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Transport of Ammonium and Methylammonium Ions by *Azotobacter vinelandii*

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Ammonium and methylammonium are rapidly taken up by cultures of *Azotobacter vinelandii* respiring in the presence of succinate. The rate of methylamine uptake increased with external pH from 5.5 to 7.5 but increasing the pH further to 8.5 had little effect on activity, indicating that methylammonium cation rather than uncharged methylamine is the permeant species. The kinetics of methylammonium entry followed the Michaelis-Menten relationship, yielding a K_m of 25 μM and a V_{max} of 3.8 nmol/min per mg of cell protein. At saturating concentrations ammonium was taken up at rates 30-fold higher than those for methylammonium. Ammonium was a competitive inhibitor of methylammonium uptake and gave an inhibition constant of 1 μM. Ammonium derivatives were inhibitors of methylammonium entry in order of effectiveness: hydrazine > methylhydrazine > formamidine > guanidine > dimethylamine > ethylamine; amides and amino acids did not block uptake. Likewise, metal cations inhibited in the order Tl^+ > Cs^+ > Rb^+, whereas Na^+, K^+, and Li^+ produced no significant effect. Methylammonium uptake was blocked in cells exposed to an uncoupler, *p*-trifluoromethoxycarbonyl cyanide-phenyl hydrazone or gramicidin D, but not with dicyclohexylcarbodiimide or arsenate. Valinomycin stimulated methylammonium entry into cells in a K^+-free medium but prevented entry in the presence of 10 mM K^+. Monensin and nigericin had little effect on transport. These results indicate that methylammonium and ammonium ions enter *A. vinelandii* electrogenically via a specific transporter.

The transport of ammonium is of special interest in nitrogen-fixing bacteria because it is a powerful repressor of nitrogenase. Kleiner (7) found that cultures of *Azotobacter vinelandii* can maintain a 100-fold concentration gradient of ammonium, indicating activity of an energy-linked transport system. Recently, Laane et al. (8) have shown that addition of ammonium chloride to cultures of *A. vinelandii* produces a rapid, complete inhibition of nitrogenase activity in vivo. They suggested that ammonium entry, via a potassium transporter, results in depolarization of the membrane potential and a decrease in the reducing equivalents necessary for nitrogen fixation. Little information is available concerning the specificity and mechanism of ammonium transport in bacteria. Stevenson and Silver (13) used radioactive methylamine as substrate to describe two energy-linked uptake systems in *Escherichia coli*; although inhibited by ammonium, these systems proved difficult to characterize kinetically. Bellion et al. (2) found an inducible uptake process for methylamine by *Pseudomonas* sp. MA, but it was not sensitive to inhibition by ammonium. Ammonium transport has been studied more extensively in eucaryotic microorganisms (5, 10, 11) in which uptake required expenditure of metabolic energy. The mechanism of energy coupling has not been elucidated in these cases.

In this paper we demonstrate that methylammonium is a suitable substrate for the characterization of ammonium transport in *A. vinelandii*. This system is distinct from those for transport of other monovalent cations and provides an electrogenic pathway for ammonium entry into the cell.

MATERIALS AND METHODS

A. vinelandii OP (ATCC 13705) was grown at 30°C on a medium previously described (14) except that 1% succinic acid (sodium salt) was the carbon source. Cells from an overnight culture were diluted 250-fold with fresh medium and grown out to mid-log phase (40 to 60 Klett turbidity units with a no. 66 filter). The cells were pelleted by centrifugation for 5 min at 3,000 × *g* and suspended in an equal volume of 20 mM sodium phosphate buffer (pH 7.4) containing 0.2 mM $MgCl_2$. This washing procedure was repeated once, and the cells were resuspended at a protein concentration of 0.3 to 0.4 mg/ml in the same buffer and stored on ice.

The assay method for measuring uptake of methylammonium was a modification of a method already described (1). The assay mixture (0.1 ml) contained 10 mM sodium phosphate (pH 7.4), 0.1 mM $MgCl_2$, 2 mM sodium succinate, and 15 to 20 μg of cell protein. These mixtures were incubated at 25°C for 5 min under an atmosphere of water-saturated O_2. Then [^{14}C]methylamine hydrochloride (61.1 Ci/mol) was added at a final concentration of 20 μM, and the incubation was continued for the time specified. The reaction was terminated by dilution with 10 mM sodium phosphate (pH 7.4) and filtration onto a polycarbonate filter with 0.8-μm pores (Bio-Rad Laboratories, Richmond, Calif.). The nonspecific binding of [^{14}C]methylamine to these filters was <0.5% of that usually retained by the cells. In some cases the reaction was terminated by centrifugation of cells through a layer of oil composed of a mixture of 41.25 g of bromododecane and 0.749 g of dodecane (3). An Eppendorf 5412 centrifuge which reached 15,600 × *g* in 5 s was used in these experiments. Filters or cell pellets were counted by liquid scintillation. Values for methylamine uptake agreed within 10% for the two methods. The uptake of ammonium was measured with a larger assay mixture (1 ml), termination of reaction by oil centrifugation, and determination of ammonium in the supernatant by use of glutamic dehydrogenase (15). All results for uptake were expressed as nanomoles per milligram of cell protein as determined by the method of Lowry et al. (9).

The intracellular metabolism of [^{14}C]methylamine was detected by extraction of filters with boiling water and thin-layer chromatography of the extracts on silica gel plates developed with methanol-water-acetic acid, 60:38:2 (vol/vol/vol). Radioactive zones were detected by autoradiography and identified by cochromatography of ninhydrin-reactive authentic methylamine. Methylamine (R_f = 0.52) was converted to a less polar metabolite (R_f = 0.67). The purity of [^{14}C]methylamine hydrochloride (Amersham Corp., Arlington Heights, Ill.) was checked by dansylation (12) and thin-layer chromatography on silica gel plates developed with benzene-cyclohexane-methanol, 85:10:5 (vol/vol/vol). Two batches of this material were found to be radiochemically pure but significantly contaminated with ammonium. The extent of ammonium contamination was determined by enzymatic assay (15) to be 0.28 mol of ammonium per mol of methylamine. Since the uptake of ammonium by *A. vinelandii* is much more rapid than uptake of methylammonium (see Results), the ammonium contaminant in [^{14}C]-methylamine was removed by incubation with cells. After removal of cells, the [^{14}C]methylamine was radiochemically pure, but ammonium content was reduced to <0.006 mol/mol of methylamine.

Nigericin and monensin were gifts of Lilly Laboratories, Indianapolis, Ind. Valinomycin was purchased from Calbiochem, La Jolla, Calif. Gramicidin D, bromododecane, and dodecane were from Sigma Chemical Co., St. Louis, Mo. Pierce Chemical Co., Rockford, Ill., was the source of *p*-trifluoromethoxycarbonyl cyanide-phenyl hydrazone (FCCP). Methylamine hydrochloride, obtained from J. T. Baker Chemical Co., Phillipsburg, N.J., contained <0.1% ammonium and was used without further purification.

RESULTS

Cultures of *A. vinelandii* OP rapidly accumulate [^{14}C]methylamine when respiring in the presence of succinate (Fig. 1). Washed cells without energy source took up methylamine at <2% of the rate observed with incubations containing succinate. The amount of methylamine uptake is proportional with time for the first 5 or 10 min (Fig. 1) and with cell protein from 20 to 100 μg (not shown). Under the conditions of Fig. 1, the entry of methylamine was blocked essentially completely by KCN (2 mM), excess unlabeled methylamine (1 mM), or ammonium (0.2 mM), but these agents failed to produce exodus of previously accumulated [^{14}C]methylamine (not shown). Loss of methylamine during the filtration assay does not appear to be a complication, since duplicate experiments using rapid centrifugation of cells through oil (see Materials and Methods) agreed within 10%. A substantial portion of the methylamine taken up by *A. vinelandii* was converted to a less polar unidentified metabolite which accounted for 66% of the total radioactivity taken up in 1 min and for 91% after 5 min; the remaining cellular radioactivity was chemically unaltered methylamine.

The dependence of methylamine uptake on the external pH is illustrated in Fig. 2. Although the amount of accumulation increased nearly threefold between pH 5.5 and 7.5, little effect

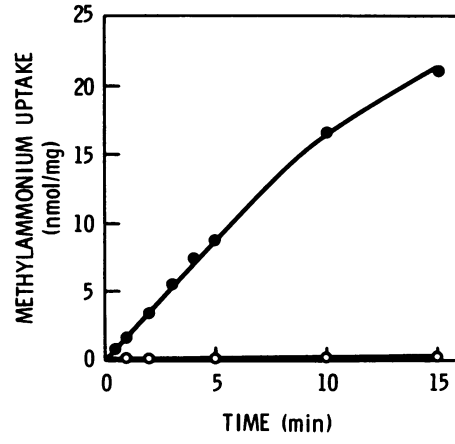

FIG. 1. *Effect of succinate on methylammonium uptake by A. vinelandii. Incubation mixtures (0.1 ml) contained 10 mM sodium phosphate (pH 7.4), 0.1 mM $MgCl_2$, 20 μM [^{14}C]methylammonium chloride (61 Ci/mol), 18 μg of cell protein, and 2 mM sodium succinate (where indicated). Reactions were terminated by dilution and filtration at the times shown, and radioactivity was counted as described in the text. Values for methylammonium uptake are expressed as nanomoles per milligram of cell protein. Symbols:* (○) *control;* (●) *2 mM succinate.*

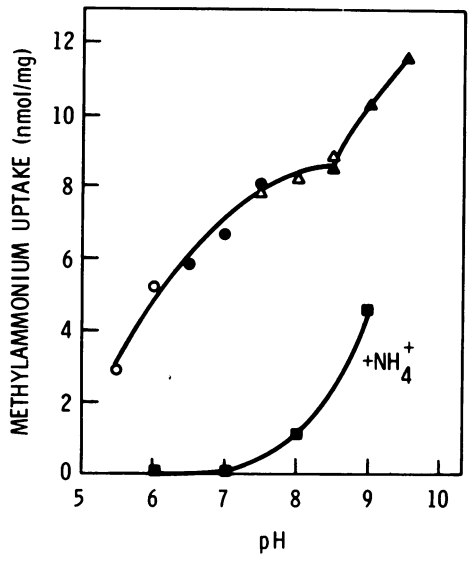

FIG. 2. *Effect of external pH on methylammonium uptake by A. vinelandii. Incubations were carried out as described in the legend to Fig. 1 except for addition of the indicated buffer (10 mM) adjusted to the pH shown with NaOH. Uptake was terminated at 10 min. Symbols:* (○) *2-(N-morpholino)ethanesulfonate (MES);* (●) *phosphate;* (△) *N-2-hydroxyethylpiperizine-N'-2-ethanesulfonate (HEPES);* (▲) *2-(cyclohexylamino)ethanesulfonate (CHES);* (■) *buffers as above with 50 μM NH_4Cl added.*

was noted between pH 7.5 and 8.5. At pH 9.0 to 9.5 a second uptake component was noted which was insensitive to inhibition by ammonium. This latter probably represents passive methylamine accumulation due to a transmembrane pH gradient (1). Since methylamine has a pK_a value of 10.6, methylammonium ion is the predominant species at pH 7.4 (99.9%). Although increasing the external pH from 7.0 to 8.5 would be expected to increase the ratio of methylamine to methylammonium by over 30-fold, the uptake of radioactivity increased by only 22%. This suggests that methylammonium rather than methylamine is the permeant species.

The initial rate of methylammonium uptake by *A. vinelandii* obeys the Michaelis-Menten relationship since linear monophasic Eadie-Hofstee plots (not shown) were obtained. This analysis gave a K_m value of 25 μM for methylammonium and a V_{max} of 3.8 nmol/min per mg of cell protein. Ammonium ion was a competitive inhibitor of methylammonium uptake and yielded a K_i value of 1.1 μM. Of the metal cations tested, Tl^+ was the most potent inhibitor and gave a K_i value of 13 μM, with the inhibition being of the mixed type. A nonspecific effect of Tl^+ is unlikely since $^{45}Ca^{2+}$ uptake measured under similar conditions was not affected by 100 μM Tl^+. At levels of 1 mM, Cs^+ and Rb^+ produced 44 and 35% inhibition, respectively, of methylammonium uptake; Na^+, K^+, or Li^+ failed to block significantly. Of the ammonium derivatives tested (Table 1), hydrazine and hydroxylamine were the most potent inhibitors of methylammonium uptake. Because of the reactivity of these substances, the inhibition kinetics were analyzed further (not shown). Hydrazine proved to be a competitive inhibitor (K_i = 2.3 μM), whereas hydroxylamine gave inhibition of the mixed type (K_i = 6.3 μM). Methylhydrazine was a less effective inhibitor of methylammonium accumulation, followed by formamidine and guanidine. Of the other analogs tested (not shown), dimethylamine and ethylamine gave 51 and 21% inhibition, respectively, at 1 mM concentrations; allylamine, propylamine, trimethylamine, and the higher homologs produced less than 20% inhibition under these conditions. Likewise, amides (acetamide and formamide) and amino acids (Gly, Ala, Arg, Asn, Asp, Glu, Gln) were not inhibitory.

A series of ionophores (Table 2) was tested for effect on uptake of methylammonium by *A. vinelandii*. The uncouplers FCCP and gramicidin D were very potent inhibitors of methylammonium accumulation. The presence of EDTA was shown to be required for the inhibition by gramicidin and was included in this series of experiments. EDTA produced no significant effect on control values. Valinomycin stimulated accumulation of methylammonium in the absence of added K^+ but blocked uptake dramatically in the presence of 10 mM external K^+. By contrast, monensin and nigericin were poor inhibitors. Agents known to block phosphorylation, 0.2 mM arsenate or 0.2 mM dicyclohexylcarbodiimide, had no significant effect on the accumulation of methylammonium (not shown).

The properties of ammonium uptake by cultures of *A. vinelandii* were also investigated

TABLE 1. *Nitrogenous inhibitors of methylammonium uptake by A. vinelandii*[a]

Inhibitor	I_{50} (μM)
Hydroxylamine	3
Hydrazine	6
Methylhydrazine	24
Formamidine	46
Guanidine	620

[a] Assays were carried out as described in the legend to Fig. 1, except that the compounds listed were added. Uptake was terminated after 5 min. The concentration of inhibitor necessary to produce a 50% inhibition of methylamine uptake (I_{50}) was determined from plots of uptake versus log of the inhibitor concentration.

TABLE 2. *Effect of ionophores on methylammonium uptake by A. vinelandii*[a]

Addition	Concn (M)	Methylammonium uptake (%)
None		100
FCCP	1×10^{-7}	1.3
Gramicidin D	1×10^{-6}	4.7
Valinomycin	1×10^{-7}	130
Valinomycin + KCl	1×10^{-7} 1×10^{-2}	13
Monensin	1×10^{-7}	70
Nigericin + KCl	1×10^{-7} 1×10^{-2}	83
KCl	1×10^{-2}	85

[a] Incubations were carried out as in the legend to Fig. 1 except that 1 mM EDTA (sodium salt) and the compounds listed were added; uptake was terminated after 5 min. Uptake values are expressed as a percentage of controls without inhibitor. Ionophores were added as aliquots of ethanol or dimethyl sulfoxide solutions. Solvent concentrations did not exceed 1% in the assay and had no effect on methylammonium uptake.

briefly. Ammonium, present at an initial concentration of 0.1 mM, was removed from the medium by respiring cells at a rate of 49 nmol/min per mg of cell protein. Methylammonium was taken up at only 4% of this rate under comparable conditions. Like methylammonium uptake, the removal of ammonium from the medium required succinate and was inhibited by 0.1 μM FCCP but not by 1 mM K^+ (not shown). The inhibition of ammonium uptake by 1 mM methylammonium (20%) was in the range predicted (28%) by the Michaelis-Menten equation for competitive inhibition. For this calculation it was assumed that the K_m for ammonium transport is identical to the K_i (1 μM) for ammonium inhibition of methylammonium uptake and, furthermore, that the K_i for methylammonium inhibition is the K_m for methylammonium uptake (25 μM).

DISCUSSION

The studies described demonstrate that methylammonium is a useful substrate for investigation of ammonium transport in *A. vinelandii*. The uptake of ammonium and methylammonium is energy dependent, and both substrates mutually compete for translocation. Ammonium has greater apparent affinity for the transporter than methylammonium, as was previously found with a fungal system (5). These two substrates were transported at similar rates in fungi, whereas we found ammonium entry to be much more rapid than methylammonium entry in *A. vinelandii*. There are precedents for large differences in V_{max} values for a single transporter, e.g., an eightfold difference for lactose and thiodigalactoside uptake rates by the β-galactoside transport system of *Escherichia coli* (6).

The use of methylammonium as a substrate in *A. vinelandii* is complicated by its conversion to a less polar unknown metabolite. Identification of this compound is in progress. The most significant question raised by this observation is whether transport or metabolism is rate limiting for uptake of [^{14}C]methylammonium by the cells. The suggestion that translocation is rate limiting rests on four lines of evidence. (i) The uptake of methylammonium is strongly dependent on external pH (Fig. 2), with the ammonium-sensitive component of uptake being optimal at neutral pH. Such a dependence on external pH would not be expected if metabolism was rate limiting. (ii) Potent inhibition by Tl^+ of methylammonium uptake is consistent with an effect on an ion transporter but less likely for a metabolic conversion. (iii) The effects of valinomycin on methylammonium uptake are more consistent with a membrane transport process (see below). (iv) After 1 min, methylamine constitutes 34% of the intracellular radioactivity; this declines to <5% after 10 min. This behavior suggests that the onset of metabolism is delayed. Since the initial rate of uptake is the same as the rate after 5 or 10 min (Fig. 1), it is unlikely that metabolism controls the overall transport rate within this time span.

The effect of ammonium derivatives and metal cations on methylammonium uptake suggests that the transport system is quite specific for ammonium. Ammonium had the lowest inhibition constant (1 μM) of any compound tested; the next most potent competitive inhibitor was hydrazine, a closely related derivative. The order of effectiveness for metal cations as inhibitors of methylammonium uptake was: $Tl^+ > Cs^+ > Rb^+ > Na^+, K^+, Li^+$. This seems to rule out significant ammonium transport via a potassium transporter, as proposed by Laane et al. (8). Furthermore, the substrate selectivity of the ammonium transporter cannot be strictly based on ionic radius because of the discrimination between ions of similar radii (e.g., NH_4^+ versus Tl^+ versus Rb^+ and dimethylammonium versus ethylammonium). The selectivity in favor of pseudoalkalis (NH_4^+ and Tl^+) with an asymmetrical charge distribution may, according to the principles of Eisenman and Krasne (4), suggest a tetrahedral arrangement of liganding groups in the binding site of the transporter.

The effect of ionophores (Table 1) and other inhibitors on methylammonium uptake by *A.*

vinelandii indicates that the mechanism of energy coupling involves facilitated movement of methylammonium down the electrical gradient. The uncouplers FCCP and gramicidin D were potent inhibitors of methylammonium uptake but agents which block phosphorylation, dicyclohexylcarbodiimide and arsenate, were ineffective. Valinomycin stimulated methylammonium uptake in the absence of added K^+ but blocked uptake in the presence of K^+. This is consistent with a hyperpolarizing effect of K^+ exodus and a depolarizing effect of K^+ entry. Furthermore, monensin and nigericin, which perturb proton gradients, had little effect on methylammonium accumulation. In addition, the shape of the pH profile (Fig. 2) indicates that methylammonium rather than uncharged methylamine is the permeant species. Under the conditions reported here, *A. vinelandii* cells develop a transmembrane electrical potential of 170 to 180 mV negative-inside (measured by the accumulation of [^{3}H]tetraphenylphosphonium), indicating that a substantial driving force is available for electrogenic movement of ammonium. But in contrast to the findings of Laane et al. (8), no evidence for a depolarization by methylammonium or ammonium was found (E. M. Barnes, Jr., unpublished data). We are currently investigating possible compensatory movements of other ions which could account for these differences.

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LITERATURE CITED

1. **Barnes, E. M., Jr.** 1980. Proton-coupled calcium transport by intact cells of *Azotobacter vinelandii*. J. Bacteriol. **143**:1086–1089.
2. **Bellion, E., M. Y. A. Khan, and M. J. Romano.** 1980. Transport of methylamine by *Pseudomonas* sp. MA. J. Bacteriol. **142**:786–790.
3. **Cornell, N. W.** 1980. Rapid fractionation of cell suspensions with the use of brominated hydrocarbons. Anal. Biochem. **102**:326–331.
4. **Eisenman, G., and S. J. Krasne.** 1975. The ion selectivity of carrier molecules, membranes, and enzymes, p. 27–59. *In* C. F. Fox (ed.), MTP international review of science. Biochemistry series, Vol. 2. Butterworths, London.
5. **Hackette, S. L., G. E. Syke, C. Burton, and I. H. Segel.** 1970. Characterization of an ammonium transport system in filamentous fungi with methylammonium-^{14}C as the substrate. J. Biol. Chem. **245**:4241–4250.
6. **Kepes, A., and G. N. Cohen.** 1962. Permeation, p. 179–221. *In* I. C. Gunsalis and R. Y. Stanier (ed.), The bacteria, vol. 4. Academic Press, Inc., New York.
7. **Kleiner, D.** 1975. Ammonium uptake by nitrogen fixing bacteria. I. *Azotobacter vinelandii*. Arch. Microbiol. **104**:163–169.
8. **Laane, C., W. Krone, W. Konings, H. Haaker, and C. Veeger.** 1980. Short-term effect of ammonium chloride on nitrogen fixation by *Azotobacter vinelandii* and by bacteroids of *Rhizobium leguminosarum*. Eur. J. Biochem. **103**:39–46.
9. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
10. **Pateman, J. A., E. Dunn, J. R. Kinghorn, and E. C. Forbes.** 1974. The transport of ammonium and methylammonium in wild type and mutant cells of *Aspergillus nidulans*. Mol. Gen. Genet. **133**:225–236.
11. **Roon, R. J., H. L. Even, P. Dunlop, and F. Larimore.** 1975. Methylamine and ammonia tranport in *Saccharomyces cerevisiae*. J. Bacteriol. **122**:502–509.
12. **Seiler, N., and M. Wiechmann.** 1965. Zum Nachweis von Aminen im 10^{-10} Mol-Maßstab. Trennung von 1-Dimethylaminonapthalin-5-sulfonsaureamiden auf Dunnschichtchromatogrammen. Experientia **21**:203–204.
13. **Stevenson, R., and S. Silver.** 1977. Methylammonium uptake by *Escherichia coli*: evidence for a bacterial NH^+ transport system. Biochem. Biophys. Res. Commun. **75**:1133–1139.
14. **Strandberg, G. W., and P. W. Wilson.** 1968. Formation of the nitrogen-fixing enzyme system in *Azotobacter vinelandii*. Can. J. Microbiol. **14**:25–31.
15. **Tabor, C. W.** 1970. The determination of NH_3 with the use of glutamic dehydrogenase. Methods Enzymol. **17A**:955.