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Ammonium and Methylammonium Transport by the Nitrogen-Fixing Bacterium *Azotobacter vinelandii*

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Azotobacter vinelandii, grown with NH_4^+ as nitrogen source, was shown to possess an active transport system which can take up NH_4^+ against a concentration gradient of 58-fold. The properties of the NH_4^+ uptake system were investigated with the NH_4^+ analog $CH_3NH_3^+$. The use of this analog was justified on the basis of the conclusion that the uptake of NH_4^+ and $CH_3NH_3^+$ involves a common binding site, as shown by the competitive inhibition of $CH_3NH_3^+$ uptake by NH_4^+ (K_i approximately 3 μM). A Lineweaver-Burk plot for $CH_3NH_3^+$ uptake revealed a biphasic curve, suggesting the existence of two $CH_3NH_3^+$ (NH_4^+) uptake systems with apparent K_m's for $CH_3NH_3^+$ equal to 61 μM and 661 μM. The uptake of $CH_3NH_3^+$ was inhibited by arsenate, as well as by cyanide or carbonyl cyanide-*m*-chlorophenyl hydrazone, indicating that phosphate bond energy is required.

All free-living N_2-fixing bacteria are known to respond to the presence of fixed nitrogen in the environment by shutting off synthesis of nitrogenase. The regulatory mechanism which responds to NH_4^+ is highly efficient and accomplishes the immediate repression of nitrogenase synthesis in *Azotobacter vinelandii* (28), resulting in a greater than 10^4-fold decrease in the enzyme level (12). The nature of the regulatory mechanism is not well understood. A hypothesis which has not yet been investigated is that NH_4^+ uptake plays a role in regulating nitrogenase synthesis. The observation that catabolite repression can result from the transport of sugars across the inner membrane (23, 24) suggests that transport mechanisms may play a more direct role in regulating enzyme synthesis than previously thought. The original model proposed for the regulation of nitrogenase synthesis specified a role for the *glnA*-encoded NH_4^+ assimilatory enzyme glutamine synthetase (33, 34). Because of the recent evidence that two cistrons closely linked to *glnA* are involved in the regulation of glutamine synthetase and other enzymes of nitrogen metabolism (20, 22), this model may have to be modified. There are, as yet, no data available regarding the effect of mutations in these cistrons on nitrogenase synthesis. Interaction between the NH_4^+ transport system and the NH_4^+ assimilatory system has not been studied.

Although little is presently known about NH_4^+ transport mechanisms, some details have been reported about the properties of the uptake systems in *Escherichia coli* (30), *Klebsiella pneumoniae* (17), *A. vinelandii* (16, 21), and *Clostridium pasteurianum* (18, 19). Eucaryotic NH_4^+ uptake mechanisms have been more extensively characterized than the procaryotic mechanisms. Data on the kinetics of NH_4^+ uptake, substrate affinity, and control of uptake activity are available for *Penicillium chrysogenum* (13), *Aspergillus nidulans* (7), *Saccharomyces cerevisiae* (26), and *Stemphylium botryosum* (4). The dearth of information on bacterial NH_4^+ transport systems prompted us to study the properties of the NH_4^+ uptake system in *A. vinelandii* in an attempt to define the requirements for transport and the nature of the proteins involved. It is hoped that this information will enable us to investigate the role of NH_4^+ transport in the regulation of N_2 fixation. Furthermore, the characterization of NH_4^+ transport systems in N_2-fixing bacteria should facilitate the isolation of NH_4^+-excreting mutants which would mimic the symbiotic N_2-fixing bacterium *Rhizobium* in providing fertilizer nitrogen to plants.

(A preliminary account of this work has been presented elsewhere [R. A. Moore and J. K. Gordon, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, K119, p. 146].)

MATERIALS AND METHODS

Bacterial strains. *A. vinelandii* OP (5), obtained from the American Type Culture Collection, was the wild-type organism used in these studies. Mutant strains UW2 (12) and UW10 (11, 29) are derived from *A. vinelandii* OP and are unable to fix N_2.

Media and culture conditions. The organisms

were grown on a modified Burk medium (31). Ammonium was supplied as ammonium acetate at a concentration of 29 mM (400 μg of nitrogen per ml) and was filter sterilized before use. Cultures were grown at 30°C on a rotary shaker to a density of 100 Klett units (0.3 mg of protein per ml) unless otherwise specified.

Chemicals. [^{14}C]methylamine hydrochloride, obtained from New England Nuclear (Lachine, Quebec), was diluted with unlabeled methylamine hydrochloride from Sigma Chemical Co. (St. Louis, Mo.). The scintillation cocktail was prepared with PPO (2,5-diphenyloxazole) obtained from Mallinckrodt, Inc. (Paris, Ky.). All other chemicals used were reagent grade, available commercially.

Measurement of NH_4^+ uptake. Cells were harvested by centrifugation, washed twice, and suspended to the original density in Burk N-free medium. The washed cell suspension was incubated aerobically at 30°C for 10 min to allow for depletion of remaining extracellular NH_4^+. At that time, NH_4^+ (as ammonium sulfate or ammonium acetate) was added to a final concentration of 0.8 mM. At 1-min intervals, 1-ml samples were removed and filtered through a 0.45-μm-pore-size filter (Gelman, Inc., Ann Arbor, Mich.). The filtrate collected was assayed for NH_4^+ content with a glutamate dehydrogenase assay (32).

Determination of intracellular NH_4^+ level. The intracellular level of NH_4^+ was estimated by a modification of the procedure described by Kleiner (16). The pellet from a 100-ml culture of density equal to 100 Klett units was collected by centrifugation at 0 to 5°C and washed two times with Burk N-free medium before being suspended in 10-ml of Burk N-free medium with 0.1% toluene. The cells were then collected by centrifugation at 12,000 × *g* for 5 min, the supernatant was removed, and the pellet was dislodged by blending in a Vortex mixer. One milliliter of Burk phosphate buffer was added, the tube was placed in a boiling water bath for 5 min, and the suspension was subjected to ultrasonic disruption for 4 min with a Bronwill Biosonik at a setting of 55 to 70. Cell debris was removed by centrifugation at 12,000 × *g* for 20 min, and the supernatant was assayed for NH_4^+ with a glutamate dehydrogenase assay (32).

Transport of $CH_3NH_3^+$. Unless otherwise noted, cultures were grown with NH_4^+ as nitrogen source to a density of 100 Klett units. For assay of $CH_3NH_3^+$ uptake, all traces of extracellular NH_4^+ were removed before initiation of the assay. This was done by collecting the cells on a 0.45-μm-pore-size filter and washing twice with Burk N-free medium. When a nonphosphate-containing medium was required, 5 mM glycylglycine supplemented with Burk salts (final pH 7.45) was used in place of Burk N-free medium. The filter was removed, and the cells were suspended in N-free medium to the original density. Cell suspensions were kept at 0 to 5°C until assayed. A 10-min aerobic incubation at 30°C preceded the addition of $^{14}CH_3NH_3^+$ to initiate the reaction. Assays were performed in 8.5-ml serum vials or 20-ml scintillation vials in volumes ranging from 1.2 to 1.6 ml. When cyanide, carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP), or arsenate was used, incubation for 5 min in the presence of the inhibitor preceded the addition of substrate. Assays of $CH_3NH_3^+$ uptake in the presence of NH_4^+ were initiated by the addition of the cell suspension to vials containing both NH_4^+ and $CH_3NH_3^+$. The specific activity of the $^{14}CH_3NH_3^+$ was adjusted to permit accurate quantitation of the uptake rates and ranged from 0.2 to 44 μCi/μmol. At various times within a 2-min period, at least four samples of 200-μl volume were removed, and the cells were collected by vacuum filtration on a 0.45-μm-pore-size filter (25-mm diameter). The wash buffer used was the same as that used in the uptake assay and contained a concentration of unlabeled $CH_3NH_3^+$ equivalent to that in the assay. Wash buffers, maintained at 30°C, were added in at least 20-fold excess of the sample volume. The filters containing bacterial cells were dried at 55°C for 60 min, and the radioactivity was determined by counting in a Nuclear Chicago Isocap 300 liquid scintillation counter. All data were fitted to straight lines by linear regression analysis.

Whole-cell protein determination. A modified Lowry procedure (27) was used to measure whole-cell protein. The cell suspension was centrifuged, and the pellet was washed with Burk buffer before being suspended to the original density in distilled water. A 1-ml sample of the washed cell suspension was mixed with 1 ml of 1 N NaOH and immersed in a boiling water bath for 5 min. After cooling, a sample was removed, and the protein content was determined.

Determination of ATP. Intracellular ATP levels were determined by the luciferin-luciferase system (15). Extraction of ATP was performed by the method of Cole et al. (6). Measurement of luminescence was accomplished with a Nuclear Chicago 720 series liquid scintillation counter operated at ambient temperature.

Paper chromatography. Paper chromatography was used to determine whether $CH_3NH_3^+$ was metabolized by *A. vinelandii*. Cells incubated in the presence of $^{14}CH_3NH_3^+$ were extracted by boiling for 10 min. A portion of the boiled cell suspension was spotted onto Whatman no. 1 filter paper. Samples of $^{14}CH_3NH_3^+$, unlabeled $CH_3NH_3^+$, alanine, glycine, glutamate, glutamine, and serine were also spotted onto the filter paper to serve as references. A solvent system of butanol–formic acid–water (75:15:15) was employed as described by Hackette et al. (13). Compounds were visualized by spraying with ninhydrin. The radioactivity in 1-cm strips of the chromatogram was determined, as was the mobility of the ^{14}C compared to that of the reference compounds.

RESULTS

Uptake of NH_4^+ and $CH_3NH_3^+$ against a concentration gradient. Substrate uptake against a concentration gradient is one criterion for an active transport system. Data indicating the ability of NH_4^+-grown cultures of *A. vinelandii* to concentrate NH_4^+ are shown in Table 1. The intracellular pool of NH_4^+ was determined to be 2.9 mM. When the cells were suspended in medium containing 0.17 mM NH_4^+, exogenous NH_4^+ was taken up at a high rate (80.2 nmol/min per mg of protein). Thus, these cells were capable of uptake of NH_4^+ against a concentration gradient of at least 17-fold. The

rate of uptake remained linear until the depletion of detectable exogenous NH_4^+. Because the lower limit of detection of exogenous NH_4^+ in the assay was 0.05 mM, one can conclude that the cells are capable of concentrating NH_4^+ at least 58-fold.

Similarly, the ability of NH_4^+-grown cells of *A. vinelandii* to take up $CH_3NH_3^+$ against a concentration gradient was demonstrated as shown in Table 1. Cultures suspended in 0.02 mM $CH_3NH_3^+$ took up the substrate at a rate of 0.9 nmol/min per mg of protein, incorporating 32,500 cpm/ml within 20 min. The radioactively labeled carbon appeared to remain mainly in $CH_3NH_3^+$ as greater than 82% of the radioactivity comigrated with $CH_3NH_3^+$ during paper chromatography. Although several amino acids comigrate with $CH_3NH_3^+$, it is unlikely that much of the label was present in amino acids because no label was detectable in trichloroacetic acid-precipitable material. On the basis of these observations, the intracellular $CH_3NH_3^+$ concentration was calculated to be 1.3 mM, indicating that the cells could concentrate $CH_3NH_3^+$ at least 65-fold.

Transport of NH_4^+ and $CH_3NH_3^+$ by a common system. If NH_4^+ and $CH_3NH_3^+$ share a transport system, the rate of uptake of one substrate will be diminished in the presence of the other. Uptake of $CH_3NH_3^+$ was assayed in the presence of various concentrations of NH_4^+. The uptake of 0.02 mM $CH_3NH_3^+$ in the absence of NH_4^+ commenced immediately and proceeded linearly (Fig. 1). When NH_4^+ was included in the assay mix, there was a lag before $CH_3NH_3^+$ uptake began. The lag was proportional to the concentration of NH_4^+ added and corresponded to the amount of time required for depletion of NH_4^+ from the medium. The results suggest that the presence of extracellular NH_4^+ interferes with the uptake of $CH_3NH_3^+$. Methylamonium uptake can be accomplished by cells with measurable NH_4^+ pools (R. Moore, unpublished data), indicating that intracellular NH_4^+ does not interfere with $CH_3NH_3^+$ uptake. There are two possible explanations for the lag. A likely

TABLE 1. *Ability of A. vinelandii to take up $CH_3NH_3^+$ and NH_4^+ against a concentration gradient*[a]

Substrate	Extracellular concn (mM)	Intracellular concn achieved (mM)
$CH_3NH_3^+$	0.02	1.3
NH_4^+	0.17	2.9

[a] The rate of $CH_3NH_3^+$ uptake was 0.9 nmol/min per mg of protein. The rate of NH_4^+ uptake was 80.2 nmol/min per mg of protein.

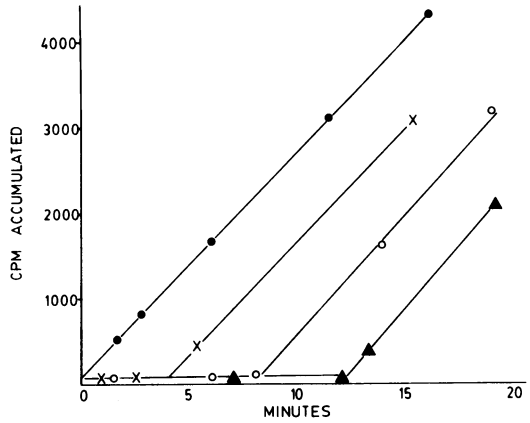

FIG. 1. *Uptake of $^{14}CH_3NH_3^+$ in the presence of different amounts of NH_4^+. Bacteria were added to assay vials containing 20 μM $^{14}CH_3NH_3^+$ (5 μCi/μmol) and NH_4^+ at the concentrations indicated. Symbols: ●, no NH_4^+; ×, 100 μM NH_4^+; ○, 200 μM NH_4^+; ▲, 300 μM NH_4^+.*

possibility is that NH_4^+ and $CH_3NH_3^+$ are transported by the same carrier (the carrier exhibiting greater affinity for NH_4^+ than for $CH_3NH_3^+$). It is also possible that NH_4^+ inhibits $CH_3NH_3^+$ uptake in some other way. To distinguish between these possibilities, we determined whether or not NH_4^+ was a competitive inhibitor of $CH_3NH_3^+$ uptake, altering the apparent affinity of the transport system for $CH_3NH_3^+$, but leaving the V_{max} unchanged. Rates of $CH_3NH_3^+$ uptake were measured in the presence and absence of 150 μM NH_4^+ at concentrations of $CH_3NH_3^+$ ranging from 0.7 to 4.0 mM. Attempts to use low concentrations of $CH_3NH_3^+$ (<0.3 mM) resulted in barely detectable rates of uptake when NH_4^+ was present. A Lineweaver-Burk plot for rates of uptake in the presence and absence of NH_4^+ is shown in Fig. 2. The curve representing uptake rates in the absence of NH_4^+ is also shown in Fig. 3 on a different scale. The two curves in Fig. 2 have the same y intercept, indicating that the V_{max} is unaltered by the presence of NH_4^+. An Eadie-Scatchard plot ($v/[S]$ versus v) confirms that the V_{max} in the presence of NH_4^+ (9.0 nmol/min per mg of protein) is the same as the V_{max} in the absence of NH_4^+ (9.1 nmol/min per mg of protein) and that NH_4^+ is a competitive inhibitor of $CH_3NH_3^+$ uptake. The competitive inhibition of $CH_3NH_3^+$ uptake by NH_4^+ indicates that NH_4^+ and $CH_3NH_3^+$ bind to a common site for uptake and that one can therefore use $CH_3NH_3^+$ as a substrate to study this reaction.

Kinetic evidence for multiple uptake systems. Rates were determined for the uptake of $CH_3NH_3^+$ at concentrations ranging from 5 μM

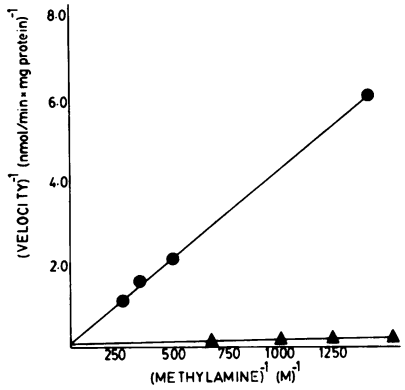

FIG. 2. *Competitive inhibition of $^{14}CH_3NH_3^+$ uptake by NH_4^+. The curve representing rates of $CH_3NH_3^+$ uptake in the presence of 150 μM NH_4^+ (●) is shown to have the same y intercept as the plot for uptake rates in the absence of NH_4^+ (▲). The K_i for NH_4^+ is approximately 3 μM.*

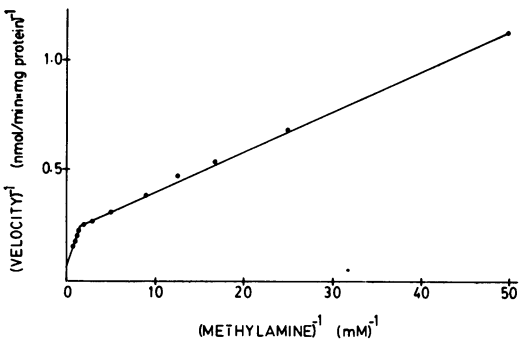

FIG. 3. *A Lineweaver-Burk plot for rates of $CH_3NH_3^+$ uptake.*

to 1.5 mM. A Lineweaver-Burk plot of the data reveals two linear segments (Fig. 3). This result suggests that more than one transport system exists or that there is a single system which can exhibit a different K_m at different substrate concentrations. The different K_m and V_{max} values were determined from the Lineweaver-Burk plot. The apparent K_m for the higher-affinity system postulated (K_{m1}) is 61 μM; the V_{max1} is 3.7 nmol/min per mg of protein. The lower-affinity system postulated displays an apparent K_m (K_{m2}) of 661 μM. The reciprocal of the y intercept of this portion of the curve represents $V_{max1} + V_{max2}$ and is equal to 9.1 nmol/min per mg of protein. Consequently, V_{max2} was estimated to be 5.4 nmol/min per mg of protein. A plot of $v/[S]$ versus v, which weights points at high and low $[S]$ more equally than the $1/v$ versus $1/[S]$ plot, also showed two linear segments. The K_{m1} from this plot was calculated as 55 μM; K_{m2} was calculated to be 638 μM, in good agreement with the results obtained from the double-reciprocal plot. If there were two systems, the high-affinity system (K_{m1}) would make the major contribution to the observed velocity at low substrate concentrations. It would therefore be possible to study system 1 independently of system 2 by using low substrate concentrations. For this reason, determination of the nature of the energy requirement was done with a low (20 μM), as well as a high (1 mM), substrate concentration.

Energy requirement. To establish the nature of the energy requirement for NH_4^+/$CH_3NH_3^+$ uptake, we determined the effect of various metabolic inhibitors on $CH_3NH_3^+$ uptake (Table 2). Because *A. vinelandii* utilizes aerobic respiration to generate energy, it was expected that an inhibitor of respiration, such as cyanide, or an uncoupler, such as CCCP, would inhibit $CH_3NH_3^+$ uptake, regardless of whether transport is dependent on ATP or on a component of the proton motive force. Indeed, treatment with KCN or CCCP at 0.2 mM inhibited uptake of both 20 μM and 1 mM $CH_3NH_3^+$, whereas 0.2 mM KCl had little effect (Table 2).

The selective inhibition of ATP synthesis can be accomplished by arsenate addition. Barnes (1) has shown that treatment with 20 mM arsenate does not disrupt respiration-linked glucose transport by membrane vesicles from *A. vinelandii*. However, ATP synthesis by NH_4^+-grown or N_2-fixing *A. vinelandii* was strongly inhibited by treatment with 15 mM arsenate (Table 3). The incubation of cells washed and suspended in a non-phosphate-containing buffer with 15 mM arsenate resulted in a 51% decrease in intracellular ATP levels within 10 s. A 94% decrease was observed within 1 min of addition, and a 95% decrease was observed within 10 min. Interestingly, the effect of arsenate on intracellular ATP was also quite pronounced when the arsenate treatment was performed in Burk medium (approximately 6 mM phosphate), as shown in Table 3. The ability of arsenate to inhibit the uptake of $CH_3NH_3^+$ by NH_4^+-grown or N_2-fixing *A. vinelandii* is shown in Table 2. Rates less than 11% of the control rate were observed for the uptake of 20 μM or 1 mM $CH_3NH_3^+$ in both phosphate-free medium and Burk medium. An equivalent concentration of phosphate (15 mM) or NaCl (30 mM) inhibited uptake only slightly. These results indicate that $NH_4^+/CH_3NH_3^+$ uptake is dependent on the presence of ATP or an equivalent high-energy phosphate compound.

Regulation of transport in response to nitrogen starvation. The response of $CH_3NH_3^+$ transport activity to nitrogen starvation was determined with the non-N_2-fixing mutant strains of *A. vinelandii*, UW2 (12) and

TABLE 2. *Effect of inhibitors of energy generation on $CH_3NH_3^+$ uptake*

Nitrogen source for growth	Assay buffer	Addition (mM)	% Inhibition of uptake rate	
			20 μM $CH_3NH_3^+$	1 mM $CH_3NH_3^+$
NH_4^+	Burk[a] (PO_4^{3-})	None	0	0
		KCN, 0.2	99	94
		CCCP, 0.2	99	90
		Na_2As, 15	97	89
		NaCl, 30	27	13
		PO_4^{3-}, 15	35	0
		KCl, 0.2	13	13
	Glycylglycine[a]	None	0	0
		Na_2As, 15	98	90
		NaCl, 30	24	15
N_2[b]	Burk[c] (PO_4^{3-})	None	0	0
		Na_2As, 15	99	94
		NaCl, 30	30	0
		PO_4^{3-}, 15	25	0
	Glycylglycine[c]	None	0	0
		Na_2As, 15	0	0
		NaCl, 30	24	20

[a] Control rates for NH_4^+-grown cultures assayed in Burk buffer or glycylglycine were 0.9 nmol/min per mg of protein (20 μM $CH_3NH_3^+$) and 5.3 nmol/min per mg of protein (1 mM $CH_3NH_3^+$).

[b] N_2-grown cultures were washed and suspended in the appropriate buffer supplemented with Burk salts to a density of 100 Klett units after growth to a density of 150 Klett units.

[c] Control rates for N_2-grown cultures assayed in Burk buffer or glycylglycine were 0.6 nmol/min per mg of protein (20 μM $CH_3NH_3^+$) and 3.7 nmol/min per mg of protein (1 mM $CH_3NH_3^+$).

TABLE 3. *Effect of arsenate and CCCP on intracellular ATP levels*

Nitrogen source for growth	Buffer	Addition (mM)	% Decrease in ATP
NH_4^+	Burk (PO_4^{3-})	None[a]	0
		Na_2As, 15	92
		CCCP, 0.2	86
	Glycylglycine	None[a]	0
		Na_2As, 15	91
N_2[b]	Burk (PO_4^{3-})	None[c]	0
		Na_2As, 15	92
	Glycylglycine	None[c]	0
		Na_2As, 15	90
		NaCl, 60	0

[a] ATP was approximately 17 nmol/mg of protein.

[b] N_2-grown cultures were washed and suspended in the appropriate buffer supplemented with Burk salts to a density of 100 Klett units after growth to a density of 150 Klett units.

[c] ATP was approximately 15 nmol/mg of protein.

UW10 (11, 29). The mutant strains were grown in NH_4^+-supplemented medium, washed, and suspended in N-free medium. A $CH_3NH_3^+$ uptake assay done at time zero indicated uptake rates characteristic of wild-type *A. vinelandii* grown under similar conditions. After aerobic incubation for 3 h, the cultures of nitrogen-starved cells exhibited a rate of uptake approximately 1.5-fold greater than that observed at time zero. In no case was the activity in nitrogen-starved cultures greater than twice that observed in NH_4^+-sufficient cultures. Because of the possibility that nitrogen-starved cells might be unable to synthesize new or additional transport proteins, the experiment was repeated with adenine as a limiting nitrogen source (generation time of 6.8 h compared to 1.8 h for NH_4^+-sufficient cultures). Under these conditions, the rate of $CH_3NH_3^+$ uptake was no greater than that observed for NH_4^+-sufficient cultures. Thus, it appears that the rate of $NH_4^+/CH_3NH_3^+$ transport does not increase significantly as a result of nitrogen limitation or starvation.

DISCUSSION

Our results demonstrate the existence of an active transport system for NH_4^+ in cultures of *A. vinelandii* which have been grown with NH_4^+ as nitrogen source. This system can establish an NH_4^+ concentration gradient of at least 58-fold. Kleiner (16) has reported the ability of N_2-grown cultures of *A. vinelandii* to concentrate NH_4^+ approximately 100-fold.

To define the properties of the NH_4^+ uptake system, we used the NH_4^+ analog $CH_3NH_3^+$, which has been used by other investigators to

characterize NH_4^+ transport systems (4, 7, 13, 18, 21, 26, 30). The fact that NH_4^+ and $CH_3NH_3^+$ are transported by a common system has been demonstrated for several eucaryotic microorganisms (4, 13, 26) in which competitive inhibition was observed. Competition between NH_4^+ and $CH_3NH_3^+$ in procaryotes has been reported previously only for N_2-grown cultures of *C. pasteurianum* (19). The demonstration that NH_4^+ is a competitive inhibitor of $CH_3NH_3^+$ uptake by NH_4^+-grown cultures of *A. vinelandii* was accomplished with high $CH_3NH_3^+$ concentrations (0.7 to 4.0 mM) and a concentration of NH_4^+ (150 μM) that permitted detection of $CH_3NH_3^+$ uptake yet did not change too rapidly during the assay.

A double-reciprocal plot (Fig. 3) or a plot of $v/[S]$ versus v for $CH_3NH_3^+$ uptake over the range of substrate concentrations 5 μM to 1.5 mM revealed a biphasic curve. This result is consistent with the existence of two transport systems, although other possible explanations exist, as indicated below. A high-affinity apparent K_m of 61 μM and a low-affinity apparent K_m of 661 μM were observed. Kinetic and genetic evidence for multiple $NH_4^+/CH_3NH_3^+$ transport systems has been reported for *Saccharomyces cerevisiae* (9). Genetic evidence for the existence of multiple transport systems in *A. vinelandii* is currently being sought in our laboratory with putative NH_4^+-transport-defective strains.

Although competition between NH_4^+ and $CH_3NH_3^+$ could be demonstrated with high $CH_3NH_3^+$ concentrations, a direct demonstration of competition specifically for the high-affinity system could not be accomplished because of the difficulty in detecting $CH_3NH_3^+$ uptake in the presence of NH_4^+ at low $CH_3NH_3^+$ concentration. However, the observation (Fig. 1) that the lag before the uptake of 20 μM $CH_3NH_3^+$ commenced corresponded to the time required for depletion of NH_4^+ is consistent with competition between the two substrates for a common site. Furthermore, it is likely that there is competition between NH_4^+ and $CH_3NH_3^+$ for both systems because of the observation that the presence of NH_4^+ did not alter the $(V_{max1} + V_{max2})^{-1}$.

It is possible, however, that the biphasic plots observed are not due to the existence of multiple $CH_3NH_3^+$ transport systems. There may be a single system, displaying different K_m's, depending on the substrate concentration. Wilson (36) has described an interaction between the galactose and β-methyl galactoside transport systems which results in a threefold reduction of the K_m for transport by the galactose system in the presence of high galactose concentrations. Also, Ezzell and Dobrogosz (10) have reported that the K_m for hexose phosphate transport is affected by the level of phosphate in the uptake environment.

It was found that the ability to take up $CH_3NH_3^+$ did not increase significantly upon nitrogen limitation or starvation of *A. vinelandii*. Stevenson and Silver (30) reported that the $CH_3NH_3^+$ transport system in *E. coli* is constitutive, and Breiman and Barash (4) reported a 60% decrease in the rate of $CH_3NH_3^+$ uptake in nitrogen-starved *S. botryosum*. However, the rate of $CH_3NH_3^+$ uptake does increase with nitrogen starvation in *P. chrysogenum* (13), *S. cerevisiae* (26), *A. nidulans* (7), and *K. pneumoniae* (unpublished data).

The nature of the energy requirement for $CH_3NH_3^+$ uptake was determined with the use of the inhibitors cyanide, CCCP, and arsenate. The inhibition of $CH_3NH_3^+$ uptake was observed upon addition of cyanide or CCCP (Table 2). Because *A. vinelandii* is an obligate aerobe, dependent on aerobic respiration for ATP synthesis, these results would be expected for an active transport system which requires either proton motive force or ATP. The observation that arsenate inhibits $CH_3NH_3^+$ uptake indicates a requirement for ATP or an equivalent high-energy phosphate compound. Arsenate was found to inhibit uptake by both NH_4^+-grown and N_2-grown cultures (Table 2).

Plate (25) has shown that glutamine transport by *E. coli* requires not only ATP but also proton motive force. It is possible that $CH_3NH_3^+$ uptake requires a component of proton motive force in addition to ATP. Because CCCP caused a strong reduction in the ATP pool of this organism (Table 3), we cannot conclude whether a pH gradient is required for the transport of $CH_3NH_3^+$. It is interesting to note that Laane et al. (21) have reported that $CH_3NH_3^+$ uptake by N_2-fixing cultures of *A. vinelandii* is driven by $\Delta\psi$. We observed that N_2-fixing cultures require ATP for $CH_3NH_3^+$ uptake, but our results do not rule out the possibility that $\Delta\psi$ is required in addition to ATP. However, the conclusion reached by Laane et al. was based primarily on the observation that the addition of K^+ and valinomycin disrupts the uptake of $CH_3NH_3^+$ and results in the efflux of accumulated $CH_3NH_3^+$ from *A. vinelandii*. But if the addition of K^+ and valinomycin results in a decreased ATP pool, as is found in *E. coli* (25), the same results would be expected if only ATP were required for uptake. The demonstration that $\Delta\psi$ is required in addition to ATP for glutamine transport by *E. coli* (25) required the use of *unc* mutants which do not deplete ATP pools upon

addition of K^+ and valinomycin because they lack a functional ATPase. Laane et al. (21) also based their conclusion regarding the involvement of $\Delta\psi$ on the stimulation of $CH_3NH_3^+$ uptake by nigericin. However, the data shown are not very strongly indicative of stimulation.

There appears to be a correlation between dependence on ATP and involvement of periplasmic binding proteins. The observation that phosphate bond energy is required for transport by osmotic-shock-sensitive systems was originally reported by Berger (2) for the periplasmic-binding-protein-dependent glutamine transport system. The observation was later extended to other amino acid transport systems for which binding proteins have been found (3). However, a requirement for phosphate bond energy has been reported for some shock-sensitive systems for which binding proteins have not yet been found (8, 14, 35). Furthermore, one of the potassium transport systems in *E. coli* exhibits dependence on phosphate bond energy yet is shock resistant (D. Rhoads and W. Epstein, Fed. Proc. **35**:1168, 1976), suggesting either that a periplasmic protein is not involved or that it is not easily released. Thus, although systems involving binding proteins apparently require ATP (or equivalent phosphate bond energy), it is not known whether all ATP-requiring systems involve a periplasmic binding protein. An attempt to isolate a $NH_4^+/CH_3NH_3^+$ binding protein is currently under way in this laboratory.

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