THESIS

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OF NITROUS OXIDE

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NON-DENITRIFYING BIOLOGICAL SOURCES OF NITROUS OXIDE

By

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ABSTRACT

NON-DENITRIFYING BIOLOGICAL SOURCES OF NITROUS OXIDE

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Possible non-denitrifying sources of N_2O were investigated. Microbes found to produce N_2O from NO_3^- but not consume it were: (i) all of the facultatively anaerobic dissimilatory reducers of nitrate to ammonium examined, Escherichia coli K12, Serratia marcescens, Klebsiella pneumoniae, Enterobacter aerogenes, Erwinia caratovora and Bacillus subtilis: (ii) a few of the assimilatory nitrate-reducing bacteria examined, e.g. Azotobacter vinelandii 12837, Azotobacter vinelandii nif-12, and Azotobacter vinelandii rif^r nif-64: (iii) some but not all of the assimilatory nitrate-reducing yeasts and fungi, Hansenula sp., Rhodotorula sp., Aspergillus sp., Alternaria sp., and Fusarium sp. Neither of the two NO_3^--reducing obligate anaerobes examined (Clostridium KDHS2 and Vibrio succinogenes) produced N_2O. Production of N_2O occurred only in stationary phase. The enteric bacteria and Bacillus achieved the highest conversions of NO_3^- to N_2O, reaching up to 36% of the NO_3^--N recovered as N_2O-N. Production of N_2O was apparently not regulated by ammonium; enzymes produced during secondary metabolism could be the N_2O source. Nitric oxide (NO) was not detected from enteric bacteria or yeasts.

N_2O was also found to arise from some damaged plant tops, probably due to microbial growth. Levels of N_2O above the ambient level in the atmosphere were found in human breath samples.

To Mom, Dad, and Robert
the pack
I will always run with.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION AND EXPERIMENTAL OBJECTIVES

Both nitrate and nitrite can be converted to nitrogenous gases by chemical and biological processes. One such gas, N_2O, has received much attention, since it may act to deplete the Earth's ozone layer (12), and help promote an atmospheric greenhouse effect (40).

Production of N_2O from several chemical mechanisms involving NO_2^- (27, 28, 34, 39, 45) or NH_2OH (6) in laboratory experiments has been reported. The significance of these mechanisms in nature has not been demonstrated. It may be that most of the N_2O produced in nature is due to biological processes. Of these, nitrification and denitrification have received the most attention.

Although it has been known for some time that nitrifying bacteria can produce N_2O (46), it has not been intensively investigated until recently (16, 20). Studies by Blackmer and Bremner (4) indicate that application of ammoniacal fertilizers to aerobic soils can result in significant losses of N_2O.

Denitrifying bacteria are those which use nitrogenous oxides as electron acceptors to generate ATP under anaerobic conditions. In the process, the majority of NO_3^- or NO_2^- is converted to N_2 or N_2O (29), with the proportion of N_2O produced being dependent on several environmental factors. Firestone *et al.* (14) found that increases in nitrate, nitrite, oxygen and soil acidity cause N_2O production to increase relative to N_2. Studies with pure cultures of denitrifiers have shown NO in addition to N_2O and N_2 to be produced during denitrification (3), but the role of NO in denitrification is still controversial.

Some bacteria can accomplish the dissimilatory

reduction of NO_2^- to NH_4^+ (10, 11, 33), apparently done to reoxidize reduced pyridine nucleotides during fermentation. Work by Yoshida and Alexander (46) and in our laboratory (37) led to the suspicion that these bacteria could produce N_2O as well as NH_4^+, and that the presence of carbon could reduce N_2O production.

Yoshida and Alexander (46) and Bollag and Tung (5) had found that certain fungi could produce N_2O from NO_2^-. Work with green plants has indicated that nitrogenous oxides might be released from their foliage (35, 36, 41). Kaspar and Tiedje's finding that NO_3^- and NO_2^- are dissimilated to NH_4^+ and N_2O in the bovine rumen (22), coupled with the existence of similar organisms in the gastrointestinal tract, raised the question of whether N_2O might be found in animal breath.

This study was conducted as a survey of possible sources of N_2O that had not received prior attention. Organisms reported to have the capacity to assimilate NO_3^- into cell material (18, 29) were thought worthy of investigation. I report here on various microbes that produced N_2O but not NO, as well as the production of N_2O by damaged plant tops and in human breath.

My research centered on the following questions:

For pure cultures of microorganisms,

1. Which physiological groups of organisms are able to produce N_2O from NO_3^- or NO_2^-?
2. At what stage of growth does N_2O production occur?
3. What is the effect of carbon source on N_2O production?
4. Is the production of N_2O by these organisms regulated by ammonium?
5. Is NO produced by any of these organisms?
6. What are the rates of N_2O production from NO_2^- by these organisms?

For green plants,

1. Is there evidence to support the production of N_2O by plant tissue?

For human breath,

1. Is N_2O found in human breath at levels exceeding the ambient concentration of the atmosphere?
2. Does the consumption of NO_3^-/NO_2^- in the diet cause a change in the level of N_2O in breath?

MATERIALS AND METHODS

Microorganisms

The bacteria studied included Escherichia coli K12, Serratia marcescens, Enterobacter aerogenes, Klebsiella pneumoniae, Erwinia caratovora, Bacillus subtilis, and Acinetobacter sp. Clostridium KDHS2 was isolated by W. H. Caskey (8). Vibrio succinogenes was from the laboratory of Dr. C. A. Reddy. Dr. Harold Sadoff provided cultures of Azotobacter vinelandii strains A. vinelandii 12837, A. vinelandii nif-12, A. vinelandii rif^r nif-64; and Azotobacter macrocytogenes strains A. macrocytogenes 8700 and A. macrocytogenes 9129. The following fungi and yeasts were obtained from Dr. A. Rogers: Alternaria sp., Aspergillus sp., Fusarium sp., Helminthosporium sp., Penicillium sp., Actinomucor elegans, Candida tropicalis, Rhodotorula sp., and Hansenula sp.

^{13}N-studies

Pure cultures of bacteria were grown aerobically

in 500 ml Erlenmeyer flasks which contained 250 ml of 5% tryptic soy broth (Difco) with 3.5 mM KNO_3. Cells were grown at 30° C on a rotary shaker at 150 rpm. After 12 h cells were harvested by centrifugation, washed in 0.05 M Tris buffer (pH 7.0), and resuspended. Cell suspensions of 0.5 ml were injected by syringe into serum vials containing 5% tryptic soy broth without nitrate, under a helium headspace, with Ti(III)citrate to establish a low Eh. Autoclaved cells were prepared in a similar manner and served as a sterile control. To initiate the experiment, $^{13}NO_3^-/^{13}NO_2^-$ (approx. 1 mCi) produced at the MSU cyclotron (38) and mixed with unlabeled KNO_3 was injected into each vial to achieve a nitrate concentration of 10 μM. The vials were agitated on a rotary shaker for 20 min at 25° C, after which the headspace gas was analyzed for ^{13}N-gases by gas chromatography-proportional counting (38). Each vial was then opened, and the medium clarified by filtration through a 0.22 μm filter. The medium was analyzed for ^{13}N-ions by radio-HPLC (38).

Conditions for assessment of N_2O production in batch culture

Pure cultures of bacteria, yeasts and fungi were grown in 26 ml Balch tubes (Bellco Glass, Vineland, NJ), which contained 5 ml of the respective media. Media were amended with 5 mM KNO_3, unless stated otherwise.

The enteric bacteria and Bacillus were usually grown in 1.5% (w/v) tryptic soy broth. Potato dextrose broth (Difco) was used to culture all yeasts and fungi. Selected yeasts and fungi were also grown on a synthetic NH_4^+-free medium, prepared as follows. The following stocks (g/l) were prepared and autoclaved separately:

Solution A-- K_2HPO_4, 160.0; KH_2PO_4, 40.0; NaCl, 10.0: Solution B-- $MgSO_4 \cdot 7H_2O$, 20.0: Solution C-- $CaCl_2 \cdot 2H_2O$, 2.5; $FeCl_3 \cdot 6H_2O$, 0.25. Stock vitamin and trace mineral solutions as described in (1) were prepared and sterilized separately. The synthetic medium was prepared by adding 10 ml of each stock solution to one liter of double-distilled water containing KNO_3 and glucose, and adjusting the pH to 5.1 with 1 N HCl.

The Azotobacter strains were grown in Burk's medium (42), with KNO_3 substituted for NH_4NO_3. Acinetobacter was grown on a medium of (g/l): Na acetate, 2.0; KNO_3, 2.0; and $MgSO_4 \cdot 7H_2O$, 0.2, prepared in 0.04 M KH_2PO_4 and Na_2HPO_4 buffer (pH 6.0). To this was added 1% (v/v) of the same trace mineral solution as above.

Clostridium KDHS2 was grown on the medium of Caldwell and Bryant (7) except that soluble starch and cellobiose were omitted, and KNO_3 was added. Vibrio was grown on the medium of Wolin, Wolin and Jacobs (44).

The fungi, yeasts, Azotobacter and Acinetobacter cultures were incubated under air. The enteric bacteria, Bacillus, yeasts and obligate anaerobes were incubated under O_2-free argon, achieved by evacuating and flushing each tube three times. Tubes were inoculated with a 1-6% inoculum from a seed culture. Tubes were positioned horizontally, and shaken at 100 rpm on a rotary shaker. Incubation was at 25° C in the dark, except for the enteric organisms, Bacillus and Vibrio which were incubated at 31° C.

The fungi were grown as above, inoculated either via syringe from sporulating slant cultures flooded with sterile saline, or by an inoculating loop scraped across such cultures. Culture tubes of fungi were incubated stationary and vertical, except prior to gas analysis when they were shaken to ensure gaseous equilibrium.

Resting cell studies

Seed cultures of Escherichia coli K12, Klebsiella pneumoniae and Enterobacter aerogenes were grown in 40 ml of tryptic soy broth without glucose, with 5 mM KNO_3. Incubation was at 25° C with shaking at 100 rpm in Erlenmeyer sidearm flasks. Cultures were grown for 1 day (early stationary phase), then their entire contents were aseptically transferred to 450 ml of the same medium in 500 ml Erlenmeyer flasks. These flasks were capped with rubber stoppers pierced by one-way check valves (Nupro Co., Willoughby, OH), to relieve gas pressure. Incubation was stationary, at 25° C, for 1-2 days.

Since Hansenula sp. grew best aerobically, its volume of medium was the same as above, but in one liter flasks. It was grown in potato dextrose broth with 5 mM KNO_3.

Stationary phase cultures were harvested by centrifugation at 10,000 x g for 10 min. Pelleted cells were resuspended in 10 ml of the same medium without nitrate, plus 200 μg ml^{-1} chloramphenicol. Cells were kept on ice for no more than 12 h until used.

Cells were added to 40 ml of the initial growth medium without KNO_3, plus 200 μg ml^{-1} chloramphenicol, in presterilized 125 ml Erlenmeyer flasks having Hungate sidearms. Each flask was connected to the recirculating gas assay system described by Kaspar and Tiedje (21). The system was modified to allow the semicontinuous sampling of gases from four flasks. Magnetic stirrers afforded continuous agitation of the cultures, and aided maintenance of equilibrium between gaseous and liquid phases.

After making the flasks anaerobic by flushing with argon, 2 ml of sterile 5 mM $NaNO_2$ was added to each culture. Gas samples were usually taken

every 20 min. At termination of each experiment, cells were saved for protein analysis.

Green plants

Since plants accomplish the light-driven reduction of NO_2^- to NH_4^+ within their chloroplasts (13, 24, 26), plant tissue was investigated as a possible source of N_2O.

For plant top analyses, seedlings in the field were uprooted, and their roots kept in water during transport to the laboratory. The seedlings were rinsed under tap water to remove as much soil adhering to foliage as possible, and blotted on paper toweling. Plant tops were removed and placed into serum bottles, then sealed with butyl rubber septa and aluminum crimp caps. The headspace of some bottles was air, termed aerobic. Others were evacuated and flushed three times with argon gas, and are termed anaerobic. Incubation was at 32° C.

For the diced leaf experiments, fresh spinach was purchased at local markets and refrigerated until washing. Leaves selected for their wholeness and fresh appearance were rinsed under cold tap water to remove soil, then blotted on paper toweling. Leaves were then placed flat on plastic trays, covered with clear plastic wrap, and incubated in a growth chamber at 5-10° C under incandescent lights for 4-12 h, to revitalize their photosynthetic apparatus (9).

Selected leaves were cut into approx. 1 cm^2 pieces. Leaves were large enough so that eight pieces could be cut from each. Four pieces of leaf were put into serum bottles, each bottle containing pieces from only one leaf. Ten milliliters of 5 mM KNO_3 or $NaNO_2$ was pipetted into each bottle; chloramphenicol, when included in this solution, was at 200 $\mu g\ ml^{-1}$.

Bottles were sealed, then evacuated and flushed three times with argon to afford infiltration of the nitrogen solutions into the leaf tissue; a slight modification of the method used by Klepper (23). Evidence of infiltration was taken as bubbles forming on the leaf surface under vacuum. After drawing the third vacuum, bottles were brought to atmospheric pressure, and reopened. After the aqueous phase was poured off, the bottles were resealed under room air. Dark treatment bottles were covered with aluminum foil. All bottles were incubated under incandescent lights at 35° C.

Human breath

The effect of high nitrate/nitrite levels on N_2O in breath was examined in five individuals by comparing N_2O content of breath before and after eating. Samples of breath were obtained by having subjects hold their breath for 15-20 sec, then exhaling into the plastic inlets of one liter Saran bags (Markson Scientific Inc., Del Mar, CA) capped with rubber septa. Each person used a separate bag throughout the experiment. Bags were evacuated and flushed three times with argon between samplings to eliminate any N_2O carryover.

At two hours and one hour before eating, samples of each subject's breath were taken to provide individual background N_2O values. These two values varied little for each person; so the two values were averaged and equated to one. The data reported are the change in N_2O at each post-meal sampling, referenced to the pre-meal mean for that individual.

The five subjects ate a high NO_3^-/NO_2^- lunch of spinach-and-bacon salad. Fresh spinach is reported to contain 69-541 ppm NO_3^- on a fresh weight basis (25),

and bacon 20-50 ppm NO_2^-. Each individual ate approximately 100 g of spinach.

Analytical methods

Except for the resting cell experiments, gas sampling was done by removing 0.25 ml gas samples with 0.5 ml glass syringes fitted with 25 gauge stainless steel needles (Becton, Dickinson and Co., Rutherford, NJ). Needle tips were capped with rubber stoppers to prevent leakage until samples were analyzed.

Unless otherwise noted, injections were made onto a Perkin-Elmer Model 910 gas chromatograph, with Porapak Q columns at 50° C, and dual ^{63}Ni electron capture detectors operated at 300° C. Carrier gas was 5% CH_4-95% Ar with a flow rate of 15 ml min^{-1}. Peak areas were determined with computing integrators. The lower level detection limit for N_2O on this gas chromatograph was approx. 0.1 ng N/ml gas; for NO, the lower level detection limit was approx. 1.0 ng N/ml gas (21).

The Carle gas chromatograph used for some analyses had a Porapak Q column at 30° C and a microthermister detector. Carrier gas was helium with a flow rate of 15-19 ml min^{-1}. Its lower level detection limit for N_2O was 560 ng N/ml gas.

Presence of NO_3^-/NO_2^- in cultures was determined by spot tests with diphenylamine reagent (30). Detection limits for this reagent were 100 μM NO_3^- and 10 μM NO_2^-.

Protein determination was by Lowry method (19).

Growth of microbial cultures was monitored turbidimetrically with a spectrophotometer at 640 nm wavelength.

RESULTS

Evidence that nitrate-respiring bacteria produced N_2O is shown in Table 1. The results in Table 2 show that the presence of glucose reduced the amount of N_2O produced by the Escherichia and Enterobacter species, and that neither N_2 nor NO were produced. Failure to detect N_2 indicates that these bacteria are not denitrifiers. Absence of ^{13}N gas production by autoclaved cells confirms that the mechanism was biological. Further evidence of the non-denitrifying nature of enteric organisms and Bacillus is provided by the work of Smith (33). Whereas acetylene blocks the reduction of N_2O to N_2 by denitrifying organisms (2, 47, 48), Smith (33) found no increase in N_2O production by Citrobacter and Bacillus isolates in the presence of acetylene.

Evidence for the dissimilation of NO_3^- to NH_4^+ by nitrate-respiring bacteria is shown in Table 3. Under these conditions N_2O did not constitute more than 5% of the ^{13}N gaseous products (Table 2). Ammonium was the major product (Table 3), and label associated with the cells was insignificant.

Growth studies with two enteric bacteria showed that they produced N_2O, but only after reaching stationary phase. This is shown by the Serratia growth study (Figure 1). Glucose slowed the rate of N_2O production in Escherichia coli (Table 4). With glucose, production of N_2O after 2 days was slightly more than that after 5 h without glucose.

The yeasts, like the enteric bacteria, produced N_2O only in the stationary phase. Hansenula started producing N_2O a few hours after growth ceased (Table 4), but its production was three orders of magnitude below that of the enteric bacteria.

Generally, the enteric bacteria produced micromolar

Table 1. Production of nitrous oxide by several bacteria.[a]

Organism	% NO_3^--N recovered as N_2O-N[b]	
	12 h	2.5 days
Escherichia coli	16	36
Klebsiella pneumoniae	11	30
Erwinia caratovora	3	19
Serratia marcescens	5	12
Enterobacter aerogenes	10	6
Bacillus subtilis	5	3

[a]Grown in 3% tryptic soy broth with 3.5 mM KNO_3.

[b]Analyses done with a Carle gas chromatograph with microthermister detector.

Table 2. Production of ^{13}N gases by enteric bacteria as influenced by glucose.

Organism	Glucose	$^{13}N_2O$ (counts)	$^{13}N_2 + {}^{13}NO$ (counts)
Escherichia coli	---	25,581	0
Escherichia coli	+	0	0
Escherichia coli, autoclaved	---	0	0
Enterobacter aerogenes	---	19,237	0
Enterobacter aerogenes	+	263	0

Table 3. Reduction of $^{13}NO_3^-$ and $^{13}NO_2^-$ to $^{13}NH_4^+$ by several bacteria.[a]

Sample	Ratio of ^{13}N ions found		
	$^{13}NH_4^+$	$^{13}NO_2^-$	$^{13}NO_3^-$
Source	0	20	80
Enterobacter aerogenes, autoclaved	0	22	78
Escherichia coli	100	18	82
Enterobacter aerogenes	100	0	0
Klebsiella pneumoniae	100	0	0
Bacillus subtilis	100	0	0
Erwinia caratovora	100	0	0

[a]Cultures incubated 20 min with $^{13}NO_3^-$ diluted with 10 uM unlabeled KNO_3. Due to different geometries and efficiencies of the several ^{13}N detectors, it was difficult to achieve accurate mass balances for the added ^{13}N; the recoveries of ^{13}N as $\frac{^{13}N_2O + ^{13}NH_4^+ \text{ produced}}{^{13}NO_2^- + ^{13}NO_3^- \text{ added}}$ were generally 0.7 to 1.1.

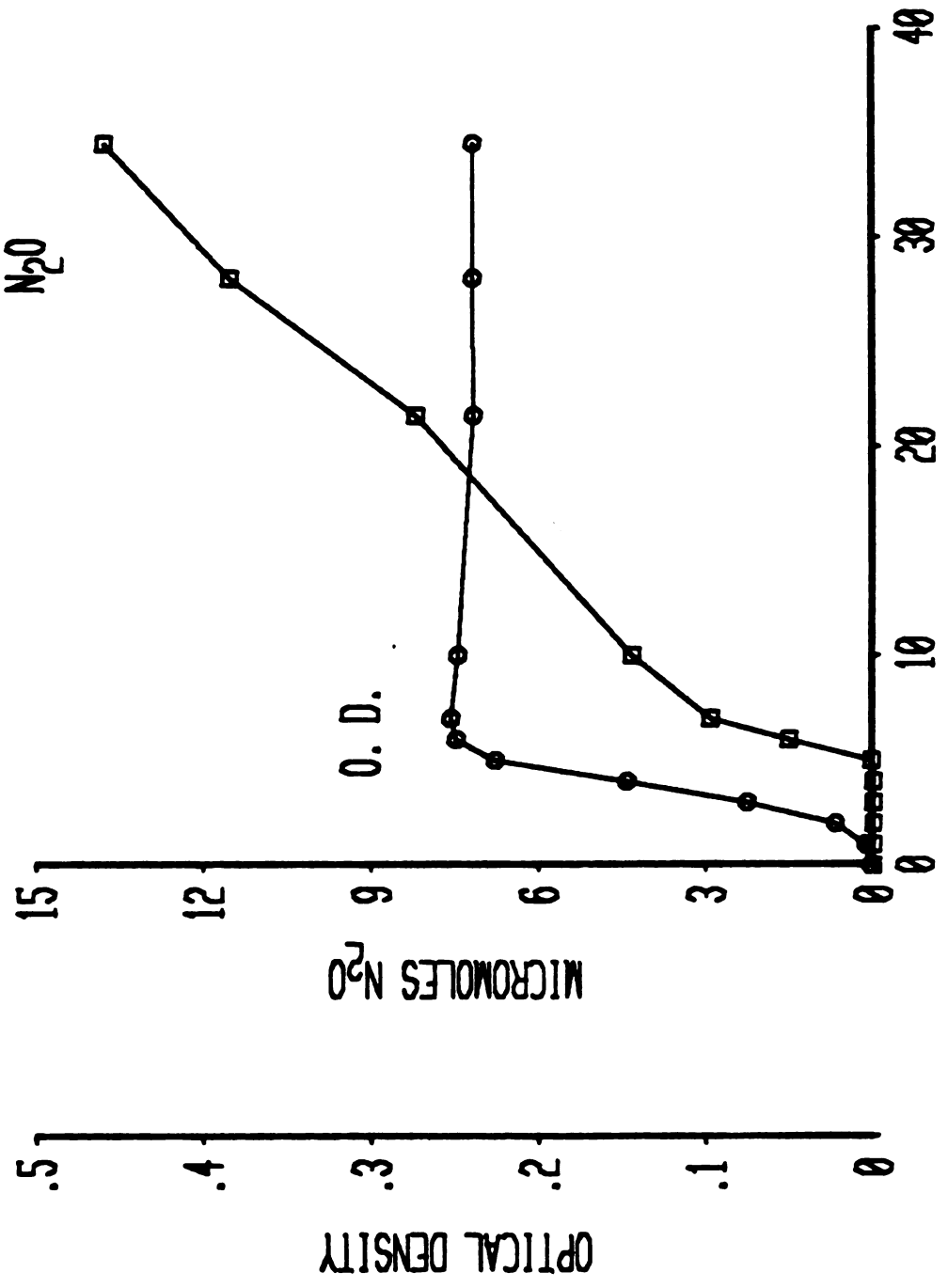

Figure 1. Relation between N_2O production and phase of growth in Serratia marcescens.

Table 4. Production of N_2O in early stationary phase by several microbes in complex media containing 5 mM KNO_3.

Organism and medium[a]	Carbon source	Time in stationary phase (hours)[b]	μ moles N_2O	Conversion of NO_3^--N to N_2O-N (%)
Escherichia coli K 12	No glucose	2	4.5 (0.88)[c]	2.2
in tryptic soy broth		5	6.8 (1.4)	3.4
		11	15 (0.34)	7.5
	Glucose[d]	20	2.5 (1.7)	1.2
		32	5.6 (0.57)	2.8
		55	8.1 (2.4)	4.1
Serratia marcescens	No glucose	1	2.9 (0.63)	1.5
in tryptic soy broth		4	4.3 (0.75)	2.2
		16	8.2 (1.4)	4.1
		29	14 (2.4)	6.9
	Glucose	0	2.3 (0.003)	1.2
		1	3.8 (0.64)	1.9
		10	7.9 (1.7)	4.0
Hansenula sp.	Glucose[e]	4	0	0
in potato dextrose broth		17	0.0021 (0.0015)	0.0085
		28	0.0039 (0.0018)	0.016
Vibrio succinogenes	Na formate[f]	2	0	0

[a]Bacteria incubated at 31°C; yeasts incubated at 25°C; all cultures shaken at 100 rpm.
[b]No appreciable N_2O detected during exponential phase of growth.
[c]Values are means, + (in parentheses) standard deviations.
[d]Contained 0.25% (w/v) glucose.
[e]Contained 2% (w/v) glucose.
[f]Contained 0.6% (w/v) Na formate.

amounts of N_2O concomitantly with the onset of stationary phase. For other organisms, the production was in nanomolar quantities, and the production started a few days after growth ceased. Rhodotorula, for example, did not produce N_2O until about 3-4 days after entering stationary phase (Figure 2). It did not produce much more N_2O at 31° C than at 25° C (Table 5). Failure of autoclaved Rhodotorula cells to produce N_2O indicates that the mechanism of N_2O production by this yeast was biological (Table 5).

All the yeasts were grown both aerobically and anaerobically; tabulated results are for aerobically grown cells, since only these incubations resulted in N_2O production. Candida was the only yeast to reach high and roughly equal densities (O. D. 1.0 aerobically and 0.92 anaerobically) under both conditions. It was also the only yeast that did not produce N_2O (Table 5). Hansenula and Rhodotorula both achieved far higher cell densities when grown aerobically than anaerobically (O. D. 1.1 vs 0.49, and 0.93 vs 0.33, respectively), and both produced N_2O (Table 5).

The fungi seemed to have the weakest N_2O generating ability of any group studied (Table 6). The initial amounts of N_2O assayed in these cultures did not increase much over time.

When grown in potato dextrose broth, every N_2O-producing organism still had NO_3^- or NO_2^- left at termination of the assay. But when Hansenula, Aspergillus and Alternaria were grown in a NH_4^+-free synthetic medium, NO_3^- and NO_2^- were consumed completely, and no N_2O was formed. Only after Hansenula received additional NO_2^- did N_2O production start. This may indicate that potato dextrose broth is high enough in reduced nitrogen compounds so that nitrate and nitrite were not assimilated into cell mass.

Table 5. Production of N_2O in late stationary phase by several yeasts in media containing glucose and 5 mM KNO_3.

Organism and medium[a]	Glucose concentration (% w/v)	Time in stationary phase (days)	n moles N_2O	Conversion of NO_3^--N to N_2O-N (%)
Hansenula sp. in potato dextrose broth	2	4	4.8 (3.2)[b]	0.019
		8	5.0 (2.9)	0.020
		10	5.8 (4.0)	0.023
		11	6.0 (3.0)	0.024
		36	22 (3.0)	0.089
in synthetic NH_4^+-free medium	1	18[c]	7.4 (2.6)	0.029
Rhodotorula sp. in potato dextrose broth	2	7	2.4 (1.7)	0.010
		9	5.8 (3.4)	0.023
		11	5.7 (1.8)	0.023
		2[d]	14 (5.7)	0.0071
		5	35 (14)	0.017
		10	61 (22)	0.030
Rhodotorula sp., autoclaved in potato dextrose broth[e]	2	6	0	0
Candida tropicalis in potato dextrose broth	2	15	0	0
in synthetic NH_4^+-free medium	1	8	0	0

[a] Incubated at 25°C unless otherwise noted.
[b] Values are means, ± (in parentheses) standard deviations.
[c] Tubes at 10 days had produced no N_2O, and diphenylamine test showed no NO_3^- or NO_2^- remained; so 0.5 ml of sterile 5 mM $NaNO_2$ was added to each tube on day 10. Amount of N_2O shown is for 11 days after addition of nitrite.
[d] Incubated at 31°C.
[e] Autoclaved soon after beginning of stationary phase.

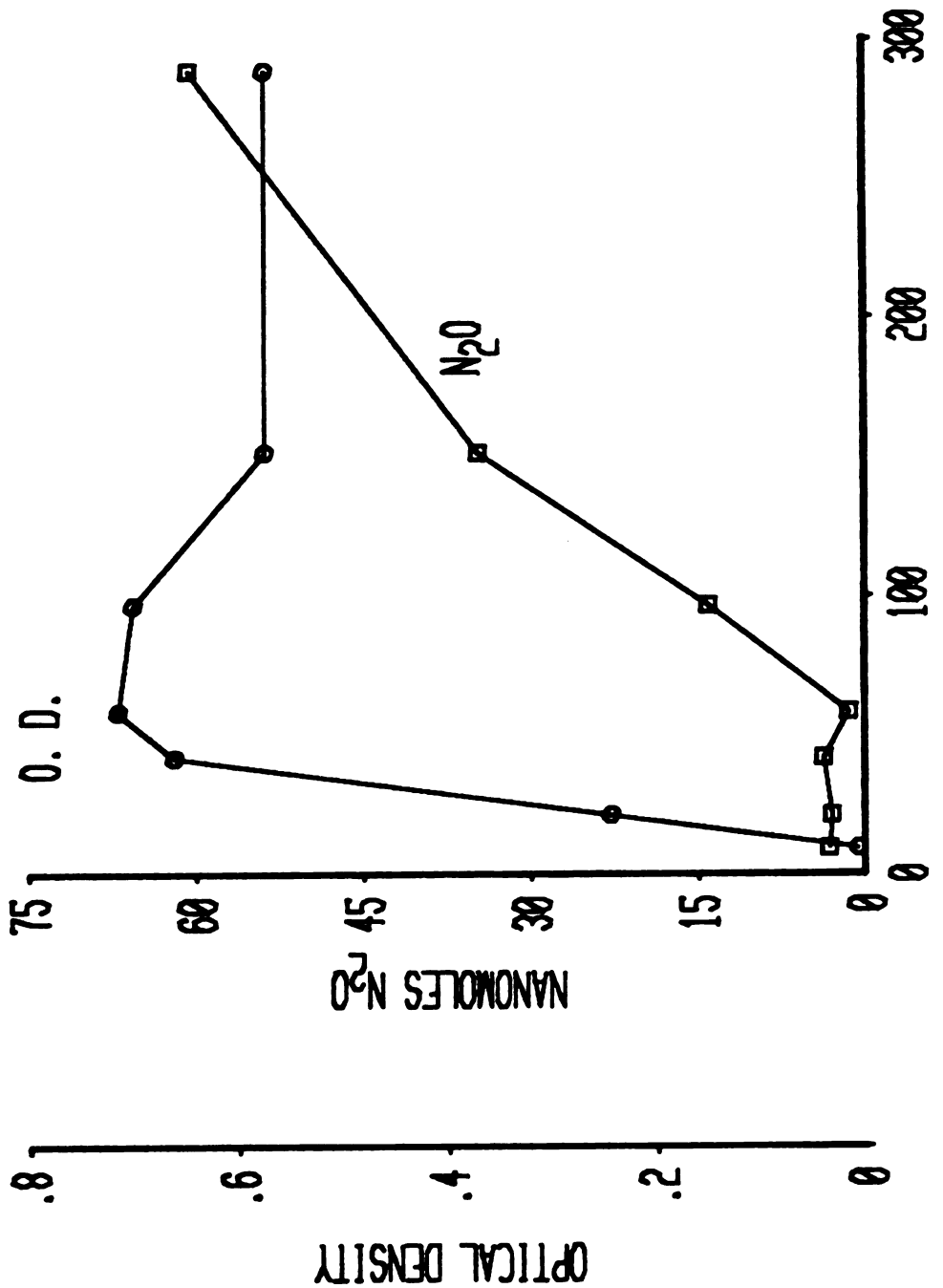

Figure 2. Relation between N_2O production and phase of growth in Rhodotorula sp.

Table 6. Production of N_2O in late stationary phase by several fungi in media containing glucose and 5 mM KNO_3.

Organism and medium[a]	Glucose concentration (% w/v)	Time in stationary phase (days)[b]	n moles N_2O	Conversion of NO_3^--N to N_2O-N (%)
Aspergillus sp. in potato dextrose broth	2	11	2.8 (1.6)[c]	0.011
		21	2.8 (1.5)	0.011
		39	3.6 (1.6)	0.014
in synthetic NH_4^+-free medium	1	15	0	0
Alternaria sp. in potato dextrose broth	2	21	1.8 (0.63)	0.007
		70	3.5 (1.1)	0.014
		90	3.9 (1.3)	0.015
in synthetic NH_4^+-free medium	1	16	0	0
Fusarium sp. in potato dextrose broth	2	90	3.9 (0.72)	0.016
Helminthosporium sp. in potato dextrose broth	2	90	0	0
Actinomucor elegans in potato dextrose broth	2	90	0	0
Penicillium sp. in potato dextrose broth	2	90	0	0

[a]All incubations at 25°C. Fungi in potato dextrose broth were stationary for most of incubation, but shaken at 100 rpm for at least 15 minutes before gas sampling. Cultures in synthetic medium were shaken continuously from time of inoculation.

[b]Time after which no further growth became evident in the tubes.

[c]Values are means ± (in parentheses) standard deviations.

The two obligate anaerobes, Vibrio (Table 4) and Clostridium (Table 7) failed to produce N_2O. The Acinetobacter (Table 7) and several fungi (Table 6) did not produce N_2O, either. The role of ammonium as a regulator of N_2O production is not obvious from these results.

All the Azotobacter cultures had NO_3^- or NO_2^- remaining at the end of the experiment. But the three A. vinelandii strains produced N_2O in stationary phase (Table 7), while the A. macrocytogenes strains produced none.

Rates of N_2O production by resting cells of several of the N_2O producing cultures are summarized in Table 8. After addition of nitrite, most of the organisms exhibited a linear rate of N_2O production, followed by a plateau region. The linear regions were used to estimate rates of N_2O production. When K. pneumoniae was given a second nitrite addition, it exhibited another linear rise, then leveled off again (Figure 3). This was interpreted to mean that all the NO_2^- had been dissimilated to NH_4^+ and N_2O by the time a plateau was reached. The data confirmed that the organisms studied produced N_2O but did not consume it.

The rate studies were done in the recirculating system of the gas chromatograph, where O_2-free conditions can be carefully maintained and monitored (21). This is necessary for a sensitive assay of NO, since NO quickly breaks down when it reacts with O_2 (15). None of the organisms exhibited any measurable NO production.

Plant tops incubated in bottles often produced N_2O (Table 9). Most long-term anaerobic incubations produced more N_2O than short-term ones. Studies which included leaves treated with chloramphenicol

Table 7. Production of N_2O in late stationary phase by several NO_3^--reducing bacteria in synthetic media containing 5 mM KNO_3.

Organism and medium[a]	Carbon source	Time in stationary phase (days)	n moles N_2O	Conversion of NO_3^--N to N_2O-N (%)
Azotobacter vinelandii nif 12	Glucose[b]	12	12 (8.3)[c]	0.047
in Burk's medium		19	35 (49)	0.14
Azotobacter vinelandii rif^{r}nif 64	Glucose	12	16 (9.0)	0.064
in Burk's		19	18 (11)	0.070
Azotobacter vinelandii 12837	Glucose	12	11 (9.0)	0.045
in Burk's		19	13 (10)	0.052
Azotobacter macrocytogenes 9129	Glucose	19	0	0
Azotobacter macrocytogenes 8700	Glucose	19	0	0
in Burk's				
Acinetobacter sp.	Na-Acetate[d]	8	0	0
in synthetic NH4+-free medium				
Clostridium KDHS2	Glucose[f]	24	0	0
in synthetic medium[e]				

[a]All incubated at 30°C; cultures shaken at 100 rpm.
[b]Contained 0.4% (w/v) glucose.
[c]Values are means, ± (in parentheses) standard deviations.
[d]Contained 0.2% (w/v) Na acetate.
[e]Reduced NO_3^- in a dissimilatory manner. All other organisms were assimilatory NO_3^--reducers.
[f]Contained 0.8% (w/v) glucose.

Table 8. Rates of N_2O production from NO_2^- by several microbes.

Organism	Rate of N_2O production (n mol N_2O min^{-1} mg $protein^{-1}$)[a]
Escherichia coli K12	0.28
Klebsiella pneumoniae	0.14
Enterobacter aerogenes	0.11
Hansenula sp.	0.04

[a]Protein was measured by the method of Lowry (19), with bovine serum albumin as the standard.

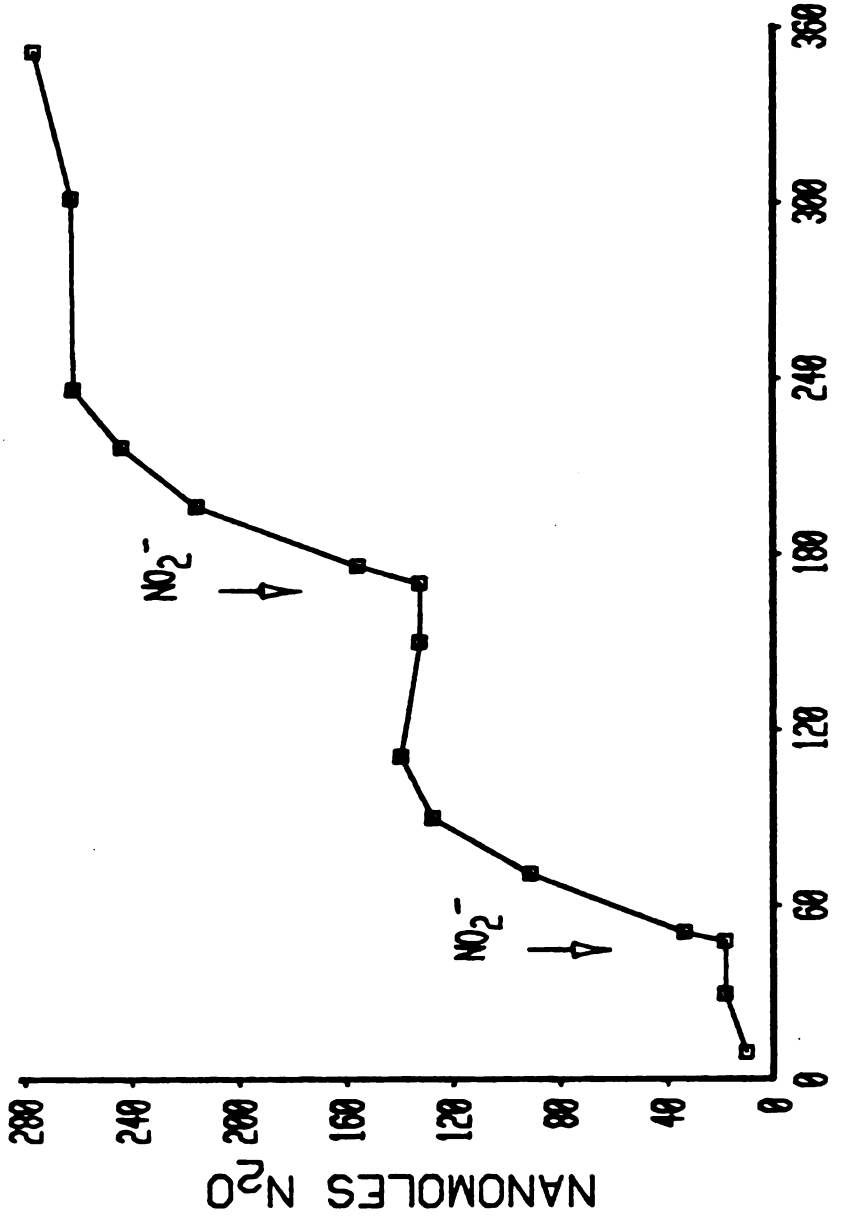

Figure 3. Pattern of N_2O production from NO_2^- by resting cells of Klebsiella pneumoniae taken in early stationary phase. Arrows indicate points of NO_2^- addition.

Table 9. Production of N_2O by plant tops.

Species[a]	Atmosphere	Incubation period (hours)	nmoles N_2O in bottle
Amaranthus retroflexus (Redroot pigweed)	Air	8[b]	18
Capsella bursa-pastoris (Shepherd's purse)	Air	6	23
Rumex sp. (Dock)	Argon	72	164
Plantago sp. (Plantain)	Argon	72	157
Stellaria media (Common chickweed)	Argon	72	102
Acer negundo (Boxelder)	Argon	72	3
Atmosphere	Argon		1

[a]All incubations at 32°C. Each bottle contained one plant top.

[b]Incubated under incandescent lights. All others incubated in the dark.

seemed to bear out that microbes, not the plant tissue itself, were producing N_2O (Table 10). In the diced leaf experiments, the illuminated samples produced less N_2O than did the dark incubations. This may have been due to production of O_2 by the chloroplasts in the illuminated samples, which could inhibit NO_3^- reduction.

Results of the human breath experiment are shown in Table 11. Random spot testing of people's breath had previously shown that some samples exceeded ambient atmospheric N_2O levels. This experiment was designed to see if a meal high in NO_3^-/NO_2^- could raise the N_2O levels of breath. A statistically significant increase in breath N_2O content was noted after the meal. The most dramatic increase seen in a subject is shown in Figure 4.

DISCUSSION

The Bacillus and enteric bacteria that dissimilated nitrate to ammonium in tryptic soy broth were the most rapid and prolific producers of N_2O from NO_3^-. N_2O production by Escherichia and Enterobacter was slowed in the presence of glucose. Such an effect makes sense if the glucose allowed fermentation to proceed to a greater extent, delaying the onset of stationary phase and N_2O production. Smith (32) found this effect in tryptic soy broth, but not in nutrient broth. It would appear that the effect of glucose upon N_2O production by enteric bacteria can vary with nutrition.

Every organism which produced N_2O did so only after growth had ceased. In addition, the presence of reduced forms of nitrogen did not seem to affect N_2O production. The production of N_2O did not seem

Table 10. Influence of chloramphenicol on N_2O production by diced spinach leaves in air.

Incubation length (hours)	Treatment	N_2O production (nmoles per bottle)	
		Without chloramphenicol	With chloramphenicol[a]
9	Light[b]	0	0
	Dark[c]	0	0
15	Light	32 (51)[d]	0
	Dark	158 (93.6)	0.002 (0.0006)
21	Light	not done	0
	Dark	not done	2.2 (0.76)

[a]200 μg ml^{-1} chloramphenicol.
[b]Bottles in growth chamber at 32°C with two incandescent lights.
[c]Bottles covered with aluminum foil in the growth chamber.
[d]Values are means, $\pm$ (in parentheses) standard deviations.

Table 11. Levels of N_2O in human breath before and after a meal containing NO_3^- and NO_2^-.

Time (hours)	N_2O	Level of significance (%)[a]
--Before eating--		
-2	1 (0.06)[b]	
-1	1 (0.06)	
--After eating--		
1	1.30 (0.25)	90
2	1.37 (0.46)	80
3	1.32 (0.28)[c]	80

[a]Evaluated by two-tailed t test.

[b]Values are means $\pm$ (in parentheses) standard deviations.

[c]Calculated from four subjects; all other values from five subjects.

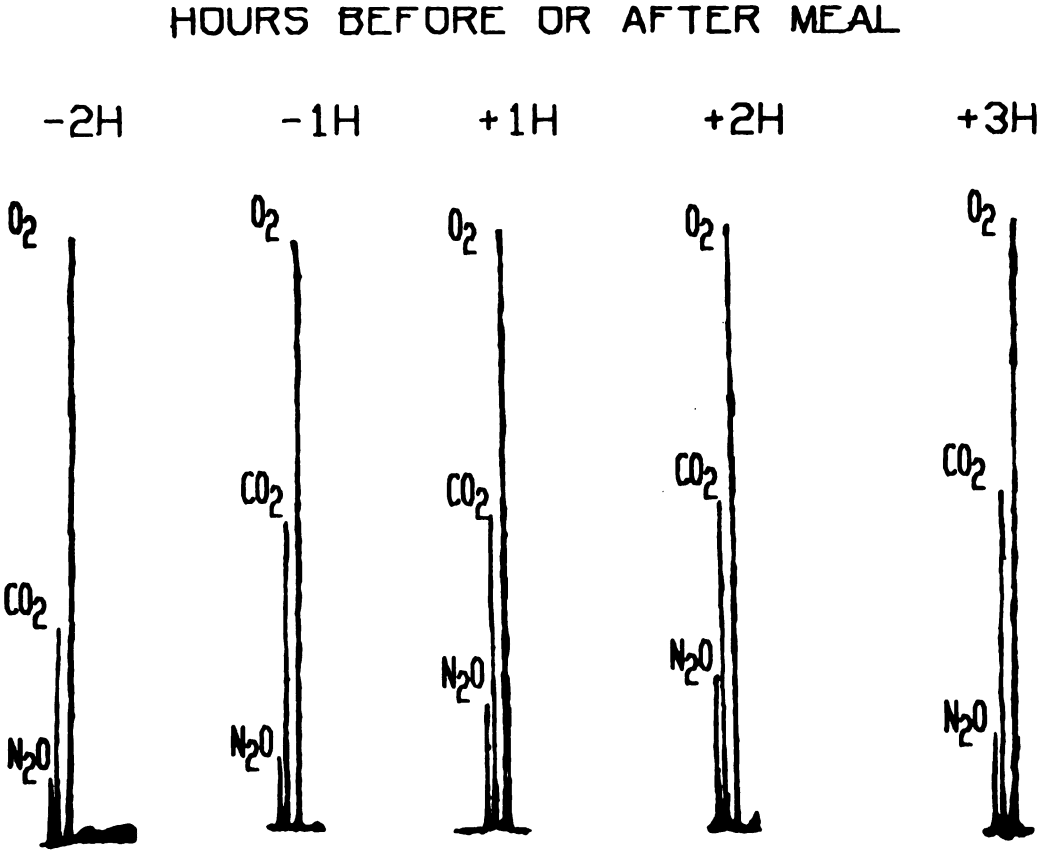

Figure 4. N_2O content of breath before and after ingesting a meal high in NO_3^- and NO_2^-.

to be related to the assimilatory NO_3^--reduction pathway, since this pathway is repressed by NH_4^+ (29). Instead, nitrous oxide could be produced by enzymes independent of any previously described. Smith (32) found that chloramphenicol prevented the induction of N_2O-producing activity in a Citrobacter soil isolate, indicating that it produces N_2O enzymatically. He also found that three E. coli mutants lacking NADH-dependent dissimilatory nitrite reductase produced N_2O at rates equal to the wild type, but released NH_4^+ at a much slower rate. Satoh et al. (31) isolated mutant strains of K. pneumoniae that were defective in the reduction of NO_2^- to NH_4^+, but which produced N_2O at rates comparable to the wild type. These findings suggest that N_2O is not a side product of dissimilatory nitrite reduction to ammonium.

Although the mechanism of N_2O production is uncertain, the fact that it is produced only in stationary phase suggests that it may be produced by enzymes of secondary metabolism. Since most soil microorganisms grow very slowly, existing essentially in stationary phase, N_2O production in nature by the microbes I examined seems reasonable.

The level of detection for NO on the gas chromatograph should have allowed me to detect 2% conversion of NO_2^--N to NO-N. However, no strong evidence for NO was found. This indicates that free NO is not involved in the pathway to N_2O of these organisms; whereas NO has been found under the same assay conditions for denitrification (3).

If any of the plant materials had begun production of N_2O soon after incubation started, the role of the plant itself might have been more at issue. But production of N_2O was never noticeable before 6 to 8 h,

most likely because it was not until then that microbial populations reached adequate levels to produce detectable amounts of N_2O from nitrate present in plant tissue. The role of microbes in producing N_2O from plants was best supported by the chloramphenicol experiment, in which inclusion of chloramphenicol with the nitrite solution prevented significant N_2O production.

The plant tissue examined was damaged. The act of pushing the plant tops through the narrow mouths of serum bottles almost always resulted in visible bruising or laceration of the plant tissue. In the diced leaf experiments the tissue was exposed to a vacuum, also causing damage. Damaged plant tissue may be the rule in nature rather than the exception, due to insects, winds and other influences. Making a cautious extrapolation to field situations, it may be that microbes growing upon damaged plants can account for some N_2O production. In a recent study (17), 167 strains of Serratia were isolated from 623 plant samples. If such bacteria were to colonize damaged plant tissue rich in nitrates, the release of N_2O from within anaerobic sites in plant tissue might ensue.

Might plant tissue ever produce N_2O by itself? Using the microbial studies as a model, perhaps the ability exists in some plant tissues at a physiological stage corresponding to stationary phase in microbes. Autumn might be the best time to look for such activity, in senescing plant tissues.

The analysis of breath samples showed that the level of N_2O in human breath can rise significantly above that of the atmosphere. The point in the human body from which N_2O originates is problematic. Microbial flora in the gut or oral cavity could be

two possible sources; different diets and levels of dental hygiene may account for differences in N_2O levels in the breath of different people. Perhaps the purported nitrifying activity of human tissue itself (43) can lead to release of N_2O.

This study found that N_2O can be released from microbial, plant and animal sources. It is possible that microbes are the true producers in each case. The flux of N_2O from these sources may significantly contribute to the N_2O flux into the Earth's atmosphere. Although the percentages of N_2O these sources produce are small, the extent of these sources is large.

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APPENDIX

APPENDIX

FATE OF $^{15}NO_3^-$ IN TWO COMPLEX MEDIA USED FOR MPN ESTIMATES OF SOIL DENITRIFIER POPULATIONS

The most-probable-number (MPN) method allows estimation of microbial populations by the use of dilution series. Given the highest dilution at which positive results are seen, statistical tables allow one to say what the most probable number of organisms in the inoculum was (1).

I participated in an effort undertaken by the laboratory to validate the best method of providing an estimate of soil denitrifier populations by the MPN procedure. The procedure developed is as follows: 10 g of refrigerated or fresh soil was placed in a blender containing 90 ml of 0.85% sterile saline solution plus one drop of Tween 80. The suspension was blended for 2 min, poured into a dilution bottle with a rubber stopper, and shaken to ensure suspension of the soil. One milliliter was withdrawn by syringe and injected into 9 ml of sterile saline solution. This tube was labeled the 10^{-2} dilution. One milliliter of its suspension was removed and injected into 9 ml of sterile saline solution, designated the 10^{-3} dilution. This procedure was continued until the 10^{-6} dilution was reached, so that five tubes containing dilutions of soil in saline solution were prepared. These were used to inoculate a five tube dilution series. One tenth milliliter was withdrawn from each saline dilution and used to inoculate 10 ml of sterile medium. The 10^{-2} saline

dilution was used to inoculate the five 10^{-3} culture tubes, and so on.

The media was contained in Hungate tubes, allowing injection of sterile acetylene into the tubes before inoculation with soil. Acetylene inhibits nitrous oxide reduction by denitrifying bacteria (2, 6, 7), and is used to confirm that the NO_3^- is removed by denitrification.

Tubes were incubated in stationary position at 20-30° C for 14 days. Tubes were then assayed for N_2O production with a Carle gas chromatograph having a Porapak Q column at 30° C and a microthermister detector. The carrier gas was helium with a flow rate of 15-19 ml min^{-1}. Later, the medium in the tubes was assayed with diphenylamine reagent (4) for the presence of NO_3^-/NO_2^-.

After many trials, it was found that two media, tryptic soy broth and nutrient broth, afforded high estimates of soil denitrifier populations. However, when the two media were inoculated with the same soil, there would often be an order of magnitude difference between the populations estimated by each.

The fate of nitrate in the two media appeared to be different. Usually if 20% or more of the NO_3^--N in a nutrient broth tube was recovered as N_2O-N, all the NO_3^-/NO_2^- was gone. However, in tubes of tryptic soy broth, although NO_3^-/NO_2^- would be totally consumed in a tube, the amount of NO_3^--N converted to N_2O-N could vary widely, but was usually well below 20% of the NO_3^--N.

To see if the two media did indeed promote two different fates of nitrate, an experiment using ^{15}N as a tracer was designed. The MPN procedure was conducted as previously described, except that $K^{15}NO_3$ was used.

The soil used as an inoculum was a Sloan loam (Fluventic Haplaquoll).

After 14 days incubation, the tubes were analyzed for N_2O production by gas chromatography. After steam distillation, cultures were assayed for the total amount of NH_4^+ present by Solorzano method (5). Ratio mass spectroscopy was then performed as described in (3), to find the amount of $^{15}NO_3^-$ that was converted to $^{15}NH_4^+$.

The results are shown in Table 12 and Table 13. Cultures in nutrient broth (Table 12) consistently produced a considerable amount of N_2O, with little ammonium being formed from nitrate as a rule. Tryptic soy broth, on the other hand (Table 13), usually fostered the production of large amounts of ammonium, but little N_2O, from nitrate.

The correlation between 20% or more of NO_3^--N converted to N_2O-N, and the disappearance of NO_3^-/NO_2^- was very good in nutrient broth (Table 12), making nutrient broth the medium of choice for enumerating soil denitrifier populations.

The correlation between appearance of N_2O and disappearance of NO_3^-/NO_2^- was not good in tryptic soy broth (Table 13). The large amounts of ammonium produced from nitrate in these tubes indicated that this medium selected for dissimilatory ammonium-producing bacteria instead of denitrifiers.

Each medium may prove useful to enumerate a different population in soil; nutrient broth to enumerate denitrifiers, and tryptic soy broth to enumerate bacteria that dissimilate nitrate to ammonium.

Table 12. ^{15}N MPN experiment in nutrient broth using Sloan loam, with 14 day incubation at 25°C.

Dilution	Tube	Nutrient broth + 5 mM KNO_3 + 1 ml acetylene[a]				
		% conversion of NO_3^--N to N_2O-N	% conversion of $^{15}NO_3$-N to $^{15}NH_4$-N	mg NH_4-N/tube[b]	% recovery of NO_3^- as (N_2O + NH_4)	Presence (-) or absence (+) of NO_3^- and/or NO_2^- at 2 weeks[c]
10-3	1	74.0	7.2	.8	81.2	+
	2	75.1	3.8	.6	78.9	+
	3	74.8	11.4	1.1	86.2	+
	4	77.0	7.9	.87	84.9	+
	5	76.9	11.6	1.2	88.5	+
10-4	1	73.3	5.1	.9	78.4	+
	2	82.6	3.3	1.0	85.9	+
	3	74.6	4.1	1.0	78.7	+
	4	74.5	3.7	1.2	78.2	+
	5	65.0	17.9	2.1	82.9	+
10-5	1	67.6	20.0	7.0	87.6	+
	2	71.1	6.3	2.6	77.4	+
	3	65.1	19.1	2.8	84.2	+
	4	75.4	7.8	3.1	83.2	+
	5	6.4	3.3	1.9	9.7	-
10-6	1	86.0	-[d]	-	86.0	+
	2	1.7	0.3	1.0	2.0	-
	3	0.4	0	0.4	0.4	-
	4	0	0.1	0.4	0.1	-
	5	3.8	2.5	1.9	6.3	-
10-7	1	0.1	0	0.4	0.1	-
	2	0.1	0	0.4	0.1	-
	3	0	0	0.4	0	-
	4	0	0	0.8	0	-
	5	0	0	0.3	0	-

[a]1 ml acetylene added to 6 ml headspace over 10 ml medium.
[b]Determined by method of Solorzano (5).
[c]Determined by diphenylamine reagent (4).
[d]Tube was broken before analysis.

Table 13. ^{15}N MPN experiment in tryptic soy broth using Sloan loam, with 14 day incubation at 25°C.

Dilution	Tube	Tryptic soy broth + 0.25% (w/v) glucose + 5 mM KNO_3 + 1 ml acetylene[a]				
		% conversion of NO_3^--N to N_2O-N	% conversion of $^{15}NO_3$-N to $^{15}NH_4$-N	mg NH_4-N/tube[b]	% recovery of NO_3^- as (N_2O + NH_4)	Presence (-) or absence (+) of NO3- and/or NO2- at 2 weeks[c]
10-3	1	5.2	72.1	3.6	77.3	+
	2	5.0	59.3	5.2	64.3	+
	3	5.4	83.9	4.0	89.3	+
	4	5.8	81.8	4.1	87.6	+
	5	5.1	77.9	4.2	83.0	+
10-4	1	3.1	65.3	4.9	68.4	+
	2	5.7	53.5	6.1	59.2	+
	3	9.6	72.8	12.8	82.4	+
	4	5.4	85.2	6.6	90.6	+
	5	6.1	72.2	8.7	78.3	+
10-5	1	2.7	49.5	6.9	52.2	+
	2	16.5	55.3	5.9	71.8	+
	3	1.9	75.7	4.3	77.6	+
	4	3.4	84.0	8.4	87.4	+
	5	1.3	47.0	7.7	48.3	+
10-6	1	87.3	0	0.4	87.3	+
	2	2.2	47.0	7.7	49.2	+
	3	6.6	87.4	18.4	94.0	+
	4	12.4	79.3	21.7	91.7	-
	5	5.1	89.4	17.9	94.5	+
10-7	1	0	0.1	0.4	0.1	-
	2	0.1	0	0.4	0.1	-
	3	0	0	0.4	0	-
	4	0	0.1	0.4	0.1	-
	5	0	0	0.4	0	-

[a]1 ml acetylene added to 6 ml headspace over 10 ml medium.

[b]Determined by method of Solorzano (5).

[c]Determined by diphenylamine reagent (4).

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