# ACCUMULATION OF INTERMEDIATES DURING DENITRIFICATION--KINETIC MECHANISMS AND REGULATION OF ASSIMILATORY NITRATE UPTAKE

By

Michael Richard Betlach

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1979

ABSTRACT

ACCUMULATION OF INTERMEDIATES
DURING DENITRIFICATION--KINETIC MECHANISMS
AND
REGULATION OF ASSIMILATORY NITRATE UPTAKE

By

Michael Richard Betlach

The generally accepted pathway for denitrification is as follows: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$, but the role of NO as an intermediate is still disputed. The other intermediates, $NO_2^-$ and $N_2O$, frequently accumulate during denitrification. The recent discovery that $N_2O$ can catalyze the destruction of stratospheric ozone has led to a reexamination of factors contributing to its production in soil, which include aerobic conditions and high concentrations of $NO_3^-$ and $NO_2^-$.

I examined the kinetics of denitrification in pure cultures of three numerically dominant denitrifiers to avoid the heterogeneous denitrifier populations and confounding chemical and physical effects, such as diffusion, present in soil. A closed gas recirculation system connected to a gas chromatograph with an electron capture detector facilitated work with NO and $N_2O$.

An Alcaligenes species and a strain of Pseudomonas fluorescens accumulated significant amounts of $NO_2^-$ during denitrification; the Flavobacterium species did not. Such accumulation was not due to a specific inhibitory mechanism, because $NO_3^-$ had no effect on $NO_2^-$ reduction in cultures grown on tungstate to inactivate $NO_3^-$ reductase.

In all three isolates neither $NO_3^-$ nor $NO_2^-$ affected the rate of $N_2O$ reduction. Instead, the $N_2O$ concentration dropped rapidly to steady state values, which were maintained until the nitrogenous ions had been reduced. All organisms produced NO during denitrification. Its concentration also reached a steady state, then dropped after $NO_3^-$ and $NO_2^-$ had been reduced. NO was not detected in the absence of cell suspensions. Such behavior is consistent with schemes which include NO as an obligate intermediate of denitrification.

Increased $O_2$ concentrations reduced the amount of gas produced by Alcaligenes and P. fluorescens, but had no effect on total gas production by Flavobacterium. However, all three isolates produced higher ratios of $N_2O$ to $N_2$ at higher concentrations of $O_2$, as found in soils.

The $K_m$ values for nitrate reduction by all three denitrifiers were less than 10 μM, based on linear rates observed at higher nitrate concentrations. The $K_m$ values for nitrite reduction were determined from progress curves to be 12.5, 5.7, and 5.4 μM for Alcaligenes, Flavobacterium, and P. fluorescens, respectively. The $K_m$ value for nitrous oxide reduction by Flavobacterium was 0.5 μM.

A model of denitrification based on Michaelis-Menten kinetics can account for the patterns of $NO_2^-$, NO, and $N_2O$ accumulation observed. Accumulation of the intermediates is a result of different rates of reduction in this model, rather than of specific inhibition of any of the reductive steps. With the assumption that oxygen is a nonspecific inhibitor of all reductive steps in denitrification, the model can simulate the increase in the ratio of $N_2O$ to $N_2$ and the decrease in gas production observed at increased $O_2$ concentrations. The simplicity of this kinetic model and its power to unify disparate observations should

provide a useful guide to future research on denitrifier physiology and behavior.

In contrast to the vast amount of research on denitrification, relatively little has been done to investigate the mechanism of nitrate assimilation in bacteria. The lack of research may reflect lack of a suitable isotopic label: $^{15}N$ methods are not sufficiently sensitive to detect small amounts of nitrogen incorporated into bacterial cells in short time periods characteristic of most uptake studies. I overcame this difficulty by using standard membrane filtration techniques in uptake studies with $^{13}NO_3^-$ generated at the MSU Cyclotron Laboratory. This isotope of nitrogen has a half-life of 10 min.

P. fluorescens can actively transport $NO_3^-$ against a concentration gradient, as demonstrated by finding $^{13}NO_3^-$ internally in cells grown on tungstate to inactivate nitrate reductase. Incorporation of $^{13}NO_3^-$ in cells grown on $NO_3^-$ was inhibited by 1 mM azide and cyanide as well. An internal pool of $^{13}NO_3^-$ was not detectable in cells with an active nitrate reductase; instead all the $^{13}N$ was present as ammonia or amino acids.

Cells grown on $(NH_4)_2SO_4$ and $NH_4NO_3$ were unable to assimilate $NO_3^-$. Cells grown on $NH_4^+$ demonstrated a diauxic lag when transferred to medium containing $NO_3^-$ as the sole nitrogen source. $NO_3^-$ reduction began at the end of the lag phase. These observations indicate that excess ammonia repressed synthesis of the assimilatory enzymes and transport protein. Both nitrate and nitrite stimulated synthesis of the assimilatory enzymes in the absence of ammonia.

Ammonia inhibited $NO_3^-$ transport in cells grown with or without tungstate. Glutamine inhibited $NO_3^-$ incorporation as well, but glutamate

had no effect, even though high voltage electrophoresis demonstrated that both were early organic products of $NO_3^-$ assimilation.

Chlorate, a chemical analog of $NO_3^-$, did not affect $NO_3^-$ transport or incorporation in P. fluorescens, in contrast to previous reports of inhibition of both the assimilatory and dissimilatory $NO_3^-$ reductases of other bacteria.

The Michaelis constants for $NO_3^-$ uptake by cells grown on $NO_3^-$ and cells grown on $NO_2^-$ with tungstate were similar, 7.1 and 6.6 μM, respectively. This similarity and the lack of an internal $NO_3^-$ pool in cells with an active nitrate reductase suggest that nitrate assimilation in P. fluorescens is limited by the rate of nitrate transport, rather than by the rate of nitrate reduction.

**To Paulanne and my parents**

**In love this too is theirs**

## ACKNOWLEDGMENTS

I am most grateful to Dr. James Tiedje for creating a favorable environment for research and giving me the freedom to explore it fully. His trust and friendship and the comraderie and support of Bill Caskey, Mary Firestone, and Scott Smith have made the past years a very special experience for me. I deeply appreciate the contributions they and the other members of the lab, especially Henry Kaspar, have made to my scientific and personal growth.

I thank the members of my committee--Drs. J. A. Breznak, T. R. Corner, P. Filner, and H. L. Sadoff--for their encouragement and excellent advice throughout my tenure at MSU.

Without the enthusiasm and cooperation of Dr. Richard Firestone there would not have been an opportunity to use $^{13}N$ in this research.

My wife Paulanne had the faith to support me when my will failed, and the patient trust to go to San Diego in a time of uncertainty. Words alone are insufficient to express my gratitude; at least we will be together again soon.

I thank April Norton for her superb typing and charming smile through many long days and numerous revisions.

During this research I was supported by an NSF Graduate Fellowship, an NIH Traineeship, and a departmental assistantship. This research was supported by USDA Regional Research Project NE-39 and a grant from the National Science Foundation.

TABLE OF CONTENTS

## LIST OF TABLES

| Figure | APPENDIX A | Page |
|---|---|---|
| 1 | Effect of nitrite on nitrate determination using Szechrome NAS reagent ..... | 115 |

LIST OF FIGURES

# INTRODUCTION

The central importance of nitrogen to life on this planet has become a hackneyed justification for research dealing with any aspects of the nitrogen cycle. The vast mountain of research being conducted today--whether to obtain estimates of global nitrogen fluxes (30) or to investigate a particular enzyme involved in a particular pathway (31)--is a monument to the time-worn relevance of such endeavors. Unfortunately, it also prevents most scientists from obtaining a sense of perspective for their own efforts. In an attempt to maintain such a perspective I have restricted myself to a discussion of the fates of nitrate in soil. The reader desiring more detailed information about various aspects of the nitrogen cycle may wish to consult any of several recent reviews (2,9,12,20,22,28,30).

Increased costs of fertilizer, concern over accelerated eutrophication and pollution, and the recent discovery that nitrous oxide may be an important regulator of stratospheric ozone all have renewed interest in soil nitrogen processes (28). Figure 1 illustrates the various transformations of nitrate in soil. Of these, assimilation is probably the most likely fate since nitrogen is cycled within the soil much more than it is transported in and out (28). Even though ammonia per se can repress synthesis of the assimilatory nitrate enzymes, the relative unavailability of ammonia in soil due to its adsorption on negatively charged clays and organic matter (1) makes nitrate the usual

**Figure 1. Fates of nitrate in soil.**

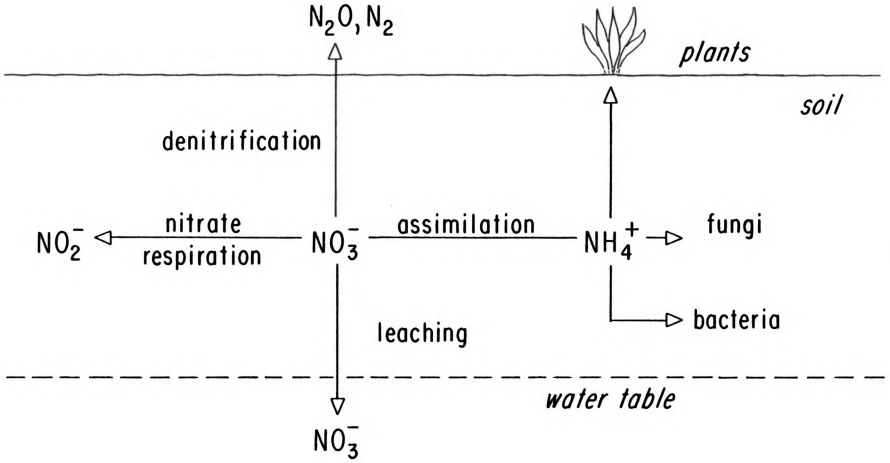

$N_2O, N_2$
plants
soil
denitrification
$NO_2^-$
nitrate
respiration
$NO_3^-$
assimilation
$NH_4^+$
fungi
bacteria
leaching
water table
$NO_3^-$

FIGURE 1

nitrogen source for plants and microorganisms.

The other fates of nitrate in soil are significant only if the soil or a portion of it is anaerobic. In muck soils the conditions which are necessary for leaching, high nitrate concentrations and heavy rains, are also those which favor denitrification (15). Both denitrification, the reduction of nitrogenous oxides to gaseous end products, and nitrate respiration, the reduction of nitrate only to nitrite, provide more energy for bacteria than does fermentation (20). Both have a common first product, nitrite. However, nitrite accumulation is rarely observed in soil, which is puzzling considering that nitrate respiring organisms outnumber denitrifiers about ten to one in most soils. Even when nitrite accumulation is observed, it is usually a result of inhibition of nitrite oxidation at alkaline pH and high ammonia concentrations (4). It is possible the end product of nitrate respiration is ammonia. I have not included the process in Figure 1 since it has only recently been recognized and apparently occurs only in highly reducing environments, such as flooded soils (3). Ammonia apparently does not inhibit the dissimilatory reduction of nitrate (20).

Several different approaches have been taken to clarify the dynamics of nitrate in soil. These can be summarized briefly as the thermodynamic, kinetic, and tracer approaches. Table 1 presents the free energies and redox potentials of the common reductive steps. The thermodynamic approach is primarily a theoretical one constructed to account for observations of nitrous oxide accumulation during denitrification in soils that were partially aerobic. Focht and Verstraete found the redox potential of the nitrous oxide-nitrogen couple was approximately 250 mV when calculated from molar growth yields of -

Table 1. Free energies and redox potentials for reduction of inorganic nitrogen compounds.

| Reaction | $\Delta G_o'$ (KJ/mol)[a] | $E_o'$ (mV)[a] | Reference |
|---|---|---|---|
| Nitrate Respiration | | | |
| $NO_3^- + [H_2] \rightarrow NO_2^- + H_2O$ | -161.0 | +420 | Zumft (35) |
| Nitrate Assimilation | | | |
| $NO_3^- + 2H^+ 4[H_2] \rightarrow NH_4^+ + 3H_2O$ | -590.8 | +350 | Zumft (35) |
| Denitrification | | | |
| $2NO_3^- + 2H^+ + 5[H_2] \rightarrow N_2(g) + 6H_2O$ | -1121.2 | +749 | Zumft (35) |
| Reactions of intermediates | | | |
| $NO_2^- + 2H^+ + 3[H_2] \rightarrow NH_4^+ + 2H_2O$ | -436.4 | +341[b] | Thauer et al. (34) |
| $NO_2^- + 1\frac{1}{2}[H_2] + H^+ \rightarrow NO(g) + H_2O$ | -76.2 | +374 | Zumft (35) |
| $2NO(g) + [H_2] \rightarrow N_2O(g) + H_2O$ | -306.3 | +1177 | Zumft (35) |
| $N_2O(g) + [H_2] \rightarrow N_2(g) + H_2O$ | -339.5 | +1352 | Zumft (35) |
| Oxygen Respiration | | | |
| $O_2 + 2[H_2] \rightarrow 2H_2O$ | -474.5 | +816[b] | Thauer et al. (34) |

[a] pH 7.0

[b] Calculated from the free energy change as described by Stumm and Morgan (32).

bacteria (9). They felt this low value might account for $N_2O$ accumulation at high Eh found in aerobic soils. However, they did not discuss why this value is much lower than the one calculated from free energies of formation (Table 1). On thermodynamic grounds alone nitric oxide and nitrous oxide should rarely be observed, since they are more powerful oxidants than oxygen. The reactions most likely to be inhibited by aerobic conditions are the reduction of nitrate and nitrite to ammonia. But such an examination of thermodynamic values only indicates where equilibrium eventually will lie, rather than the rate at which that equilibrium will be attained, and so may be of little practical value.

The kinetic approach does not have that disadvantage since it concerns itself with the dynamics of nitrate use. It is attractive because it offers the possibility to model the processes studied, and so to be able to predict what will happen under given conditions. Measurement of $K_m$ values and evaluation of competitive ability among organsims based on such values is an attractive alternative to monitoring the distribution of nitrate in the soil. Yet of the large number of organisms capable of nitrate assimilation and denitrification (12,20,22), $K_m$ values for relatively few have been obtained (Table 2). Many of these values are for purified enzymes, and so may be of little use when considering intact organisms. Furthermore, extrapolation of such laboratory determinations to natural environments is risky, because the determinations themselves reflect complex physiological control in the organisms (33). In addition, measurement of the concentration of a single compound, such as nitrate, and comparison with $K_m$ values to determine if the nutrient is limiting ignores the synergism of other

Table 2. Michaelis constants reported for biological fates of nitrate in soils.

| Organism | $K_m$ (mM) | References |
|---|---|---|
| Assimilation--$NO_3^-$ reductase | | |
| *Azotobacter chroococcum* | 0.25 | Guerrero *et al.* (14) |
| *Aspergillus nidulans* | 0.83 | Downey (7) |
| --$NO_3^-$ transport | | |
| *Neurospora crassa* | 0.25 | Schloemer and Garrett (29) |
| *Penicillium chrysogenum* | <0.01 | Goldsmith *et al.* (13) |
| *Arabidopsis thaliana* | 0.04, 25 | Doddema and Telkamp (5) |
| Barley | 0.11 | Rao and Rains (23) |
| Maize | 0.02 | Honert and Hooymans (17) |
| Rye-grass | 0.03 | Lycklama (19) |
| Tobacco | 0.40 | Heimer and Filner (16) |
| Respiration--$NO_3^-$ reductase | | |
| *Bacillus licheniformis* | 0.11 | Riet *et al.* (26) |
| *Escherichia coli* | 1.50 | Forget (11) |
| *Klebsiella aerogenes* | 0.10 | Riet and Planta (25) |
| Denitrification--$NO_3^-$ reductase | | |
| *Paracoccus denitrificans* | 0.29 | Forget (10) |
| *Paracoccus halodenitrificans* | 1.3 | Rosso *et al.* (27) |
| *Pseudomonas aeruginosa* | .02 | Fewson and Nicholas (8) |

chemical and environmental limiting factors so apparent in heterogeneous soil (6).

If application of kinetic values obtained in the laboratory is difficult, what of determinations made in the soils themselves? Such estimates have been primarily of denitrification, for which $K_m$ values vary from 0.12 mM to 12 mM (18,35). The kinetics of denitrification assays in soils may not reflect the kinetics of nitrate reduction itself. For instance, denitrification rates are frequently found to be carbon-limited (18,35). Kohl et al. (18) pointed out that it was not possible to determine a kinetic mechanism by comparing how well several mathematical models fit the data. The kinetics observed may not reflect a biological process at all. As Reddy, Phillips, and Patrick found (21,24), many previous reports of first-order denitrification kinetics may actually have been due to diffusion of nitrate from solution to the sites of nitrate reduction.

Tracer studies using $^{15}N$ have been used infrequently to determine kinetics; rather, their strength is in the ability to follow nitrate movement through the many pools of soil nitrogen. Most commonly, such studies have been with labeled fertilizer, to determine how much is taken up by the crop and how much is leached through the soil profile into the water table (1). The major disadvantages of such studies are that $^{15}N$-enriched material is expensive to obtain and to analyze and also that large amounts have to be used to avoid problems detecting enrichment against the 0.366% natural abundance of $^{15}N$. Furthermore, to determine whether the labeled nitrate is assimilated by bacteria and fungi, a way must be found to separate the organisms from the much larger pool of soil organic matter. Recent reports of fumigation

techniques to partition biomass $^{15}N$ from the active $^{15}N$ pool offer hope that tracer techniques may be of greater use as better methods become available (R. P. Voroney, N. G. Juma, and E. A. Paul, Abstr. Annu. Meet. Am. Soc. Micriobiol., 1979, N21, p. 183; and N. G. Juma and E. A. Paul, Abstr. Annu. Meet. Am. Soc. Agron., 1979, p. 158).

The three approaches described above to determine the fate of nitrate in soils all have sever shorcomings. Probably the greatest problem is the complexity of the soil system itself. This difficulty is certainly confounded by our ignorance of the ways in which the participants--plants, fungi, and bacteria--can respond to nitrate. At least these boxes within the larger black box of soil can be examined separately from the various factors that obscure investigations with soil. More detailed knowledge of the ways in which the components regulate their use of nitrate may make it easier to figure out how the intact assemblage functions, though it is apparent from what has been said before (6,33) that any extrapolation from studies in culture to behavior in soil must be made cautiously.

I chose to examine two components of the nitrate cycle: denitrification and the aerobic assimilation of nitrate by bacteria. For the denitrification study I selected three organisms representative of the diverse groups found in soil. My research goals were:

1. To determine the $K_m$ values for nitrate, nitrite, and nitrous oxide reduction in each organism;
2. To determine whether the organisms accumulated nitrite, and if so whether such accumulation was a result of nitrate inhibition of nitrite reduction;
3. To determine if nitrate or nitrite inhibited nitrous oxide

reduction; and

4. To determine whether nitrous oxide production increased at higher oxygen tensions.

Since so little is known about nitrate assimilation in bacteria (2), I cose to stydy only one of the above isolates. My research goals were:

1. To determine whether synthesis of the enzymes in the pathway for nitrate assimilation was regulated and if it was, in what manner;
2. To determine how the activity of the assimilation pathway was regulated, particularly whether ammonia inhibited nitrate assimilation; and
3. To determine the mechanism of nitrate transport during nitrate assimilation.

I was very fortunate to have access to facilities for production and use of $^{13}N$, the longest-lived radioisotope of nitrogen--half-life, ten minutes. Without $^{13}N$, I would not have investigated nitrate transport or assimilation.

I have attempted to discuss the significance of my results in relation to the fate of nitrate in soil. I have also sketched some directions for future research. In such discussion I fear I have been overcome with zeal to push back the veil of ignorance for a glimpse of Nature's beauty. I ask you to smile knowingly should you find such passages; please forgive my rapture.

## LITERATURE CITED


1. Bartholomew, W. V., and F. E. Clark (eds.) Soil Nitrogen. Am. Soc. Agron., Inc. Madison, Wis. 1965.

2. Brown, C. M., and B. Johnson. 1977. Inorganic nitrogen assimilation in aquatic microoganisms. Adv. Aquatic Microbiol. 1: 49-114.

3. Buresh, R. J., and W. H. Patrick, Jr. 1978. Nitrate reduction to ammonium in anaerobic soil. Soil Sci. Soc. Am. J. 42: 913-918.

4. Chapman, H. D., and G. F. Liebig, Jr. 1952. Field and laboratory studies of nitrite accumulation in soils. Soil Sci. Soc. Am. Proc. 16: 276-282.

5. Doddema, H., and G. P. Telkamp. 1979. Uptake of nitrate by mutants of Arabidopsis thaliana, disturbed in uptake or reduction of nitrate II. Kinetics. Physiol. Plant. 45: 332-338.

6. Dommergues, Y. R., L. W. Belser, and E. L. Schmidt. 1978. Limiting factors for microbial growth and activity in soil. Adv. Microbial Ecol. 2: 49-104.

7. Downey, R. J. 1971. Characterization of the reduced nicotinamide adenine dinucleotide phosphate-nitrate reductase in Aspergillus nidulans. J. Bacteriol. 105: 759-768.

8. Fewson, C. A., and D. J. D. Nicholas. 1961. Nitrate reductase from Pseudomonas aeruginosa. Biochim. Biophys. Acta. 49: 335-349.

9. Focht, D. D., and W. Verstraete. 1977. Biochemical ecology of nitrification and denitrification. Adv. Microbial Ecol. 1: 135-214.

10. Forget, P. 1971. Les nitrate-reductases bacteriennes, solubilisation, purification et propertes de l'enzyme A de Micrococcus denitrificans. Eur. J. Biochem. 18: 442-450.

11. Forget, P. 1974. The bacterial nitrate reductases. Solubilization, purification and properties of the enzyme. Eur. J. Biochem. 42: 325-322.

12. Garcia, J.-L. 1975. La denitrification dans les sols. Bull. Inst. Pasteur. 73: 167-193.

13. Goldsmith, J., J. P. Livoni, C. L. Norberg, and I. H. Segel. 1973. Regulation of nitrate uptake in Penicillium chrysogenum by ammoniun ion. Plant Physiol. 52: 362-367.

14. Guerrero, M. G., J. M. Vega, E. Leadbetter, and M. Losada. 1973. Preparation and characterization of a soluble nitrate reductase from Azotobacter chroococcum. Arch. Mikrobiol. 91: 287-304.

15. Guthrie, T. F., and J. M. Duxbury. 1978. Nitrogen mineralization and denitrification in organic soils. Soil Sci. Soc. Am. J. 42: 908-912.

16. Heimer, Y. M., and P. Filner. 1971. Regulation of the nitrate assimilation pathway in cultured tobacco cells. III. Nitrate uptake system. Biochim. Biophys. Acta. 230: 362-372.

17. Honert, T. H. van den, and J. J. M. Hooymans. 1955. On the absorption of nitrate by maize in water culture. Acta Bot. Neerl. 4: 376-384.

18. Kohl, D. H., F. Vithayathil, P. Whitlow, G. Shearer, and S. H. Chien. 1976. Denitrification kinetics in soil systems: the significance of good fits of data to mathematical forms. Soil Sci. Soc. Am. J. 40: 249-253.

19. Lycklama, J. C. 1963. The absorption of ammonium and nitrate by perennial rye-grass. Acta Bot. Neerl. 12: 361-423.

20. Payne, W. J. 1973. Reduction of nitrogenous oxides by micro-organisms. Bacteriol. Rev. 37: 409-452.

21. Phillips, R. E., K. R. Reddy, and W. H. Patrick, Jr. 1978. The role of nitrate diffusion in determining the order and rate of denitrification in flooded soil. II. Theoretical analysis and interpretation. Sol Sci. Soc. Am. J. 42: 272-278.

22. Pichinoty, F. 1973. La reduction bacterienne des composes oxygenes mineraux de l'azote. Bull. Inst. Pasteur. 71: 317-395.

23. Rao, K. P., and D. W. Rains. 1976. Nitrate absorption by barley I. Kinetics and energetics. Plant Physiol. 57: 55-58.

24. Reddy, K. R., W. H. Patrick, Jr., and R. E. Phillips. 1978. The role of nitrate diffusion in determining the order and rate of denitrification in flooded soil: I. Experimental results. Soil Sci. Soc. Am. J. 42: 268-272.

25. Riet, J. Van'T, and R. J. Planta. 1969. Purification and some properties of the membrane-bound respiratory nitrate reductase of Aerobacter aerogenes. FEBS Letters. 5: 249-252.

26. Riet, J. Van'T, F. B. Wientjes, J. van Doorn, and R. J. Planta. 1979. Purification and characterization of the respiratory nitrate reductase of Bacillus licheniformis. Biochim. Biophys. Acta. 576. 347-360.

27. Rosso, J. P., P. Forget, and F. Pichinoty. 1973. Les nitrate-reductases bacteriennes. Solubilisation, purification et proprietes de l'enzyme A de Micrococcus halodenitrificans. Biochim. Biophys. Acta. 321: 443-455.

28. Rosswall, T. 1976. The internal nitrogen cycle between micro-organisms, vegetation and soil, in B. H. Svensson and R. Soderlund (eds.) Nitrogen, Phosophorus and Sulphur--Global Cycles. SCOPE Report 7. Ecol. Bull. (Stockholm) 22: 157-167.

29. Schloemer, R. H., and R. H. Garrett. 1974. Nitrate transport system in Neurospora crassa. J. Bacteriol. 118: 259-269.

30. Soderlund, R., and B. H. Svensson. 1976. The global nitrogen cycle, in B. H. Svensson and R. Soderlund (eds.) Nitrogen, Phosphorus and Sulphur--Global Cycles. SCOPE Report 7. Ecol. Bull. (Stockholm) 22: 23-73.

31. Stouthamer, A. H. 1976. Biochemistry and genetics of nitrate reductase in bacteria. Adv. Mirobial Physiol. 14: 315-375.

32. Stumm, W., and J. J. Morgan. 1970. Aquatic Chemistry. John Wiley & Sons, Inc. New York.

33. Tempest, D. W., and O. M. Neijssel. 1978. Eco-physiological aspects of microbial growth in aerobic nutrient-limited environments. Adv. Microbial Ecol. 2: 105-153.

34. Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41: 100-180.

35. Yoshinari, T., R. Hynes, and R. Knowles. 1976. Acetylene inhibition of nitrous oxide reduction and measurement of denitrification and nitrogen fixation in soil. Soil Biol. Biochem. 8: 1-7.

36. Zumft, W. G. 1979. The inorganic biochemistry of nitrogen bioenergetic processes. Naturwissenschaften 66: 81-88.

# CHAPTER I

## A KINETIC EXPLANATION FOR ACCUMULATION OF NITRITE, NITRIC OXIDE, AND NITROUS OXIDE DURING BACTERIAL DENITRIFICATION

The generally accepted pathway for denitrification is as follows: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$, though the role of nitric oxide as an intermediate is still under challenge (7,16,21,28,41,52). The other intermediates, nitrite and nitrous oxide, frequently accumulate in denitrifying cultures; only nitrous oxide is detected in soils. The reason for this difference is not known. The recent discovery that nitrous oxide can catalyze the destruction of stratospheric ozone has led many scientists to determine the amount of nitrous oxide produced during denitrification and reexamine factors contributing to its production. Aeration status apparently regulates whether soil is a source or a sink for nitrous oxide (3,5,13,15). In addition, high concentrations of nitrate and nitrite favor nitrous oxide production, which varies with time after onset of anaerobic conditions (11,27,39,40).

Any of several hypotheses may account for the effects of nitrate, nitrite and oxygen on nitrous oxide production. They are: (i) differential synthesis of the enzymes for production and reduction of nitrous oxide; (ii) specific inhibition of the nitrous oxide reductase; (iii) preferred use of nitrate, nitrite or oxygen to nitrous oxide as a terminal electron acceptor; and (iv) different rates of nitrous oxide production and consumption. One or more of these explanations may account for accumulation of nitrite during denitrification by bacterial

cultures, though different species may have different regulation. For instance, nitrate accumulation may result from delayed synthesis of nitrite reductase in Micrococcus (Paracoccus) denitrificans and Pseudomonas aeruginosa (24,46), whereas nitrite accumulation may be due to nitrate inhibition of a specific reductase in Pseudomonas perfectomarinus (29,30).

In order to resolve which explanation accounts for accumulation of intermediates during denitrification, I selected three isolates--an Alcaligenes sp., a Flavobacterium sp., and Pseudomonas fluorescens--from a previous study of numerically dominant denitrifers (12). By working with pure cultures I avoided difficulties of interpretation due to heterogeneous denitrifier populations present in soil or confounding chemical and physical effects, such as diffusion. I used resting cell suspensions throughout and so did not test the hypothesis of differential synthesis. During the course of this study I also determined the apparent $K_m$ values for nitrite reduction in the three isolates, and that for nitrous oxide reduction in Flavobacterium. I found that different rates of reduction of intermediates of denitrification could explain the accumulation of nitrite, nitric oxide, and nitrous oxide observed. A kinetic model of denitrification summarizing this result is presented in the discussion section. The effects of oxygen on total gas production and on the proportion of $N_2O$ formed are reported as well as a mechanism which can account for the observed effects.

## MATERIALS AND METHODS

Organisms. Organisms were selected from a collection obtained during a study to assess numerically dominant groups of denitrifiers in

world soils (15). T. N. Gamble (M.S. Thesis, Michigan State University, 1976) has characterized the isolates. Isolate 72, from a poorly drained agricultural soil in Lamberton, Minnesota, has been identified as Pseudomonas fluorescens biotype II; it is representative of the most prevalent group of denitrifiers isolated (15). Isolate 175, from a fallow field in Parana, Argentina, has been asigned to the genus Flavobacterium, which until recently was not considered capable of denitrification (33). Isolate 17, from a poultry waste oxidation ditch, is a member of a heterogeneous group typical of Alcaligenes species in its inability to utilize simple organic compounds as carbon sources for growth. However, it has a single polar flagellum and so may actually be a Pseudomonas pseudoalcaligenes capable of denitrification. For convenience, I have referred to isolate 17 as Alcaligenes. I hoped such a selection might point out similarities and differences in denitrification among diverse soil species. Such differences would be important for those attempting to interpret the behavior of complex natural assemblages of denitrifers.

Alcaligenes odorans was obtained from ATCC (No. 15554). This species cannot reduce nitrate, but readily denitrifies if supplied with nitrite (37). It was used to examine whether nitrate inhibited nitrous oxide reduction directly.

Media. Organisms were grown on nitrate broth (10 mM $KNO_3$ in 8 g Difco nutrient broth per liter). For A. odorans, 5 mM $KNO_2$ was used instead of $KNO_3$. Seed cultures of 50 ml volume in 50 or 125 ml flasks were grown for 24 h at 30°C, then used to inoculate 450 ml nitrate broth in 500 ml Erlenmeyer flasks. A rubber stopper was wired in place on each flask and the cultures incubated at 30°C for a day. This procedure

allowed cultures to use residual oxygen in the medium and small headspace while shifting to anaerobic metabolism. Nitrate and nitrite were not detected in the spent medium. That harvested cells were capable of denitrification was verified by the color of the cell pellets, salmon pink for Alcaligenes and Pseudomonas and deep orange for Flavobacterium, which is typical of the shift in cytochrome content required for denitrification. In contrast, cultures grown aerobically were tan or, for Flavobacterium, yellow.

To differentiate between nitrate inhibition of nitrite reduction and use of nitrate as a preferential electron acceptor it was necessary to obtain cells incapable of nitrate reduction. Cells lacking nitrate reductase activity were obtained by substituting 5 mM $KNO_2$ for $KNO_3$ and including 5 mM $Na_2WO_4$ in the growth medium, which prevented formation of an active nitrate reductase (43).

Resting cell suspensions. Cells were harvested by centrifugation at 10,000 x g for 10 min. Cell pellets were resuspended in 10 ml nutrient broth (Difco) containing 200 µg chloramphenicol per ml. This concentration of chloramphenicol was sufficient to prevent growth of the least sensitive organism, P. fluorescens, as determined by the serial dilution procedure. Resuspended cells were centrifuged and washed again. After two washings, the cell pellet was suspended in nutrient broth with chloramphenicol. This stock was kept refrigerated or on ice until use, but never longer than 12 h.

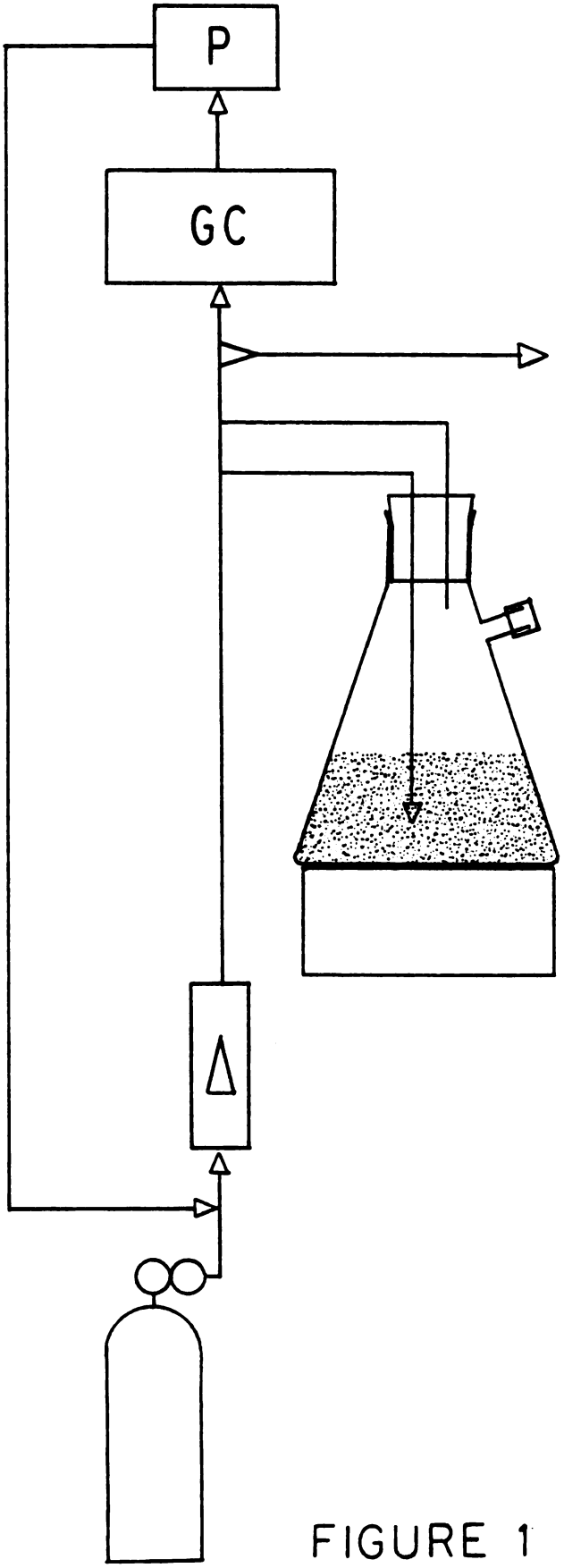
Nitrate and nitrite reduction. A stock suspension was diluted prior to use to give convenient rates of reduction. The diluted cell suspension, generally 18 ml, was placed in a 125 ml DeLong culture flask (Bellco Glass Co., Vineland, NJ) containing a stirring bar. A large serum stopper was placed over the top of the flask and the headspace

gases evacuated and replaced three times with argon. Slight overpressure was maintained in the flask to minimize oxygen leakage. Cell suspensions were preincubated 5 min with magnetic stirring at room temperature (25°C), then 2 ml of nitrate or nitrite solution was added to start the reaction. At regular intervals the flask was inverted and samples removed by syringe. After the flask had been placed back on the magnetic stirrer a volume of argon equal to that of the sample was injected to maintain the overpressure. Cells were removed by filtration through 0.4 μm polycarbonate filters with glass fiber prefilters (both from Nuclepore). Cellulose acetate-cellulose nitrate filters were unsuitable because nitrate could be leached from them during filtration.

Nitrite concentrations in the samples were determined by diazotization (2) and comparison of sample absorbances at 520 nm with those of nitrite standards run daily. Nitrate was determined with Szechrome NAS reagent or Szechrome NB reagent (Research and Development Authority, Ben-Gurion University of the Negev, Beer-Sheva, Israel; see Appendix A), which form stable diphenylamine or benzidine complexes, respectively, with nitrate in concentrated mineral acids (these reagents now can be obtained from Polysciences Inc., Warrington, Penn.). Samples were treated with 0.1 volume 5% (wt/vol) sulfamic acid to remove nitrite prior to nitrate determination. For some experiments (Figures 2 and 3) nitrate and nitrite were determined using a Technicon Autoanalyzer equipped with a cadmium reduction column.

NO and $N_2O$ measurement. A sealed, continuous gas circulating system designed by Dr. Heinrich Kaspar, Michigan State University, was used to monitor accumulation and reduction of gaseous intermediates during denitrification (Figure 1). The incubation vessel, a 125 ml

Figure 1. Diagram of a system for continuous gas circulation through an incubation flask and the sampling loop of a gas chromatograph.




MEMBRANE PUMP

GAS CHROMATOGRAPH

3-WAY-VALVE, VENT

FLASK

SAMPLE

MAGNETIC STIRRER

FLOW METER

GAS TANK

FIGURE 1

flask equipped with a side injection port, held 50 ml nutrient broth. It was placed on a magnetic stirrer to facilitate gas exchange. Gas was circulated through the flask via a pump (Neptune Dyna-Pump, Universal Electronics Company, Owosso, Michigan) connected to a sample loop, 0.12 ml volume, on a Perkin Elmer 910 gas chromatograph equiped with a $^{63}Ni$ electron capture detector. Nitrate or nitrite was included in the nutrient broth prior to cell addition. Oxygen and other gases were purged from the system by flushing it with argon for 5 min. After a known volume of nitrous oxide had been added to the headspace and allowed to equilibrate for 20 min, the reaction was started by adding sufficient stock suspension to give a final absorbance of 0.25. Injections onto the gas chromatrographic column could be made every 8 min, which allowed sufficient time for water to elute from the column and detector. Total gas volume in the system was calculated from the amount of $N_2O$ added and its concentration after equilibration.

The gas chromatograph was equipped with a Porapak Q column (3 mm by 1.87 m) connected to the sampling valve, which was in turn connected to the injection port. Column temperature was 55°C; the manifold temperature, 90°C; and the detector temperature, 300°C. The flow rate of the carrier gas, argon with 5% methane, was 16 ml per min. Under these conditions the retention times of NO and $N_2O$ were 0.90 and 2.35 min, respectively. Since electron capture units cannot detect $N_2$, it did not interfere with NO measurements. Integrated peak areas determined by a computing integrator (Supergrator-1, Columbia Scientific Industries, Austin, Texas) were compared with standards run within a day of the experiments. All concentrations were corrected for changes in volume due to removal of gas and liquid samples.

Liquid samples were withdrawn from the flask through an 18 gauge spinal tap needle connected to 1 ml syringe through a one-way valve (Becton-Dickinson; Rutherford, NJ) and immediately were placed in the acidic colorimetric reagents, which stopped denitrification.

Progress curve analysis for $K_m$ values. The $K_m$ values for nitrite reductase and for nitrous oxide reductase were estimated by analyzing progress curves in which the disappearance of substrate was monitored. I chose this procedure to avoid problems with nonlinearity in initial velocity measurements at low substrate concentrations. The integrated Michaelis-Menten equation for a single substrate irreversible reaction was fitted by an interative method using the Newton-Raphson procedure (9,26), and Wilkinson's procedure (48) to estimate the standard error. The $K_m$, $V_{max}$, and error estimates were obtained using a program written for the IMSAI 8080 computer in our laboratory (Appendix B).

Oxygen effect on nitrous oxide reduction. The effect of oxygen on nitrous oxide reduction was investigated using $^{13}NO_3^-$ generated by the MSU Sector-Focused Cyclotron (47). After a 0.8 ml water target had been bombarded with 15 MeV protons for 10 min, the water was removed and placed in a 50 ml pear-shaped flask. NaOH was added and the source evaporated to dryness on a flash evaporator to remove radioactive gases and $^{13}NH_3$. The source was neutralized with HCl, then 8 ml of a 30 μM $KNO_3$ solution was added.

Five milliliters of cell suspension were pipetted into Hungate-type screw-capped tubes, approximately 16 ml total volume, (Bellco Glass Inc.; Vineland, NJ) several hours before the experiment. The tubes were capped and the atmosphere replaced with helium by evacuation and flushing five times. Immediately before nitrate addition, a volume of gas was

removed from each tube and replaced with an equal volume of air. One ml of nitrate solution was injected into the tube, which then was placed horizontally on a rotary shaker set at 250 rpm. After incubation for 5 and 10 min, 1 ml of the headspace gas was drawn into a tuberculin syringe with a Mininert valve (Precision Scientific Co., Baton Rouge, Louisianna).

The $^{13}N$ gases were separated and quantified using a gas chromatograph-proportional counter system described previously (47). (M. K. Firestone has presented a more detailed description of the electronics setup in an appendix to her Ph.D. thesis, Michigan State University, 1978). Both the Porapak Q and the Molecular Sieve 13X columns were used to separate oxygen from nitrogen in order to check that oxygen had not been depleted during incubation. Radioactive counts in the gas peaks were corrected for background and $^{13}N$ decay, then the peak areas and centroids were determined by a modification of FASTFIT, a program written by Dr. R. B. Firestone to analyze nuclear spectra (Appendix B).

## RESULTS

Patterns of nitrite accumulation. The extent of nitrite accumulation in resting cell suspensions varied from organism to organism. Flavobacterium (Figure 2) produced almost undetectable quantities of nitrite during nitrate reduction. In contrast, P. fluorescens (Figure 3) produced much higher amounts of nitrite; frequently nitrite accumulation was almost stoichiometric (Figure 6). Alcaligenes resembled P. fluorescens in the pattern of nitrite accumulation it exhibited (data not shown).

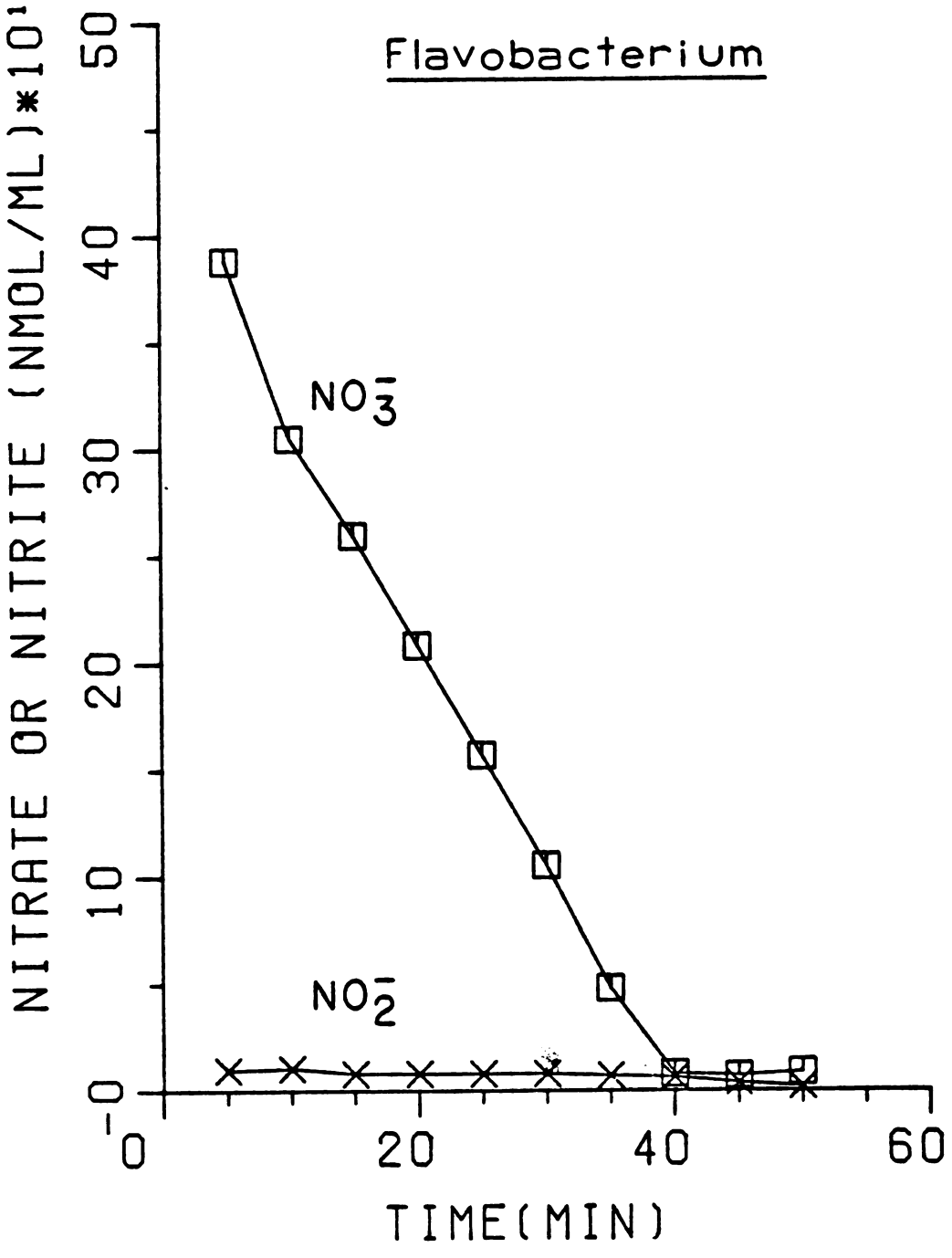



Figure 2. Lack of nitrate accumulation during denitrification by Flavobacterium.

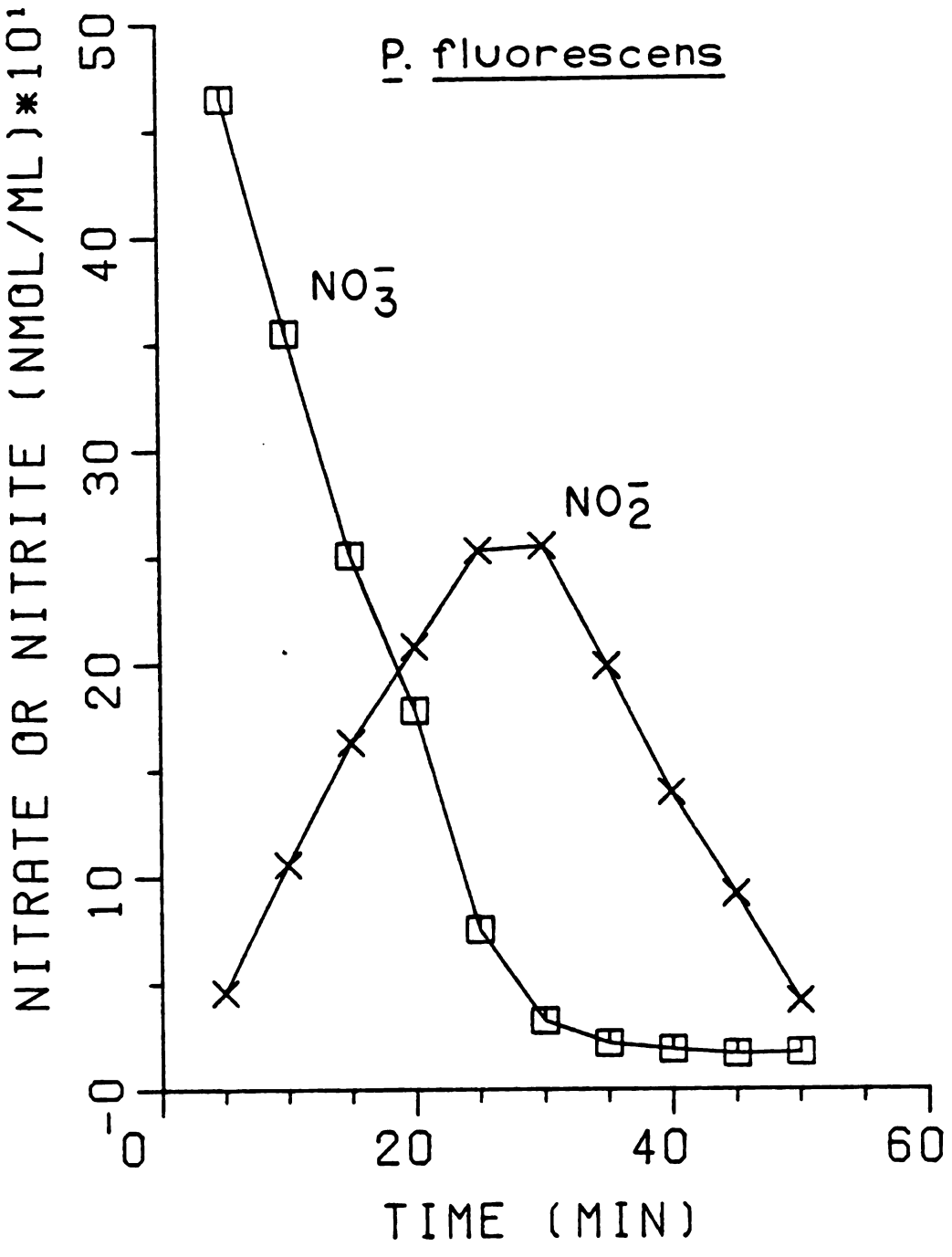



Figure 3. Pattern of accumulation of nitrite during denitrification by P. fluorescens.

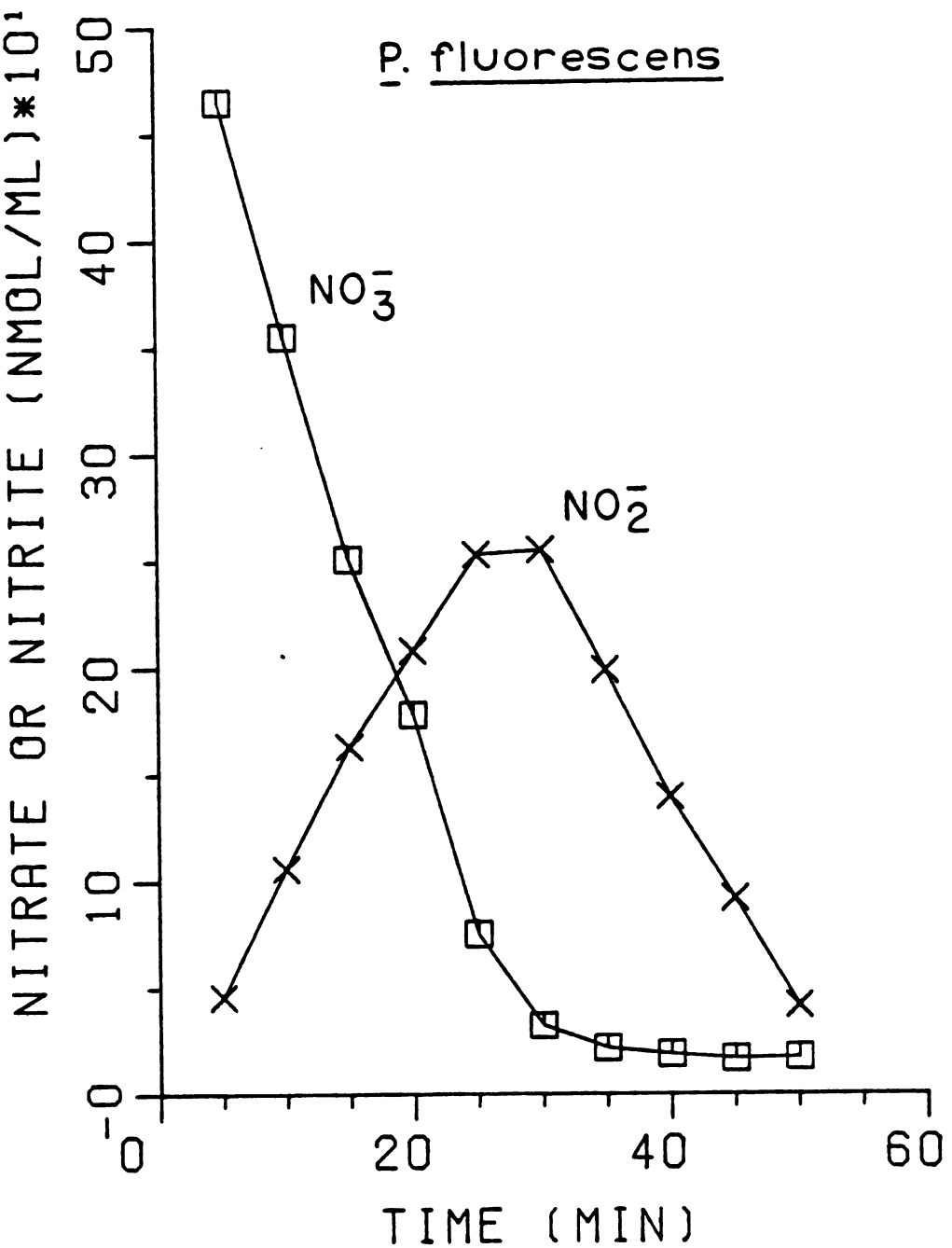



Figure 3. Pattern of accumulation of nitrite during denitrification by P. fluorescens.

Table 1. Calculated and measured rates of nitrite accumulation during denitrification by P. fluorescens, from data shown in Figure 3.

| Process | Time interval for rate estimate (min) | Rate ($\mu M\ min^{-1}$) |
|---|---|---|
| Nitrate reduction | 5 to 25 | 19.1±2.28[a] |
| Nitrite reduction | 30 to 50 | 10.0±1.35 |
| Nitrite accumulation | | |
| calculated[b] | -- | 8.8±2.65 |
| measured | 5 to 25 | 10.7±0.85 |

a Rates determined by linear regression; confidence limits are $t_{0.05}s_b$; 3 degrees of freedom.

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

The pattern observed in Pseudomonas and Alcaligenes may have resulted from nitrate inhibition of nitrite reduction. A difference in the rates of nitrate and nitrite reduction also might have caused nitrite accumulation. Comparison of the rates of nitrate and nitrite reduction in the experiment with P. fluorescens (Table 1) supported the latter explanation: the rate of nitrite accumulation observed was not significantly different from that calculated as the difference between rates of nitrate and nitrite reduction.

If nitrate inhibited nitrite reduction by acting on the nitrite reductase itself, such inhibition should still have been apparent in suspensions of 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 on 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 up to 7 mM, the highest tested, had no effect on the rate of nitrite reduction in Flavobacterium and P. fluorescens (Table 2). Hence, nitrate did not inhibit nitrite reduction directly in either organism.

Table 2. Effect of nitrate concentration on the rate of nitrite reduction by Flavobacterium and P. fluorescens lacking nitrate reductase activity.

| Nitrate concentration (mM) | Rate of nitrite reduction (μM $min^{-1}$) | |
|---|---|---|
| | Flavobacterium | P. fluorescens |
| 0 | 17.2±1.44 (4)[a] | 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 Rates determined by linear regression; confidence limits are $t_{0.05}s_b$; numbers in parentheses are degrees of freedom.

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. If either ion inhibited reduction, the nitrous oxide concentration should have increased. Addition of nitrate to Flavobacterium suspensions did not affect the rate of nitrous oxide reduction or lead to nitrous oxide

accumulation (Figure 4). Inclusion of 0.28 mM nitrite also did not affect nitrous oxide reduction. Similar results were obtained with the other two isolates. Gas leakage did not cause the decrease in nitrous oxide concentration, since the nitrous oxide concentration prior to addition of the cell suspension was constant (e.g., Figure 8) and no oxygen was detected in samples taken after addition.

A. odorans cannot reduce nitrate but can reduce nitrite (37), and so can be considered physiologically similar to cells grown in the presence of tungstate. As shown in Table 3, neither nitrate nor nitrite affected the rate of nitrous oxide reduction in this organism. However, net reduction of nitrous oxide did not begin until 25 and 47 min after cells were added to broth containing 0.2 and 0.5 mM nitrite, respectively. The lag probably represented a phase during which the rate of nitrite reduction to nitrous oxide matched the rate of nitrous oxide reduction to nitrogen, i.e., one in which the concentration of nitrous oxide did not change even though there was a flux of nitrogen through the $N_2O$ pool. No nitrite or nitrous oxide was detected in the medium at the end of either experiment, indicating both substrates had been reduced to dinitrogen gas.

Nitric oxide and nitrous oxide accumulation. When nitrate or nitrite was included along with $N_2O$ in the medium for any of the isolates, the nitrous oxide concentration decreased until it reached a constant value. This low nitrous oxide concentration was maintained until all the ionic species had been used (Figures 5 and 6). As shown in Figure 6, nitrous oxide added after the steady state had become established also was reduced rapidly until its concentration reached the previous value. Maintenance of a relatively constant $N_2O$ concentration even

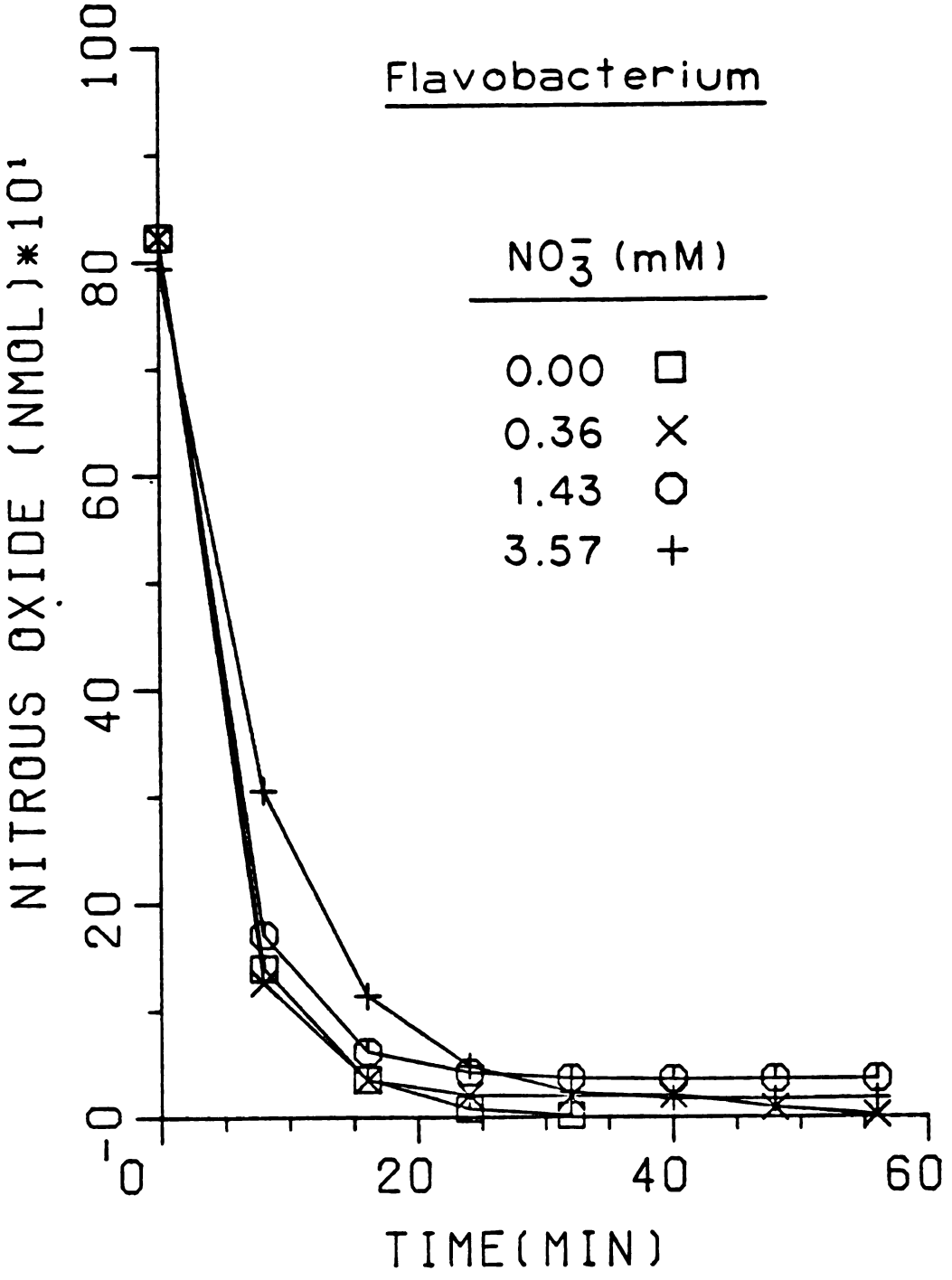



Figure 4. Failure of different nitrate concentrations to inhibit nitrous oxide reduction by Flavobacterium.

Table 3. Effect of nitrate concentration and of nitrite concentration on the rate of nitrous oxide reduction by Alcaligenes ordorans.

| Concentration (mM) | First order rate constant, k ($min^{-1}$) |
|---|---|
| Nitrate | |
| 0 | 0.147 ± 0.020[a] |
| 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 Rate constants 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 degrees of freedom.

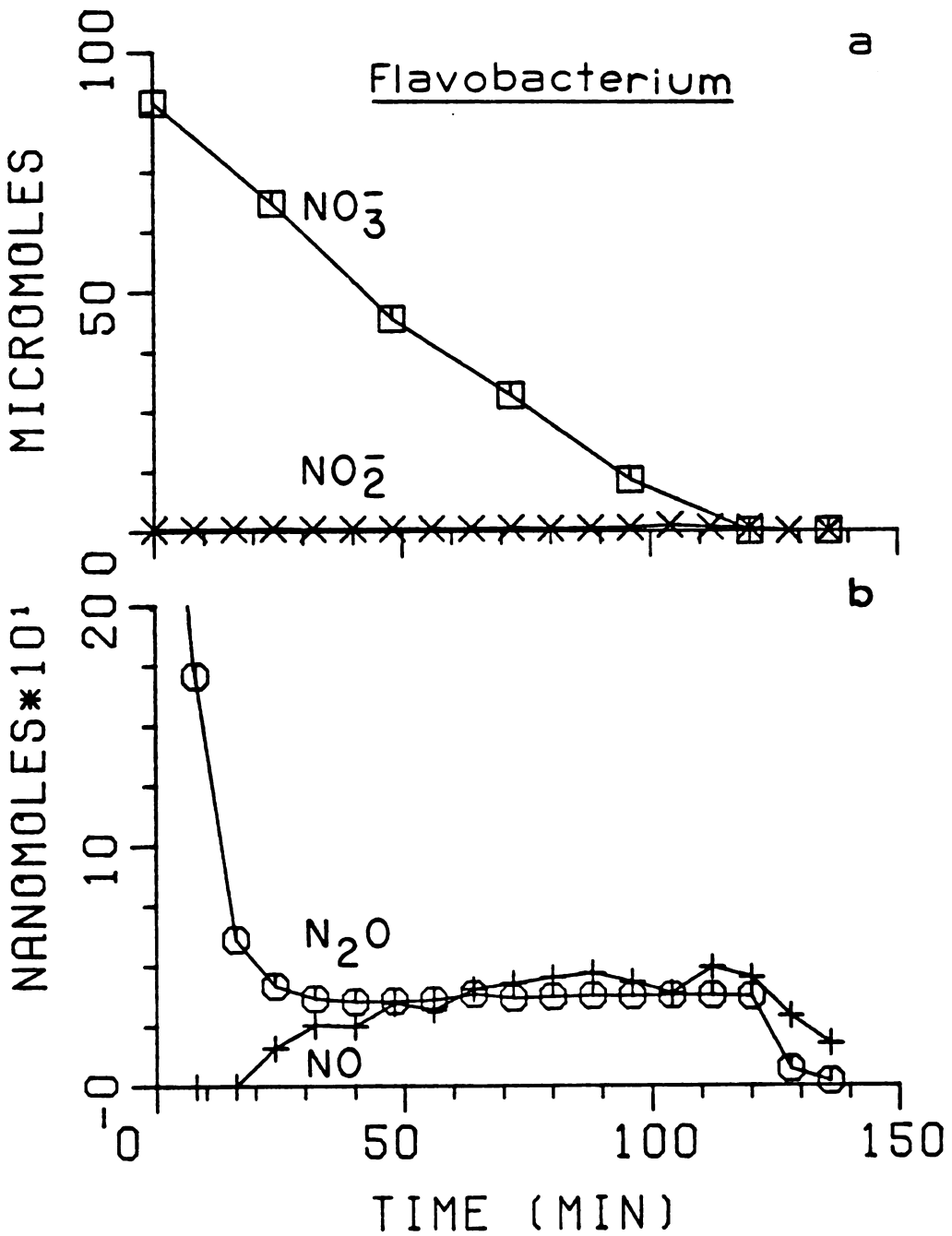



Figure 5. Pattern of nitrite and gas accumulation during denitrification by Flavobacterium.

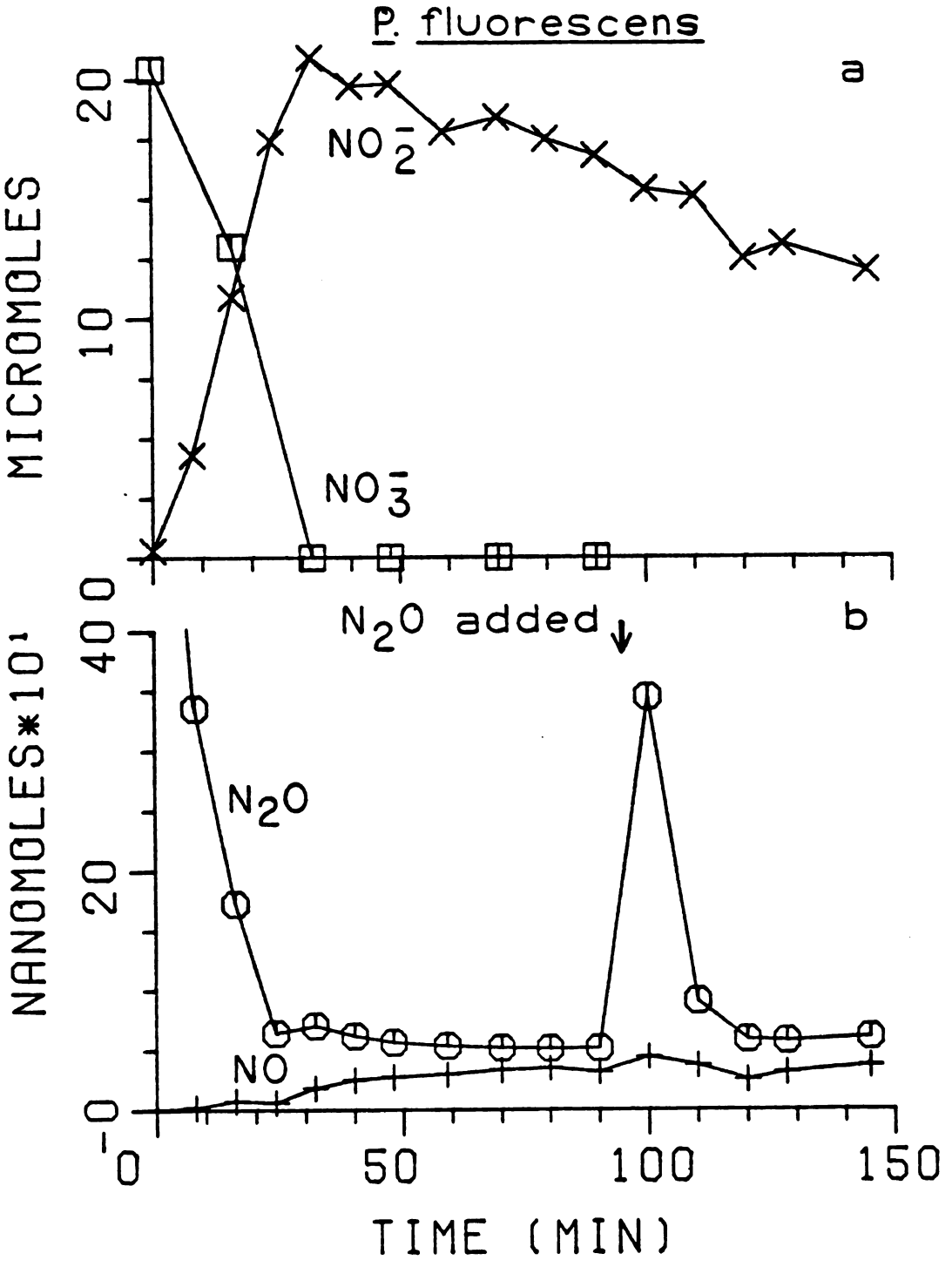



Figure 6. Pattern of nitrite and gas accumulation during nitrate reduction by P. fluorescens, showing the return of nitrous oxide concentration to a steady state value after perturbation.

after perturbation was evidence that the steps in denitrification were in kinetic equilibrium.

All three isolates accumulated another gaseous product in addition to nitrous oxide. This product appeared shortly after cell suspensions had been added to incubation vessels containing nitrate or nitrite. Its concentration also became constant until all the nitrate or nitrite had been used. We identified the gas as nitric oxide based on its retention time on the Porapak Q column and its behavior during incubation. 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 (compare Figures 5 and 6). Nitric oxide production thus could not be attributed to chemical decomposition of nitrite. A. odorans also produced nitric oxide when incubated with nitrite, though not when incubated with nitrate since this organism is incapable of nitrate reduction.

Effect of oxygen on nitrous oxide accumulation. I 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 (Figure 7, data for Alcaligenes not shown). Samples taken from the same cell suspensions after additional incubation had reduced percentages of nitrous oxide, but the relationship between percentage of nitrous oxide and oxygen concentration remained the same. Higher oxygen concentrations inhibited denitrification by P. fluorescens and Alcaligenes. However, no decrease in total gas production was observed in several experiments with Flavobacterium. Apparently nitrous oxide reduction in this organism was more sensitive to oxygen than the other steps in denitrification.

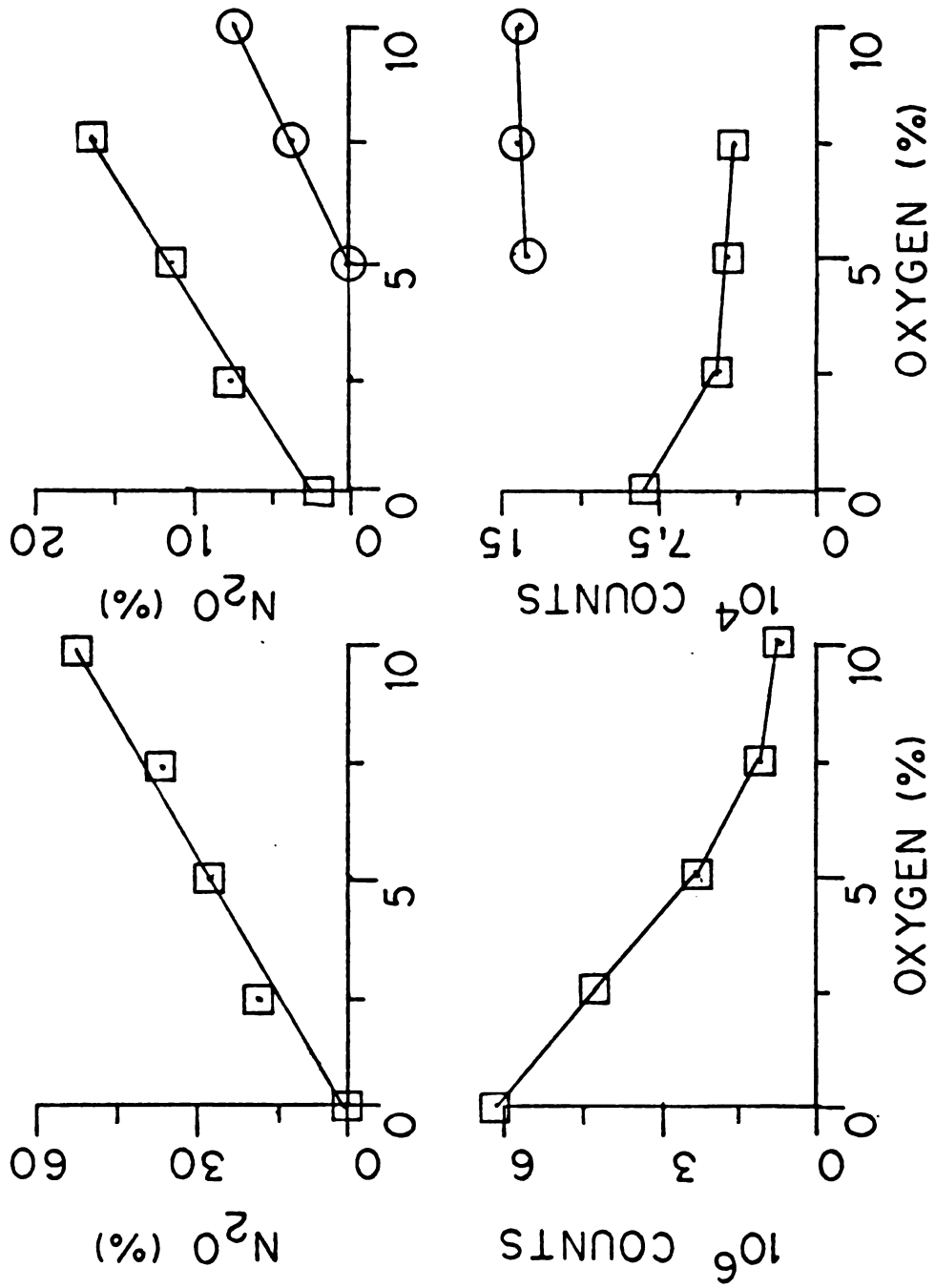



Figure 7. Effects of increasing oxygen concentration on composition and amount of gases produced during denitrification by Flavobacterium and P. fluorescens.

Michaelis constants for nitrate, nitrite, and nitrous oxide reduction. Values of the Michaelis constant for nitrite reduction were determined by analysis of progress curves. The values for Alcaligenes, Flavobacterium, and P. fluorescens were similar: 12.5±0.5 μM, 5.7±0.9 μM, and 5.4±0.1 μM ($K_m$±S.E.), respectively. Nitrite concentrations on which these determinations were based were near the detection limits of the procedure used. Though not exact values, the constants are of the correct magnitude: the rate of nitrite reduction at concentrations above 30 μM was linear.

I was unable to obtain $K_m$ estimates directly for nitrate reduction, due to limited sensitivity and precision of the colorimetric procedures used. Based on the linear rate of nitrate reduction observed at concentrations above 15 μM, the $K_m$ values for nitrate reduction were at least as low as those for nitrite reduction.

The apparent $K_m$ value for nitrous oxide reduction by Flavobacterium was 458±4 nM (2±0.017 Pa). This estimate was determined from experiments starting at several initial partial pressures of nitrous oxide; data from a typical experiment are shown in Figure 8. To insure I measured the biological rate of $N_2O$ reduction and not the rate of $N_2O$ transfer from the gas to the liquid phase, I increased the transfer rate by sparging gas through the continuously stirred medium. Still, I had to reduce the rate of $N_2O$ reduction by decreasing cell density to an absorbance of 0.005 and by decreasing the liquid volume to 25 ml.

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

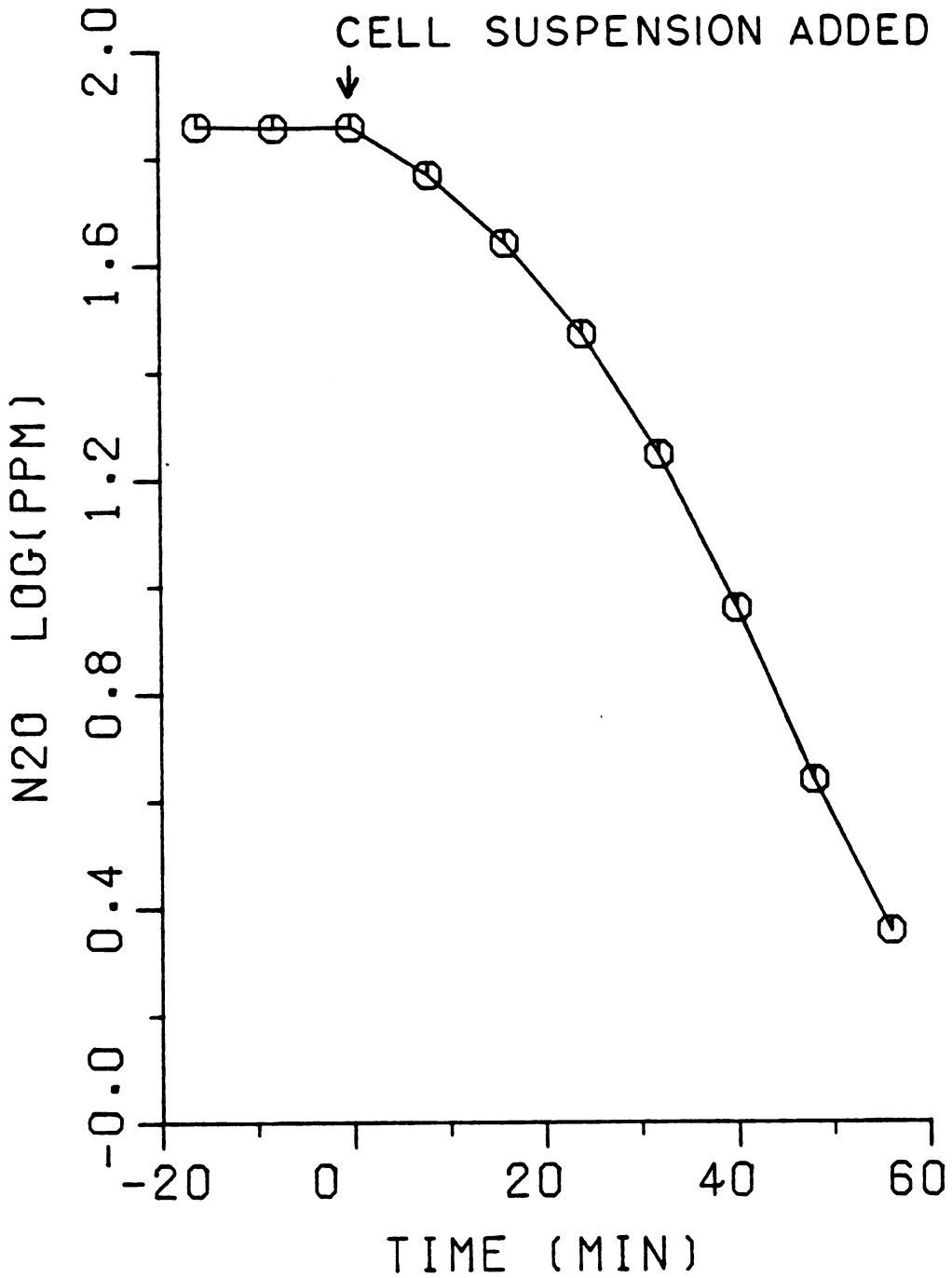



Figure 8. An experiment to determine the $K_m$ value for nitrous oxide reduction by Flavobacterium.

of reduction had always been greater than the rate of gas transfer, there should have been a single exponential curve only, reflecting diffusion of $N_2O$ into the aqueous phase. Also, in the latter case the $K_m$ value obtained should have been at least as high as the initial nitrous oxide concentration, assuming the iterative procedure for the progress curve analysis would converge. I did not observe a single or biphasic exponential curve (Figure 8), nor did I obtain a $K_m$ value as high as the initial $N_2O$ concentration. Thus I am confident that I measured only the biological process.

## DISCUSSION

Although there have been numerous measurements of denitrification kinetics in soils, relatively little in comparison has been done with pure cultures, and that primarily with purified enzymes. I worked with pure cultures of denitrifiers to avoid difficulties of interpretation caused by the heterogeneous chemical and physical structure of soil.

Michaelis constants for the nitrate, nitrite and nitrous oxide reductases of the bacteria in this study were all below 15 μM. These values are much lower than those previously reported for purified enzymes. For instance, with purified nitrate reductase preparations from both species of Paracoccus the values obtained were 0.25 mM and 1.3 mM (45). The value reported for the nitrite reductase isolated from a photosynthetic denitrifier was 51 μM (42). Estimates of $K_m$ values for denitrification of nitrate in soils are much higher than those I obtained: for instance, 0.23 mM (22), 0.29 mM and 3.5 mM (23), and 0.13 mM and 1.2 mM (51). As Kohl et al. (23) pointed out, the kinetic mechanism of a reaction cannot always be determined in such a complex system as soil merely by examining the goodness of fit of the data to a given model.

The increase in denitrification rate they observed after a carbon amendment suggested that denitrification was actually carbon-limited; the apparent $K_m$ values for denitrification they obtained may have reflected the kinetics of carbon limitation more than those of denitrification.

Recently, Reddy, Phillips and Patrick (32,38) questioned whether most kinetic analyses in soil actually measure denitrification. They concluded that the typical procedure used, measurement of nitrate reduction in a flooded soil, frequently measured the rate of nitrate diffusion from solution into the soil matrix, rather than denitrification itself. If denitrification were dominated by diffusion, one would expect to observe first order rates at concentrations higher than those obtained with denitrifiers themselves. Such confounding factors as carbon limitation and diffusion likely account for the discrepancy between values of denitrification reported in soils and those we found for nitrate and nitrite reduction.

Previous attempts to measure the $K_m$ value for nitrous oxide reduction may also have been affected by diffusion from the gas to the liquid phase. St. John and Hollocher (41) reported the value for P. aeruginosa was less than 100 μM. Matsubara and Mori (25) obtained an estimate of 30 to 60 μM for Pseudomonas denitrificans. In soil Yoshinari et al. (51) found the nitrous oxide $K_m$ value was 0.7 to ].0 mM, though it is not clear whether they attempted to maximize the rate of gas transfer into solution. The value I obtained for Flavobacterium, 0.5 μM on a solution basis, is much lower than those cited above, but is not confounded by limited phase transfer. Though low, this value is still 50 times greater than the ambient nitrous oxide concentration calculated

from its atmospheric abundance and solubility in water.

It is appropriate to compare the values obtained here with those for oxygen respiration in cultures, since these substances and oxygen serve as terminal electron acceptors for the respiratory system. $K_m$ values for oxygen are less than 16 μM (6); those reported here are similar in magnitude. The $K_m$ value for respiration in soils was 2 to 4 μM (18). Such low values for the terminal electron acceptors insure that respiration rate and growth are not limited by the low concentrations of these substances frequently found in soils.

The diversity of denitrifiers found in natural environemnts (15,16, 28) also may affect estimates of kinetic constants in soils. That diversity is apparent in the patterns of nitrite accumulation I observed: Flavobacterium did not accumulate significant quantities of nitrite (Figure 2), whereas Alcaligenes and P. flourescens (Figure 3) did. Other investigators also have observed nitrite accumulation in cultures of denitrifying bacteria (24,29,46,49). They have found such accumulation occurs because the synthesis of nitrite reductase lagged behind that of nitrate reductase (24,46,49), or else because nitrate inhibited nitrite reduction (29). Differential synthesis of the denitrifying enzymes cannot account for the nitrite accumulation I observed, because I used cell suspensions blocked in protein synthesis. Yet I did not find any evidence that nitrate directly affected nitrite reduction in P. fluorescens lacking an active nitrate reductase. Rather, nitrite accumulation in P. fluorescens, and the lack of nitrite accumulation in Flavobacterium, appeared due to different rates of nitrate and nitrite reduction.

To demonstrate how different rates of reduction could lead to the patterns of nitrite accumulation observed, I have constructed a kinetic model of denitrification. Since I did not see any direct effect of nitrate on nitrite reduction and since the reductive steps in denitrification are essentially irreversible reactions, I have modelled all reaction rates with the simple Michaelis-Menten equation: $v = \frac{V \cdot S}{K_m + S}$. I assumed $K_m$ values for nitrate reduction and nitrite reduction were 1 and 2, respectively. (The units are arbitrary; I selected values to demonstrate the qualitative behavior of such a model, rather than to model the experimental data exactly.) After choosing suitable maximum velocities for each reaction and selecting the initial nitrate and nitrite concentrations, I simulated denitrification on a computer by calculating the rates of reduction during short time intervals, then updating the substrate concentrations.

Figure 9 presents the results of two such simulations. In Figure 9A the maximum rate of nitrite reduction was five times that of nitrate reduction; as a result, little nitrite accumulated. This case is similar to the behavior of Flavobacterium (Figure 3). In Figure 9B the maximum rate of nitrite reduction is one-fifth that of nitrate reduction; most of the nitrate accumulated as nitrite before being reduced further. This case is similar to the behavior of P. fluorescens (Figure 6).

Of course, the maximal rate of reduction (V in the model) actually depends not only on the specific activity of each reductase molecule, but also on the number of such molecules present. Therefore, it is possible to incorporate differential synthesis of nitrate and nitrite reductases into the model by making V a function of time. Such a change would account for the mechanism nitrite accumulation observed in some

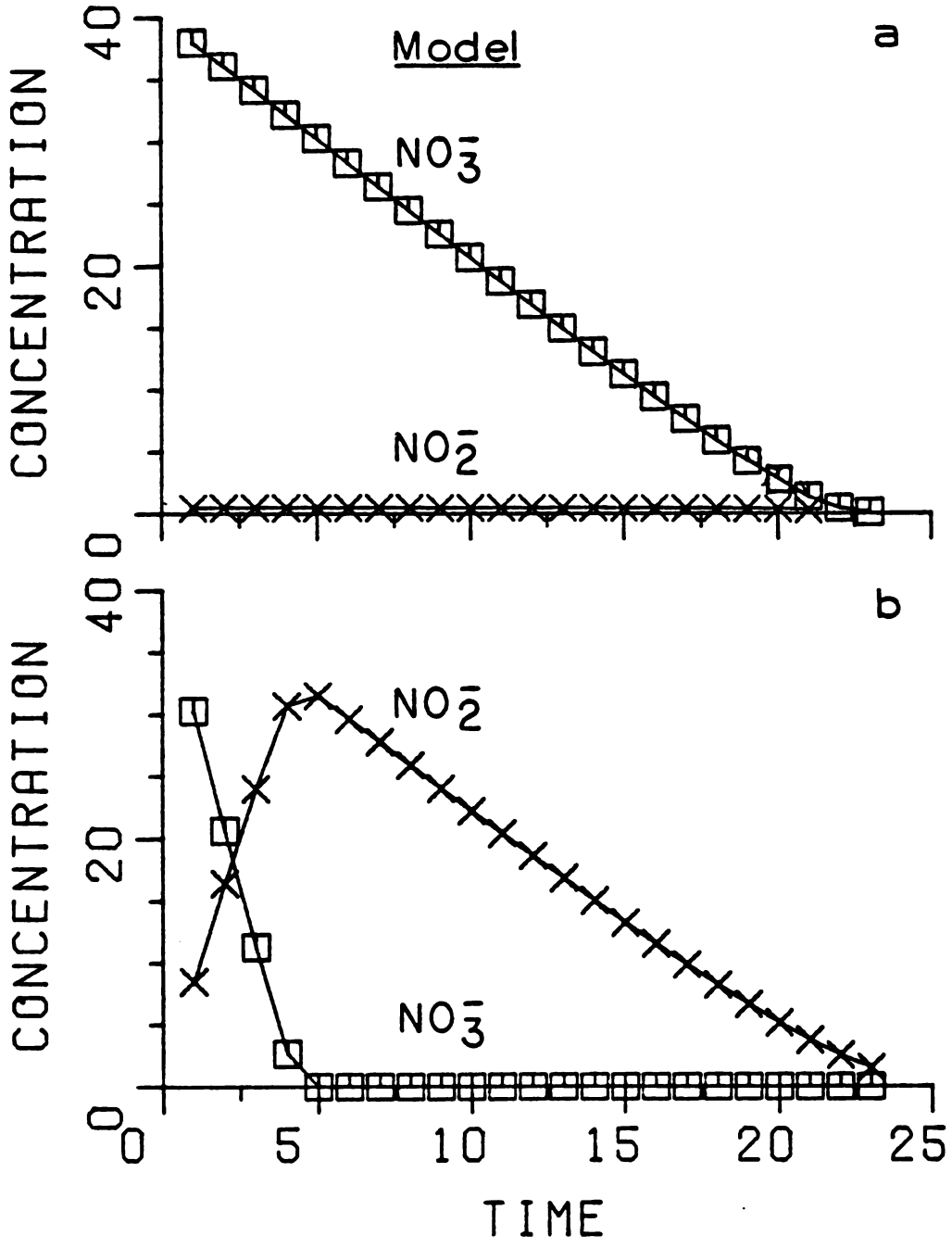



Figure 9. Pattern of nitrite accumulation as a function of the rates of nitrate and nitrite reduction in a model of denitrification.

bacterial cultures. Since nitrite rarely accumulates in soils under denitrifying conditions, the rate of nitrite reduction must be greater than the rate of nitrite formation. To the best of my knowledge this hypothesis has not been tested directly.

The role of nitric oxide as an intermediate in denitrification is still not clear (8,16,28,46,52), despite numerous reports that denitrifers can reduce NO to $N_2O$ and $N_2$ (1,34,35,36,37). St. John and Hollocher (21,41) concluded nitric oxide was not a freely diffusible intermediate after studying $^{15}N$ isotope scrambling in cultures of Pseudomonas aeruginosa exposed to both nitrite and nitric oxide. I observed accumulation of nitric oxide and its subsequent reduction in suspensions of all three isolates and also in suspensions of A. odorans. The behavior of nitric oxide was similar to that of nitrous oxide. As discussed later, the pattern of nitric oxide accumulation I observed can be explained by assuming that nitric oxide is an intermediate in denitrification and that its production is regulated kinetically in a manner similar to other denitrification intermediates.

Nitrous oxide, the other gaseous intermediate, frequently accumulates during denitrification in soils and cultures (7,8,16,28). Some denitrifiers even produce nitrous oxide rather than nitrogen as the terminal product (18). Other bacteria, including nitrifiers and enteric organisms (50, research in this laboratory) also can produce nitrous oxide. How much these groups may contribute to $N_2O$ production in soils has not been determined yet. By adding nitrate to soils several investigators have found they can increase the ratio of $N_2O$ to $N_2$ (4,10,27, 39,40). Firestone et al. (10) postulated that nitrite produced during the nitrate reduction was the true inhibitor, because they found the

same effect could be obtained with lower concentrations of nitrite than nitrate. Delwiche (7), on the other hand, suggested the nitrate effect in Pseudomonas denitrificans was due to preferential use of $NO_3^-$ as the terminal electron acceptor.

In my experiments with the three isolates and with A. odorans neither nitrate nor nitrite at concentrations similar to those used by other investigators (4,10) had any effect on the rate of nitrous oxide reduction (Figures 5 and 6, Table 3). The lack of inhibition observed here contrasts sharply with that which occurs in soils. One possible explanation for different behavior may be that synthesis of nitrous oxide reductase lags behind that of the other reductases in soils, so that addition of nitrate or nitrite causes production of $N_2O$ which cannot be rapidly reduced. Such differential synthesis is supported by the observation that nitrous oxide accumulates in soils shortly after they become anaerobic, but then declines after longer periods of anaerobiosis (11,27,39,40). With soils, Firestone and Tiedje (11) found the transition to a phase of net nitrous oxide consumption required nitrate or nitrous oxide, but could be blocked by addition of the protein synthesis inhibitor, chloramphenicol. They observed a similar effect of chloramphenicol in a culture of Flavobacterium transferred to anaerobic conditions. Similarly, chloramphenicol blocked the increase in denitrification rate observed after anaerobic conditions had been established (44). In this study I used cultures harvested in late exponential phase of growth; they were probably already in a state where nitrous oxide reduction was more rapid than nitrous oxide production, so that $N_2O$ would not accumulate.

In the three isolates I used the concentrations of both nitric oxide and nitrous oxide appeared to be under kinetic control. Such

control seemed especially evident when the nitrous oxide concentration in a P. fluorescens suspension rapidly returned to its steady state value after perturbation (Figure 6). I have extended the kinetic model presented earlier to include nitric oxide and nitrous oxide reduction. The $K_m$ values used were identical with that for nitrite, but the relative velocities of reduction were 1:2:4:3 for nitrate reduction, nitrite reduction, and so on. As shown in Figure 10, this model responded in the same way to addition of nitrous oxide after a steady state had been reached as the suspension of P. fluorescens did (Figure 6). Note too the accumulation of low amounts of nitric oxide during this simulation. Qualitatively, the behavior of NO and $N_2O$ in the model is similar to that observed with all three isolates.

A simple kinetic mechanism may not explain why increased oxygen concentrations increased the relative amount of nitrous oxide produced by each isolate. Firestone et al. (10) observed a similar response to oxygen in soils. Indeed, aeration status of a soil may determine whether it is a source or a sink for nitrous oxide (3,5,13,14). Yet oxygen itself is known to inhibit denitrification and repress synthesis of the denitrifying enzymes (8,16,28). Such inhibition of total gas production has been observed both in soils (10) and with the Alcaligenes and P. fluorescens here. In contrast, oxygen did not inhibit total gas production in Flavobacterium, even though the ratio of $N_2O$ to $N_2$ increased at higher oxygen concentrations. Apparently the nitrous oxide reductase in this organism was more sensitive to oxygen than were the other enzymes in the denitrification pathway.

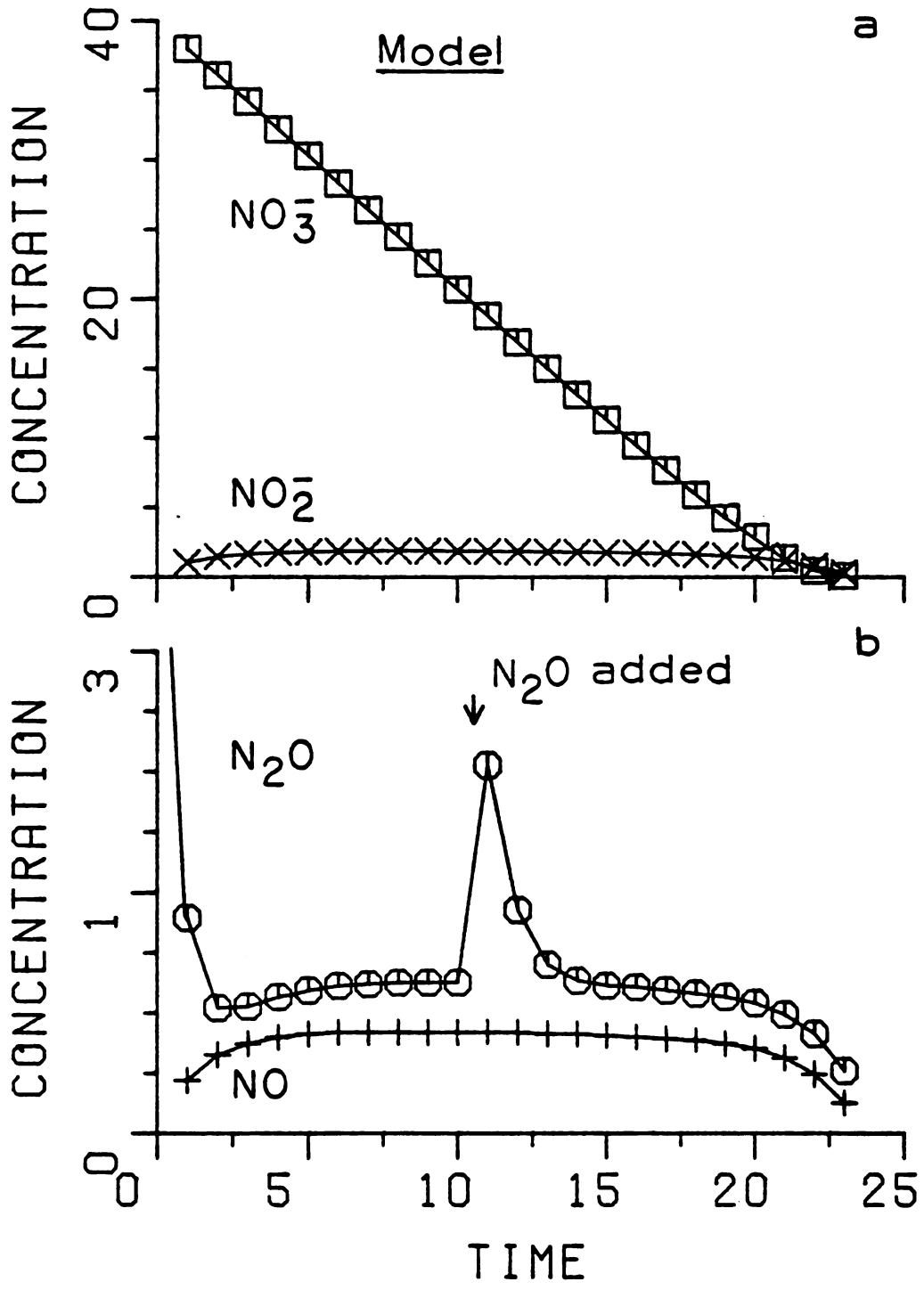



Figure 10. Pattern of nitrite and gas accumulation in a model of denitrification, showing the response of nitrous oxide concentration to perturbation.

Did oxygen affect $N_2O$ production in the other two isolates by nonspecifically inhibiting denitrification, e.g., as a preferred electron acceptor, or did it instead specifically inhibit the nitrous oxide reductase? The first alternative is supported by Hartingsveldt and Stouthamer's observation (19) that a mutant of Pseudomonas aeruginosa unable to synthesize heme aerobically could denitrify when transferred from anaerobic to aerobic conditions. Under aerobic conditions, the electron transport chain to oxygen was incomplete, so oxygen could not act as the preferential electron acceptor. To examine this alternative I modified the kinetic model of each reductive step to include a term, P, which ranged from 0 to 1: $v = P \cdot \frac{V \cdot S}{K_m + S}$. P is the fraction of the suspension that is anaerobic at any given time. In a stochastic model, it would be analogous to the probability that any small volume in the suspension was anaerobic at a given time. As the oxygen concentration in the headspace increased, the fraction of the solution that was anaerobic should decrease. As the solution became less anaerobic, the rate of each reductive step would decrease.

Results of this model are presented in Figure 11. As anaerobic volume decreased (that is, as the oxygen concentration increased), the relative percent of nitrous oxide to total gas production increased. This result is similar to that observed with all three isolates (Figure 7). In addition, the total amount of gas production in the simulation decreased with increased oxygen concentration, as observed with Alcaligenes and P. fluorescens. At longer incubation times the relationship between oxygen concentration and percentage of gas as nitrous oxide still held, though the absolute percentage at any concentration of oxygen was less.

An untested prediction of this model is that the percentage of nitrite relative to the more reduced nitrogen compounds should increase

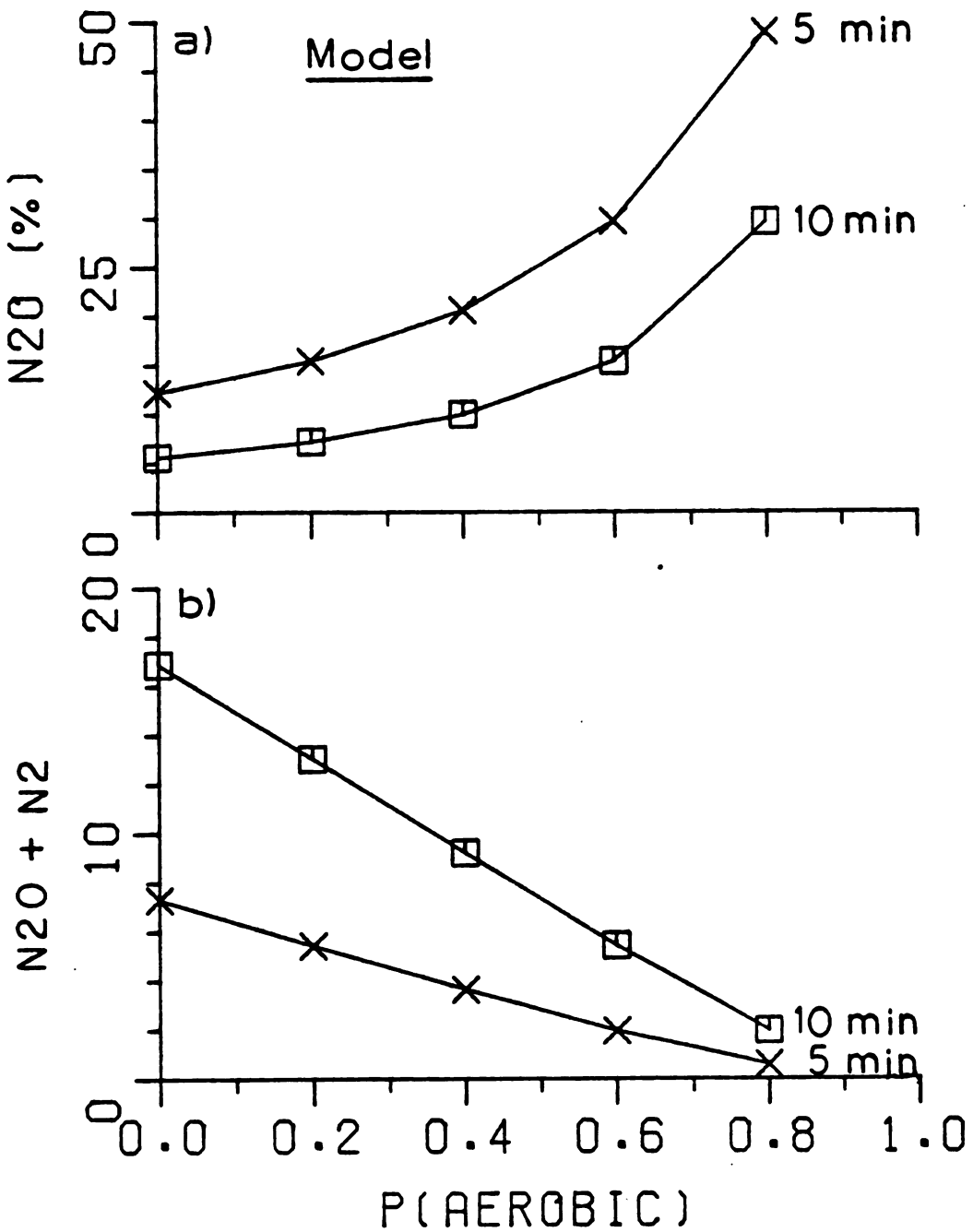



Figure 11. Effects of increasing oxygen concentration on composition and amount of gases produced in a model of denitrification.

with increasing oxygen concentration as did the ratio of $N_2O$ to $N_2$. Also, any factor which inhibits all steps in denitrification equally, for instance decreased temperature, should elicit a response similar to that observed with increased oxygen concentrations since the mathematics of the model would be unchanged.

## FUTURE RESEARCH

In the discussion I have outlined a kinetic mechanism which can account for the patterns of nitrite and nitrous oxide accumulation observed in resting cell suspensions. Verification that this mechanism is correct would require measurement of rates of reduction and of $K_m$ values for the various reductases. The most difficult aspect of such research would be to devise sensitive methods for measuring the concentrations of substrates and products. Procedures for improving nitrate analysis are discussed in Appendix A.

I was able to obtain the value for nitrous oxide reduction using the gas recirculation system described. This system has the advantage of reproducible and frequent sampling. Sampling frequency could be increased by using parallel GC columns, or possibly by including a trap to remove water vapor. However, such a trap might also absorb $N_2O$ in the water vapor retained, and so complicate the analysis. Gas transfer across the phase boundary may limit the rate of $N_2O$ reduction, masking the enzymatic rate. I minimized this problem by using very low cell densities, which prolonged the analysis time. The gas phase could be eliminated entirely. Nitrous oxide in solution could be measured by injecting a liquid sample onto a column warmer than $100^{\circ}C$. However cells and organic compounds in the sample might plug the column. A

replaceable guard column would minimize this problem. A longer column to separate $CO_2$ from $N_2O$ might be required at higher temperatures as well.

One should be able to model observed patterns of accumulation knowing the $K_m$ values and the rates of reduction for each of the reductases. Specific inhibition of any reductive steps by other substrates would lead to disagreement between the predicted and observed concentrations. The same sort of disagreement might result from preferential use of one substrate as an electron acceptor. Distinguishing between direct inhibition and preferential use would require comparison of normal cells with cultures unable to reduce the supposed inhibitor. I used tungstate-grown cells to investigate the effects of nitrate on nitrite reduction. A mutant strain unable to reduce nitrate would have worked also.

An alternative to use of mutants might be to examine substrate use in carbon-limited cultures growing in chemostats. Here various nitrogen compounds, for instance nitrate and nitrous oxide, could be present in excess. Reduction of one compound only would indicate preferential use, assuming that specific inhibition had already been excluded.

The discrepancy between results reported here and those obtained in soils pointed out the difficulty of extending work with cultures to more complex systems. It also highlights the importance of examining cells in more than one physiological state. Numerous investigators have found that the 'physiology' of denitrification in soil systems changes with time after transition from an aerobic to an anaerobic state. Firestone and Tiedje (11) also found such a change with Flavobacterium, reflected in its pattern of $N_2O$ production and consumption. The observations with

soil systems and that with Flavobacterium can be explained by assuming a sequential or at least differential synthesis of denitrifying enzymes after oxygen depletion. However, there are only a few studies in which the pattern of enzyme synthesis during this transition have been examined. Payne and Riley (30) found coordinate derepression of all enzymes in the pathway, though nitrite still accumulated in Pseudomonas perfectomarinus. Williams et al. (49) also observed nitrite accumulation in P. aeruginosa. However, they found nitrite reductase synthesis lagged behind that of nitrate reductase. Examination of other denitrifier species would not be difficult, but would add much to our understanding of denitrifier physiology, its diversity, and its applicability to soils.

The apparent lack of oxygen inhibition of all steps but nitrous oxide reduction in Flavobacterium is intriguing. Previous reports had found lack of inhibition only of the nitrate reductase (20,24,31). The lack of general inhibition of denitrification in this organism may reflect a difference in the branch point(s) of its cytochrome system when compared with the cytochrome systems of other denitrifiers. Alternately, nitrous oxide reduction could be inhibited by direct interaction of oxygen with the reductase rather than by diversion of electron flow from nitrous oxide to oxygen. A comparison of spectra in cells incubated with and without nitrous oxide should identify the cytochromes oxidizable by nitrous oxide. Similar bleaching studies with oxygen could also be done. If oxygen inhibited nitrous oxide reduction by interacting directly with the reductase, then those cytochromes associated only with nitrous oxide recuction should remain reduced in the presence of both nitrous oxide and oxygen. If instead electron flow was diverted to oxygen, the cytochromes associated with nitrous oxide reduction should become oxidized. Nitrous oxide and oxygen pulse experiments

in a rapid-scanning dual beam spectrophotometer should provide the answers to such questions. Of course, a similar strategy could be employed to examine the general pattern of oxygen inhibition proposed above to account for nitrous oxide accumulation by P. fluorescens and Alcaligenes.

## SUMMARY

I have tried to outline experiments here which may account for the discrepancies between my results and those obtained in soils. I should point out that these proposed experiments will determine only the range of behaviors possible by particular denitrifiers. I feel the kinetic model I have constructed offers the advantage of simplicity while maintaining the power to unify numerous phenomena within one system. I do not intend that the hypothesis that accumulation of intermediates of denitrification reflects different rates of reduction of those intermediates should obscure different behaviors possible among denitrifiers. Such differences include the pattern of nitrite accumulation I observed, which could be explained by the model, and the lack of oxygen inhibition of gas production by Flavobacterium compared to the other isolates, which could not.

These results emphasize the importance of further comparative research on denitrifier physiology. With such an arsenal of knowledge we may begin to attack the Gordian knot of soil denitrification; without it, we are likely to end up only with loose ends.

## LITERATURE CITED


1. Baldensperger, J., and J.-L. Garcia. 1975. Reduction of oxidized inorganic nitrogen compounds by a new strain of Thiobacillus denitrificans. Arch. Microbiol. 103: 31-36.

2. Black, C. A. 1965. Methods of soil analysis. Part 2, Chemical and microbiological methods. American Society of Agronomy, Inc., Madison, Wis.

3. Blackmer, A. M., and J. M. Bremner. 1976. Potential of soil as a sink for atmospheric nitrous oxide. Geophys. Res. Let. 3: 739-942.

4. Blackmer, A. M., and J. M. Bremner. 1978. Inhibitory effect of nitrate on reduction of $N_2O$ to $N_2$ by soil microorganisms. Soil Biol. Biochem. 10: 187-191.

5. Brice, K. A., A. E. J. Eggleton, and S. A. Penkett. 1977. An important ground surface sink for atmospheric nitrous oxide. Nature. 268: 127-129.

6. Chance, B. 1957. Cellular oxygen requirements. Fed. Proc. 16: 671-680.

7. Delwiche, C. C. 1959. Production and utilization of nitrous oxide by Pseudomonas denitrificans. J. Bacteriol. 77: 55-59.

8. Delwiche, C. C., and B. A. Bryan. 1976. Denitrification. Ann. Rev. Microbiol. 30: 241-262.

9. Duggleby, R. G., and J. F. Morrison. 1977. The analysis of progress curves for enzyme-catalyzed reactions by non-linear regression. Biochim. Biophys. Acta. 481: 297-312.

10. Firestone, M. K., M. S. Smith, R. B. Firestone, and J. M. Tiedje. 1979. The influence of nitrate, nitrite and oxygen on the composition of the gaseous products of denitrification in soil. Soil Sci. Soc. Am. J. (in press)

11. Firestone, M. K., and J. M. Tiedje. 1979. Temporal change in nitrous oxide and dinitrogen from denitrification following onset of anaerobiosis. Appl. Environ. Microbiol. (in press)

12. Focht, D. D. 1974. The effect of temperature, pH, and aeration on the production of nitrous oxide and gaseous nitrogen--a zero-order kinetic model. Soil Sci. 118: 173-179.

13. Freney, J. R., O. T. Denmead, and J. R. Simpson. 1978. Soil as a source or sink for atmospheric nitrous oxide. Nature. 273: 530-532.

14. Freney, J. R., O. T. Denmead, and J. R. Simpson. 1979. Nitrous oxide emission from soils at low moisture contents. Soil Biol. Biochem. 11: 167-173.

15. Gamble, T. N., M. R. Betlach, and J. M. Tiedje. 1978. Commonality of numerically dominant denitrifying bacteria from world soils. Appl. Environ. Microbiol. 33: 926-939.

16. Garcia, J.-L. 1975. La denitrification dans les sols. Bull. Inst. Pasteur. 73: 167-193.

17. Greenberg, E. P., and G. E. Becker. 1977. Nitrous oxide as end product of denitrification by strains of fluorescent pseudomonads. Can. J. Microbiol. 23: 903-907.

18. Greenwood, D. J. 1962. Nitrification and nitrate dissimilation in soil. II. Effect of oxygen concentration. Plant Soil. 17: 378-391.

19. Hartingsveldt, J. van, and A. H. Stouthamer. 1974. Properties of a mutant of Pseudomonas aeruginosa affected in aerobic growth. J. Gen. Microbiol. 83: 303-310.

20. Higashi, T. 1960. Physiological study on the oxygen- and nitrate-respiration of Pseudomonas aeruginosa. J. Biochem. (Tokyo) 47: 327-334.

21. Hollocher, T. C. 1978. Isotope studies of denitrification: role of NO and $N_2O$ in the pathway from nitrite to $N_2$ in Pseudomonas aeruginosa, in D. Schlessinger, ed. Microbiology--1978. American Society for Microbiology.

22. Klemedtsson, L., B. H. Svensson, T. Lindberg, and T. Rosswall. 1977. The use of acetylene inhibition of nitrous oxide reductase in quantifying denitrification in soils. Swedish J. Agric. Res. 7: 179-185.

23. Kohl, D. H., F. Vithayathil, P. Whitlow, G. Shearer, and S. H. Chien. 1976. Denitrification kinetics in soil systems: the significance of good fits of data to mathematical forms. Soil Sci. Soc. Am. J. 40: 249-253.

24. Lam, Y., and D. J. D. Nicholas. 1969. Aerobic and anaerobic respiration in Micrococcus denitrificans. Biochim. Biophys. Acta. 172: 450-461.

25. Matsubara, T., and T. Mori. 1968. Studies on denitrification: IX. Nitrous oxide, its production and reduction to nitrogen. J. Biochem. (Tokyo) 64: 863-871.

26. Nimmo, I. A., and G. L. Atkins. 1974. A comparison of two methods for fitting the integrated Michaelis-Menten equation. Biochem. J. 141: 913-914.

27. Nommik, H. 1956. Investigations on denitrification in soil. Acta Agr. Scand. 6: 195-228.

28. Payne, W. J. 1973. Reduction of nitrogenous oxides by microorganisms. Bacteriol. Rev. 37: 409-452.

29. Payne, W. J., and P. S. Riley. 1969. Supression by nitrate of enzymatic reduction of nitric oxide. Proc. Soc. Exp. Biol. Med. 132: 258-260.

30. Payne, W. J., P. S. Riley, and C. D. Cox, Jr. 1971. Separate nitrite, nitric oxide, and nitrous oxide reducing fractions from Pseudomonas perfectomarinus. J. Bacteriol. 106: 356-361.

31. Pfitzner, J., and H. G. Schlegel. 1973. Denitrifikation bei Hydrogenomonas eutropha Stamm H16. Arch. Microbiol. 90: 199-211.

32. Phillips, R. E., K. R. Reddy, and W. H. Patrick Jr. 1978. The role of nitrate diffusion in determining the order and rate of denitrification in flooded soil: II. Theoretical analysis and interpretation. Soil Sci. Soc. Am. J. 42: 272-278.

33. Pichinoty. F., J. Bigliardi-Rouvier, M. Mandel, B. Greenway, G. Metenier, and J.-L. Garcia. 1976. The isolation and properties of a denitrifying bacterium of the genus Flavobacterium. Antonie van Leeuwenhoek. 42: 349-354.

34. Pichinoty, F., and L. D'Ornano. 1961. Recherches sur la reduction de protoxyde d'azote par Micrococcus denitrificans. Annal. Inst. Pasteur. 101: 418-426.

35. Pichinoty, F., J.-L. Garcia, M. Mandel, C. Job, and M. Durand. 1978. Isolement de bacteries utilisant en anaerobiose l'oxyde nitrique comme accepteur d'electrons respiratoire. Comptes Rendus Acad. Sci. Paris. 286: 1403-1405.

36. Pichinoty, F., M. Mandel, and J.-L. Garcia. 1977. Etude physiologique et taxonomique de Paracoccus denitrificans. Ann. Microbiol. Inst. Pasteur. 128B: 243-251.

37. Pichinoty, F., M. Veron, M. Mandel, M. Durand, C. Job, and J.-L. Garcia. 1978. Etude physiologique et taxonomique du genre Alcaligenes: A. denitrificans, A. odorans et A. faecalis. Can. J. Microbiol. 24: 743-753.

38. Reddy, K. R., W. H. Patrick, Jr., and R. E. Phillips. 1978. The role of nitrate diffusion in determining the order and rate of denitrification in flooded soil: I. Experimental results. Soil Sci. Soc. Am. J. 42: 268-272.

39. Ryden, J. C., L. J. Lund, and D. D. Focht. 1979. Direct measurement of denitrification loss from soils: I. Laboratory evaluation of acetylene inhibition of nitrous oxide reduction. Soil Sci. Soc. Am. J. 43: 104-110.

40. Ryden, J. C., L. J. Lund, J. Letey, and D. D. Focht. 1979. Direct measurement of denitrification loss from soils: II. Development and application of field methods. Soil Sci. Soc. Am. J. 43: 110-118.

41. St. John, R. T., and T. C. Hollocher. 1977. Nitrogen 15 tracer studies on the pathway of denitrification in Pseudomonas aeruginosa. J. Biol. Chem. 252: 212-218.

42. Sawada, E., T. Satch, and H. Kitamura. 1978. Purification and properties of a dissimilatory nitrite reductase of a denitrifying phototrophic bacterium. Plant Cell Physiol. 19: 1339-1351.

43. Scott, R. H., G. T. Sperl, and J. A. DeMoss. 1979. In vitro incorporation of molybdate into demolybdoproteins in Escherichia coli. J. Bacteriol. 137: 719-726.

44. Smith, M. S., M. K. Firestone, and J. M. Tiedje. 1979. Phases of denitrification following oxygen depletion in soil. Soil. Biol. Biochem. 11: 261-267.

45. Stouthamer, A. H. 1976. Biochemistry and genetics of nitrate reductase in bacteria. Adv. Microbial Physiol. 14: 315-375.

46. Swain, H. M., and H. J. Somerville. 1978. Denitrification during growth of Pseudomonas aeruginosa on octane. J. Gen. Microbiol. 107: 103-112.

47. Tiedje, J. M., R. B. Firestone, M. K. Firestone, M. R. Betlach, M. S. Smith, and W. H. Caskey. 1979. Methods for production and use of $^{13}N$-$NO_3^-$ for studies of denitrification. Soil Sci. Soc. Am. J. (in press)

48. Wilkinson, G. N. 1961. Statistical estimations in enzyme kinetics. Biochem. J. 80: 324-332.

49. Williams, D. R., J. J. Rowe, P. Romero, and R. G. Eagon. 1978. Denitrifying Pseudomonas aeruginosa: Some parameters of growth and active transport. Appl. Environ. Microbiol. 36: 257-263.

50. Yoshida, T., and M. Alexander. 1970. Nitrous oxide formation by Nitrosomonas europaea and heterotrophic microorganisms. Soil Sci. Soc. Am. Proc. 34: 880-882.

51. Yoshinari, T., R. Hynes, and R. Knowles. 1976. Acetylene inhibition of nitrous oxide reduction and measurement of denitrification and nitrogen fixation in soil. Soil Biol. Biochem. 8: 1-7.

52. Zumft, W. G. 1979. The inorganic biochemistry of nitrogen bioenergetic processes. Naturwissenschaften. 66: 81-88.

# CHAPTER II

## NITRATE UPTAKE AND ASSIMILATION BY PSEUDOMONAS FLUORESCENS

Nitrogen in its reduced oxidation state is of fundamental importance to biology on this planet. The assimilation of ammonia, amino acids, or proteins can support the nitrogen requirements of most organisms. Yet without the existence of nitrogen-fixing bacteria the vast reservoir of atmospheric nitrogen would not be available to biology. It is no wonder that a great deal of research has been directed toward an understanding of biological nitrogen fixation and the pathways of ammonia utilization. Many organisms, notably green algae and higher plants, also can grow with nitrate as a nitrogen source. Nitrate often is more plentiful than ammonia, as in the open ocean; or more readily available, as in a soil solution. Assimilatory nitrate reduction in plants has received a great deal of attention, since nitrate is an important alternate nitrogen source for these primary producers.

In contrast, assimilation of nitrate by bacteria has not been extensively investigated. Possibly the small number of bacteria reported to grow with nitrate as the sole nitrogen source (43) is due to this lack of systematic investigation. Nevertheless, the ability of relatively few bacteria to utilize a nitrogen source not available to others should confer a competitive advantage in those environments where nitrate is more abundant than ammonia. Elucidation of the mechanism of nitrate uptake and assimilation in bacteria is also of interest for

comparison with eucaryotic systems more intensively studied, since such a property and associated regulatory activities ought to be conserved during evolution. Furthermore, the study of nitrate assimilation may elucidate the structure of anion transport systems, which have been neglected in favor of cation transport systems.

For these reasons I have chosen to study the mechanisms of transport and assimilation of nitrate in a common soil bacterium, Pseudomonas fluorescens. The availability of the longest-lived radioisotope of nitrogen, $^{13}N$, for this research has permitted the use of experimental techniques which would be impossible or much more cumbersome if only the stable isotope, $^{15}N$, were available. In this research I have found that P. fluorescens can actively transport nitrate against a concentration gradient. I also have determined the $K_m$ value for transport as well as the effects of chlorate and ammonia on this process. A portion of this research was presented at the annual meeting of the American Society for Microbiology in Los Angeles (M. R. Betlach and J. M. Tiedje, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, I86, p. 109).

## MATERIALS AND METHODS

Organism and Growth Medium. The strain of Pseudomonas fluorescens (isolate 72) used is representative of the dominant group of denitrifiers in soils (19). The growth medium for this organism contained: 50 mM glucose; 50 mM phosphate buffer, potassium and sodium salts, pH 7.0; 0.8 mM $MgSO_4 \cdot 7H_2O$; 0.35 mM $CaCl_2 \cdot 2H_2O$; 0.005 mM $MnSO_4 \cdot 4H_2O$; 0.02 mM $FeSO_4 \cdot 7H_2O$ with 0.025 mM citric acid; and 0.1 or 0.6 mM $Na_2MoO_4 \cdot 2H_2O$. Glucose was added after autoclaving. The standard nitrogen source was 10 mM $KNO_3$.

All nitrate reductases which have been characterized are molybdoproteins (69). An inactive nitrate reductase is formed when tungstate

is included in the growth medium instead of molybdate (22,28,57). Replacement of molybdate with 10 mM $Na_2WO_4$ prevented cells from forming an active nitrate reductase. Since such cells could not grow on nitrate, 5 or 10 mM $KNO_2$ was used as a nitrogen source instead.

After addition of a 5% seed culture to 200 ml medium in 2 l Erlenmeyer flasks, the flasks were placed on a rotary shaker, 250 rpm, in a 30°C incubator. Cells were harvested while in late exponential or early stationary phase of growth 8 to 12 h after inoculation.

Several experiments were done to determine whether nitrate assimilation was induced by nitrate or repressed by ammonia. (Throughout this chapter the term 'ammonia' is used to refer to both ammonia and ammonium ion, though the latter is the prelavent species at pH 7.) For these experiments 10 mM $NH_4NO_3$ or 5 mM $(NH_4)_2SO_4$ was provided instead of $KNO_3$. In one series, a culture was grown on $(NH_4)_2SO_4$ until it had reached late exponential phase. After being harvested and washed, the cells were inoculated into fresh media with different nitrogen sources. The new cultures were sampled at 15 min intervals until they had been growing exponentially for several hours. The sample absorbance was measured at 660 nm, then the sample was filtered and the concentrations of ammonia and nitrate in the filtrate were determined.

Cultures also were grown on limiting ammonia to see if synthesis of nitrate reductase could be derepressed. A solution of $(NH_4)_2SO_4$ was added at 1.5 h intervals so that the ammonia concentration in the growth medium did not exceed 0.5 mM. After 12 h, the culture was harvested and used to determine whether cell suspensions could reduce nitrate to nitrite.

Preparation of cell suspensions. Cells harvested by centrifugation at 10,000 g for 10 min were resuspended in 10 ml of a buffer solution

(hereafter refered to as standard buffer) containing 50 mM potassium and sodium phosphate salts, pH 7.0, and 200 µg chloramphenicol (grade B, Sigma Chemical Co., St. Louis, MO) per ml. This concentration of chloramphenicol was sufficient to inhibit growth of the organism in nutrient broth, as determined by the serial dilution method. The resuspended cells were centrifuged for 5 min at 10,000 g, then resuspended and centrifuged again. The pellet of washed cells was resuspended in 5 ml standard buffer and kept on ice. Appropriate volumes of this stock suspension were added to incubation mixtures at the start of each experiment.

Production of $^{13}NO_3^-$. Procedures for the production and purification of $^{13}N$-containing inorganic compounds have been described previously (64). Proton irradiation of a water target produces $^{13}N$ free radicals which react with water to form nitrate, nitrite, ammonia, and some gaseous products. Radioactive gases and ammonia were removed under vacuum after placing the water in a flash evaporator with 0.1 ml of 0.01 N NaOH. The salts remaining were neutralized with HCl and brought to a suitable volume by addition of a $^{14}NO_3^-$ solution.

$^{13}NO_3^-$ uptake. Standard membrane filtration techniques were used to follow uptake of radioactive nitrate with time. Volumes of stock cell suspension chosen to give an absorbance at 660 nm of 0.25 or 0.50 in a final volume of 10 ml were placed in a 125 ml flask with standard buffer. Glucose in solution was added to a final concentration of 50 mM. Cell suspensions were preincubated for 5 min at room temperature on a magnetic stirrer. The reaction was started by adding carrier nitrate and the $^{13}NO_3^-$ solution. Inhibitors of uptake including methionine sulfoxime (Sigma Chemical Co., St. Louis, MO) and azaserine (Calbiochem,

LaJolla, CA) were added at the start of preincubation or during the course of uptake, as indicated in the results. All solutions used were prepared fresh in standard buffer.

At various times 1 ml samples were removed and rapidly filtered through 0.45 μm cellulose acetate-cellulose nitrate membrane filters (Metricel GA-6, Gelman Instrument Co., Ann Arbor, MI). Filters were held in 200 ml filter funnels (Gelman) which were inserted into 125 ml suction flasks containing 16 by 100 mm test tubes to receive the filtrate. Filtered cells were washed by passing 2 ml standard buffer through the filters. The filters were removed, placed in scintillation vials, and 10 ml of scintillation cocktail were added (FilterSolv, Beckman Instrument Co., Fullerton, CA). Vials were shaken for 10 min to digest the filters, then counted in a Beckman 8100 liquid scintillation counter. Each sample set was counted twice with the discriminator window wide open (0 to 1000). Background--less than 40 counts per minute (cpm)--was ignored since all samples had at least 1500 cpm at the end of counting. No quench correction was applied since all samples showed constant but minimal quenching. Samples of the $^{13}N$ stock solution also were counted to determine the specific activity of the nitrate solution used.

High pressure liquid chromatography detection system. A high pressure liquid chromatography (HPLC) system to separate and identify radioactive nitrogen ions was described previously (64). Effluent from an anion exchange column (Partisil 10-SAX, Whatman) was passed between two NaI crystals attached to photomultiplier tubes in a coincidence circuit. Counts were determined at 2 s intervals by reading a scalar connected to either a Xerox Sigma 7 or a PDP 11/45 computer. Since 50 mM potassium phosphate buffer at pH 3.0 was used to elute the ions, any

amino acids injected onto the column would co-chromatograph with ammonia.

Internal form of $^{13}N$. Cell suspensions were incubated as described above, except that the total volume was 5 ml in a 50 ml flask. After cells were filtered and washed once with 2 ml standard buffer, the filter apparatus was transferred to a new receiving flask. A 2 ml volume of hot methanol (50°C) was placed on the filter and incubated at least 30 seconds, after which suction was applied. The methanol extract was removed, transfered to a 15 ml conical bottom test tube, and dried using a Buchler Evapo-Mix unit. The dried extract was dissolved in 200 µl distilled water and a sample injected onto the HPLC. For electrophoresis, the extract was redissolved in 20 µl ethanol, 5 µl of amino acid standards were added, and then appropriate volumes were spotted on a cellulose-coated thin layer chromatography plate. Each plate was sprayed with buffer and subjected to electrophoresis for 8 to 12 min at 2800 volts (63), then scanned with a plate scanner (Berthold Varian Aereograph Radioscanner). The distribution of radioactivity among the amino acids was determined by cutting out peaks and weighing them, then adjusting the weights to take $^{13}N$ decay into account. Peaks were identified by relative position and by comparison with standards which were visualized by spraying with ninhydrin.

Nitrate and nitrite reduction. In several experiments nitrate and nitrite reduction rates were measured using unlabeled compounds and standard colorimetric procedures. The experimental design was similar to that for $^{13}NO_3^-$ uptake, except that filtered cells were not washed. Significant amounts of nitrate could be leached from the cellulose acetate-cellulose nitrate filters, so polycarbonate filters or glass fiber prefilters (Nuclepore) were used instead. Nitrate in the filtrate

was analyzed using Szechrome NAS reagent (Research and Development Authority, Ben-Gurion University of the Negev, Beer-Sheva, Israel; see Appendix A). Subsamples of the filtrate were treated with 0.1 volume of 5% (wt/vol) sulfamic acid to remove nitrite, which also reacted with the reagent. Nitrite was determined by diazotization (2). After adjustment of the sample volume to 1 ml, 1 ml each of sulfanilamide and of naphthylethylene diamine (NEDD) was added and the absorbance measured at 520 nm in a Turner Model 350 spectrophotometer. Ammonia was determined by a modified indophenol procedure (67).

Nitrate reduction in toluene-treated cells. Toluene-treated cells were used to determine whether or not chlorate inhibited nitrate reductase or whether chlorate simply could not penetrate the cell membrane. Cell suspensions were treated with toluene added to a final concentration of 2% (vol/vol). Treated suspensions were kept at room temperature for at least 5 min prior to use. Addition of a few crystals of sodium dithionite to the supernatant fluid from cells prepared in this way resulted in the appearance of a very strong absorption peak at 340 nm, indicative of reduction of NAD(P). The release of NAD(P) from the cells demonstrated that the toluene treatment had destroyed the permeability barrier of the cytoplasmic membrane.

Cells treated with toluene no longer reduced nitrate to nitrite when energized by glucose. Instead, the procedure of Lowe and Evans (36) was modifed to measure nitrate reduction in such cell suspensions. The reaction vessels, 15 ml centrifuge tubes, contained 0.1 ml each of the following solutions: 0.15 M phosphate buffer, pH 7.0; 23 mM sodium dithionite in 48 mM $NaHCO_3$; 0.02% (wt/vol) methyl viologen; and 100 mM $KNO_3$. Inhibition of nitrate reduction in toluene-treated cells was

examined after addition of 0.1 ml of either 60 mM $(NH_4)_2SO_4$ or 60 mM $KClO_3$ to the reaction mixtures, and 0.1 ml phosphate buffer to the blank. After all reagents had been added the tubes were preincubated for 5 min at room temperature (25°C).

The reaction was started by adding 0.1 ml of the toluene-treated cell suspension to each tube. It was stopped by vigorous shaking to oxidize the dithionite and methyl viologen. Each solution was brought to 1 ml volume with buffer, then 1 ml sulfanilamide was added. The suspension was centrifuged, the supernatant fluid was decanted, and nitrite in the supernatant fluid was determined by adding 1 ml NEDD reagent.

$^{14}C$ uptake experiments. Uniformly labeled D-glucose, L-glutamic acid, and L-glutamine were obtained from Amersham and diluted with unlabeled materials to reduce the specific activity to 1 μCi per μmol. Procedures used were identical to those for uptake of $^{13}NO_3^-$ except that the reaction was started by adding $^{14}C$-labeled material rather than nitrate solution. The final concentration of glucose used was 1 mM. The final concentrations of glutamate and glutamine were each 100 μM.

Analysis of $^{13}N$ counts. The radioactive isotope of fluorine, $^{18}F$, is a normal contaminant of $^{13}N$ solutions prepared by bombardment of water targets. The fluoride, and some $^{13}NO_3^-$, adsorbed to the membrane filters. Use of filters of different chemical composition did not prevent binding, nor did an increase in the wash volume to 60 times the sample volume remove bound counts (J. C. Meeks, personal communication).

To determine the contribution of $^{18}F$ to total radioactivity and to correct for $^{13}N$ decay, I solved the following pair of simultaneous

equations:

1) $CPM(t_1) = N_0 \cdot D_N(t_1) + F_0 \cdot D_F(t_1)$;

2) $CPM(t_2) = N_0 \cdot D_N(t_2) + F_0 \cdot D_F(t_2)$.

In these equations $CPM(t_1)$ represents the total counts in the sample vial recorded at elapsed time, $t_1$. $N_0$ and $F_0$ refer to the cpm attributable to $^{13}N$ and $^{18}F$, respectively, in the sample at the start of counting the sample set. $D_N(t_1)$ refers to the fraction of $^{13}N$ radioactivity remaining at time $t_1$. It is determined by evaluating the expression $\exp(-\ln 2 \cdot t/t_{\frac{1}{2}})$, where $t_{\frac{1}{2}}$ is the half-life of $^{13}N$, 9.96 min. A similar expression is represented by $D_F(t_1)$, except that the half-life of $^{18}F$ is 110 min. Since the counts per minute and the elapsed time (hence fraction remaining) are known for each sample, the equations need only be solved for $N_0$ and $F_0$. This was done using a program (Appendix B) written for an IMSAI 8080 microcomputer in our laboratory. This computer was connected to the scintillation counter (via a Beckman RS232C C ommunications Interface) to receive counting data directly. Such a configuration permitted analysis of an experiment within a few minutes after the last sample had been counted, so that subsequent experiments could be modified as needed.

Since $^{18}F$ has a much longer half-life than $^{13}N$, its contribution to total counts increased continuously after bombardment. In all experiments where cells had incorporated radioactivity, the contribution of fluorine was lower than in the source used, indicating that there was a selective enrichment for $^{13}N$ in the filtered cells. In contrast, filters through which samples of the source were passed to measure nonspecific adsorption always had a higher percentage of counts attributable to fluorine than the source or the filtered cells, indicating $^{18}F$

was prefentially bound to the filters. Results of a typical experiment which showed these effects are presented in Table 1.

Other mathematical analyses. The lag time for cells transferred from ammonia- to nitrate-containing medium was estimated from the equation of Hinshelwood (29):

$$N(t) = N(t_0) \exp [\mu_{max}(t - L)],$$

where L is the lag time; N(t) and $N(t_0)$ are the absorbances of the culture at time t and immediately after transfer, respectively.

Growth rates were calculated by linear regression procedures after performing a logarithmic transformation of the absorbance readings (59). Linear uptake rates at different nitrate concentrations were calculated by standard linear regression procedures as well. Slopes of the regression lines and the corresponding initial nitrate concentrations were used to estimate $K_m$ and $V_{max}$ from the nonparametric direct linear plot procedure of Eisenthal and Cornish-Bowden (15). Intersection points and median values were calculated with a program written in BASIC for an IMSAI 8080 microcomputer instead of being estimated from the graph. Confidence limits for $K_m$ and $V_{max}$ were determined using the procedure of Porter and Trager (48). Their nonparametric procedure gives asymmetric upper and lower bounds for the confidence limits. The total probability enclosed by these bounds depends on the number of intersection points obtained in the direct linear plot.

## RESULTS

Repression by ammonia. Evidence for repression of the enzymes for nitrate assimilation by ammonia is presented in Figure 1. Only cells transferred to the medium where nitrate was the sole nitrogen source

Table 1. Corrected data for uptake of 1 μM $NO_3^-$ by cells grown on nitrite and tungstate, illustrating $^{18}F$ contribution to total radioactivity on filters.

| Incubation time (min) | Disintegrations per minute[a] $^{18}F$ | $^{13}N$ | Percent $^{18}F$ | Percent of possible $^{13}N$ incorporation |
|---|---|---|---|---|
| 0.5 | 1092 | 18667 | 5.53 | 4.56 |
| 1.0 | 1432 | 22756 | 5.92 | 5.56 |
| 1.5 | 906 | 29213 | 3.01 | 7.14 |
| 2.0 | 1407 | 35392 | 3.82 | 8.65 |
| 2.5 | 1443 | 40889 | 3.41 | 9.89 |
| 3.0 | 2166 | 41253 | 4.99 | 10.08 |
| 3.5 | 1979 | 43459 | 4.35 | 10.62 |
| 4.0 | 1024 | 45643 | 2.19 | 11.15 |
| 4.5 | 1871 | 45232 | 3.97 | 11.05 |
| Adsorption to filter | 347 | 494 | 41.27 | 0.12 |
| $^{13}N$ solution | 144635 | 409303 | 26.11 | 100.00 |

[a] Values reported as disintegrations per minute since they have been calculated back to the time I began counting the sample set.

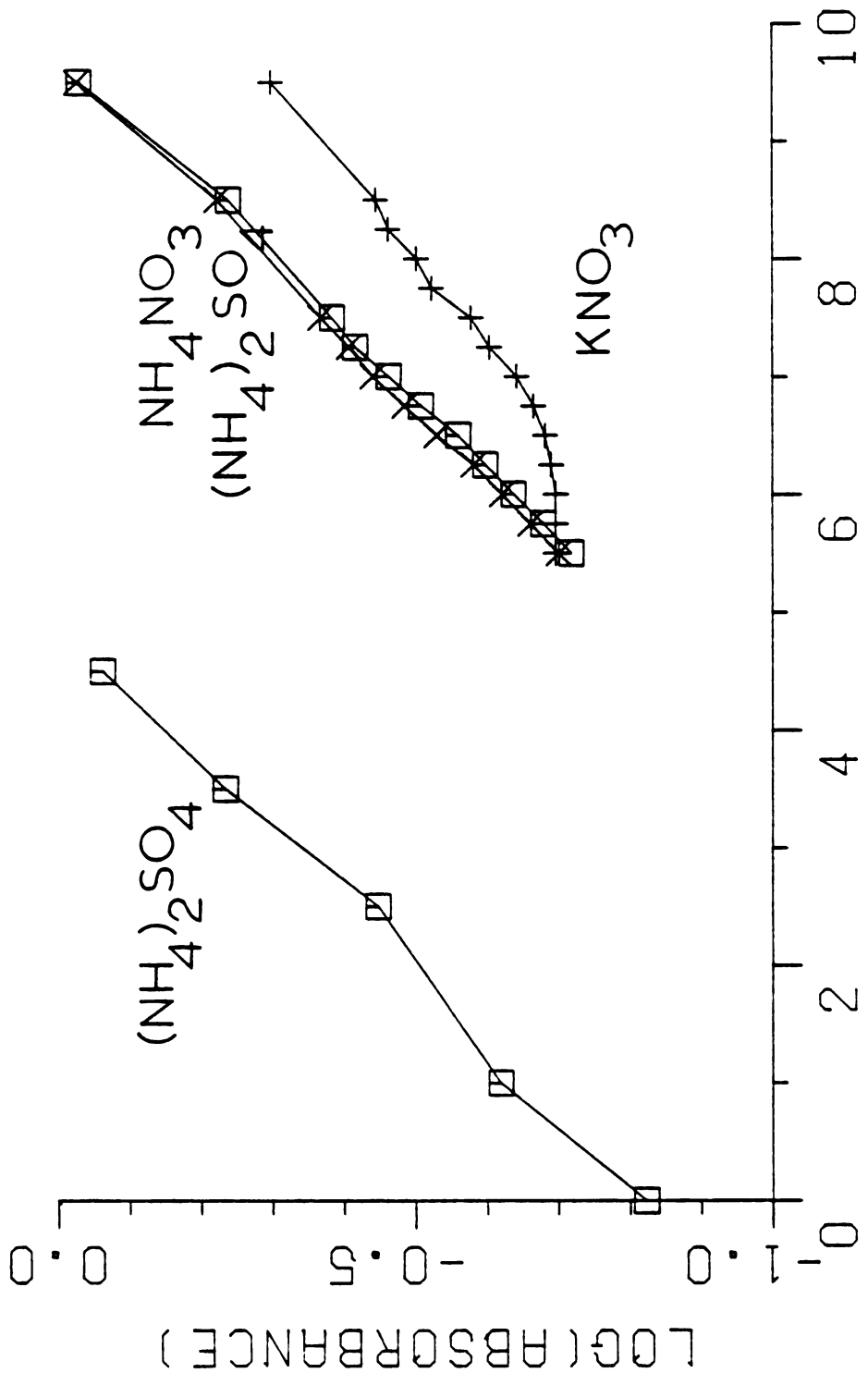



Figure 1. Growth of P. fluorescens on $KNO_3$, $NH_4NO_3$, and $(NH_4)_2SO_4$ after growth on $(NH_4)_2SO_4$.

exhibited a lag in growth; those transferred to a medium containing ammonium sulfate or ammonium nitrate did not. The lag time was 1.06 h. The generation time for cells growing on nitrate was 2.22 h, whereas it was 1.76 h for cells growing on ammonium sulfate or ammonium nitrate.

The lag in growth observed, approximately one half a generation, is consistent with the requirement for de novo synthesis of the enzymes for nitrate assimilation. Nitrate consumption did not begin until the end of the lag phase in cells transferred to medium with nitrate only. No nitrate consumption was observed in cells transferred to ammonium nitrate-containing medium until ammonia was depleted.

In addition to the lag in growth and nitrate consumption observed, cells grown on ammonia did not take up $^{13}NO_3^-$ (Figure 2). Cells grown on ammonium nitrate did not incorporate $^{13}NO_3^-$ either (Figure 2), indicating that repression by ammonia overrode any induction by nitrate.

Induction by nitrate and nitrite. As illustrated in Figure 2, cells grown on nitrate readily incorporated $^{13}NO_3^-$. Experiments with unlabeled nitrate (Table 2) demonstrated that these cells had an active nitrate reductase, as expected. Cells grown on nitrite also synthesized nitrate reductase, though the amount of enzyme formed was only 50% of that in nitrate-grown cells. As expected, cells grown with tungstate in the medium had little capacity to reduce nitrate (Table 2).

Derepression in absence of ammonia. Nitrite appeared to induce synthesis of the complete nitrate assimilation pathway. This apparent induction may have been derepression instead, since the nitrite- and nitrate-containing media both lacked ammonia. Cells grown on limiting ammonia were able to reduce nitrate to nitrite (Table 2), though the rate of reduction was much less than that in cells grown on nitrate or

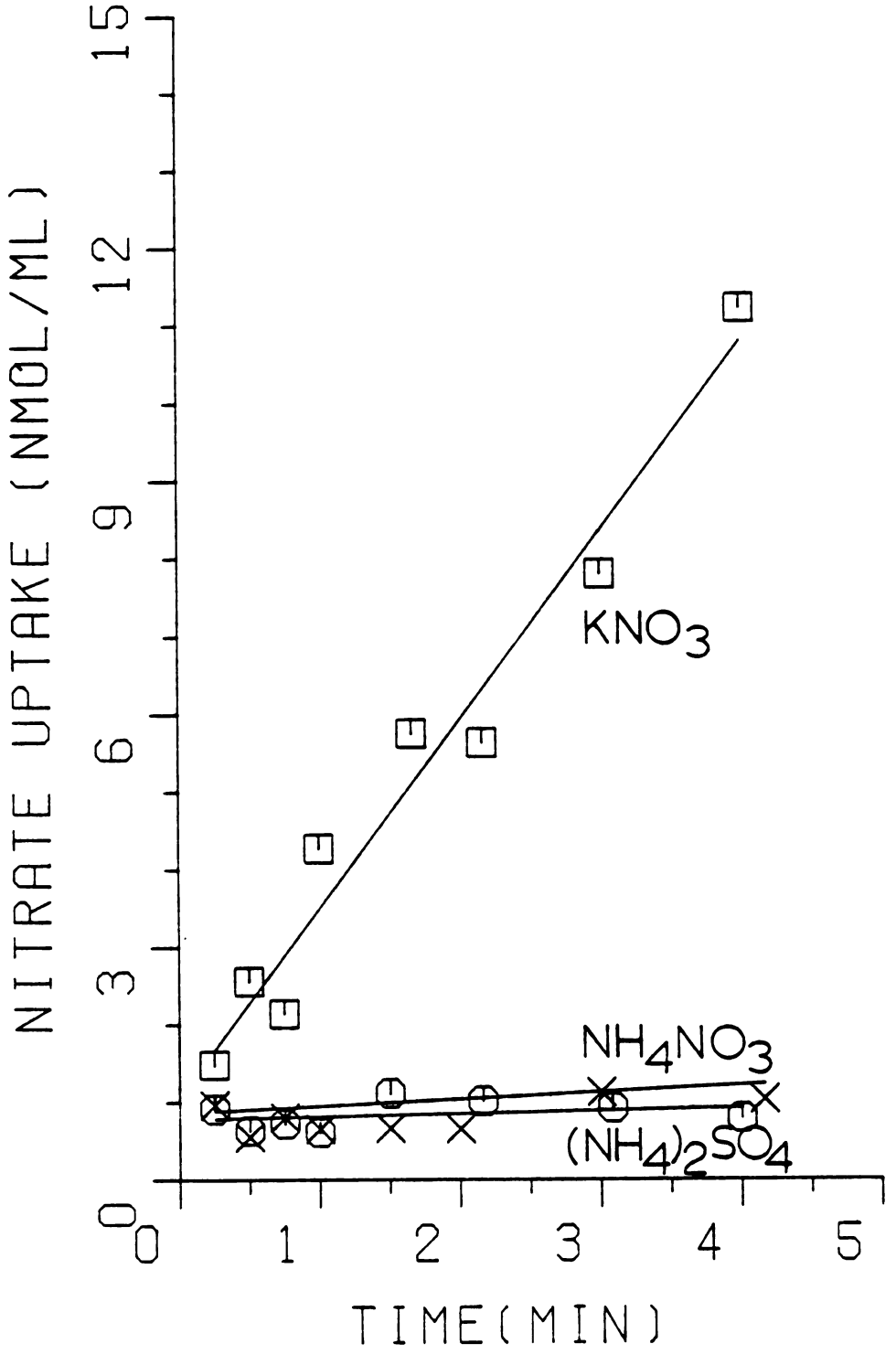



Figure 2. Incorporation of $^{13}NO_3^-$ by P. fluorescens grown on nitrate, ammonium nitrate, or ammonium sulfate.

Table 2. Rate of nitrate reduction in cells grown on different nitrogen sources.

| Growth medium | Rate of $NO_3^-$ reduction ($\mu M\ min^{-1}$) |
|---|---|
| 10 mM $NO_3^-$ | 15.90 ± 6.50 (3)[a] |
| 10 mM $NO_2^-$ | 7.54 ± 0.35 (7) |
| 10 mM $NO_2^-$ and 10 mM $Na_2WO_4$ | 1.32 ± 0.42 (7) |
| 0.5 mM $NH_4^+$, replenished every 1.5 h | 0.95 ± 0.16 (6) |

[a] Limits are $t_{0.05}s_b$; numbers in parentheses are degrees of freedom.

nitrite. Derepression in the absence of ammonia, coupled with stimulation of synthesis in the presence of nitrate or nitrite, apparently accounted for the pattern of regulation observed.

Internal form of $^{13}NO_3^-$. Cells grown on nitrate rapidly incorporated $^{13}NO_3^-$ (Figure 2). Examination of methanol extracts of cells incubated with labeled nitrate revealed that the $^{13}N$ was present internally as ammonia or amino acids (Table 3). Cells grown on nitrite and tungstate were incapable of significant nitrate reduction. As shown in Table 3, the internal form of $^{13}N$ in these cells was primarily nitrate. In other experiments $^{13}NO_3^-$ was the only product detected in extracts of tungstate-grown cells.

The cell density used in these experiments was 300 μg (wet weight) per ml. Since approximately 3% of the nitrate was taken up as nitrate, these cells were able to concentrate nitrate at least 100-fold (0.03/0.0003, assuming a cell density of 1 gram per milliliter). This ability to concentrate nitrate indicated P. fluorescens could actively transport

Table 3. Comparison of internal form of $^{13}N$ in cells able to reduce nitrate with that in cells grown with tungstate or incubated with ammonia.

| Cell treatment | Incubation time (min) | Percent of label in $NH_4^+$ | $NO_2^-$ | $NO_3^-$ | Percent taken up[a] |
|---|---|---|---|---|---|
| Growth on $NO_3^-$ | 1 | 100.0 | 0.0 | 0.0 | 5.2 |
| | 3 | 91.5 (0.0)[b] | 8.5 (7.3) | 0.0 (92.7) | 12.8 |
| Growth on $NO_2^-$ and $WO_4^=$ | 2 | 11.5 | 0.0 | 88.5 | 2.4 |
| | 5 | 12.9 (0.0) | 0.0 (tr) | 87.1 (100) | 3.1 |
| Growth on $NO_3^-$; incubation with 10 mM $NH_4^+$ | 1 | 6.5 | 18.5 | 75.0 | 0.50 |
| | 3 | 3.5 (0.0) | 13.7 (7.3) | 82.0 (92.7) | 0.56 |

[a] Compared to total $^{13}N$ added.

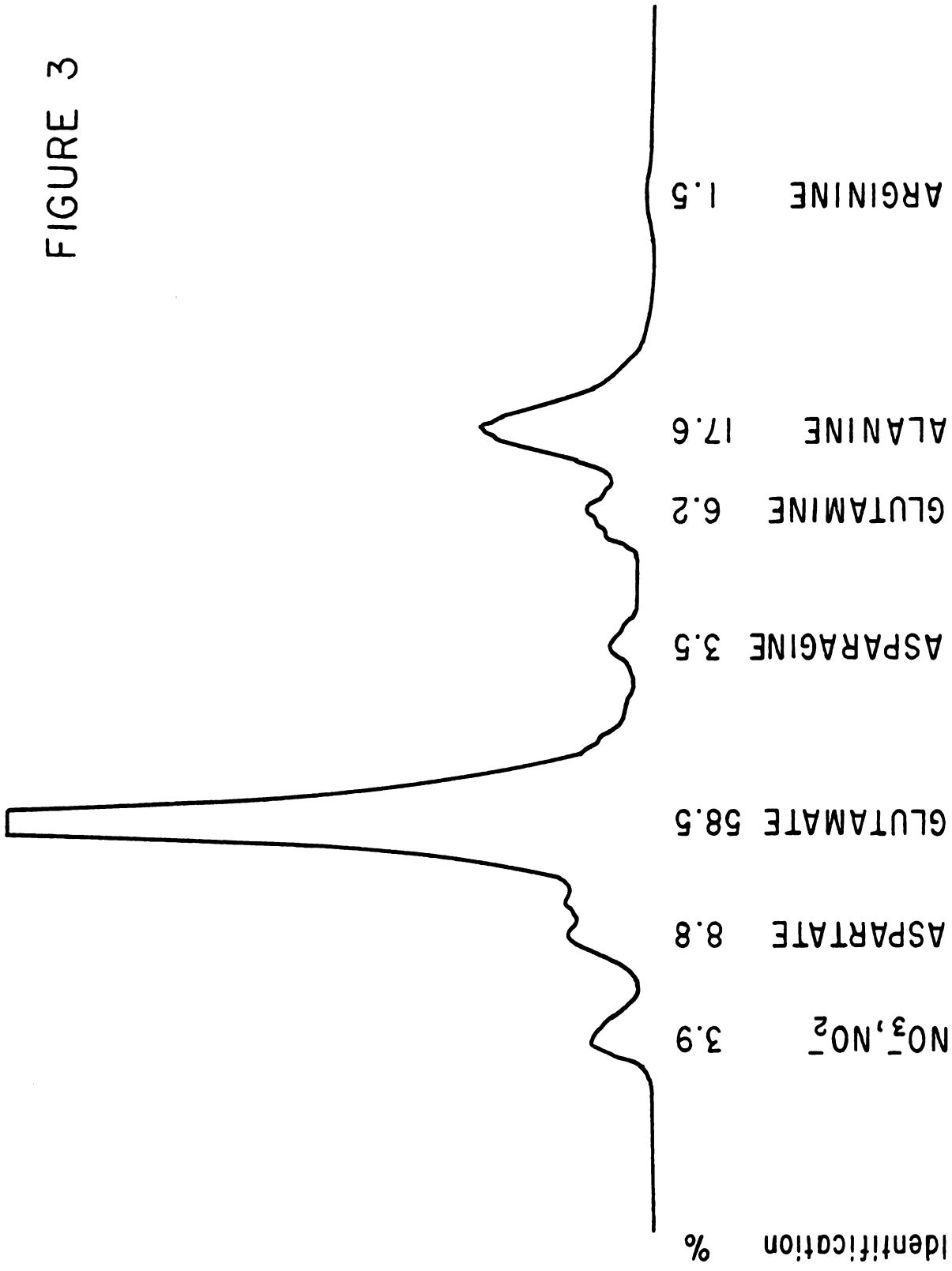
[b] Numbers in parentheses refer to distribution of $^{13}N$ in solution before addition to cell suspensions.

nitrate into its cytoplasm.

The distribution of $^{13}N$ in amino acid pools was examined using high voltage electrophoresis of methanol extracts of the cells. Results are presented in Figure 3 for the distribution after 2.5 min exposure to labeled nitrate. In preliminary experiments designed to examine the short-term sequence of labeling, the distribution of $^{13}N$ in amino acids was the same from 0.5 to 5 min. Those samples were left wet on the filters prior to extraction with methanol, which permitted time for labeled nitrogen to redistribute among the amino acids. Experiments with more rapid extraction procedures revealed that the label in glutamine was 74.3% of that in glutamate after 10 s, but only 35.1% of that in glutamate after 75 s, suggesting that $^{13}NO_3^-$ was incorporated into amino acids through the glutamine synthetase-glutamate synthase pathway after

Figure 3. Distribution of $^{13}N$ after high voltage electrophoresis of P. fluorescens incubated with labeled nitrate.

FIGURE 3


Identification %
$NO_3^-, NO_2^-$ 3.9
ASPARTATE 8.8
GLUTAMATE 58.5
ASPARAGINE 3.5
GLUTAMINE 6.2
ALANINE 17.6
ARGININE 1.5

reduction to ammonia (66).

Energy dependence of $^{13}NO_3^-$ uptake. Cells exposed to labeled nitrate did not take up $^{13}NO_3^-$ when incubated with 1 mM cyanide or azide (Figure 4). These observations provided further evidence that nitrate was taken up by an active transport mechanism. Since the energy for nitrate transport was provided by glucose metabolism in the cell suspensions used, I examined the effect that these inhibitors had on incorporation of $^{14}C$-glucose. Addition of cyanide or azide to a final concentration of 1 mM completely inhibited further glucose uptake. Since glucose is actively transported in Pseudomonas (10,23), these data indicate that the inhibitors interfered with energization of transport.

Nitrite inhibition. I also examined inhibition of label incorporation by nitrite. As shown in Figure 5, addition of two different concentrations of nitrite decreased or stopped label incorporation. Nitrite addition also led to loss of label from the cells, suggesting that there was a nitrite pool with which the added nitrite exchanged. When I examined filtrates of cell suspensions by HPLC the amount of labeled ammonia or amino acids in the filtrate increased after nitrite addition, but there was little change in the amount labeled nitrite. Such a result could be due to dilution of $^{13}N$ specific activity and acceleration of the rate of ammonia formation upon addition of $^{14}NO_2^-$ instead of $^{14}NO_2^-$ exchange with a $^{13}NO_2^-$ internal pool.

Chlorate inhibition. Chlorate, a chemical analog of nitrate, inhibits both the dissimilatory and assimilatory nitrate reductases (43,44,45). Since several experiments indicated nitrate was actively transported into the cells, I examined the specificity of the nitrate carrier by trying to inhibit nitrate uptake with chlorate. Chlorate

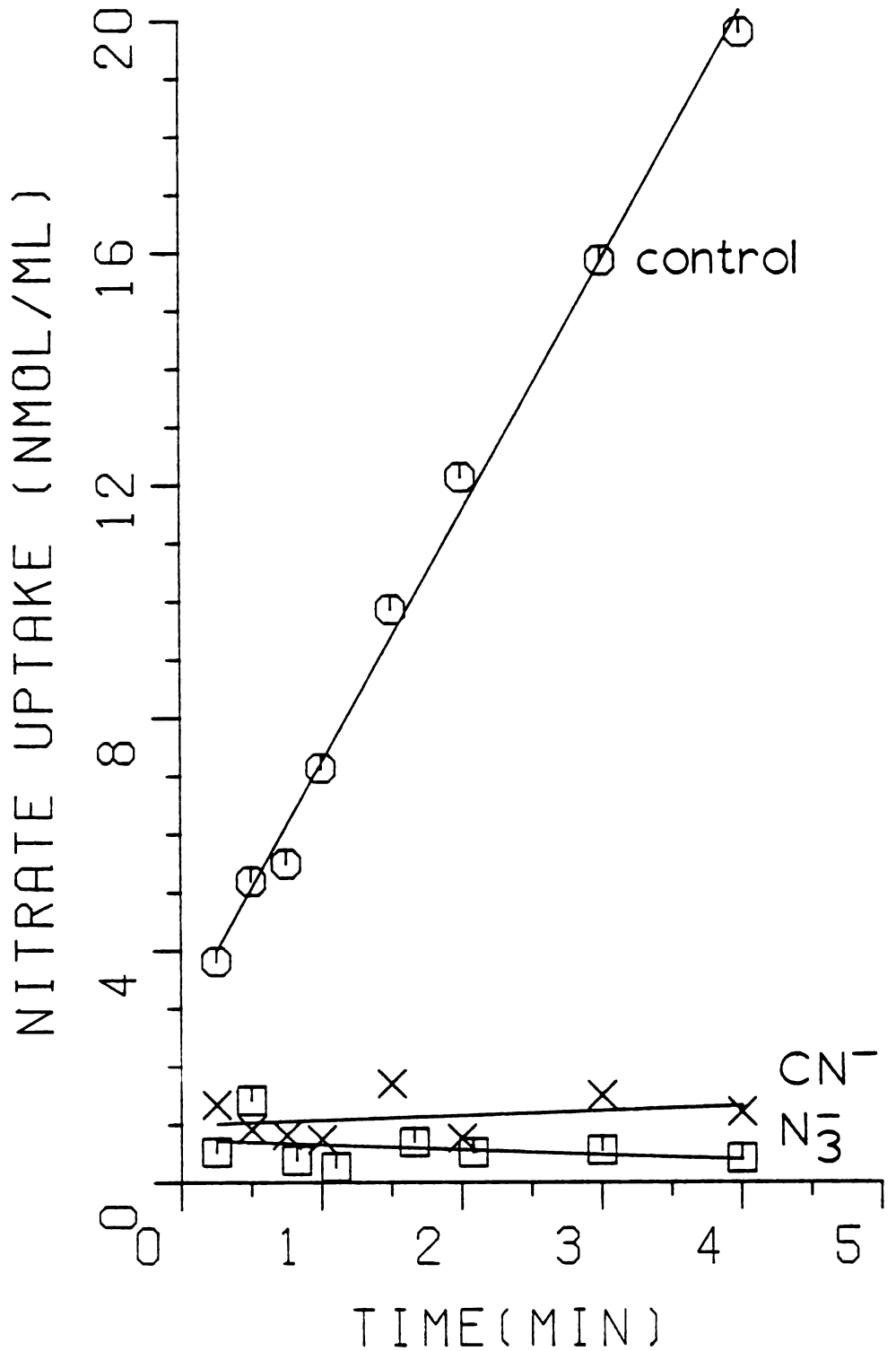



Figure 4. Inhibition of nitrate uptake by 1 mM cyanide and azide.

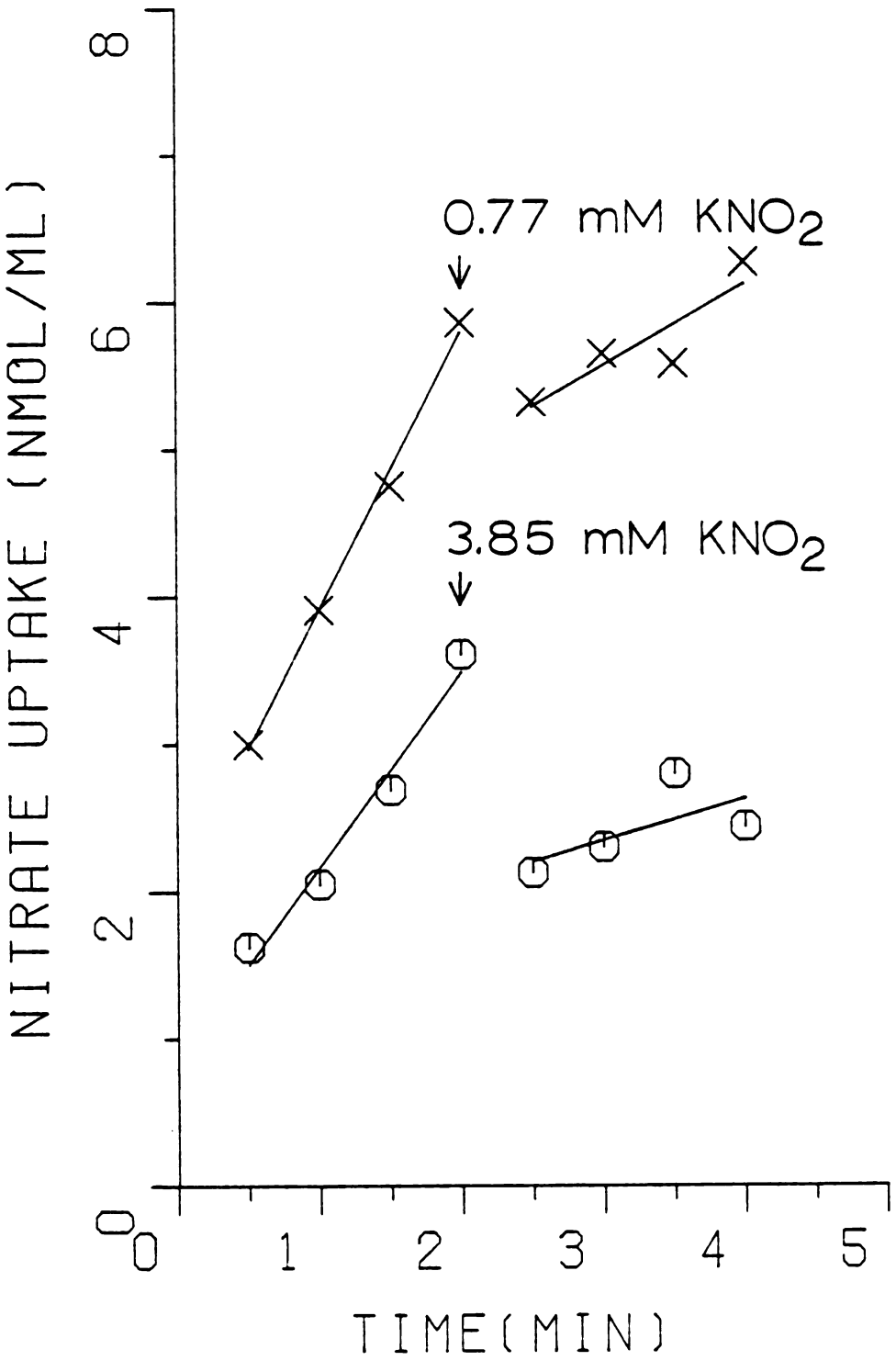



Figure 5. Decrease in rate of nitrate uptake and loss of radioactivity from cells after addition of nitrite.

addition had no effect on the rate of nitrate uptake (Figure 6). Cells grown on nitrite and tungstate also readily took up $^{13}NO_3^-$ when incubated with chlorate (Figure 7). This lack of inhibition in both systems indicates that the nitrate transport system is highly specific for nitrate. Interestingly, in the dithionite-reduced methyl viologen system chlorate also failed to inhibit nitrate reduction (Table 4) in normal or toluenized cells.

Ammonia inhibition. Addition of ammonia to cell suspensions of P. fluorescens grown on nitrate prevented further incorporation of $^{13}NO_3^-$ (Figure 6). Cells incubated with ammonia prior to nitrate addition also did not take up labeled nitrate.

Addition of ammonia may prevent incorporation of $^{13}NO_3^-$ by at least three processes: 1) inhibition of nitrate transport; 2) inhibition of nitrate reduction; and 3) apparent inhibition by dilution of $^{13}NH_4^+$ formed via nitrate reduction due to exchange with a large external ammonia pool. The loss of $^{13}N$ label from cells after ammonia addition (Figure 6) indicated there was a significant internal pool of exchangeable ammonia. Examination of methanol extracts from cells incubated with ammonia (Table 3) suggested that nitrate reduction was inhibited as well, since nitrate was the primary labeled component in the extract. Experiments using unlabeled nitrate and colorimetric procedures confirmed that ammonia addition prevented nitrate reduction.

To distinguish whether the ammonia effect was due to inhibition of nitrate transport or to inhibition of nitrate reduction, I examined the effect of ammonia on nitrate reduction in toluene-treated cells. Ammonia did not inhibit nitrate reduction in such cells (Table 4). Ammonia also did not inhibit nitrate reduction in intact cells assayed

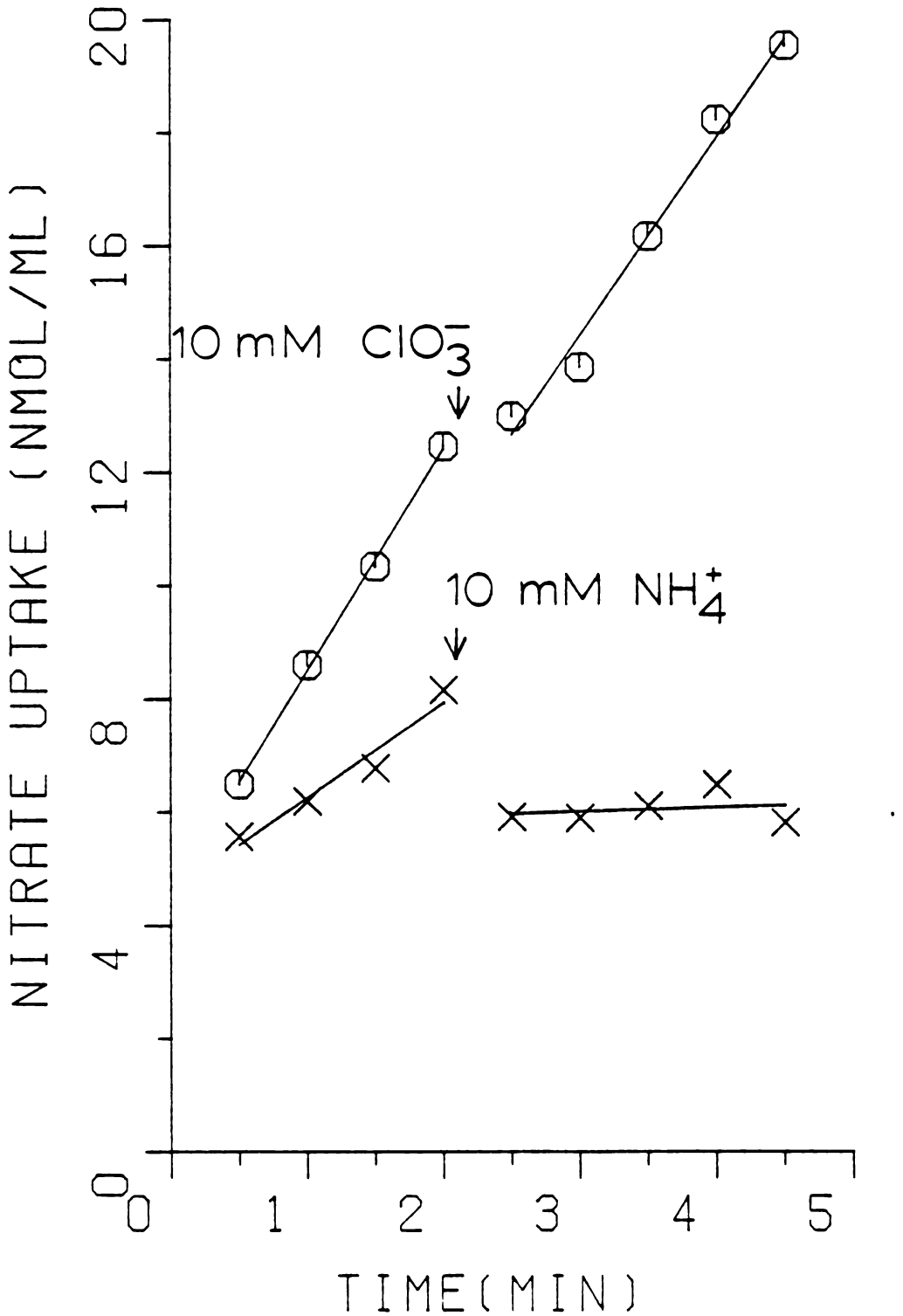



Figure 6. Effects of chlorate and ammonia on incorporation of labeled nitrate.

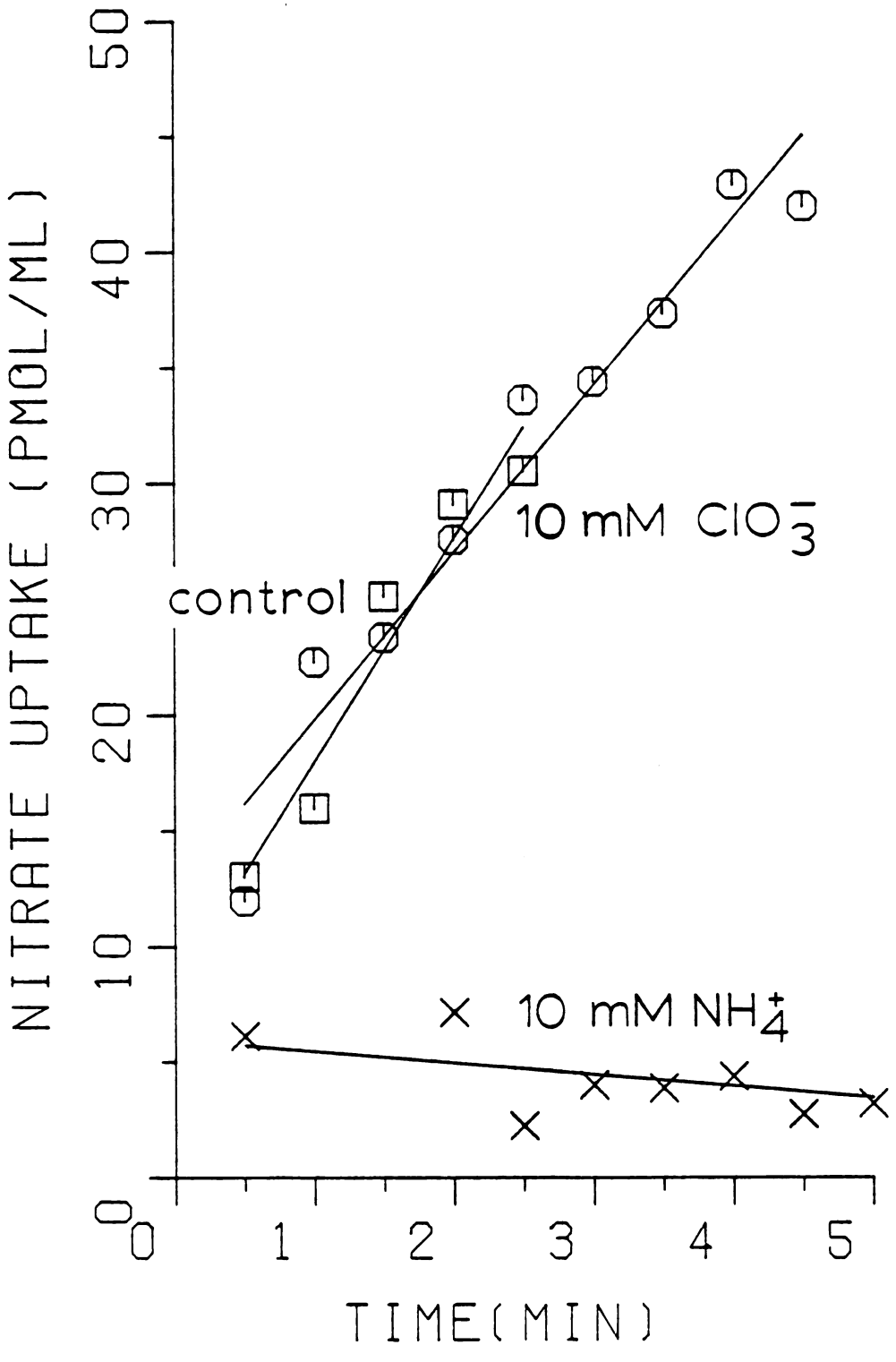



Figure 7. Effects of chlorate and ammonia on nitrate transport in cells grown on nitrite and tungstate.

Table 4. Rate of $NO_3^-$ reduction to $NO_2^-$ in the presence of methyl viologen and various inhibitors.

| Additions | Rate of $NO_2^-$ formation ($\mu M \cdot min^{-1}$) Cells with toluene | Cells without toluene |
|---|---|---|
| None | 33.2 ± 2.9[a] | 8.4 ± 4.1 |
| 10 mM $NH_4^+$ | 26.4 ± 5.7 | 8.0 ± 0.9 |
| 10 mM $ClO_3^-$ | 51.9 ± 11.0 | 5.5 ± 1.6 |

[a] Limits are $t_{0.05}s_b$; 3 degrees of freedom.

with reduced methyl viologen (Table 4). This lack of inhibition may be an artifact of the artificial electron donor system, rather than an indication that ammonia actually inhibited by blocking nitrate transport.

However, if ammonia prevented incorporation of $^{13}NO_3^-$ only by blocking nitrate reduction, addition of ammonia to cells grown on nitrite and tungstate should have had little effect. Incubation of tungstate-grown cells with ammonia completely prevented uptake of $^{13}NO_3^-$ (Figure 7). I concluded from this result and the inability of ammonia to inhibit nitrate reduction in the methyl viologen system that ammonia inhibited nitrate incorporation and reduction in intact cells primarily by inhibiting nitrate transport.

Inhibition by glutamate and glutamine. To determine whether ammonia or an early product of ammonia assimilation was the actual inhibitor, I examined whether glutamate and glutamine affected nitrate assimilation. Glutamine blocked further nitrate uptake; glutamate had no effect (Figure 8). Glutamine gave similar results when nitrate reduction was examined by colorimetric procedures. The ammonia

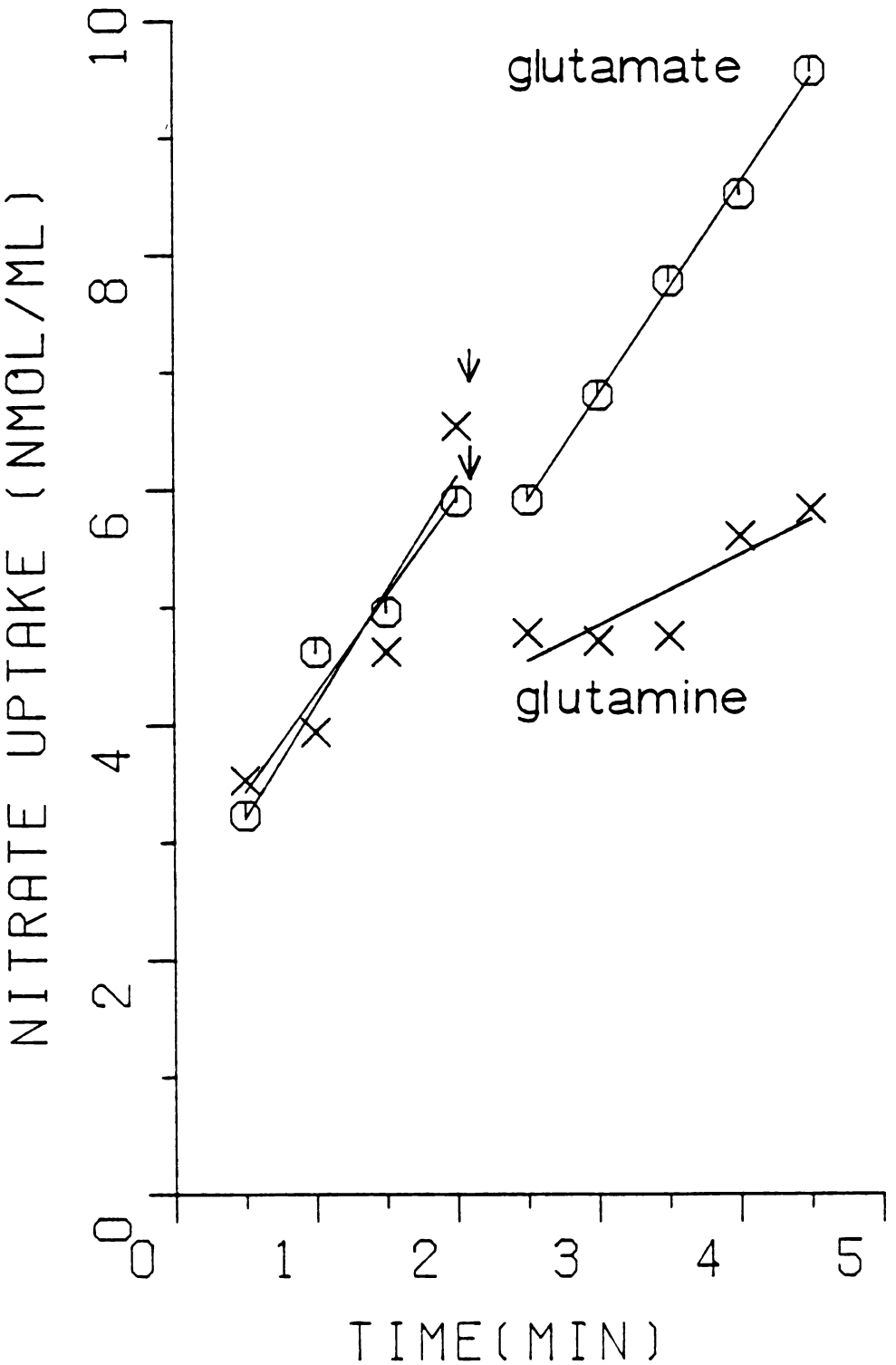



Figure 8. Effects of glutamine and glutamate additions on incorporation of labeled nitrate.

concentration in the filtrates increased from 25 μM to 190 μM due to glutamine addition. Since the glutamine solution was ammonia-free, this increase was probably due to rapid deamination of glutamine.

To rule out the possibility that glutamine but not glutamate was transported into the cells, I examined the uptake of $^{14}$C-glutamine and $^{14}$C-glutamate. The results presented in Figure 9 indicate that both glutamine and glutamate were taken up by cells grown on nitrate. (These results correspond with those of Kay and Gronlund (32), who found that transport of amino acids was constitutive in Pseudomonas aeruginosa.) Thus, lack of glutamate transport cannot explain the failure of glutamate to inhibit $^{13}NO_3^-$ incorporation.

Effect of inhibitors of ammonia assimilation. Short-term labeling experiments had suggested that ammonia was assimilated first into glutamine, then glutamate, in nitrate-grown cells. To verify that this pathway was the one functioning, I intended to examine the distribution of label in amino acids in the presence of the inhibitors methionine sulfoximine and azaserine. The former should block incorporation of ammonia into glutamine; the latter should prevent the transfer of amide nitrogen from glutamine into glutamate (58). Neither should exert a significant effect on ammonia assimilation catalyzed by glutamate dehydrogenase.

Azaserine inhibited incorporation of $^{13}NO_3^-$, but methionine sulfoximine had no effect (Figure 10). W. H. Caskey (Ph.D. Dissertation, Michigan State University, 1978) had demonstrated previously that glutamine synthetase in cell-free extracts of this strain of P. fluorescens was inhibited by methionine sulfoximine, but that cultures could grow in medium containing methionine sulfoximine. Perhaps these cells

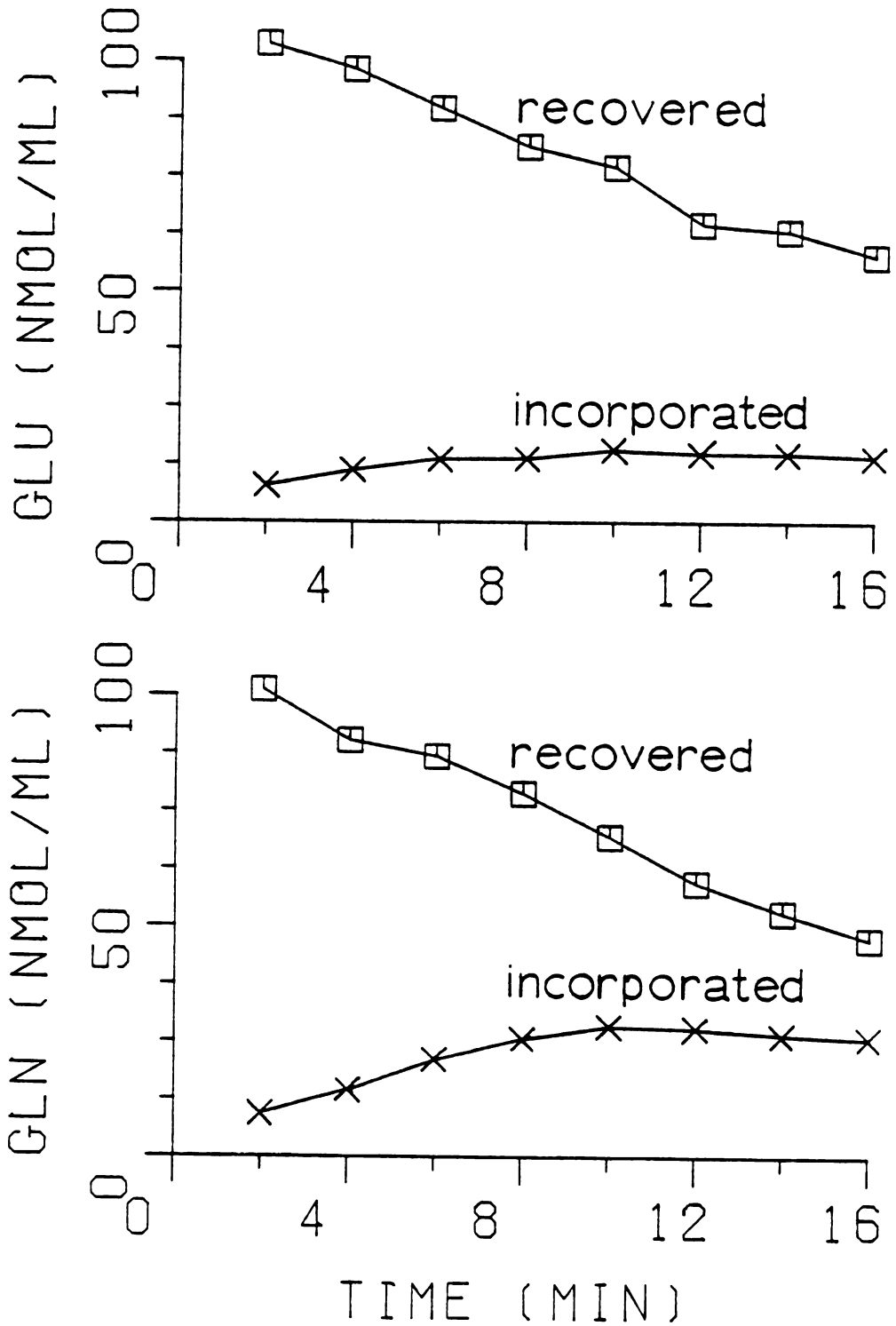



Figure 9. Uptake and respiration of $^{14}C$-labeled glutamine and glutamate by P. fluorescens.

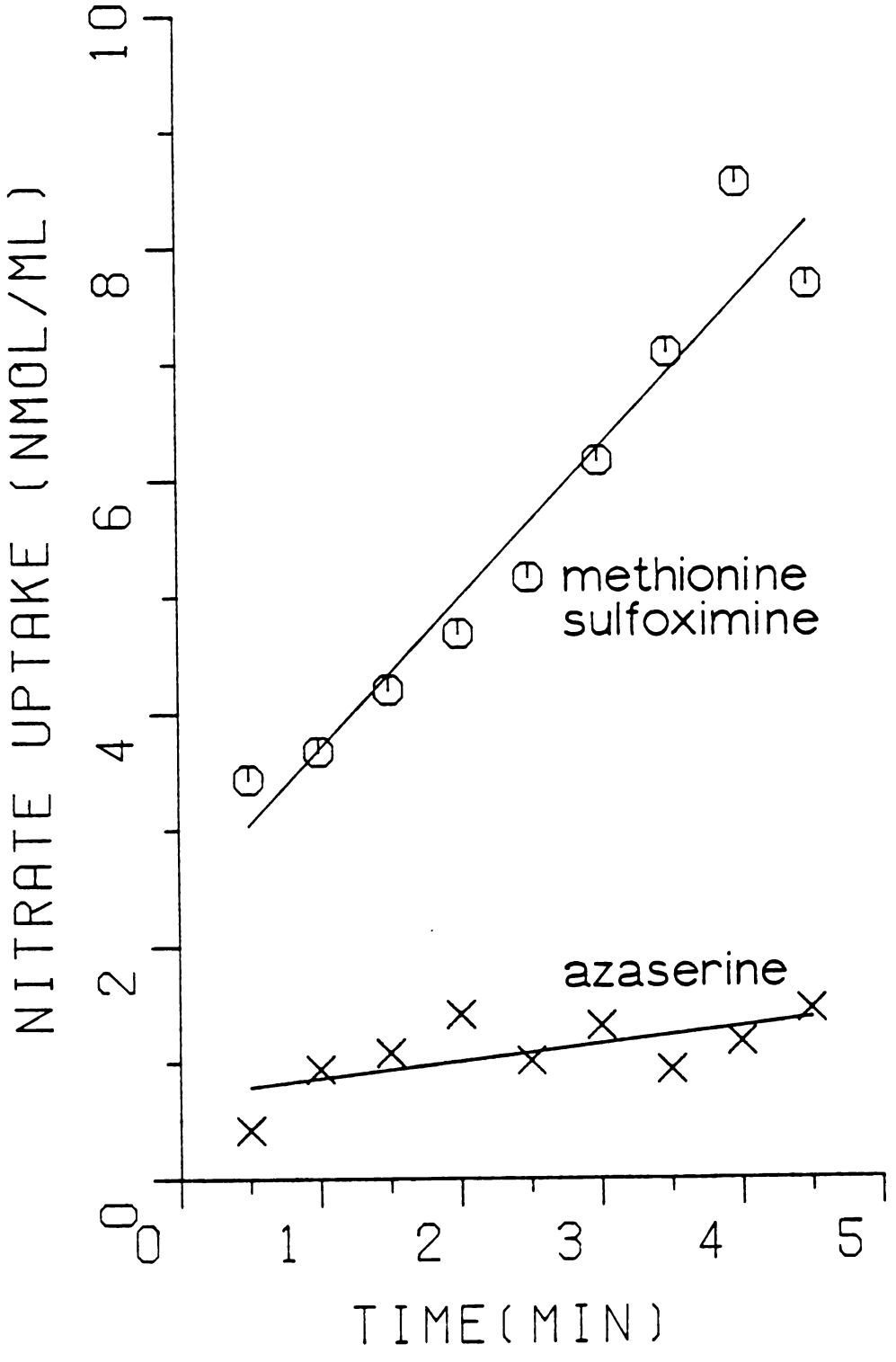



Figure 10. Effects of azaserine and methionine sulfoximine on incorporation of labeled nitrate.

are impermeable to methionine sulfoximine. The alternate explanation, that glutamate was the first product of assimilation of ammonia produced from nitrate, seems to be ruled out by the complete inhibition observed with azaserine. Filtrates of cells incubated with azaserine contained only ammonia or a co-chromatographing substance when examined by HPLC, indicating that azaserine did not inhibit transport and reduction of nitrate through some nonspecific process.

Kinetics of nitrate uptake. I determined the rate of $^{13}NO_3^-$ uptake in nitrate-grown cells at four different nitrate concentrations (Figure 11). Rates calculated by linear regression analysis (59) are listed in Table 5. Regression lines are indicated on the graph. A plot of rates versus initial nitrate concentration, shown in the inset of Figure 11, is that of a rectangular hyperbola expected if uptake followed Michaelis-Menten kinetics. The $K_m$ value calculated from a direct linear plot (Figure 12) was 7.11 μM, with lower and upper bounds for 91.6% confidence limits of 3.21 μM and 12.53 μM. The $V_{max}$ value was 4.356 $\mu M \cdot min^{-1} \cdot mg^{-1}$.

I performed a similar experiment with cells grown on nitrite and tungstate to determine the $K_t$ for transport alone. Rates calculated at various nitrate concentrations are presented in Table 5. The $V_{max}$ obtained, 0.318 $\mu M \cdot min^{-1} \cdot mg^{-1}$, was much lower than that in nitrate-grown cells. The $K_t$, 6.66 μM, (91.6% confidence limit bounds: 1.03 μM and 9.80 μM) was essentially identical to that for nitrate assimilation, indicating that the rate-limiting step in nitrate assimilation was nitrate transport into the cytoplasm.

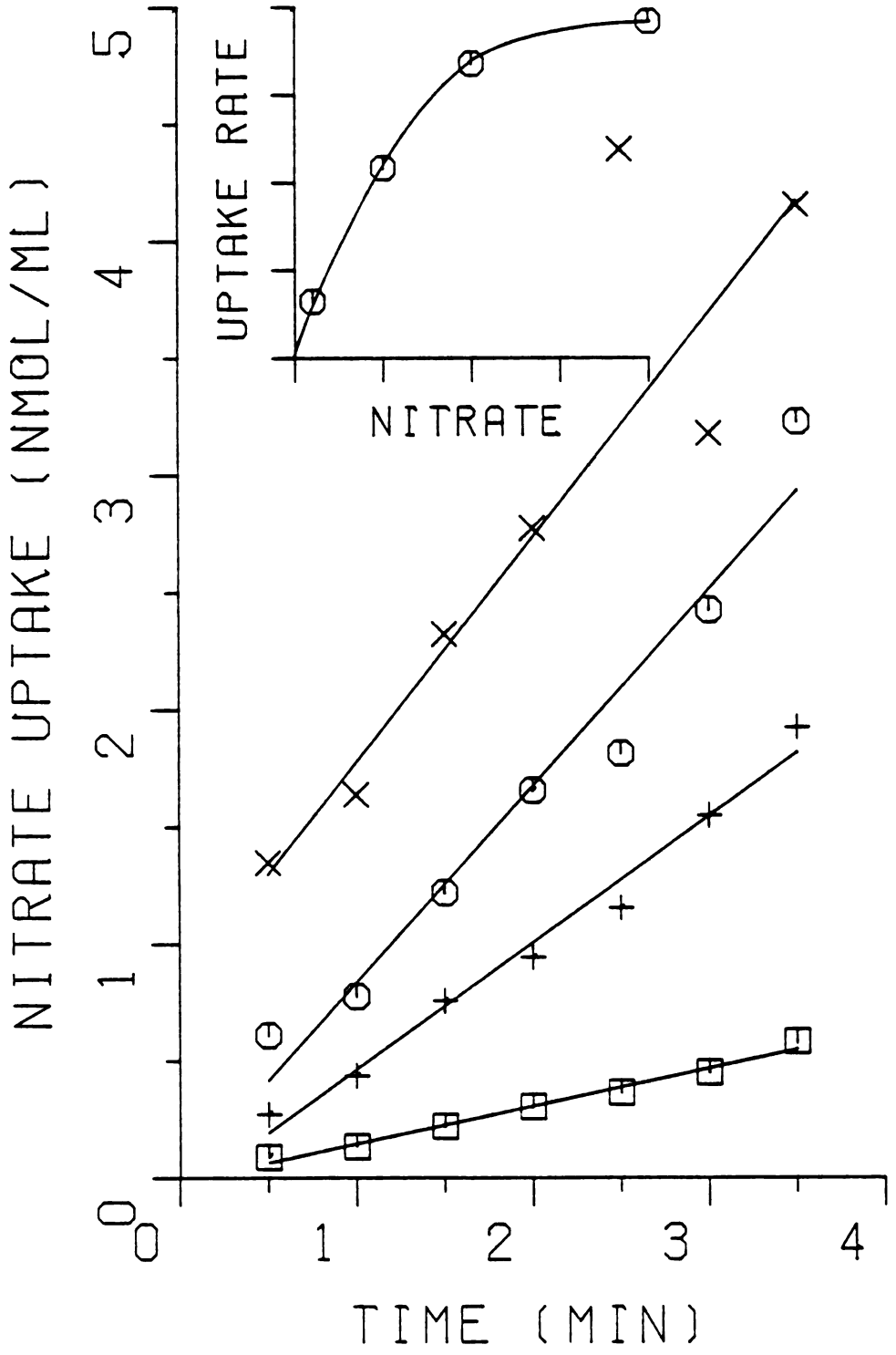



Figure 11. Rate of nitrate uptake at different nitrate concentrations, demonstrating hyperbolic rate response to increasing nitrate concentrations.

Table 5. Rate of nitrate uptake in cells exposed to different nitrate concentrations.

| Initial nitrate concentration (μM) | $NO_3^-$ uptake rate ($\mu M \cdot min^{-1}$) | |
|---|---|---|
| | Cells grown on $NO_3^-$ | Cells grown on $NO_2^-$ and $WO_4^=$ |
| 1 | 0.161 ± 0.021[a] | 0.0125 ± 0.0026 |
| 5 | 0.541 ± 0.084 | 0.0422 ± 0.0043 |
| 10 | 0.839 ± 0.202 | 0.0632 ± 0.0245 |
| 20 | 0.958 ± 0.133 | 0.0662 ± 0.0262 |

[a] Limits are $t_{0.05}s_b$; 5 degrees of freedom (d.f.) for cells grown on $NO_3^-$; 6 d.f. for cells grown on $NO_2^-$ and $WO_4^=$.

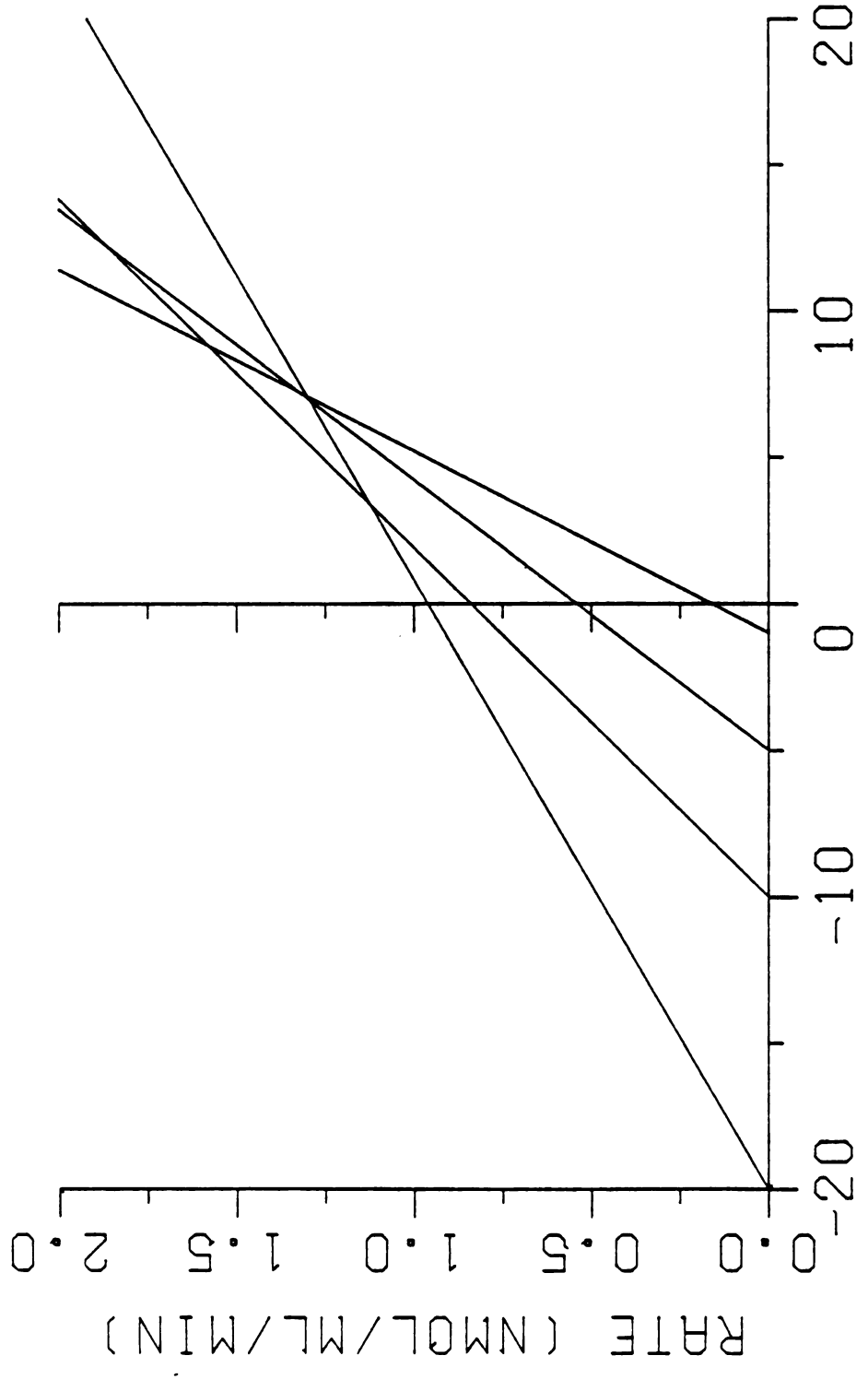



Figure 12. Direct linear plot of rates of nitrate uptake at different substrate concentrations as shown in Figure 11.

# DISCUSSION

Three mechanisms for the eventual incorporation of $^{13}N$ from labeled nitrate can be proposed; only the last requires a specific nitrate carrier protein. 1) Nitrate is reduced externally and nitrite or some more reduced compound is then transported into the cytoplasm. 2) Nitrate is transported into the cytoplasm by nitrate reductase at the same time it is being reduced to nitrite. 3) Nitrate is transported into the cytoplasm as nitrate by a specific carrier.

If the first scheme is correct, then $^{13}NO_3^-$ should never be observed in the cytoplasmic contents. My observation that label was present in nitrate-grown cells only as ammonia or amino acids supports this hypothesis. However, cells grown on nitrite with tungstate also took up $^{13}NO_3^-$; the internal $^{13}N$ was found almost exclusively as nitrate. Thus, reduction to nitrite or a more reduced compound is not required prior to transport into the cytoplasm.

If the second proposed mechanism were operative, an intracellular nitrate pool should not be detectable either. Butz and Jackson (8) proposed such a nitrate transport mechanism for chloroplasts. In their scheme a membrane-bound dimer of nitrate reductase simultaneously reduced nitrate to nitrite and transported it across the membrane. In barley (51) and Neurospora crassa (56) tungstate apparently inhibits nitrate transport as well as nitrate reduction, supporting the proposal that nitrate reductase is also a transport protein. However, if tungstate inactivated the nitrate-reducing activity but not the transport activity of nitrate reductase, then an intracellular pool of nitrate might be observed.

Several observations argue against nitrate transport via nitrate reductase in bacteria. The assimilatory nitrate reductase is generally considered a cytoplasmic (soluble) enzyme (43), although both cytoplasmic and membrane-bound reductases in soil bacteria have been reported (M. S. Schneider, H. L. Gibson, and E. R. Leadbetter, Abstr. Annu. Meet. Am. Soc. Microbiol., 1979, K153, p. 170). If the reductase were membrane-bound and served as a nitrate carrier as well, then one would not expect to find cryptic mutants capable of nitrate reduction only in cell-free extracts. E. A. Beck and J. J. Rowe (Abstr. Annu. Meet. Am. Soc. Microbiol., 1979, I81, p. 108) recently reported characterizing exactly such cryptic mutants in Pseudomonas. Rowe (personal communication) considers them to be permease-deficient. Even in dissimilatory nitrate reduction, where the nitrate reductase does span the membrane (3), the site of nitrate reduction appears to be on the interior of the cytoplasmic membrane (31,34,35), rather than on the exterior as previously proposed (20). This site would be unavailable for nitrate recognition and transport across the membrane. Scheme 2, then, does not appear to be the likely mechanism for nitrate transport.

What evidence is there for transport by a carrier protein, as proposed in the third mechanism? Would not nitrate diffusion be sufficient to account for nitrate detectable in the cytoplasm in the absence of nitrate reduction? Garland et al. (20) found that nitrate diffusion across the plasma membrane was too slow to support the rate of dissimilatory nitrate reduction they observed in Escherichia coli. However, they argued that such evidence supported nitrate reduction on the exterior of the plasma membrane, rather than the existence of a nitrate transport mechanism. Yet if nitrate transport was only a diffusive

process, even a facilitated one, the highest concentration possible internally would be comparable to that of the external medium. My data showing that P. fluorescens could concentrate nitrate at least 100-fold indicate diffusion is not the mechanism of nitrate transport. Similarly, a diffusive mechanism would not demonstrate the energy dependence apparent from my results. My evidence indicates that nitrate is actively transported across the cytoplasmic membrane, which requires the existence of a specific nitrate carrier protein as a component of the nitrate assimilation pathway.

How specific is the carrier protein for nitrate? Chlorate, a nitrate analog, did not inhibit nitrate transport even when present in 100-fold excess of the nitrate concentration. This lack of inhibition may reflect the lack of structural similarity between the two anions--nitrate is a planar molecule while chlorate is pyramidal. If so, then the carrier protein has greater specificity for nitrate than do most nitrate reductases, where chlorate is either a substrate or an inhibitor (43,44,45).

Addition of nitrite did inhibit incorporation of labeled nitrate, but the mechanism of inhibition has not been determined. The nitrite effect may simply indicate trapping of $^{13}NO_2^-$ in the large exogenous pool of nitrite. It may also be attributed to rapid reduction of nitrite to ammonia, which in turn inhibited uptake. Thacker and Syrett (62) proposed that the nitrite inhibition they observed was due to preferential use of nitrite as an electron acceptor. More rapid reduction of nitrite than nitrate would prevent nitrite from reaching toxic concentrations. Still, the nitrite inhibition I observed may be of little significance in natural environment where nitrite concentrations rarely,

if ever, approximate nitrate concentrations. In any case, examination of nitrite inhibition in cells with an inactive nitrate reductase should differentiate between trapping label as nitrite and some other mechanism. It may be necessary to compare the inhibition constants for nitrite and ammonia in order to determine whether the effect is due to ammonia formed by nitrite reduction, though in such cases one might expect the ammonia formed to be detectable also by colorimetric methods.

How does regulation of synthesis of the assimilatory nitrate pathway in P. fluorescens compare with that found in other organsims? I found synthesis of the assimilatory enzymes in P. fluorescens was repressed by growth on excess ammonia. Nitrite as a nitrogen source enhanced synthesis of the pathway in the absence of ammonia. Nitrate-grown cells had the highest nitrate-reducing activity. Brown et al. (7) reported similar results for a marine bacterium, though they did not examine cells grown on nitrite. Riet et al. (52) found that ammonia repressed synthesis of the assimilatory nitrate reductase in Aerobacter (now Enterobacter) aerogenes while nitrate induced its synthesis. In several cyanobacteria (26,60) a similar mechanism of regulation has been observed. Only Pichinoty (46) did not observe repression or inhibition of bacterial nitrate reductase by ammonia; he used Pseudomonas putida.

The pattern of regulation in eucaryotic organisms is generally similar to that which I observed. In algae (17,62), fungi (9,11,21,41, 47, 49,56), and higher plants (12,27) the enzymes are repressed by ammonia. In contrast to the bacterial systems, nitrogen limitation or starvation in eucaryotes does not result in derepression (17,21,27,63). Arabidopsis (12) may be an exception to this generalization.

Nitrite can serve as an inducer instead of nitrate in Neurospora crassa. This induction prompted Coddington (11) to propose that nitrite is actually the true coinducer, not nitrate. He felt that nitrate was only capable of induction after it had been reduced to nitrite by low amounts of a constitutive nitrate reductase. If his hypothesis is correct then mutants incapable of nitrate reduction should not induce for other activities associated with the pathway when exposed to nitrate.

Recently, Premakumar et al. (49) reported that glutamine is the actual repressor in Neurospora. They worked with mutants lacking glutamine synthetase and found that glutamine repressed synthesis of the nitrate reduction system, whereas glutamate and ammonia had only a slight effect. However, Hattori (26) and Stevens and Baalen (60) found that glutamate repressed synthesis as effectively as glutamine in cyanobacteria. Since glutamate and glutamine are early fixation products of ammonia in bacteria (4,66), they may actually be the true effectors in bacterial systems as well.

If ammonia or an early product of ammonia assimilation regulates synthesis of the assimilatory pathway in P. fluorescens, does it also regulate activity of the synthesized enzymes? Results presented here show that inhibition by ammonia was rapid and complete. Furthermore, ammonia inhibited nitrate assimilation by blocking nitrate transport. Ammonia did not inhibit nitrate reduction in toluene-treated cells lacking a permeability barrier to nitrate. Nitrate transport appears to be the site of ammonia inhibition in Penicillium chrysogeneum (21) and N. crassa (56) as well. In other organisms (17,37,53,60,62) ammonia or amino acids have no effect on transport but instead block nitrate reductase activity. In still another study with N. crassa (49), ammonia

had no inhibitory effect at all though the nitrate reductase activity decayed due to prevention of further synthesis. Based on these few research results, some organisms apparently control nitrate assimilation at the levels of enzyme synthesis and of activity, while for others repression of synthesis alone is sufficient.

Is ammonia the true inhibitor of nitrate transport in P. fluorescens, or is some other product of ammonia assimilation? Glutamate certainly is not such an inhibitor, as my results indicate. Glutamine may be an inhibitor, although the relatively high amounts of ammonia detectable after glutamine addition serve only to confuse the issue. Glutamine inhibition might be expected since the sequence of amino acid labeling observed suggests it is the first organic product of nitrate assimilation. Brown et al. (5) previously examined glutamine synthetase (GS), glutamate dehydrogenase (GDH), and glutamate synthase (GOGAT) activities in P. fluorescens and other bacteria grown on nitrate or limiting ammonia. Under those conditions GS and GOGAT activities were high, but GDH was barely detectable. Hence glutamine would be the first organic nitrogen product formed. While glutamine appears to be the corepressor in N. crassa, it does not inhibit nitrate reduction in that organism. However, amino acids do inhibit nitrate reduction in other organisms (21,27,41,56).

Assuming that glutamine is the true inhibitory substance, how do azaserine and methionine sulfoximine exert their effects on nitrate uptake? Inhibition of glutamine synthetase by methionine sulfoximine, which Caskey (Ph.D. dissertation, Michigan State University, 1978) previously demonstrated in extracts of P. fluorescens, should prevent formation of glutamine and would cause the concentration of ammonia to

build up in the cytoplasm. Unless some other reasonably efficient mechanism for ammonia assimilation exists in cells grown on nitrate, the labeled ammonia should rapidly diffuse out of the cells. Little uptake of label would be expected. Since Caskey found growth of cultures was not inhibited by inclusion of methionine sulfoximine in the medium, the cells may be impermeable to methionine sulfoximine. Unfortunately, any attempt to make the cells permeable to methionine sulfoximine would also make them permeable to nitrate and other small molecules. It would be impossible with such cells to test whether methionine sulfoximine itself would have the expected effect.

Azaserine included in the preincubation mixture caused complete inhibition of $^{13}N$ incorporation from labeled nitrate. Azaserine should cause glutamine and ammonia to accumulate in the cytoplasm, where they would quickly equilibrate with the external medium. The accumulation of glutamine would be expected to block further nitrate transport. It is possible, too, that such inhibition could be caused by azaserine itself, since it is a glutamine analog. However, since I found ammonia or a co-chromatographing compound in the filtrate from cells incubated with azaserine, azaserine apparently did not inhibit either nitrate transport or nitrate reduction to ammonia. Resolution of the mechanisms of azaserine and methionine sulfoximine action will require further work, preferably with organisms blocked in nitrate reduction so that the effect on transport can be examined directly.

Why doesn't chlorate inhibit nitrate reduction in P. fluorescens? John (31) and Kristjansson and Hollocher (34) found that the cell membrane was impermeable to chlorate, but chlorate reduction proceeded rapidly in spheroplasts and cell extracts from organisms capable of

dissimilatory nitrate reduction. This reduction of chlorate by nitrate reductase is the criterion for Pichinoty's reductase type A (43,45). In assimilatory nitrate reduction, a reductase B is supposed to be involved. Type B reductase is inhibited by chlorate. Perhaps chlorate could not enter the cytoplasm of P. fluorescens, since it did not affect nitrate transport in tungstate-grown cells. However, nitrate reduction in toluene-treated cells--cells now permeable to chlorate--was not inhibited by chlorate either. A recent study (M. S. Schneider, H. L. Gibson, and E. R. Leadbetter, Abstr. Annu. Meet. Am. Soc. Microbiol., 1979, K153, p. 170) found similar lack of inhibition of assimilatory reductases from some other soil bacteria, and questioned the validity and utility of Pichinoty's classification scheme for nitrate reductases. The lack of inhibition I observed supports their argument.

What are the implications of the mechanism of assimilatory nitrate reduction reported here for P. fluorescens? The ability to grow with nitrate as a sole nitrogen source is widespread among eucaryotic organisms, but has been observed in relatively few bacterial species (43,45). Brown, however, regards the ability to reduce nitrate to be common among marine bacteria (4). Since most soil organisms apparently cannot use nitrate P. fluorescens should have a competitive advantage in soils. Nitrate assimilation in this organism is apparently controlled at the level of nitrate transport. The $K_m$ for transport, 6.6 μM, is essentially the same as that for nitrate assimilation into organic products, 7.0 μM. The inhibition of nitrate transport by ammonia has the advantage that no energy is expended to transport what cannot be reduced. There is no evidence to suggest this organism sequesters nitrate internally for later reduction, the way some algae (65) and bacteria (1) accumulate

phosphate in a storage form.

Few other kinetic values for assimilatory nitrate reduction have been reported for common soil bacteria. The purified reductase from Azotobacter chroococcum has a $K_m$ for nitrate of 0.25 mM (22). Brown et al. found a value of 0.26 mM in marine pseudomonad, 0.29 mM in the cell-free system. Values for nitrate assimilation in fungi are also scarce. Schloemer and Garret (56) found a transport constant of 0.25 mM in N. crassa (0.3 mM in cells grown on vanadate instead of molybdate). However, Goldsmith et al. (21) considered the $K_m$ in Penicillium to be less than 10 μM. They based their conclusion on the observation of similar rates at 0.05 mM and 1 mM, suggesting saturation of the assimilatory enzymes even at the lower concentration. Since 0.05 mM was the limit of nitrate detection in their system, they could not extend their observations into a more useful range.

Investigators working with higher plants have found a similar wide range for transport and assimilation of nitrate, varying from 0.022 mM to 0.4 mM (27,30,37,50). This is also true of algal species where values ranged from 0.1 to 5 μM (16,18) to 0.67 mM in Chlamydomonas (40). Eppley et al. (18) attempted to correlate the values they observed in chemostat cultures with the competitive ability of the species examined. The $K_s$ values for growth correlated quite well with the distribution of species observed in marine samples, particularly when other factors such as response to light intensity were taken into account.

I am tempted to promote a similar argument here. P. fluorescens should have a competitive advantage in soils because of its low $K_t$ for nitrate assimilation. This advantage may be less marked than commonly

believed, especially if the lower estimates reported for plant nitrate uptake are typical of most plants. However, this study has not been designed to determine what advantage such a low kinetic value and regulatory pattern confer on P. fluorescens. A much more detailed comparison of nitrate uptake and growth in different soil bacteria is required, preferably using chemostat cultures in a procedure similar to that of Eppley's group (18).

Of course, knowledge of the response in culture does not guarantee a knowledge of the response of the bacteria in soil. Ammonia availability in soil is dependent on many factors, such as cation exchange capacity. Ammonia presence may result in almost continual repression of assimilatory nitrate reduction. The mobility of nitrate in the soil solution would affect its availability both to plants and to micro-organisms. Rates of organic matter mineralization, resulting in ammonia release, and rates of oxidation of ammonia to nitrate all may regulate nitrate assimilation. Such considerations already offer sufficient complexity to prevent generalizing from a survey of nitrate uptake kinetics to competitive ability in natural environments, yet do not begin to take into account other factors affecting microbial growth and competition in soils.

## FUTURE RESEARCH

The ability to generate $^{13}N$ at the MSU Cyclotron Laboratory has permitted an examination of nitrate transport and assimilation in bacteria possible only with great difficulty by other techniques. The sensitivity provided by the high activity of such a short-lived isotope makes it relatively easy to measure very low activity, such as transport

in tungstate-treated cells, without the requirement for high cell volumes necessitated by other nitrate assays. Following the redistribution of labeled nitrogen in various inorganic and organic compounds using $^{15}N$ might be possible, but would entail large amounts of cells and possibly nonphysiological concentrations of $^{15}N$-containing material to obtain sufficient label for analysis. The analysis itself would require access to a mass spectrometer after separation of the components of interest. Use of $^{13}N$ makes possible techniques similar to those used with $^{14}C$, but at much lower concentrations than with that tracer. The major limitations to research with $^{13}N$ include access to facilities to generate the isotope (though van de Graaf accelerators of sufficient energy may be as plentiful as high resolution ratio mass spectrometers). The development of relatively rapid techniques for preparing the $^{13}N$ compounds of interest and for separating and detecting the products is also necessary, since even a few extra minutes required represents a significant loss of radioactivity. Yet the ability to generate $^{13}N$ is not essential for much of the research needed to verify and extend the observations reported here.

The genetics of nitrate assimilation has been studied in reasonable detail in fungi (11,49,56) and algae (40), but little has been done with bacterial systems. Clearly the nature of regulation of the various compounds of the nitrate assimilation pathway is an area open to such genetic research. The possible involvement of glutamine synthetase in a regulatory function similar to that found for control of nitrogen assimilation in enteric organisms (66) would be one area of possible research. The development of a well-characterized set of mutants with which to examine in isolation the response of different components of

the assimilatory pathway to various inducers and inhibitors is also desirable. Of course, genetic linkage analysis would be invaluable in establishing that there is indeed a separate nitrate transport protein in P. fluorescens. However, P. fluorescens may not be the organism of choice for such research, since its genetic system has not been studied extensively. P. aeruginosa, a closely related species, offers a more thoroughly characterized system but has the disadvantage shared with P. fluorescens of being able to denitrify under appropriate conditions. (The procedures for aerobic growth used in this study did not result in synthesis of dissimilatory nitrate reductase and the other enzymes of denitrification.) P. putida also has a well-characterized genetic system but is incapable of denitrification; it may be the organism of choice. However, Pichinoty's observation that ammonia neither inhibits nor represses nitrate assimilation in P. putida (46) would need to be examined in detail.

The results presented indicate that nitrate transport is inhibited by general respiratory poisons such as cyanide and azide. According to the chemiosomotic hypothesis nitrate transport should occur via a proton symport mechanism (33). The alkaline transients reported by Hollocher's group during their studies of denitrification and nitrate respiration (30,31) support such a mechanism. Measurement of pH transients during assimilatory nitrate transport or the use of selected ionophores such as carbonyl cyanide, m-chlorophenyl hydrazone (CCCP) should be able to resolve the mechanism of energy linkage to transport. Hollocher's group did find CCCP inhibition of nitrate transport and reduction during denitrification in Paracoccus denitrificans and also in Pseudomonas denitrificans, though in the latter case the concentration used did not inhibit completely (35).

Another approach to investigate energy linkage to nitrate transport and to demonstrate the function of a nitrate carrier protein would be to examine transport in membrane vesicles. I tried to obtain such vesicles during this research, but was unable to get gentle lysis and release of the wall components. Since procedures for lysis of Pseudomonas aeruginosa have been worked out (23), such research should be possible. Of course, there is a wealth of literature on transport in membrane vesicles, so such research should be able to draw on well-established techniques.

Another possible line of research would be to attempt to purify the carrier protein and to examine the mechanism of nitrate binding to the protein. Determination of the metal cofactors, if any, involved in the selective recognition of nitrate would be most interesting. This is especially true since molybdate seems not to be required for nitrate transport in P. fluorescens, as reported here, and in other organisms (27,49,51,56). Molybdate has been identified in all the nitrate reductases examined (69), but its role there may be as an electron carrier rather than a component of the nitrate recognition center.

The physiological and genetic studies outlined above should elucidate the nature of the nitrate transport system and its regulation in P. fluorescens. They do not address the ecological implications of such a system. As stated above, the contribution an assimilatory nitrate uptake system of high affinity makes to the competitive ability of this common soil organism is not easy to assess. Certainly a more comprehensive survey of the ability of soil bacteria to utilize nitrate as a nitrogen source should be undertaken. A characterization of the kinetics of growth under nitrate limitation, in a manner similar to Eppley's study (18), would provide a first approximation of the role nitrate

assimilation may play in competition among soil bacteria.

A more direct approach to the advantage nitrate assimilation may confer on P. fluorescens would be to make use of antibiotic resistant mutants as marked populations. Such a procedure has been used previously to monitor survival of a plant pathogen (68) and also to investigate growth of denitrifiers under simulated field conditions (M. S. Smith, and J. M. Tiedje, Agron. Abstr., 1979, pp. 163-164).

I envision an approach somewhat similar to those used previously. Antibiotic-resistant parent strains and mutants unable to assimilate nitrate would be inoculated into a model soil system and their numbers monitored under simulated field conditions. Comparison of plate counts on antibiotic-containing media with either nitrate or ammonia as a nitrogen source should make enumeration relatively easy. If nitrate assimilation does give P. fluorescens a competitive advantage in soil, the organisms with a functional nitrate assimilation system should become more numerous than the mutants unable to utilize nitrate. Such an experiment does not address the broader question of competitive advantage with respect to other species, but that itself is complicated by other physiological differences, such as the capability to use a wide range of carbon sources. By limiting the comparison to organisms derived from the same parent strain and subjected to the same conditions--including the demand that the organisms be able to establish themselves when inoculated into a preexisting community--I should be able to determine whether it is likely nitrate assimilation can play a competitive advantage at all. Of course, this advantage may be apparent only in certain environments: if ammonia were always available in excess there would be no chance to demonstrate an advantage for organisms

capable of assimilatory nitrate reduction.

## SUMMARY

This is the first report of a specific nitrate transport system in bacteria. A model summarizing the results of this study is presented in Figure 13. There are five essential components of this model. First, there is a nitrate carrier protein which can actively transport nitrate against a concentration gradient. Second, ammonia inhibits nitrate transport. Third, chlorate, an analog of nitrate, has no effect either on its transport or reduction. Fourth, ammonia present in non-limiting concentrations represses synthesis of the enzymes for nitrate assimilation. Fifth, nitrate and nitrite appear to be positive effectors on synthesis of the pathway. It is not yet clear whether ammonia or an organic product of assimilation, such as glutamine, is the actual corepressor and inhibitor of the pathway. Nor has the role of nitrite as a possible inhibitor of nitrate uptake been determined. Much research still must be done before we have a detailed understanding of the regulation of the pathway, the mechanism of energy coupling to transport, and the role nitrate assimilation plays in the ecology of P. fluorescens.

Figure 13. Model of regulation of synthesis and activity of the nitrate assimilation pathway in P. fluorescens.

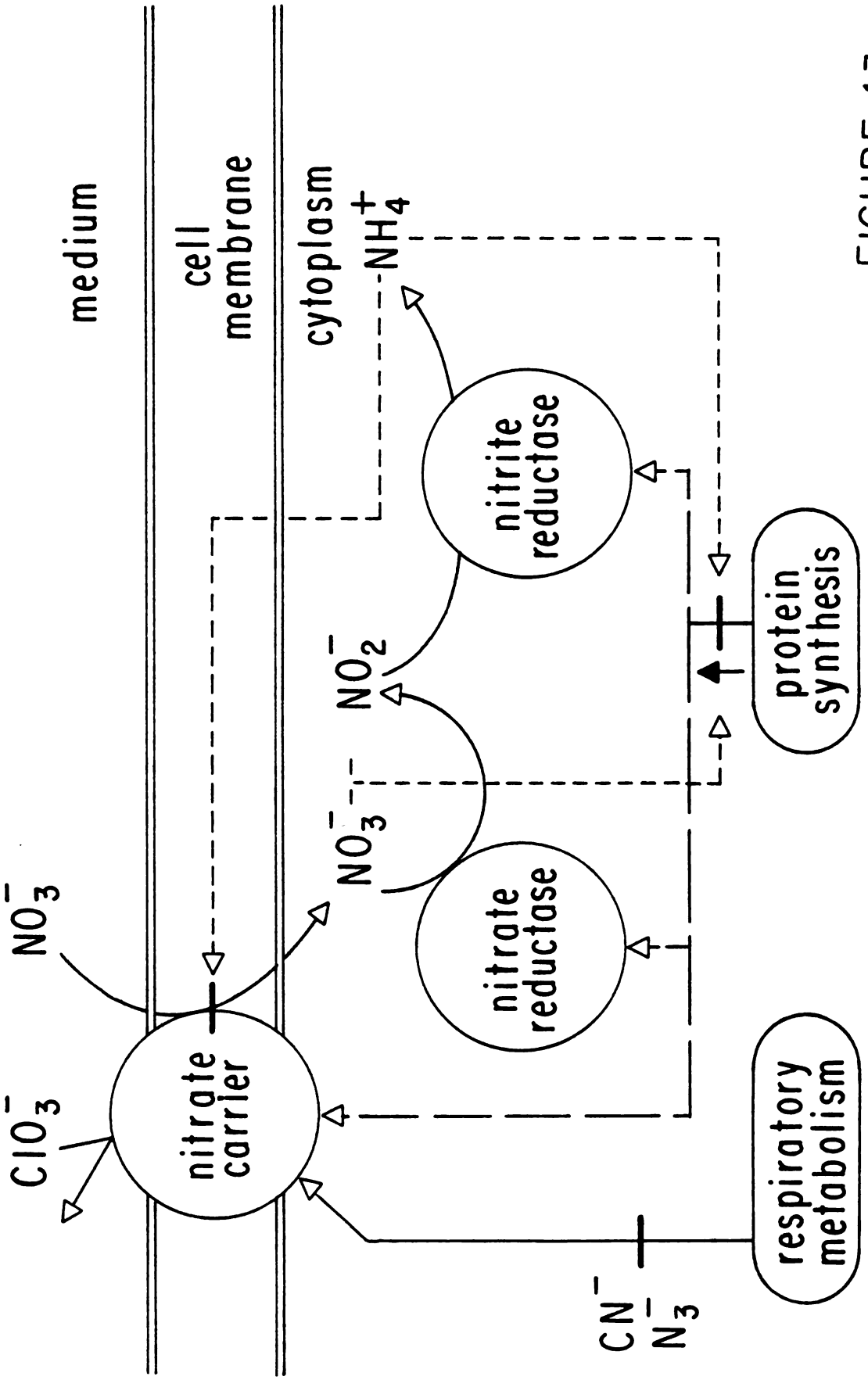

medium
cell membrane
cytoplasm
$NH_4^+$
$NO_3^-$
$ClO_3^-$
nitrate carrier
nitrite reductase
nitrate reductase
$NO_2^-$
$NO_3^-$
protein synthesis
respiratory metabolism
$CN^-$
$N_3^-$

FIGURE 13

## LITERATURE CITED


1. Alfasi, H., D. Friedberg, and I. Friedberg. 1978. Phosphate transport in arsenate resistant mutants of Micrococcus lysodeikticus. J. Bacteriol. 137: 69-72.

2. Black, C. A. 1965. Methods of Soil Analysis. Part 2, Chemical and Microbiological Methods. American Society of Agronomy, Inc., Madison, WI.

3. Boxer, D. H., and R. A. Clegg. 1975. A transmembrane location for the proton-translocating reduced ubiquinone nitrate reductase segment of the respiratory chain of Escherichia coli. FEBS Letters 60: 54-57.

4. Brown, C. M., and B. Johnson. 1977. Inorganic nitrogen assimilation in aquatic microorganisms. Adv. Aquatic Microbiol. 1: 49-114.

5. Brown, C. M., D. S. Macdonald-Brown, and S. O. Stanley. 1972. Inorganic nitrogen metabolism in marine bacteria: nitrogen assimilation in some marine pseudomonads. J. Mar. Biol. Ass. U. K. 52: 793-804.

6. Brown, C. M., D. S. Macdonald-Brown, and S. O. Stanley. 1973. The mechanisms of nitrogen assimilation in Pseudomonads. Antonie van Leeuwenhoek. 39: 89-98.

7. Brown, C. M., D. S. Macdonald-Brown, and S. O. Stanley. 1975. Inorganic nitrogen metabolism in marine bacteria: nitrate uptake and reduction in a marine pseudomonad. Marine Biol. 31: 7-13.

8. Butz, R. G., and W. A. Jackson. 1977. A mechanism for nitrate transport and reduction. Phytochemistry. 16: 409-417.

9. Choudary, V. and G. R. Rao. 1976. Regulatory properties of yeast nitrate reductase in situ. Can. J. Microbiol. 22: 35-42.

10. Clarke, P. H. and L. N. Ornston. 1975. Metabolic pathways and regulation. In P. H. Clarke and M. H. Richmond, (eds.) Genetics and Biochemistry of Pseudomonas, Vol. 1. p. 191-261, John Wiley and Sons, New York.

11. Coddington, A. 1976. Biochemical studies on the Nit mutants of Neurospora crassa. Molec. Gen. Genet. 145: 195-206.

12. Doddema, H., J. J. Hofstra, and W. J. Feenstra. 1978. Uptake of nitrate by mutants of Arabidopsis thaliana, disturbed in uptake or reduction of nitrate. I. Effect of nitrogen source during growth on uptake of nitrate and chlorate. Physiol. Plant. 43: 343-350.

13. Doddema, H. and H. Otten,. 1979. Uptake of nitrate by mutants of Arabidopsis thaliana, disturbed in uptake or reduction of nitrate. III. Regulation. Physiol. Plant. 45: 339-346.

14. Doddema, H. and G. P. Telkamp. 1979. Uptake of nitrate by mutants of Arabidopsis thaliana, distrubed in uptake or reduction of nitrate. II. Kinetics. Physiol. Plant. 45: 322-338.

15. Eisenthal, R., and A. Cornish-Bowden. 1974. The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters. Biochem. J. 139: 715-720.

16. Eppley, R. W., and J. L. Coatsworth. 1968. Uptake of nitrate and nitrite by Ditylum brightwellii--kinetics and mechanisms. J. Phycol. 4: 151-156.

17. Eppley, R. W., J. L. Coatsworth, and L. Solorzano. 1969. Studies of nitrate reductase in marine phytoplankton. Limnol. Oceanog. 14: 194-205.

18. Eppley, R. W., J. N. Rogers, and J. J. McCarthy. 1969. Half-saturation constants for uptake of nitrate and ammonium by marine phyto-plankton. Limnol. Oceanogr. 14: 912-920.

19. Gamble, T. N., M. R. Betlach, and J. M. Tiedje. 1978. Numerically dominant denitrifying bacteria from world soils. Appl. Environ. Microbiol. 33: 926-939.

20. Garland, P. B., J. A. Downie, and B. A. Haddock. 1975. Proton translocation and the respiratory nitrate reductase of Escherichia coli. Biochem. J. 152: 547-559.

21. Goldsmith, J., J. P. Livoni, C. L. Norberg, and I. H. Segel. 1973. Regulation of nitrate uptake in Penicillium chrysogenum by ammonium ion. Plant Physiol. 52: 362-367.

22. Guerrero, M. G., J. M. Vega, E. Leadbetter, and M. Losada. 1973. Preparation and characterization of a soluble nitrate reductase from Azotobacter chroococcum. Arch. Mikrobiol. 91: 287-304.

23. Guyman, L. F., and R. G. Eagon. 1974. Transport of glucose, gluconate, and methyl α-D-glucoside by Pseudomonas aeruginosa. J. Bacteriol. 117: 1261-1269.

24. Hamilton, W. A.. 1973. Energy coupling in microbial transport, Adv. Microbial Physiol. 13: 1-53.

25. Hattori, A. 1962. Light-induced reduction of nitrate, nitrite and hydroxylamine in a blue green algae Anabaena cylindrica. Plant Cell Physiol. 3: 355-369.

26. Hattori, A. 1962. Adaptive formation of nitrate reducing system in Anabaena cylindrica. Plant Cell Physiol. 3: 371-377.

27. Heimer, Y. M., and P. Filner. 1971. Regulation of the nitrate assimilation pathway in cultured tobacco cells. III. Nitrate uptake system. Biochem. Biophys. Acta. 230: 362-372.

28. Heimer, Y. M., J. L. Wray, and P. Filner. 1969. The effect of tungstate on nitrate assimilation in higher plant tissues. Plant Physiol. 44: 1197-1199.

29. Hinshelwood, C. N. 1946. The Chemical Kinetics of the Bacterial Cell. Clardendon. Oxford.

30. Honert, T. H. van den, and J. J. M. Hooymans. 1955. On the absorption of nitrate by maize in water culture. Acta Bot. Neerl. 4: 376-384.

31. John, P. 1977. Aerobic and anaerobic bacterial respiration monitored by electrodes. J. Gen. Microbiol. 98: 231-238.

32. Kay, W. W., and A. F. Gronlund. 1969. Amino acid transport in Pseudomonas aeruginosa. J. Bacteriol. 97: 273-281.

33. Kepes, A., and G. N. Cohen. 1962. Permeation, in The Bacteria Vol. 4: The Physiology of Growth. J. C. Gunsalus and R. Y. Stanier (eds.), Academic Press. New York.

34. Kristjansson, J. K., and T. C. Hollocher. 1979. Substrate binding site for nitrate reductase of Escherichia coli is on the inner aspect of the membrane. J. Bacteriol. 137: 1227-1233.

35. Kristjansson, J. K., B. Walter, and T. C. Hollocher. 1978. Respiration-dependent proton translocation and the transport of nitrate and nitrite in Paracoccus denitrificans and other denitrifying bacteria. Biochemistry. 17: 5014-5019.

36. Lowe, R. H., and H. J. Evans. 1964. Preparation and some properties of a soluble nitrate reductase from Rhizobium japonicum. Biochim. Biophys. Acta. 85: 377-389.

37. Lycklama, J. C. 1963. The absorption of ammonium and nitrate by perennial rye-grass. Acta Bot. Neerl. 12: 361-423.

38. Morris, I., and P. J. Syrett. 1963. The development of nitrate reductase in Chlorella and its repression by ammonium. Arch. Mikrobiol. 47: 32-41.

39. Minotti, P. L., D. C. Williams, and W. A. Jackson. 1969. The influence of ammonium on nitrate reduction in wheat seedlings. Planta. 86: 267-271.

40. Nichols, G. L., S. A. M. Shehata, and P. J. Syrett. 1978. Nitrate reductase deficient mutants of Chlamydomonas reinhardii. J. Gen. Microbiol. 108: 79-88.

41. Oaks, A., M. Aslam, and I. Boesel. 1977. Ammonium and amino acids as regulators of nitrate reductase in corn roots. Plant Physiol. 59: 391-394.

42. Pardee, A. B., and K. Watanbee. 1968. Location of sulfate-binding protein in Salmonella typhimurium. J. Bacteriol. 96: 1049-1054.

43. Payne, W. J.. 1973. Reduction of nitrogenous oxides by microorganisms. Bacteriol. Rev. 37: 409-452.

44. Pichinoty, F. 1964. A propos des nitrate-reductases d'une bacterie denitrifiante. Biochim. Biophys. Acta. 89: 378-381.

45. Pichinoty, F. 1973. La reduction bacterienne des composes oxygenes mineraux de l'azote. Bull. Inst. Pasteur. 71: 317-395.

46. Pichinoty, F. 1970. Les nitrate-reductases bacterinnes. IV. Regulation de la biosynthese et de l'activite de l'enzyme B. Arch. Mikrobiol. 71: 116-122.

47. Pichinoty, F., and G. Metenier. 1966. Contribution a l'etude de la nitrate-reductase assimilatrice d'une levure. Anal. Inst. Pasteur. 111: 282-313.

48. Porter, W. R., and W. F. Trager. 1977. Improved non-parametric statistical methods for the estimation of Michaelis-Menten kinetic parameters by the direct linear plot. Biochem. J. 161: 293-302.

49. Premakumar, R., G. J. Sorger, and D. Gooden. 1979. Nitrogen metabolite repression of nitrate reductase in Neurospora crassa. J. Bacteriol. 137: 1119-1126.

50. Rao, K. P., and D. W. Rains. 1976. Nitrate absorption by barley. I. Kinetics and energetics. Plant Physiol. 57: 55-58.

51. Rao, K. P., and D. W. Rains. 1976. Nitrate absorption by barley. II. Influence of nitrate reductase activity. Plant Physiol. 57: 59-62.

52. Riet, J. Van'T., A. H. Stouthamer, and R. J. Planta. 1968. Regulation of nitrate assimilation and nitrate respiration in Aerobacter aerogenes. J. Bacteriol. 96: 1455-1464.

53. Rigano, C., G. Aliotta, and U. Violante. 1974. Reversible inactivation by ammonia of assimilatory nitrate reductase in Cyanidium caldarum. Arch. Microbiol. 99: 81-90.

54. Rigano, C., V. di M. Rigano, V. Vona, A. Fuggi, and G. Aliotta. 1978. Studies in vivo on the control by ammonia of nitrate reduction to nitrite in the unicellular alga Cyanidium caldarium. Plant Sci. Let. 13: 301-307.

55. Rosenberg, H., R. G. Gerdes, and K. Chegwidden. 1977. Two systems for the uptake of phosphate in Escherichia coli. J. Bacteriol. 131: 505-511.

56. Schloemer, R. H., and R. H. Garrett. 1974. Nitrate transport system in Neurospora crassa. J. Bacteriol. 118: 259-269.

57. Scott, R. H., G. T. Sperl, and J. A. DeMoss. 1979. In vitro incorporation of molybdate into demolybdoproteins in Escherichia coli. J. Bacteriol. 137: 719-726.

58. Skokut, T. A., C. P. Wolk, J. Thomas, J. C. Meeks, P. W. Shaffer, and W.-S. Chien. 1978. Initial organic products of assimilation of [$^{13}$N]ammonia and [$^{13}$N]nitrate by tobacco cells cultured on different sources of nitrogen. Plant Physiol. 62: 299-304.

59. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Iowa State University Press. Ames.

60. Stevens, S. E., Jr., and C. Van Baalen. 1974. Control of nitrate reductase in a blue-green alga. The effect of inhibitors, blue light, and ammonia. Arch. Biochim. Biophys. 161: 146-152.

61. Syrett, P. J., and J. Morris. 1963. The inhibition of nitrate assimilation by ammonium in Chlorella. Biochim. Biophys. Acta. 67: 566-575.

62. Thacker, A., and P. J. Syrett. 1972. The assimilation of nitrate and ammonium by Chlamydomonas reinhardii. New Phytol. 71: 423-433.

63. Thomas, J., C. P. Wolk, P. W. Shaffer, S. M. Austin, and A. Galonsky. 1975. The initial organic products of fixation of $^{13}$N-labeled nitrogen gas by the blue-green alga Anabaena cylindrica. Biochem. Biophys. Res. Commun. 67: 501-507.

64. Tiedje, J. M., R. B. Firestone, M. K. Firestone, M. R. Betlach, M. S. Smith, and W. H. Caskey. 1979. Methods for the production and use of $^{13}$N in studies of denitrification. Soil Sci. Soc. Am. J. 43: (in press)

65. Tilman, D. and S. S. Kilham. 1976. Phosphate and silicate growth and uptake kinetics of the diatoms Asterionella formosa and Cyclotella meneghiniana in batch and semicontinuous culture. J. Phycol. 12: 375-383.

66. Tyler, B. 1978. Regulation of the assimilation of nitrogen compounds. Ann. Rev. Biochem. 47: 1127-1162.

67. Verdouw, H., C. J. A. Van Echteld, and E. M. J. Dekkers. 1978. Ammonia determination based on indophenol formation with sodium salicylate. Water Res. 12: 399-402.

68. Weller, D. M., and A. W. Saettler. 1978. Rifampin-resistant Xanthomonas phaseoli var. fuscans and Xanthomonas phaseoli: Tools for field study of bean blight bacteria. Phytopathology. 68: 778-781.

69. Zumft, W. G. 1976. Anorganische Biochemie des Stickstoffs die Mechanismen der Stickstoffassimilation. Naturwissenschaften. 63: 457-464.

# APPENDICES

## APPENDIX A

## EVALUATION OF SZECHROME NAS REAGENT TO MEASURE NITRATE CONCENTRATIONS IN BACTERIAL CULTURES

Numerous procedures have been established for measurement of nitrate concentrations in soils, plant extracts, and aqueous solutions (1,6). Most of the reported methods have at least one of the following disadvantages: other substances, particularly nitrite and organic matter, interfere with color development; large sample sizes are required; the procedures are time-consuming; or the procedures are relatively insensitive, with limits of detection no better than 50 to 100 μM. In order to obtain accurate estimates of the $K_m$ for nitrate reduction I sought a method that would be sensitive to micromolar concentrations of nitrate and easy to adapt for routine processing of ten or more samples.

The Szechrome NAS and NB reagents developed by Dr. E. Szekely (Research and Development Authority, Ben-Gurion University of the Negev, Beer-Sheva, Israel) appeared to meet these criteria. Instructions provided with free samples indicated that the limits of detection for nitrate were 16 μM and 0.8 μM, respectively. In addition, full color was supposed to develop in 5 to 30 min and nitrite and other ions were not supposed to affect analysis. The NAS and NB powders contain the choromogens diphenylamine-4-sulfonic acid (8) and diphenylbenzidine, respectively, as well as other chemicals to stabilize the colored complexes formed. The reagents are prepared by dissolving 5 g of powder in one liter of concentrated phosphoric and sulfuric acids, mixed one to

one for NAS powder and four to six for NB powder. Samples of 0.5 ml containing up to 320 μM nitrate are mixed with 5 ml NAS solution, then allowed to stand for 20 min at room temperature. For determination of lower nitrate concentrations, samples of 1.0 ml containing up to 16 μM nitrate are mixed with 5 ml NB solution, then allowed to stand for 20 min at room temperature. I followed these procedures but found results were more reproducible if I extended the development time to 30 min at room temperature for NAS solutions, and to 1 h at 30°C for NB solutions.

Others had found $K_m$ values for nitrate reductase from 0.1 mM to 1.5 mM (7). I wanted to determine the $K_m$ values in intact bacteria, rather than enzyme systems, but expected the values to be comparable. Therefore I concentrated on evaluation of the NAS reagent, rather than the more sensitive NB reagent.

Nitrite is not supposed to affect nitrate analysis with NAS reagent. Unfortunately, nitrite concentrations typical of those I expected to observe during denitrification did interfere. To test the significance of nitrite interference with the NAS reagent, I used a factorial design with four nitrate concentrations (0, 80, 160, and 240 μM), four nitrite concentrations (0, 87, 174, and 260 μM), and two replicates of each combination. As shown in Table 1, nitrite did not react as strongly with NAS reagent as nitrate did, but the nitrite interference was still very highly significant.

To remove nitrite I mixed 0.1 ml of a 5% (wt/vol) sulfamic acid solution with 0.5 ml sample for a few seconds before addition of NAS solution. I added the same amount of sulfamic acid to the reagent blank and standards as well. This concentration of sulfamic acid was sufficient to remove nitrite from the samples, but reduced color development

Table 1. Effect of nitrite on nitrate determination using Szechrome NAS reagent.

| Source of variance | Sum of squares | Degrees of freedom | Mean square | F statistic | Level of significance |
|---|---|---|---|---|---|
| Nitrate concentration | 1.251002 | 3 | .41700070 | 310.7235 | <0.0005 |
| Nitrite concentration | .04578284 | 3 | .01526095 | 11.371529 | <0.0005 |
| Interaction of nitrate and nitrite | .01085578 | 9 | .00120620 | .89878527 | .548 |
| Residual error | .02147250 | 16 | .00134203 | | |
| Total | 1.329113 | 31 | | | |

slightly (Figure 1).

By removing nitrite with sulfamic acid and incubating samples for 30 min to allow full color development I was able to use NAS reagent for routine nitrate determinations in glucose and buffer solutions. However, samples containing full strength nutrient broth (8 g Difco nutrient broth per liter) severely inhibited color development (Figure 1). Such interference probably was similar to that caused by organic matter in nitrate determinations with phenoldisulfonic acid in concentrated sulfuric acid (1). I found little interference in broth solutions which had been diluted 10-fold, but such dilution was only possible at higher nitrate concentrations.

Nitrite and organic matter also interfered with nitrate analysis in NB reagent solutions. Nitrite concentrations which caused slight interference in NAS solutions caused more color development in NB solutions, probably due to the greater sensitivity of this reagent. Most components of the glucose and mineral salts medium I used in studies of nitrate assimilation did not interfere with nitrate analysis, but molybdate reacted to form a colored product. The NB reagent may be sufficiently sensitive to examine changes in nitrate concentration due to transport in defined media, though I did not investigate that application.

Measurement of nitrate with the NAS and NB reagents was not the ideal method for nitrate analysis I had hoped to find, but proved reasonably workable. Both reagents should be quite suitable for their intended purpose, the analysis of nitrate concentrations in natural waters, where nitrate and organic matter concentrations would be too low to interfere. These reagents now can be purchased from an American

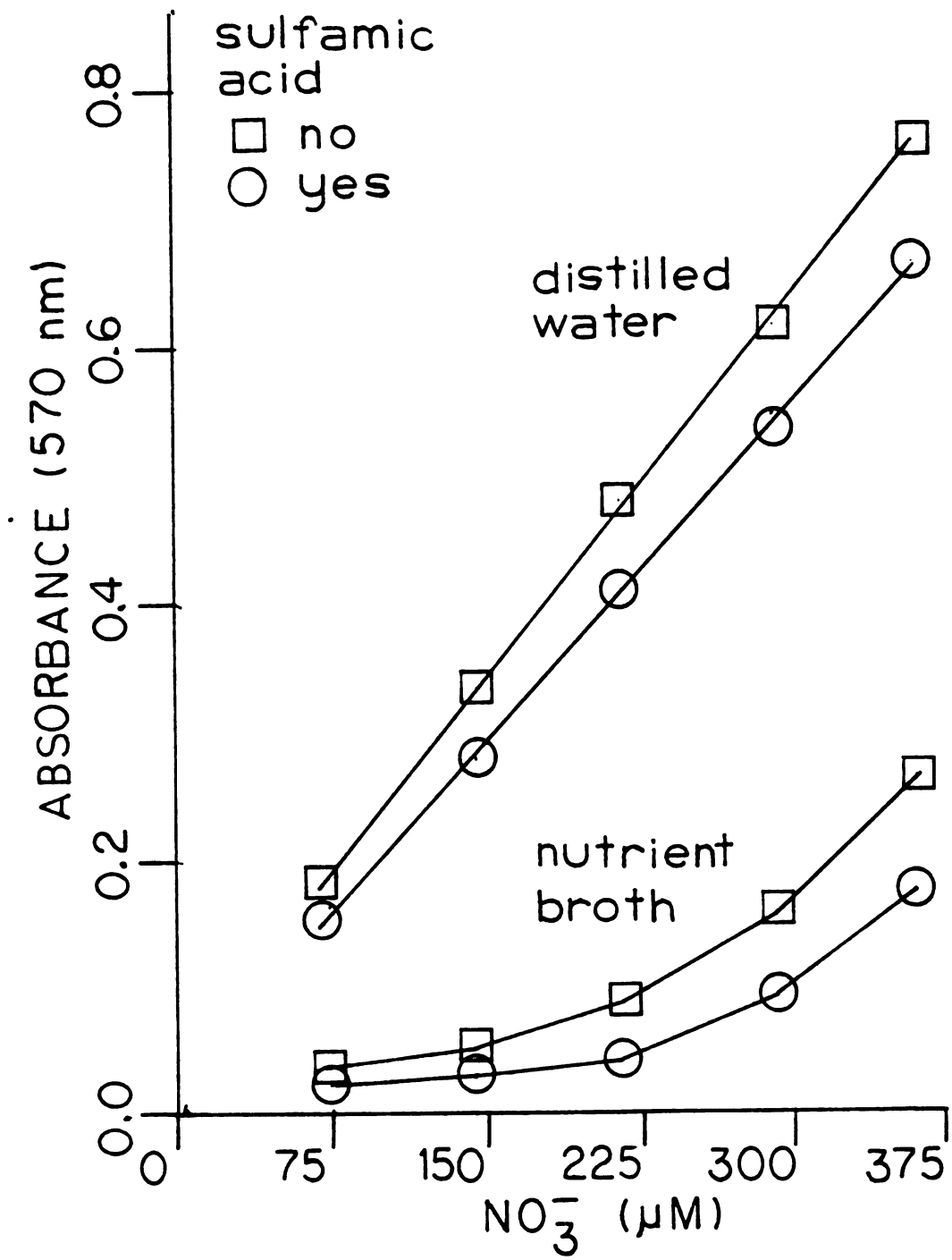



Figure 1. Effects of sulfamic acid and nutrient broth on nitrate detection using Szechrome NAS reagent.

supplier (Polysciences, Inc., Warrington, Penn.).

Based on my experiences here and a survey of other methods for nitrate determination, I feel the method of choice is to measure nitrate after its reduction to nitrite. Of course, nitrite would have to be determined separately or else removed from the samples prior to reduction. Measurement of nitrate as nitrite has the advantage of high sensitivity with few interferences that is characteristic of the diazotization procedure for nitrite analysis (1). However, most enzymatic methods for reduction of nitrate to nitrite have the disadvantage that they are slow (5) or require special handling procedures to maintain activity (4). I would select nitrate reduction by copper-treated cadmium (2), which is quicker and does not require the special precautions typical of enzymatic procedures. Although the spectrofluorimetric procedure for nitrate determination based on oxidation of NADH during enzymatic reduction (3) is rapid (3 min or less) and uses quite small sample sizes (50 µl), I feel its requirement for elaborate equipment and attention to detail would make it too inconvenient for routine use.

## LITERATURE CITED


1. Barker, A. V. 1974. Nitrate determinations in soil, water, and plants. Res. Bull. 611. Mass. Agr. Experiment Station. Amherst, Mass.

2. Gardner, W. S., D. S. Wynne, and W. M. Dunstan. 1976. Simplified procedure for the manual analysis of nitrate in seawater. Marine Chem. 4: 393-396.

3. Kiang, D.-H., S. S. Kuan, and G. G. Guilbault. 1978. Enzymatic determination of nitrate: fluorometric detection after reduction with nitrate reductase. Anal. Chem. 50: 1323-1325.

4. Lowe, R. H., and J. L. Hamilton. 1967. Rapid method for determination of nitrate in plant and soil extracts. J. Agr. Food Chem. 15: 359-361.

5. McNamara, A. L., G. B. Meeker, P. D. Shaw, and R. H. Hageman. 1971. Use of a dissimilatory nitrate reductase from Escherichia coli and formate as a reductive system for nitrate assays. J. Agr. Food Chem. 19: 229-231.

6. Standard Methods for the Examination of Water and Wastewater, 13th Ed. 1971. Am. Public Health Assoc. Washington, D. C.

7. Stouthamer, A. H. 1976. Biochemistry and genetics of nitrate reductase in bacteria. Adv. Microbial Physiol. 14: 315-375.

8. Szekely, E. 1967. Spectrophotometric determination of nitrate with p-diaminodiphenylsulphone and diphenylamine-p-diamino-diphenylsulphone. Talanta 14: 941-950.

# APPENDIX B

## COMPUTER PROGRAMS USED TO ANALYZE KINETIC AND $^{13}N$ DATA

The computer programs included in this appendix have been developed during the course of this research to facilitate data analysis. I hope the brief descriptions provided are sufficient to orient the reader to the program structure and relevant mathematics involved. Because computer systems vary in their implementations of FORTRAN and especially BASIC, these programs probably cannot be used without modification. All have been written specifically for the applications decribed in this dissertation, but the solution procedures should be generally applicable to similar problems.

With the exception of FASTFIT, developed by Dr. Richard Firestone, (who is now at UC-Berkeley Livermore Laboratory) all programs were written for an IMSAI 8080 Microcomputer equipped with two North Star disk drives, the corresponding operating system, and North Star BASIC. Major differences between this and other versions of BASIC most likely are found in the input/output routines. The ! is an abbreviated form of "PRINT"; % specifies a format string, for instance, %10F2 is equivalent to the FORTRAN specification 10F2.0.

Progress curve analysis. This program implements procedures for determination of $K_m$ and $V_{max}$ values based on progress curve analysis using the integrated Michaelis-Menten equation for a single substrate uninhibited reaction(2,3,4). As inputs the program requires the initial

substrate concentration and the time and substrate concentration at each sampling period. These substrate concentrations are converted into product concentrations (lines 60 to 90) which are transformed to provide initial estimates for the $K_m$ and $V_{max}$ values (lines 90 to 180, [4]). Next the desired precision of product estimates and convergence criteria for $K_m$ and $V_{max}$ values are input. Lines 340 to 430 calculate new product concentrations based on the current values of the kinetic constants, using an iterative Newton-Raphson procedure (2). If the data do not fit the integrated equation well, the program will usually abort in line 380, when 1-P(I)/SO becomes negative. This condition can occur if the rate of reduction is approximately linear, or if there is substantial error in the estimate of initial substrate concentration.

After new product concentrations have been calculated they are compared with the observed values and used to calculate the changes to make in $K_m$ and $V_{max}$ estimates (lines 450 to 720), according to Wilkinson's multiple linear regression procedure (5). Once the kinetic constants have been updated (lines 690 and 700) the program checks to see if the estimates have converged (lines 280 and 290). If not, the process is repeated; otherwise the kinetic constants and their standard errors are printed. The program usually converges after four or five passes; it does not check for lack of convergence, though this is apparent from the intermediate results printed out (line 710).

PROGRESS CURVE ANALYSIS LISTING

```
 10  REM    PROGRESS CURVE ANALYSIS FOR KM,VMAX ESTIMATES
 20  REM    GET DATA POINTS AND TRANSFORM TO PRODUCT CONCENTRATIONS
 30  INPUT "ENTER NUMBER OF DATA POINTS AND SO ",N,SO
 40  DIM A(N,2),P(N),S(N),T(N),PO(N)
 50  !"ENTER EACH DATA PAIR, TIME FIRST, ONE PAIR PER LINE"
 60  FOR I=1 TO N
 70  INPUT T(I),S(I)
```

```
 80  P(I)=SO-S(I)
 90  PO(I)=P(I)
100  Y=P(I)/T(I)
110  X=-LOG(SO/S(I))/T(I)
120  REM    FORM PARTIAL SUMS FOR LINEAR REGRESSION
130  Y1=Y1+Y
140  X1=X1+X
150  Y2=Y2+Y*Y
160  X2=X2+X*X
170  X9=X9+X*Y
180  NEXT I
190  REM    CALCULATE INITIAL KM AND VMAX ESTIMATES
200  K=(X9-X1*Y1/N)/(X2-X1*X1/N)
210  V=Y1/N-K*X1/N
220  !"FIRST ESTIMATE FOR K IS ",K,"   FOR V IS ",V
230  !"ENTER PRECISION DESIRED FOR P ESTIMATION AND "
240  INPUT " FOR KM,VMAX ESTIMATES ",E0,E1
250  GOSUB 350
260  GOSUB 450
270  REM    CHECK FOR CONVERGENCE, IF NOT REPEAT PROCEDURE
280  IF ABS(V9) <E1 THEN 290 ELSE 250
290  IF ABS(K9)<E1 THEN 310 ELSE 250
300  REM    PRINT RESULTS
310  !"KM ESTIMATE IS ",K," +- ",SQRT(K2)
320  !"VMAX ESTIMATE IS ",V," +- ",SQRT(V2)
330  END
340  REM    UPDATE PRODUCT CONCENTRATIONS USING NEWTON-RAPHSON PROCEDURE
350  F9=0
360  FOR I=1TON
370  A(I,1)=(SO-P(I)+K)/(SO-P(I))
380  A(I,2)=(V*T(I)-P(I)+K*LOG(1-P(I)/SO))/A(I,1)
390  P(I)=P(I)+A(I,2)
400  IF ABS(A(I,2))>=E0 THEN F9=1
410  NEXT I
420  IF F9=1 THEN 350
430  RETURN
440  REM    CALCULATE NEW VMAX AND KM ESTIMATES AND ERROR
450  DATA 0,0,0,0,0,0
460  RESTORE 450
470  READ A1,A2,A3,P1,P2,P3
480  REM    TRANSFORM DATA FOR MULTIPLE LINEAR REGRESSION
490  FOR I=1TON
500  D=1+K/(SO-P(I))
510  Y=PO(I)-P(I)
520  X1=LOG(1-P(I)/SO)/D
530  X2=T(I)/D
540  REM    FORM PARTIAL SUMS FOR REGRESSION ANALYSIS
550  A1=A1+X1*X1
560  A2=A2+X2*X2
570  A3=A3+X1*X2
580  P1=P1+X1*Y
590  P2=P2+X2*Y
600  P3=P3+Y*Y
610  NEXT I
```

```
620  REM    CALCULATE NEW KM AND VMAX, AND ERROR FOR EACH
630  D9=A1*A2-A3*A3
640  K9=(A2*P1-A3*P2)/D9
650  V9=(A1*P2-A3*P1)/D9
660  S2=(P3-K9*P1-V9*P2)/(I-2)
670  K2=A1*S2/D9
680  V2=A2*S2/D9
690  K=K+K9
700  V=V+V9
710  !"INTERMEDIATE VALUES:  K ",%10F2,K,"  AND V ",%10F2,V
720  RETURN
730  END
```

Nuclear spectra analysis (FASTFIT). This program was written by Dr. Richard Firestone for the XEROX Sigma 7 computer. I modified it slightly to take advantage of the dynamic file assignment capability (CALL ASSIGN) of that FORTRAN implementation. It is designed to analyze data in compressed binary format (CALL CREAD) obtained by flowthrough systems, such as the liquid chromatograph and gas chromatograph used in this research, but will accomodate data taken sequentially from any time- or position-dependent apparatus. A detailed description of the instrument setup employed is included as an Appendix to M. K. Firestone's Ph.D. thesis, Michigan State University, 1978. The program is designed to analyze $^{13}N$ or $^{11}C$ materials, determined by the flag ISTOPE, with a base counting interval of 2 s. (ALAM is the decay constant for this 2 s period).

Program input consists of a card with the data file name and type of isotope used, followed by several cards which specify the type of half-life correction, lower and upper background limits, and peak limits. Half-life correction defaults to the first data interval on file, but can be overridden by appropriate modification of the data card. Field definitions on the data card, format (2I1,2I4,14I5) are:

| | |
|---|---|
| col. 1 | number of peaks; if zero, get new file or end |
| col. 2 | time constant flag: 0, 2 s data; 1, no $t_{\frac{1}{2}}$ correction; 2, count interval in seconds is $0.1^{2}$ x ITIME |
| col. 3-6 | ITIME, used only for different count interval |
| col. 7-10 | ISCALE, number of interval relative to first count interval which should be used for time zero |
| col. 11-15 | lower left background limit |
| col. 16-20 | upper left background limit |
| col. 21-25 | lower right background limit |
| col. 26-30 | upper right background limit |
| col. 31-35 | lower limit for first peak |
| col. 36-40 | upper limit for first peak |
| col. 41-80 | lower and upper limits for other peaks |

The program calculates an average lower and upper background, and then adjusts all counts within a peak interval by subtracting a linear interpolation of the average background values. Counts are then corrected for decay and summed within the peak limits. Partial sums are also formed with which to calculate the error in area estimates (1) and the interval which defines the "center of mass" of each peak.

FASTFIT LISTING

```
 1.  C PROGRAM FASTFIT
 2.  C
 3.  C
 4.        DIMENSION IDATA(10000),TITLE(20),PU(5),PL(5),B(10000)
 5.        DIMENSION NAME(9)
 6.        REAL LSUM
 7.        INTEGER BLL,BLU,BRU,BRL,PL,PU
 8.  111   READ(,5,END=999)NAME,ISTOPE
 9.  5     FORMAT(12,8A4,6X,A4)
10.        ALAM=0.001160
11.        IF(ISTOPE.EQ.4HC11 )ALAM=0.0005635
12.        CALL ASSIGN(5,1,NAME,8,2)
13.        REWIND 5
14.        READ(5,1)TITLE
15.        FORMAT(16X,20A4)
16.        CALL CREAD(IDATA,NCHAN,NRUN,NERR,NSTART,5)
17.        PRINT 52,TITLE
18.  52    FORMAT(10X,20A4)
19.        PRINT 51
20.  51    FORMAT('0',2X,'CENTROID    PEAK AREA    AREA ERROR
21.       +   BACKGROUND AND PEAK LIMITS')
22.  100   READ(1,2,END=999) N,M,ITIME,ISCALE,BLL,BLU,BRL,BRU,PL(1),PU(1),PL(
23.       +2),PU(2),PL(3),PU(3),PL(4),PU(4),PL(5),PU(5)
24.  2     FORMAT(211,214,1415)
25.        IF(N.EQ.0)GO TO 111
```

```
      LSUM=0.
      RSUM=0.
      TIME=ITIME
      SCALE=ISCALE
      IF(M.EQ.0) TCON=2.
      IF(M.EQ.1) TCON=0.0
      IF(M.GT.1) TCON=0.1*TIME
C
C CALCULATE BACKGROUND
      DO 10,I=BLL,BLU
      ADATA=IDATA(1)
      LSUM=LSUM+ADATA
10    CONTINUE
      DO 11,I=BRL,BRU
      ADATA=IDATA(I)
      RSUM=RSUM+ADATA
11    CONTINUE
      NL=BLL+(BLU-BLL)/2
      NR=BRL+(BRU-BRL)/2
      BDIFF=NR-NL
      SUMD=RSUM/(BRU-BRL)-LSUM/(BLU-BLL)
      CDIFF=SUMD/BDIFF
      B(NL)=LSUM/(BLU-BLL)
      DO 12,I=NL+1,NR
      B(I)=B(I-1)+CDIFF
12    CONTINUE
C FIT PEAKS AND ERRORS
      DO 13,I-1,N
      PEAK=0.
      BSUM=0.
      PSUM=0.
      SERR=0.
      XERR=0.
      DO 14,J=PL(I),PU(I)
      ADATA=IDATA(J)
      BSUM=BSUM+B(J)
      PSUM=PSUM+ADATA
      AJ=J
      T=TCON*(AJ-SCALE)
      PEAK=(ADATA-B(J))*EXP(ALAM*T)+PEAK
      IF((ADATA-B(J)).EQ.0.)  GO TO 14
      SERR=SERR+1.0*(ADATA-B(J))
      XERR=XERR+AJ*(ADATA-B(J))
14    CONTINUE
      ERR=PEAK*SORT(PSUM+BSUM)/(PSUM-BSUM)
      CENT=XERR/SERR
      PRINT 50,CENT,PEAK,ERR,BLL,BLU,PL(I),PU(I),BRL,BRU
50    FORMAT('0',3X,F9.3,3X,F9.0,3X,F9.0,10X,6(15,2X))
      TO=SCALE*TCON
      IF(M.NE.1) PRINT 53,TO
53    FORMAT('+', 90X,'T(1/2) CORRECTED, T(0) = ',F7.1,'SEC')
13    CONTINUE
      GO TO 100
999   CONTINUE
      END
```

Denitrification model. The listing for the denitrification model developed in Chapter 1 follows. The subscript, I, for each of the arrays refers to the first, second, etc. intermediate in the denitrification pathway: that is, $NO_3^-$, $NO_2^-$, NO, and so forth. At each time interval the change in substrate concentration is calculated from the equation: $\Delta S = P \cdot \Delta t \cdot \frac{V \cdot S}{K_m + S}$. After all changes have been calculated the substrate concentrations are adjusted and the time is checked to see if intermediate values are to be printed. This program could of course be readily adapted to a simulation language such as IBM's CSMP or CDC's MIMIC, where the built-in plotting capability would permit a rapid interpretation of the effects changes in the kinetic constants might have. To simulate $N_2O$ addition at the beginning and during an experiment, I interrupted the program and changed the concentration of S(4), then continued program execution.

DENITRIFICATION MODEL LISTING

```
 10  REM    DENITRIFICATION MODEL BASED ON IRREVERSIBLE MICHAELIS-MENTEN
              KINETICS
 20  REM    INITIALIZE KM AND VMAX VALUES
 30  FOR I=1 TO 4
 40  V(I)=2.
 50  K(I)=2
 60  NEXT I
 70  K(1)=1
 80  REM       GET VARIABLE INPUT AND ADJUST CONSTANTS
 90  INPUT  "ENTER ENZYME AMOUNTS FOR 4 REDUCTASES ",E(1),E(2),E(3),E(4)
100  INPUT  "ENTER NO3 CONCENTRATION AND ANAER. PROBABILITY ",S(1),P
110  FOR I=1 TO 4
120  F(I)=V(I)*E(I)
130  NEXT I
140  REM    WRITE COLUMN HEADINGS
150  !"TIME",TAB(10),"NO3",TAB(25),"NO2",TAB(40),"NO",TAB(55),"N2O",
          TAB(70),"N2"
160  REM    BEGINNING OF DISCRETE TIME KINETIC MODEL LOOP
170  IF S(1)<1 THEN STOP
```

```
180  T=T+.025
190  REM    LOOP TO CALCULATE RATE OF REDUCTION OF ITH COMPOUND
200  FOR I=1 TO 4
210  C(I)=.025*P*F(I)*S(I)/(K(I)+S(I))
220  NEXT I
230  REM    LOOP TO UPDATE CONCENTRATIONS OF INTERMEDIATES
240  FOR I=1 TO 4
250  S(I)=S(I)+C(I-1)-C(I)
260  NEXT I
270  S(5)=S(5)+C(4)
280  REM    PRINT INTERMEDIATE VALUES
290  IF T-INT(T)=0 THEN !T,TAB(5),S(1),TAB(20),S(2),TAB(35),S(3),
          TAB(50),S(4),
300  IF T-INT(T)=0 THEN !TAB(65),S(5)
310  GOTO 170
320  END
```

$^{13}$N scintillation counter analysis. The mathematics upon which this program is based have been described in the Materials and Methods section of Chapter 2. The first section of the program is data entry, either by hand (lines 110 to 240) or from a disk file (lines 270 to 330) created through an interface program to the scintillation counter. Each sample set must be counted twice in order to provide sufficient information for solution of the simultaneous equations. Data are entered in order of counting--elapsed time and counts per sample. Since the samples were counted twice the number of samples, N9, equals the number of data pairs, I9, divided by two.

The next section of the program calculates the decay factors for $^{13}$N and $^{18}$F, based on elapsed time and the appropriate half-life (lines 360 to 390). Next the $^{13}$N and $^{18}$F counts in each sample when the sample set began counting are determined by solving the simultaneous equations (lines 440 to 490). Any corrections for dilution, e.g. due to addition of inhibitor, are made next by entering the sample range to be corrected and the dilution factor (lines 500 to 590). Finally, the percent of $^{13}$N added to each sample which has been incorporated is calculated (line 630)

and the results are printed.

This program could be modified for simultaneous determination of two or more isotopes provided their half-lives are sufficiently different to permit a unique solution. One would only need to change the decay constants and, if more than two isotopes were counted, to change the simultaneous solution equations in lines 450 to 480.

### $^{13}$N SCINTILLATION COUNTER LISTING

```
 10  REM       13N SCINTILLATION CORRECTION PROGRAM
 20  REM       SIMULATANEOUS EQUATION SOLUTION BASED ON 13N AND 18F DECAY
 30  DIM N1(100),NO(50),T(100),FO(50),N2(100),F2(100),PO(50)
 40  REM       DECAY CONSTANT CALCULATION
 50  L=LOG(2)
 60  N2=L/9.96
 70  F2=L/110
 80  REM       MANUAL DATA ENTRY SECTION
 90  INPUT "ARE DATA ALREADY ON FILE? ",Y$
100  IF Y$="YES" THEN 270
110  I=0
120  PRINT "ENTER DATA PAIRS:  TIME,COUNTS.  END BY ENTERING 0,0"
130  I=I+1
140  INPUT T(I),N1(I)
150  IF T(I)>0 THEN 130
160  I9=I-1
170  N9=I9/2
180  INPUT "ENTER DISK UNIT TO WHICH DATA WILL BE WRITTEN ",D$
190  INPUT "ENTER FILE NAME OR DATA FILE ",A$
200  OPEN #0,A$+",+D$
210  FOR I=1 TO I9
220  WRITE #0,T(I),N1(I)
230  NEXT I
240  CLOSE #0
250  GOTO 350
260  REM       READ DATA FROM DISK FILE
270  INPUT "ENTER DISK UNIT WITH DATA FILE ",D$
280  INPUT "ENTER DATA FILE NAME ",A$
290  OPEN #0,A$+","+D$
300  READ #0%0,I9
310  FOR I=1 TO I9
320  READ#0%15*I,N9,T(I),N1(I)
330  NEXT I
340  N9=I9/2
350  REM       CALCULATE FRACTION OF ACTIVITY REMAINING DUE TO DECAY
360  FOR I=1 TO I9
370  N2(I)=EXP(N2*T(I))
380  F2(I)=EXP(F2*T(I))
```

```
390  NEXT I
400  PRINT N9," SAMPLES COUNTED."
410  PRINT "ENTER WHICH ONE WILL BE USED FOR COUNTS ADDED "
420  INPUT "AND SCALE FACTOR TO USE ",N,S
430  REM       CALCULATE 13N and 18F COUNTS AT ZERO TIME
440  FOR I=1 TO N9
450  D=N2(I)*F2(I+N9)-N2(I+N9)*F2(I)
460  NO(I)=(N1(I)*F2(I+N9)-N1(I+N9)*F2(I))/D
470  FO(I)=(N2(I)*N1(I+N9)-N2(I+N9)*N1(I))/D
480  FO(I)=100*FO(I)/(FO(I)+NO(I))
490  NEXT I
500  REM       MAKE CORRECTIONS FOR ANY DILUTIONS DURING EXPERIMENT
510  INPUT "ARE ANY DILUTION CORRECTIONS TO BE MADE? ",Y$
520  IF Y$ <>"YES" THEN 610
530  PRINT "ENTER SAMPLE RANGE TO CORRECT AND CORRECTION FACTOR"
540  INPUT I1,I2,C
550  FOR I-I1 TO I2
560  NO(I)=NO(I)*C
570  NEXT I
580  INPUT "MORE CORRECTIONS TO BE MADE? ",Y$
590  IF Y$="YES" THEN 540
600  REM       PRINT RESULTS
610  PRINT "SAMPLE  PERCENT 18F    13N AT TO      FRACTION OF SOURCE"
620  FOR I=1 TO N9
630  PO(I)=NO(I)/(NO(N)*S)
640  PRINT $4I,I,TAB(11),%7F2,FO(I),TAB(26),%10E4,NO(I),TAB(45),PO(I)
650  NEXT I
660  END
```

## LITERATURE CITED


1. Bevington, P. R. 1969. Data Reduction and Error Analysis for the Physical Sciences. pp. 247-254, McGraw Hill, New York.

2. Duggleby, R. G., and J. F. Morrison. 1977. The analysis of progress curves for enzyme-catalyzed reactions by non-linear regression. Biochim. Biophys. Acta. 481: 297-312.

3. Fernley, H. N. 1974. Statistical estimations in enzyme kinetics: the integrated Michaelis equation. Eur. J. Biochem. 43: 377-378.

4. Nimmo, I. A., and G. L. Atkins. 1974. A comparison of two methods for fitting the integrated Michaelis-Menten equation. Biochem. J. 141: 913-914.

5. Wilkinson, G. N. 1961. Statistical estimations in enzyme kinetics. Biochem. J. 80: 324-332.