METHYLAMMONIUM UPTAKE BY ESCHERICHIA COLI: EVIDENCE FOR A BACTERIAL NH_4^+ TRANSPORT SYSTEM

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Summary- Energy-dependent concentrative uptake of $^{14}CH_3NH_3^+$ by cells of Escherichia coli provides preliminary evidence for one or more transport systems for NH_4^+ uptake. NH_4^+, but not glutamic acid, inhibited the uptake of $^{14}CH_3NH_3^+$. Varying the pH for the uptake assays exposed two apparent systems: one maximally functioning at pH 7 that was strongly inhibited by cyanide or by the uncoupler m-chlorophenyl carbonylcyanide hydrazone and another maximally functioning at pH 9 and resistant to cyanide or m-chlorophenyl carbonylcyanide hydrazone. Kinetic analysis showed considerable experimental variability from day to day. Often simple Michaelis-Menten kinetics were not followed, but NH_4^+ was reproducibly a stronger inhibitor of uptake of $^{14}CH_3NH_3^+$ than was nonradioactive $CH_3NH_3^+$.

There are no reports of ammonium transport systems in bacteria, partially because of the lack of a suitable radioactive isotope of N and partially because of the common assumption that NH_3 passively diffuses across the cellular membranes. However, with a pK_a of 9.25 more than 99% would be present as NH_4^+ and not as NH_3 under physiological growth conditions. Considering the selective advantages of a specific NH_4^+ transport system under conditions of nitrogen-limited growth, it seemed reasonable to expect that such systems would have evolved. We undertook to characterize the putative NH_4^+ transport system in Escherichia coli using the available radioactive analogue for NH_4^+ methylammonium ion ($^{14}CH_3NH_3^+$) that (with a pK_a of 10.7) would also be almost exclusively ionized at normal pHs. $^{14}CH_3NH_3^+$ has been previously used to characterize ammonium transport systems with eukaryotic microbes (1-3).

Abbreviation: CCCP, m-chlorophenyl carbonylcyanide hydrazone.

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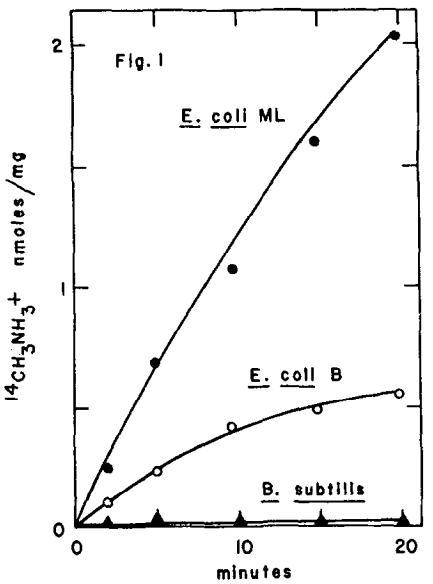
MATERIALS AND METHODS

E. coli strain ML308-225 was used in most studies. Comparable results in many cases were obtained with E. coli strains K-12 or B. The bacteria were grown in M9 minimal medium (4) with 0.5% glucose as carbon source and with the usual NH_4Cl replaced with either 2 or 20 mM glutamate or NH_4Cl as indicated. Log phase cells or cells after nitrogen-limited growth were centrifuged, often washed once with N-free M9 and finally resuspended for transport assays in either N-free M9 medium with 0.4% glucose or in 50 mM Tris-HCl buffer plus 72 mM NaCl and 0.2% glucose. Uptake assays at 37°C used $^{14}CH_3NH_3^+$ from ICN Pharmaceuticals (specific activity 4.2 mCi/mmole). For studies requiring high substrate concentrations, the specific activity was lowered with nonradioactive CH_3NH_3Cl from Sigma Chemical Co. Conventional Millipore filtration was followed by washing the cells on the filter twice with 37°C non-radioactive suspension medium. Results are presented as nmoles accumulated per mg dry weight cells. Between 0.25 to 0.45 mg dry weight cells per ml was used and the precise amount of cells was calculated from a standard curve of turbidity vs. dry weight.

RESULTS

E. coli cells grown with glutamate as N-source accumulate $^{14}CH_3NH_3^+$ as a function of time (Fig. 1) generally to levels approaching 10% of that added--with low external concentrations. This represents about a 100-fold concentration gradient with 0.25 mg cells per ml and assuming four-times as much cell water as dry weight. E. coli strains ML308-225,B (Fig. 1) and K-12 (data not shown) took up approximately similar amounts of $^{14}CH_3NH_3^+$, whereas Bacillus subtilis strain W23 did not accumulate $CH_3NH_3^+$ under tested conditions (Fig. 1). Similar levels of uptake were obtained in M9 medium with 2 mM glutamate or with no N source. However, 2 mM NH_4^+ completely inhibited $CH_3NH_3^+$ uptake.

Because of the central question of whether uptake was of the protonated or unprotonated form of methylammonia, the effect of pH on uptake was measured



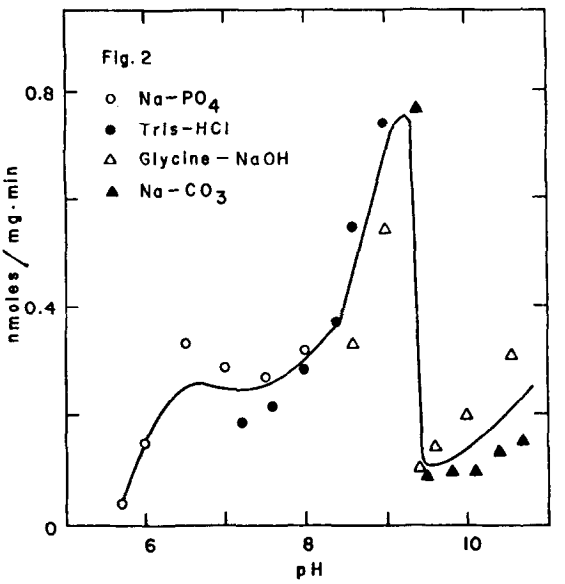
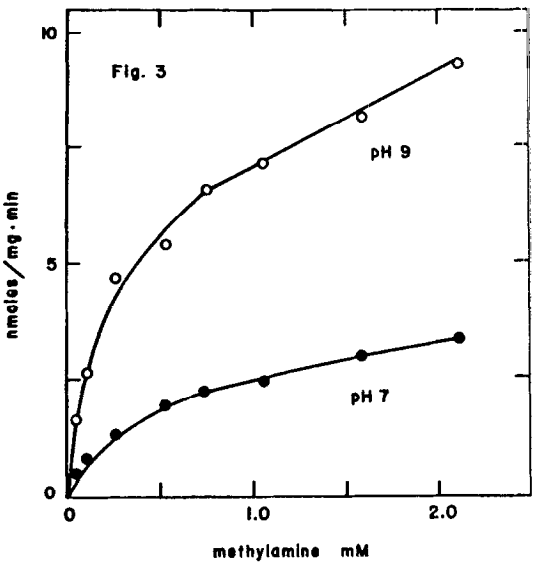


Fig. 1 (left). Accumulation of $^{14}CH_3NH_3^+$. E. coli cells were grown, washed and suspended in M9 medium with 2 mM glutamate. B. subtilis W23 was grown, washed and suspended in a similar medium containing citrate. 6 μM $^{14}CH_3NH_3^+$ was added and 0.5 ml samples filtered and washed.

Fig. 2 (right). Effect of pH on $CH_3NH_3^+$ uptake. E. coli ML308-225 was grown into log phase in M9 medium with 2 mM glutamate, washed and suspended. The cells were diluted 4-fold into 0.15 M (final concentration) phosphate, Tris, glycine or bicarbonate buffer containing 72 mM NaCl and 0.2% glucose. After 10 min incubation at 37°C with 6 μM $^{14}CH_3NH_3^+$, samples were filtered, washed and counted.

(Fig. 2). The results of many such experiments indicated the existence of two systems for $^{14}CH_3NH_3^+$ uptake: first a shoulder of activity near pH 7 and then a peak with 3-4 times the net rate as the pH 7 system and with a pH optimum around pH 9.0--1.7 units below the 10.7 pK_a for methylammonium.

Attempts to determine the kinetic parameters of $^{14}CH_3NH_3^+$ uptake have yielded variable results, the reasons for which are not clear. Initial uptake rates both at pH 7 and at pH 9 showed some evidence of saturation (Fig. 3), but were not fully saturated even at 2 mM $CH_3NH_3^+$. Reciprocal coordinate Lineweaver-Burk plots of data such as in Fig. 3 show signs of a high affinity system with a K_m varying from experiment to experiment from 40 μM to 200 μM



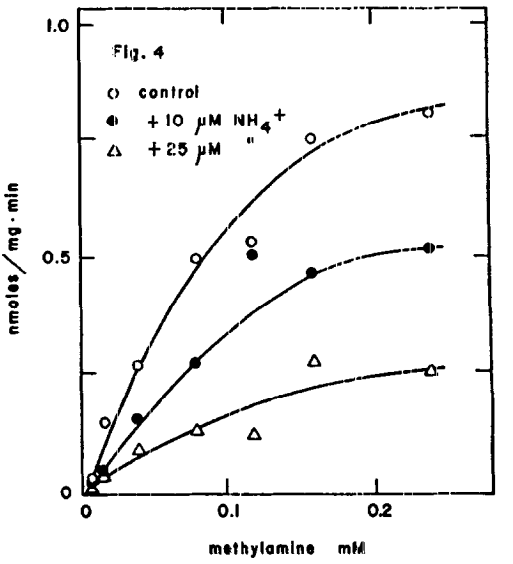
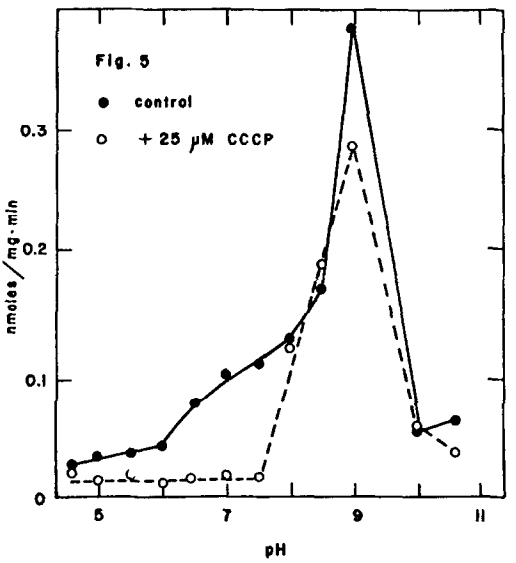


Fig. 3 (left). Concentration dependence of $CH_3NH_3^+$ uptake: 5 min uptake at 37°C in 50 mM Tris-HCl (pH 7 or pH 9) containing 72 mM NaCl plus 0.2% glucose.

Fig. 4 (right). Inhibition of $CH_3NH_3^+$ uptake by NH_4^+. ML308-225 grown (with 2 mM glutamate), washed and suspended in M9 medium with no added N source, were exposed for 5 min at 37°C to $^{14}CH_3NH_3^+$ with or without added NH_4^+.

$CH_3NH_3^+$ plus another system with a K_m of 1 mM $CH_3NH_3^+$ or more. However, the apparent half-saturation constants were the same at both pH's and there was no suggestion that the pH 7 system has a higher or lower affinity for substrate than does the pH 9 system (Fig. 3 and additional data). With the lack of simple kinetics, it is difficult to interpret the results of inhibition of $CH_3NH_3^+$ uptake by NH_4^+ (Fig. 4), which we have measured repeatedly. NH_4^+ was a strong inhibitor of $^{14}CH_3NH_3^+$ uptake. 10 μM NH_4^+ inhibited the uptake of $^{14}CH_3NH_3^+$ by about 50%. Concentrations of NH_4^+ above 10 μM caused greater inhibition of uptake (Fig. 4). In other experiments, however, inhibition did not increase with added NH_4^+ between 10 and 100 μM. Both types of inhibition response were obtained at pH 7 and at pH 9 in Tris buffer, as well as in the PO_4^{3-} -buffered M9 medium (Fig. 4). This lack of reproducibility of results and the failure of the results of single experiments to fit simple kinetic



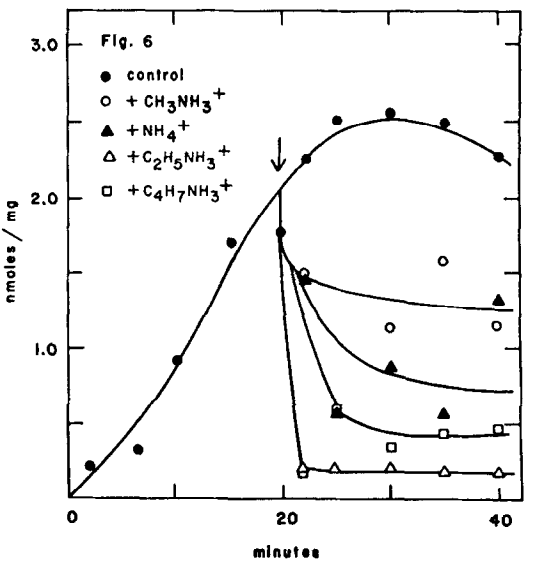


Fig. 5 (left). Effect of the uncoupler CCCP on $^{14}CH_3NH_3^+$ uptake by glutamate-grown E. coli ML308-225 in Tris-NaCl-glucose at different pH's. Uptake assays were run for 10 min at 37°C with 6 μM $^{14}CH_3NH_3^+$ plus or minus 25 μM CCCP.

Fig. 6 (right). Release of acumulated $^{14}CH_3NH_3^+$. Strain ML308-225 accumulated $^{14}CH_3NH_3^+$ (32 μM) for 20 min at 37°C in M9 medium without N. At that time (arrow) 100 mM NH_4^+ or alkylamines were added to different aliquots.

models precludes any conclusion at this time except that NH_4^+ was generally a stronger inhibitor of uptake of $^{14}CH_3NH_3^+$ than was a comparable level of nonradioactive $CH_3NH_3^+$.

The pH 7 system was very sensitive to CCCP, while the pH 9 system was essentially resistant to CCCP (Fig. 5) even up to levels of 100 μM CCCP (data not shown). The pH 7 activity was also completely sensitive to 10 mM KCN, which only inhibited the pH 9 activity by perhaps 50%. 10 mM KCN plus 50 μM CCCP together inhibited pH 9 activity by 80% and activity at pH 9.5 completely (data not shown). These results provide preliminary evidence that energy-dependent uptake may be involved at both pH 7 and pH 9 but that the energy-coupling mechanism may differ in the two cases. Analogous differences in energy coupling with more than one transport system has been found for both phosphate transport (5-7) and potassium transport (5, 8), as well as for many

systems for transport of organic nutrients. $^{14}CH_3NH_3^+$ uptake at both pH's was temperature dependent, but the pH 9 uptake showed a greater "Q_{10}" of 2.4 corresponding to an Arrhenius plot slope of 14 kcal per mole. The pH 7 uptake was less temperature dependent (Q10 of 1.5; data not shown).

The $CH_3NH_3^+$ accumulated by E. coli cells was not incorporated into proteins and $CH_3NH_3^+$ would not serve as N source for growth. All of the accumulated $CH_3NH_3^+$ was removed by treatment with ice-cold 5% trichloroacetic acid (data not shown). Chloramphenicol, the inhibitor of protein synthesis, did not inhibit $^{14}CH_3NH_3^+$ uptake when added at 20 μg/ml. Furthermore, the accumulated $^{14}CH_3NH_3^+$ could be chased from the cells by addition of high levels of NH_4^+, or nonradioactive alkylamines (Fig. 6). We do not understand why the relative efficacy in this chase experiment appeared to be $C_2H_5NH_3^+ > C_4H_7NH_3^+ > NH_4^+ > CH_3NH_3^+$, which is not related to the chain length of the alkyl groups. The 100 mM concentration of "chasing" (alkyl)ammonium added in this experiment was 3,000 the concentration of radioactive methylammonium. 100 mM $CH_3NH_3^+$ was not inhibitory for E. coli growing in M9 medium with either glutamate or NH_4^+ as N source (data not shown).

DISCUSSION

The results presented in this note provide the first preliminary report for an NH_4^+ transport process in bacterial cells, as far as we are aware. Using the ammonium analogue $^{14}CH_3NH_3^+$, we readily demonstrated energy-dependent concentrative uptake of methylammonium in the apparent absence of subsequent metabolism of this material. Extensive kinetic analysis suggested two systems with differing pH optima and energy-coupling. However, complex kinetics and lack of conditions for studying the systems one at a time limited the conclusions that could be drawn as to NH_4^+ as an alternative substrate for $^{14}CH_3NH_3^+$ uptake process.

Although such experiments are new for bacterial cells, extensive studies of $^{14}CH_3NH_3^+$ transport have been reported with several eukaryotic microbes. In three diverse species, Penicillium chrysogenum, Aspergillus nidulans and

Saccharomyces cerevisiae, energy dependent $^{14}CH_3NH_3^+$ transport showing simple Michaelis-Menten kinetics was found (1-3). The $CH_3NH_3^+$ transport system in P. chrysogenum and A. nidulans was repressible by growth in rich nitrogen sources (1,2) whereas those of S. cerevisiae (3) and E. coli (data not shown) appeared largely constitutive. Methylammonium-resistant mutants altered in NH_4^+ transport were isolated with A. nidulans (2) and S. cerevisiae (3), but this does not appear practical in our case, since E. coli is resistant to 100 mM $CH_3NH_3^+$. The properties of these fungal NH_4^+ transport systems and the predicted properties of trace-level NH_4^+ scavenging systems in bacteria were recently discussed in a broader context of inorganic nutrient transport in bacteria (5).

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