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The bioenergetics of ammonia and hydroxylamine oxidation in *Nitrosomonas europaea* at acid and alkaline pH

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Abstract. Autotrophic ammonia oxidizers depend on alkaline or neutral conditions for optimal activity. Below pH 7 growth and metabolic activity decrease dramatically. Actively oxidizing cells of *Nitrosomonas europaea* do not maintain a constant internal pH when the external pH is varied from 5 to 8. Studies of the kinetics and pH-dependency of ammonia and hydroxylamine oxidation by *N. europaea* revealed that hydroxylamine oxidation is moderately pH-sensitive, while ammonia oxidation decreases strongly with decreasing pH. Oxidation of these exogenous substrates results in the generation of higher proton motive force which is mainly composed of a $\Delta\Psi$. Hydroxylamine, but not ammonia, is oxidized at pH 5, which leads to the generation of a high proton motive force which drives energy-dependent processes such as ATP-synthesis and secondary transport of amino acids.

Endogenous substrates can be oxidized between pH 5 to 8 and this results in the generation of a considerable proton motive force which is mainly composed of a $\Delta\Psi$. Inhibition of ammonia-mono-oxygenase or cytochrome *aa3* does not influence the magnitude of this gradient or the oxygen consumption rate, indicating that endogenous respiration and ammonia oxidation are two distinct systems for energy transduction.

The results indicate that the first step in ammonia oxidation is acid sensitive while the subsequent steps can take place and generate a proton motive force at acid pH.

Key words: *Nitrosomonas europaea* – pH Dependency – solute transport – bioenergetics – Ammonia/hydroxylamine oxidation

Microbial nitrification is a two step process carried out by two distinct groups of bacteria, placed together in the *Nitrobacteriaceae* (Bock et al. 1986). The first step, the oxidation of ammonia to nitrite, is performed by ammonia-oxidizing bacteria such as *Nitrosomonas europaea*. The second reaction, the conversion of nitrite to nitrate, is performed by nitrite oxidizers, like *Nitrobacter* sp.

The strong effect of the external pH on the activity of autotrophic nitrifiers, both in soils and in liquid cultures, is a well documented phenomenon (Focht and Verstraete 1977). Although there is accumulating evidence that autotrophic nitrification does take place in acid soils (Bhuiya and Walker 1977; De Boer et al. 1988, 1989; Hankinson and Schmidt 1984; Stams et al. 1990; Walker and Wickramasinghe 1979), ammonia-oxidizing bacteria isolated from these soils are not acid-tolerant (De Boer and Laanbroek 1989; Hankinson and Schmidt 1984; Martikainen and Nurmiaho-Lassila 1985). It has been suggested that the sensitivity of ammonia oxidizers at low pH is due to the absence of NH_3, the proposed substrate for ammonia mono-oxygenase (AMO) (Suzuki et al. 1974). This enzyme catalyses the first step of ammonia oxidation (Bédard and Knowles 1989; Wood 1986, 1988), which leads to the formation of hydroxylamine:

$$NH_4^+ + XH_2 + O_2 \rightarrow NH_2OH + H_2O + X + H^+ \quad (1)$$

The cell invests reducing power in this reaction that has a ΔG_0 close to zero, and this consequently does not result in the generation of metabolic energy. In the subsequent reaction hydroxylamine is converted to nitrite with the release of four electrons by the enzyme hydroxylamine oxidoreductase (HAO).

$$NH_2OH + H_2O + 2X \rightarrow NO_2^- + 1H^+ + 2XH_2 \quad (2)$$

This enzyme is the in vivo source of reductant for AMO. Under steady-state conditions, the concentration of hydroxylamine will be constant, and of the four reducing equivalents formed by HAO, two will return to the AMO and two will be used in the electron transport chain (Bédard and Knowles 1989; Hyman and Wood 1983;

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Wood 1986, 1988). Electrons can flow down the electron transport chain to the terminal oxidase cytochrome *aa3*. However, since *N. europaea* uses reversed electronflow to synthesize NADH, several reducing equivalents are used for NAD reduction (Hyman and Wood 1983). In the absence of exogenous substrates *N. europaea* has a low level of respiration. Although the chemical nature of the compounds that are oxidized is not known, endogenous respiration does yield energy but has properties distinct from ammonia oxidation (Drozd 1976).

It was reported that *N. europaea* does not regulate the intracellular pH (Kumar and Nicholas 1983). Although this indicates that *N. europaea* is not adapted to be active a low pH, we show that energy transduction can take place at pH 5 when hydroxylamine replaces ammonia as an energy source.

Materials and methods

Organism and growth conditions

Nitrosomonas europaea ATCC 19178 was grown in an aerated 10 litre continuous culture vessel equipped with a pH electrode and an automatic titration unit. The dilution rate (D) was 0.04 h^{-1}, and the temperature was kept at 28 °C. The mineral medium, described by De Boer and Laanbroek (1989), was supplemented with $(NH_4)_2SO_4$ to a final concentration of 20 mM NH_4^+. The culture was kept at a constant pH of 7.8 by the addition of sterile Na_2CO_3. Purity was tested regularly by inoculating a 0.1% glucose-peptone-yeast extract medium (De Boer and Laanbroek 1989).

Cells were harvested from the culture vessel by centrifugation (10.000 × g for 15 min) and washed in 100mM K/Na-Phosphate pH 7.8 if not indicated otherwise.

O_2uptake measurements

O_2 uptake rates were measured at 28 °C using a Clark-type oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, USA) in a water-jacketed reaction vessel with a volume of 4.0 ml.

Electrical potential measurements

The electrical potential ($\Delta\Psi$, interior negative) was determined from the distribution of tetraphenylphosphonium-ion (TPP^+) across the cytoplasmic membrane using a TPP^+-sensitive electrode (Shinbo et al. 1978). Measurements were performed at 28 °C and were corrected for non-specific probe binding to the cells (Lolkema et al. 1982).

In order to permeabilise the outer membrane, cells were washed once in 100 mM Tris-HCl, pH 8.0, incubated with 5 mM Potassium-EDTA (Kumar and Nicholas 1983) for 10 min at 30 °C, and resuspended in the assay buffer before the $\Delta\Psi$ measurements.

The standard assay was done in 1 ml oxygen saturated buffer (100 mM K/Na-phosphate or 50 mM Na-citrate plus 50 mM K-phosphate) in the presence of 4 µM TPP^+.

Hydroxylamine or ammoniumchloride was added to a final concentration of 5 mM. Nigericin (potassium/proton exchanger) and valinomycin (potassium ionophore) were added to final concentrations of 50 and 200 nM, respectively.

ATP measurements

Cells were washed and resuspended in 10 ml buffer of pH 5 or 8 and composition described above. After 10 min of preincubation at 28 °C with forced aeration, substrate was added. Samples (1 ml) were taken and rapidly centrifuged through a layer of silicon oil into 50 µl of 14% (w/v) perchloric acid (PCA). After neutralisation samples were assayed for ATP (Lundin and Thore 1975).

Table 1. Effect of pH on the endogenous respiration rate and the V_{max} and K_m for ammonia and hydroxylamine oxydation by *Nitrosomonas europaea*[a]

NH_4^+					
pH	$K_m$app [mM $NH_4^+ + NH_3$]	K_m [µM NH_3]	V_{max}		
			[nmol O_2/ mg · min]	[nmol S/ mg · min]	[nmol e^-/ mg · min]
5.0	n.d.	n.d.	n.d.	n.d.	n.d.
7.0	8.7	49	660	440	880
7.5	2.2	39	568	379	757
8.0	0.86	48	505	337	673

NH_2OH					Endogenous
pH	K_m [µM NH_2OH]	V_{max}			V
		[nmol O_2/ mg · min]	[nmol S/ mg · min]	[nmol e^-/ mg · min]	[nmol O_2/ mg · min]
5.0	198	53	53	212	36
7.0	182	169	169	676	27
7.5	189	222	222	888	37
8.0	149	164	164	656	43

[a] Rates were measured as oxygen-consumption/mg protein. min at 28 °C in 50 mM Na-citrate/50 mM KPi at pH 5.0, pH 7.0, pH 7.5, and pH 8.0 respectively. Calculations were made using $O_2 : NH_4^+$ and $O_2 : NH_2OH$ ratio's of 1.5 : 1 and 1 : 1 respectively; n.d. · not detectable

Transport studies

Uptake of [^{14}C]-labelled L-alanine or 2-amino-isobutyric acid (AIB) was assayed by a filtration method. Cells (100–300 µg protein) were suspended in 1 ml buffer. Energy-source and subsequently after 1 min [^{14}C]-labelled compounds were added to the magnetically stirred suspension. At intervals 100 µl was sampled, 2 ml of ice-cold 100 mM LiCl added, and the sample filtered immediately over 0.45 µm cellulose-nitrate filters (BA 85, Schleicher & Schüll, Dassel, FRG). Filters were washed once with 2 ml 100 mM LiCl, and transferred to scintillation vials. Scintillation fluid (4 ml) was added and radioactivity was measured with a liquid scintillation counter (Packard Tri-Carb-460 CD, Packard Instruments Comp., Downers Grove, Ill., USA).

Analytical procedure

Protein was determined according to the method of Lowry et al. (1951) using bovine serum albumin as standard.

Chemicals

L-[^{14}C]-Alanine and [^{14}C]-α-amino-isobutyric acid (AIB) (174 and 59 mCi/mmol, respectively) were obtained from the Radiochemical Centre, Amersham, UK. All other chemicals were reagent grade and obtained from commercial sources.

Results

O_2 uptake rates

To verify that ammonia hydroxylation is the acid-sensitive part of ammonia oxidation to nitrite in *Nitrosomonas europaea*, the kinetics of the oxidation of ammonia and hydroxylamine were studied at different pH values. When the pH was lowered from 8 to 7 the maximal rate of ammonia oxidation did not change, but the apparent affinity for ammonia decreased about 10-fold (Table 1). The calculated affinity constant for NH_3 is the same within this interval. Hydroxylamine oxidation was found to be far less acid-sensitive than ammonia oxidation (Table 1) and could take place at pH 5, which is below the limit for growth of *N. europaea* on ammonia. The oxygen-consumption by endogenous respiration is low compared to ammonia oxidation, but it is hardly affected by the external pH.

Electrical potential measurements

Fresh, washed, endogenously respiring cells maintained a considerable $\Delta\Psi$ which was fully dissipated by valinomycin. At pH 8 inhibition of cytochrome *aa3*, the terminal oxidase, by cyanide (100 µM), did not affect the endogenous respiration and the $\Delta\Psi$ (Table 2) but completely repressed ammonium respiration. Pretreatment of the cells for 15 min with 2–10 mM KCN was necessary to block endogenous respiration and collapse $\Delta\Psi$ (Table 2). The $\Delta\Psi$ has been measured in the presence of nigericin to collapse the pH gradient (ΔpH) (Fig. 1). Upon the addition of nigericin the $\Delta\Psi$ in endogenously respiring cells decreased at pH 7–8 and increased at pH 5–6.

Both the addition of ammonia or hydroxylamine at pH 8 yielded a similar increase in $\Delta\Psi$. Under these conditions, diethyl-dithiocarbamate (DIECA) inhibited stimulation of $\Delta\Psi$ by ammonia but not by hydroxylamine oxidation (Table 2). Below pH 7 ammonia was oxidized slowly and no increase of the $\Delta\Psi$ was observed (data not shown). However, hydroxylamine could be used to energize cells over the whole pH range 5–8. As long as oxygen and hydroxylamine were present the high $\Delta\Psi$ remained (Fig. 1). Addition of nigericin to cells oxidizing hydroxylamine did not affect the $\Delta\Psi$ at pH 7–8. However, upon addition of nigericin at pH 5–6 there was an increase of $\Delta\Psi$ under these conditions.

Table 2. Effects of inhibitors on the $\Delta\Psi$ in *N. europaea* cells in the presence or absence of external substrates (no nigericin added)

pH 8			
Addition	$\Delta\Psi$ Endogenous	$\Delta\Psi$ $+NH_4Cl$	$\Delta\Psi$ $+NH_2OH$
None	−138 mV	−157 mV	−155 mV
100 µM DIECA	−135 mV	−134 mV	−158 mV
100 µM KCN	−134 mV	−130 mV	−130 mV
500 µM KCN	−125 mV	−132 mV	−131 mV
5 mM KCN (15' pretreated)	0 mV	0 mV	0 mV

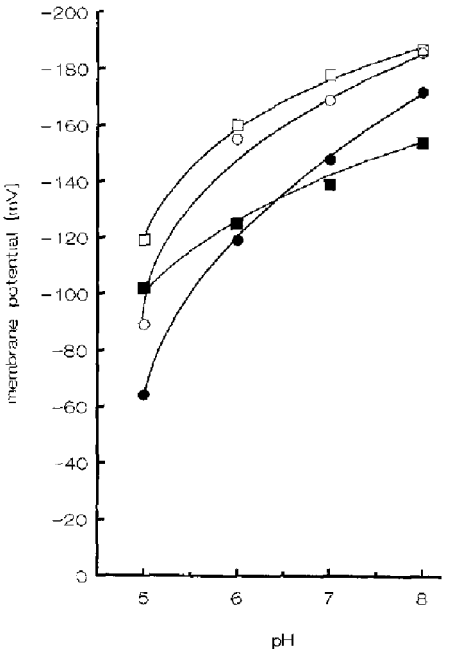

Fig. 1. Membrane potential measured in 50 mM Na-citrate/50 mM KPi by means of a TPP^+-sensitive electrode at different pH values in the presence or absence of 5 mM hydroxylamine and with or without 20 nM nigericin. No substrate, no nigericin (●), no substrate, + nigericin (■), + substrate, no nigericin (○), + substrate, + nigericin (□)

Internal ATP levels

The proton-motive force is used to drive secondary energy-dependent processes, like ATP synthesis by the H^+-ATPase (Kumar and Nicholas 1982).

At pH 8, addition of ammonia or hydroxylamine to an aerated cell suspension caused an increase of the internal ATP concentration *N. europaea* cells (Fig. 2A). With ammonia the ATP concentration slowly decreased after the initial increase, while with hydroxylamine a faster decrease was observed.

As expected at pH 5, addition of ammonia caused no change in the internal ATP concentration (Fig. 2B). However, at this pH-value hydroxylamine could be used to drive ATP synthesis, but again the ATP levels were transient.

The internal ATP concentration tended to increase when cells were diluted from a concentrated stock, kept on ice, to the incubation buffer (28 °C). Because of this effect we chose a preincubation time of 10 min. This initial increase could be completely inhibited by DCCD (100 μM).

Transport experiments

We investigated whether *N. europaea* can actively accumulate amino-acids and used the uptake rates as a bioenergetic parameter in our study.

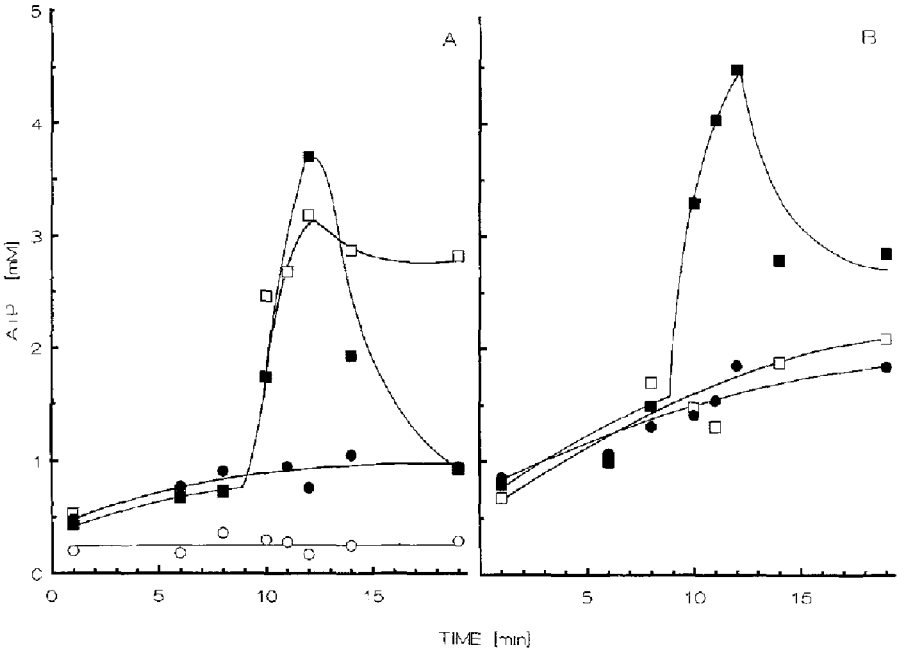

Fig. 2. Internal ATP concentration at pH 8.0 (**A**) and pH 5.0 (**B**). At $t = 9$ min 5 mM ammonia (□) or 5 mM hydroxylamine (■ was added. The controls were (●) no additions and (○) 100 μM DCCD at $t = 0$

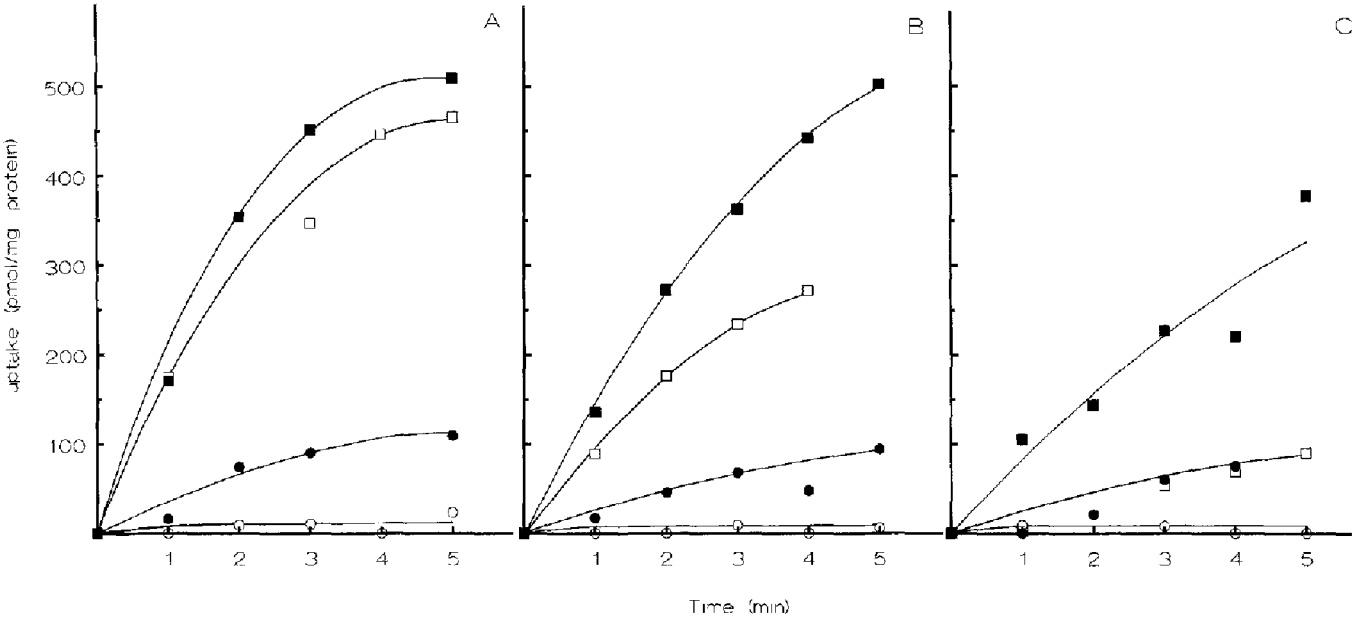

Fig. 3. Influence of pH on the uptake of AIB. Cells were washed twice in buffer (50 mM Na-citrate/50 mM KPi) of the appropriate pH and diluted in the assay buffer containing 5 mM ammonia (□), 5 mM hydroxylamine(■), 5 mM hydroxylamine + 50 μM CCCP (○), or no additions (●). The pH values were 8.0 (**A**), 6.5 (**B**) and 5.0 (**C**)

Endogenously respiring cells maintained a $\Delta\Psi$ of considerable magnitude, and could take up L-alanine (not shown) and the L-alanine analog AIB at a significant rate in the absence of ammonia or hydroxylamine (Fig. 3). At pH 8, ammonia and hydroxylamine oxidation increased the uptake rate to a similar extent (Fig. 3A). When the pH of the medium decreased, the uptake rate of AIB in the presence of ammonia or hydroxylamine also decreased. However, in the presence of ammonia this decrease was much more pronounced and hydroxylamine-oxidation was still capable to support a high AIB-uptake rate at pH 5 (Fig. 3B, C).

Pretreatment of the cells with the protonophore CCCP (100 μM) resulted in a complete dissipation of the Δp and a complete inhibition of AIB uptake (data not shown).

Discussion

Although the acid-sensitivity of the ammonia oxidizers seems to restrict nitrification to neutral and alkaline environments, there is massive evidence that it does take place in acid soils (Bhuiya and Walker 1977; De Boer et al. 1988, 1989; Hankinson and Schmidt 1984; Stams et al. 1990; Walker and Wickramasinghe 1979). Because no acid-insensitive ammonia oxidizer has been isolated in pure culture thusfar we used *Nitrosomonas europaea*, the best studied ammonia oxidizer, to gain more insight in what actually prevents ammonia oxidation by this organism at low pH.

Compared to ammonia oxidation, the oxidation of hydroxylamine, an intermediate in the oxidation of ammonia to nitrite, turned out to be far less acid-sensitive and the results clearly show that energy-transduction can take place under acid conditions. Even at pH 5, hydroxylamine is oxidized, which leads to the generation of a Δp, synthesis of ATP, and energy-dependent transport activity. However, our attempts to grow *N. europaea* in batch- and chemostatcultures on hydroxylamine failed, possibly because of the toxic nature of this substrate.

Per ammonia molecule oxidized 2 electrons, and per hydroxylamine 4 electrons are released which all react with oxygen at cytochrome *aa3* when no electrons are consumed by reversed electron flow. In terms of reducing equivalents or electrons generated, the maximal rates of ammonia and hydroxylamine oxidation were very similar (Table 1), indicating that ammonia hydroxylation by AMO is not rate-limiting. Compared to hydroxylamine, twice as much ammonia molecules have to be oxidized to generate the same number of electrons. The oxidation of hydroxylamine by HAO (which is twice as fast under ammonia oxidizing conditions compared to hydroxylamine oxidizing conditions) thus can also not be rate-limiting. The actual rate-limiting step must therefore be the electron transport to cytochrome *aa3*.

Although the oxygen consumption rate is low, endogenous respiration is sufficient to maintain a considerable Δp during starvation in *N. europaea* cells. The existence of a relatively high Δp in the absence of an external substrate such as ammonia might be of survival value in an energy limited environment such as the soil.

When an external substrate is supplied, a significant increase of Δp is found. In our experiments DIECA (Bhandari and Nicholas 1979) prevented the stimulation of $\Delta\Psi$ induced by ammonia oxidation. Because AMO is the target enzyme, hydroxylamine-induced stimulation of $\Delta\Psi$ was as expected not affected by DIECA. A low concentration of potassium cyanide also did not affect the $\Delta\Psi$ built up by endogenous respiration but prevented stimulation of $\Delta\Psi$ by external substrates. These effects of DIECA and KCN indicate that there are two distinct systems for energy generation in *N. europaea*. To block endogenous respiration a high concentration of KCN was necessary. This is in agreement with the results reported for cytochrome *o* (Miller and Wood 1983), and suggests that this oxidase is involved in the endogenous respiration.

In the presence of ammonia, Kumar and Nicholas (1983) found no effect of DIECA on the $\Delta\Psi$ in whole cells. Even more surprising, in their experiments the Δp was the same (−150 mV) at pH 6 and 8, although at pH 6 no ammonia oxidation was detectable in oxygen uptake experiments, whereas at pH 8 ammonia oxidation was optimal. Therefore it is unlikely that these measurements were performed under ammonia-oxidizing conditions.

Endogenously respiring *N. europaea* cells behave similar to what was reported previously for ammonia-oxidizing cells (Kumar and Nicholas 1983): there is a slight ΔpH, inside alkaline, below pH 7 and a slight ΔpH, inside acid, above pH 7. Hydroxylamine-oxidizing cells have a slight ΔpH, inside alkaline, only at acid external pH. Altogether it is clear that the presence of oxidizable substrates does not improve the poor abilities of *N. europaea* to maintain a constant internal pH.

Our observation that hydroxylamine and not ammonia is oxidized at pH 5 and stimulates the $\Delta\Psi$, ATP synthesis, and solute transport, suggests that ammonia hydroxylation is the predominant acid-sensitive process in *N. europaea*. 25 years ago Clark and Schmidt (1967a and b) showed that *N. europaea* cells are capable of incorporating exogenously supplied amino acids. In their experiments they mainly focused on growth response of cultures and the metabolic fate of the amino acids. Until now the presence of active transport systems in autotrophic ammonia-oxidizing bacteria such as *N. europaea* has not been reported. The uptake experiments presented in this study, reveal that L-alanine and AIB can be taken up by *N. europaea* at pH 5 with the hydroxylamine as energy source.

In summary: *N. europaea* is energetically capable of functioning at pH 5, provided hydroxylamine is present, while there is no accurate regulation of the internal pH under any of the conditions studied. In the pH-range studied when conditions do not allow ammonia oxidation, cells can depend on endogenous substrates to maintain a considerable Δp. However, external substrates are needed to drive energy-dependent processes such as ATP-synthesis and solute transport.

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