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Energy transduction efficiencies in nitrogenous oxide respirations of *Azospirillum brasilense* Sp7

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Abstract. For *Azospirillum brasilense* Sp7, the energy transformation efficiencies were measured in anaerobic respirations with either nitrate, nitrite or nitrous oxide as respiratory electron acceptors by determining the maximal molar growth yields and the H^+-translocations using the oxidant pulse method. In continuous cultures grown with malate limiting, the maximal molar growth yields (Y_s^{max}-values) were essentially the same with O_2 or N_2O but were 1/3 and 2/3 lower with NO_2^- or NO_3^-, respectively, as respiratory electron acceptors. Both the maximal molar growth yields and the maintenance energy coefficients were surprisingly high when *Azospirillum* was grown with nitrite as the sole electron acceptor and source for N-assimilation. Growth under N_2-fixing conditions drastically reduced the Y_s^{max}-values in the N_2O and O_2-respiring cells. In the H^+-translocation measurements, the $\vec{H}^+$/oxidant ratios were 5.6 for $O_2 \rightarrow H_2O$, 2.5–2.8 for $NO_3^- \rightarrow NO_2^-$, 2.2 for $NO_2^- \rightarrow N_2O$ and 3.1 for $N_2O \rightarrow N_2$ respirations when the cells were preincubated with valinomycin and K^+. All the values were enhanced when the experiments were performed with valinomycin plus methyltriphenylphosphonium (= $TPMP^+$) cation. The uncoupler carbonyl cyanide-m-chlorophenylhydrazone diminished the H^+-excretion indicating that this translocation was due to vectorial flow across the membrane. In the absence of any ionophore, nitrate and nitrite respirations were accompanied by a H^+-uptake ($NO_3^- \rightarrow N_2 = -2.9\,\vec{H}^+/NO_3^-$ and $NO_2^- \rightarrow N_2 = -2.5\,\vec{H}^+/NO_2^-$). Any significant H^+-translocation could not be detected in N_2O- and O_2-respirations under these conditions. It is concluded that nitrate reduction proceeds inside the cytoplasmic membrane, whereas nitrite is reduced extramembraneously. The data are not conclusive for the location of nitrous oxide reductase. The maximal molar growth yield determinations and the absence of any H^+-uptake in untreated cells indicate a cytoplasmic orientation of the enzyme similar to the terminal cytochrome oxidase of respiration. The low H^+-extrusion values for N_2O-respiration compared to O_2-respiration in cells treated with valinomycin plus $TPMP^+$ are, however, not in accord with such an interpretation.

Key words: Denitrification – Growth yield measurements – Nitrate respiration – Nitrogen fixation – Proton translocations in respirations – *Azospirillum*

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In denitrification, bacteria reduce the respiratory electron acceptor nitrate to NO_2^-, N_2O and N_2 (Bryan 1981; Knowles 1982; Stouthamer 1988). Evidence is now accumulating that NO is also an obligate intermediate in denitrification (Zumft et al. 1987; Goretski and Hollocher 1988). Most denitrifying bacteria can also grow with either NO_2^- or N_2O as the sole respiratory electron acceptor when neither O_2 nor NO_3^- are available. The energy transformation efficiencies in O_2, NO_3^-, NO_2^- and N_2O respirations were determined from proton translocation experiments using the oxidant pulse method (Scholes and Mitchell 1970) or from maximal molar growth yield measurements under carbon limiting conditions (Pirt 1965). Such experiments were performed with relatively few bacterial species. The data allowed to draw conclusions about the orientation of the enzymes involved in denitrification on the cytoplasmic membrane. In *Paracoccus denitrificans*, nitrite reductase and nitrous oxide reductase are situated at the periplasmic side, whereas nitrate reductase is at the cytoplasmic face (Boogerd et al. 1981, 1984; Alefounder and Ferguson 1980; Parsonage and Ferguson 1983). In contrast, the experiments of Kristjansson et al. (1978) indicated a reduction of NO_3^-, NO_2^- and N_2O inside the cytoplasmic membrane in the same bacterium. The investigations with *Pseudomonas denitrificans* (Koike and Hattori 1975b), *Wolinella succinogenes* (Shapleigh and Payne 1986) and the photosynthetic bacteria *Rhodopseudomonas sphaeroides f. denitrificans* (Kundu and Nicholas 1985) and *Rhodobacter capsulatus* (McEwan et al. 1985, 1987) indicated that all the three enzymes are located at the periplasmic side of the cytoplasmic membrane. It is not yet clear whether these differences are species specific or are a consequence of technical difficulties in performing the experiments.

Investigations of this type have not yet been performed with *Azospirillum*. Bacteria of this genus are currently much discussed as potential biofertilizers because of their capability to perform N_2-fixation (Döbereiner and Pedrosa 1987) and to produce phytohormones (Tien et al. 1979; Zimmer and Bothe 1988; Zimmer et al. 1987, 1988). *Azospirillum* cannot utilize common organic substrates like malate or succinate by fermentation. It can grow anaerobically with either NO_3^- (Nelson and Knowles 1978; Bothe et al. 1981), NO_2^- (Zimmer et al. 1984) or N_2O (Penteado Stephan et al. 1984) as terminal respiratory electron acceptor both in batch and continuous cultures as well as with nitrate in model experiments in wheat associations (Neuer et al. 1985). *Azospirillum* converts N_2O to molecular nitrogen without

the formation of ammonia. The bacterium must, therefore, synthesize nitrogenase from protein reserves when no other nitrogen source besides N_2O is available in the medium (Penteado Stephan et al. 1984). In contrast, nitrate or nitrite in the medium represses nitrogenase synthesis and induces the formation of the assimilatory nitrate and nitrite reductases (Zimmer et al. 1984; Penteado Stephan et al. 1984).

The present paper compares the energy transformation efficiencies in O_2-respiration and denitrification in *Azospirillum brasilense* Sp7 by determining the molar growth yields in carbon limited cultures and the proton translocation efficiency using the oxidant pulse method. The orientation of the enzymes involved in denitrification on the cytoplasmic membrane will be evaluated.

Materials and methods

Growth of the organism

The type strain, *Azospirillum brasilense* Sp7, was used in all the experiments and was grown in batch and continuous cultures as described previously (Zimmer et al. 1984; Zimmer 1988). For the growth yield determinations, the continuous cultures were grown at 30° C and pH 7.8 with 4 mM L-malate as the limiting nutrient and with either NO_3^-, NO_2^-, N_2O or O_2 as respiratory electron acceptor. The fermenter was continuously flushed with N_2 (purity 99.995%) or with a mixture of 90% N_2 and 10% O_2 or 90% N_2 and 10% N_2O (v/v), respectively (rate approx. 38 cm^3 gas/min). The concentrations of O_2 and N_2O were monitored by a conventional Clark-type electrode as described previously (Zimmer et al. 1985). The concentration of dissolved O_2 was less than 0.05% under anaerobic growth with either NO_3^-, NO_2^- or N_2O as respiratory electron acceptor. When added, the concentration of NH_4Cl was 5 mM in the medium.

When *Azospirillum brasilense* Sp7 was grown in batch cultures with excess of malate (10 mM) and at pH 7.0 under gassing with air, cells did not express dissimilatory nitrite and nitrous oxide reductases (Penteado Stephan et al. 1984). Therefore such cells reduced nitrate only to nitrite.

Proton translocation experiments (for details see Danneberg 1987). Cells from the continuous cultures grown with limiting amounts of malate as the carbon source and with nitrite as respiratory electron acceptor were centrifuged ($16000 \times g$/10 min) and washed twice with a 150 mM KCl-solution. The cells were suspended in 150 mM KCl plus 1 mM DL-malate and made O_2-free by repeatedly evacuating and flushing with argon. The anaerobic suspension of bacteria (routinely 1 ml of about 4 mg protein) was injected into a 4.2 ml Plexiglas cuvette equipped with a pH-electrode (Type GK 2401 C, Radiometer Comp., Copenhavn). The electrode was connected to a pH-meter (Type PHM 61, Radiometer). The pH-changes were monitored with a conventional recorder (Kipp and Zonen BD 40). A full scale response of 20 cm coresponded to 0.2 pH units.

The measurements of the H^+-translocations were performed almost exactly as described by Boogerd et al. (1981). Before the start of the actual experiment the cuvette was gassed with argon for several min. All the following manipulations were performed under continuous flushing with argon to avoid diffusion of O_2 from air into the cuvette. After the addition of the bacteria to the cuvette, the suspension was made up to 3 ml by adding an anaerobic solution of 150 mM KCl. The suspension did not contain extra buffer, but was made up to pH 7 by adding either HCl or KOH. The cells were then equilibrated for 15–30 min until no significant base line drift was observed any more. The experiments were started by adding the pulses of electron acceptors (10 µl each, dissolved in an anaerobic solution of 150 mM KCl, corresponding to 2.37 nmol O_2, 10 nmol KNO_3, 10 nmol KNO_2 and 11.04 nmol N_2O, respectively). In control experiments, the addition of anaerobic KCl solutions to the cell suspensions did not alter the pH. For the calculations of the data, the $\vec{H}^+$/electron acceptor ratios were corrected by extrapolating the tangent of the initial decay phase back to the moment of the electron acceptor addition (see Boogerd et al. 1981). The system was calibrated by adding known amounts of anaerobic HCl to the bacterial suspension in the cuvette.

Other determinations

N_2 and O_2 concentrations were determined in a gaschromatograph equipped with a thermal conductivity detector and a molecular sieve 0.5 nm column. N_2O was also measured in a gaschromatograph equipped with the same detector using a Porapak Q column (see Danneberg 1987). Poly-β-hydroxybutyrate content was determined as described by Law and Slepecky (1961), nitrite with Griess-Ilosvay's reagent (Snell and Snell 1949) and nitrate with salicylic acid (Cataldo et al. 1975). The protein concentrations in cell suspensions were assayed by a modified Lowry method (Herbert et al. 1971).

Results

Molar growth yield determinations

When *Azospirillum* was grown in continuous culture at different dilution rates with malate limiting and with a surplus of one of the respiratory electron acceptors O_2, NO_3^-, NO_2^- or N_2O, at the utmost 1% of the dry weight of the cells consisted of the storage compound poly-β-hydroxybutyrate (Zimmer et al. 1984). The reducing equivalents for respiration were apparently limiting in such cultures, which can also be deduced from experiments with nitrate in which 96% of the nitrate added was recovered as nitrite. Similarly, upon growth with nitrite, reduction almost completely stopped at N_2O and did not proceed to N_2 as were to be expected at an excess of reductant. In addition, the N_2O formed was immediately removed from the cultures by the continuous gassing with N_2. Exact stoichiometries between NO_2^--utilization and N_2O-formation were difficult to be determined, but it could be estimated that at best 1/6 of the reducing equivalents were utilized for a reduction of N_2O to N_2. The efficiencies in energy transformations of either $NO_3^- \rightarrow NO_2^-$, $NO_2^- \rightarrow N_2O$, $N_2O \rightarrow N_2$ or $O_2 \rightarrow H_2O$ respirations were, therefore, directly proportional to the molar growth yields of the cultures (Y_s) which were determined as g dry weight of cells formed per mol malate consumed. As in the preceeding publications (Zimmer et al. 1984; Penteado Stephan et al. 1984), steady state conditions at the different dilution rates could be maintained for 3–4 weeks at least as indicated by the constant protein content in the cell suspension during this period. In the dilution range assayed, plots of $1/Y_s$ against 1/D gave straight lines for the growth with ammonia as the nitrogen source and with either O_2, NO_3^- or NO_2^- as the respiratory electron acceptor and for

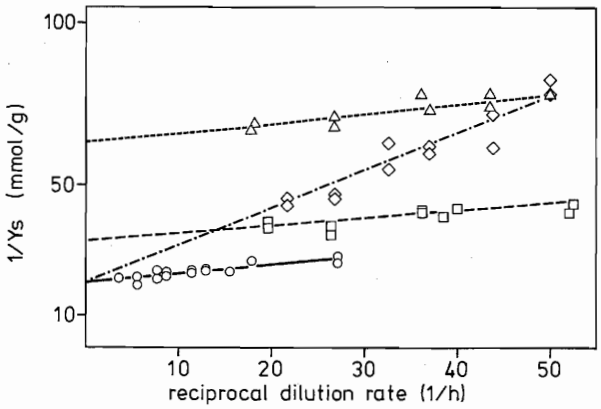

Fig. 1. Determination of Y_s^{max} of chemostat cultures grown with malate limiting and with either O_2, NO_3^- or NO_2^- as respiratory electron acceptor. For experimental details see Materials and methods. NH_4Cl as the source for nitrogen assimilation. Respiratory electron acceptor: ○——○ O_2; △·····△ NO_3^-; □–––□ NO_2^-; ◇–·–·–◇ NO_2^- as respiratory electron acceptor and as source for nitrogen assimilation

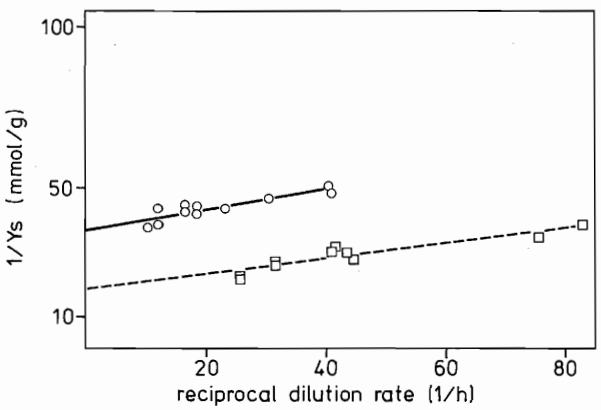

Fig. 2. Determination of Y_s^{max} of chemostat cultures with malate limiting and with N_2O as respiratory electron acceptor: □–––□ cells grown with NH_4Cl as source for N-assimilation; ○——○ cells grown with N_2 as source for N-assimilation

the culture grown with nitrite both as N-source and electron acceptor (Fig. 1). The same was true for continuous cultures grown with N_2O as respiratory electron acceptor, both under N_2-fixing conditions and with ammonia as the N-source (Fig. 2). Such a direct relationship between molar growth yield (Y_s) and dilution (D) was predicted from the Pirt equation (Pirt 1965)

$$1/Y_s = 1/Y_s^{max} + m/D$$

with Y_s^{max} as the maximal molar growth yield extrapolated to infinite dilution rate and m as the maintenance energy coefficient in mol malate consumed/(h × mg dry weight of cells). The intercept of the straight lines with the ordinate gave the reciprocal Y_s^{max}-data, and the maintenance energy coefficient was determined from the slopes of the lines.

When the cells were grown with ammonia as the N-source, the Y_s^{max}-values were essentially the same with O_2 and N_2O as respiratory electron acceptors. The Y_s^{max}-yields with NO_2^- and NO_3^- were 1/3 and 2/3 lower, respectively, than those with O_2 or N_2O. When the cultures had to use

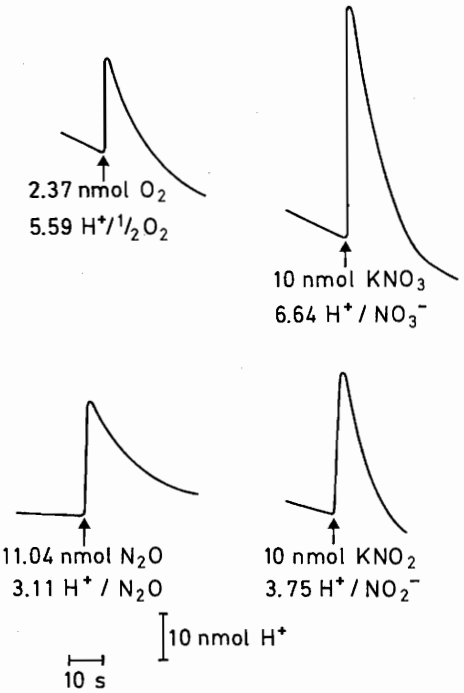

Fig. 3. Proton translocations by valinomycin-treated *Azospirillum* in dependence of pulses of different respiratory electron acceptors. These oxidant pulse experiments were performed with cells grown in continuous culture with nitrite as respiratory electron acceptor. Prior to the experiment, *Azospirillum* was incubated with valinomycin (50 µg/ml cells) for 3 h. Cells with a total protein content in the cuvette of 3.8 mg were assayed. For other experimental details see Materials and methods

nitrite both as respiratory electron acceptor and as source for N-assimilation, the Y_s^{max}-value was 48.0 and thus surprisingly high. The yield was reduced to 30.5 when the cells were supplemented with both nitrite and ammonia. Under N_2-fixing conditions, the molar growth data were reduced to one half as compared to growth with ammonia, but were the same for N_2O- and O_2-respiring cells (Table 1). The determined Y_s^{max}-value for N_2O-respiring cells grown under N_2-fixing conditions (= 27.0) closely matched that determined by Kloss et al. (1983) for N_2-fixing and O_2-respiring *Azospirillum* (= 26.8).

The maintenance energy coefficients with each of the electron acceptors were more or less the same under all continuous culture conditions employed (Table 1). A remarkable exception, however, was the growth with NO_2^- as electron acceptor and N-source, where the coefficient was 3–4-fold higher than in other cultures. Similarly, the maintenance coefficient was 0.6 mmol/h × g dry weight when the cultures utilized both NO_3^- and NO_2^- in the medium (Zimmer, Diplomarbeit, The University of Köln, 1984).

Proton translocation determinations by the oxidant pulse method

In this type of experiments, cells from continuous cultures grown with nitrite were supplemented with 1 mM malate after washing. In contrast to the experimental approach in the molar growth determinations, such cells were not reductant limited. Reductant (1 µmol malate in the cuvette) was nearly 100-fold in excess over oxidant (less than 15 nmol,

Table 1. Molar growth yields (Y_S^{max}) of chemostat cultures of *Azospirillum brasilense* Sp 7 using different respiratory electron acceptors and different nitrogen sources

Respiratory electron acceptor	NO_3^-	NO_2^-	NO_2^-	N_2O	N_2O	O_2	O_2
Source for N-assimilation	NH_4^+	NH_4^+	NO_2^-	NH_4^+	N_2	NH_4^+	N_2
Y_S^{max} (g mol^{-1})[1]	15.9	30.5	48.0	54.1	27.0	49.9	26.8[3]
m(mmol g^{-1} h^{-1})[2]	0.29	0.25	1.08	0.24	0.33	0.30	0.33[3]
Y_S^{max}:Y_S^{max} of the control[4]	0.32:1	0.61:1	0.96:1	1.08:1	0.54:1	–	0.54:1

[1] Y_S^{max}, maximum growth yield, extrapolated to infinite dilution rate (see Pirt 1965)
[2] m, maintenance energy coefficient
[3] Data from Kloss et al. (1983)
[4] Control = culture grown with NH_4^+ as the nitrogen source and O_2 as the respiratory electron acceptor
The continuous cultures were grown with malate as the limiting nutrient and with an excess of the respiratory electron acceptors

Table 2
Stoichiometry between H^+-translocation and consumption of respiratory electron acceptors in valinomycin treated *Azospirillum*

Electron acceptor and reaction	Determined ratio $\vec{H}^+$/electron acceptor	Calculated ratio $\vec{H}^+$/electron acceptor
1. $0.5\,O_2 + 2\,e^- + 2\,H^+ \rightarrow H_2O$	5.6 ± 0.8 (18)	
2. $NO_3^- + 5\,e^- + 6\,H^+ \rightarrow 0.5\,N_2 + 3\,H_2O$	6.6 ± 0.8 (15)	
3. $NO_2^- + 3\,e^- + 4\,H^+ \rightarrow 0.5\,N_2 + 2\,H_2O$	3.8 ± 0.9 (14)	
4. (NO_3^- minus NO_2^-) = (line 2 minus line 3) $NO_3^- + 2\,e^- + 2\,H^+ \rightarrow NO_2^- + H_2O$		2.8
5. $N_2O + 2\,e^- + 2\,H^+ \rightarrow N_2 + H_2O$	3.1 ± 0.8 (16)	
6. (NO_2^- minus N_2O) = (line 3 minus 0.5 line 5) $NO_2^- + 2\,e^- + 3\,H^+ \rightarrow 0.5\,N_2O + 1.5\,H_2O$		2.2
7. $NO_3^- + 2\,e^- + 2\,H^+ \rightarrow NO_2^- + H_2O$	2.5 ± 0.5 (5)	

The experiments (line 1, 2, 3 and 5) were performed with *Azospirillum* cells grown in continuous cultures with nitrite as respiratory electron acceptor (at $D = 0.026\ h^{-1}$ and pH = 7.8). These cells reduced NO_3^- to N_2, NO_2^- to N_2 and N_2O to N_2 without the formation of free intermediates (NO_2^-, N_2O) in the oxidant pulse experiments. The experiment of line 7 was done with cells grown under batch culture conditions and with O_2 as the respiratory electron acceptor. Such cells reduced NO_3^- to NO_2^-, but not to N_2O or N_2. The concentration of the oxidant pulses were in µmol: KNO_3 10, KNO_2 10, N_2O 11.04, O_2 2.37. The total protein content of the cells in the cuvette varied between 3.5 and 4.2 mg. Prior to the experiments the cells were preincubated with valinomycin (50 µg/ml) for 3 h. For other experimental details see Material and methods. The number of experiments performed are given in brackets

see Fig. 3). The cells had expressed dissimilatory nitrate, nitrite and nitrous oxide reductases and quantitatively reduced NO_3^-, NO_2^- or N_2O to N_2 without any lag phase. The addition of pulses of either O_2, KNO_3, KNO_2 or N_2O resulted in a sharp raise of the signal caused by respiration-dependent H^+-extrusion. This was followed by a decay of the signal with a half time of 20–30 s due to flow back of H^+ (Fig. 3). The size of the H^+-extrusion signal did not change significantly when up to 20 further oxidant pulses were added after the recovery from the preceeding one (not documented) which also indicates that the reductant was not limiting in the cells. The experiments were performed with valinomycin which collapsed the membrane potential and increased the ion permeability (Nicholls 1982). A concentration of 50 µg valinomycin/ml cells with 4 mg protein and a preincubation period of 3 h was optimal for maximal H^+-extrusion. In similar experiments by others (Scholes and Mitchell 1970; Kristjansson et al. 1978; Kundu and Nicholas 1985), the H^+/oxidant ratios were dependent on the amount of oxidant added with the smallest oxidant pulses giving the maximal ratios. In the present study, smallest oxidant pulses possible were, therefore, applied for obtaining statistically sound data (Table 2).

A direct comparison of the $\vec{H}^+$/oxidant ratios determined (5.6 for $O_2 \rightarrow H_2O$, 6.6 for $NO_3^- \rightarrow 1/2\ N_2$, 3.8 for $NO_2^- \rightarrow 1/2\ N_2$, 3.1 for $N_2O \rightarrow N_2$, Table 2) was not valid, since the conversion of only O_2 to H_2O and N_2O to N_2 involved $2e^-$-transfers. To get the data for the $\vec{H}^+$/$NO_3^- \rightarrow NO_2^-$ ratio, cells were assayed which were grown aerobically in batch cultures at pH 7.0 and which had not expressed dissimilatory nitrite and nitrous oxide reductases (see Materials and methods). Such cells reduced nitrate quantitatively to nitrite, and the H^+/NO_3^- determined was 2.5 (Table 2). For measuring the H^+-translocation stoichiometries for the transitions $NO_3^- \rightarrow 1/2\ N_2O$ and $NO_2^- \rightarrow 1/2\ N_2O$, cells from the continuous cultures grown with NO_2^- were assayed in the presence of rhodanide which is known to block nitrous oxide reduction completely (Boogerd et al. 1981; Kundu and Nicholas 1985). The inhibitor, indeed, totally blocked H^+-extrusion in N_2O respiration and did not affect NO_2^--reduction also in the case of *Azospirillum* (Table 3). The $\vec{H}^+/NO_2^- \rightarrow 1/2\ N_2O$ ratio was 2.2 measured in the presence of KSCN (Table 3) which matches the difference between nitrite and nitrous oxide reduction (see difference between line 3 and 5 in Table 2). Thus the presented ratios were consistent with each other. For the conversion

Table 3. Inhibition of the H^+-translocation by rhodanide in valinomycin treated *Azospirillum*

Electron acceptor and reaction	Determined ratio $\vec{H}^+$/ electron acceptor
1. $0.5\ O_2 + 2\ e^- + 2\ H^+ \rightarrow H_2O$	5.7 ± 0.7 (14)
2. $NO_3^- + 4\ e^- + 5\ H^+ \rightarrow 0.5\ N_2O + 2.5\ H_2O$	4.2 ± 0.5 (13)
3. $NO_2^- + 2\ e^- + 3\ H^+ \rightarrow 0.5\ N_2O + 1.5\ H_2O$	2.2 ± 0.4 (13)
4. $N_2O + 2\ e^- + 2\ H^+ \rightarrow N_2 + H_2O$	0 (5)

The same experimental conditions were employed as in Table 2. The final concentration of KSCN was 30 mM which was directly injected into the cuvette 15 min prior to the addition of the oxidants. Cells from the continuous cultures grown with nitrite were used. For experimental details see Table 2 and Materials and methods

of nitrate to N_2O, the difference between nitrate and nitrous oxide reduction (line 2 minus 1/2 line 5 of Table 2) gave a value of 5.0 which was slightly higher than the ratio determined in the experiment with KSCN (4.2, see Table 3). This latter lower value might be explained by the fact that nitrate reductase was slightly affected by this inhibitor in *Azospirillum* as in other organisms (Boogerd et al. 1981; van't Riet et al. 1975).

Uncouplers like carbonyl cyanide m-chlorophenylhydrazone (= CCCP) diminish the capability of the cytoplasmic membrane to exclude protons. When valinomycin-treated cells were preincubated with 10 µM CCCP for 15 min, no pH-changes were detected with both O_2 or N_2O as the electron acceptor. The uncoupler induced alkalisations with stoichiometries of $\vec{H}^+/NO_3^- \rightarrow N_2 = -1.3$ and $\vec{H}^+/NO_2^- \rightarrow N_2 = -1.4$ (Danneberg 1987). Such roughly 1:1 stoichiometries might reflect the charge compensation during the reduction of the anions nitrate and nitrite to electroneutral N_2. The controls with CCCP plus valinomycin indicate that the H^+-translocations observed with valinomycin alone were, indeed, due to a vectorial flow across the cytoplasmic membrane in nitrogenous oxides dependent respirations.

The lipophilic methyltriphenylphosphonium cation ($TPMP^+$) can also be used to measure the membrane potential and to determine the maximal H^+-extrusion (Boogerd et al. 1981; Nicholls 1982). As valinomycin, $TPMP^+$ at an optimal concentration of 10 mM caused a rapid translocation followed by a slow flow back of H^+ (not documented). Higher concentrations of $TPMP^+$ were slightly inhibitory (Fig. 4a), probably because of the damaging effect of high amounts of this cation on the cytoplasmic membrane (Demura et al. 1985). It should be pointed out that the relative contributions of O_2, NO_3^-,NO_2^- and N_2O respirations in the H^+-efflux were the same when either $TPMP^+$ or valinomycin were applied.

The addition of both valinomycin and $TPMP^+$ enhanced the $\vec{H}^+/2e^-$ ratios slightly with each of the respiratory electron acceptors but left the relative contributions unchanged (Fig. 4b). The $\vec{H}^+/2e^-$ ratio reached 7.2 for O_2-respiration (Fig. 4b). As already noted by others (Boogerd et al. 1981), neither valinomycin nor $TPMP^+$ alone could completely diminish the membrane potential and cause maximal H^+-extrusion, which was, however, achieved by the addition of both compounds.

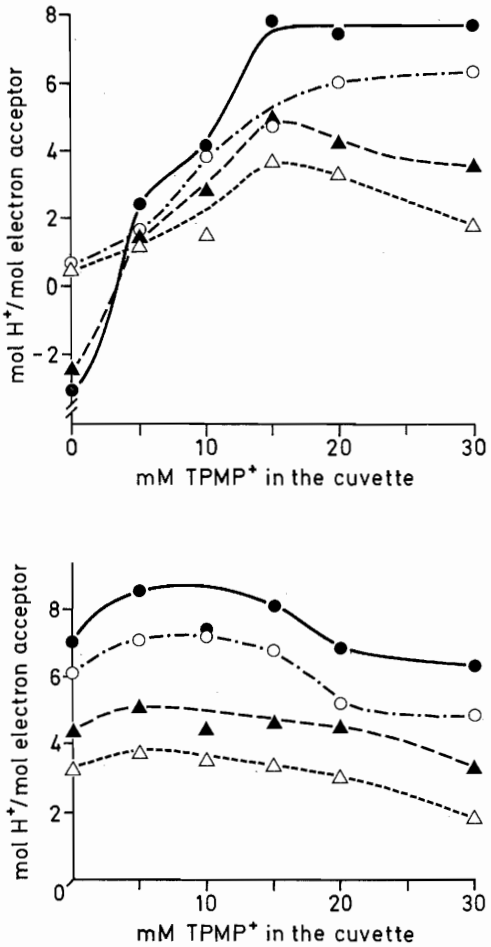

Fig. 4. a Proton translocations by *Azospirillum* assayed in the presence of $TPMP^+$. *Azospirillum* from continuous cultures grown with nitrite was tested. The cells were injected into the cuvette (altogether 3.5 mg protein) which was then supplemented with the different concentrations of $TPMP^+$. After 15 min of incubation with $TPMP^+$, the assay was started by adding the pulses of the respiratory electron acceptors: ●——● KNO_3 (10 nmol added per cuvette); ▲----▲ KNO_2 (10 nmol); △·····△ N_2O (11.04 nmol); ○–·–·–○ O_2 (2.37 nmol). **b** Proton translocation by *Azospirillum* assayed in the presence of $TPMP^+$ plus valinomycin. Same experimental conditions as in Fig. 4b, but the cells were preincubated with valinomycin (50 µg/ml) for 3 h

In the absence of any ionic species mobile across the membrane, the potential across the cytoplasmic membrane limits the H^+-efflux which is under 0.5 pH units in most bacteria (Nicholls 1982; Harold 1986). As the membrane is impermeable for a H^+-diffusion, only the protons consumed extramembraneously during the reduction of the respiratory electron acceptors should be seen. With O_2 or N_2O as respiratory electron acceptor, a H^+-extrusion in *Azospirillum* could virtually not be detected in these experiments performed without valinomycin (Fig. 5). The addition of either KNO_2 or KNO_3 resulted in a rapid alkalisation followed by a slow recovery afterwards (Fig. 5). The values were -2.5 for $\vec{H}^+/NO_2^- \rightarrow N_2$ and -2.9 for $\vec{H}^+/NO_3^- \rightarrow N_2$. Such difference between NO_3^- and NO_2^- respirations was, however, not statistically significant (Table 4). For both NO_3^- and NO_2^--respirations, the ratios did not reduce significantly when up to 20 further oxidant pulses were applied

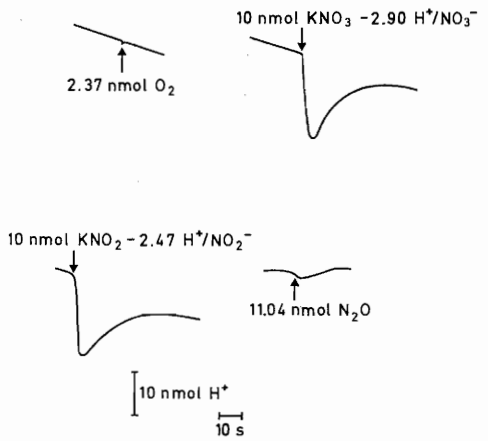

Fig. 5. Proton uptake by *Azospirillum* not treated by an ionophore in dependence of pulses of the different respiratory electron acceptors. Same experimental conditions as in Fig. 3, but the cells were not preincubated with valinomycin. The total protein content in the cuvette was 3.7 mg in this experiment

Table 4. The H^+-translocation by *Azospirillum* not treated with an ionophore

Electron acceptor and reaction	$\vec{H}^+$/electron acceptor
1. $0.5\ O_2 \rightarrow H_2O$	0 (14)
2. $NO_3^- \rightarrow N_2$	-2.9 ± 0.8 (14)
3. $NO_2^- \rightarrow N_2$	-2.5 ± 0.6 (14)
4. $N_2O \rightarrow N_2$	0 (14)

The experimental conditions are the same as in Table 2. Cells from the continuous cultures grown with nitrite were directly assayed without treatment with valinomycin or any other ionophore

(not documented). *Azospirillum* from the continuous culture grown with nitrite were used for this type of experiments. When cells were assayed from batch cultures which reduced nitrate only to nitrite, the extent of the H^+-extrusion was less than 0.2 $\vec{H}^+/NO_3^-$ (not documented).

Discussion

The Pirt equation was successfully applied in the past for determining the maximal molar growth yields and the maintenance energy coefficients in bacterial cultures grown under carbon limiting conditions (Koike and Hattori 1975a, b; Mell et al. 1982; Kloss et al. 1983). There is some debate about the validity of the equation, and it is now accepted that the equation needs modifications under carbon sufficient growth conditions where the term maintenance energy is more complicated (Pirt 1982). Even under energy source limitations, deviations from the Pirt equations were described for the extremes, in the very high and very low dilution range (van Verseveld et al. 1984). In the dilution range assayed in the present study, the data fit the Pirt equation (see straight lines in Fig. 1 and 2), and extrapolation to infinite dilution gives approximations for the maximal growth yields with the different electron acceptors.

The present study shows also for *Azospirillum* that molar growth yields are much lower when the N-demand is met by N_2-fixation and not by ammonia. The data are very close to those determined for *Rhizobium* ORS 571 at a specific growth rate of 0.1 h^{-1} (Stam et al. 1984). These differences in the growth yields between N_2-fixing and ammonia grown cells reflect the high energy costs required for N_2-fixation. The ATP/N_2 ratio in intact cells was determined as 20 for *Clostridium pasteurianum* (Daesch and Mortenson 1967) or 30 for *Klebsiella pneumoniae* (Hill 1976).

In the present study the Y_s^{max}-value for NO_3^- was 2/3 lower than that for O_2. A lower energy yield in nitrate respiration is to be expected from the normal potentials which are $E_o' = +0.43$ V for the NO_2^-/NO_3^- and $E_o' = +0.81$ V for the H_2O/O_2 couple (Thauer et al. 1977). In experiments with other bacteria, the extent of the energy conservation during nitrate respiration was higher (Koike and Hattori 1975b; Boogerd et al. 1984) than with *Azospirillum* in the present study. In vesicles from *Paracoccus* (= *Micrococcus*) *denitrificans*, using NADH as the electron donor, the ATP/$2e^-$-ratios for O_2 and NO_3^- respirations were 1.5 and 0.9, respectively (John and Whatley 1970). The lower energy yield determined in the present study for NO_3^- respiration in *Azospirillum* may be explained by the findings that the continuous cultures formed 8 mM nitrite in the steady state. Nitrate is reduced inside the bacteria (see below), and a large part of the energy available may be expended to remove toxic nitrite from the cells.

As far as we are aware of, this is the first report of a molar growth yield determination for a continuous culture growing with nitrite as respiratory electron acceptor and under carbon limitation. When nitrite is both respiratory electron acceptor and source for N-assimilation, both the Y_s^{max}-value and the maintenance energy coefficient (see Table 1) are high. The E_o' for N_2O/NO_2^- is + 0.94 V (Thauer et al. 1977) which is in accord with such a high Y_s^{max}-value. Such results were consistently obtained but cannot be explained convincingly. The lower Y_s^{max}-value for NO_2^--respiration when the medium is supplemented with ammonia can possibly be due to the uncoupling effect of ammonia. NH_4^+ is actively transported into the cells but NH_3 is rapidly diffusing across the cytoplasmic membrane. A cyclic in/out movement of NH_4^+/NH_3 is, therefore, an energy expensive process for the cells (Kleiner 1985).

The high maintenance energy required for the growth with nitrite may possibly be due to the damaging effect of nitrite. Dissimilatory nitrite reductase is at the periplasmic side of the cytoplasmic membrane, but for assimilation, nitrite must come into the cells. Nitrite may harm a lot of proteins or other cell components inside the cells (Azhar et al. 1986). A rapid and energy expensive resynthesis of the cell components might be necessary to maintain cell metabolism.

Growth yields with N_2O or O_2 were the same when compared to each other in either N_2-fixing or in NH_4^+-grown cells. Energy generation in N_2O and O_2 respiration can thermodynamically be the same, because the E_o' for N_2/N_2O is + 1.36 V and is thus even more positive than that for $H_2O/1/2\ O_2$ (Thauer et al. 1977). To obtain such high growth yields, the number of H^+-generating loops must be the same for N_2O and O_2 respiration. This is a strong indication that nitrous oxide reductase is located at the cytoplasmic face as the terminal cytochrome oxidase of O_2-respiration.

In the H^+-translocation experiments, the maximal $\vec{H}^+/O$ value obtained by incubating with valinomycin plus $TPMP^+$ was 7.2. Thus the $\vec{H}^+/O$ quotient might be 8–9 and not 6

Table 5. Calculated proton consumptions during dissimilatory reduction of different N-oxides in the absence of ionophores

	Assumed locations of the different reductions							
NO_3^--reductase	periplasmic	periplasmic	periplasmic	periplasmic	cytoplasmic	cytoplasmic	cytoplasmic	cytoplasmic
NO_2^--reductase	periplasmic	periplasmic	cytoplasmic	cytoplasmic	periplasmic	periplasmic	cytoplasmic	cytoplasmic
N_2O-reductase	periplasmic	cytoplasmic	periplasmic	cytoplasmic	periplasmic	cytoplasmic	periplasmic	cytoplasmic
Reaction								
$NO_3^- \rightarrow N_2$	6	5	3	2	4	3	1	0
$NO_2^- \rightarrow N_2$	4	3	1	0	4	3	1	0
$N_2O \rightarrow N_2$	2	0	2	0	2	0	2	0

in *Azospirillum* as in other bacteria (Scholes and Mitchell 1970; Meijer et al. 1977; Kristjansson et al. 1978; Stam et al. 1984). The H^+-yields for the respirations with the other electron acceptors were considerably lower ($\vec{H}^+/2e^-$ ratio in the presence of valinomycin for $NO_3^- \rightarrow NO_2^- = 2.8$, for $N_2O \rightarrow N_2 = 3.1$ and for $NO_2^- \rightarrow 1/2\ N_2O = 2.2$, the latter determined with KSCN, compare Tables 2 and 3). All the data are slightly lower but otherwise agree well with those for *Paracoccus denitrificans* published by Boogerd et al. (1981) but not with those by Kristjansson et al. (1978).

A consistency in the results presented here can be deduced if one assumes a periplasmic orientation of nitrate reductase, nitrite reductase and nitrous oxide reductase *for a moment*. The following considerations are based on the argument (see Boogerd et al. 1981) that scalar H^+-consumptions during the reductions of nitrogenous oxides should change the measured $\vec{H}^+/2e^-$ translocation ratios only if occurring at the periplasmic face of the cytoplasmic membrane. If occurring extramembraneously, H^+-consumptions have to be taken into account according following equations:

(1) $NO_2^- + 2e^- + 3\ H^+ \rightarrow 0.5\ N_2O + 1.5\ H_2O$
(2) $N_2O + 2e^- + 2\ H^+ \rightarrow N_2 + H_2O$
(3) $NO_3^- + 2e^- + 2\ H^+ \rightarrow NO_2^- + H_2O$
(4) $NO_2^- + 2e^- + 4\ H^+ \rightarrow 0.5\ N_2 + 2.0\ H_2O$
(5) $NO_3^- + 5e^- + 6\ H^+ \rightarrow 0.5\ N_2 + 3.0\ H_2O$
(6) $NO_3^- + 4e^- + 5\ H^+ \rightarrow 0.5\ N_2O + 2.5\ H_2O$

Therefore the corrected $\vec{H}^+/2e^-$ ratios were 5.2 for $NO_2^- \rightarrow 0.5\ N_2O$, 5.1 for $N_2O \rightarrow N_2$, 4.8 for $NO_3^- \rightarrow NO_2^-$, 5.2 for $NO_2^- \rightarrow 0.5\ N_2$, 5.0 for $NO_3^- \rightarrow 0.5\ N_2$ and 4.6 for $NO_3^- \rightarrow 0.5\ N_2O$. The H^+-translocation for $NO_3^- \rightarrow N_2O$ had to be determined in the presence of KSCN which is known to severely affect nitrous oxide reductase and to slightly block nitrate reductase (Boogerd et al. 1981; Kundu and Nicholas 1985). Therefore the $\vec{H}^+/2e^-$ ratio = 4.6 for $NO_3^- \rightarrow 0.5\ N_2O$ is expected to be lower than for the others. For the other reductions (equations 1 – 5), the $\vec{H}^+/2e^-$ ratios strikingly vary only between 4.8 and 5.2 which could suggest a periplasmic orientation of all three enzymes involved.

Several arguments suggest that such a conclusion cannot be drawn from these calculations. The $\vec{H}^+/2e^-$ ratios cannot be the same for all the transitions if one of the reductions were to proceed at the inner face of the membrane. The calculations above also imply that the electrons transported to N_2O, NO_2^- and NO_3^- share a common route. This must not be the case in *Azospirillum*. Little is known about the electron carrier composition in this bacterium. Dissimilatory nitrite reductase is a cytochrome c, d containing enzyme (Danneberg et al. 1986). Data from this laboratory (Danneberg 1987) have also shown that nitrous oxide reductase is expressed in dependence of Cu in the medium and crossreacts with antibodies to the Cu-containing enzyme from *Pseudomonas perfectomarina* (Zumft and Matsubara 1982). For nitrate reductase, other data of the present investigation indicate that this enzyme is orientated inwards. In the H^+-uptake measurements performed with cells from the batch cultures which reduced NO_3^- only to NO_2^-, the reduction in the absence of ionophores was not accompanied by a significant H^+-consumption. The H^+-uptake determinations with the cells from continuous cultures gave ratios for $\vec{H}^+/NO_3^- \rightarrow N_2 = -2.9$ and $\vec{H}^+/NO_2^- \rightarrow N_2 = -2.5$ which indicates that nitrite and not nitrate reductase is located periplasmatically. The $\vec{H}^+$/electron acceptor determinations are also in accord with a cytoplasmic orientation of nitrous oxide reductase (Table 5).

The H^+-uptake measurements also allow the conclusion that NO_3^- and NO_2^- are not taken up by H^+-symport mechanism as suggested by Kristjansson et al. (1978). The $\vec{H}^+/NO_3^-$-value (−2.9) is slightly higher than that for $\vec{H}^+/NO_2^-$ (−2.5), but such difference is not statistically sound to conclude an uptake of 1 H^+ per molecule of nitrate transported into the cells. In all the experiments, the $\vec{H}^+/NO_3^-$ or $\vec{H}^+/NO_2^-$ ratios never approached 1 as in the experiments with *Paracoccus* and *Pseudomonas denitrificans* (Kristjansson et al. 1978). In addition, as mentioned earlier, the reduction of nitrate was not accompanied by any H^+-uptake in batch cultures which had expressed nitrate reductase only. Thus nitrate uptake might proceed via an energy-independent nitrate/nitrite antiport system in *Azospirillum* as in *Paracoccus denitrificans* (Boogerd et al. 1983, 1984).

In the absence of any ionophore, the reduction of N_2O was not accompanied by a H^+-uptake which should be expected if N_2O were to be reduced at the periplasmic side. Similarly, the same Y_s^{max}-value measured in O_2 and N_2O respiration indicates that both terminal cytochrome oxidase and nitrous oxide reductase are located inside. Then the $\vec{H}^+/O_2$ and $\vec{H}^+/N_2O$ ratios in the presence of valinomycin or valinomycin plus $TPMP^+$ should be the same which was, however, not observed. There is no plausible explanation for this discrepancy. Perhaps the $\vec{H}^+/2e^-$ ratio is underestimated for N_2O, because the experiments had to be performed under a continuous stream of argon which could have blown away partly the N_2O. The same argument applies to O_2 which was also supplied as airsaturated 150 mM KCl solution. Perhaps valinomycin and $TPMP^+$ specifically affected H^+-translocations in N_2O respiration. Other explanations are not obvious. Thus it cannot be concluded from this set of experiments that N_2O-reductase resides inside,

which, however, follows from the Y_s^{max} data and the H^+-uptake measurements in the absence of ionophores.

Conclusive evidence for the suggested distributions of the enzymes on the cytoplasmic membrane (nitrate and nitrous oxide reductases inside, nitrite reductase outside) could come from experiments with the electron donors TMPD/ascorbate or reduced benzylviologen which allow to measure the scalar H^+-consumptions with the terminal respiratory oxidases (Stam et al. 1984; Kundu and Nicholas 1985). Such an experimental approach requires the total blockage of the respiratory electron flow from endogenous substrates by using inhibitors like rotenone, HOQNO, antimycin A or malonate. All attempts failed to block endogenous respiration completely by these four inhibitors (Danneberg 1987). Dibromothymoquinone (DBMIB) was the only one which could block respiration totally but DBMIB chemically interfered with TMPD/ascorbate (Danneberg 1987). Reduced benzylviologen was not tried seriously because it appears to affect the cell viability (Kundu and Nicholas 1985). Thus such studies are hampered by the fact that common inhibitors of respiration are ineffective. In this respect, *Azospirillum* resembles *Azorhizobium caulinodans* (Stam et al. 1984; de Vries et al. 1988).

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