

# Growth Yields in Bacterial Denitrification and Nitrate Ammonification<sup>∇</sup>

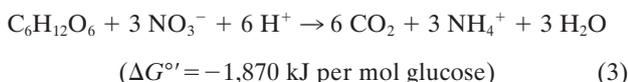
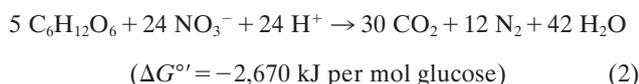
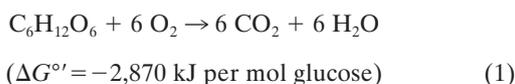
Tobin O. Strohm,<sup>1</sup> Ben Griffin,<sup>1†</sup> Walter G. Zumft,<sup>2</sup> and Bernhard Schink<sup>1\*</sup>

Fachbereich Biologie, Universität Konstanz, D-78457 Konstanz, Germany,<sup>1</sup> and Lehrstuhl für Mikrobiologie, Universität Karlsruhe, D-76128 Karlsruhe, Germany<sup>2</sup>

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**Denitrification and nitrate ammonification are considered the highest-energy-yielding respiration systems in anoxic environments after oxygen has been consumed. The corresponding free energy changes are 7 and 35% lower than that of aerobic respiration, respectively. Growth yield determinations with pure cultures of *Paracoccus denitrificans* and *Pseudomonas stutzeri* revealed that far less energy is converted via ATP into cell mass than expected from the above calculations. Denitrification with formate or hydrogen as electron donor yielded about 2.4 to 3.0 g dry matter per mol formate or hydrogen and 15 to 18 g dry matter per mol acetate. Similar yields with acetate were obtained with *Pseudomonas stutzeri*. *Wolinella succinogenes* and *Sulfurospirillum deleyianum*, which reduce nitrate to ammonia, both exhibited similar yield values with formate or H<sub>2</sub> plus nitrate. The results indicate that ATP synthesis in denitrification is far lower than expected from the free energy changes and even lower than in nitrate ammonification. The results are discussed against the background of our present understanding of electron flow in denitrification and with respect to the importance of denitrification and nitrate ammonification in the environment.**

Oxidation of biomass proceeds preferentially with oxygen as electron acceptor, and only after its consumption in deeper sediment layers are alternative electron acceptors such as nitrate, manganese(IV), ferric iron, sulfate, or CO<sub>2</sub> reduced (15, 30). After oxygen reduction, reduction of nitrate to N<sub>2</sub> (denitrification) or to ammonia (nitrate ammonification) is the highest-energy-yielding process as shown by the following equations calculated with glucose as a representative of biomass (calculated based on the tables in reference 25):



Even if we assume substrate and product concentrations more appropriate to natural conditions, i.e., 1 μM glucose and 10 μM nitrate and ammonium, the free energies of reactions 1 and 3 change by +34 kJ and that of equation 2 changes by +171 kJ, which still leaves a substantially higher amount of free energy with the denitrification process. From this comparison, one would expect that ATP synthesis as measured via growth yields in denitrification should come close to that of aerobic respiration, and yields in nitrate ammonification should be substantially lower. Thus, denitrification is expected

to be the preferred process over nitrate ammonification, especially if the overall energy yield of a metabolic process is low. Contrary to this assumption, nitrate ammonification was reported to be the preferred process in anoxic environments in which the nitrate supply is limiting (26, 27). Moreover, nearly all recently described lithotrophic bacteria oxidizing sulfide with nitrate as electron acceptor at neutral pH, e.g., *Thioploca* sp. or *Thiomargarita* sp., convert nitrate to ammonia (12, 16, 17), although denitrification should provide them with more energy.

The energetics of nitrate reduction and the mechanisms of energy conservation in this process have been discussed in the past in great detail (22, 24). Growth yields of nitrate-reducing bacteria have been determined repeatedly but always with complex substrates such as sugars, succinate, or glutamate (9, 10, 23), and not in all cases were the experimental conditions documented clearly enough to identify the limiting substrate and to verify whether nitrate was reduced completely. Later the structural organization of the electron transfer components within and outside the cytoplasmic membrane was resolved (6, 32), but the implications of these findings for the overall energy balance of denitrification remained unclear. We therefore undertook a comparative evaluation of growth yields with well-described denitrifying and ammonifying bacteria in mineral media with simple substrates such as acetate, H<sub>2</sub>, and formate under directly comparable conditions in order to obtain reliable data for a comparison of growth yields of these two important anaerobic respiratory processes.

## MATERIALS AND METHODS

**Organisms and cultivation.** *Paracoccus denitrificans* (DSM 65<sup>T</sup>), *Pseudomonas stutzeri* (DSM 5190<sup>T</sup>), and *Sulfurospirillum deleyianum* (DSM 6946<sup>T</sup>) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. *Wolinella succinogenes* (DSM 1740<sup>T</sup>) was a kind gift of the lab of Achim Kröger, Frankfurt, Germany.

*P. denitrificans* and *P. stutzeri* were grown in oxygen-free nonreduced bicarbonate-buffered mineral salts medium (13). *S. deleyianum* and *W. succinogenes* were grown in bicarbonate-buffered sulfide-reduced mineral salts medium as

\* Corresponding author. Mailing address: Fachbereich Biologie, Universität Konstanz, D-78457 Konstanz, Germany. Phone: (49) 7531 882140. Fax: (49) 7531 884047. E-mail: Bernhard.Schink@uni-konstanz.de.

† Present address: Department of Microbiology, University of Illinois Urbana-Champaign, Urbana, IL 61801.

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TABLE 1. Growth experiments with *P. denitrificans*<sup>a</sup>

Substrate	Net OD <sub>546</sub>	Dry wt (mg · liter <sup>-1</sup> )	Limiting substrate	Yield	
				g · (mol e <sup>-</sup> donor) <sup>-1</sup>	g · (mol e <sup>-</sup> acceptor) <sup>-1</sup>
2 mM acetate + 10 mM NO <sub>3</sub> <sup>-</sup>	0.13	31.2	Acetate	17.9	11.2
5 mM acetate + 10 mM NO <sub>3</sub> <sup>-</sup>	0.27	64.8	Acetate	15.0	9.4
10 mM formate + 10 mM NO <sub>3</sub> <sup>-</sup>	0.11	26.4	Formate	3.03	7.6
20 mM formate + 10 mM NO <sub>3</sub> <sup>-</sup>	0.22	52.8	Formate	3.03	7.6
40 mM H <sub>2</sub> <sup>b</sup> + 5 mM NO <sub>3</sub> <sup>-</sup>	0.13	31.2	Nitrate	2.8	7.2
5 mM acetate + 5 mM NO <sub>3</sub> <sup>-</sup>	0.235	56.5	Nitrate	18.1	11.3
20 mM formate + 5 mM NO <sub>3</sub> <sup>-</sup>	0.13	30	Nitrate	2.4	6.0
5 mM acetate + 2 mM NO <sub>2</sub> <sup>-</sup>	0.08	19.2	Nitrite	25.4	9.6
5 mM acetate + 5 mM NO <sub>2</sub> <sup>-</sup>	0.20	46.8	Nitrite	21.0	10.5
20 mM formate + 2 mM NO <sub>2</sub> <sup>-</sup>	0.05	12	Nitrite	4.0	6.0
20 mM formate + 5 mM NO <sub>2</sub> <sup>-</sup>	0.11	26.4	Nitrite	2.4	4.6
5 mM acetate + 10 mM N <sub>2</sub> O <sup>c</sup>	0.13	31.1	N <sub>2</sub> O	12.6	3.15
10 mM formate + 10 mM N <sub>2</sub> O <sup>c</sup>	0.095	22.7	Formate	2.6	2.3
10 mM formate + 4 mM N <sub>2</sub> O <sup>d</sup>	0.053	12.7	N <sub>2</sub> O	3.2	3.2

<sup>a</sup> Growth yields were calculated for the respective limiting substrate (electron donor or electron acceptor) and converted stoichiometrically to the corresponding cosubstrate. Results are documented as means of at least two independent growth experiments (mostly four to six) which differed by less than 15%. Where electron donors were limiting, the dissimilated substrate was calculated from the total substrate conversion via correction for assimilated substrate, which was 20.6 μmol acetate or 82.4 μmol formate or H<sub>2</sub> per mg dry matter.

<sup>b</sup> Eighty percent H<sub>2</sub> in a 12-ml headspace in a 22-ml test tube.

<sup>c</sup> N<sub>2</sub>O at 2.4 ml over 10 ml medium.

<sup>d</sup> One milliliter N<sub>2</sub>O over 10 ml medium.

described earlier (14, 29). Growth experiments were carried out at least in duplicate (most in four to six independent assays) in 22-ml Hungate tubes sealed with butyl rubber stoppers and filled with 10 ml culture medium under a headspace of N<sub>2</sub>/CO<sub>2</sub> (80%/20%) or in tubes completely filled with medium and closed with latex-sealed screw caps. With gaseous substrates, tubes were incubated lying on a shaker at about 60 rpm. Growth was followed via turbidity measurement with a Spectronics 70 spectrophotometer at a 546-nm wavelength (Bausch & Lomb, Rochester, NY). Turbidity was followed at intervals of 4 to 12 h for up to 10 days. Growth yield determinations were calibrated by gravimetric determination of dry cell mass grown in 1-liter cultures.

**Biochemical analysis of acetate activation.** Cells of *P. denitrificans* and *P. stutzeri* were harvested in 1-liter cultures in the late exponential growth phase and washed with oxygen-free 100 mM Tris-HCl buffer, pH 7.8, containing 5 mM MgCl<sub>2</sub> and 2 mM dithiothreitol. Cells were broken by repeated French press treatment anoxically under N<sub>2</sub>, and cell debris was removed by centrifugation at 100,000 × g for 10 min. The membrane fraction was removed by centrifugation at 100,000 × g for 45 min. The supernatant contained the cytoplasmic and the periplasmic fraction. Synthesis of acetyl coenzyme A (acetyl-CoA) from acetate, ATP, and coenzyme A was followed in a coupled photometric assay including myokinase, phosphoenolpyruvate, and NADH (31). The stoichiometry of NADH oxidation over acetate consumption was assayed with limiting amounts (0.1 mM) of acetate.

**Chemicals.** Chemicals used were of analytical grade and were obtained from Fluka (Buchs, Switzerland), Riedel-de Haën (Seelze, Germany), and Merck (Darmstadt, Germany). Biochemicals were purchased from Sigma (München, Germany).

## RESULTS

**Determination of conversion factors.** Conversion factors for calculation of cell yields from optical density (OD) values were determined in 1-liter cultures. The conversion factors obtained were very similar for all bacteria used. An OD value at 546 nm (OD<sub>546</sub>) of 0.1 measured against a medium blank corresponded to 24.0 mg (dry weight) of cells per liter with *P. denitrificans*, 23.5 mg (dry weight) of cells per liter with *P. stutzeri*, 24.8 mg (dry weight) of cells per liter with *W. succinogenes*, and 24.3 mg (dry weight) of cells per liter with *S. deleyianum*. These calibration factors were identical also after growth with different substrates because the cell shape of these

bacteria is very constant. These values were used for calculations of cell yields in the subsequent experiments.

**Growth experiments.** *P. denitrificans* grew with acetate plus nitrate with doubling times of 4 to 6 h. With formate, doubling times were about 10 h, and with hydrogen they were about 20 h. Growth yields were determined with *P. denitrificans* in static cultures with either acetate, formate, or hydrogen as electron donor and either nitrate, nitrite, or N<sub>2</sub>O as electron acceptor. As reported in Table 1, growth yields with acetate plus nitrate were about 15 to 18 g per mol acetate or 9.4 to 11.3 g per mol nitrate, no matter whether acetate or nitrate was the limiting substrate, indicating that nitrate was completely reduced to N<sub>2</sub>. With formate or hydrogen as electron donor, yields were considerably smaller, with 2.4 to 3.0 g per mol formate or H<sub>2</sub> and 6.0 to 7.6 g per mol nitrate. With nitrite as electron acceptor, the yields were substantially higher, 18 to 25 g per mol acetate, and were lower with N<sub>2</sub>O. Similar yield determinations were carried out with *Pseudomonas stutzeri*, another well-studied nitrate reducer which oxidizes only organic compounds such as acetate. The growth yields obtained (Table 2) were similar to those found with *P. denitrificans* on acetate, indicating that these results may be taken as representative of denitrifying bacteria in general.

Growth yields were also determined with the nitrate-ammonifying bacteria *W. succinogenes* and *S. deleyianum*. As shown in Table 3, nitrate reduction with formate or H<sub>2</sub> as electron donor by *W. succinogenes* yielded about 4 g cell mass per mol formate and 1.7 g per mol H<sub>2</sub> with nitrate as electron acceptor; with fumarate or nitrite as electron acceptor, the obtained yields were slightly higher or lower, respectively. Since *W. succinogenes* has to be grown in a sulfide-reduced medium, care has to be taken with growth studies in the presence of excess electron acceptor: Turbidity develops quickly after transfer, but this turbidity is caused not by cell growth but by partial oxidation of sulfide to elemental sulfur. *W. succino-*

TABLE 2. Growth experiments with *P. stutzeri*<sup>a</sup>

Substrate	Net OD <sub>546</sub>	Dry wt (mg · liter <sup>-1</sup> )	Limiting substrate	Yield	
				g · (mol e <sup>-</sup> donor) <sup>-1</sup>	g · (mol e <sup>-</sup> acceptor) <sup>-1</sup>
2 mM acetate + 10 mM NO <sub>3</sub> <sup>-</sup>	0.12	28.8	Acetate	16.6	10.3
5 mM acetate + 5 mM NO <sub>3</sub> <sup>-</sup>	0.22	53	Nitrate	16.9	10.6
5 mM acetate + 2 mM NO <sub>2</sub> <sup>-</sup>	0.11	26.4	Nitrite	35.2	13.2
5 mM acetate + 5 mM NO <sub>2</sub> <sup>-</sup>	0.17	41	Nitrite	21.8	8.2
5 mM acetate + 10 mM N <sub>2</sub> O <sup>b</sup>	0.23	55	N <sub>2</sub> O	22.1	5.5

<sup>a</sup> Growth yields were calculated for the respective limiting substrate (electron donor or electron acceptor) and converted stoichiometrically to the corresponding cosubstrate. Results are documented as means of at least two independent growth experiments which differed by less than 15%. Where electron donors were limiting, the dissimilated substrate was calculated from the total substrate conversion via correction for assimilated substrate, which was 20.6 μmol acetate or 82.4 μmol formate or H<sub>2</sub> per mg dry matter.

<sup>b</sup> N<sub>2</sub>O at 2.4 ml over 10 ml medium.

*genes* also turned out to be quite sensitive to enhanced nitrite additions: no growth was observed at nitrite concentrations higher than 3 mM nitrite, and growth was inhibited by nitrite accumulating in cultures with excess nitrate. Growth yields obtained with *S. deleyianum* with formate as electron donor and nitrate as electron acceptor were nearly identical to those obtained with *W. succinogenes* (results not shown).

**Acetate activation by *P. denitrificans* and *P. stutzeri*.** The enzyme system responsible for activation of acetate to acetyl-CoA was identified in *P. denitrificans* cytoplasm. The coupled assay system (31) revealed that 0.206 mM NADH was oxidized per 0.1 mM acetate supplied, indicating that two ATP equivalents were consumed in acetate synthesis, as typical of an acetate-CoA ligase enzyme (acetyl-CoA synthetase, EC 6.2.1.1) forming AMP and pyrophosphate (with subsequent hydrolysis) from ATP in acetyl-CoA synthesis. Similar results were obtained with extracts of *P. stutzeri*.

## DISCUSSION

Our results clearly document that the energy yields of nitrate reduction are far lower than one would expect from the free energy changes of the overall redox reactions (see above). Although similar studies have been carried out in the past, yield determinations in denitrification and nitrate ammonification under directly comparable conditions in defined mineral media have not been carried out so far. Detailed studies of the bioenergetics of *Wolinella succinogenes* have been performed in the past, and our yield values confirm those obtained in those studies (2, 20). These authors documented in detail that

formate-dependent growth of *W. succinogenes* with nitrate as electron acceptor yields less cell mass than formate oxidation with fumarate does, although the free energy change of the latter reaction is substantially lower than that of nitrate ammonification. The corresponding ATP yields were calculated to be 0.43 ATP per mol formate with nitrate or 0.72 ATP per mol formate with fumarate as electron acceptor (2, 20). Hydrogen-dependent nitrate ammonification by the sulfate reducer *Desulfovibrio desulfuricans* yields 5.2 g cell mass per mol hydrogen in static culture conditions, which is equivalent to about 0.5 ATP per mol (19).

Growth yields in denitrification have been determined in the past mainly with sugars and other more complex substrates (9, 10, 22, 23). Phosphorylation efficiencies measured with membrane vesicles of *P. denitrificans* were found to be 0.9 P per NADH (8, 11).

The molecular organization of the nitrate-reducing enzyme apparatus of denitrifying bacteria has been worked out in regard to its biochemistry and its molecular organization (4, 6, 32); a general concept of the electron transport system is depicted in Fig. 1, which is based on similar schemes by Ferguson (6) and Zumft (32). For comparison, one should take into account that in the aerobic respiratory chain of mitochondria and many aerobic bacteria, a total of 10 H<sup>+</sup> are transported across the membrane per NADH electron pair, 4 H<sup>+</sup> in the NADH dehydrogenase reaction, 2 H<sup>+</sup> in the quinone cycle, 2 H<sup>+</sup> in the cytochrome *bc*<sub>1</sub> complex, and 2 H<sup>+</sup> in the cytochrome oxidase reaction (7, 28). Reduction of nitrate to N<sub>2</sub>, on the other hand, proceeds through four different reductase en-

TABLE 3. Growth experiments with *W. succinogenes*<sup>a</sup>

Substrate	NO <sub>2</sub> <sup>-c</sup> (mM)	Net OD <sub>546</sub>	Dry wt (mg · liter <sup>-1</sup> )	Limiting substrate	Yield	
					g · (mol e <sup>-</sup> donor) <sup>-1</sup>	g · (mol e <sup>-</sup> acceptor) <sup>-1</sup>
10 mM formate + 5 mM NO <sub>3</sub> <sup>-</sup>	1.47					
20 mM formate + 5 mM NO <sub>3</sub> <sup>-</sup>	0.09	0.35	84	Formate	4.2	16.8
20 mM formate + 10 mM NO <sub>3</sub> <sup>-</sup>	0.06	0.34	82	Formate	4.1	14.4
20 mM formate + 25 mM fumarate		0.50	120	Formate	6.0	6.0
20 mM formate/2 mM NO <sub>2</sub> <sup>-</sup>		0.07	16.8	Nitrite	2.8	8.4
20 mM formate/5 mM NO <sub>2</sub> <sup>-</sup>	2.7/4.6					
40 mM H <sub>2</sub> <sup>b</sup> /5 mM NO <sub>3</sub> <sup>-</sup>		0.28	67.4	H <sub>2</sub>	1.7	6.8

<sup>a</sup> Growth yields were calculated for the respective limiting substrate (electron donor or electron acceptor) and converted stoichiometrically to the corresponding cosubstrate. Results are documented as means of at least two independent growth experiments which differed by less than 15%.

<sup>b</sup> Eighty percent H<sub>2</sub> in a 12-ml headspace in a 22-ml test tube.

<sup>c</sup> Nitrite present after incubation.

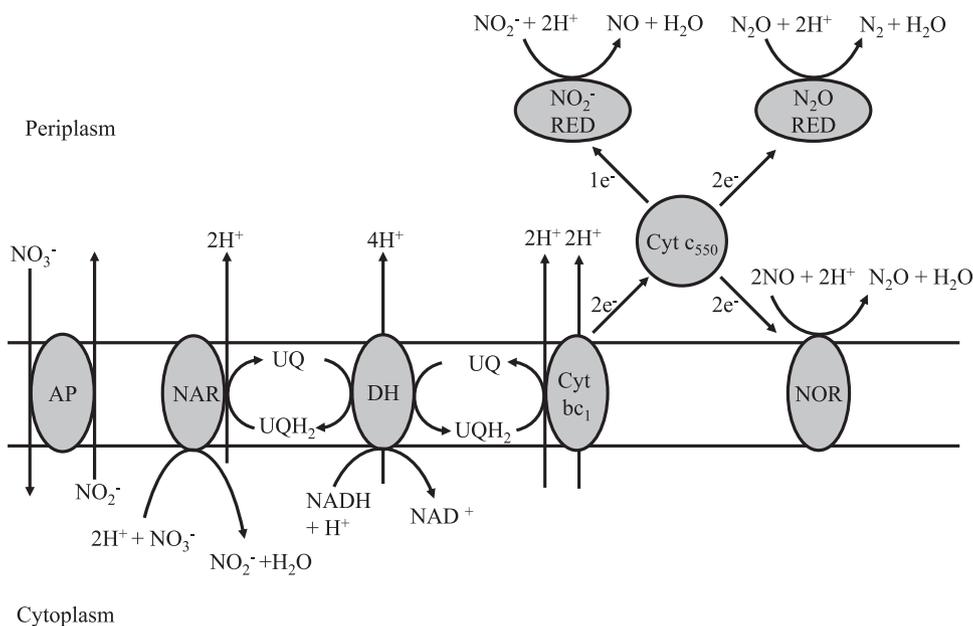


FIG. 1. Organization of respiratory elements involved in denitrification by *Paracoccus denitrificans* and the impact of proton translocation across the membrane. The figure is redrawn from similar schemes by Ferguson (6) and Zumft (32). Abbreviations: AP, antiporter; NAR, nitrate reductase; UQ, ubiquinone; DH, NADH dehydrogenase; Cyt  $bc_1$ , cytochrome  $bc_1$  complex; NOR, NO reductase;  $\text{NO}_2^-$  RED, nitrite reductase;  $\text{N}_2\text{O}$  RED,  $\text{N}_2\text{O}$  reductase; Cyt  $c_{550}$ , cytochrome  $c_{550}$ .

zymes: nitrate reductase, NADH dehydrogenase, and NO reductase are integral membrane proteins whereas nitrite reductase and  $\text{N}_2\text{O}$  reductase are located in the periplasm. NADH dehydrogenase and the cytochrome  $bc_1$  complex are considered to be active proton pumps, and the ubiquinone cycles connecting either to nitrate reductase or to the cytochrome  $bc_1$  complex are supposed to translocate protons as well. The other reductases do not contribute to proton translocation; rather, they consume protons in the periplasmic space. Nonetheless, this proton consumption does not influence the ATP yield significantly because the pH in the periplasm is maintained by the medium buffer.

Reduction of two molecules of nitrate to  $\text{N}_2$  with five NADH molecules as electron donor translocates a total of 36 protons across the membrane. If we assume that four protons are required for synthesis of one ATP molecule (3, 5, 18), nine ATP molecules can be synthesized with the electrons of five NADH molecules, i.e., 1.8 ATP is synthesized per NADH, which would be twice as much as the value (0.9) obtained by the study in reference 8.

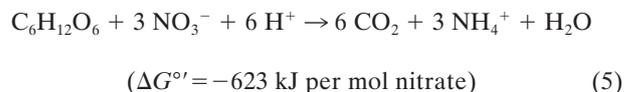
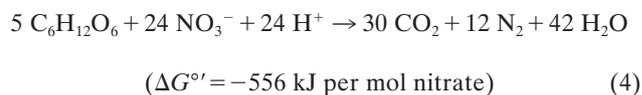
Hydrogen and formate are oxidized on the outer face of the cytoplasmic membrane and donate electrons to quinones, leaving two protons per two electrons in the periplasm (20); the proton balances and ATP yields with these substrates are therefore lower than those obtained with NADH as electron donor (26  $\text{H}^+$  per 10  $\text{e}^-$ , or 1.3 ATP per two electrons). From the average molar growth yield with formate or  $\text{H}_2$ ,  $Y_{\text{ATP}}$  can be estimated to be about 2.3 g per mol ATP. This value is typical of a bacterium which has to synthesize its cell material autotrophically from  $\text{CO}_2$  or formate via the Calvin cycle (21).

Acetate oxidation in the citric acid cycle yields three electron pairs at the NADH level and one at the ubiquinone level. The total proton balance for ubiquinol-dependent nitrate reduction

is 16 protons translocated per five ubiquinol molecules oxidized. This leads to a total of 24.8 protons per acetate oxidized, corresponding to 6.2 ATP formed by electron transport phosphorylation. Two ATP equivalents are consumed in acetate activation as confirmed in our study, and one ATP molecule is gained in the citric acid cycle in the succinate thiokinase reaction, leaving a total of 5.2 ATP per mol acetate. Our growth yields agree with this assumption, giving a  $Y_{\text{ATP}}$  value of 3.3 g per mol ATP, which is low for heterotrophic growth in a mineral medium with a simple substrate like acetate (21).

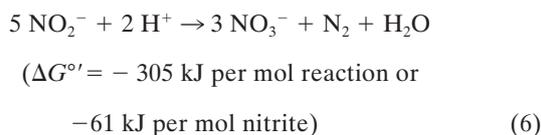
Growth yields with nitrite or  $\text{N}_2\text{O}$  as electron acceptor were in the same range as those with nitrate. Indeed, reduction of nitrite, NO, and  $\text{N}_2\text{O}$  translocates similar amounts of protons per NADH or ubiquinol oxidized as does nitrate reduction to  $\text{N}_2$  (Fig. 1). These results confirm basically earlier findings (9, 10) that the three main steps in denitrification release about equal amounts of energy.

A comparison of growth yields between *P. denitrificans* and *W. succinogenes* makes it obvious that denitrification yields slightly lower rather than higher cell amounts than nitrate ammonification does. This becomes even more pronounced if the yields are calculated per mol electron acceptor: since reduction of nitrate to ammonia consumes eight electrons rather than five in denitrification, the energy yield per nitrate reduced is higher for nitrate ammonification than for denitrification:



In our experiments it appears that about twice as much cell mass can be synthesized per mol nitrate by nitrate ammonification as by denitrification (15.6 versus 7.6 g per nitrate reduced with formate as electron donor; Tables 1 and 3). Thus, it is not surprising that nitrate ammonification is the preferred nitrate respiration process under conditions of nitrate limitation (26) and that especially lithoautotrophic sulfide oxidation by, e.g., *Thioploca* sp. or *Thiomargarita* sp. prefers nitrate ammonification over denitrification (12, 16, 17).

The observed inefficient energy conservation in the reduction of nitrite to nitrogen gas may also explain why no bacterium has been described so far which grows by dismutation of nitrite to nitrate plus N<sub>2</sub> although this reaction yields substantial amounts of energy:



The analogous dismutation of sulfite to sulfate plus sulfide, yielding a similar amount of energy ( $\Delta G^\circ = -59 \text{ kJ per mol sulfite}$ ), was described many years ago as a novel type of "inorganic fermentation" (1). This appears not to work out with nitrite dismutation to N<sub>2</sub> plus nitrate, obviously because the reduction of the most positive electron acceptor systems (reduction of NO and N<sub>2</sub>O, at  $E^\circ = +1,180$  and  $+1,350 \text{ mV}$ , respectively) proceeds in the periplasm, without concomitant energy conservation.

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