JOURNAL OF BACTERIOLOGY, Oct. 1984, p. 204–210
0021-9193/84/100204-07$02.00/0

Vol. 160, No. 1

Methylammonium Transport in *Anacystis nidulans* R-2

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Received 21 May 1984/Accepted 16 July 1984

Methylammonium was taken up rapidly by illuminated cells of *Anacystis nidulans* R-2, leading to internal concentrations of 1.3 ± 0.1 mM within 1 min, and a gradient of up to 200 between the cells and medium. Accumulation of $^{14}CH_3NH_3^+$ required at least 5 mM NaCl, but the uptake rate was independent of medium pH between 6.5 and 9. The kinetics of uptake could be resolved into an initial fast phase lasting less than 1 min (approximate K_m, 7.2 μM; V_{max}, 12.5 nmol min^{-1} mg of protein^{-1} at 15°C). A second, slower phase associated with product formation was eliminated by preincubation with methionine sulfoximine, a specific inhibitor of glutamine synthetase; the rapid phase was unaffected by this treatment. Ammonium ions competed with $^{14}CH_3NH_3^+$ for entry, and addition of 5 μM NH_4^+ or 100 μM $CH_3NH_3^+$ released $^{14}CH_3NH_3^+$ accumulated during the rapid phase of entry. Small additions of NH_4^+ made at the same time as additions of $^{14}CH_3NH_3^+$ delayed the start of radioactivity uptake by a time which corresponded accurately with the period needed for the complete removal of the added NH_4^+. The effects of inhibitors on accumulation and carbocyanine dye fluorescence suggest that ATP-dependent membrane potential was needed to drive $^{14}CH_3NH_3^+$ transport. Spheroplasts were as active as whole cells in accumulating NH_4^+ and $^{14}CH_3NH_3^+$, indicating that soluble periplasmic components are not involved in the translocation. Some significant differences between the translocation of $^{14}CH_3NH_3^+$ and that of NH_4^+ were observed: growth with NH_4^+ in place of NO_3^- repressed $^{14}CH_3NH_3^+$ accumulation ability without affecting the NH_4^+ uptake rate, Na^+ was not required for NH_4^+ uptake, and concentrations of KCl inhibitory with $^{14}CH_3NH_3^+$ did not reduce NH_4^+ uptake.

Ammonia is the inorganic nitrogen source most widely used by microorganisms and, in those which are also able to use nitrate, nitrite, or dinitrogen, is also an obligate intracellular intermediate in nitrogen assimilation. An ability to take up externally available or internally generated ammonia efficiently would therefore be of considerable advantage to free-living cells. A number of recent investigations have demonstrated the existence and defined some of the properties of methylammonium transport systems in microorganisms (1, 5, 7, 11–15, 18, 21, 23). Since ammonium generally inhibits methylammonium entry competitively, it has been assumed that NH_4^+ is in fact the natural substrate of the system.

Cyanobacteria resemble other microorganisms in taking up ammonia even when other nitrogen sources are available, and the characteristics of ammonium uptake and retention in some of the easily manipulated unicellular types have been examined (1a). A concentration gradient of at least 10^3 could be maintained between the cells and the surrounding medium, but net uptake occurrs only in illuminated cells supplied with bicarbonate. Uptake is blocked not only by energy poisons but also by the specific glutamine synthetase inhibitor methionine sulfoximine. These results suggest that net uptake was observed only when conditions permitted continuing amidation and amino acid synthesis.

In an effort to distinguish between the process of transport across the cytoplasmic membrane and subsequent metabolic conversion of entered molecules, the uptake of methylammonium was investigated. In *Anacystis nidulans* R-2, as in microorganisms studied in other laboratories, methylammonium could be accumulated against a concentration gradient of 150- to 200-fold. Kinetic parameters and other characteristics were established. Transport could be separated from conversion to a metabolic derivative kinetically and by insensitivity to methionine sulfoximine and low concentrations of −SH reagents. Methylammonium entry was much slower in cells grown with NH_4^+ as a nitrogen source and required at least 5 mM Na^+ for maximal activity. Neither of these phenomena could be demonstrated in parallel determinations of NH_4^+ uptake. Methylammonium transport and ammonium uptake in this cyanobacterium thus appear to differ in some significant way.

MATERIALS AND METHODS

Organisms and growth conditions. *A. nidulans* R-2 was obtained from J. Williams, Boyce Thompson Institute, Cornell University, Ithaca, N.Y. Cultures were grown in BG-11 medium (20) containing 5 or 10 mM Na or KNO_3. When 5 mM NH_4Cl was substituted for NO_3^- as a nitrogen source, the medium was supplemented with 5 mM Na or $KHCO_3$. The cultures were maintained at 30°C and illuminated and gassed with 3% CO_2 in air as previously described (1a).

Uptake measurements. (i) Methylammonium. Cells from exponentially growing cultures (50 to 100 μg of protein ml^{-1}) were washed once in 20 mM potassium buffer (pH 7.1) and resuspended in the same buffer, usually containing 10 mM $KHCO_3$ and other additions as noted, to a cell concentration generally between 60 and 130 μg of protein ml^{-1}. After 15 min of preequilibration in light from a 100-W lamp at a distance of 30 cm at 30°C, $^{14}CH_3NH_3Cl$ (1 to 50 μM; specific activity, 3.4 to 56 mCi mmol^{-1}) was added. Samples of 100 μl were filtered through polycarbonate membranes (diameter, 25 mm; pore size, 1 μm; Nuclepore Corp., Pleasanton, Calif.), and the radioactivity retained was determined with a Beckman LS 100 liquid scintillation spectrometer. Samples

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of the total isotope-containing cell suspensions, counted under identical conditions, were used to compute the quantities of radioactive compounds retained on the filters. The counts in the samples taken 5 s after the addition of $^{14}CH_3NH_3^+$ corresponded with the estimates of extracellular fluid retained by the filters when samples of suspensions containing [^{14}C]dextran (5) were treated in the same way.

The effects of inhibitors were determined as described in individual experiments.

(ii) Ammonium. Uptake of ammonium was measured by a chemical assay as described elsewhere (1a), except the cell concentration was increased to 0.2 to 0.3 mg of protein ml^{-1} and samples were taken after shorter intervals over a total period of 2 to 4 min, as indicated in individual experiments.

Pool extraction. Samples containing 120 to 240 μg of protein ml^{-1} were incubated with $^{14}CH_3NH_3^+$ and sampled as described above, and the filters were then transferred within 5 s to 100 μl of 0.1 N HCl at 0°C. After 15 min, the samples were centrifuged. Portions of the supernatants were applied to Polygram CEL 300 (Macherey-Nagel, Düren, FRG) cellulose thin-layer plates and chromatographed with propan-2-ol–formic acid–water (40:2:10, vol/vol). Radioactive areas were cut out and counted. A single metabolic derivative of $^{14}CH_3NH_3^+$ (R_f, 0.54; R_f for authentic methylammonium, 0.48) was found in extracts.

Spheroplast formation. Exponentially growing cells were taken from cultures containing less than 100 μg of protein ml^{-1}, washed in 20 mM PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)–Tris buffer (pH 7.1), and then resuspended in the same buffer containing, in addition, 0.25 M sucrose, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, and 10 mM EDTA (spheroplast medium) to a final concentration of 7.5 mg of cell protein ml^{-1}. Solid lysozyme (2 mg ml^{-1}) was added, and the suspension was incubated at 37°C. The progress of digestion was followed by diluting samples into 10 mM potassium phosphate buffer (pH 7.2); after 30 to 90 min of incubation with lysozyme, more than 90% of the spheroplasts burst within 5 min after dilution. The spheroplasts were centrifuged and washed once in spheroplast medium without lysozyme. Samples were diluted to 110 to 130 μg of protein ml^{-1} for uptake assays.

Alkaline phosphatase release. Enzyme activity released into the supernatant from samples centrifuged at intervals during spheroplast formation was measured as previously described (6).

Growth. Growth was followed by measuring the increase in absorbance at 750 nm. Cell protein was determined by the procedure of Lowry et al. (16), with bovine serum albumin as standard.

Membrane potential changes. The carbocyanine dye 3,3′-dipentyloxocarbocyanine (22) was used as a probe for observing light-induced changes in membrane potential. Exponential cells from cultures containing less than 120 μg of protein ml^{-1} were washed once in 20 mM PIPES-Tris buffer (pH 7.0) and then resuspended in 20 mM PIPES-Tris–10 mM $MgCl_2$–10 mM EDTA (pH 7) to a density of 6 mg of protein ml^{-1}. The suspension was incubated for 1 h at 38°C and then centrifuged. The sedimented cells were resuspended to the same concentration in 10 mM sodium phosphate buffer–10 mM $NaHCO_3$ (pH 7). No fluorescence changes were seen unless suspensions were preincubated with EDTA. For measurement of changes in fluorescence, the suspension was diluted in the phosphate-bicarbonate buffer to a final concentration of 60 μg of protein ml^{-1}, and 1 μM dipentyloxocarbocyanine iodide was added from a stock solution in ethanol. After preequilibration in the dark for 30 min, changes in fluorescence at 500 nm on excitation with 450-nm wavelength light (incident intensity, 5×10^6 ergs cm^{-2} s^{-1}) were followed with a Farrand 801 spectrofluorometer. The exciting light source was a quartz halogen lamp, with output passed through an Oriel LP 03 filter; wavelengths above 610 nm were prevented from reaching the photomultiplier by insertion of an Oriel M-11 filter in the light path.

Chemicals. $^{14}CH_3NH_3Cl$ (specific activity, 56 mCi $mmol^{-1}$) was obtained from Amersham Corp., Arlington Heights Ill.; [^{14}C]dextran (specific activity, 1.4 mCi g^{-1}) and $^{14}C_2H_5NH_3Cl$ (specific activity, 14.2 mCi $mmol^{-1}$) were obtained from New England Nuclear Corp., Boston, Mass. 3,3′-Dipentyloxocarbocyanine iodide was purchased from Molecular Probes Inc., Junction City, Oreg.

RESULTS

Effect of assay medium. When low concentrations (5 to 25 μM) of $^{14}CH_3NH_3^+$ were added to illuminated suspensions of *A. nidulans* R-2 in phosphate buffers containing $NaHCO_3$, a rapid uptake into the cells was observed. When the value of 5.1 μl of internal volume mg of $protein^{-1}$ (9) was used, internal concentrations averaged 1.3 ± 0.1 mM and exceeded the external concentrations by factors of 100 to 200 within 1 min. Table 1 shows the buffer mixtures in which $^{14}CH_3NH_3^+$ accumulation was tested. The rate of uptake was the same in the presence and absence of bicarbonate in freshly harvested and washed suspensions; it declined slowly, however, over a period of 60 min when the suspensions were kept illuminated in the absence of HCO_3^-, but not in its presence. In most experiments, therefore, HCO_3^- was included in the preincubation medium. Uptake was strongly stimulated by Na^+ in either potassium phosphate or PIPES-Tris buffer (Fig. 1). Maximum stimulation was observed in 5 to 20 mM Na^+, but

TABLE 1. Effect of composition of suspending medium on $^{14}CH_3NH_3^+$ accumulation[a]

Buffer[b]	Addition	Uptake rate (nmol min^{-1} mg of $protein^{-1}$)[c]
Potassium phosphate (pH 7.1)	None	0.5 ± 0.3
	10 mM $KHCO_3$[d]	1.4 ± 0.3
	10 mM $NaHCO_3$[d]	6.4 ± 0.4
	10 mM $KHCO_3$ + 20 mM NaCl	6.6 ± 0.6
	10 mM KCl	1.5
PIPES-Tris (pH 7.1)	None	1.2
	20 mM $KHCO_3$	1.6
	10 mM $KHCO_3$ + 20 mM NaCl	6.0
Tris-hydrochloride (pH 7.1)	None	0
	10 mM $KHCO_3$	0.2
	10 mM $KHCO_3$ + 20 mM NaCl	0.2
Choline-bicarbonate (pH 7.8)	None	1.5
	20 mM NaCl	1.6

[a] Suspensions were prepared and preincubated as described in the legend to Fig. 1. Final cell concentration was 120 μg of protein ml^{-1}.

[b] Concentration of all buffers was 20 mM.

[c] Measured over the first minute after adding 9 μM $^{14}CH_3NH_3Cl$ (specific activity, 56 mCi $mmol^{-1}$).

[d] Na or $KHCO_3$ was added to suspensions which were then incubated for 15 min in light before $^{14}CH_3NH_3Cl$ was added. In all other experiments, cells were preincubated with $KHCO_3$, and NaCl was added 5 to 10 s before $^{14}CH_3NH_3Cl$.

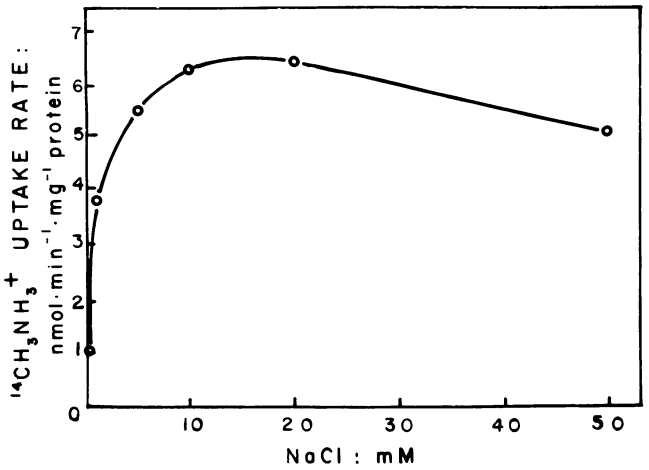

FIG. 1. Effect of Na^+ on methylammonium uptake rate. Exponentially growing cells were washed twice in 20 mM potassium phosphate buffer and resuspended to 135 μg of protein ml^{-1} in 20 mM potassium phosphate–10 mM $KHCO_3$ (pH 7.1). Suspensions were preincubated for 15 min at 30°C with illumination from a 100-W lamp at a distance of 30 cm. NaCl additions were made just before the assay was started by the addition of 9 μM $^{14}CH_3NH_3Cl$ (specific activity, 56 mCi mmol^{-1}). Samples were taken at 5 and 60 s and assayed for retained activity as described in the text.

higher concentrations up to 100 mM were only slightly inhibitory. The effect of Na^+ was specific, and no more than 20% stimulation was given by 20 mM Li^+, Rb^+, Ca^{2+}, or Mg^{2+}. No preference for the accompanying anion was apparent. Little or no $^{14}CH_3NH_3^+$ accumulation was observed in suspensions in Tris-hydrochloride or choline buffers, even when 10 mM $NaHCO_3$ was also added. When suspensions were kept in darkness, $^{14}CH_3NH_3^+$ uptake was barely detectable (Fig. 2). The uptake rate was independent of external pH between pH 6.5 and 9.0 (Table 2).

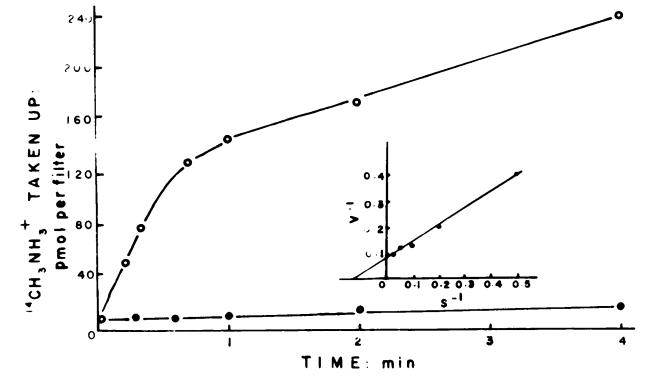

FIG. 2. Kinetics of methylammonium transport. Exponentially growing cells were washed and resuspended in 20 mM potassium phosphate–10 mM $NaHCO_3$ (pH 7.1) as described in the legend to Fig. 1. After 15 min of preincubation at 30°C, suspensions were transferred to 15°C and kept at this temperature for 5 min before addition of $^{14}CH_3NH_3Cl$ and sampling for radioactivity taken up. Shown in the main figure is the time course of uptake: $^{14}CH_3NH_3Cl$, 9 μM; specific activity, 56 mCi mmol^{-1}. Symbols: ○, light; ●, dark. Inset: Double reciprocal plot of data (15°C) using 2 to 20 μM $^{14}CH_3NH_3^+$ (specific activity, 56 mCi mmol^{-1}) and 40 μM $^{14}CH_3NH_3Cl$ (specific activity, 3.6 mCi mmol^{-1}). S, Micromolar; V, nanomoles per minute per milligram of protein.

TABLE 2. Effect of nitrogen source for growth and of assay pH on uptake of methylammonium and ammonium

N source	Assay pH	Uptake rate (nmole min^{-1} mg of protein^{-1}) of:	
		$^{14}CH_3NH_3^+$[a]	NH_4^+[b]
$NaNO_3$	6.5	7.3	27
	7.1	7.2	40
	9.0	7.5	32
NH_4Cl	7.1	0.9	37

[a] Exponential cells were washed and resuspended in 20 mM potassium phosphate–10 mM $NaHCO_3$ as described in the legend to Fig. 1. $^{14}CH_3NH_3Cl$ (9 μM; specific activity, 56 mCi mmol^{-1}) was added after 15 min of preincubation in light. The uptake rate was calculated from time points at 5 and 60 s.

[b] Cells from exponential cultures were washed and resuspended in 20 mM potassium phosphate–10 mM $NaHCO_3$ to a final concentration of 240 μg of protein ml^{-1}. After preincubation, NH_4Cl (50 μM) was added and five samples were filtered over a period of 2 min. The NH_4^+ in the filtrate was assayed chemically.

Effect of growth medium. Cultures supplied with NH_4Cl as nitrogen source during growth took up $^{14}CH_3NH_3^+$ much more slowly than did those in the normal, $NaNO_3$-containing medium (Table 2). Other changes, such as variations in the level of CO_2 or maintenance of culture pH at 7.0 or 9.5, had no effect on the ability of suspensions to concentrate $^{14}CH_3NH_3^+$. The rates at which NH_4^+ was taken up were not affected by growth nitrogen source (Table 2) (1a).

Uptake kinetics and gradient formation. The time course of methylammonium uptake was clearly biphasic; an initial rapid phase lasting 40 to 50 s was followed by a slower, rather more variable rate which was sustained for many minutes. The distinction between the phases was emphasized when the temperature at which the measurements was carried out was lowered to 15°C (Fig. 2). The rapid phase was then almost linear for 40 to 50 s and showed saturation kinetics, and an apparent K_m of 7.2 μM and a V_{max} of 12.5 nmol min^{-1} mg of protein^{-1} were determined from double reciprocal plots of initial uptake rates over a concentration range of 2 to 40 μM (Fig. 2, insert). Chromatography of acid extracts of cell samples taken at different time points during $^{14}CH_3NH_3^+$ uptake showed that they contained a single radioactive component corresponding to unaltered $^{14}CH_3NH_3^+$ up to 40 s; after 1 min, 10 to 30% of the counts recovered were in a second component which moved faster than $^{14}CH_3NH_3^+$ (Fig. 3). With longer times, the radioactivity in this second component of the pool increased, whereas that of $^{14}CH_3NH_3^+$ remained constant. Methylammonium thus reached a maximum concentration inside the cells within about 45 s, and the second phase of uptake was accounted for by its conversion to a metabolite, presumably the γ-methyl analog of glutamine (5, 15). This interpretation of the uptake kinetics is supported by the effects of inhibitors (see Table 3); preincubation of suspensions with 100 μM methionine sulfoximine for 10 min before the addition of $^{14}CH_3NH_3^+$ did not affect the rapid phase of entry but eliminated the slow phase. All of the entered radioactivity could be chased out of the cells by the addition of 5 μM NH_4^+ (Fig. 3; see below). Chromatography of extracts prepared from such suspensions after 1 or even 4 min still showed a single radioactive component (Fig. 4). These results suggest that a maximum internal concentration of 1.0 to 1.3 mM $CH_3NH_3^+$ could be achieved and that further net entry occurred only to replace molecules removed from the pool by the action of glutamine synthetase.

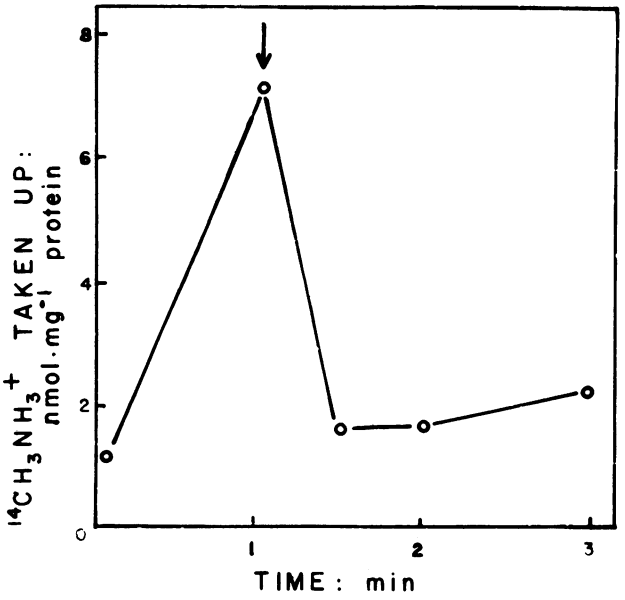

FIG. 3. Effect of NH_4^+ addition on $^{14}CH_3NH_3^+$ accumulated in the presence of methionine sulfoximine. Time course of $^{14}CH_3NH_3^+$ accumulation is shown. Exponentially growing cells were washed and resuspended in potassium phosphate–10 mM $NaHCO_3$ (pH 7.1) and equilibrated in light at 30°C for 10 min; 100 μM methionine sulfoximine was then added and incubation was continued for 10 min. $^{14}CH_3NH_3Cl$ (9 μM; specific activity, 56 mCi mmol^{-1}) was added, followed 1 min later (at arrow) by the addition of 5 μM NH_4Cl. Samples were taken at the times indicated.

TABLE 3. Effect of inhibitors on $^{14}CH_3NH_3^+$ accumulation and retention[a]

Addition[b]	Concn (μM)	% Inhibition[c]		% Counts released[d]
		Fast phase	Slow phase	
MSX	100	20	100	
CCCP	5	10–20	100	
	20	100	100	55
Monensin	25	100	100	80
Gramicidin	0.5	40		60
	1	100		
DCCD	5	20		ND
	20	100	100	
NEM	100	10	100	ND
	250	80	100	
PCMB	1,000	100		

[a] Suspensions were prepared from exponential cultures and preincubated in 20 mM potassium phosphate–10 mM $NaHCO_3$ for 15 min at 30°C.

[b] Abbreviations: MSX, methionine sulfoximine; CCCP, carbonyl cyanide-*m*-chlorophenyl hydrazone; DCCD, dicyclohexylcarbodiimide; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate.

[c] Carbonyl cyanide-*m*-chlorophenyl hydrazone, monensin, and gramicidin were added 5 s before $^{14}CH_3NH_3Cl$ (9 μM; specific activity, 5 μCi μmol^{-1}). Dicyclohexylcarbodiimide and methionine sulfoximine were added 10 min before $^{14}CH_3NH_3Cl$; *N*-ethylmaleimide was added 5 min before $^{14}CH_3NH_3^+$. Uptake rates in control suspensions were 6.2 to 7.5 nmol min^{-1} mg of protein^{-1}.

[d] Uptake was followed for 1 min and inhibitor added at 65 s. Release was determined from samples filtered after a further 30 s. ND, Not determined.

Specificity. The effects of a number of possible analogs on $^{14}CH_3NH_3^+$ uptake are shown in Table 3. Ethylammonium, glutamine, and leucine had no effect on the time course of uptake, regardless of whether they were added at the same time as $^{14}CH_3NH_3^+$ or after uptake had proceeded for 1 to 2 min. Radioactive $C_2H_5NH_3^+$ (10 or 100 μM) was not concentrated in the cells under these experimental conditions. Approximately 75% of the total counts in the cells 1 min after uptake was initiated was rapidly lost when 5 μM NH_4^+ or 100 μM $CH_3NH_3^+$ was added to the suspension at this time. Additions of NH_4^+ made at the same time as $^{14}CH_3NH_3^+$ eliminated radioactivity uptake for a time period that was related to the concentration of added NH_4^+. When the duration of the lags was used to calculate the rate of NH_4^+ uptake on the assumption that all of this compound must be removed by the cells before $^{14}CH_3NH_3^+$ uptake could start, NH_4^+ uptake rates were almost identical to those obtained in direct measurements and to values obtained previously (Tables 4 and 5) (1a). The system which is responsible for the concentrative uptake of $^{14}CH_3NH_3^+$ thus appears to recognize only NH_4^+ among the compounds tested but to have a substantially higher affinity for NH_4^+ than for $CH_3NH_3^+$. This precluded measurement of a K_i. The specificity of the transport system was also investigated in counterflow experiments (26), in which poisoned suspensions were preloaded with high concentrations of NH_4Cl. Such suspensions accumulated $^{14}CH_3NH_3^+$ transiently after removal of external NH_4^+, but $^{14}C_2H_5NH_3^+$ uptake did not differ in preloaded and control poisoned suspensions preincubated without NH_4Cl (Fig. 5).

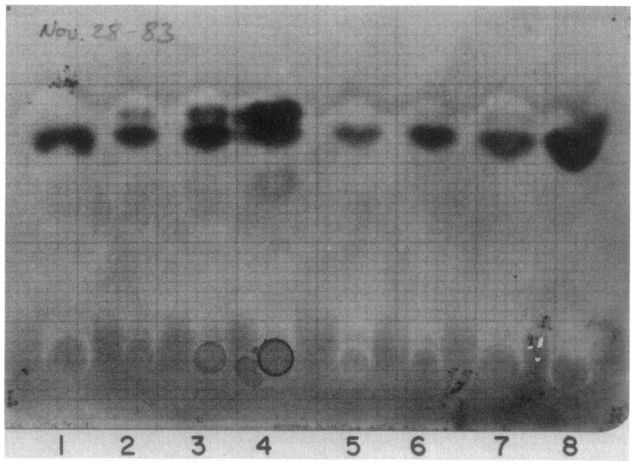

FIG. 4. Autoradiogram of thin-layer chromatogram of acid extracts. Samples were filtered, extracted, and chromatographed as described in the text. Lanes 1 and 8: $^{14}CH_3NH_3Cl$ standards. Lanes 2, 3, and 4: samples from control suspension taken at 0.5, 1, and 4 min, respectively; the calculated intracellular concentrations of $^{14}CH_3NH_3^+$ and product at these times were 0.8, 1.2, and 1.1 and 0.16, 0.3, and 1.4 mM, respectively. Lanes 5, 6, and 7: corresponding samples taken from suspensions preincubated with methionine sulfoximine. The calculated intracellular concentrations of $^{14}CH_3NH_3^+$ and product at 0.5, 1, and 4 min were 0.5, 0.8, and 0.9 and not detectable, 0.05, and 0.05 mM, respectively.

Energy requirement. An energy requirement for $^{14}CH_3NH_3^+$ accumulation was to be expected from the large gradients which can be established, and, as already noted, uptake was barely detectable in suspensions which were not illuminated (Fig. 2). The effects of a number of inhibitors are shown in Table 3. The ionophores carbonyl cyanide-*m*-

TABLE 4. Effect of possible competitors on $^{14}CH_3NH_3^+$ accumulation[a]

Addition	Concn (μM)	% Inhibition
Ethylammonium	100	0
Glutamine	50	0
Leucine	50	0
Ammonium	5	100
NO_3^-	1,000	0

[a] Exponentially growing cells were washed, suspended, and preincubated in 20 mM potassium phosphate–10 mM $NaHCO_3$ as described in the legend to Fig. 1, and uptake was measured at 5 and 60 s. Other additions were made 5 to 10 s before $^{14}CH_3NH_4Cl$ (9μM; specific activity, 56 mCi mmol^{-1}). Control rate, 6.5 nmol min^{-1} mg of protein^{-1}.

chlorophenyl hydrazone, monensin, and gramicidin prevented uptake when added at the same time as $^{14}CH_3NH_3^+$ and caused rapid efflux when added 1 min after uptake was initiated, confirming the energy requirement. The ATPase inhibitor dicyclohexylcarbodiimide was also an extremely effective inhibitor, suggesting that ATP was an intermediate in energizing the transport of $^{14}CH_3NH_3^+$. Addition of ≥100 mM KCl inhibited the rapid phase of $^{14}CH_3NH_3^+$ accumulation and resulted in efflux of accumulated radioactivity when added after 1 min of uptake (data not shown). These observations suggest that membrane potential could be involved in establishing the concentration differences between the cells and medium. Support for this possibility was obtained from measurements of the fluorescence of the carbocyanine dye 3,3′-dipentyloxocarbocyanine. When added to cells pretreated with EDTA, dye fluorescence was quenched by illumination (Fig. 6), suggesting a light-induced increase in membrane potential. Addition of 100 mM KCl eliminated the light-induced change for about 15 min, after which the signal gradually returned. The permeant cation triphenylmethyl phosphonium at 1 mM inhibited $^{14}CH_3NH_3^+$ accumulation by 90% (data not shown) and eliminated light-induced fluorescence quenching. Parallel effects on fluorescence changes and on $^{14}CH_3NH_3^+$ uptake were also observed with other inhibitors (Table 3 and Fig. 6). All the findings are consistent with the involvement of membrane potential, generated by ATP hydrolysis, as the driving force in $^{14}CH_3NH_3^+$ accumulation. Attempts to drive the transport by means of artificially imposed pH gradients over the range pH 4.5 to 9 were not successful. Sulfhydryl reagents such as *N*-ethylmaleimide or *p*-chloromercuribenzoate at low concentrations affected primarily the slow phase of $^{14}CH_3NH_3^+$ uptake, although at higher concentrations all accumulation was inhibited. It is not clear at present, therefore, whether the −SH reagents act indirectly, perhaps through effects on light energy conversion, or whether −SH groups are involved in the actual translocation process.

TABLE 5. Lag in onset of $^{14}CH_3NH_3^+$ uptake produced by simultaneous addition of NH_4^+[a]

NH_4^+ added (μM)	Duration of lag (min)	Calculated NH_4^+ uptake rate (nmol min^{-1} mg of protein^{-1})
0	0	
5[b]	1	37
10[c]	1	41
20[c]	2	42
30[c]	4	31

[a] Exponentially growing cells were washed and resuspended in 20 mM potassium phosphate–10 mM $NaHCO_3$ (pH 7.1) and preincubated as described in the legend to Fig. 1. NH_4^+ and $^{14}CH_3NH_3Cl$ (9 μM; specific activity, 56 mCi mmol^{-1}) were added simultaneously, and sampling was carried out for 3 to 6 min. The lag time represents the delay noted before $^{14}CH_3NH_3^+$ accumulation started.

[b] Suspension contained 120 μg of protein ml^{-1}.

[c] Suspension contained 250 μg of protein ml^{-1}.

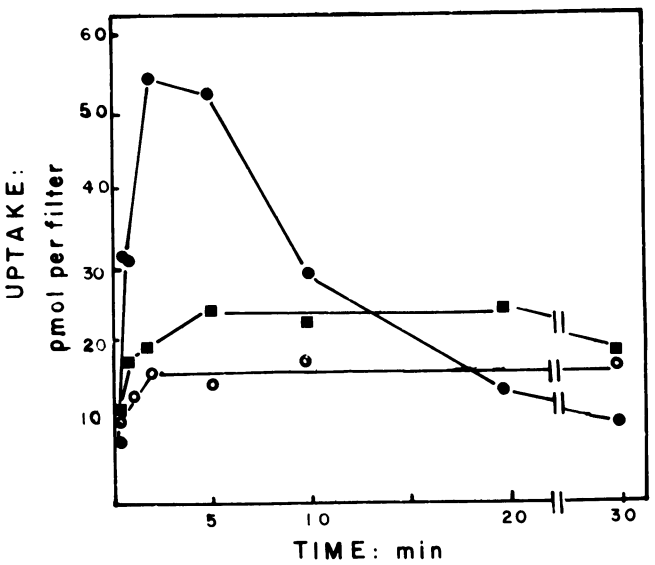

FIG. 5. Counterflow of $^{14}CH_3NH_3^+$ or $^{14}C_2H_5NH_3^+$ in poisoned cells preloaded with 200 mM NH_4Cl. Suspensions from exponentially growing cultures were washed and resuspended in 20 mM potassium phosphate, pH 7.1. Carbonyl cyanide-*m*-chlorophenyl hydrazone (20 μM), dicyclohexylcarbodiimide (200 μM), and NH_4Cl (200 mM) were added, and the suspensions were incubated in darkness for 1 h. At the end of this period, the cells were sedimented rapidly and resuspended to a concentration of 620 μg of protein ml^{-1}. Radioactive compounds wre added immediately, and the suspensions were sampled at the times indicated. Shown are control cells preincubated with inhibitors but without NH_4Cl with $^{14}CH_3NH_3Cl$ added (9 μM; specific activity, 56 mCi mmol^{-1}) (○) and preloaded cells with $^{14}CH_3NH_3Cl$ (9 μM; specific activity, 56 mCi mmol^{-1}) (●) or $^{14}C_2H_5NH_3Cl$ (■) (20 μM; specific activity, 14.2 mCi mmol^{-1}) added.

Methylammonium uptake in spheroplasts. Conversion of *A. nidulans* cells to spheroplasts did not affect their ability to accumulate $^{14}CH_3NH_3^+$ over short periods of time (Fig. 7), although the periplasmically located enzyme alkaline phosphatase (6, 10, 24) was released almost quantitatively during spheroplast formation. The decrease in transport ability at the last time point appeared to be due to some cell lysis, as a small quantity of phycocyanin was also released into the spheroplast medium at that time. The rate of NH_4^+ uptake was the same in whole cells and after conversion to spheroplasts (data not shown). Methylammonium transport thus does not appear to involve soluble periplasmic components.

DISCUSSION

The process of methylammonium transport in *A. nidulans* was separated clearly from metabolic conversion of the entered molecules by limiting the time period over which uptake measurements were made or by using selected inhibitors. Unaltered $^{14}CH_3NH_3^+$ could be accumulated to give a concentration gradient of at least 150 between illuminated cells and their environment. As might be expected from the very low respiration rate of *A. nidulans*, transport in darkness was, at most, 10% of that seen in light. The driving

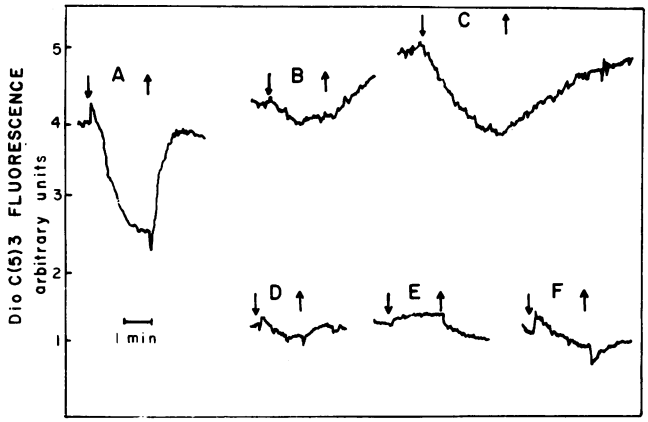

FIG. 6. Light-induced fluorescence changes: 3,3'-dipentyloxocarbocyanine. Suspensions were prepared and preincubated with dye as described in the text. ↓, Exciting light on; ↑, exciting light off. (A) Control: suspension in buffer only; (B) KCl (100 mM) added 1 min before light turned on; (C) KCl (100 mM) added 6 min before light turned on; (D) triphenylmethyl phosphonium (1 mM) added 10 min before light turned on; (E) dicyclohexylcarbodiimide (100 μM) added 10 min before light turned on; (F) *N*-ethylmaleimide (200 μM) added 15 min before light turned on. Relative position of traces A, B, and C reflects baseline changes on adding KCl.

force for concentration gradient formation appeared to be the membrane potential component of protonmotive force, since additions of high concentrations of K^+ caused a reduction in methylammonium transport rate and also of light-induced quenching of the fluorescence of a carbocyanine dye, and the penetrant cation triphenylmethyl phosphonium eliminated both. The ΔpH as such did not appear to be involved, since both the rate and extent of transport were unaffected by variations in the external pH over a range that would change not only the magnitude but also the sign of the ΔpH across the cell boundary (4, 17). The ATPase inhibitor dicyclohexylcarbodiimide drastically reduced transport and fluorescence changes, showing that ATP is an essential intermediate in energizing transport. This emphasizes the difference in membrane functional organization in *Anacystis* and *Plectonema* spp., in which dicyclohexylcarbodiimide does not affect glucose analog transport (19).

The specificity, reversibility, and repressibility of methylammonium transport in this cyanobacterium resemble findings with other microorganisms (e.g., 1, 5, 13, 14). Inhibition of $^{14}CH_3NH_3^+$ accumulation by dicyclohexylcarbodiimide, which enhanced the pH gradient across the thylakoid membranes, as judged from quinacrine fluorescence changes (unpublished data), ruled out the possibility that the accumulation was due simply to internal pH changes. This was also confirmed by the distribution of $^{14}C_2H_5NH_3^+$, which was not a transport substrate, and has given results close to those with dimethyloxazolidinedione when used as a pH probe (4). Differences between the cyanobacterial $^{14}CH_3NH_3^+$ transport system and those in other microorganisms include a lower apparent K_m and insensitivity to external pH changes between pH 6.5 and 9. The specific requirement for millimolar concentrations of Na^+ has not been described in other systems. A detailed explanation for this requirement is not yet available, but a possible analogy lies in the involvement of Na^+ binding to a component of the glutamate transport system of *E. coli* (2, 3).

A careful comparison of the uptake of $CH_3NH_3^+$ and NH_4^+ in *A. nidulans* indicates that $CH_3NH_3^+$ is concentrated within the cyanobacterial cell by a transport system which also handles NH_4^+ and has a higher affinity for the latter, and presumably natural, substrate. It does not follow, however, that this system is the only mechanism involved in establishing and maintaining NH_4^+ gradients in these cells, and some apparently major differences were found between the translocation of $^{14}CH_3NH_3^+$ and that of NH_4^+. The Na^+ requirement, repressibility by growth in NH_4^+, and inhibition by high KCl concentrations could be demonstrated only in measurements of $^{14}CH_3NH_3^+$ accumulation, whereas NH_4^+ uptake was unaffected by these factors.

At least two explanations for these differences can be suggested. *A. nidulans* may possess two distinct systems which are responsible for NH_4^+ transport, one of which is highly specific for NH_4^+ and constitutive and the other of which recognizes both NH_4^+ and $CH_3NH_3^+$, but not $C_2H_5NH_3^+$, and is repressible. It is also possible that there is in fact only one system, and that the differences observed experimentally result from kinetic considerations. As has been demonstrated here, $^{14}CH_3NH_3^+$ transport can be separated from conversion to a metabolic product both in time and by the use of inhibitors, so that the entry rate can be clearly visualized and measured. The net rate of NH_4^+ entry, on the other hand, may be governed by the amidation rate, as suggested by the need for bicarbonate during the incubation period preceding uptake measurements (1a). The potential transport rate may thus be very much higher than the net rate actually observed. If this were so, even extensive repression of the system might go unnoticed in the type of measurements made here. The obvious difference in Na^+ requirement for transport of the two compounds could have a comparable explanation, and even 90% reduction in potential activity may still fail to make this stage in the overall uptake process rate limiting. This suggestion implies that the inward transport capability of *A. nidulans* is much greater

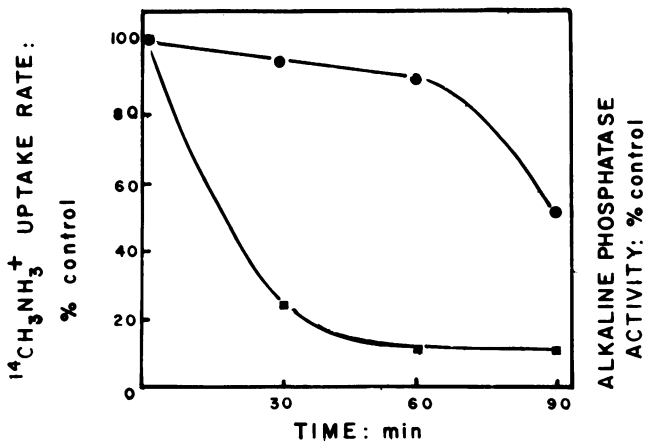

FIG. 7. Methylammonium transport in spheroplasts. Spheroplasts were made by lysozyme treatment as described in the text. Samples were taken at the start and at intervals during the incubation for determination of $^{14}CH_3NH_3^+$ accumulation rate and for measurement of alkaline phosphatase activity retained by the cells. $^{14}CH_3NH_3^+$ uptake: cell concentration, 130 μg of protein ml^{-1}; 9 μM $^{14}CH_3NH_3Cl$ (specific activity, 56 mCi $mmol^{-1}$). Uptake was measured between 5 and 60 s. The initial rate was 6.5 nmol min^{-1} mg of $protein^{-1}$. Alkaline phosphatase activity in resuspended cells was measured as described in the text. The initial activity was 15 nmol of *p*-nitrophenylphosphate hydrolyzed min^{-1} mg of $protein^{-1}$. Symbols: ●, $^{14}CH_3NH_3^+$ uptake rate; ■, alkaline phosphatase activity.

than that needed to maintain the growth rate and may account for the finding that NH_4^+ is taken up as rapidly in the presence of much higher concentrations of NO_3^- as when it constitutes the only available nitrogen source (1a). It could also support the suggestion that an important normal function of an NH_4^+ transport system is to recapture NH_4^+ generated internally from other inorganic nitrogen sources, such as NO_3^- or N_2. Genetic analysis will be needed to establish unequivocally whether one or several transport systems are involved in NH_4^+ and $CH_3NH_3^+$ translocation. The possibility of such an analysis (e.g., 25) was an important factor in the choice of organism for this study. The investigations described here emphasize the need for caution in extrapolating conclusions about normal growth and survival from results obtained with a convenient analog of an essential nutrient.

ACKNOWLEDGMENTS

This work was supported in part by grant PCM-8112490 from the National Science Foundation and by funds provided through the New York State Experimental Station.

We are grateful to R. E. McCarty and his research group for use of the fluorometer and for much stimulating discussion.

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