Energy Yield of Denitrification: An Estimate from Growth Yield in Continuous Cultures of *Pseudomonas denitrificans* under Nitrate-, Nitrite- and Nitrous Oxide-limited Conditions

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SUMMARY

The molar growth yields of *Pseudomonas denitrificans*, for nitrate, nitrite and nitrous oxide, were determined in chemostat culture under electron acceptor-limited conditions. Glutamate was used as the source of energy, carbon and nitrogen. The catabolic pattern was identical, irrespective of the terminal electron acceptors.

The molar growth yields, corrected for maintenance energy, were 28.6 g/mol nitrate, 16.9 g/mol nitrite and 8.8 g/mol nitrous oxide. The energy yield, expressed on an electron basis, was proportional to the oxidation number of the nitrogen: nitrate (+5), nitrite (+3) and nitrous oxide (+1). It was concluded that oxidative phosphorylation occurs to a similar extent in each of the electron transport chains associated with the reduction of nitrate to nitrite, nitrite to nitrous oxide and nitrous oxide to nitrogen.

INTRODUCTION

In the denitrification performed by *Pseudomonas denitrificans* and *P. perfectomarinus*, nitrate is reduced stepwise to nitrogen gas via nitrite, nitric oxide and nitrous oxide as intermediates (Barbaree & Payne, 1967; Matsubara & Mori, 1968; Miyata & Mori, 1968; Payne, Riley & Cox, 1971).

Phosphorylation coupled to nitrate reduction to nitrite has been demonstrated in *Micrococcus denitrificans*, *P. denitrificans* and *P. aeruginosa* (Yamanaka, Ota & Okunuki, 1962; Ohnishi, 1963; Naik & Nicholas, 1966). Some denitrifying bacteria grow anaerobically with nitrite or nitrous oxide as terminal electron acceptor, suggesting that phosphorylation also takes place coupled to nitrite reduction to nitrogen gas (Pascal, Pichinoty & Bruno, 1965; Kodama, Shimada & Mori, 1969; Matsubara, 1971). However, neither the steps nor the sites of coupling of electron transport in the reduction of nitrate with phosphorylation have been determined.

The energy yielded by *P. denitrificans* denitrification is about half that of aerobic respiration (Koike & Hattori, 1975). The low efficiency of denitrification can be explained by the lack of oxidative phosphorylation in certain step(s) of denitrification and/or by a reduction in the number of phosphorylation sites in the electron transport chains associated with denitrification.

The present study examines which steps of the denitrification sequence are coupled to oxidative phosphorylation and the energy yield in the electron transport chain associated with each step in the reduction of nitrogenous oxides. *P. denitrificans* grows well with nitrate, nitrite or nitrous oxide as terminal electron acceptor. The growth yields per mole of terminal electron acceptors reduced were used as an index.

METHODS

Organism. *Pseudomonas denitrificans* was isolated from the bottom mud of a brackish lake (Koike & Hattori, 1975).

Culture media and conditions. The mineral components of the medium were those used in the previous study (Koike & Hattori, 1975), except for KNO_3 . Glutamate (12 mM) was used as carbon, energy and nitrogen source. The organism was grown in a chemostat with limiting terminal electron acceptor of 10 mM- KNO_3 or $-\text{NaNO}_2$.

Anaerobiosis was maintained by bubbling argon (50 ml/min) through the medium. When nitrous oxide was used, the argon was replaced by a mixture of 10.3 % nitrous oxide and 89.7 % argon (Takachiho Co., Tokyo). The flow rate was kept constant with a peristaltic pump at values from 5.8 ml/min at dilution rate (D) = 0.05, to 8.5 ml/min at D = 0.11, and checked daily. To eliminate traces of oxygen, argon was bubbled through the medium in the reservoir at regular intervals.

The culture apparatus and details of procedures are described elsewhere (Koike, 1975).

Determination of molar growth yield. The growth yield (g/mol) for nitrate, nitrite or glutamate was calculated using the equation:

$$Y = \frac{x}{S_1 - S_0},$$

where x is the bacterial concentration (dry weight, g/l) in the medium at steady-state, S_1 is the substrate concentration in the feeding medium (mol/l), and S_0 is the substrate concentration in the culture vessel (mol/l).

The growth yield for nitrous oxide ($Y_{\text{N}_2\text{O}}$, in g/mol) was calculated from the rate of cell production (g/l/h) and the rate of nitrous oxide consumption (mol/l/h):

$$Y_{\text{N}_2\text{O}} = \frac{\text{rate of cell production}}{\text{rate of } \text{N}_2\text{O} \text{ consumption}} \\ = \times D/(C_1 - C_2) \times \frac{273}{T + 273} \times \frac{f}{100} \times \frac{1}{22.4},$$

where D is the dilution rate (h^{-1}), C_1 the nitrous oxide content in the feeding gas (%), C_2 the nitrous oxide content in the effluent gas corrected for volume change (%), T the temperature of the gas mixture ($^{\circ}\text{C}$), and f the flow rate of gas (l/h).

Analytical methods. Volatile and non-volatile organic acids in the culture media were determined by a Hitachi model K 53 gas chromatograph equipped with a hydrogen flame ionization detector. A 2 m glass column was used, packed with Chromosorb W on which 10 % Carbowax 20 M terminated with 2-nitro-terephthalic acid plus 3 % phosphoric acid had been adsorbed (Gaschro Industry Co., Tokyo). The column temperature was 150 $^{\circ}\text{C}$. Nitrogen was used as the carrier gas. Samples to be assayed were neutralized and concentrated fivefold under reduced pressure. For volatile acids, 1.0 ml portions of the concentrated samples were acidified by adding 0.2 ml of 25 % metaphosphate in 5 N- H_2SO_4 . Non-volatile organic acids were methylated by boiling 0.4 ml of the concentrated samples with an equal volume of 14 % (w/v) boron-trifluoride in methanol for 10 min. The methyl esters were then extracted with chloroform (0.2 ml). Crotonic acid and fumaric acid, for volatile and non-volatile acids respectively, were used as internal standards. These two acids were not present in the original samples.

The concentration of CO_2 and N_2O in the inflow and outflow gases was determined with a Shimadzu model GC-3 AL gas chromatograph fitted with a thermal conductivity detector.

Samples were collected in a gas reservoir (40 ml capacity) with two stop-cocks, after first passing 1 l of gas through the reservoir. Portions (1 ml) were introduced at a pressure of 1 atm by a mercury Toepler pump into the gas chromatograph with a 1 m column of Porapak T (Waters Associates Inc., Massachusetts, U.S.A.). Chromatography was carried out at 50 °C, using helium as carrier gas. Since the retention time of CO₂ was almost identical with that of N₂O, samples treated with 2 ml of 40 % (w/v) potassium hydroxide were run simultaneously to correct for N₂O. CO₂ and N₂O from Takachiho Co., Tokyo, were used as reference compounds. No CO₂ was detectable in the inflow argon gas. The rate of CO₂ production was therefore calculated from the concentration of CO₂ in the outflow gas and the flow rate as measured by a Roger-Gilmont flowmeter. Correction was made for dissolved CO₂ in the effluent medium.

When N₂O was used as electron acceptor, the Porapak T column was replaced by a 3 m column of silica gel. Separation of N₂O from CO₂ was satisfactory at a column temperature of 30 °C.

The concentration of NO in the effluent gas was determined mass-spectrometrically as described by Koike & Hattori (1975).

Nitrate and nitrite were determined by the methods of Wood, Armstrong & Richards (1967) and Bendschneider & Robinson (1952), respectively. Other analytical procedures were as described previously (Koike & Hattori, 1975).

RESULTS

Molar growth yield for glutamate under electron donor- or acceptor-limited conditions

Using glutamate as sole organic source, growth yields of *P. denitrificans* were compared under the conditions of anaerobiosis and limitation of supply of nitrate, nitrite, N₂O or glutamate (Fig. 1). The specific growth rate changed from about 0.12 (near the maximum specific growth rate, $\mu_{\max}$) to about 0.04. Under anaerobic conditions, nitrate, nitrite and N₂O supported bacterial growth. The growth yield per mole of glutamate decreased with decrease in the specific growth rate (μ). The highest growth yield was obtained under glutamate-limited conditions. Complete oxidation of glutamate under glutamate-limited conditions was shown previously (Koike & Hattori, 1975). The dependence of growth yield on specific growth rates under N₂O-limited conditions differed somewhat from those observed with nitrate and nitrite. The growth yield was invariably higher when growth was limited by glutamate than when limited by the supply of terminal electron acceptors.

Carbon balances under terminal electron acceptor-limited conditions

Table 1 summarizes the carbon balances in nitrate-, nitrite- and nitrous oxide-limited growth. Large amounts of glutamate remained in the effluent medium. About half of the glutamate-carbon was oxidized to CO₂ and 15 to 20 % was incorporated into cell materials, irrespective of the limiting factors. The rest was accounted for by acetate and succinate. Total recovery was more than 90 %.

Under nitrate-limited conditions, the proportion of acetate to consumed glutamate was almost unaltered, irrespective of the specific growth rate (Table 2), whereas the proportion of succinate decreased considerably with increase in specific growth rate. The same trend was observed when nitrate was replaced with nitrite or N₂O (Table 2). Transient accumulation of acetate in batch culture of *P. denitrificans* under denitrifying conditions using glutamate as substrate has already been reported (Koike & Hattori, 1975).

Fig. 1

Fig. 2

Fig. 1. Growth yield per mole of glutamate of *P. denitrificans* under anaerobic conditions as a function of the specific growth rate, μ . The growth yield with glutamate-limitation is cited from Koike & Hattori (1975). Culture temperature was 30 °C; μ was obtained from the dilution rate. ■, Glutamate limitation; ○, nitrate limitation; ▽, nitrite limitation; ●, nitrous oxide limitation.

Fig. 2. Growth yield per mole of nitrate, nitrite or nitrous oxide of *P. denitrificans* under anaerobic conditions as a function of specific growth rate, μ . Culture temperature was 30 °C; μ was obtained from the dilution rate. ○, Nitrate limitation; ▽, nitrite limitation; ●, nitrous oxide limitation.

Table 1. Carbon balance during the steady-state growth of *P. denitrificans* under nitrate-, nitrite- or nitrous oxide-limited conditions in the presence of excess glutamate as carbon source

Figures in parentheses represent the ratio of each of the carbon products to consumed glutamate on a carbon basis.

Carbon source	Production or consumption of carbon (mg C/h)		
	Nitrate-limited ($\mu = 0.049$)	Nitrite-limited ($\mu = 0.053$)	Nitrous oxide-limited ($\mu = 0.057$)
Inflow			
Glutamate	34.7	38.4	44.0
Outflow			
Glutamate	11.3	21.5	15.6
Cell	3.84 (17 %)	2.72 (16 %)	5.28 (19 %)
CO ₂	11.9 (51 %)	7.80 (45 %)	16.6 (59 %)
Acetate	2.54 (11 %)	1.28 (7 %)	1.32 (5 %)
Succinate	4.16 (18 %)	5.68 (32 %)	1.75 (6 %)
Total	33.7	39.0	40.6
Percentage recovery	97.1 %	102 %	92.3 %

Acetate and succinate are known to be common products of carbohydrate fermentation (Wood, 1961). The genus *Clostridium* can also ferment glutamate to yield CO₂, acetate and butyric acid (Barker, 1961). Harrison & Pirt (1967) reported that large amounts of metabolites from glucose accumulated during the oxygen-limited continuous growth of *Aerobacter aerogenes*.

Table 2. *Acetate and succinate concentrations in culture medium of P. denitrificans under nitrate-, nitrite- or nitrous oxide-limited conditions*

Figures in parentheses refer to the ratio of the organic acids to the total amount of glutamate consumed on a molar basis. Culture temperature was 30 °C.

Terminal electron acceptors	Specific growth rate, μ (h ⁻¹)	Acetate production (mM)	Succinate production (mM)
Nitrate	0.049	2.15 (27 %)	1.76 (22 %)
	0.086	2.20 (28 %)	0.58 (7.4 %)
	0.110	2.32 (30 %)	0.01 (0.13 %)
Nitrite	0.053	1.00 (19 %)	2.22 (42 %)
	0.075	1.25 (25 %)	0.52 (10 %)
	0.110	0.82 (19 %)	0.21 (4.8 %)
Nitrous oxide	0.057	0.97 (11 %)	0.64 (7.6 %)
	0.089	0.87 (15 %)	0.61 (10 %)
	0.107	0.48 (9.1 %)	0.09 (1.7 %)

From a study of the TCA cycle enzymes and inhibitor experiments, Foget & Pichinoty (1965) concluded that the TCA cycle is fully operative in the denitrifying bacterium, *Micrococcus denitrificans*, under denitrifying conditions. This is probably true also of *P. stutzeri* (Spangler & Gilmour, 1966). The operation of the TCA cycle in our strain of *P. denitrificans* is suggested by the complete oxidation of glutamate under glutamate-limited denitrifying conditions (Koike & Hattori, 1975). The molar composition of the products observed in the present experiment was distinct from that of the fermentation products of glutamate in *Clostridium*. It is unlikely that the accumulation of metabolites under denitrifying conditions is caused by the fermentative degradation of glutamate. Glutamate oxidation in *P. denitrificans*, in the presence of excess glutamate, may proceed at a rate much higher than that of electron flow through the respiration system when the supply of electron acceptors is limited. Under these circumstances, the concentration of NADH in the cell would be expected to increase. Weitzman & Jones (1968) showed that citrate synthase of Gram-negative bacteria, including *Pseudomonas*, is inhibited by NADH. If so this would explain the observed accumulation of acetate.

The identical pattern of carbon distribution among the products under these different conditions strongly suggested that the catabolism of glutamate in this bacterium was not altered by a change of terminal electron acceptors.

Molar growth yield for nitrogenous oxides

Figure 2 shows the molar growth yields for nitrate, nitrite and nitrous oxide as functions of the specific growth rates. No accumulation of intermediary products of nitrate or nitrite reduction to nitrogen were detected under either nitrate- or nitrite-limited conditions. Nitrogenous oxides were not reduced beyond the nitrogen level when glutamate was the substrate (Koike & Hattori, 1975). The concentrations of nitrate and nitrite in the culture were always less than 2 μ M. Under N₂O-limited conditions, the N₂O content of the effluent gas was less than 2 % of that of the inflow gas.

The highest molar growth yield was obtained under nitrate-limited conditions. With nitrite-limitation, it was about 35 % lower. The growth yield for N₂O was the lowest, being about 65 % lower than that with nitrate-limitation.

Fig. 3. Double reciprocal plots of Y against μ in nitrate-, nitrite- or nitrous oxide-limited growth of *P. denitrificans*. Growth yields are expressed on an electron basis. Linear regression analysis was applied to determine $Y_{\max}$ and m . $\circ$, Nitrate limitation; ∇ , nitrite limitation; $\bullet$, nitrous oxide limitation.

Table 3. $Y_{\max}$ and maintenance energy coefficients (m) of *P. denitrificans* for nitrate, nitrite and nitrous oxide

Calculated from the data given in Fig. 3.

Limiting substance	$Y_{\max}$		$10^{-3} \times$ Maintenance energy coefficient, m (electron equiv./g/h)
	g/mol	g/electron equiv.	
Nitrate	28.6	5.72	6.48
Nitrite	16.9	5.63	4.95
Nitrous oxide	8.80	4.40	5.36

Maintenance energy requirements and maximum growth yields

The catabolic process is closely conjugated with the anabolic process under electron acceptor-limited conditions in this bacterium, because the flow rate of electrons through the respiratory systems of denitrification controls the overall process of bacterial growth (Senez, 1962). The growth yield increased with increase in the supply of terminal electron acceptor or the specific growth rate (Fig. 2).

Maintenance energy requirements (m) and maximum growth yield ($Y_{\max}$) under nitrate-, nitrite- and N_2O -limited conditions can be determined from a plot of $1/Y$ versus $1/\mu$ (Pirt, 1965) (Fig. 3). This determination was made with respect to the terminal electron acceptors reduced. The $Y_{\max}$ values for nitrate, nitrite and N_2O were estimated to be 28.6, 16.9 and 8.8 g/mol, respectively (Table 3). However, the $Y_{\max}$ values expressed on an electron basis were almost identical with each other (Table 3). Values for maintenance energy coefficients were also almost identical and agreed well with those obtained under glutamate-limited conditions in the presence of oxygen or nitrate (Koike & Hattori, 1975).

DISCUSSION

There is a controversy as to which step(s) in the sequential reduction of nitrate to nitrogen is associated with phosphorylation in bacterial denitrification. Nitrite reductase of *P. denitrificans* is a soluble enzyme (Iwasaki, Shidara, Suzuki & Mori, 1963), and this suggests that phosphorylation is not coupled with nitrite reduction (Yamanaka, 1964). The same conclusion was obtained by Naik & Nicholas (1966) from ^{32}P experiments using cell-free extracts of *P. denitrificans*.

On the other hand, the occurrence of phosphorylation coupled with nitrite reduction has been reported in the Iwasaki strain of *P. denitrificans* (Ohnishi & Mori, 1960; Matsubara, Miyata, Terai & Mori, 1969). In *M. denitrificans*, phosphorylation is associated with nitrite reduction but not with N_2O reduction (Naik & Nicholas, 1966). They suggested that only nitrogenous oxides containing at least two atoms of oxygen are effective in phosphate esterification.

Cox & Payne (1973) found that nitrite and NO reductases of *P. perfectomarinus* are solubilizable, but nitrate and N_2O reductases are particulate-bound. They inferred that throughout the whole process of denitrification only two steps, the reduction of nitrate to nitrite and the reduction of N_2O to N_2 , function as energy-yielding systems.

The data obtained with cell-free systems are sometimes biased by artefacts produced during preparation and do not necessarily reflect the events occurring *in vivo*. Assessment of energy yield based on growth yield in chemostat cultures is advantageous, because there is no risk of introducing such effects.

In the oxidation of glutamate, ATP would be produced mainly by oxidative phosphorylation coupled to electron transport of denitrification, except for the ATP formation associated with acetate production. One mole of ATP is synthesized per mole of acetate, if acetate is produced via acetyl-CoA. The contribution to energy production associated with acetate production (Y_{acetate}) was estimated, assuming $Y_{\text{ATP}} = 10 \text{ g/mol}$ (Forrest, 1969). The fraction of Y_{acetate} in the total growth yield was $10 \pm 3 \%$ (w/w). Therefore, the effect of acetate production can be disregarded. Under these circumstances, the Y_{max} values for nitrate, nitrite and N_2O can be correlated with energy yields associated with the reduction of these nitrogenous oxides to nitrogen.

The relative energy yields calculated from the data given in Table 3 for reduction of nitrate, nitrite and N_2O to nitrogen were 5.0, 3.0 and 0.8, respectively. These values are proportional to the oxidation number of the nitrogenous oxides: nitrate (+5), nitrite (+3) and N_2O (+1). We take growth yield per half mole of N_2O , because the N_2O molecule contains two nitrogen atoms. The reduction of nitrite to N_2O consists of two consecutive steps: nitrite to NO, and NO to N_2O (Payne, 1973). Unfortunately, the growth yield under NO-limitation has not been determined because of technical difficulties. It is therefore uncertain whether phosphorylation is associated with either of these reduction steps, although circumstantial evidence suggests with both. In any event, the present data (Table 3) show that phosphorylation is coupled with the reduction of nitrite to N_2O in our strain of *P. denitrificans*, a finding incompatible with the view presented by Yamanaka (1964), Naik & Nicholas (1966) and Cox & Payne (1973) on results of experiments using cell-free systems.

The Y_{max} values and the maintenance energy coefficients for these nitrogenous oxides are almost identical if expressed on an electron basis (Table 3). The extent of coupling of electron transport chains with phosphorylation, or the number of phosphorylation sites in the electron transport chain in each step of the denitrification sequence, seem to be identical. The energy yield of *P. denitrificans* associated with denitrification is approximately half that with aerobic respiration on an electron basis (Koike & Hattori, 1975). We therefore conclude that the number of sites of oxidative phosphorylation in the electron transport systems associated each step of denitrification is about half that in the electron transport system associated with oxygen respiration. This view is consistent with the observation that, in extracts of *M. denitrificans*, ATP synthesis coupled with oxygen is about 70 % more efficient than that coupled with nitrate reduction to nitrite (John & Whatley, 1970).

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