Kinetic Explanation for Accumulation of Nitrite, Nitric Oxide, and Nitrous Oxide During Bacterial Denitrification[†]

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The kinetics of denitrification and the causes of nitrite and nitrous oxide accumulation were examined in resting cell suspensions of three denitrifiers. An *Alcaligenes* species and a *Pseudomonas fluorescens* isolate characteristically accumulated nitrite when reducing nitrate; a *Flavobacterium* isolate did not. Nitrate did not inhibit nitrite reduction in cultures grown with tungstate to prevent formation of an active nitrate reductase; rather, accumulation of nitrite seemed to depend on the relative rates of nitrate and nitrite reduction. Each isolate rapidly reduced nitrous oxide even when nitrate or nitrite had been included in the incubation mixture. Nitrate also did not inhibit nitrous oxide reduction in *Alcaligenes odorans*, an organism incapable of nitrate reduction. Thus, added nitrate or nitrite does not always cause nitrous oxide accumulation, as has often been reported for denitrifying soils. All strains produced small amounts of nitric oxide during denitrification in a pattern suggesting that nitric oxide was also under kinetic control similar to that of nitrite and nitrous oxide. Apparent K_m values for nitrate and nitrite reduction were 15 μM or less for each isolate. The K_m value for nitrous oxide reduction by *Flavobacterium* sp. was 0.5 μM. Numerical solutions to a mathematical model of denitrification based on Michaelis-Menten kinetics showed that differences in reduction rates of the nitrogenous compounds were sufficient to account for the observed patterns of nitrite, nitric oxide, and nitrous oxide accumulation. Addition of oxygen inhibited gas production from $^{13}NO_3^-$ by *Alcaligenes* sp. and *P. fluorescens*, but it did not reduce gas production by *Flavobacterium* sp. However, all three isolates produced higher ratios of nitrous oxide to dinitrogen as the oxygen tension increased. Inclusion of oxygen in the model as a nonspecific inhibitor of each step in denitrification resulted in decreased gas production but increased ratios of nitrous oxide to dinitrogen, as observed experimentally. The simplicity of this kinetic model of denitrification and its ability to unify disparate observations should make the model a useful guide in research on the physiology of denitrifier response to environmental effectors.

Denitrification is the reduction of nitrate through the intermediates nitrite, perhaps nitric oxide, and nitrous oxide to form dinitrogen gas (7, 14, 24, 31, 39). Nitrate-amended soils and denitrifying bacterial cultures often accumulate nitrite and nitrous oxide but not nitric oxide. Renewed interest in accumulation of nitrite and nitrous oxide during denitrification reflects concern for their roles in nitrosamine formation and destruction of stratospheric ozone, respectively (5).

Many factors affect the accumulation of denitrification intermediates, but there have been few attempts to develop a unifying explanation. We report here on a kinetic mechanism which could explain a number of the observations summarized below. Nitrite accumulation may result from a lag in synthesis of nitrite reductase (20, 34, 37) or from nitrate inhibition of nitric oxide reductase (25). Nitrate and nitrite amendments enhance production of nitrous oxide (2, 8, 29, 30), perhaps by inhibiting nitrous oxide reductase or by acting as preferred electron acceptors. Net nitrous oxide production in soils varies with time after onset of anaerobiosis (9, 23, 29, 30), possibly owing to a lag in synthesis of nitrous oxide reductase (9). Aerobic soils are sources of

rather than sinks for nitrous oxide (10, 11, 12, 23), suggesting that nitrous oxide reduction is more sensitive to oxygen inhibition than were the other steps in denitrification, but nitrous oxide production during nitrification may account for such observations as well (3).

Measurement of denitrification rates and nitrous oxide production in soils is complicated by the presence of heterogeneous communities of denitrifying organisms (13) and by the chemical and physical complexity of soils (14). For instance, lack of suitable carbon sources (18, 38) and slow diffusion of nitrate to the sites of denitrification (26, 28) may alter not only the rate of denitrification but also the estimates of its kinetic parameters. In situ rate measurements integrate the effects of these extrinsic factors on denitrification and so are suitable for estimates of global nitrogen flux. However, such integrated measurements mask the basic dynamics of the denitrification process.

To avoid the complex and uncertain effect of soils on denitrification, we examined causes for accumulation of nitrite and nitrous oxide in pure cultures of denitrifiers. Our results suggest that the transient accumulation and steady-state pools of intermediates can in many cases be determined by reaction rates rather than any specific inhibition of a step in the denitrification sequence. We developed a model of denitrification based on Michaelis-Menten kinetics to demonstrate how different rates of reduction and general inhibition of denitrification, e.g., by O_2, could lead to the observed patterns of accumulation. The kinetic model should be a useful tool with which to examine accumulation of denitrification intermediates in other systems and in response to other environmental variables.

MATERIALS AND METHODS

Organisms. An *Alcaligenes* species, isolate 17 (ATCC 33513); a *Flavobacterium* species, isolate 175 (ATCC 33514); and a strain of *Pseudomonas fluorescens* biotype II, isolate 72 (ATCC 33512) were selected from a collection of numerically dominant denitrifiers from world soils (13). The isolates had been characterized previously (T. N. Gamble, M.S. thesis, Michigan State University, East Lansing, 1976). *Alcaligenes odorans* was obtained from the American Type Culture Collection, Rockville, Md. (no. 15554). This species, wich does not reduce nitrate, readily denitrifies if supplied with nitrite (27). It was used to determine whether nitrate directly inhibited nitrous oxide reduction.

Media. Organisms were grown on nutrient broth (Difco Laboratories, Detroit, Mich.) containing 10 mM KNO_3. For *A. odorans*, 5 mM KNO_2 was used instead of KNO_3. Seed cultures (50-ml volumes) were grown for 24 h at 30°C and then used to inoculate 450 ml of nitrate broth in 500-ml Erlenmeyer flasks. A rubber

stopper was wired in place on each flask, and the cultures were incubated at 30°C for 24 h. This procedure allowed cultures to use residual oxygen in the flask while shifting to anaerobic metabolism. Nitrate and nitrite were not detected in the spent media. When desired, organisms incapable of nitrate reduction were obtained by substituting 5 mM KNO_2 for KNO_3 and including 10 mM Na_2WO_4 in the growth medium to prevent formation of an active nitrate reductase (32).

Resting cell suspensions. Cells were harvested by centrifugation, washed twice in nutrient broth containing 200 μg of chloramphenicol ml^{-1}, and resuspended in nutrient broth containing chloramphenicol. The chloramphenicol concentration was sufficient to prevent growth of the least-sensitive organism, *P. fluorescens*. Stock cell suspensions were refrigerated or kept on ice and used within 12 h after harvest.

Nitrate and nitrite reduction. A stock cell suspension was diluted before use to give desired rates of reduction. The diluted cell suspension, 18 ml, was placed in a 125-ml flask containing a stirring bar. A large serum stopper was placed over the top of the flask, and the headspace gases were replaced three times with argon. Slight overpressure was maintained in the flask to minimize oxygen entry. The cell suspension was preincubated for 5 min at room temperature (25°C). Two milliliters of nitrate or nitrite solution was injected through the serum stopper to start the reaction. At regular intervals, the flask was inverted, and a sample was removed by syringe. A volume of argon equal to that of the sample was injected to maintain overpressure. Cells were removed by filtration through 0.4 μM polycarbonate filters with glass fiber prefilters (Nucleopore Corp., Pleasanton, Calif.).

Nitrite was determined by diazotization (1). Nitrate was determined with Szechrome NAS reagent and Szechrome NB reagent (Polysciences Inc., Warrington, Penn.), which form stable diphenylamine and benzidine complexes, respectively, with nitrate in concentrated mineral acids. Samples were treated with 0.1 volume of 5% (wt/vol) sulfamic acid to remove nitrite before nitrate determination. For some experiments (see Fig. 1 and 2), nitrate and nitrite were determined with a Technicon Autoanalyzer equipped with a cadmium reduction column.

NO and N_2O measurements. A sealed, continuous gas-circulating system (15) connected to a Perkin Elmer 910 gas chromatograph equipped with a Porapak Q column and ^{63}Ni electron capture detector was used to monitor accumulation and reduction of gaseous intermediates during denitrification. The incubation vessel was a 125-ml flask equipped with a side injection port. Nitrate or nitrite was added to 50 ml of nutrient broth in the flask, which then was flushed with argon for 5 min. A known volume of nitrous oxide was added and allowed to equilibrate for 20 min. The reaction was started by adding a volume of cell suspension sufficient to give a final absorbance at 600 nm of 0.1. NO and N_2O concentrations were calculated with a digital integrator; all concentrations were corrected for changes in volume resulting from removal of gas and liquid samples.

Liquid samples for nitrate and nitrite analyses were

withdrawn from the flask through an 18-gauge spinal tap needle connected to a 1-ml syringe through a one-way valve. Samples were immediately placed in the acidic colorimetric reagents, which stopped denitrification.

Progress curve analysis for estimation of K_m values. The K_m values for nitrite and nitrous oxide reduction were estimated by aalyzing progress curves in which the disappearance of substrate was monitored. The integrated Michaelis-Menten equation for a single-substrate irreversible reaction was fitted by an iterative method, using the Newton-Raphson procedure (6, 22) and Wilkinson's procedure (36) to estimate the standard error.

Oxygen effect on nitrous oxide reduction. The effect of oxygen on nitrous oxide reduction was investigated by using $^{13}NO_3^-$ generated at the Michigan State University sector-focused cyclotron and purified of ^{13}N gases as previously described (35). A 5-ml volume of cell suspension (absorbance at 600 nm, 0.2) was pipetted into Hungate-type screw-capped tubes (Bellco Glass, Inc., Vineland, N.J.). The tubes were capped, and the atmosphere was replaced with helium. Immediately before nitrate addition, a volume of gas was removed from each tube and replaced with an equal volume of air. One milliliter of a 30-μM ^{13}N solution was injected into the tube, which was then placed horizontally on a rotary shaker set at 250 rpm. During incubation, headspace gas was sampled by a syringe and analyzed by a gas chromatograph-proportional counter system described previously (35). Radioactive counts in the gas peaks were corrected for background and ^{13}N decay, and the peak areas and centroids were calculated with FASTFIT, a program written by R. B. Firestone, based on statistical procedures used to analyze nuclear spectra.

Mathematical model of denitrification. To see whether the results we obtained could be accounted for by simple kinetic processes, we developed a denitrification model for computer simulation. We assumed that denitrification took place according to the sequence: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$. Each reductive step was modeled by Michaelis-Menten kinetics:

$$v_i = \frac{V_{max_i} S_i}{K_{m_i} + S_i} \qquad (1)$$

where the subscript i refers to the appropriate constants and concentration for each substrate in the sequence. Rather than derive the exact solution for this four-reaction sequence, we approximated solutions by numerical integration, using Euler's method. The model was not intended to predict denitrification in resting cell cultures but rather to demonstrate how simple kinetics could lead to the behavior observed. For this reason, we deliberately used dimensionless units for substrate concentration and time in the figures presenting results of the simulations.

To examine what effect oxygen may have if it is a general inhibitor of each reductive step in the denitrification pathway, we modified the model so that:

$$v_i = P \frac{V_{max_i} S_i}{K_{m_i} + S_i} \qquad (2)$$

In this equation, P represents the fraction of the bulk solution that was anaerobic at any given time. All reaction rates took place at 100 P percent of the rate under completely anaerobic conditions. P was held constant throughout the simulation for each oxygen concentration. Computer program listings for this simulation model, the progress curve analysis, and FAST-FIT are included in M. R. Betlach's Ph.D. dissertation, Michigan State University, East Lansing, 1979, D.A. no. 80-06083.

RESULTS

Patterns of nitrite accumulation. The extent of nitrite accumulation in resting cell suspensions varied from organism to organism. *Flavobacterium* sp. produced almost undetectable quantities of nitrite during nitrate reduction under the conditions used here (Fig. 1). In contrast, *P. fluorescens* (Fig. 2) and *Alcaligenes* sp. produced much larger quantities of nitrite.

Nitrite accumulation by *P. fluorescens* and *Alcaligenes* sp. might have resulted either from nitrate inhibition of nitrite reduction or from a difference in the rates of nitrate and nitrite reduction. Comparison of the rates of nitrate and nitrite reduction for *P. fluorescens* (Table 1) supported the latter explanation: the rate of nitrite accumulation observed was not significantly different from that calculated as the dif-
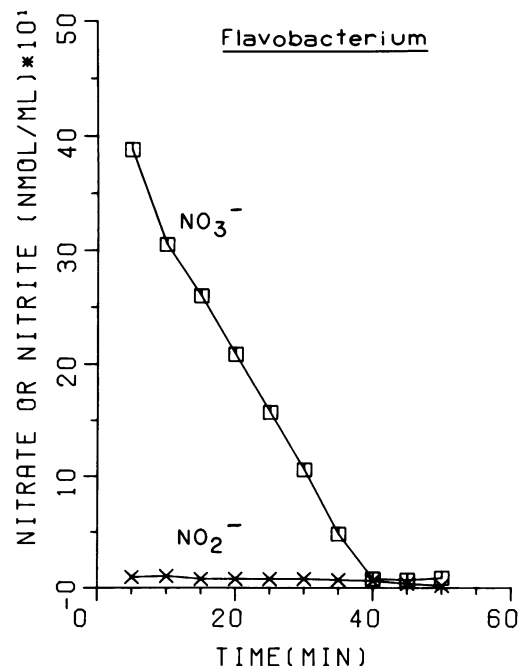

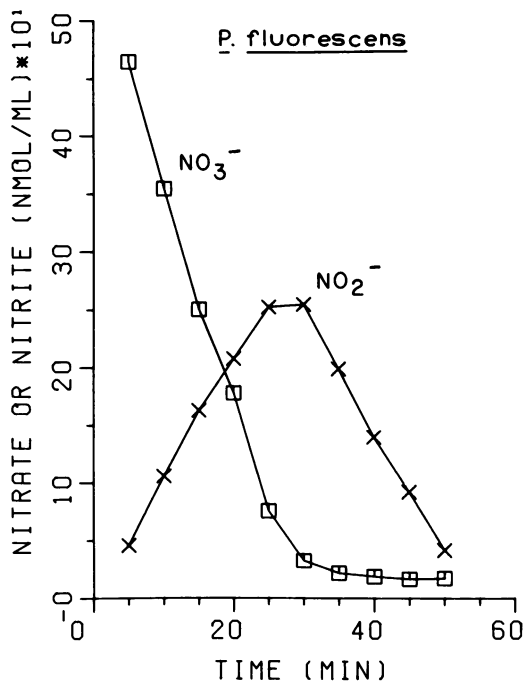FIG. 1. *Lack of nitrite accumulation during denitrification by Flavobacterium sp. (160 μg of protein ml⁻¹). The initial nitrate concentration was 0.5 mM.*

FIG. 2. *Pattern of nitrite accumulation during denitrification by P. fluorescens (160 µg of protein ml^{-1}). The initial nitrate concentration was 0.5 mM.*

TABLE 1. *Calculated and measured rates of nitrite accumulation during denitrification by P. fluorescens (160 µg of protein ml^{-1}); derived from data shown in Fig. 2*

Process	Time interval for rate estimate (min)	Rate (μM min^{-1})[a]
Nitrate reduction	5–25	19.1 ± 2.28
Nitrite reduction	30–50	10.7 ± 0.85
Nitrite accumulation		
Calculated[b]		8.4 ± 3.13
Measured	5–25	10.3 ± 1.35

[a] Rates determined by linear regression; confidence limits are $t_{0.05}s_b$; 3 df.

[b] Difference between measured rates of nitrate reduction and nitrite reduction.

ference between rates of nitrate and nitrite reduction. Furthermore, if nitrate had inhibited nitrite reduction by acting on the nitrite reductase itself, such inhibition should have been observed in cells incapable of nitrate reduction. We obtained such suspensions by growing cultures anaerobically in medium containing nitrite and tungstate. Cells grown with nitrite alone reduced nitrate as rapidly as those grown with nitrate (29.0 ± 7.5 μM min^{-1} and 29.3 ± 4.6 μM

min^{-1}, respectively), but cells grown with nitrite and tungstate were unable to reduce nitrate (0.35 ± 0.30 μM min^{-1}; rate ± $t_{0.05}s_b$). Nitrate concentrations as high as 8 mM had no effect on the rate of nitrite reduction in *Flavobacterium* sp. *P. fluorescens* (Table 2). Hence, nitrate did not directly inhibit nitrite reduction in either organism.

Since nitrite accumulation appeared to be due to different rates of nitrate and nitrite reduction, we used a series of computer simulations to test whether the aforementioned model of denitrification could generate behavior similar to that observed (Fig. 3). The K_m values for nitrate reductase and nitrite reductase were the same in both simulations, but the relative maximal rate of reduction, V_{max}, varied. When the V_{max} for nitrate reduction was set to one-fifth that for nitrite reduction, little nitrite accumulated (Fig. 3a), as had occurred with *Flavobacterium* sp. (Fig. 1). When the rate of nitrate reduction was five times that of nitrite reduction, nitrite accumulated in almost stoichiometric amounts before being reduced (Fig. 3b), thus following a pattern similar to that observed for *P. fluorescens* (Fig. 2). Thus, the kinetic explanation for nitrite accumulation is consistent with the experimental results.

Nitrate and nitrite inhibition of nitrous oxide reduction. To test whether nitrate or nitrite inhibited nitrous oxide reduction in any of the isolates, we monitored reduction of added nitrous oxide in suspensions exposed to different concentrations of nitrate or nitrite. The nitrous oxide concentration should have increased if either ion inhibited reduction. Addition of nitrate to *Flavobacterium* suspensions neither affected the rate of nitrous oxide reduction nor resulted in nitrous oxide accumulation (Fig. 4).

TABLE 2. *Effects of nitrate concentration on the rate of nitrite reduction by Flavobacterium sp. and P. fluorescens lacking nitrate reductase activity*[a]

Nitrate concn (mM)	Nitrite reduction rate (μM min^{-1})[b]	
	Flavobacterium sp.	*P. fluorescens*
0	17.2 ± 1.44 (4)	9.0 ± 0.60 (6)
1.43	19.1 ± 3.01 (3)	8.8 ± 0.24 (6)
3.57	14.5 ± 1.59 (5)	8.6 ± 0.36 (6)
7.14	17.7 ± 2.80 (5)	10.0 ± 0.35 (6)

[a] Cells were grown in nutrient broth containing 5 mM KNO_2 and 5 mM Na_2WO_4. The initial nitrite concentration was 0.125 mM in the assay system. *Flavobacterium* sp. had 32 μg of protein ml^{-1} and *P. fluorescens* had 160 μg of protein ml^{-1}.

[b] Rates were determined by linear regression; confidence limits are $t_{0.05}s_b$; numbers in parentheses are degrees of freedom.
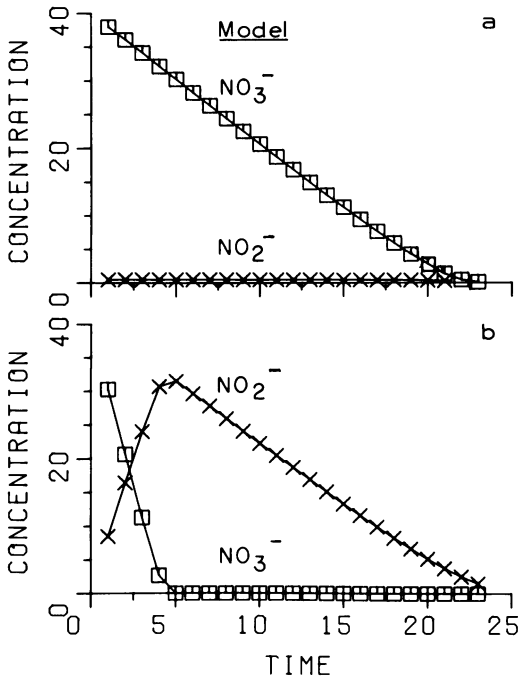
FIG. 3. *Pattern of nitrite accumulation as a function of the rates of nitrate and nitrite reduction in a model of denitrification. The K_m for nitrate reduction was 1, the K_m for nitrite reduction was 2, and the initial nitrate concentration was 40. The maximum velocities for nitrate and nitrite reduction were 2 and 10, respectively, for (a) and 10 and 2, respectively, for (b).*

Inclusion of 0.28 mM nitrite also did not affect the rate of nitrous oxide reduction (data not shown). Nitrous oxide reduction by the *Alcaligenes* isolate and *P. fluorescens* also was not inhibited by addition of nitrate or nitrite at concentrations similar to those given above. Furthermore, the low steady-state concentrations of N_2O (see below) were independent of the amount of nitrate or nitrite added.

Since *A. odorans* reduces nitrite but not nitrate (23), it can be considered physiologically similar to cells grown anaerobically in the presence of tungstate to inhibit nitrate reductase. Neither nitrate nor nitrite affected the rate of nitrous oxide reduction in this organism (Table 3). Net reduction of nitrous oxide began immediately after addition of cells to nitrate broth, but not until 25 and 47 min after cells were added to broth containing 0.2 and 0.5 mM nitrite, respectively. This apparent lag can be attributed to a period during which the rate of nitrite reduction to nitrous oxide matched the rate of nitrous oxide reduction to nitrogen. Neither nitrite nor nitrous oxide was detected in the me-

dium at the end of either experiment, indicating that both substrates had been reduced to dinitrogen.
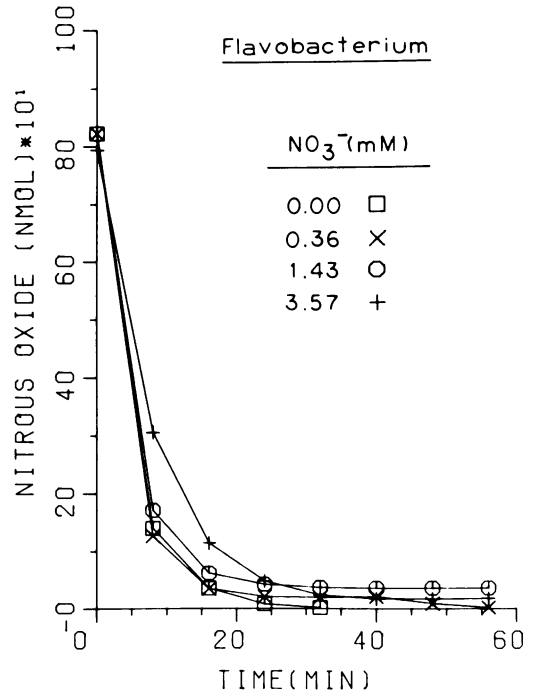

FIG. 4. *Failure of various nitrate concentrations to inhibit nitrous oxide reduction by Flavobacterium sp. (32 μg of protein ml^{-1}). The initial nitrous oxide amount was 820 nmol of N_2O-N (100 ppm, vol/vol, in gas phase). In this and the following figures, amounts of nitrogenous oxides as N per flask are used rather than concentrations in solution to simplify comparison between gaseous and ionic species.*

TABLE 3. *Effect of nitrate concentration and nitrite concentration on the rate of nitrous oxide reduction by A. odorans[a]*

Concn (mM)	First-order rate constant, k (min^{-1})[b]
Nitrate	
0	0.147 ± 0.020
1	0.153 ± 0.044
10	0.169 ± 0.025
100	0.158 ± 0.016
Nitrite	
0	0.143 ± 0.019
0.2	0.154 ± 0.036
0.5	0.149 ± 0.020

[a] The initial nitrous oxide concentration was approximately 100 ppm (100 μl l^{-1}), and the cell concentration was 32 μg of protein ml^{-1}.

[b] Rate constants were determined by linear regression on the transformation: $\ln(N_2O)_t = \ln(N_2O)_0 - kt$; confidence limits are $t_{0.05}s_b$; 3 df.

Nitric oxide and nitrous oxide accumulation. When nitrate or nitrite was included in the medium along with 820 nmol of N_2O, each isolate still reduced the nitrous oxide concentration to a constant value. A low nitrous oxide concentration was maintained until all ionic species had been depleted (Fig. 5 and 6). Nitrous oxide added after the steady state had become established also was reduced rapidly until its concentration reached the previous value (Fig. 6). Maintenance of a relatively constant N_2O concentration even after perturbation was evidence that the steps in denitrification were in kinetic equilibrium.

All three organisms accumulated nitric oxide shortly after cell suspensions had been added to incubation vessels containing nitrate or nitrite. The concentration of nitric oxide also became constant until all the nitrate or nitrite had been used. No nitric oxide was detected in nitrite broth before addition of the cell suspensions, nor did the amount produced correlate with the amount of nitrite present (cf. Fig. 5 and 6). Hence, nitric oxide production could not be attributed to chemical decomposition of nitrite.

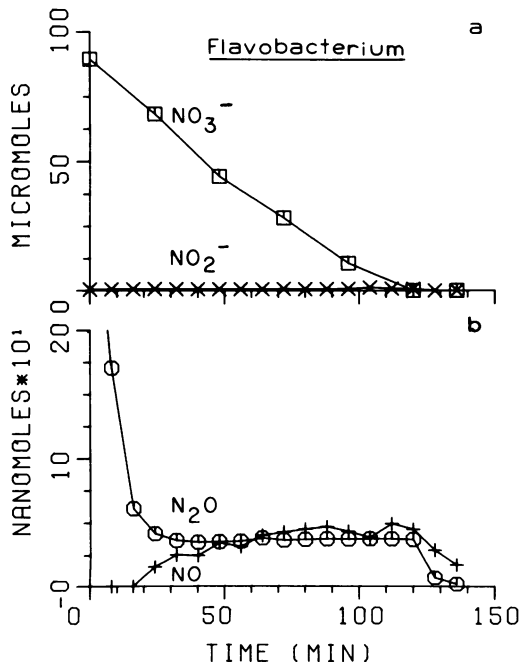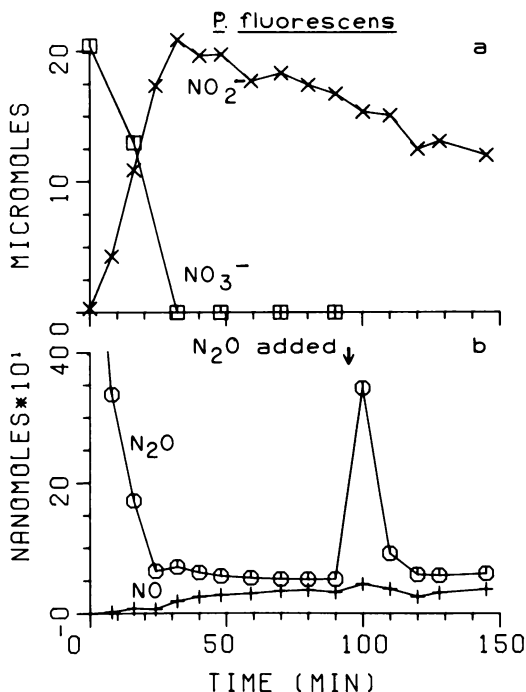The transient appearance of low amounts of nitric oxide and nitrous oxide during denitrifi-

FIG. 6. *Pattern of nitrite and gas accumulation during denitrification by P. fluorescens (32 µg of protein ml⁻¹). The figure shows the return of nitrous oxide to a steady-state value after perturbation. Initial amounts were 20.5 µmol of KNO₃ (0.40 mM) and 820 nmol of N₂O-N. At 95 min (arrow), 410 nmol of N₂O-N was added to the flask.*

cation and the rapid return of nitrous oxide to steady-state amounts after perturbation suggested again that simple kinetics could have accounted for the behavior observed. Addition of these two steps to the denitrification model resulted in the simulation results presented in Fig. 7. The high initial N_2O concentration was rapidly reduced to a steady-state value, which was also reestablished after more N_2O had been added. Such relaxation kinetics were similar to the pattern observed for *P. fluorescens* (Fig. 6). In the simulation, nitric oxide also appeared as an intermediate which was maintained at relatively constant amounts until the more-oxidized compounds had been depleted. The qualitative behavior of NO and N_2O in this mode was similar to the experimental results obtained for all four bacterial species.

Effect of oxygen on nitrous oxide accumulation. We used $^{13}NO_3^-$ to investigate the effect of oxygen on nitrous oxide production and consumption. At higher oxygen concentrations, all three isolates produced a higher percentage of nitrous oxide in the gases released than that produced at lowered oxygen concentrations (Fig.
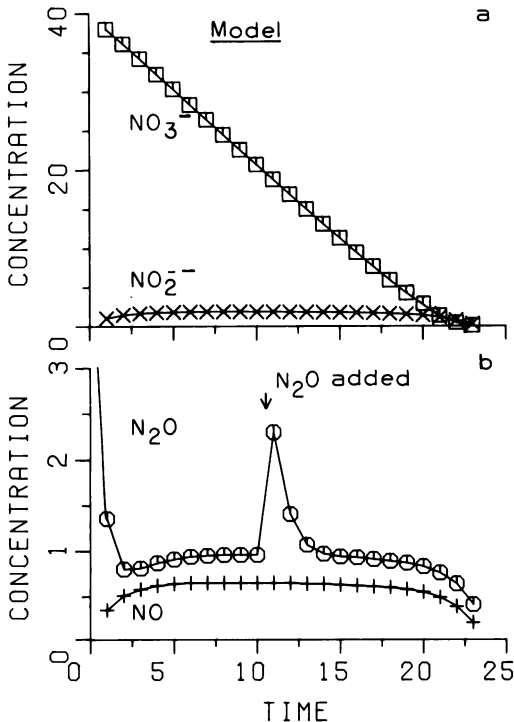

FIG. 5. *Pattern of nitrite and gas accumulation during denitrification by Flavobacterium sp. (32 µg of protein ml⁻¹). The initial amounts were 86 µmol of KNO₃ (1.72 mM) and 820 nmol of N₂O-N (N₂O, 120 ppm [vol/vol]).*

FIG. 7. *Patterns of nitrite and gas accumulation in a model of denitrification, showing the response of nitrous oxide concentration to perturbation. The initial NO_3^- concentration was 40; the initial N_2O-N concentration was 4. At the point indicated by the arrow, the N_2O-N concentration was increased by 3. For reduction of NO_3^-, NO_2^-, NO, and N_2O-N, the K_m values were 1, 2, 2, and 2, respectively; the V_{max} values were 2, 4, 8, and 6, respectively.*

8a and 9a; data for *Alcaligenes* sp. not shown). After additional incubation, cell suspensions had reduced percentages of nitrous oxide, yet the relationship between the percentage of nitrous oxide and oxygen concentration remained the same (Fig. 9; 20 min). Higher oxygen concentrations inhibited denitrification by *P. fluorescens* (Fig. 8b) and *Alcaligenes* sp. However, no decrease in total gas production was observed in several experiments with *Flavobacterium* sp. (Fig. 9b). Nitrous oxide reduction in this organism appeared to be more sensitive to oxygen than were the other steps in denitrification.

Could general inhibition of denitrification by oxygen have caused increased production of nitrous oxide relative to dinitrogen as oxygen tension increased, or was nitrous oxide reduction more sensitive to oxygen than the other reductive steps? As a preliminary approach to solving this problem, we modified the denitrification model to include a term for oxygen inhibition of each step in the pathway, as described in equa-

tion 2. The contribution of N_2O to total gas production was calculated at various times for each of the four values for P. As the system became more aerobic the percentage of N_2O produced increased (Fig. 10a), as observed for all three isolates. In addition, the time-dependent decrease in N_2O contribution at different oxygen tensions resembled the behavior observed in the bacterial cultures (Fig. 9a). As expected, the total amount of gas produced decreased at higher oxygen tensions (Fig. 10b).

K_m values for nitrate, nitrite, and nitrous oxide reduction. K_m values for nitrite reduction were determined by analysis of progress curves. The values for *Alcaligenes* sp., *Flavobacterium* sp., and *P. fluorescens* were similar: 12.9 ± 2.6, 5.6 ± 1.6, and 5.5 ± 1.1 μM (K_m ± standard error), respectively.

It was not possible to obtain K_m estimates for nitrate reduction directly, owing to the limited sensitivity and precision of the colorimetric procedures used. Since rates of nitrate reduction observed at concentrations greater than 15 μM were linear, the K_m values for nitrate reduction ought to have been at least as low as those for nitrite reduction. Recent results of Edwards and Tiedje confirm that nitrate K_m values are in the
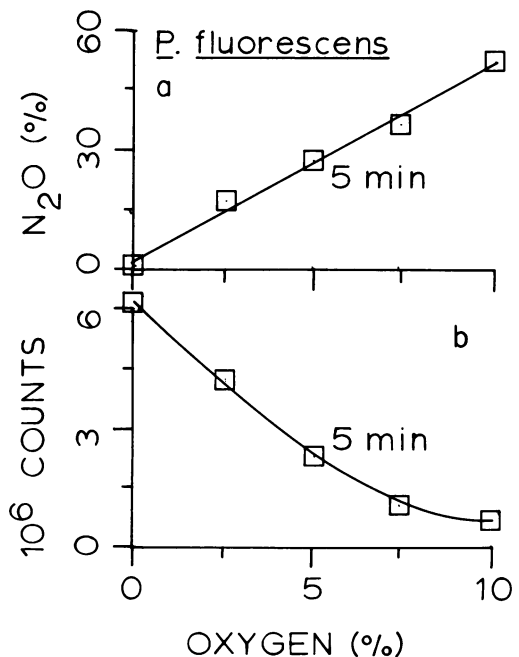

FIG. 8. *Effects of increasing oxygen concentration on composition and amount of gases produced by P. fluorescens (65 μg of protein ml^{-1}) 10 min after addition of $^{13}NO_3^-$. The initial NO_3^- concentration was 5 μM. (a) Percentage of gaseous products (N_2O and N_2) as N_2O; (b) total ^{13}N in N_2O and N_2 per 1-ml sample.*
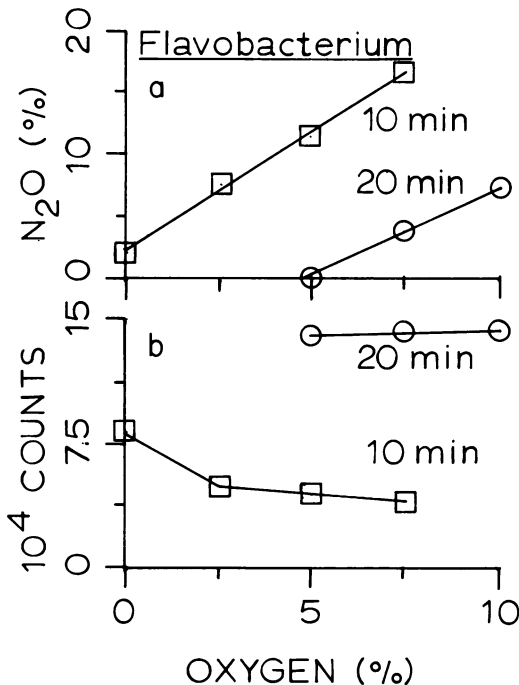
FIG. 9. *Effects of increasing oxygen concentration on composition and amount of gases produced by Flavobacterium sp. (65 μg of protein ml^{-1}) 10 and 20 min after addition of $^{13}NO_3^-$. The initial NO_3^- concentration was 5 μM. (a) Percentage of gaseous products (N_2O and N_2) as N_2O; (b) total ^{13}N in N_2O and N_2 per 1-ml sample.*

range of 5 to 10 μM (R. M. Edwards and J. M. Tiedje, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, N48, p. 181).

The estimated K_m value for nitrous oxide reduction by *Flavobacterium* sp. was 440 + 11 nM (1.92 + 0.048 Pa). This value was determined from experiments starting at several initial partial pressures of nitrous oxide. Data from a typical experiment are shown in Fig. 11. To ensure that the biological rate of N_2O reduction was measured rather than the rate of N_2O transfer from the gas to the liquid phase, we increased the transfer rate by sparging gas through the continously stirred medium. In addition, the rate of N_2O reduction was reduced by decreasing cell density to an absorbance of 0.005 and the liquid volume to 25 ml.

If phase transfer had limited the initial rate of nitrous oxide reduction, we should have obtained a biphasic exponential curve: the first portion of the curve would represent the rate of transfer into the liquid phase, and the second portion would represent the rate of biological reduction when transfer was sufficiently rapid to support the reduction rate. If the biological rate of reduction had always been greater than the

rate of gas transfer, there should have been only one exponential curve, reflecting diffusion of N_2O into the aqueous phase. Also, if the iterative procedure for the progress curve analysis would converge in the latter case, the K_m value obtained should have been at least as high as the initial nitrous oxide concentration. We did not observe a single or a biphasic exponential curve (Fig. 11), nor did we obtain a K_m value as high as the initial N_2O concentration. Thus we are confident that only the biological process was measured in these experiments.

DISCUSSION

The results presented above permit us to rule out direct inhibition of nitrite reduction by nitrate as a mechanism of nitrite accumulation: the rates of nitrite reduction by *P. fluorescens* were similar in the presence and absence of nitrate (Table 1), and nitrate did not inhibit nitrite reduction in tungstate-grown bacteria lacking a functional nitrate reductase (Table 3). Apparently, different rates of reduction of the intermediates of denitrification caused their accumulation, rather than any specific inhibitory mechanism. The kinetic model of denitrification

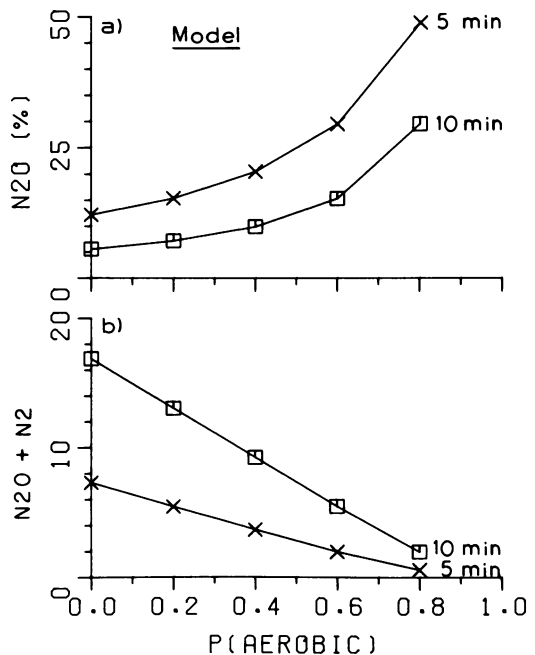FIG. 10. *Effects of increasing oxygen concentration on composition and amount of gases produced in a model of denitrification 5 and 10 min after NO_3^- addition. The initial NO_3^- concentration was 40. The rate constants are as specified for Fig. 7. (a) Percentage of gaseous products (N_2O and N_2) as N_2O; (b) total amount of N_2O and N_2 produced.*
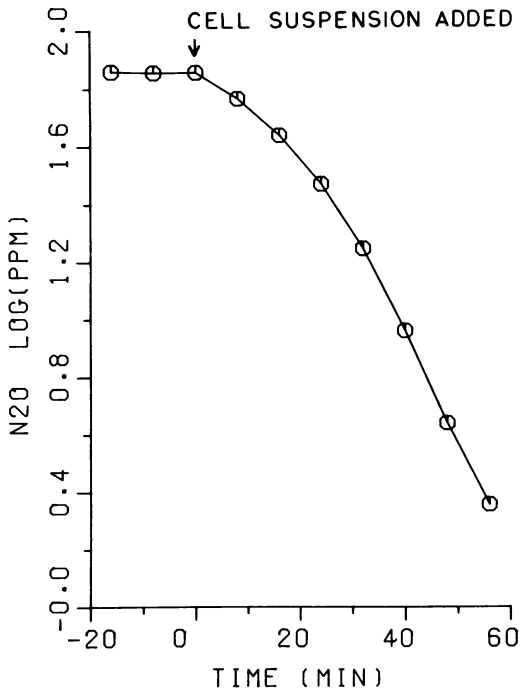
FIG. 11. *Progress curve used to determine the* K_m *value for nitrous oxide reduction by Flavobacterium sp. N_2O concentration in the gas phase at start of incubation was 72 ppm (vol/vol), and cell concentration after addition (arrow) was 1.6 μg of protein ml^{-1}.*

(equation 1) was constructed to demonstrate how simple kinetics may cause the patterns of nitrite accumulation observed. Varying the relative rates of nitrate and nitrite reduction in the model determined the extent of nitrite accumulation (Fig. 3). In the bacterial cultures, the maximal velocity of nitrate or nitrite reduction would have depended not only on substrate concentration but also on the number of reductase molecules present. Variations in the extent of nitrite accumulation by *P. fluorescens*—compare Fig. 2 and 6—suggest that the relative amounts of nitrate and nitrite reductase were not constant. Differences may reflect both the time at which the cultures were harvested relative to the time they became anaerobic as well as corresponding lags in synthesis of nitrite reductase relative to nitrate reductase, as Williams et al. reported (37).

Nitrous oxide frequently accumulates in soils during experimental studies of denitrification. The ratio of N_2O to N_2 may be increased by adding nitrate to soils (2, 8, 29, 30), suggesting that nitrate specifically inhibits nitrous oxide reduction. Firestone et al. (8) postulated that nitrite produced during nitrate reduction may

be the actual inhibitor of N_2O reduction, since N_2O accumulation is greater with nitrite than with nitrate. However, the experiments reported here showed that neither nitrate nor nitrite at concentrations comparable to those used by Firestone et al. (8) had any effect on the rate of nitrous oxide reduction in pure cultures of denitrifiers. The explanation for the discrepancy between our results and the previous results for soils may be due to one or both of the following. We used cultures harvested in late exponential phase of growth which probably had more N_2O reductase relative to the previous enzymes in the sequence than is the case in soils (9). Secondly, the more-oxidized N-oxides may be preferentially reduced when the supply of electron donor is limited. Kohl et al. (18) and Yoshinari et al. (38) found that addition of glucose to soil increased the denitrification rate and also affected the K_m estimated for denitrification. Use of more-oxidized electron acceptors when carbon was limiting could have prevented reduction of nitrous oxide, an effect difficult to distinguish from direct inhibition of the nitrous oxide reductase. Use of a rich medium in the experiments reported here prevented such carbon limitation.

As noted, carbon availability could affect the estimation of K_m values for denitrification in soil. Phillips et al. (26) and Reddy et al. (28) recently demonstrated that the physical structure of soil also may influence the kinetics of denitrification. They concluded that the typical procedure which uses flooded soil to determine the rate of nitrate reduction actually measures the rate of nitrate diffusion from the overlying solution into the soil matrix. Such mass transfer resistance would result in apparent first-order denitrification rates at nitrate concentrations higher than those required to saturate enzymes in a system not limited by diffusion and consequently would give higher K_m estimates (33). Both carbon limitation and effects of diffusion may explain why K_m values for denitrification in soils: 0.23 mM (17), 0.29 and 3.5 mM (18), and 0.13 and 1.2 mM (38), are so much higher than the values we obtained for nitrate and nitrite reduction by pure cultures, which were <0.015 mM.

Diffusion limitations may also have affected measurements of the K_m value for nitrous oxide reduction. The value we obtained for *Flavobacterium* sp., 0.5 μM, was much smaller than those reported previously: 5 μM for a lysate of *Paracoccus denitrificans* (19), 30 to 60 μM for *Pseudomonas denitrificans* (21), less than 100 μM for *P. aeruginosa* (31), and 0.7 to 1.0 mM for a soil (38). We had to use very low cell concentrations and rapid mixing to ensure that our K_m measurement was not affected by diffusion of

N_2O across the gas-liquid interface. Though lower than previous estimates, the K_m value for *Flavobacterium* sp. was still 50 times higher than the N_2O concentration in solutions equilibrated with the atmosphere.

In the three isolates we used, the concentration of nitrous oxide appeared to be under kinetic control. Such control was especially evident when the nitrous oxide concentration rapidly returned to a steady-state value after addition of more N_2O (Fig. 6). The slight accumulation during denitrification of nitric oxide suggested that it, like nitrite and nitrous oxide, was also under kinetic control. The increase in NO after addition of the substrate and its depletion after nitrate and nitrite were consumed was the pattern expected if NO had been an intermediate or in equilibrium with an enzyme-bound intermediate in the denitrification pathway. Use of an electron capture detector and a system designed to exclude oxygen may account for our being able to readily detect NO during denitrification and other investigators not being able to do so. The kinetic model of denitrification represented by equation 1 could account for the transient appearance of nitric oxide and nitrous oxide during denitrification as well as the accumulation of nitrite (Fig. 7).

Increased oxygen in soils enhances the ratio of N_2O to N_2 produced during denitrification. However, oxygen also inhibits denitrification and represses synthesis of the denitrifying enzymes (5, 14, 24). Such general inhibition was apparent in the decrease in total gas production by *Alcaligenes* sp. and *P. fluorescens* exposed to increasing oxygen concentrations (Fig. 8). Gas production by *Flavobacterium* was not inhibited by oxygen (Fig. 9), although the proportion of gas as N_2O increased with increased oxygen. Focht (10) developed a zero-order kinetic model of denitrification based on data from Nommik (23), in which increased production of N_2O at higher soil aeration results from oxygen inhibition of N_2O reduction greater than that of nitrate reduction. Since gas production by *Flavobacterium* sp. was not decreased by oxygen, this organism appears to correspond to Focht's model. Nitrous oxide reductases of the other two organisms might also have been more sensitive to oxygen than were the enzymes catalyzing nitrous oxide formation, but the decrease in gas production at increased oxygen concentrations suggested that general inhibition of denitrification by oxygen may cause similar behavior. Results of the model in which a general oxygen inhibition term, P, was included gave results (Fig. 10) qualitatively similar to those obtained for *P. fluorescens* (Fig. 8).

An untested prediction of the model represented by equation 2 is that the percentage of nitrite relative to the more-reduced nitrogen compounds should also increase with increased oxygen concentration. Furthermore, since the mathematics of the model would remain unchanged, any factor which inhibited all steps in denitrification equally should elicit a response similar to that observed with increased oxygen concentration. Recently, Keeney et al. (16) reported that soils incubated at 5 and 15°C produced a higher proportion of gas as N_2O rather than N_2 when compared with soils incubated at 25°C. Total gas production was lower at the lower temperatures. Data presented by Bollag and Kurek (4) showed that the effects of pesticide metabolites on denitrification were similar to those of decreased temperature: in the presence of pesticide metabolites, more nitrite and nitrous oxide are produced during denitrification, which takes place at a reduced rate compared with that of unamended controls. The time course data presented in both papers show that the effects of temperature and pesticide metabolites are less pronounced with longer incubation, as we found for the oxygen effect on N_2O, both by the bacteria (Fig. 9) and in the model (Fig. 10).

The kinetic model presented in this paper has the advantage of being simple without sacrificing any power to unify disparate observations regarding accumulation of intermediates during denitrification. We developed the model based on work with pure cultures, and we were able to use it for isolating the physiology of the denitrifiers from the limitations imposed upon them by complex environments. There is no less a need to investigate how the physicochemical environment modifies this physiological potential (e.g., by controlling substrate availability through limited diffusion). We do not intend the hypothesis that accumulation of intermediates reflects different rates of reduction to obscure phenotypic differences among denitrifying bacteria (e.g., O_2 on *Flavobacterium* spp.), nor is it meant to discount the possibility of specific inhibition in some cases. However, we do feel that the kinetic model can be a useful guide in research on the physiology and ecology of denitrification.

ADDENDUM IN PROOF

The isolate termed *Alcaligenes* in this paper has

recently been classified as *Pseudomonas alcaligenes* by the American Type Culture Collection. We did not originally consider this identification, since *P. alcaligenes* is reported not to denitrify in *Bergey's Manual* (8th ed., p. 219).

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