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Proton Electrochemical Gradients in Washed Cells of *Nitrosomonas europaea* and *Nitrobacter agilis*

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The components of the proton motive force (Δp), namely, membrane potential (ΔΨ) and transmembrane pH gradient (ΔpH), were determined in the nitrifying bacteria *Nitrosomonas europaea* and *Nitrobacter agilis*. In these bacteria both ΔΨ and ΔpH were dependent on external pH. Thus at pH 8.0, *Nitrosomonas europaea* and *Nitrobacter agilis* had ΔΨ values of 173 mV and 125 mV (inside negative), respectively, as determined by the distribution of the lipophilic cation [^{3}H]tetraphenyl phosphonium. Intracellular pH was determined by the distribution of two weak acids, ^{14}C-benzoic and ^{14}C-acetyl salicylic, and the weak base [^{14}C]methylamine. *Nitrosomonas europaea* accumulated ^{14}C-benzoic acid and ^{14}C-acetyl salicylic acid when the external pH was below 7.0 and [^{14}C]methylamine at alkaline pH. Similarly, *Nitrobacter agilis* accumulated the two weak acids below an external pH of about 7.5 and [^{14}C]methylamine above this pH. As these bacteria grow best between pH 7.5 and 8.0, they do not appear to have a ΔpH (inside alkaline). Thus, above pH 7.0 for *Nitrosomonas europaea* and pH 7.5 for *Nitrobacter agilis*, ΔΨ only contributed to Δp. In *Nitrosomonas europaea* the total Δp remained almost constant (145 to 135 mV) when the external pH was varied from 6 to 8.5. In *Nitrobacter agilis*, Δp decreased from 178 mV (inside negative) at pH 6.0 to 95 mV at pH 8.5. Intracellular pH in *Nitrosomonas europaea* varied from 6.3 at an external pH of 6.0 to 7.8 at external pH 8.5. In *Nitrobacter agilis*, however, intracellular pH was relatively constant (7.3 to 7.8) over an external pH range of 6 to 8.5. In *Nitrosomonas europaea*, Δp and its components (ΔΨ and ΔpH) remained constant in cells at various stages of growth, so that the metabolic state of cells did not affect Δp. Such an experiment was not possible with *Nitrobacter agilis* because of low cell yields. The effects of protonophores and ATPase inhibitors on ΔpH and ΔΨ in the two nitrifying bacteria are considered.

The nitrifying bacteria *Nitrosomonas europaea* and *Nitrobacter agilis* oxidize the inorganic nitrogen compounds NH_4^+ and NO_2^-, respectively, thus generating energy (ATP) and reducing equivalents for growth (3–7). Although some aspects of the metabolism of these bacteria have been extensively studied and reviewed on several occasions (1, 2, 27, 39, 43, 47, 49), the role of components of the proton motive force (Δp) associated with cell membranes is relatively unexplored. *Nitrosomonas europaea* translocates protons during respiration (8, 9, 14, 19) with an effective $\rightarrow H^+/O$ ratio of 4 for either NH_4^+, NH_2OH, or NH_2NH_2 as the oxidizable substrate (19). However, proton ejection during NO_2^- oxidation by *Nitrobacter agilis* was not detected (19). According to Mitchell's chemiosmotic hypothesis (37), the electrochemical gradient of protons gives rise to Δp, which is responsible for the coupling of metabolic energy to the transport of a number of nutrients and to ATP synthesis (18, 21, 33, 51). This proton gradient consists of an electrical potential (ΔΨ) and a pH gradient (ΔpH) across the cytoplasmic membrane. The two components of Δp have the following relationship (37): $\Delta p = \Delta\Psi - 2.3RT/F\ \Delta pH$; or, in millivolts at 25°C: $\Delta p = \Delta\Psi - 59\ \Delta pH$.

Whereas Δp has been determined in a variety of bacteria (13, 15, 17, 20, 22–24, 26, 36, 41), no information is available for the nitrifiers. We now report on the measurements of Δp in two nitrifying bacteria, *Nitrosomonas europaea* and *Nitrobacter agilis*.

MATERIALS AND METHODS

Bacteria and growth conditions. The strain of *Nitrosomonas europaea* used was obtained from Jane Meiklejohn of Rothamsted Experimental Station, Harpenden, U.K. Cultures were grown in either 8- or 40-liter

batches at 28°C with vigorous aeration for 3 days (midexponential phase) in an inorganic medium (8). The pH was maintained at 7.8 throughout the growth by titration of the medium with sterile 20% (wt/vol) K_2CO_3, using an automatic pH stat unit (Radiometer, Copenhagen, Denmark). The cells harvested by continuous-flow centrifugation (Ivan Sorvall, Inc., Norwalk, Conn.) at 4°C as described previously (8, 30), were washed several times with cold 100 mM Tris-hydrochloride buffer (pH 7.5) and finally suspended in the appropriate buffer. *Nitrobacter agilis* ATCC 14123 was grown in 8-liter batches for 5 days with vigorous aeration in an inorganic medium described by Wallace et al. (48). Cells were harvested and washed as for *Nitrosomonas europaea*. The typical cell yields were about 150 and 50 mg (wet weight) liter^{-1} for *Nitrosomonas europaea* and *Nitrobacter agilis*, respectively.

EDTA treatment of cells. Cells of both *Nitrosomonas europaea* and *Nitrobacter agilis* were suspended in 100 mM Tris-hydrochloride buffer (pH 8.0; 20 mg ml^{-1}) and treated with EDTA adjusted to pH 7 with KOH (5 mM for *Nitrosomonas europaea* and 10 mM for *Nitrobacter agilis*) for 10 min at 37°C. The cells, collected by centrifugation, were washed once in the buffer and suspended in the appropriate buffer. The EDTA-treated cells were used within 2 h.

Intracellular space. Intracellular space was determined by using ^3H_2O, [^{14}C]sucrose, and [^{14}C]inulin (34, 46). Thus, for *Nitrosomonas europaea* and *Nitrobacter agilis*, the intracellular water spaces were 1.7 ± 0.2 and 1.2 ± 0.2 μl mg^{-1} (dry weight), respectively.

Uptake of labeled probes. Untreated or EDTA-treated cells were incubated at 25°C in Na^+ phosphate (100 mM) or Tris-hydrochloride (50 mM) buffer at the appropriate pH. The cell suspensions were either vigorously oxygenated for 10 min with pure oxygen or mixed with catalase (0.05 mg ml^{-1}) and H_2O_2 (1 μl ml^{-1}). The substrate was 5 mM NH_4Cl for *Nitrosomonas* and 5 mM $NaNO_2$ for *Nitrobacter*. Then the isotopically labeled compound was added, and incubation was continued for a further 5 to 15 min. Samples (1 ml) were then centrifuged in an Eppendorf microfuge at 13,000 × *g* for 1 min. Portions of the supernatant (100 μl) and of the cell pellet were added to 1 ml of 3 M perchloric acid in 15-ml scintillation glass vials. After 30 min, when cell proteins were completely dissolved, 5 ml of a scintillation counting fluid (PCS; Amersham, Australia) was added to each vial, and the contents were mixed thoroughly and radioassayed in a Packard Tri-Carb 460 CD liquid scintillation spectrometer. In the standard protocol, two consecutive experiments were carried out in which $\Delta\Psi$ and ΔpH were measured. For $\Delta\Psi$ determination, [^{3}H]tetraphenyl phosphonium ([^{3}H]TPP$^+$) bromide (20 to 50 nCi ml^{-1}) was added to a cell suspension (1 to 1.5 mg [dry weight] ml^{-1}). For ΔpH determination, ^{14}C-benzoic acid (2 μCi ml^{-1}), ^{14}C-acetyl salicylic acid (2 μCi ml^{-1}) or [^{14}C]methylamine hydrochloride (1 μCi ml^{-1}) was added. ^3H_2O was used to determine total pellet water.

The calculations of $\Delta\Psi$ and ΔpH were made by using the Nernst equation as described previously (24) after correcting for nonspecifically bound [^{3}H]TPP$^+$ and extracellular counts of ^{14}C-benzoic acid, ^{14}C-acetyl salicylic acid, and [^{14}C]methylamine, respectively.

Oxygen uptake. The oxidation of NH_4^+ by *Nitrosomonas europaea* and NO_2^- by *Nitrobacter agilis* was measured in an oxygen electrode (Rank Bros., Cambridge, U.K.). For this purpose, cells (40 mg, wet weight) were suspended in 5 ml of 50 mM Tris-hydrochloride buffer (pH 7.8). The reaction was started by adding 10 μmol of NH_4Cl for *Nitrosomonas* or 10 μmol of $NaNO_2$ for *Nitrobacter*. The response of the electrode was monitored with a Rikadenki chart recorder, and oxygen uptake values were calculated as described before (19).

Materials. Carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), *N*,*N*′-dicyclohexylcarbodiimide (DCCD), and diethylstilbestrol (DESB) were purchased from Sigma Chemical Co., St. Louis, Mo. All other chemicals used were of the highest grade available. The radioisotopes [^{3}H]TPP$^+$ bromide (23.7 Ci mmol^{-1}), acetyl [*carboxyl*-^{14}C]salicylic acid (20 mCi mmol^{-1}), and [^{14}C]inulin (5.6 Ci mmol^{-1}) were from Amersham International Ltd. ^3H_2O (1 Ci mol^{-1}), 7-^{14}C-benzoic acid (22.6 mCi mmol^{-1}), [^{14}C]methylamine hydrochloride (51.8 mCi mmol^{-1}), and [^{14}C]sucrose (1 mCi mmol^{-1}) were purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Uptake of radioactive probes. All probes used to determine $\Delta\Psi$ and ΔpH were readily taken up by cells of *Nitrosomonas europaea* and *Nitrobacter agilis*, and an equilibrium state was reached within 5 min. The EDTA treatment of bacteria was necessary to make them permeable to the radioactive compounds. High concentrations of EDTA (5 mM for *Nitrosomonas* and 10 mM for *Nitrobacter*), relative to those used for *Escherichia coli* (41), were employed because of the complex cell membrane structures of these bacteria (38). EDTA-treated cells were metabolically active, since oxygen uptake values were similar to those of untreated cells.

Measurement of ΔpH as a function of pH°. The uptake of ^{14}C-benzoic acid, ^{14}C-acetyl salicylic acid, and [^{14}C]methylamine, respectively, by *Nitrosomonas europaea* and *Nitrobacter agilis* was observed over a range of external pH (pH°). The uptake of all three compounds was pH° dependent. *Nitrosomonas europaea* accumulated the two weak acids only when the pH° was below 7.0, and accumulated the weak base, methylamine, when the pH° was above 7.0, indicating that the intracellular pH (pH^i) of the bacterium was maintained around neutrality. The ΔpH was almost 0 at pH° 7, but when pH° was >7.0 the pH^i became acidic in relation to pH° (inside acidic). For *Nitrobacter agilis* the pH° at which neither of the two weak acids nor the weak base was taken up by the cells was about 7.5. Benzoic and acetyl salicylic acids were not metabolized by either strain (checked by thin-layer chromatography). *Nitrosomonas europaea*, however, slowly utilized methylamine when the external pH was greater than 7.5.

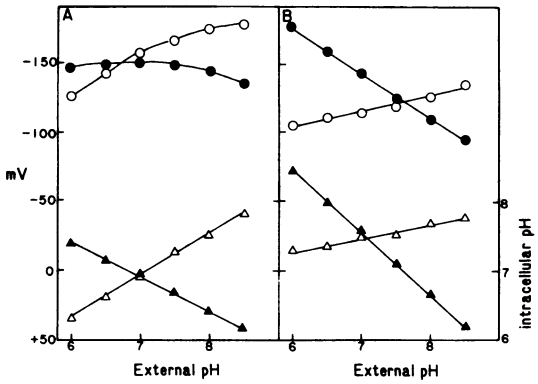

FIG. 1. Effects of external pH on pH^i ΔpH, ΔΨ, and Δp in EDTA-treated cells of (A) *Nitrosomonas europaea* and (B) *Nitrobacter agilis*. EDTA-treated cells were suspended in 50 mM sodium phosphate buffer at the pH values indicated. Uptake studies were carried out as described in the text. Intracellular pH (△) and ΔpH represented in terms of millivolts (59 × ΔpH) (▲) were determined with ^{14}C-benzoic acid and [^{14}C]methylamine. ΔΨ values (○) were calculated from the uptake of [^{3}H]TPP^+. Δp (●) was calculated from ΔpH and ΔΨ as described in the text.

Because methylamine was utilized slowly by *Nitrosomonas europaea*, the uptake studies with the probes, which were completed within 5 min, were unaffected by this metabolism.

Nitrosomonas europaea had limited capacity to maintain a constant pH^i, and thus it increased from 6.3 to 7.8 when the external pH (pH^o) was varied over the range from 6.0 to 8.5 (Fig. 1A). On the other hand, in *Nitrobacter agilis* (Fig. 1B) pH^i increased from 7.3 to 7.8 when the pH^o was increased from 6.0 to 8.5. Thus at pH 6.0 *Nitrosomonas europaea* and *Nitrobacter agilis* had ΔpH values of 0.3 and 1.3 pH units, respectively. As these bacteria respire optimally between pH 7.5 and 8.0, it appears that they do not have a ΔpH (inside alkaline), but instead their pH^i values are either similar to their pH^o or more acidic (inside acid). Over a range of pH^o (from 6.0 to 8.5, the pH^i in *Nitrobacter agilis* increased only by about 0.5 units, whereas in *Nitrosomonas europaea* it increased by 1.5 units.

Measurement of ΔΨ as a function of pH^o. The variations in ΔΨ as determined by [^{3}H]TPP^+ uptake over a pH^o range from 6 to 8.5 are shown in Fig. 1. In both bacteria ΔΨ increased with increasing pH^o. Thus in *Nitrosomonas europaea* ΔΨ increased from 125 mV at pH^o 6 to 178 at pH^o 8.5. In *Nitrobacter agilis* the effect of pH^o on ΔΨ was less pronounced than in *Nitrosomonas europaea*; thus it increased from 105 mV at pH^o 6.0 to 135 mV at pH^o 8.5, an increase of approximately 10 mV for each pH unit. The increase in ΔΨ in *Nitrosomonas europaea* was nonlinear and approached a plateau at pH^o 8.0, whereas in *Nitrobacter agilis* the increase was almost linear.

Total Δp. Since Δp is a function of ΔΨ and ΔpH, it is clear from Fig. 1A that it remained almost constant (135 to 145 mV) in *Nitrosomonas europaea* over a range of external pH. This was largely the result of an increase in ΔΨ and a decrease in ΔpH when the pH^o was increased from 6.0 to 8.5; thus a decrease in ΔpH was compensated by an increase in ΔΨ. In *Nitrobacter agilis* (Fig. 1B), however, the contribution of ΔpH decreased rapidly when the pH^o was increased (−73 mV at pH 6.0 to +40 mV at pH 8.5), while ΔΨ increased by 30 mV only from pH^o 6 to 8.5, thus decreasing the total Δp from 177 mV at pH^o 6 to 95 mV at pH^o 8.5.

Δp in cells of *Nitrosomonas europaea* harvested at various stages of growth. *Nitrosomonas europaea* grows slowly (mean generation time, 10 to 12 h). About 24 h after inoculation, the exponential stage of growth started, and it lasted for another 4 days (Fig. 2). Because the cell yields were low, it was not possible to conduct uptake studies with the probes to determine ΔpH and ΔΨ in growing cultures as described by Kashket and co-workers for a number of bacteria (22–24). To assess whether there were any changes in ΔpH and ΔΨ (at various stages of growth), cultures (1 liter) were harvested at various times as shown in Fig. 2. Thus ΔpH and ΔΨ were determined at two pH^o values (6 and 8) after the washed cells were suspended in a fresh culture medium. The intracellular water volume was reasonably constant during growth (1.6 ± 0.2 μl mg^{-1} [dry weight]). Cells harvested at different stages of growth maintained a fairly constant ΔΨ and pH^i (Fig. 2). Thus, at pH^o6 ΔΨ was approximately 122 mV and ΔpH was 0.3 units (inside alkaline), and at pH^o8 ΔΨ was approximately 165 mV and ΔpH was 0.5 units (inside acid). A similar experiment was not possible with *Nitrobacter agilis* because of the exceptionally low cell yields (40 to 50 mg [wet weight] $liter^{-1}$ of culture after 5 days of growth).

Effects of some inhibitors on the components of Δp. To determine the relevance of respiratory potential to Δp maintenance, the effects of respiratory inhibitors on ΔΨ and ΔpH in *Nitrosomonas europaea* and *Nitrobacter agilis* were investigated. Diethyldithio-carbamate, a potent inhibitor of NH_4^+ oxidation by *Nitrosomonas europaea* (8, 9), completely inhibited respiration at 20 μM, but did not have any substantial effect on Δp (Table 1). In *Nitrobacter agilis* (Table 2), sodium azide at 50 μM completely inhibited NO_2^- oxidation (also see references 30 and 40); although it had no effect on ΔΨ, it dissipated ΔpH, thus lowering Δp by about 20 mV. Be-

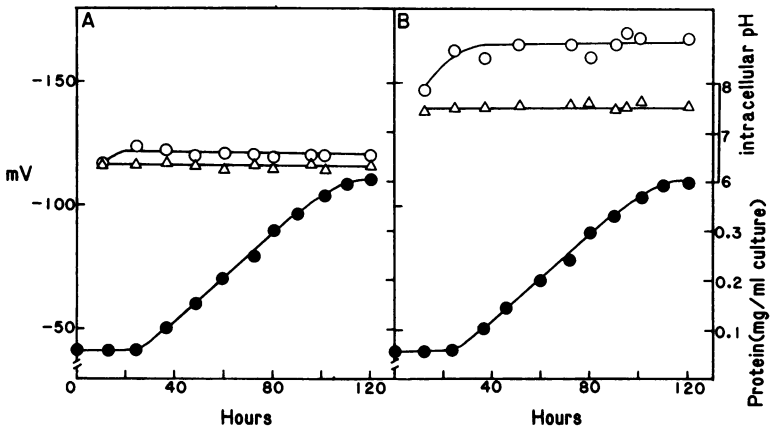

FIG. 2. Intracellular pH and $\Delta\Psi$ in cells of *Nitrosomonas europaea* at various stages of growth determined at pH° values of 6.0 (A) and 8.0 (B). Cultures (18 liters) were grown in 20-liter Pyrex glass bottles at constant temperature (28°C) and pH (8.0). Growth of bacteria (●) was monitored throughout the growth period by the rate of NH_4^+ oxidation and by determining the protein contents of cells. At the time intervals indicated, 1- to 2-liter samples, withdrawn aseptically from the cultures, were harvested by centrifugation (10,000 × *g* for 30 min) in 250-ml polycarbonate bottles. The cells were then washed and suspended in fresh growth medium at either pH 6.0 (A) or pH 8.0 (B). Uptake studies were carried out as described in the text. $\Delta\Psi$ (○) was calculated from the uptake of [^{3}H]TPP$^+$. Intracellular pH (△) was determined by the uptake of ^{14}C-benzoic acid and [^{14}C]methylamine at pH 6.0 and 8.0, respectively.

cause respiration in nitrifiers has been shown to be inhibited by uncouplers (2, 4, 7, 8, 11, 12, 30) and this effect was related to the collapse of $\Delta\Psi$ in *Nitrobacter*, we investigated the effect of the classical uncoupler CCCP on Δp in both *Nitrosomonas europaea* (Table 1) and *Nitrobacter agilis* (Table 2). The respiration of both nitrifiers was completely inhibited by CCCP concentrations of more than 50 μM, but Δp was only partially collapsed (Table 1 and 2). Recently we have found that several ATPase inhibitors re-

TABLE 1. Effects of some inhibitors on respiration and Δp in *Nitrosomonas europaea*[a]

Inhibitor	Concn (μM)	% Inhibition of respiration[b]	$\Delta\Psi$ (mV)	ΔpH^c	Δp (mV)
None			−147	0.18	−158
DIECA[d]	20	100	−148	0.00	−148
CCCP	10	80	−110	0.10	−116
	100	100	−80	0.00	−80
DCCD	200	50	−117	0.16	−128
DESB	50	25	−187	0.50	−216

[a] Washed cell suspensions in 50 mM Tris-hydrochloride (pH 6.9) were employed for $\Delta\Psi$ and ΔpH determination.
[b] Determined at pH 7.8 by the oxygen electrode technique described in the text. The control rate of O_2 uptake was approximately 850 ng-atoms of O min^{-1} mg^{-1} of protein.
[c] Determined by ^{14}C-benzoic acid distribution.
[d] DIECA, Diethyldithiocarbamate.

TABLE 2. Effects of some inhibitors on respiration and Δp in *Nitrobacter agilis*[a]

Inhibitor	Concn (μM)	% Inhibition of respiration[b]	$\Delta\Psi$ (mV)	ΔpH^c	Δp (mV)
None			−115	0.34	−138
Sodium azide	50	100	−114	0.05	−117
CCCP	10	70	−82	0.20	−94
	50	100	−75	0.12	−82
DCCD	100	55	−116	0.38	−138
	250	100	−118	0.08	−113
DESB	20	45	−124	0.31	−142
	50	85	−133	0.09	−139

[a] Washed cell suspensions in 50 mM Tris-hydrochloride buffer at pH 7.0 were employed for $\Delta\Psi$ and ΔpH determination.
[b] Determined at pH 7.8 by the oxygen electrode technique described in the text. The control rate of O_2 uptake was approximately 650 ng-atom of O min^{-1} mg^{-1} of protein.
[c] Determined by ^{14}C-benzoic acid distribution.

strict respiration in nitrifying bacteria to an extent similar to that for ATPase itself. To investigate whether this inhibition was related to a collapse of Δp, the effects of two ATPase inhibitors, DCCD and DESB, on ΔΨ and ΔpH were investigated. DCCD at high concentrations (>200 μM) affected Δp in both nitrifiers by lowering ΔΨ in *Nitrosomonas europaea* (Table 1) and ΔpH in *Nitrobacter agilis* (Table 2). DESB had little or no effect on Δp in *Nitrobacter agilis* (Table 2), but at 50 μM concentration it elevated Δp by about 60 mV (inside negative) in *Nitrosomonas europaea*.

Effects of NH_4^+ and NH_2OH on ΔΨ and ΔpH. Permeant amines and amine-like compounds have a tendency to redistribute across the membrane towards the acidic side in response to a pH gradient (16, 29). It has been shown that high concentrations of substrate (NH_4^+, NH_2OH, or $N_2H_5^+$) in *Nitrosomonas europaea* tend to diminish proton pumping (19). Both NH_4^+ and NH_2OH at high concentrations diminished completely the small pH gradient (≈0.1 unit) across the cell membranes of *Nitrosomonas europaea* (Fig. 3). Moreover, NH_4^+ also decreased ΔΨ (170 mV to 140 mV at 100 mM NH_4^+), but relatively small concentrations of NH_2OH (≈20 mM) rapidly decreased ΔΨ by about 60 mV. Increasing the NH_2OH beyond 20 mM did not dissipate ΔΨ any further. *Nitrobacter agilis* does not oxidize NH_4^+ or NH_2OH, but it assimilates small amounts (2 mM) of NH_4Cl (31). High concentrations of NH_4^+ (>10 mM) dissipated ΔpH completely and ΔΨ partially (20 mV at 100 mM NH_4^+).

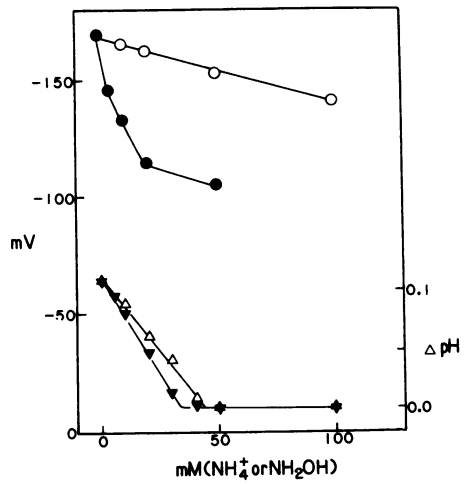

FIG. 3. Effects of NH_4^+ and NH_2OH on ΔΨ and ΔpH in washed cells of *Nitrosomonas europaea*. EDTA-treated washed cells were suspended in 50 mM Tris-hydrochloride (pH 6.9). ΔΨ was determined from the uptake data for $[^3H]TPP^+$ after the treatment of suspensions with either NH_4Cl (○) or NH_2OH (●) at special concentrations. ΔpH was calculated from the distribution of ^{14}C-benzoic acid in the presence of either NH_4Cl (▼) or NH_2OH (△).

DISCUSSION

The results indicate that at a pH^o of 7.0 for *Nitrosomonas europaea* and 7.5 for *Nitrobacter agilis* there was no transmembrane pH gradient (inside alkaline) because at these pH values neither weak acids nor weak bases were concentrated by the bacteria. The pH optimum for NH_4^+ and NO_2^- oxidation by *Nitrosomonas europaea* and *Nitrobacter agilis*, respectively, was between 7.5 and 8.0, indicating that optimally respiring cells of these bacteria do not have a ΔpH. *Nitrosomonas europaea* actively translocates protons (8, 9, 14, 19) during respiration, with $\rightarrow H^+/O$ ratios of up to 4.0 for either NH_4^+, NH_2OH, or NH_2NH_2 as the substrate (19). The results of this study indicate that for pH^o values above 7.0, the pH^i of *Nitrosomonas europaea* becomes more acid than the pH^o. Moreover, its pH^i did not remain constant as the pH^o was varied, so that *Nitrosomonas europaea* had a limited capacity to maintain a constant pH^i. This result contrasts with those reported for *Micrococcus lysodeikticus* (15) and *E. coli* under aerobic conditions (41), but is comparable to anaerobic bacteria, namely, *Methanospirillum hungatei* (20), *Clostridium pasteurianum* (45), and even *E. coli* grown under anaerobic conditions (25). In *Nitrobacter agilis* the pH^i remained relatively constant (7.3 to 7.8) over a range of pH^o values (6 to 8.5), which is similar to the results for *E. coli* (41), *Micrococcus lysodeikticus* (15), *Thiobacillus acidophilus* (36), and *Bacillus subtilis* (28) (for a recent review, see reference 42).

The weak base methylamine, used as a probe for the determination of ΔpH, is not oxidized by either nitrifier. Since ammonia and its analogs are probably taken up by *Nitrosomonas europaea* as neutral species (14, 19), it is unlikely that the cells would accumulate methylamine in response to a ΔΨ (inside negative) as reported for *Azotobacter vinelandii* (32).

At a pH^o of 6.0, neither *Nitrosomonas europaea* nor *Nitrobacter agilis* oxidized its respective substrate, but they still maintained a reasonable ΔpH and ΔΨ, and thus Δp. In fact, in *Nitrobacter agilis*, Δp was maximum at pH 6.0 (or less than 6.0), and it decreased linearly with an increase in pH^o. However, at pH 7.0, when both *Nitrosomonas europaea* and *Nitrobacter agilis* retain about half of their respiratory activities, the small pH gradient was dissipated by uncouplers and compounds which inhibit respiration. It is known that *Nitrosomonas europaea*

and *Nitrobacter agilis* have appreciable rates of endogenous respiration (19) involving complex organic substrates, e.g., poly-β-hydroxybutyric acid in *Nitrobacter agilis* (7). In *Nitrosomonas europaea*, endogenous respiration has been shown to be coupled to proton translocation (19). It is likely that this endogenous respiration enables the cells to maintain a reasonable Δp in the absence of exogenous substrates, or when the exogenous respiration is inhibited. This phenomenon could be of ecological significance for nitrifiers, because these soil bacteria in their natural habitat may encounter conditions that preclude respiration for extended periods of time.

In *Nitrosomonas europaea* the uncoupler CCCP severely inhibited respiration (80% at 10 μM CCCP) but lowered Δp by about 40 mV. At higher concentrations (100 μM CCCP) the respiration was completely inhibited, but the Δp was lowered by only 78 mV (from 158 to 80 mV). In *Nitrobacter agilis*, CCCP (50 μM) completely restricted respiration, but nonrespiring cells still maintained a Δp of 82 mV (inside negative). Uncouplers are known to restrict respiration in nitrifying bacteria (2, 4, 7, 8, 30), but it is only recently that they have been shown to inhibit respiration in other bacteria, e.g., *T. acidophilus* (36). Besides the nitrifiers, uncouplers have been shown to inhibit respiration in denitrifying bacteria (50). Thus in *Pseudomonas aeruginosa* and *Pseudomonas denitrificans* the effects of uncouplers on respiration were not linked to a collapse of Δp but rather to their detergent-like effects on cell membranes (50). It is possible that the mechanism of inhibition of respiration by CCCP and other uncouplers in *Nitrosomonas europaea* and *Nitrobacter agilis* is similar to that in the denitrifying bacteria.

Because the inhibitors known to collapse membrane potential also restricted nitrite oxidation in *Nitrobacter winogradskyi*, Cobley (11, 12) predicted that respiration was ΔΨ dependent. The way in which nitrite oxidation is mediated by a ΔΨ is, however, not understood. He also reported (11, 12) that NO_2^- oxidation by membrane particles of *Nitrobacter winogradskyi* was stimulated by NH_4^+, a compound known to collapse ΔpH (11). The results reported in this paper indicate that NH_4^+ diminished the ΔpH in *Nitrobacter agilis*, but in a recent study (31) with washed cells of *Nitrobacter agilis* we found that NH_4^+ inhibited NO_2^--dependent O_2 uptake and that NH_4^+ competed with either NO_2^- translocation or NO_2^- oxidation, because NH_4^+ inhibition could be reversed by increasing nitrite. Cobley's prediction that NH_4^+ stimulation of NO_2^- oxidation resulted from a collapse of ΔpH is not substantiated by our data.

DCCD and DESB, inhibitors of ATPase (10), severely restricted respiration in both nitrifying bacteria, indicating a close functional relationship between ATPase and oxidoreductase systems of these bacteria. Whether this inhibition was associated with a collapse of Δp is not clear, because DESB elevated Δp in *Nitrosomonas europaea* rather than lowering it. It is possible, however, that in washed cells these inhibitors are nonspecific and affect other metabolic functions. Recently DCCD has been shown to inhibit beef heart cytochrome oxidase function (44).

The results reported in this paper indicate that the total Δp at pH 7.5 would be approximately 150 mV and 125 mV (inside negative) in *Nitrosomonas europaea* and *Nitrobacter agilis*, respectively. These values are comparable to those reported for *E. coli* (41) and *Methanobacterium thermoautotrophicum* (20). In *E. coli*, Δp is composed of both ΔΨ and ΔpH; however, ΔpH appears to be absent in *Methanobacterium thermoautotrophicum* and *Methanospirillum hungatei* (20). The overall behavior of ΔΨ and ΔpH in nitrifying bacteria is quite similar to that reported for other bacteria. By using isolated membrane vesicles from *Bacillus alcalophilus* it has been shown (35) that respiration results in proton extrusion, whereas cation H^+ antiporters (K^+/H^+ and Na^+/H^+) catalyze inward proton movements. These cation H^+ antiporters have also been found in *E. coli* (see reference 42 for review) and can explain how an electrical potential (inside negative) is supported in the absence of a ΔpH or when the pH^i of the bacterium is lower than the pH^o (inside acid). A similar mechanism has been suggested for *Methanobacterium thermoautotrophicum* and *Methanospirillum hungatei* (20). This is also a likely mechanism for nitrifiers, although the existence of such systems in nitrifying bacteria has yet to be determined.

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