during the second phase (fig1).

Immediate effects of MSX on $^{14}\text{CH}_3\text{NH}_3^+$ uptake in both cyanobacteria are presented in fig. 6. Simultaneous addition of both MSX and $^{14}\text{CH}_3\text{NH}_3^+$ resulted in an unaltered first phase in *Anabaena* 7120 while in *Nostoc* ANTH, MSX affected the first phase (fig 6.) The uptake rate in the presence of MSX, in *Nostoc* ANTH, upto 30 seconds was similar to that observed in control cells followed by a slower uptake rate till the ^{14}C -label in the cells reached a level similar to that at the end of first phase of $^{14}\text{CH}_3\text{NH}_3^+$ uptake in control cells (fig 6b).

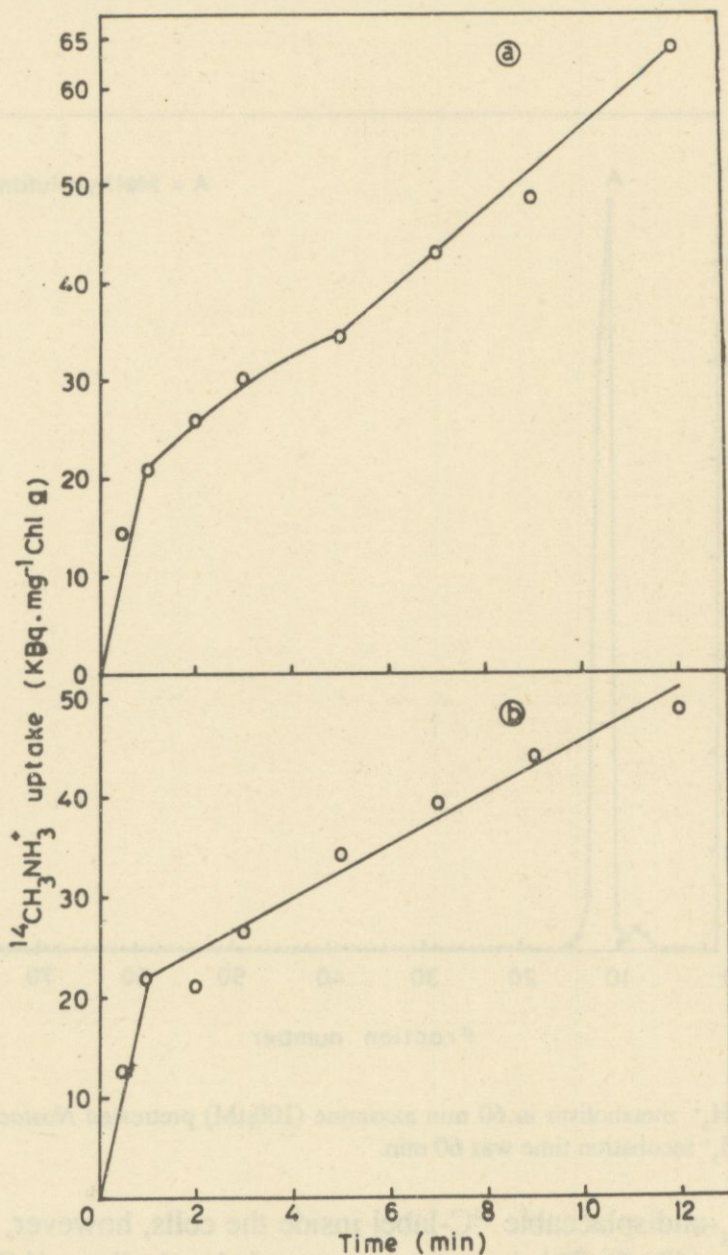


Fig 4. $^{14}\text{CH}_3\text{NH}_3^+$ uptake by *Anabaena* 7120 (a) and *Nostoc* ANTH (b) filaments. In this and all other experiments (fig 5-8) the data are means of four replicates obtained from two repeat experiments. The variations range was between 5-10% from the average.

When MSX was added after $^{14}\text{CH}_3\text{NH}_3^+$ addition, during the second phase of $^{14}\text{CH}_3\text{NH}_3^+$ uptake, it caused an immediate inhibition of $^{14}\text{CH}_3\text{NH}_3^+$ uptake both in *Anabaena* 7120 and *Nostoc* ANTH (fig 6). However, in the case of *Nostoc* ANTH, MSX caused an efflux of $16 \text{ Bq} \cdot \mu\text{g}^{-1} \text{ Chl } a$ ^{14}C -label (fig 6b). This immediate $^{14}\text{CH}_3\text{NH}_3^+$ uptake inhibition by MSX concentration ($10 \mu\text{M}$) used here showed no GS inactivation upto 15 min (data not shown). Thus, MSX seems to have two inhibitory targets in these cyanobacteria: (1) at transport level and (2) at GS inactivation level. These results are similar to those in *Anabaena doliolum* (Singh *et al.*, 1986) and indicate that ATS may have a regulatory site of MSX as proposed in bacteria (Kleiner and Castorh, 1982). Inhibition of only second uptake phase by

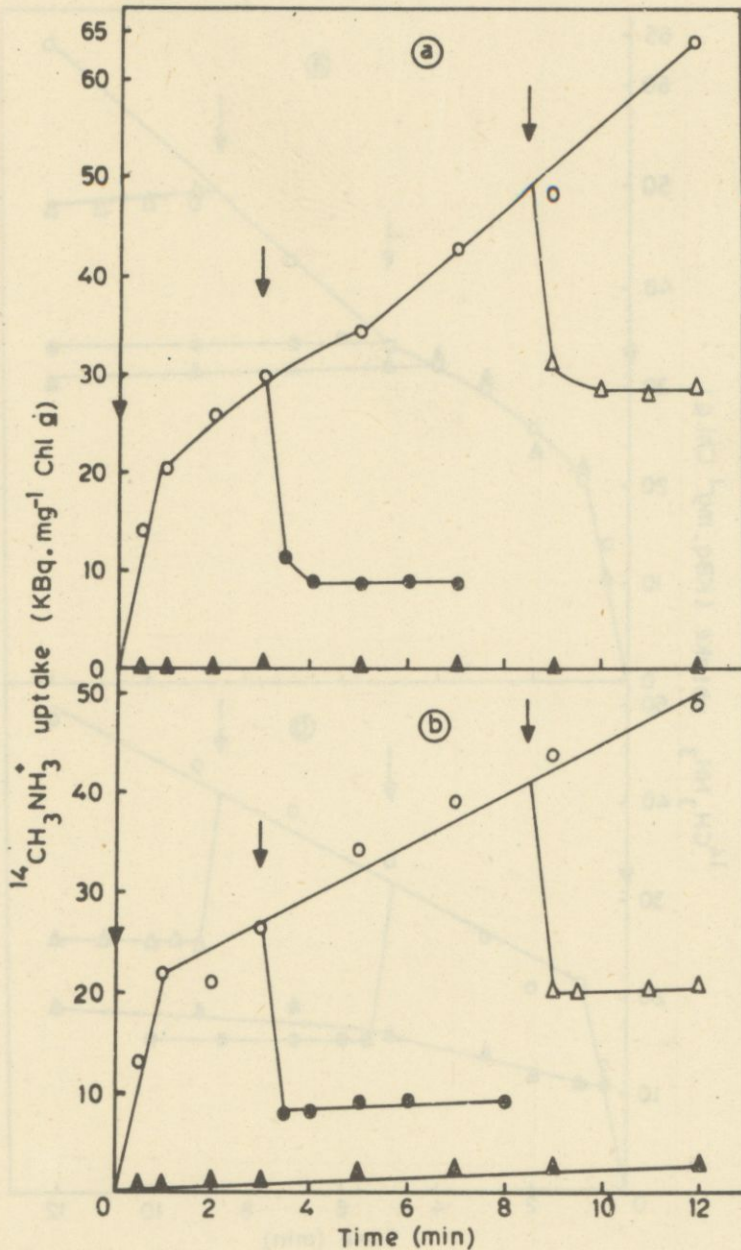


Fig 5. Effect of NH_4Cl ($200 \mu\text{M}$) addition on $^{14}\text{CH}_3\text{NH}_3^+$ uptake by *Anabaena* 7120 (a) and *Nostoc* ANTH (b). NH_4Cl was added at times indicated (arrows). O, control, ●, NH_4Cl added at 3 min after $^{14}\text{CH}_3\text{NH}_3^+$ addition, Δ, NH_4Cl added at 8.5 min after $^{14}\text{CH}_3\text{NH}_3^+$ addition; ▲, simultaneous addition of NH_4Cl and $^{14}\text{CH}_3\text{NH}_3^+$ at zero time.

MSX, and the unaffected initial uptake phase in the presence of MSX, indicate that biphasic uptake pattern is true representation of operation of two distinct transport systems: one being MSX-insensitive rapid ATS (first phase) and the other being MSX-sensitive slower ATS (second phase). Such two distinct ATS have been reported in GS-mutant of *Anabaena cycadeae* (Singh *et al.*, 1985).

The observed difference in the effluxed pool size, in *Nostoc* ANTH, by NH_4^+ /MSX addition during second phase (fig 5b and 6b) suggested that free $^{14}\text{CH}_3\text{NH}_3^+$

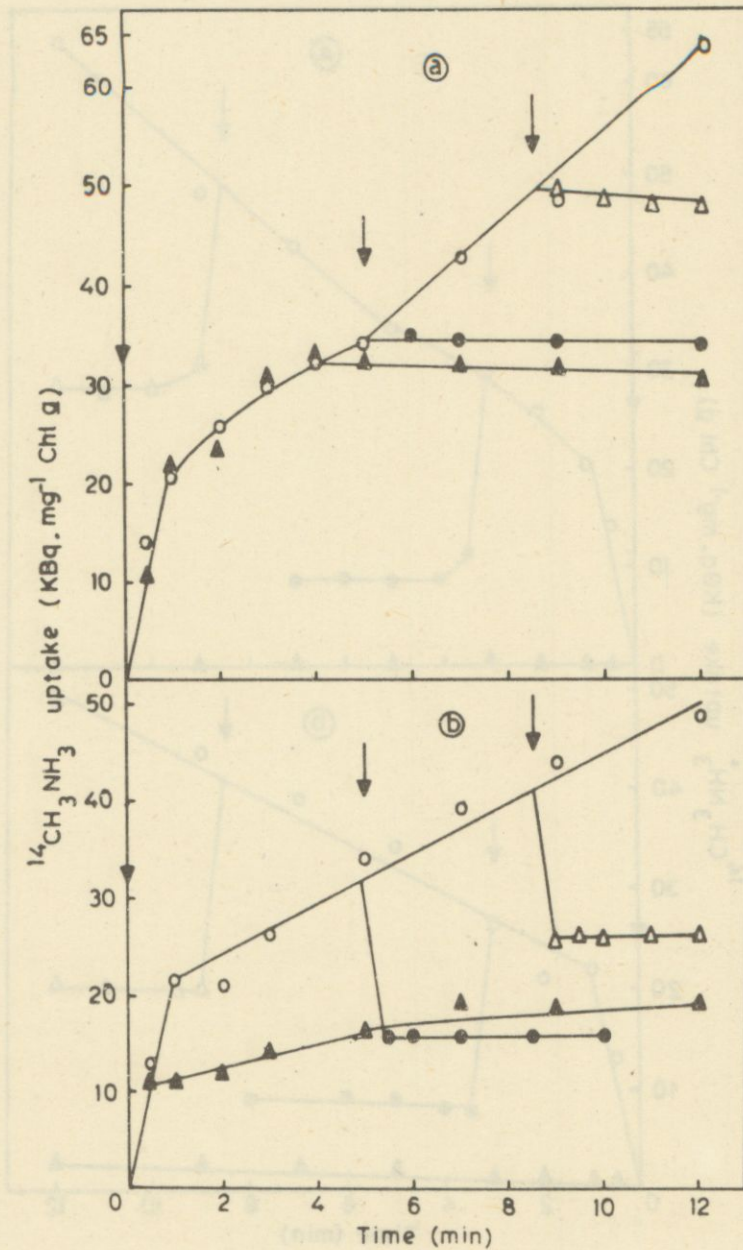


Fig 6. Effect of MSX ($10\mu\text{M}$) addition on $^{14}\text{CH}_3\text{NH}_3^+$ uptake by *Anabaena 7120* (a) and *Nostoc ANTH* (b). MSX added at times indicated (arrows). O, control; ▲, MSX and $^{14}\text{CH}_3\text{NH}_3^+$ added simultaneously at zero time; ●, MSX added at 5 min after $^{14}\text{CH}_3\text{NH}_3^+$ addition; △, MSX added at 8.5 min after $^{14}\text{CH}_3\text{NH}_3^+$ addition.

pool inside the cells, which is accumulated during initial phase, is localized at two sites (probably, one inside the thylakoids and the other in the cytoplasm). The cytoplasmic pool seems to be subjected to cyclic retention processes under normal conditions, while thylakoid pool is not.

The two ATS, in *Anabaena 7120* were further characterised by studying K_m and V_{max} in the external $^{14}\text{CH}_3\text{NH}_3^+$ concentration range of 1 - 500 μM . Both ATS exhibited a biphasic pattern of concentration dependent increase in $^{14}\text{CH}_3\text{NH}_3^+$

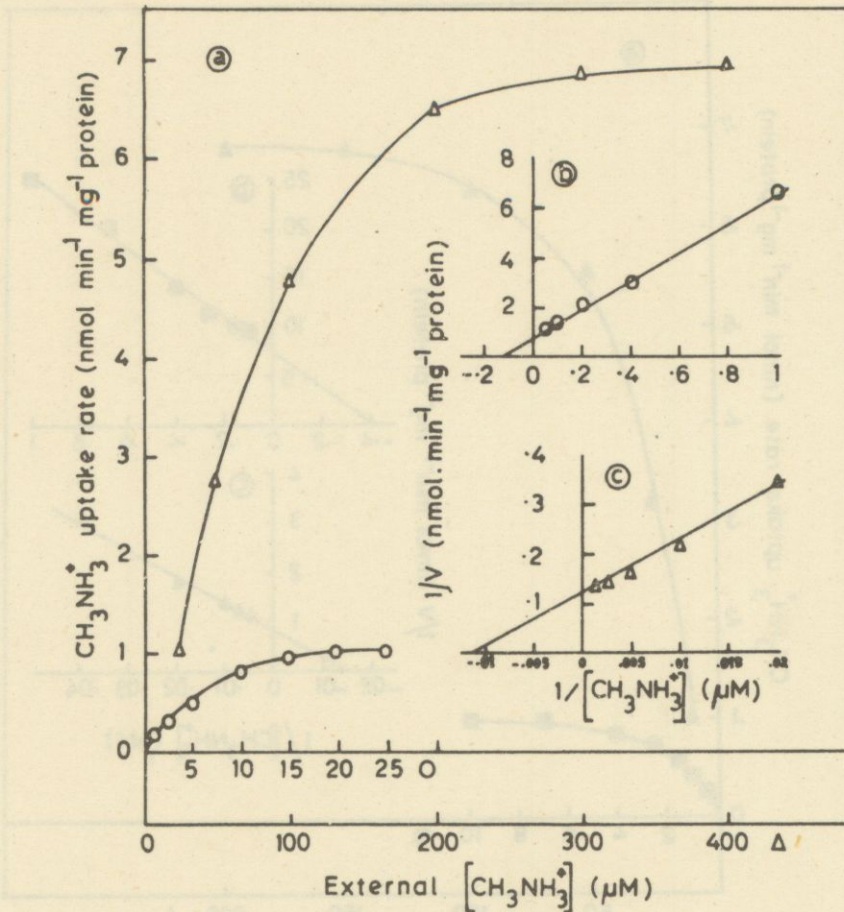


Fig 7. a) Concentration dependent $^{14}\text{CH}_3\text{NH}_3^+$ uptake rates during the initial MSX-insensitive phase by *Anabaena* 7120 filaments showing dual isotherm Michaelis-Menten Kinetics. O, uptake rates at external concentration range of 1 - 25 μM $^{14}\text{CH}_3\text{NH}_3^+$; Δ , uptake rates at external concentration range of 25 - 400 μM $^{14}\text{CH}_3\text{NH}_3^+$. b and c) Lineweaver-Burk plots for $^{14}\text{CH}_3\text{NH}_3^+$ uptake during high affinity mode (1-25 μM external $^{14}\text{CH}_3\text{NH}_3^+$ concentration, O) and low affinity mode (25 - 400 μM external $^{14}\text{CH}_3\text{NH}_3^+$ concentration, Δ).

uptake rates. MSX-insensitive ATS showed V_{max} values of 1 and 7 nmol.min⁻¹ mg⁻¹ protein in the external concentration range of 1-25 and 25 - 500 μM , respectively (fig. 7a; data beyond 400 μM not shown). The corresponding K_m values were 8 and 80 μM (fig 7b and 7c: calculated from Lineweaver-Burk plots). The observed V_{max} values for MSX-sensitive ATS were 0.1 and 0.7 nmol.min⁻¹. mg⁻¹ protein (fig 8a) in the external concentration range of 1-50 and 50 - 500 μM , respectively (data beyond 200 μM not shown). The corresponding K_m values were 2.5 and 70 μM (fig 8b and 8c: calculated from Lineweaver-Burk plots).

Thus, both ATS showed a low and high affinity mode of operation depending on the substrate concentration, and a ten-fold higher uptake rate was observed via MSX-insensitive ATS than via MSX-sensitive ATS. There was a seven-fold higher uptake rate via low affinity mode as compared to that via the high affinity mode for each transport system.

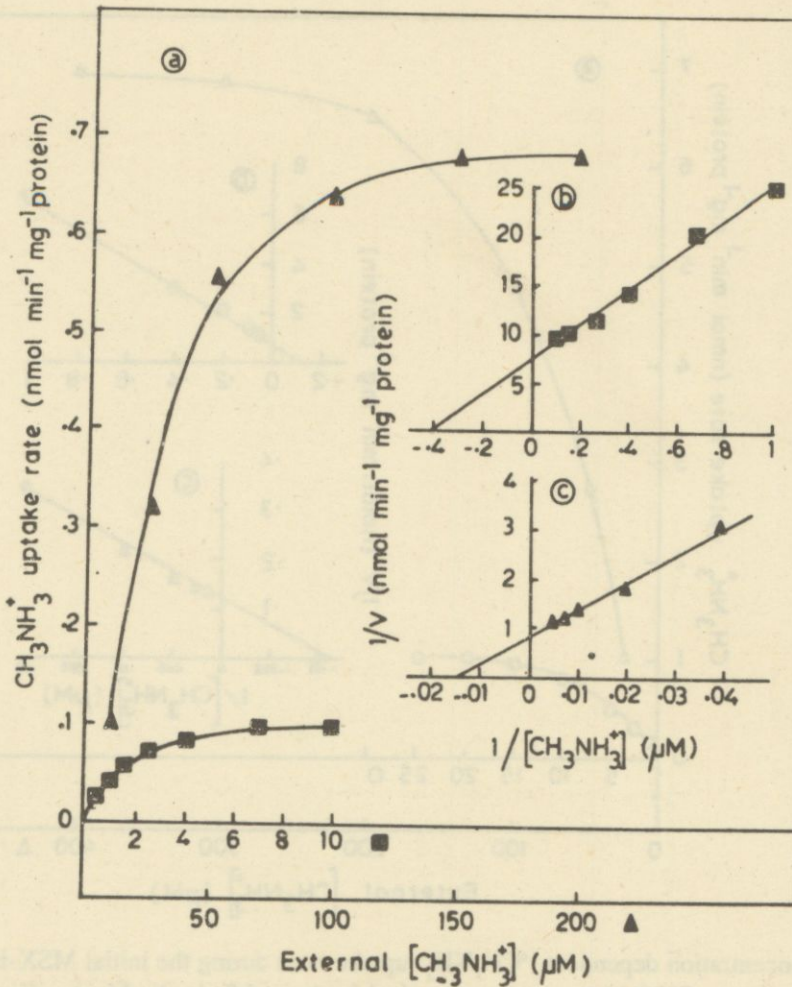


Fig 8. a) Concentration dependent $^{14}\text{CH}_3\text{NH}_3^+$ uptake rates during the second MSX-sensitive phase by *Anabaena* 7120 filaments showing dual isotherm Michaelis-Menten Kinetics. ■ uptake rates at external $^{14}\text{CH}_3\text{NH}_3^+$ concentration range of 1- 10 μM ; ▲, uptake rates at external $^{14}\text{CH}_3\text{NH}_3^+$ concentration range of 10 - 200 μM . b and c) Lineweaver-Burk plots for $^{14}\text{CH}_3\text{NH}_3^+$ uptake during high affinity mode (1 - 10 μM external $^{14}\text{CH}_3\text{NH}_3^+$ concentration ■) and low affinity mode (10-200 μM external $^{14}\text{CH}_3\text{NH}_3^+$ concentration, ▲).

Overall, our data indicate that there are two ATSS in *Anabaena* 7120 and *Nostoc* ANTH, that both these ATSS show affinity modulation which can serve as a regulatory point for CH_3NH_3^+ /Ammonium entry into the cells, and that *Nostoc* ANTH differs from *Anabaena* 7120 in terms of CH_3NH_3^+ metabolism.

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