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Evidence for the occurrence of a specific methylammonium transport system in the cultured cyanobiont of the *Anthoceros punctatus–Nostoc* association

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Methylammonium uptake and metabolism was studied in *Nostoc* sp. ANTH (a free-living cultured isolate from *Anthoceros punctatus*). Unlike other cyanobacteria, *Nostoc* sp. ANTH assimilated methylammonium as a fixed nitrogen source. While N_2-grown *Nostoc* sp. ANTH cells showed ammonium transport characteristics similar to those in other cyanobacteria, a specific energy-dependent methylammonium transport was noticed in methylammonium-grown cells. Unlike methylammonium/ammonium transport in N_2-grown cells, methylammonium transport in methylammonium-grown cells was neither affected by ammonium nor by L-methionine-DL-sulphoximine.

Introduction

In several cyanobacteria a common transport system for methylammonium and ammonium has been demonstrated (see Kleiner, 1985; Rai & Prakasham, 1989). Except in the case of an *Anabaena variabilis* mutant (Reglinski *et al.*, 1989), none of the cyanobacteria studied have the capacity to metabolize methylammonium as a nitrogen source for growth, and [^{14}C]methylammonium has been used primarily as a probe for studying ammonium transport characteristics.

Nostoc sp. ANTH was isolated and cultured by us from a symbiotic association with *Anthoceros punctatus* and was found to metabolize methylammonium as a nitrogen source. We have made a comparative study of the characteristics of methylammonium uptake/transport in methylammonium- and N_2-grown cells of *Nostoc* sp. ANTH. We report here that while N_2-grown *Nostoc* sp. ANTH has a common methylammonium/ammonium transport system with characteristics similar to those reported in other cyanobacteria, the methylammonium-grown *Nostoc* sp. ANTH has a specific energy-dependent methylammonium transport system which is, in contrast to the methylammonium/ammonium transport system, insensitive to ammonium and L-methionine-DL-sulphoximine (MSX). This is the first report of the occurrence of a specific methylammonium transport system in cyanobacteria.

Methods

Organism and growth conditions. Nostoc sp. ANTH, an isolate obtained from *Anthoceros punctatus*, and *Anabaena* sp. PCC 7120 were grown in axenic culture in BG-11_0 medium (Rippka *et al.*, 1979) at 28 ± 1 °C and at a photon fluence rate of 50 μmol m^{-2} s^{-1}. When required, NH_4Cl (1 mM), CH_3NH_2Cl (5 mM) and/or dichlorophenyl dimethylurea (DCMU, 10 μM) were added to the medium buffered with HEPES/NaOH (10 mM, pH 7·5).

Chlorophyll estimation. The chlorophyll *a* (Chl *a*) content of cyanobacteria was measured according to the method of Mackinney (1941).

Measurement of [^{14}C]methylammonium uptake. The cyanobacteria were harvested during the exponential growth phase, washed and resuspended in 10 mM-HEPES/NaOH buffer (pH 7·0), and equilibrated for 30 min at 28 ± 1 °C and at a photon fluence rate of 50 μmol m^{-2} s^{-1}. [^{14}C]methylammonium was then added to the cell suspension to a final concentration of 50 μM (specific activity 185 kBq μmol^{-1}). At different time intervals, 400 μl samples were taken and the cells were separated from the medium using the silicone-oil microcentrifugation technique (Scott & Nicholls, 1980). ^{14}C-label in cells was measured using a liquid scintillation spectrophotometer (model LS 1801, Beckman Instruments). Non-specific binding of [^{14}C]methylammonium was determined by measuring its incorporation in toluene-treated cells as described by Rai *et al.* (1984).

Measurement of nitrogenase activity. Nitrogenase activity was measured by gas chromatography using the acetylene reduction assay (Stewart *et al.*, 1967).

Abbreviations: Chl *a*, chlorophyll *a*; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCMU, dichlorophenyl dimethylurea; GS, glutamine synthetase; MSX, L-methionine-DL-sulphoximine. $TPMP^+$, triphenylmethylphosphonium.

Measurements of intracellular volume and internal pools of methylammonium. Internal cell volume and the pools of methylammonium in N_2-grown cells were estimated as described previously (Rai *et al.*, 1984). To determine the intracellular pool of methylammonium in methylammonium-grown cells, the cellular material was extracted, chromatographed, and the radioactivity of the eluted methylammonium fraction measured in a scintillation counter. The method used was essentially the same as described previously (Rai *et al.*, 1984) except that instead of an amino acid analyser, HPLC was used. The intracellular methylammonium pool was also determined from methylammonium uptake in MSX-pre-incubated cells in which glutamine synthetase (GS) had been fully inactivated and in which no methylammonium metabolism occurred. These two methods gave comparable values of 3·78 and 3·93 mM, respectively.

Chemicals. $^{14}CH_3NH_2Cl$ was purchased from Amersham. Silicone oil DC 550 and dinonylphthalate were obtained from Fluka. All other chemicals were obtained from Sigma.

Results

Growth of Anabaena sp. PCC 7120 and Nostoc sp. ANTH in N_2-medium and in methylammonium-supplemented medium

Both *Anabaena* sp. PCC 7120 and *Nostoc* sp. ANTH differentiated heterocysts, reduced acetylene and grew well at the expense of N_2 as a nitrogen source (Table 1). The observed specific growth rates were 1·065 and 0·62 doublings per day for *Anabaena* sp. PCC 7120 and *Nostoc* sp. ANTH, respectively.

Anabaena sp. PCC 7120 did not grow in methylammonium-supplemented medium. Heterocyst frequency was not determined since the inoculum became pale yellow in colour and heterocysts were difficult to distinguish. However, nitrogenase activity was measured and found to be undetectable. These data show the inability of *Anabaena* sp. PCC 7120 to utilize methylammonium as a nitrogen source. In addition, in methylammonium-supplemented medium, *Anabaena* sp. PCC 7120 could not use N_2 as a nitrogen source since nitrogenase activity was lost.

In contrast to *Anabaena* sp. PCC 7120, *Nostoc* sp. ANTH grew well in methylammonium-supplemented medium. Since there was over 92% repression of heterocyst differentiation and nitrogenase activity, these results clearly show that *Nostoc* sp. ANTH used methylammonium as a nitrogen source.

To determine whether methylammonium can be utilized as a carbon source by *Nostoc* sp. ANTH, its growth was measured in DCMU- (to inhibit photosynthesis and subsequent carbon fixation) and methylammonium-supplemented medium. Such a medium did not support the growth of *Nostoc* sp. ANTH (Table 1), indicating that methylammonium was not used as a carbon source.

[^{14}C]Methylammonium uptake by N_2-, ammonium, and methylammonium-grown cells

N_2-grown *Nostoc* sp. ANTH cells, at pH 7·0 and 50 μM-[^{14}C]methylammonium (external concentration), showed a biphasic pattern of [^{14}C]methylammonium uptake with an initial rapid phase for the first 60 s followed by a slower second phase (Fig. 1). These results were similar to those observed in other cyanobacteria for which ammonium uptake studies have been performed (Rai *et al.*, 1984; Singh *et al.*, 1987; Boussiba, 1989; Rai & Prakasham, 1989; Reglinski *et al.*, 1989). In contrast, ammonium-grown cells showed no [^{14}C]methylammonium uptake (Fig. 1), consistent with reports suggesting ammonium transport system repression in ammonium-grown bacterial and cyanobacterial cells (Kleiner, 1985; Rai *et al.*, 1986; Rai & Prakasham, 1989).

Unlike in N_2-grown cells, [^{14}C]methylammonium uptake in methylammonium-grown cells did not show as

Table 1. *Growth, heterocyst frequency and nitrogenase activity in the presence and absence of methylammonium and/or DCMU in Nostoc sp. ANTH and Anabaena sp. PCC 7120*

Heterocyst frequency is expressed as the percentage of total cells in a filament, and nitrogenase activity as nmol C_2H_2 reduced h^{-1} (μg Chl *a*)$^{-1}$ ± SE.

		Specific growth rate (d^{-1})			
Organism	Methylammonium	+DCMU	−DCMU	Heterocyst frequency	Nitrogenase activity
Nostoc sp. ANTH	+	nd	0·76	0·5 ± 0·02	nd
	−	nd	0·62	6·5 ± 0·30	5·25 ± 0·25
Anabaena sp. PCC 7120	+	nd	nd	ND	nd
	−	nd	1·06	5·4 ± 0·20	4·25 ± 0·50

ND, Not determined. nd, Not detected.

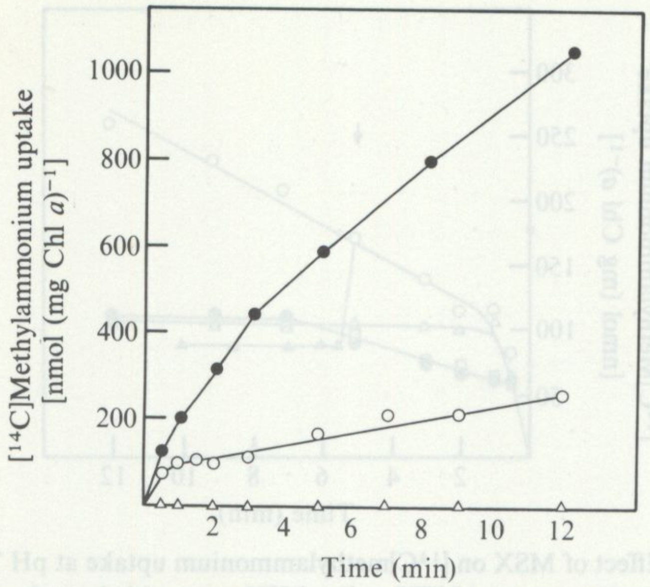

Fig. 1. $[^{14}C]$Methylammonium uptake at pH 7·0 by N_2-grown (○), methylammonium (5 mM)-grown (●), and ammonium (1 mM)-grown (△) *Nostoc* sp. ANTH cells. In this and all other experiments, non-specific binding of $[^{14}C]$methylammonium was determined by measuring $[^{14}C]$methylammonium incorporation in toluene-treated cells. These values were subtracted before plotting the data.

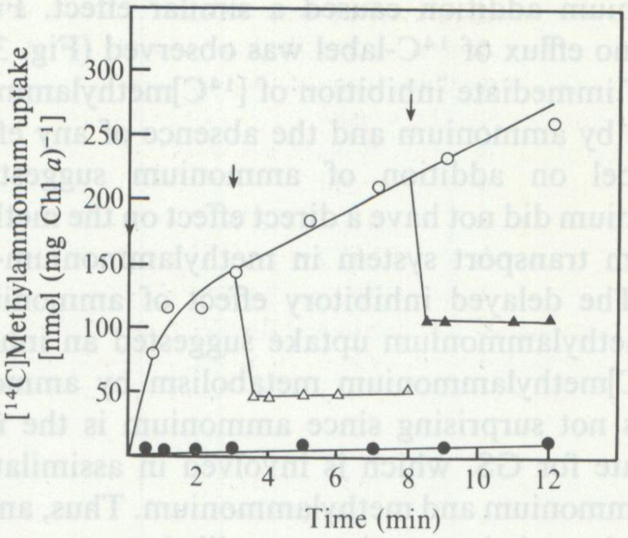

Fig. 2. Effect of NH_4Cl on $[^{14}C]$methylammonium uptake at pH 7·0 by N_2-grown *Nostoc* sp. ANTH cells. NH_4Cl was added at the times indicated (arrows) to a final concentration of 200 µM. ○, Control ($[^{14}C]$methylammonium only); ●, NH_4Cl and $[^{14}C]$methylammonium added simultaneously at zero time; △, NH_4Cl added 3 min after addition of $[^{14}C]$methylammonium; ▲, NH_4Cl added 8 min after addition of $[^{14}C]$methylammonium.

distinct a biphasic pattern, and the uptake rate in methylammonium-grown cells was about five-fold higher than that of N_2-grown cells during the second phase (Fig. 1).

Intracellular volume and the methylammonium pool

To enable calculation of the internal concentration of methylammonium, the intracellular volume of N_2- and methylammonium-grown cells was determined. This was found to be 48 µl (mg Chl *a*)$^{-1}$ in the case of N_2-grown cells of 50 µl (mg Chl *a*)$^{-1}$ in the case of methylammonium-grown cells. The pool of free intracellular methylammonium in N_2-grown cells was 2·25 mM, while that in methylammonium-grown cells 3·78 mM. These values correspond to 108 and 189 nmol methylammonium (mg Chl *a*)$^{-1}$ for N_2- and methylammonium-grown cells, respectively, and represent the level of $[^{14}C]$methylammonium taken up by these cells during the first 60 s. Thus, the intracellular pool of free methylammonium seems to be built up during the initial rapid phase.

Effect of NH_4Cl on $[^{14}C]$methylammonium uptake by N_2- and methylammonium-grown cells

Simultaneous addition of NH_4Cl and $[^{14}C]$methylammonium resulted in total inhibition of $[^{14}C]$methylammonium uptake in N_2-grown *Nostoc* sp. ANTH cells (Fig. 2). When NH_4Cl was added subsequent to the addition of $[^{14}C]$methylammonium, two effects were observed: (*a*) a sudden efflux of pre-accumulated ^{14}C-label from the cells into the medium, and (*b*) complete inhibition of further $[^{14}C]$methylammonium uptake by cells (Fig. 2). These results are similar to those reported for other cyanobacteria and indicate the existence of a common transport system for ammonium and methylammonium (Singh *et al.*, 1987; Rai & Prakasham, 1989).

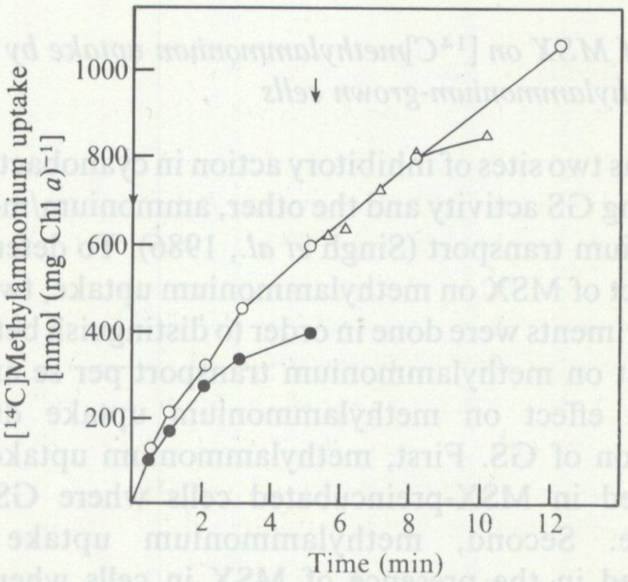

Fig. 3. Effect of NH_4Cl on $[^{14}C]$methylammonium uptake at pH 7·0 by methylammonium-grown *Nostoc* sp. ANTH cells. NH_4Cl (200 µM) was added at the times indicated (arrows). ○, Control ($[^{14}C]$methylammonium only); ●, NH_4Cl and $[^{14}C]$methylammonium added simultaneously at zero time; △, NH_4Cl added 5 min after addition of $[^{14}C]$methylammonium.

In the case of methylammonium-grown *Nostoc* sp. ANTH cells, on simultaneous addition of $[^{14}C]$methylammonium and NH_4Cl, $[^{14}C]$methylammonium uptake continued unaffected for the initial 2 min period after which further uptake was progressively inhibited (Fig. 3). Addition of NH_4Cl subsequent to $[^{14}C]$methylammonium addition caused a similar effect. Furthermore, no efflux of ^{14}C-label was observed (Fig. 3). The lack of immediate inhibition of [^{14}C]methylammonium uptake by ammonium and the absence of any efflux of ^{14}C-label on addition of ammonium suggests that ammonium did not have a direct effect on the methylammonium transport system in methylammonium-grown cells. The delayed inhibitory effect of ammonium on [^{14}C]methylammonium uptake suggested an inhibition of [^{14}C]methylammonium metabolism by ammonium. This is not surprising since ammonium is the natural substrate for GS, which is involved in assimilation of both ammonium and methylammonium. Thus, ammonium and methylammonium are likely to compete as substrates for GS. Overall, these results indicate that in methylammonium-grown cells, methylammonium uptake occurred through a specific methylammonium uptake/transport system quite distinct from the common ammonium/methylammonium transport system found in N_2-grown cells.

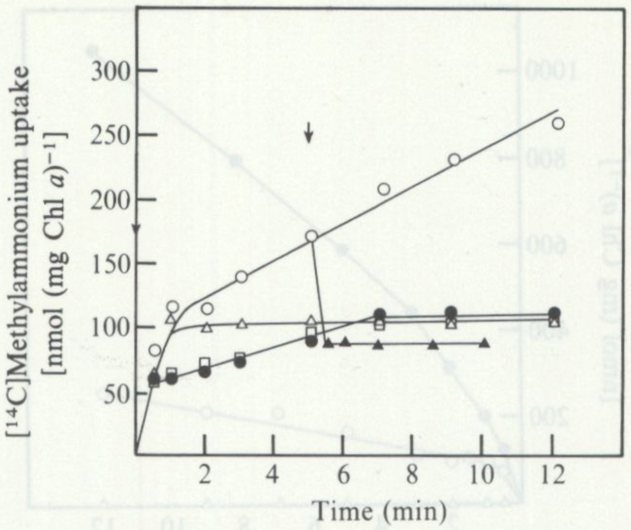

Fig. 4. Effect of MSX on [^{14}C]methylammonium uptake at pH 7·0 by N_2-grown *Nostoc* sp. ANTH cells. MSX was added at the times indicated (arrows) to a final concentration of 10 µM. ○, Control ([^{14}C]methylammonium only); ●, MSX and [^{14}C]methylammonium added simultaneously at zero time; ▲, MSX added 5 min after addition of [^{14}C]methylammonium; □, △, [^{14}C]methylammonium uptake in MSX-pre-incubated cells [cells were incubated with 10 µM-MSX for 1 h, then washed and resuspended in fresh buffer, and uptake was studied with (□) and without (△) MSX present in the medium].

Effect of MSX on [^{14}C]methylammonium uptake by N_2- and methylammonium-grown cells

MSX has two sites of inhibitory action in cyanobacteria – one being GS activity and the other, ammonium/methylammonium transport (Singh *et al.*, 1986). To determine the effect of MSX on methylammonium uptake, two sets of experiments were done in order to distinguish between its effect on methylammonium transport per se and its indirect effect on methylammonium uptake due to inhibition of GS. First, methylammonium uptake was measured in MSX-preincubated cells where GS was inactive. Second, methylammonium uptake was measured in the presence of MSX in cells where GS remained active throughout the experimental period of 12 min. GS inactivation, at an MSX concentration of 10 µM, required a minimum of 1 h and was undetectable during the initial 30 min (data not shown; see also Rai & Prakasham, 1989). Hence, [^{14}C]methylammonium uptake in the second set of experiments was studied for less than 10 min without any pre-incubation with MSX.

N_2-grown cells, in which GS was inactivated by pre-incubation with MSX (10 µM) for 1 h, did not show [^{14}C]methylammonium uptake beyond the initial 60 s period (Fig. 4). This is similar to the findings for other cyanobacteria and agrees with earlier conclusions that the first phase of [^{14}C]methylammonium uptake represents [^{14}C]methylammonium accumulation independent of its metabolism via GS, and that continued uptake of [^{14}C]methylammonium during the second phase requires metabolism of its internal pool via GS (Rai *et al.*, 1984). In the cells which were not pre-incubated with MSX, simultaneous addition of [^{14}C]methylammonium and MSX resulted in [^{14}C]methylammonium uptake for up to 7 min. However, the rate of uptake was comparatively slow and the total uptake was similar to that in MSX-pre-incubated cells (Fig. 4). [^{14}C]Methylammonium uptake in MSX-pre-incubated cells, in presence of MSX, showed a similar pattern. These data indicate that MSX partially inhibited the methylammonium transport in N_2-grown cells and that this effect was independent of the GS inactivation by MSX. Addition of MSX 5 min after the addition of [^{14}C]methylammonium caused an effect similar to that of ammonium addition, leading to an immediate efflux of ^{14}C-label from the cells and cessation of any further uptake. The efflux caused by MSX has not been noted in other cyanobacteria (Rai *et al.*, 1984; Singh *et al.*, 1985; Rai & Prakasham, 1989) and may reflect the fact that unlike other cyanobacteria, *Nostoc* sp. ANTH metabolizes methylammonium as a nitrogen source.

Methylammonium-grown cells, in which GS was inactivated by pre-incubation with MSX and [^{14}C]methylammonium uptake was studied in the absence of MSX showed similar results as for N_2-grown cells (Fig. 5), suggesting that, as in N_2-grown cells, the first phase of [^{14}C]methylammonium uptake represents accumulation of [^{14}C]methylammonium in the cells independent of its metabolism via GS, and the second phase represents assimilation of intracellular [^{14}C]methylammonium via GS. However, simultaneous addition of MSX and [^{14}C]methylammonium had no effect on [^{14}C]methylammonium uptake during the

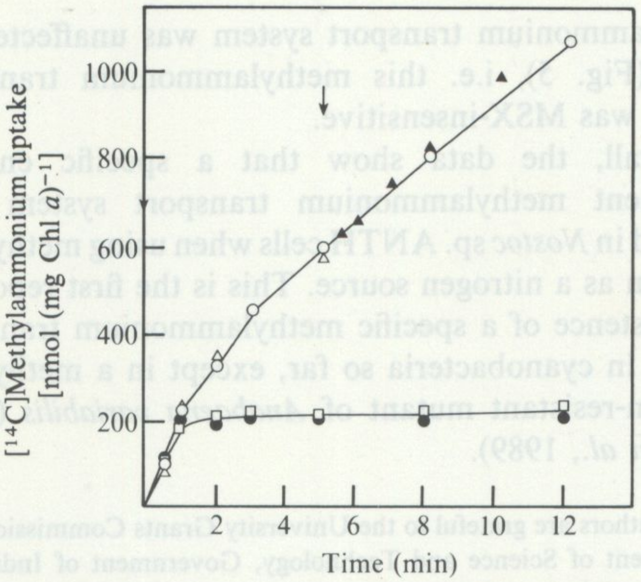

Fig. 5. Effect of MSX on [^{14}C]methylammonium uptake at pH 7·0 by methylammonium-grown *Nostoc* sp. ANTH cells. MSX (10 μM) was added at the times indicated (arrows). ○, Control ([^{14}C]methylammonium only); △, MSX and [^{14}C]methylammonium added simultaneously at zero time; ▲, MSX added 5 min after addition of [^{14}C]methylammonium; [^{14}C]methylammonium uptake in MSX-pre-incubated cells (see legend to Fig. 4) with (□) and without (●) MSX present in the medium during the uptake.

5 min experimental period. In addition, [^{14}C]methylammonium uptake by MSX-preincubated cells, in the presence of MSX, showed a pattern similar to that obtained for the [^{14}C]methylammonium uptake by such cells in the absence of MSX. These data suggest that in contrast to the N_2-grown cells, MSX did not affect the methylammonium transport system in methylammonium-grown cells. Addition of MSX 5 min after addition of [^{14}C]methylammonium also had no effect on [^{14}C]methylammonium uptake and no efflux of ^{14}C-label was observed (Fig 5). This is again in contrast to the observation in N_2-grown cells and shows the uniqueness of the methylammonium transport system as compared to the common methylammonium/ammonium transport system.

Effect of CCCP and TPMP$^+$ on [^{14}C]methylammonium accumulation by methylammonium-grown cells

Effect of CCCP (an uncoupler) and TPMP$^+$ (a lipophilic cation which collapses transmembrane electrical potential) on [^{14}C]methylammonium accumulation was studied in methylammonium-grown *Nostoc* sp. ANTH cells in which GS was inactivated by pre-incubation with MSX. This was done to distinguish the effect of CCCP and TPMP$^+$ on methylammonium accumulation independent of methylammonium metabolism. As shown in Table 2, MSX-pre-incubated cells accumulated 196·5 nmol [^{14}C]methylammonium (mg Chl *a*)$^{-1}$. Since no methylammonium metabolism occurred in such cells, these values represent the intracellular pool of free [^{14}C]methylammonium, corresponding to 3·93 mM [intracellular volume 50 μl (mg Chl *a*)$^{-1}$; specific activity 185 kBq μmol^{-1}]. In contrast the level of [^{14}C]methylammonium uptake in MSX-pre-incubated cells which were also treated with CCCP and TPMP$^+$, was nearly 70% lower. These data indicate that the process of methylammonium transport in methylammonium-grown cells is energy-dependent.

Table 2. *Effect of CCCP and TPMP$^+$ on methylammonium accumulation by methylammonium-grown Nostoc sp. ANTH cells pre-incubated with MSX*

MSX was added to *Nostoc* sp. ANTH cells growing exponentially in methylammonium-supplemented BG-11_0 medium to a final concentration of 10 μM. The cells were harvested after 1 h and resuspended in 10 mM-HEPES/NaOH buffer (pH 7·0) containing 10 μM-MSX. These resuspended cells were incubated for 30 min in the light with or without CCCP and TPMP$^+$ (final concentration 100 μM). [^{14}C]Methylammonium was then added and after 5 min incorporation of ^{14}C into the cells was determined as described in Methods.

	Methylammonium accumulation in the cells		
Treatment	nmol Methylammonium (mg Chl *a*)$^{-1}$ (±SE)	mM	Percentage of control
Control (MSX-pre-incubated cells)	196·5 ± 14·2	3·93	100
+CCCP	57·2 ± 4·7	1·14	29
+TPMP$^+$	61·6 ± 5·7	1·23	31

Discussion

The results presented in this paper indicate that methylammonium can be metabolized as a fixed-nitrogen source by *Nostoc* sp. ANTH. This conclusion was exemplified by the growth of *Nostoc* sp. ANTH in methylammonium-supplemented medium and concurrent repression of heterocyst differentiation and nitrogenase activity (Table 1). Such results are in contrast to observations with other cyanobacteria, where methylammonium could not be used as a nitrogen source since it was metabolized only up to methylglutamine, which accumulates in the cells (Rai *et al.*, 1984; Reglinski *et al.*, 1989). A common transport system for methylammonium/ammonium with characteristics similar to those observed in *Anabaena* sp. PCC 7120 (Rai & Prakasham, 1989) and other cyanobacteria (Rai *et al.*, 1984; Singh *et al.*, 1987; Boussiba, 1989) was observed in N_2-grown cells.

Both N_2- and methylammonium-grown cells showed a biphasic pattern of [^{14}C]methylammonium uptake consisting of a rapid first phase followed by a slower second phase (Fig. 1). In both these cases, the level of [^{14}C]methylammonium taken up during the first phase (60 s) corresponded to the level of the intracellular pool of free methylammonium in these cells (2·25 and 3·78 mM in N_2- and methylammonium-grown cells, respectively). In MSX-pre-incubated cells, where GS was inactivated, the first phase of [^{14}C]methylammonium uptake persisted while the second phase was absent (Figs 4 and 5). The pool of free methylammonium in MSX-pre-incubated cells was 2·3 and 3·93 mM in N_2- and methylammonium-grown cells, respectively. This again is similar to the level of [^{14}C]methylammonium taken up during the initial 60 s period. These data suggest that the first phase of [^{14}C]methylammonium uptake was intracellular accumulation of free methylammonium while the second phase was linked to assimilation of intracellular methylammonium by GS.

The methylammonium transport system in methylammonium-grown *Nostoc* sp. ANTH cells was specific for methylammonium and it did not transport ammonium. This conclusion was based on the following facts. First, unlike methylammonium accumulation by the methylammonium/ammonium transport system (Fig. 2), methylammonium accumulation by the methylammonium transport system was not inhibited by the addition of ammonium (Fig. 3). Second, in contrast to the observations in N_2-grown cells (Fig. 2), addition of ammonium did not cause efflux of pre-accumulated [^{14}C]methylammonium from methylammonium-grown cells (Fig. 3).

The methylammonium transport system seems to be an inducible system since it developed only in methylammonium-grown cells. This is supported by the following. (1) In N_2-grown cells methylammonium uptake occurred only through the methylammonium/ammonium transport system. This is because, if the specific methylammonium transport system was operative in N_2-grown cells, then ammonium should not have caused total inhibition of [^{14}C]methylammonium uptake and total efflux of the intracellular pool of free [^{14}C]methylammonium from such cells. (2) In ammonium-grown cells, no methylammonium uptake occurred (Fig. 1).

The specific methylammonium transport system had the following additional characteristics. First, methylammonium uptake through the methylammonium transport system was an energy-dependent process, driven by a transmembrane electrical potential as shown by the inhibition of methylammonium accumulation by CCCP (an uncoupler) and TPMP$^+$ (an agent causing collapse of transmembrane electrical potential) (Table 2). Second, unlike the observation in N_2-grown cells where MSX inhibited the methylammonium/ammonium transport system (Fig. 4), methylammonium uptake by the methylammonium transport system was unaffected by MSX (Fig. 5), i.e. this methylammonium transport system was MSX-insensitive.

Overall, the data show that a specific energy-dependent methylammonium transport system was induced in *Nostoc* sp. ANTH cells when using methylammonium as a nitrogen source. This is the first report of the existence of a specific methylammonium transport system in cyanobacteria so far, except in a methylammonium-resistant mutant of *Anabaena variabilis* (Reglinski *et al*., 1989).

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