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Ammonium and Methylammonium Transport in *Rhodobacter sphaeroides*

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Rhodobacter sphaeroides **maintained intracellular ammonium pools of 1.1 to 2.6 mM during growth in several fixed nitrogen sources as well as during diazotrophic growth. Addition of 0.15 mM NH_4^+ to washed, nitrogen-free cell suspensions was followed by linear uptake of NH_4^+ from the medium and transient formation of intracellular pools of 0.9 to 1.5 mM NH_4^+. Transport of NH_4^+ was shown to be independent of assimilation by glutamine synthetase because intracellular pools of over 1 mM represented NH_4^+ concentration gradients of at least 100-fold across the cytoplasmic membrane. Ammonium pools of over 1 mM were also found in non-growing cell suspensions in nitrogen-free medium after glutamine synthetase was inhibited with methionine sulfoximine. In NH_4^+-free cell suspensions, methylammonium ($^{14}CH_3NH_3^+$) was taken up rapidly, and intracellular concentrations of 0.4 to 0.5 mM were maintained. The $^{14}CH_3NH_3^+$ pool was not affected by methionine sulfoximine. Unlike NH_4^+ uptake, $^{14}CH_3NH_3^+$ uptake in nitrogen-free cell suspensions was repressed by growth in NH_4^+. These results suggest that *R. sphaeroides* may produce an NH_4^+-specific transport system in addition to the NH_4^+/$^{14}CH_3NH_3^+$ transporter. This second transporter is able to produce normal-size NH_4^+ pools but has very little affinity for $^{14}CH_3NH_3^+$ and is not repressed by growth in high concentrations of NH_4^+.**

Rhodobacter sphaeroides, formerly *Rhodopseudomonas sphaeroides* (19), is a facultatively photoheterotrophic bacterium which can utilize a variety of nitrogenous compounds as its sole source of nitrogen for growth. Under aerobic conditions, ammonia, urea, and several amino acids are used; in addition, when grown anaerobically (phototrophically) it can fix N_2. Regardless of the nitrogen source for growth, *R. sphaeroides* maintained an intracellular pool of 1.1 to 2.6 mM ammonia (M. Cordts and J. Gibson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K3, p. 172), which is comparable to the intracellular NH_4^+ concentration found in a number of other procaryotes (7, 10, 16, 21, 23, 30). During exponential growth, the pool must turn over rapidly; for cells with a 5-h doubling time, the estimated nitrogen requirement for protein synthesis alone would exhaust an intracellular pool of 2 mM NH_4^+ in less than 30 s.

The ability of growing bacteria to maintain an intracellular ammonia pool against a concentration gradient of at least 100-fold suggests that they have an efficient NH_4^+ transport system which can compensate for the outward diffusion of the membrane-permeable NH_3 (23). A recent report estimates that, in N_2-fixing *Klebsiella pneumoniae*, the recapture of NH_4^+ following its diffusion as NH_3 is energetically expensive and suggests that, on average, six cycles of diffusion/transport occur before an ammonia molecule is assimilated to the level of glutamine (24). Measured membrane permeabilities to amines are similar in *R. sphaeroides* and *K. pneumoniae* (R. J. Ritchie and J. Gibson, unpublished data), implying that a significant expenditure of energy also may be required to maintain an NH_4^+ pool in *R. sphaeroides*.

The methyl analog of NH_4^+, methylammonium ($^{14}CH_3NH_3^+$), is commonly used in studies of the NH_4^+ transport system (2, 5, 15, 18, 20; for a review, see reference 25). Both ions are generally believed to share a common transport system because uptake of the analog is completely inhibited by low concentrations of the natural substrate, NH_4^+, and because the two can be exchanged in counterflow experiments (5, 15). In the instances where $^{14}CH_3NH_3^+$ has been used to study NH_4^+ transport, the analog cannot support growth of the organism, and its intracellular accumulation is believed to be due to fortuitous recognition by the NH_4^+ transporter. *Paracoccus denitrificans* is an exception since it can transport $CH_3NH_3^+$ as a nitrogen source in the absence of NH_4^+ (17).

In the study of NH_4^+ transport in *R. sphaeroides* described here, $^{14}CH_3NH_3^+$ uptake was characterized and shown to be strongly inhibited by low concentrations of NH_4^+. The capacity for $^{14}CH_3NH_3^+$ uptake was repressed when the organism was grown in NH_4^+. In contrast, NH_4^+ was taken up at approximately the same rate regardless of the source of nitrogen during growth. When changes in the intracellular concentration of NH_4^+ were monitored as an index of transport of the natural substrate, the results suggested that NH_4^+ may be taken into the cell by two transport systems. One system recognized both NH_4^+ and $CH_3NH_3^+$ and was repressed by growth in high concentrations of NH_4^+; the second system recognized NH_4^+ but had a much lower affinity for $CH_3NH_3^+$ and was not repressed by excess NH_4^+ in the growth medium.

(A preliminary account of this work was presented at the 86th Annual Meeting of the American Society for Microbiology, Washington, D.C. [M. Cordts and J. Gibson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K73, p. 205].)

MATERIALS AND METHODS

Organism and growth conditions. *R. sphaeroides* 2.4.1 was obtained from the American Type Culture Collection, Rockville, Md. (ATCC 17023), and was grown aerobically (chemoheterotrophically) except for experiments with diazotrophic cultures. Basal medium consisted of 25 mM KH_2PO_4, 10 mM sodium succinate, and 2% (vol/vol) vitamin

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and mineral mixture [solution C (31), containing 12.9 mg of $NaMo_2O_4 \cdot 2H_2O$ in place of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$]; the pH was adjusted to 6.8 to 6.9 with NaOH before autoclaving. Nitrogen sources were added separately after autoclaving; stock solutions of $(NH_4)_2SO_4$ were autoclaved, and other nitrogen sources were filter sterilized. Aerobic cultures were grown in flasks shaken at 60 rpm at 25°C. Diazotrophic cultures were grown under N_2 in screw-capped bottles, which were three-quarters filled with basal medium, and incubated at 25°C approximately 20 cm from a 52-W incandescent light bulb.

For NH_4^+ pool experiments, a BRL Airlift Fermenter (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was filled with 2.1 liters of 2× basal medium plus nitrogen source and gassed with approximately 500 ml of air min^{-1}. Diazotrophic suspensions were gassed with 300 ml of N_2 min^{-1} and illuminated by five lamps with peak emission at 750 nm (range, 700 to 850 nm) set at 100 to 300 microeinsteins m^{-2} s^{-1}.

NH_4^+ pool determinations. Batch cultures at half their maximum density (approximately 0.1 mg of protein ml^{-1} or 10^9 CFU ml^{-1}) were either added directly to a "stop solution" or centrifuged and then suspended in nitrogen-free buffer (25 mM potassium phosphate, 10 mM sodium succinate, adjusted to pH 6.8 to 7.0 with NaOH; "succinate buffer") before experimental treatment and addition to the stop solution. The stop solution consisted of 1 g of ice per ml of cell suspension plus 5 μg of chloramphenicol ml^{-1}, 10 μM methionine sulfoximine (MSX), and 50 μM dicyclohexylcarbodiimide. After 10 min, the mixture was centrifuged, and the pellet was mixed immediately with 0.05 N $HClO_4$ at 0°C (final cell concentration, 5 to 10 mg of protein ml^{-1}). After extraction for at least 2 h, the cell pellet was removed by centrifugation, and the NH_4^+ in the supernatant fluid was determined after microdiffusion.

Microdiffusion assay. Ammonia was distilled into 55 μl of 100 mM H_2SO_4 from 1 ml of cell extract mixed with 2 ml of saturated $Na_2B_4O_7$ (final pH, approximately 10.5) by using Conway microdiffusion cells (Fisher Scientific Co., Pittsburgh, Pa.). After 4.5 h at 30°C, NH_4^+ in the acid was assayed by the phenol hypochlorite method (32) modified for small volumes as previously described (7). For each experiment, standard curves were prepared from cell extracts distilled with known additions of NH_4^+. Each NH_4^+ pool value is the average of three to six replicate distillations, and each experiment was repeated two to five times.

Uptake experiments. Cells were harvested at half maximal growth, washed once with succinate buffer, and suspended in succinate buffer to a density of 0.15 to 0.2 mg of protein ml^{-1}. After aerobic equilibration at 25 to 30°C for approximately 30 min, the pH of the suspensions was adjusted to 6.3 with H_2SO_4 (the optimum for $^{14}CH_3NH_3^+$ assays) or to 7.2 to 7.3 with NaOH (the best value for NH_4^+ uptake assays).

(i) Methylammonium uptake. Uptake was initiated by adding $^{14}CH_3NH_3Cl$ (9 μM, 0.50 μCi ml^{-1}, final concentration) to approximately 0.5 ml of equilibrated cell suspension at room temperature (20 to 23°C). Samples of 0.1 ml were filtered through 0.2-μm-pore-size polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.). The $^{14}CH_3NH_3^+$ retained per milligram of cell protein was determined by scintillation counting (5).

(ii) Ammonium uptake. Equilibrated cell suspension was added to NH_4Cl (50 μM, final concentration) and stirred rapidly at 30°C. Samples were filtered through Nylon-66 membrane filters (0.22-μm pore size, 25-mm diameter, MSI, Micronsep; Fisher Scientific Co.) by using a 30-tube filtering manifold; filtration was completed in 5 s. The NH_4^+ in each filtrate was assayed directly (without microdiffusion) by the modified phenol hypochlorite method.

Anaerobic uptake experiments. Diazotrophic cultures were harvested, washed once in succinate buffer, and suspended in N_2-flushed succinate buffer to a concentration of 0.5 to 1 mg of protein ml^{-1}. After anaerobic preincubations in the light (1 to 6 h at 25 to 30°C) in glass syringes, the cell suspension was diluted to a final concentration of approximately 0.1 mg of protein ml^{-1} with argon-flushed succinate buffer, and uptake assays were done as described above except for continued illumination and argon gassing.

Amino acid assays. The colorimetric assay of Rosen (29) was used with glutamate as the standard. Total intracellular amino pools (amino acids plus NH_4^+) were determined by using 50 μl of the $HClO_4$-treated cell extract (prepared for the NH_4^+ pool experiments) diluted with 450 μl of buffer. For amino acid uptake experiments, filtrates were assayed after sampling as described for NH_4^+ uptake.

Methylammonium pool determinations. At intervals after the addition of 9 μM $^{14}CH_3NH_3^+$, samples of cell suspension containing approximately 0.3 mg of protein ml^{-1} were filtered as described above. Acid extraction and thin-layer chromatography of the cell extracts in propan-2-ol–formic acid–water proceeded as described elsewhere (5).

Protein and cell volume. Protein concentrations were determined by Coomassie blue dye binding (8) by using dye reagent concentrate obtained from Bio-Rad Laboratories (Richmond, Calif.) and bovine serum albumin as the standard. Intracellular volume was calculated from the difference between the amount of entrapment of membrane-impermeable [^{14}C]dextran and membrane-permeable [^{14}C]ethylene glycol or ^3H_2O in duplicate samples, modified from Gaensslen and McCarty (12). Extracellular water in the pellet averaged 12 ± 2 μl (mg of protein)$^{-1}$; intracellular volumes were 6 ± 3 and 4 ± 0.5 μl (mg of protein)$^{-1}$ (averages of four and two determinations, respectively) for chemoheterotrophically and diazotrophically grown cells, respectively. For amino acid and NH_4^+ pool determinations, corrections were made for the quantity of substrate contained in the extracellular water of the cell pellet. In all cases, this correction was less than 10% of the total intracellular pool.

Chemicals. $^{14}CH_3NH_3Cl$ (specific activity, 56 mCi/mmol) was obtained from Amersham Corp., Arlington Heights, Ill., and used at this specific activity in all experiments; [^{14}C]dextran (specific activity, 1.4 mCi g^{-1}) and ethylene glycol (10 mCi $mmol^{-1}$) were purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Intracellular NH_4^+ pool. Regardless of growth nitrogen source or growth rate, the intracellular NH_4^+ amounted to 6 to 14 μmol of NH_4^+ (g of protein)$^{-1}$. This was equivalent to an average concentration of 1.1 to 2.3 mM intracellular NH_4^+ for chemoheterotrophic *R. sphaeroides* and 2.6 mM for nitrogen-fixing cultures (Table 1). The extracellular NH_4^+ concentration at the time of harvest was equal to or less than 0.015 mM, indicating a concentration gradient of at least 100-fold between the cells and the external medium.

The intracellular ammonium pool fell to 0.3 to 0.4 mM during centrifugation and resuspension of metabolically active cells, but the pool increased upon addition of NH_4^+ to the suspending medium (Fig. 1). For example, when 0.15 mM NH_4Cl was added to a nitrogen-free, aerated cell

TABLE 1. Intracellular pools of NH_4^+ in *R. sphaeroides*[a]

Nitrogen source for growth (mM)	Doubling time (h)	Intracellular NH_4^+ pool (mM); no. of expts[b]
NH_4^+ (1.25)	5	1.4 ± 0.5; 3
Glutamine (1.5)	5	2.3 ± 0.7; 2
Proline (2)	12	1.6 ± 0.1; 2
Glutamic acid (2)	14	1.1 ± 0.1; 2
N_2 (anaerobic)	53	2.6 ± 0.1; 2
$CH_3NH_3^+$	NG[c]	

[a] At mid-exponential phase, cultures (approximately 100 mg of protein) were added directly to the stop solution, and NH_4^+ was assayed after distillation of six replicate cell extracts as described.

[b] Values shown are the average of two or three experiments ± standard deviation.

[c] NG, No growth.

suspension, ammonium accumulated intracellularly to 9 μmol of NH_4^+ (g of protein)$^{-1}$ (or 1.6 mM) within 3 min and was maintained for approximately 5 min, after which it again decreased as extracellular NH_4^+ was depleted.

Two types of control experiments were performed with *R. sphaeroides* grown in high NH_4^+ (initially 5 mM) to validate the amount of NH_4^+ which was trapped in the interstitial water of the pellet. (i) Quadruplicate samples of cell suspensions in succinate buffer were treated with the stop solution at 0°C. After 10 min, 75 μM NH_4^+ was added to two of the samples, and all four were immediately centrifuged. The NH_4^+ pools then were determined as described above, and the results showed that the NH_4^+ correction calculation completely accounted for the apparent difference in NH_4^+ pool size between the samples containing 75 μM NH_4^+ and the NH_4^+-free samples. (ii) NH_4^+ (0.125 mM) was added to cell suspensions in succinate buffer, and the extracellular concentrations of NH_4^+ in the supernatant fluids (obtained after centrifugation of the stop solution-treated cells) were compared with small samples of the suspension removed by filtration. The ratio between NH_4^+ concentrations in the supernatants and values obtained in parallel uptake experiments was 1.02 ± 0.08 (n = 6), indicating that the stop solution effectively prevented further NH_4^+ uptake from the medium.

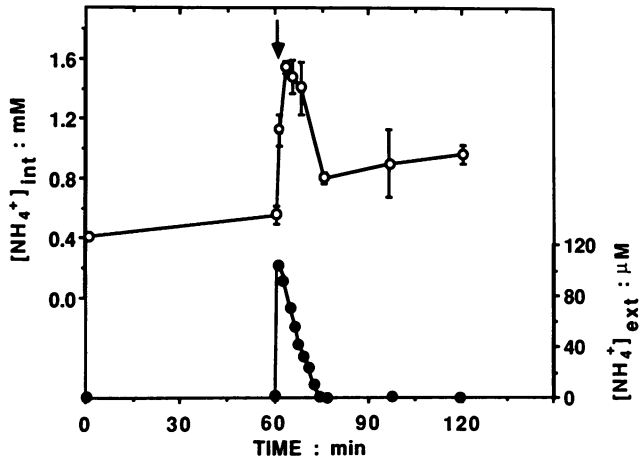

FIG. 1. Effect of NH_4^+ addition on pool size. *R. sphaeroides* was grown as described with 2.5 mM NH_4^+ and harvested by centrifugation. At time zero, the cells were resuspended in aerobic, nitrogen-free succinate buffer (pH 6.9) at a density of 0.9 mg of protein ml^{-1}. At the time indicated by the arrow, 0.15 mM NH_4^+ was added. Symbols: ○, intracellular NH_4^+ concentration determined after microdiffusion (error bars show ±1 standard deviation of triplicate distillations); ●, extracellular concentration of NH_4^+ determined in filtered subsamples.

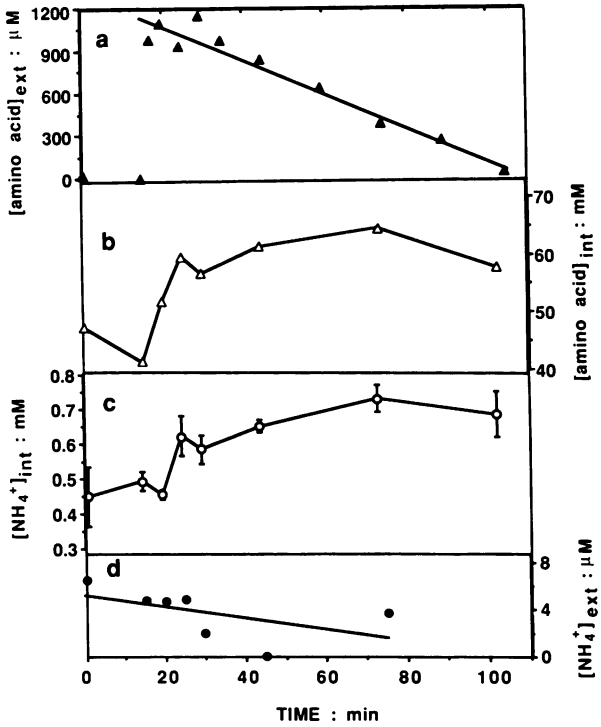

FIG. 2. Effect of amino acid addition on the NH_4^+ and amino pools. *R. sphaeroides* was harvested after growth in 4 mM glutamate and at time zero was resuspended in aerated, nitrogen-free succinate buffer to a density of 0.32 mg of protein ml^{-1}. After 15 min, a mixture of amino acids was added (Glu [750 μM]; Gln, Ala, and Ser [75 μM]; and His, Phe, Val, Ile, Leu, Met, and Tyr [38 μM]; final concentrations). (a) Extracellular amino acid concentrations, determined by chemical assay of samples of filtrate (see Materials and Methods). (b) Intracellular amino acid pools, averaged from duplicate samples of cell extracts. (c) Intracellular NH_4^+ pools, averaged from triplicate microdiffusions, ±1 standard deviation. (d) Extracellular NH_4^+ concentrations, determined by microdiffusion of 1 ml of filtrate.

The addition of a mixture of 11 amino acids (1.2 mM, final concentration; see legend to Fig. 2) resulted in a small increase in the NH_4^+ pool from 0.5 to over 0.7 mM within 1 h (Fig. 2); the total amino pool increased from 41 to 65 mM during the same period. The extracellular NH_4^+ concentration remained below 5 μM even when glutamine (75 μM) was added. Since glutamine interfered with the NH_4^+ determination, microdiffusion was used in these assays, incidentally confirming that there was no degradation of glutamine under the alkaline conditions of this process (21). Additions of single amino acids (0.3 mM proline, 1 mM serine, or 1 mM glutamine) after growth on these nitrogen sources did not change the NH_4^+ pool size within 1 h.

$^{14}CH_3NH_3^+$ uptake. When washed cells from cultures grown with glutamine as the nitrogen source were provided with $^{14}CH_3NH_3^+$ in the absence of NH_4^+, uptake began immediately (Fig. 3). An initial rapid phase of uptake showing saturation kinetics lasted approximately 20 s and was followed by a slower, linear rate which continued for at least 40 min. When micromolar concentrations of NH_4^+ were added simultaneously with 9 μM $^{14}CH_3NH_3^+$, uptake of the analog was completely inhibited for periods which corre-

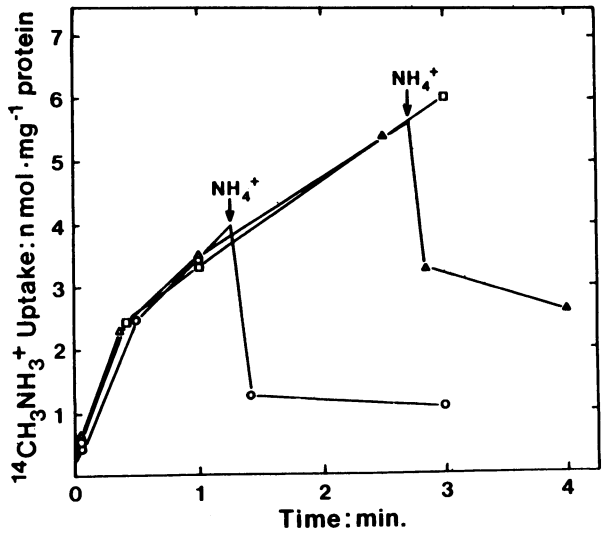

FIG. 3. Effect of NH_4^+ addition on $^{14}CH_3NH_3^+$ accumulation. Cultures grown in glutamine (1.5 mM) were harvested at half maximal growth, washed, and resuspended aerobically in succinate buffer. $^{14}CH_3NH_3^+$ (9 μM) was added at time zero, and total intracellular radioactivity was determined by filtration. Symbols: □, no NH_4^+ addition; ○, 2 mM NH_4^+ added at 65 s; △, 2 mM NH_4^+ added at 175 s.

sponded to the estimated time required to take up the NH_4^+. Even 1 μM NH_4^+ resulted in a measurable delay (approximately 3 s) of $CH_3NH_3^+$ uptake. Since the K_m for the first phase of $CH_3NH_3^+$ uptake in *R. sphaeroides* is 9.5 μM (Cordts and Gibson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985), the blockage of $CH_3NH_3^+$ uptake with low levels of NH_4^+ suggests that the transporter has a very high affinity for NH_4^+. The strong preference for NH_4^+ over the analog made it impossible to obtain values for K_i, as has also been observed in other studies (5, 15, 17).

Further evidence that NH_4^+ and $CH_3NH_3^+$ use a common transport system is shown in Fig. 3. In the first phase of $^{14}CH_3NH_3^+$ uptake, a maximum intracellular concentration of 0.4 to 0.5 mM $CH_3NH_3^+$ was reached (see below). When 2 mM NH_4^+ was added 1 min after the addition of $^{14}CH_3NH_3^+$, immediate release of the analog was equivalent to 2.6 μmol of $CH_3NH_3^+$ (g of protein)$^{-1}$, representing an intracellular pool of 0.44 mM $CH_3NH_3^+$. Addition of 2 mM NH_4^+ after 3 min caused the release of 1.9 μmol of $CH_3NH_3^+$ (g of protein)$^{-1}$, which was equivalent to 0.32 mM. Addition of 5 μM carbonyl cyano-*m*-chlorophenol hydrazone (a protonophore) 1 min after the addition of 9 μM $^{14}CH_3NH_3^+$ also resulted in the release of accumulated radioactivity equivalent to 0.4 to 0.5 mM (data not shown).

The biphasic uptake curves (Fig. 3) suggested that a metabolite was produced from $^{14}CH_3NH_3^+$ within 1 min. A single metabolite accumulated at the same rate as the slow phase of uptake and was not released upon addition of NH_4^+. Both $^{14}CH_3NH_3^+$ and the metabolite are shown in Fig. 4; in addition to the $^{14}CH_3NH_3^+$ spot (R_f 0.50), intracellular ^{14}C accumulated in a second spot (R_f 0.59; presumably [^{14}C]-γ-*N*-methyl glutamine [3, 5, 15]). At 10, 45, 90, and 150 s after the addition of 9 μM $^{14}CH_3NH_3^+$, the intracellular concentrations of $^{14}CH_3NH_3^+$ shown in Fig. 4 (lanes 1 to 4) were 0.33, 0.49, 0.47, and 0.53 mM, and those of the metabolite were 0.02, 0.16, 0.25, and 0.41 mM, respectively. In longer experiments, concentrations of the ^{14}C-metabolite reached 1 mM after 10 min and 3 mM after 40 min. When MSX (1 μM) was added 5 s before addition of $^{14}CH_3NH_3^+$ (lanes 5 to 8), intracellular $^{14}CH_3NH_3^+$ reached concentrations similar to those of uninhibited cells (0.08, 0.4, and 0.53 mM at 10, 50, and 105 s, respectively, after the addition of $^{14}CH_3NH_3^+$). However, metabolite formation in MSX-treated cells was greatly reduced, amounting to only 0.04 mM after 105 s. After the addition of 2 mM NH_4^+ (lane 8), the intracellular concentration of $^{14}CH_3NH_3^+$ decreased by 90% to 0.05 mM; the ^{14}C-labeled metabolite was not released by NH_4^+, suggesting that it could not be recognized by the transporter. The ^{14}C-labeled metabolite was presumed to be γ-*N*-methyl glutamine since it was formed only when glutamine synthetase was active.

Independence of pool maintenance from glutamine synthetase activity. When *R. sphaeroides* was resuspended in nitrogen-free succinate buffer, the intracellular NH_4^+ pool increased from 0.3 mM (immediately after harvesting and resuspension) to 0.5 to 0.6 mM and remained at that concentration for approximately 3 h; NH_4^+ was not detected in the extracellular medium at any point (Fig. 5). In contrast, cells treated with MSX contained more NH_4^+ (0.8 to 1.1 mM), and NH_4^+ was released at a rate of 43 μmol of NH_4^+ (g of protein)$^{-1}$ h^{-1}, leading to an external concentration of 20 μM within 3 h. Both the control and MSX-treated suspensions were able to retain NH_4^+ against a concentration gradient (500- and 140-fold, respectively, after 1 h). Amino pools of 40 to 60 mM were maintained for at least 3 h under aerobic conditions in nitrogen-free succinate buffer.

In similar experiments, diazotrophic cultures bubbled constantly with N_2 maintained NH_4^+ pools of 0.8 mM for at least 2 h. Within 1 h after the addition of 0.1 mM MSX, the NH_4^+ pools increased to over 2 mM. The concentration

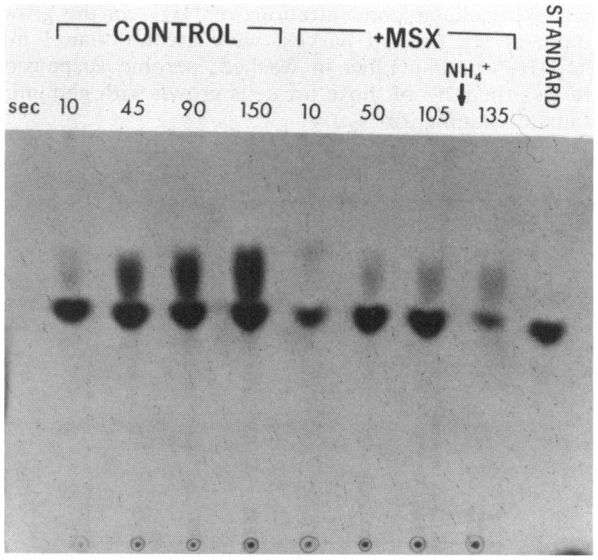

FIG. 4. Intracellular accumulation of $^{14}CH_3NH_3^+$ and ^{14}C-labeled metabolite. *R. sphaeroides* grown in 1.5 mM glutamine was harvested at half maximal growth, washed, and suspended aerobically in succinate buffer. Cell extracts were prepared, separated by thin-layer chromatography, and autoradiographed. Lanes: 1 to 4, extracts prepared 10, 45, 90, and 150 s after addition of $^{14}CH_3NH_3^+$; 5 to 8, 1 μM MSX was added to the suspension 5 s before addition of $^{14}CH_3NH_3^+$, NH_4Cl (2 mM) was added after 110 s, and cell extracts were prepared 10, 50, 105 and 135 s after addition of $^{14}CH_3NH_3^+$. The standard is $^{14}CH_3NH_3^+$ added to unlabeled cell extracts.

TABLE 2. Ammonium and methylammonium uptake

Growth nitrogen source	Assay conditions	Residual NH_4^+ (μM)	NH_4^+ uptake rate (μmol · min^{-1} · [g of protein]$^{-1}$) ± SD[a]	$CH_3NH_3^+$ uptake rate (μmol · min^{-1} · [g of protein]$^{-1}$) fast phase ± SD[b]
NH_4^+ (≥5 mM)	Aerobic	≥1,300	66 ± 6.0	0.05 ± 0.03
NH_4^+ (1.25 mM)[c]	Aerobic	600	37 ± 3.7	0.16 ± 0.03
NH_4^+ (1.25 mM)	Aerobic	~3	60 ± 2.9	1.20 ± 0.21
Glutamate, alanine, or glutamine[d]	Aerobic	<2	60 ± 5.5	4.65 ± 1.49
N_2[e]	Aerobic	<2	57 ± 7.5	3.94 ± 1.35
N_2[e]	Anaerobic	<2	42 ± 5.4	3.02 ± 1.32

[a] Linear uptake rates after addition of 50 μM NH_4^+ were determined as described in the text.
[b] Initial uptake rates after addition of 9 μM $CH_3NH_3^+$ were determined as described in the text.
[c] Culture was harvested in early exponential phase of batch growth.
[d] The amino acids tested as growth nitrogen sources were glutamate (2 mM), alanine (2 mM), and glutamine (1.5 mM).
[e] Uptake rates were determined after anaerobic equilibration.

gradient of NH_4^+ across the cytoplasmic membrane in these diazotrophs after MSX inhibition remained approximately 150-fold, even though the rate of NH_4^+ release after MSX treatment averaged 115 μmol (g of protein)$^{-1}$ h^{-1}.

Regulation of NH_4^+ and $CH_3NH_3^+$ uptake. When 50 μM NH_4^+ was added to washed cell suspensions in the presence of a suitable source of carbon and energy, it was taken up rapidly (Table 2). Uptake continued linearly in aerobic suspensions to the detection limit of the assay (approximately 5 μM) at a maximum rate of 66 μmol of NH_4^+ min^{-1} (g of protein)$^{-1}$ at 30°C. Regardless of the growth condition tested, uptake rates varied less than twofold.

Unlike NH_4^+ uptake, $^{14}CH_3NH_3^+$ uptake rates in washed suspensions varied substantially with growth source of nitrogen; significant uptake of 9 μM $^{14}CH_3NH_3^+$ (4.65 μmol min^{-1} [g of protein]$^{-1}$, fast phase; 1.5 μmol min^{-1} [g of protein]$^{-1}$, slow phase) was obtained only when cells were grown on nitrogen sources other than NH_4^+ or after exhaustion of NH_4^+ concentrations limiting for growth (Table 2). When extracellular concentrations of NH_4^+ in the growth medium at the time of harvest were greater than 1 mM, $^{14}CH_3NH_3^+$ uptake rates in washed, aerobic suspensions were less than 2% of those for cells grown with glutamine, glutamate, alanine, or N_2.

Ammonium transport activity in cells grown on high concentrations of NH_4^+. Although there was no increase in $^{14}CH_3NH_3^+$ uptake ability when NH_4^+-repressed cells were incubated in nitrogen-free succinate buffer for 3 h, NH_4^+ was taken up immediately if added to the suspending medium (0.125 mM, final concentration; Fig. 6). With each NH_4^+ addition, the pool increased transiently to approximately 1 mM NH_4^+, representing NH_4^+ concentration gradients (cellular over extracellular concentrations) of approximately 40-fold 6 min after each addition of NH_4^+. Experiments of this type demonstrated that the NH_4^+ concentration gradient across the cytoplasmic membrane, i.e., NH_4^+ transport, could be modulated even in the absence of $^{14}CH_3NH_3^+$ transport ability.

DISCUSSION

The results reported here are consistent with the existence of an NH_4^+ transport system in *R. sphaeroides*. When NH_4^+ was added to a cell suspension, intracellular NH_4^+ pools of 1 to 1.6 mM accumulated rapidly (Fig. 1 and 6). This intracellular concentration was comparable to the NH_4^+ pool found in cells harvested in mid-exponential growth on a variety of nitrogen sources (Table 1) or after treatment of a

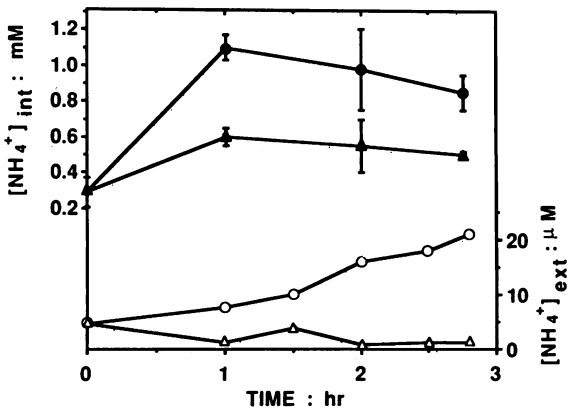

FIG. 5. Effect of inhibiting glutamine synthetase on NH_4^+ pools. *R. sphaeroides* was grown in 2.5 mM NH_4^+ and at time zero was resuspended aerobically in succinate buffer (pH 7) to a density of 0.17 mg of protein ml^{-1}. Symbols: ▲, average NH_4^+ pool values in control cells; ●, average NH_4^+ pool values in cells treated with MSX (0.1 mM) at time zero; △, extracellular NH_4^+ concentrations in control cells; ○, extracellular NH_4^+ concentrations in MSX-treated suspensions. Error bars represent ±1 standard deviation after triplicate distillations.

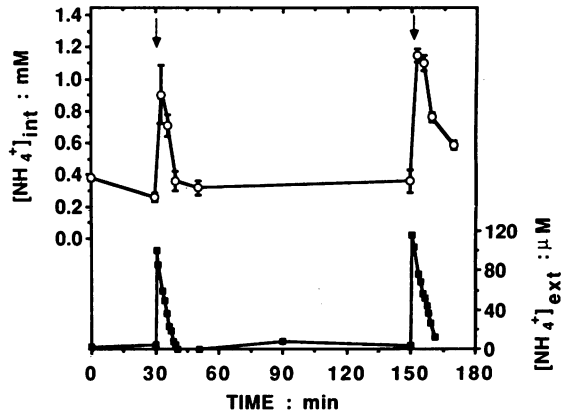

FIG. 6. Ammonium pools after growth in high NH_4^+. *R. sphaeroides* grown in excess NH_4^+ (7.5 mM) was harvested at half maximal growth and resuspended aerobically in nitrogen-free succinate buffer (pH 7) to a density of 0.3 mg of protein ml^{-1}. At each arrow, 0.125 mM NH_4^+ was added. Symbols: ■, extracellular NH_4^+ concentration, determined after filtration; ○, average intracellular NH_4^+ pool, shown ±1 standard deviation after triplicate distillations. Initial uptake rates of 9 μM $^{14}CH_3NH_3^+$ in subsamples taken at 1 and 3 h were 0.05 and 0.15 nmol min^{-1} (mg of protein)$^{-1}$, respectively.

cell suspension with MSX (Fig. 5). In washed cell suspensions provided with a mixture of amino acids (1.2 mM), intracellular concentrations of NH_4^+ were 0.6 to 0.7 mM, which was lower than that of the pools detected in growing cells, yet well in excess of the extracellular concentrations of 4 to 6 μM (Fig. 2). It is possible that under these experimental conditions, unlike during balanced growth, the rate of amino acid uptake was too low to replenish all the amino acid and NH_4^+ pools.

Intracellular NH_4^+ concentrations up to 100-fold higher than extracellular concentrations thus seem to be typical for normal growth and may represent a steady state between accumulation of the ion and leakage of unprotonated NH_3 (23). Free-living organisms such as *R. sphaeroides* thus may differ from whole cells or bacteroids of some *Rhizobium* species which neither maintain NH_4^+ pools nor accumulate $CH_3NH_3^+$ (9, 18) under conditions which lead to maximum $^{14}CH_3NH_3^+$ uptake in *R. sphaeroides*.

Two lines of evidence indicated that *R. sphaeroides* was able to take up $^{14}CH_3NH_3^+$ by using a transport system which preferentially recognized NH_4^+. (i) The addition of NH_4^+ blocked uptake of $CH_3NH_3^+$, which is consistent with competition between low- and high-affinity substrates for a common transporter. Even when a 45-fold excess of $^{14}CH_3NH_3^+$ was added simultaneously with 1 μM NH_4^+, initiation of $CH_3NH_3^+$ uptake was delayed (unpublished observations), implying that the $K_{iNH_4^+}$ for $^{14}CH_3NH_3^+$ transport was much lower than 1 μM. (ii) Intracellular accumulations of unmetabolized $^{14}CH_3NH_3^+$ were completely released when NH_4^+ was added (Fig. 3; Fig. 4, lane 8), which suggested that a common transport protein released one substrate as the other was accumulated. Energy-dependent uptake of $^{14}CH_3NH_3^+$ through the NH_4^+ transport system, competitive inhibition between the two substrates, and higher affinity for the natural substrate, NH_4^+, have been demonstrated in a variety of species (2, 5, 15, 16, 20, 25), including other purple nonsulfur bacteria (1). Uptake of NH_4^+ and $CH_3NH_3^+$ requires energy in the form of proton motive force and is insensitive to inhibition by 25 mM K^+ and the ATPase inhibitor dicyclohexylcarbodiimide (Cordts and Gibson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985). It thus differs significantly from the $K^+/CH_3NH_3^+$ antiporter recently characterized in *Escherichia coli* (20). The *R. sphaeroides* transport system contrasts with inducible, $CH_3NH_3^+$-specific transport systems found in organisms that use $CH_3NH_3^+$ as a carbon (17) or nitrogen (4, 14) source, as these latter systems are not strongly inhibited by NH_4^+.

The independence of NH_4^+ and $CH_3NH_3^+$ transport from the process of assimilation by glutamine synthetase was shown in several experiments. Bacteria treated with MSX maintained intracellular concentrations of approximately 1 mM NH_4^+, even though net uptake of NH_4^+ was prevented (Fig. 5). Transport of $CH_3NH_3^+$ also was not inhibited by MSX (Fig. 4), as intracellular $^{14}CH_3NH_3^+$ concentrations were similar in the presence or absence of the inhibitor (0.41 ± 0.09 mM $CH_3NH_3^+$ in MSX-treated cells and 0.41 ± 0.1 mM in control cells, in a total of 9 and 15 determinations, respectively). The effectiveness of MSX in such experiments is indicated by the lack of $^{14}CH_3NH_3^+$ metabolism shown in Fig. 4 (lanes 5 to 8).

The exchange of $CH_3NH_3^+$ for NH_4^+ in MSX-treated cells (Fig. 4, lane 8) clearly demonstrated that the NH_4^+ transporter was not inhibited by MSX. Glutamine synthetase activity is required for continued net uptake of NH_4^+ in this as well as in other bacteria, but the apparent loss of NH_4^+ transport activity caused by mutation of glutamine synthetase (13) or treatment with MSX (26, 33) may be adequately explained by net intracellular production of NH_4^+ from amino acid and protein turnover (6, 22, 23, 28). Thus, the processes of NH_4^+ transport and assimilation by glutamine synthetase can occur independently.

Repression of $CH_3NH_3^+$ uptake after growth in high concentrations of NH_4^+ also has been observed in other procaryotes (25). The observation that such repressed cells are able to maintain NH_4^+ pools and even supplement these pools when external NH_4^+ is added suggests strongly that a second, more specific transport system may be present under these conditions. The observed NH_4^+ pools cannot be attributed to passive entry of NH_3 followed by pH-dependent trapping because under the experimental conditions used for pool determinations the intracellular pH measured 7.4 ± 0.06 (n = 20), 0.4 pH units more alkaline than the external pH; the intracellular pH was not changed when 0.15 mM NH_4^+ was added as chloride or acetate (M. L. Cordts and R. J. Ritchie, unpublished results). Because of the limitation of experiments carried out without the aid of isotopes, we cannot exclude the possibility that increased NH_4^+ pools in these experiments arose from more rapid amino acid or protein turnover. However, the speed of the pool response and the close correlation with disappearance of NH_4^+ from the suspending medium support involvement of a transport system which has a high affinity for NH_4^+ but not for $CH_3NH_3^+$. The existence of two transport systems for NH_4^+ in *R. capsulatus* has also been suggested; one of these has a much higher affinity for $CH_3NH_3^+$ than the other has (B. J. Rapp, D. C. Landrum, and J. D. Wall, Abstr. V Int. Symp. Photosynthetic Prokaryotes 1985. VI K, p. 217).

The suggested existence of multiple transport systems for NH_4^+ in *R. sphaeroides* may reflect on the importance of this substrate to the cell and be analogous to the two K^+ transport systems known in *E. coli* (11) and *Streptococcus faecalis* (27). Maintaining intracellular NH_4^+ within certain concentration limits may be crucial to the normal functioning of the cell, possibly for purposes of ion balance, and certainly for nitrogen metabolism in this versatile phototroph. However, as with the K^+ transport systems, genetic analysis will be required to substantiate the existence of multiple NH_4^+ transporters.

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