Bacterial ammonium transport

(Futile cycle; cyclic retention; permeability coefficient for \( \text{NH}_3 \); nitrogen control; ammonium gradients; electrogenic \( \text{NH}_4^+ \) uniport)

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1. INTRODUCTION

With few exceptions (e.g., extracellular degradation of macromolecules) the metabolism of a compound starts with its transport across the cell membrane, mediated in most cases by specific transport proteins or carriers. As the starting point of a metabolic pathway, uptake is frequently strictly regulated, both on the activity and on the genetic level. Regulation of transport is almost impossible if a compound crosses the membrane by unspecific diffusion, because regulation of permeability—if occurring at all in biological systems—is only possible by biologically uncontrollable parameters (osmotic strength, temperature) and by a lengthy degradation and new synthesis of membrane lipids.

With respect to bacterial membranes, ample evidence supports the notion that most ions and 'large' polar molecules (\( M_r \) approx. 100 or more) are transported by specific carriers, while lipophilic and small, uncharged compounds (e.g., \( \text{CO}_2 \), \( \text{H}_2\text{O} \), \( \text{CH}_4 \), \( \text{H}_2 \), \( \text{O}_2 \), \( \text{N}_2 \), \( \text{NH}_3 \)) rapidly pass membranes by unspecific diffusion. Of course, dependent on their solubility in the membrane, some diffusion of otherwise specifically transported molecules must also occur, but in general this is very slow in comparison to specific transport and can be considered as biologically insignificant.

Several bacterial nutrients or metabolites with a low \( M_r \) are weak acids and bases, and thus occur in both a charged and uncharged form, e.g.:

- \( \text{CH}_3\text{COO}^- + \text{H}^+ \rightarrow \text{CH}_3\text{COOH} \; \text{pK}_a = 4.75 \)
- \( \text{NH}_3 + \text{H}^+ \rightarrow \text{NH}_4^+ \; \text{pK}_a = 9.25 \)
- \( \text{HCO}_3^- + \text{H}^+ \rightarrow \text{H}_2\text{O} + \text{CO}_2 \; \text{pK}_a = 6.37 \) (1)

According to the differences in permeability for the charged and uncharged species, unequal distribution of the non-permeating ions on both sides (1 and 2) of a membrane is observed as a consequence of a pH difference (\( \Delta \text{pH} \)):

\[
K = \frac{[X][\text{H}^+]}{[X \text{H}^+][\text{H}^+]} = \frac{[X][\text{H}^+]}{[X \text{H}^+]^2}
\]

The concentration of the diffusible species is the same on both sides. This distribution is widely used as a probe for determining \( \Delta \text{pH} \) ([1,2] and references therein).

Since these molecules in their uncharged form traverse membranes very rapidly, no necessity for specific systems transporting the ionic form seems to exist. This opinion for a long time hampered the search for such systems, and even the description of numerous \( \text{NH}_4^+ \) carriers in the last years is still often greeted with scepticism or neglected.

In this review I wish to summarize reports on different aspects of bacterial \( \text{NH}_4^+ \) transport sys-
tems using the criteria outlined previously [3]. Furthermore, I shall hypothesize on the function of these carriers by proposing a futile cycle of cyclic (NH$_3$/NH$_4^+$) retention. The hypothesis can be summarized in the statement that bacteria need NH$_4^+$ carriers for good growth under nitrogen limiting conditions just because their membranes are very permeable to NH$_3$.

This statement contradicts some of my previous opinions on the permeability of bacterial membranes to NH$_3$ [3,4].

2. ASSAYS FOR NH$_4^+$ CARRIERS

2.1. NH$_4^+$ accumulation

Specific active transport is generally assayed by determining kinetic parameters of solute accumulation in cells or vesicles. The determination of intracellular pools of metabolites, which, like NH$_4^+$, are subject to a rapid turnover, requires fast extraction methods and the avoidance of pool enlargement by decomposition of labile compounds (e.g., glutamine, carbamoyl phosphate). Since these precautions may not always have been met in the past, the reported values for the intracellular NH$_4^+$ levels show some variation (Table 1). Also, the growth conditions, in particular the amount and type of N source, probably influence the size of the NH$_4^+$ pool.

Apart from only two reports, the data in Table 1 indicate the existence of NH$_4^+$ concentration gradients across the membranes of many prokaryotes, against which NH$_4^+$ transport has frequently been observed. If permeation occurred exclusively by unspecific diffusion of NH$_3$, the direction of $\Delta$ pH across bacterial membranes...
(generally slightly alkaline on the inside [2,5]), according to Eqn. (2), should promote depletion rather than accumulation of NH$_4^+$ . From gradient formation and uphill transport, the involvement of energy-dependent uptake is therefore an inevitable conclusion.

2.2. Uptake of [$^{14}$C]methylammonium

A convenient method allowing determination of relatively fast transport kinetics is the accumulation of a radioactive substrate. Since no suitable nitrogen isotope is commercially available, many investigators have used [$^{14}$C]methylammonium ([$^{14}$C-MA) as NH$_4^+$ analog. This method was introduced in 1970 by I.H. Segel's group [18], and has been successfully applied to lower eukaryotes and later to prokaryotes ([3] and references therein). NH$_4^+$ as the natural substrate is inferred from strong competitive inhibition of 14C-MA uptake by NH$_4^+$ . The $K_i$ of NH$_4^+$ can be assumed to be equal to the $K_m$ for NH$_4^+$ uptake [18]. The accumulated $^{14}$C-MA remains only partially unmetabolized in the cell. Varying fractions are converted to $\gamma$-N-methyl glutamine [19-23]. Pulse-chase experiments or chromatographic separation of the labelled compounds enables a rapid estimation of the size of the intracellular pool of free $^{14}$C-MA.

Two pitfalls, however, have to be avoided:

(a) The carrier may be a specific MA transport system. Several methylotrophic bacteria derepress a specific carrier for MA as C-source [24-26]. Because of the low C/N ratio, MA assimilation is always accompanied by excretion of NH$_4^+$ , which does not inhibit the MA carrier. Depending on the growth conditions, Paracoccus denitrificans has recently been shown to synthesize 2 carriers mediating $^{14}$C-MA uptake [27]: a NH$_4^+$ carrier, whose MA-uptake is strongly inhibited by NH$_4^+$ but weakly by ethylamine; and a MA carrier, whose MA uptake is strongly inhibited by ethylamine, but not by NH$_4^+$. The NH$_4^+$ carrier is under nitrogen control (i.e., repressed by NH$_4^+$, see below), the MA carrier under carbon control (repressed by glucose) [27]. These inhibition and repression patterns may serve as convenient criteria for the discrimination between both transport systems. The situation, however, is additionally complicated by the discovery of carriers catalyzing uptake of MA as N source [28,29], which are only weakly inhibited by NH$_4^+$, and which also seem to be under nitrogen control [29].

(b) The NH$_4^+$ carrier may not accept MA as substrate. Recently, Gibson and coworkers provided evidence for the occurrence of 2 types of NH$_4^+$ carriers in Anacystis nidulans [30]. Only one of them also transports $^{14}$C-MA. Both carriers are distinguished by their repression patterns, Na$^+$ requirement, and inhibition by KCl. Therefore, failure to detect concentrative $^{14}$C-MA uptake by an organism does not completely preclude the existence of an NH$_4^+$ carrier.

3. DISTRIBUTION OF NH$_4^+$ CARRIERS

Since the first review on NH$_4^+$ transport appeared [3], the number of bacterial NH$_4^+$ transport systems discovered has increased from 4 to over 30 (Table 2). Many more species can be assumed to contain these carriers under certain conditions. The occurrence of NH$_4^+$ transport systems does not seem to be restricted to certain groups, since they are found in many strains with different physiologies.

The affinity of the carriers for NH$_4^+$ is rather high, with $K_m$ values ranging around $10^{-5}$ M. The $K_m$ values for the following enzyme, glutamine synthetase, generally exceeds $10^{-4}$ M [31]. Most NH$_4^+$ carriers have been detected by $^{14}$C-MA uptake. With few exceptions the MA gradient across a certain membrane (Table 2) is about the same as the NH$_4^+$ gradient (Table 1), up to about several hundred-fold. Interestingly, A. nidulans has been reported to build up more than 3000-fold NH$_4^+$ gradients [17], but only up to 200-fold MA gradients [30] under similar experimental conditions. An explanation may be the existence of the second NH$_4^+$ carrier in this organism, already mentioned above, which does not transport MA [30]. Since the size of the steady-state NH$_4^+$ pool depends on the ratio of NH$_4^+$ influx over NH$_3$ efflux plus metabolism, an additional NH$_4^+$ import should increase the NH$_4^+$ pool. Alternatively, even in the absence of a second carrier, this ratio
Table 2
Distribution and some properties of bacterial NH$_4^+$ carriers

<table>
<thead>
<tr>
<th>Strain</th>
<th>Investigation method (M)</th>
<th>$K_m$ ($\mu$M) CH$_3$NH$_2$</th>
<th>$K_m$ ($\mu$M) NH$_4^+$</th>
<th>Repression by NH$_4^+$</th>
<th>CH$_3$NH$_4^+$ gradient</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>M</td>
<td>40–200</td>
<td>–</td>
<td>no</td>
<td>100</td>
<td>[33]</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>yes</td>
<td>–</td>
<td>[34]</td>
</tr>
<tr>
<td><em>Nitrosococcus oceanus</em></td>
<td>M</td>
<td>140</td>
<td>7</td>
<td>yes</td>
<td>80</td>
<td>[35]</td>
</tr>
<tr>
<td><em>Paracoccus denitrificans</em></td>
<td>M</td>
<td>70</td>
<td>&lt;50</td>
<td>yes</td>
<td>30</td>
<td>[27]</td>
</tr>
<tr>
<td><em>Azotobacter vinelandii</em></td>
<td>M</td>
<td>25</td>
<td>1</td>
<td>partial</td>
<td>–</td>
<td>[37]</td>
</tr>
<tr>
<td><em>Azorhizophilus (Azotobacter) pspali</em></td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>[38]</td>
</tr>
<tr>
<td><em>Azomonas agilis</em></td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>[38]</td>
</tr>
<tr>
<td><em>Beijerinckia mobilis</em></td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>[38]</td>
</tr>
<tr>
<td><em>Dexia gummosa</em></td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>[38]</td>
</tr>
<tr>
<td><em>Alcaligenes latus</em></td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>[38]</td>
</tr>
<tr>
<td><em>Alcaligenes eutrophus</em></td>
<td>M</td>
<td>50–100</td>
<td>&lt;20</td>
<td>yes</td>
<td>200</td>
<td>[39]</td>
</tr>
<tr>
<td><em>Xanthobacter autotrophicus</em></td>
<td>M</td>
<td>120</td>
<td>5–10</td>
<td>yes</td>
<td>200</td>
<td>[40]</td>
</tr>
<tr>
<td><em>Azospirillum brasilense</em></td>
<td>M</td>
<td>100</td>
<td>7</td>
<td>yes</td>
<td>150</td>
<td>[41]</td>
</tr>
<tr>
<td><em>Azospirillum lipoforum</em></td>
<td>M</td>
<td>–</td>
<td>–</td>
<td>yes</td>
<td>12</td>
<td>[44]</td>
</tr>
<tr>
<td><em>Rhizobium sp. 32H1</em></td>
<td>M</td>
<td>113</td>
<td>16</td>
<td>yes</td>
<td>75</td>
<td>[44]</td>
</tr>
<tr>
<td><em>Rhizobium meliloti</em></td>
<td>M</td>
<td>2</td>
<td>2</td>
<td>yes</td>
<td>180</td>
<td>[42]</td>
</tr>
<tr>
<td><em>Rhodospirillum rubrum</em></td>
<td>M</td>
<td>100</td>
<td>7</td>
<td>yes</td>
<td>150</td>
<td>[43]</td>
</tr>
<tr>
<td><em>Rhodopseudomonas sphaeroides</em></td>
<td>M</td>
<td>113</td>
<td>16</td>
<td>yes</td>
<td>75</td>
<td>[44]</td>
</tr>
<tr>
<td><em>Rhodopseudomonas palustris</em></td>
<td>M</td>
<td>130</td>
<td>18</td>
<td>yes</td>
<td>18</td>
<td>[44]</td>
</tr>
<tr>
<td><em>Rhodopseudomonas capsulata</em></td>
<td>M</td>
<td>4</td>
<td>4</td>
<td>yes</td>
<td>22–50</td>
<td>[44]</td>
</tr>
<tr>
<td><em>Rhodomicrobium vannielli</em></td>
<td>M</td>
<td>65</td>
<td>10</td>
<td>yes</td>
<td>7</td>
<td>[44]</td>
</tr>
</tbody>
</table>

* A, ammonium uptake; M methylammonium uptake.

might be higher for NH$_4^+$ than for MA, if the natural substrate (NH$_4^+$) is transported faster than the analog (MA), as shown for some strains [9,11,50]. As will be discussed in section 6.3., the back diffusion rate of both molecules is about equal.

Similar observations have been made for transport of K$^+$ and Rb$^+$ through the K$^+$ specific Trk
A transport system in *E. coli*. Under certain experimental conditions, the highest obtainable Rb⁺ gradient was considerably lower than the K⁺ gradient. This can be explained by a slower Rb⁺ import, the leakage rate being the same for both ions [32].

### 4. ENERGY COUPLING

Apart from gradient formation, energy-dependent NH₂⁺ transport has been inferred from:

(a) dependence on an energy source; (b) inhibition by inhibitors of energy metabolism; (c) inhibition by compounds which decrease the proton motive force (p.m.f.).

Most investigators dealing with the energetics of NH₂⁺ transport propose the membrane potential Δψ as the driving force [19,30,35,37,49,45,52], which implicates that NH₂⁺ and not NH₃ is the transported species.

Arguments which favor a component of the p.m.f. are:

(d) Under anaerobic conditions, when the p.m.f. is exclusively generated by the H⁺ translocating ATPase in *C. pasteurianum* and *K. pneumoniae*, inhibition of this enzyme also eliminates NH₂⁺ transport [19,35]; while no effect was observed under aerobic conditions, where the p.m.f. is generated by respiration in *K. pneumoniae* [35].

(e) Spheroplasts of *C. pasteurianum* [19] and *A. nidulans* [30], or osmotically shocked cells of *E. coli* and *K. pneumoniae* [51] are still active in ¹⁴C-MA uptake indicating lack of requirement for binding proteins. Binding proteins are characteristic for ATP-driven carriers [2]. A specific requirement for Δψ was inferred from the following observations:

(f) The generation of an artificial Δψ drives uphill ¹⁴C-MA transport in *C. pasteurianum* [19].

(g) Addition of NH₂⁺ to whole cells partially depolarizes the membrane of *A. vinelandii* [52].

(h) Addition of lipophilic phosphonium ions selectively decreases both Δψ and ¹⁴C-MA transport in *A. cylindrica* [49] and *A. azollae* [49].

(i) Breakdown of Δψ by valinomycin induced K⁺ influx abolishes ¹⁴C-MA and NH₂⁺ accumulation in *C. pasteurianum* [19] and *A. vinelandii* [37].

Taken together, the available evidence favors a component of the p.m.f., the membrane potential Δψ as the driving force of NH₂⁺ accumulation for most, if not all NH₂⁺ carriers of Table 2. Two observations, however, are not yet fully reconcilable with this suggestion:

1. In anaerobic batch cultures of *K. pneumoniae*, a Δψ close to zero has been determined [53], but NH₂⁺ accumulation requiring a Δψ of approx. 70 mV occurs in anaerobic continuous cultures of the same organism [35]. The discrepancy may be due to different growth conditions or an underestimation of Δψ due to the method employed [54].

2. Some observations have been interpreted as indicating involvement of ATP in NH₂⁺ accumulation by *A. vinelandii* [9,23]. The observations are, however, contradictory, and may suggest that ATP serves as a positive effector as in Δψ-driven K⁺ transport of *E. coli* [55].

### 5. REGULATION

As pointed out in the first section, most active transport processes are regulated on either the activity or the genetic level, or both. NH₂⁺ transport is no exception. In most species the synthesis of the NH₂⁺ carrier is subject to 'nitrogen control', while the activity may be regulated by glutamine.

#### 5.1. Regulation of carrier synthesis

With few exceptions the synthesis of the bacterial NH₂⁺ carriers is repressed when the organisms are cultivated in a medium containing high levels of NH₂⁺ (Table 2). Similar NH₂⁺ effects on the synthesis of enzymes catabolizing nitrogen sources are widely known, and have led to the discovery of a nitrogen control (ntr) regulatory system ([56] and references therein). This system of catabolite repression is especially prominent in Enterobacteriaceae. It comprises the three regulatory genes *ntrA* (or *glnF*), *ntrB* (or *glnL*), and *ntrC* (or *glnG*). The protein products of these genes interact in a complicated and not yet fully understood manner with one another and with the promoters of the genes encoding enzymes subject to nitrogen control, especially glutamine synthetase [56–58]. Additionally, a protein or proteins of the glutamine synthetase modification system
probably participates in nitrogen control [56].

The first indication that the \textit{ntr} system may also regulate synthesis of the \textit{NH}_4^+ carrier stems from the observation that a regulatory mutant of \textit{K. pneumoniae} (strain KP060 [59]) was unable to transport MA [35]. This mutant had been characterized before as defective in glutamine synthetase (Gln^-), nitrogenase (Nif^-) and histidine utilization (Hut^+). Revertants could be produced by mild mutagenesis, which showed the characteristics of the wild-type, i.e., Gln^+, Nif^+, Hut^+, Amt^+ (ammonium transport) [35]. This pleiotropic reversion has been attributed to a mutation in a regulatory gene [35,58,59].

Recently, a more elaborate study was carried out with \textit{E. coli} strains containing mutations in different \textit{ntr} genes [34]. The authors concluded, that both the \textit{ntrA} (glnF) and the \textit{ntrC} (glnG) gene products are required to activate synthesis of the \textit{NH}_4^+ carrier, while the \textit{ntrB} (glnL) gene product plays a role in its repression. Similar effects of the \textit{ntr} genes were observed on the synthesis of glutamine synthetase, so that a strain displaying the Gin^- phenotype also was always Amt^-.

A parallel between \textit{NH}_4^+ transport and glutamine synthetase activity has also been observed in mutants of \textit{R. capsulata} displaying different degrees of activity [60]. As will be discussed below, this parallel requires critical evaluation as to the absence of \textit{NH}_4^+ carriers in the Amt^- mutants.

5.2. Regulation of activity

Under conditions of derepression of the \textit{NH}_4^+ carrier, bacteria generally assimilate \textit{NH}_4^+ via the glutamine synthetase/glutamate synthase pathway [31,56,57] as summarized by Eqns. 3–5.

\[
\text{(NH}_4^+)_{\text{extracellular}} \rightarrow \text{(NH}_4^+)_{\text{intracellular}} \quad \text{(3)}
\]

\[
\text{(NH}_4^+)_{\text{in}} + \text{glutamate} + \text{ATP} \xrightarrow{\text{glutamin synthetase}} \text{glutamine} + \text{ADP} + \text{Pi} \quad \text{(4)}
\]

\[
\text{glutamine} + 2\text{-oxo-glutarate} + \text{NAD(P)H} \xrightarrow{\text{glutamate synthase}} 2\text{glutamate} + \text{NAD(P)} \quad \text{(5)}
\]

Any metabolite appearing during the first steps of this pathway comes into consideration as a putative regulator of \textit{NH}_4^+ transport. Prerequisite is a fast response of its level to the nitrogen supply of the cell. This prerequisite is only fulfilled for glutamine, and possibly carbamyl phosphate [61]. The intracellular glutamine level in several bacteria responds fast to the external \textit{NH}_4^+ concentration [62–64]. It serves as a feedback inhibitor of several enzymes, strongly affects the modification of the glutamine synthetase [31,56–59], and is thought to regulate the modification of the nitrogenase system in phototrophs [21,65] and to be the key regulator of the \textit{ntr} system [56].

Addition of glutamine to nitrogen-limited cells of several strains decreased MA uptake to various degrees (Table 3). A fairly strong effect was found for \textit{E. coli} [34], \textit{R. leguminosarum} [11] and \textit{R. sphaeroides} [45], while inhibition in \textit{K. pneumoniae} [67], \textit{A. vinelandii} [68] and \textit{Frankia} sp. [48] was very weak. These differences, however, should not conceal the fact that the direct putative effector, the intracellular glutamine level, is unknown in these studies: it depends on, inter alia, the activity of a glutamine transport system. Except for \textit{E. coli} [5], glutamine transport has attracted little attention in the strains mentioned. An alternative explanation to a direct effect on the \textit{NH}_4^+ carrier is competition of MA and glutamine transport for a common energy source, as has been proposed for \textit{Saccharomyces cerevisiae} [66]. However, transport driven by $\Delta \psi$ is likely only for some neutral and basic amino acids [2,5], while the energy for glutamine transport in \textit{E. coli} stems from a chemical reaction [2,5].

Taken together, the studies on the inhibition of \textit{NH}_4^+ (MA) transport by glutamine do not yet rigorously prove that \textit{NH}_4^+ uptake is regulated by the intracellular glutamine pool.

Jayakumar and Barnes [68] investigated the effect of the glutamine analog $\gamma$-N-methyl glutamine on MA uptake in \textit{A. vinelandii} and found an inverse relationship between the intracellular level of this artificial metabolite and MA transport. This observation was interpreted in terms of regulation of \textit{NH}_4^+ transport by glutamine.

Several investigators [17,23,49,50,67–70] studied the effect of methionine sulfoximine (MSX) on MA and \textit{NH}_4^+ transport in various systems (Table

...
Table 3
The effect of glutamine (Gln) and methionine sulfoximine (MSX) on MA and NH\textsubscript{4} transport by several bacteria

<table>
<thead>
<tr>
<th>Strain</th>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Inhibition (%)</th>
<th>Substrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli</td>
<td>Gln</td>
<td>0.05</td>
<td>97.8</td>
<td>MA</td>
<td>[34]</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>Gln</td>
<td>4</td>
<td>50</td>
<td>MA</td>
<td>[67]</td>
</tr>
<tr>
<td></td>
<td>MSX</td>
<td>0.05</td>
<td>50</td>
<td>MA</td>
<td>[67]</td>
</tr>
<tr>
<td>Azotobacter vinelandii</td>
<td>Gln</td>
<td>1</td>
<td>25</td>
<td>MA</td>
<td>[68]</td>
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<td>50</td>
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<tr>
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<td>MSX</td>
<td>0.01</td>
<td>50</td>
<td>MA</td>
<td>[69]</td>
</tr>
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<td>Rhodospirillum rubrum</td>
<td>MSX</td>
<td>0.005</td>
<td>50</td>
<td>MA</td>
<td>[69]</td>
</tr>
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<td>Rhodopseudomonas sphaeroides</td>
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<td>50</td>
<td>MA</td>
<td>[69]</td>
</tr>
<tr>
<td></td>
<td>Gln</td>
<td>(K_i = 0.08) mM</td>
<td>20</td>
<td>NH\textsubscript{4}</td>
<td>[11]</td>
</tr>
<tr>
<td>Rhizobium leguminosarum</td>
<td>Gln</td>
<td>(K_i = 0.12) mM</td>
<td>45</td>
<td>NH\textsubscript{4}</td>
<td>[50]</td>
</tr>
<tr>
<td>Frankia sp. Cpl1</td>
<td>Gln</td>
<td>1</td>
<td>100</td>
<td>NH\textsubscript{4}</td>
<td>[17]</td>
</tr>
<tr>
<td>Anabaena variabilis</td>
<td>MSX</td>
<td>0.01</td>
<td>0</td>
<td>MA</td>
<td>[49]</td>
</tr>
<tr>
<td>Anabaena flos-aquae</td>
<td>MSX</td>
<td>0.01</td>
<td>45</td>
<td>NH\textsubscript{4}</td>
<td>[50]</td>
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<tr>
<td>Anacystis nidulans</td>
<td>MSX</td>
<td>0.05</td>
<td>0–100 *</td>
<td>MA</td>
<td>[70]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
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</tbody>
</table>

* Dependent on the incubation time.

3). This glutamine analog is known as a potent irreversible inhibitor of many enzymes of glutamine metabolism, notably glutamine synthetase [71]. As shown in Table 3, MA and NH\textsubscript{4} transport of several strains is strongly inhibited by MSX, indicating the existence of a regulatory glutamine binding site at the carrier. This interpretation, however, has been questioned at least for cyanobacteria [49,70]. In these organisms a strong inhibition is found only after prolonged incubation [50,70]. During this time, due to inhibition of glutamine synthetase by MSX, intracellular NH\textsubscript{4} from degradation of amino acids may have accumulated which in turn would inhibit MA transport [49]. In K. pneumoniae, however, inhibition of MA transport by MSX was achieved in less than 2 min [67], about the time required for MSX uptake [69], while NH\textsubscript{4} excretion started only after about 10 min (Kleiner, unpublished observations). Also, some observations of cyanobacterial systems argue for a direct effect of MSX on the NH\textsubscript{4} carrier: (a) In A. flos-aquae MSX is effective at pH 7 but not at pH 9, where import occurs largely by NH\textsubscript{3} diffusion [50]. (b) NH\textsubscript{4} transport could be blocked by MSX in a glutamine synthetase-negative mutant of Anabaena cycadeae [72].

However, the arguments proposing inhibition of MA transport by MSX due to build-up of an intracellular NH\textsubscript{4} pool [49,70] cannot be dismissed and should always be taken into consideration.

The same arguments also hold for mutants defective in glutamine synthetase and (putatively) NH\textsubscript{4} transport, especially in view of the close relationship between both processes as discussed above [34,60]. For the pleiotropic K. pneumoniae KP5060 (Gln\textsuperscript{−} Nif\textsuperscript{−} Hut\textsuperscript{−} Amt\textsuperscript{−}) mutant, however, intracellular NH\textsubscript{4} accumulation can be ruled out, since the organisms do not excrete NH\textsubscript{4} during overnight cultivation (Kleiner, unpublished observations), as expected from the absence of enzymes catalyzing nitrogen catabolism in this strain [56,59].

As mentioned in section 4, a role of ATP as stimulator of NH\textsubscript{4} transport might be inferred from results with A. vinelandii [9,23]. The necessity for an activator also would explain our observations, that energized vesicles of K. pneumoniae do not accumulate \(^{14}\)C-MA [51]. But the hypothesis of possible involvement of ATP needs further substantiation.
NH₄⁺ uptake in N. muscorum is inhibited specifically by low concentrations of copper, cysteine and dithiothreitol [73]. These results, however, are interpreted in terms of affecting the redox state of the carrier rather than being of regulatory significance.

6. FUNCTION

6.1. Evidence for cyclic retention

In general, a transport system (carrier, permease) catalyzes the uptake of biologically important molecules from the surrounding medium through a membrane, which otherwise acts as an osmotic barrier for this solute. As already outlined in the first chapter, this notion cannot be easily applied to the NH₃/NH₄⁺ system.

A clue to the function is the observation that the NH₄⁺ carrier in many strains is repressed by its own substrate (Table 2, section 5). Repression of a transport system by its cognate solute is a rare phenomenon. It is only frequent in the field of amino acid uptake by enterobacteria [74]. For instance, the high-affinity transport system for branched-chain amino acids (LIV-1) is repressed by leucine [74], but this repression is accompanied by derepression of a low-affinity transport system (LIV-II). This change from one system to another enables the organism to maintain a relatively constant internal amino acid pool, despite wide variations in the extracellular levels.

With respect to NH₄⁺ transport, however, no evidence was found by us or reported in the literature for the derepression of a second NH₄⁺ carrier under conditions of high NH₄⁺ supply. Only in N. muscorum are two NH₄⁺ transport systems synthesized [15], but the high-affinity system seems to be constitutive (A.K. Kashyap, personal communication). These negative results suggest that at high external NH₄⁺ concentrations, diffusion of NH₃ through the membrane is fast enough to support the cell's demand for nitrogen.

The rate of unspecific diffusion is described by Fick's First Law:

\[
dn/dt = -P \cdot A \cdot \Delta c
\]

where \(dn/dt\) is the number (mol) of solute molecules passing a membrane with the area \(A\) per time unit; \(\Delta c\) is the concentration difference; and \(P\) the permeability coefficient which takes into account membrane specific parameters (solubility of solute, thickness etc.). Thus, for a given membrane, the rate and direction of diffusion depends only on \(\Delta c\). Accordingly, a high internal and low external NH₃ level must result in an outward diffusion of NH₃. A gradient in this direction is found when the organisms fix N₂, reduce NO₃⁻ or degrade organic nitrogenous compounds with formation of intracellular NH₄⁺. With few exceptions, however, under these conditions no net NH₃ excretion is observed, because the organisms generally derepress the NH₄⁺ carriers, which supposedly retrieve the escaping NH₃ after protonation (Fig. 1). The ensuing diminuation of \(\Delta \psi\) by electrogenic NH₄⁺ transport has to be balanced by an energy-dependent export of protons. Thus a futile cycle of NH₃ excretion/NH₄⁺ absorption (cyclic retention) is proposed, which draws more or less severely upon the energy metabolism of the organism.

Proof for this hypothesis would be the demonstration of constant NH₃ excretion upon loss of NH₄⁺ transport, which can be achieved by selective inhibition or mutagenic alteration of the NH₄⁺ carrier.

(a) Inhibition by MSX

When MA uptake in K. pneumoniae was blocked by MSX after approaching equilibration, the cells started to lose MA after about 2 min

![Fig. 1. Cyclic NH₃/NH₄⁺ retention.](https://academic.oup.com/femsre/article-abstract/1/2/87/546448/1286756448)
Inhibition of the MA carrier, however, is not the only possible explanation, as outlined in section 5.2. Thus these experiments cannot be regarded as conclusive.

(b) NH$_3$ excretion by ammonium transport-defective mutants (Amt$^-$)

Recently, we were able to obtain a prototroph mutant of *K. pneumoniae* unable to grow on 1 mM NH$_4^+$ but able to grow on 20 mM NH$_4^+$ [75]. This Amt$^-$ strain contains all the enzymes of inorganic nitrogen metabolism except the NH$_4^+$ carrier. Possibly similar mutants of *Alcaligenes eutrophus* (*Hydrogenomonas eutropha*) were produced more than a decade ago [76], but have not been characterized with respect to NH$_4^+$ transport, glutamine synthetase or NH$_3$ excretion. When grown with nitrogen sources other than NH$_4^+$, the Amt$^-$ strain constantly excretes NH$_3$, as postulated. This constant NH$_3$ loss presumably prevents the intracellular NH$_2$ accumulation required for rapid nitrogen metabolism. Therefore the Amt$^-$ strain grows considerably slower than the wild-type, especially with N$_2$ as the sole N source. Only with abundant NH$_4^+$ does the growth rate approach that of the wild-type.

In conclusion, NH$_3$ excretion and slow growth are attributable to the loss of the NH$_4^+$ carrier, thus lending support for the hypothesis of cyclic NH$_3$/NH$_4^+$ retention in the wild-type organism.

Cyclic retention is probably not only prominent in NH$_2$ accumulation, but may also occur in amino acid retention. Mutants totally defective in the uptake of certain amino acids often lose these compounds from intracellular pools ([77] and references therein).

6.2. Cyclic NH$_3$/NH$_4^+$ retention and symbiosis

A prerequisite for good transfer of NH$_3$ from a symbiotic *Rhizobium* to the surrounding nodule may be the ability of the plant to suppress formation of the NH$_4^+$ carrier in the endosymbiont [29,52]. Since the pH of the cytosol is lower than that of the bacteria, NH$_3$ crossing the membrane is trapped on the acidic side [78]. This NH$_3$ excretion is enhanced by repression of the bacterial glutamine synthetase [79]. In contrast to the organisms living in symbiosis, free-living rhizobia induce NH$_4^+$ carriers under certain conditions (Table 2).

Suppression of the NH$_4^+$ transport system, however, has not been found in symbiotic *Anabaena azollae* [49]. Here, overflow of the NH$_4^+$ pool is apparently only caused by repression of the glutamine synthetase. No studies have yet been carried out with *Frankia* spp., the endosymbiont of nonleguminous higher plants, in the symbiotic state. In the free-living state, one *Frankia* strain has been reported to contain a powerful NH$_4^+$ carrier (Table 2) [48].

6.3. An estimation of the energy requirement for cyclic NH$_3$/NH$_4^+$ retention by *Klebsiella pneumoniae* under N$_2$-fixing conditions

As outlined above, NH$_4^+$ retention in *K. pneumoniae* is a futile cycle requiring energy for the maintenance of the proton motive force after electrogenic NH$_4^+$ influx. Under N$_2$-fixing, anaerobic conditions, the p.m.f. is maintained by the H$^+$-translocating ATPase. Taking a similar H$^+$/ATP stoichiometry as for *E. coli* [80] we expect approx. 3 protons to be expelled per ATP molecule hydrolyzed. The pertinent question is: how often does an internally produced NH$_3$ molecule pass through the futile cycle (Fig. 1) before being metabolized (trapped) by the glutamine synthetase? This question can be answered by a comparison between the rates of NH$_3$ diffusion and NH$_4^+$ assimilation. While the rate of NH$_4^+$ assimilation can be easily determined, the calculation of NH$_3$ diffusion according to Eqn. 6 requires determination of the intracellular NH$_3$ pool, the total bacterial membrane surface, and of the permeability coefficient P.

In an N$_2$-fixing continuous culture of *K. pneumoniae*, the intracellular NH$_4^+$ concentration was determined as 0.56 ± 0.25 mM (details will be published elsewhere) at an extracellular NH$_4^+$ level of 15 μM. Assuming an intracellular pH of 7.4 [53], the intracellular NH$_3$ level is 8 μM according to Eqn. 1. Since the extracellular NH$_3$ concentration was far below 0.1 μM, Δc (NH$_3$) was about 8 μM. The total surface of the bacteria was calculated from the bacterial number and the cell dimensions (length 1.5 μm, diameter 0.75 μm), and amounted to 120 cm$^2$ per ml culture.
For the estimation of P, 2 different procedures were employed (details will be published elsewhere):

(a) Cells of KP 5060 (defective in both NH₄⁺ transport and assimilation) were equilibrated with [¹⁴C]-methylamine, rapidly diluted, and the efflux rate was determined. Efflux followed first order kinetics with \( P = 1.5 \times 10^{-3} \text{ cm} \cdot \text{s}^{-1} \).

(b) An aerobic, sulfate-limited continuous culture was set up with varying extracellular NH₄⁺ concentrations. The value was determined of the extracellular NH₄⁺ concentration at which NH₄⁺ transport was just derepressed. At this point the NH₃ diffusion rate must be just sufficient to support the required nitrogen assimilation. From estimations of intra- and extracellular NH₄⁺, \( \Delta c(\text{NH}_4^+) \) and the inward NH₃ diffusion were calculated from disappearance from the medium. In this way, \( P(\text{NH}_3) \) was calculated as \( 1.8 \times 10^{-3} \text{ cm} \cdot \text{s}^{-1} \). It was considered to be in good agreement with \( P(\text{MA}) = 1.5 \times 10^{-8} \). This value is considerably higher than a previous estimation of \( P(\text{NH}_3) = 5 \times 10^{-5} \text{ cm} \cdot \text{s}^{-1} \) which was based on very crude assumptions [3]. The new values are in the same range as the \( P(\text{H}_2\text{O}) \) for bilayer lipid membranes [81]. In view of the molecular similarities between \( \text{H}_2\text{O} \) and \( \text{NH}_3 \), this is not unexpected.

Inserting \( \Delta c(\text{NH}_3) = 8 \mu \text{M}, A = 120 \text{ cm}^2 \) and \( P = 1.8 \times 10^{-3} \text{ cm} \cdot \text{s}^{-1} \) into equation (4), we arrive at a NH₃ leakage rate in the continuous N₂-fixing culture of \( \frac{dn}{dt} = -1.8 \times 10^{-3} \text{ (cm} \cdot \text{s}^{-1}) \times 120 \text{ (cm}^2) \times 8 \times 10^{-9} \text{ (mol/cm}^3) = 1.7 \times 10^{-9} \text{ mol} \cdot \text{s}^{-1} \text{ per ml culture.} \) The immobilization of nitrogen in nitrogenous compounds from N₂ amounted to \( 0.2 \times 10^{-9} \text{ mol} \cdot \text{s}^{-1} \text{ per ml culture, calculated from the dilution rate and the total nitrogen content of the culture. Thus NH₃ assimilation is about 6 times slower than NH₃ diffusion, which means, that on the average every NH₃ molecule produced by the nitrogenase passes the futile cycle about 6 times before being assimilated by the glutamine synthetase. If we take an average H⁺/ATP stoichiometry of 3, energy requirement for cyclic NH₃/NH₄⁺ retention is about 2 ATP split per NH₃ produced or 4 ATP per N₂ reduced under the conditions described. This value is only valid for the special conditions employed for this calculation. It depends solely on the ratio of NH₃ diffusion over NH₄⁺ assimilation. This ratio may be higher when the growth rate gets slower (assuming that the intracellular NH₃ level remains constant, an assumption which has yet to be verified), and it might be lower when growth is faster.

Nevertheless, the calculations indicate, that the energy costs for cyclic NH₃/NH₄⁺ retention are not negligible. This expenditure may partially resolve a long-standing problem in the energetics of N₂ fixation. Most experiments show that for the in vitro reduction of one N₂ molecule, about 16 ATP molecules have to be hydrolyzed [82]. However, from growth yield determinations, an in vivo energy expenditure of up to 29 ATP/N₂ was calculated for \( \text{K. pneumoniae} \) [83]. Part of the additional energy is used for glutamine synthesis, and probably a greater part for cyclic retention. The energy expenditure for the pathway from N₂ to glutamine can be summarized as follows:

\[
\text{N}_2 \xrightarrow{16 \text{ ATP}} \text{Nitrogenase} \xrightarrow{4 \text{ ATP}} 2 \text{NH}_3 \xrightarrow{2 \text{ATP}} \text{Glutamine synthetase} \xrightarrow{2 \text{ Gln}}
\]

A value for the in vivo reaction of approx. 20 ATP/N₂ was calculated for \( \text{Clostridium pasteurianum} \) [84], while for \( \text{Azotobacter chroococcum} \) a surprisingly low value of 4-5 ATP/N₂ was extrapolated [85].

7. CONCLUSIONS AND OUTLOOK

Because of the high permeability of bacterial membranes to NH₃, the maintenance of intracellular NH₄⁺ pools by bacteria living on N₂, NO₃⁻ or organic N sources involves energy-dependent cyclic NH₃/NH₄⁺ retention (Fig. 1). NH₄⁺ carriers play an important role in this futile cycle. In most strains, their function is reflected by an uncommon regulation of synthesis: repression during NH₄⁺ abundancy, and derepression during growth on N sources other than NH₄⁺.

Energy expenditure depends on the size of the NH₄⁺ pool and thus ultimately on the \( K_m \) value of the glutamine synthetase. If energy saving is of importance, e.g., for oligotrophic bacteria, we
would expect very low $K_m$ values of this enzyme for NH$_4^+$. Frequently the $K_m$ value of an enzyme reflects the steady-state substrate concentration [86]. This straightforward relationship, however, is not always borne out by the available data for NH$_4^+$ assimilation. A spectacular case is *Anacystis nidulans*, the intracellular NH$_4^+$ pool of which has been determined to around 2 mM [17] (Table 1), while a $K_m < 20$ μM for NH$_4^+$ was reported for the glutamine synthetase of this organism [87]. Although the reported $K_m$ values for a certain enzyme vary considerably with the experimental conditions, such a large difference is unlikely to be due to artefacts. Possibly the high NH$_4^+$ concentrations in some bacteria (Table 1) are only achieved under the special experimental set-up employed, and do not reflect the levels prominent during life under field conditions.

With regard to NH$_4^+$ transport, 2 'extremophile' bacterial groups merit special attention: the acidophiles and the alkaliphiles. Acidophiles maintain a large ΔpH with an alkaline interior across the plasma membrane, coupled to an inverse Δψ (inside positive) when the outer pH is below 3 [88,89]. This inverse Δψ prohibits a Δψ-driven NH$_4^+$ transport, and if NH$_4^+$ accumulation exists, another energy source must be tapped. To date, no reports have dealt with this problem. On the other hand, extreme alkaliphiles generally maintain a large Δψ (inside negative) together with an inverse ΔpH (inside acidic) at high pH of the environment [88]. This ΔpH, according to Eqn. 2, may be sufficient for intracellular NH$_4^+$ accumulation as a consequence of NH$_3$ diffusion, kinetically facilitated by a favorable equilibrium (shifted towards NH$_3$) at high environmental pH.

Apart from alkaliphiles, other strains which may not synthesize NH$_4^+$ carriers are bacteria which obligately live in NH$_4^+$-rich environments or which obligately use nitrogen-rich compounds as C-sources. Otherwise, it is expected that most microorganisms from terrestrial or aquatic habitats under certain conditions are able to synthesize NH$_4^+$ carriers. This conjecture is based on the hypothesis, that the inability of NH$_4^+$ accumulation leads to constant NH$_3$ excretion concomitant with slow growth on N-sources other than NH$_4^+$. Therefore a selective pressure for the evolution of NH$_4^+$ transport systems, or alternatively of high affinity NH$_4^+$ assimilating enzymes can be anticipated. The current evidence indicates, that development of NH$_4^+$ carriers was favored during evolution. The NH$_3$/NH$_4^+$ system is probably not the only one operating with cyclic retention, although it is difficult to envisage other compounds demanding a similarly high energy input. Permeable species like CO$_2$ and organic acids should be trapped in the cell after unspecific diffusion because of a favorable ΔpH. It is, however, interesting that despite this deduction, a transport system for bicarbonate exists in *Anabaena variabilis* [90].

A futile cycle seems to exist for the retention of certain amino acids [77], and, although not of major importance as an energy leak, may operate in the retention of many other nutrients.

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NOTE ADDED IN PROOF

Michel (Ph.D. thesis, Osnabrück 1985) detected a NH$_4^+$ repressible ammonium carrier in Bacillus acidocaldarius despite an inverted $\Delta \psi$, with a $K_m$ for MA of 500 $\mu$M and for NH$_4^+$ of 8 $\mu$M. The energy source is unknown.