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Nitrous Oxide Production by Organisms Other than Nitrifiers or Denitrifiers†

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Heterotrophic bacteria, yeasts, fungi, plants, and animal breath were investigated as possible sources of N_2O. Microbes found to produce N_2O from NO_3^- but not consume it were: (i) all of the nitrate-respiring bacteria examined, including strains of *Escherichia, Serratia, Klebsiella, Enterobacter, Erwinia*, and *Bacillus*; (ii) one of the assimilatory nitrate-reducing bacteria examined, *Azotobacter vinelandii*, but not *Azotobacter macrocytogenes* or *Acinetobacter* sp.; and (iii) some but not all of the assimilatory nitrate-reducing yeasts and fungi, including strains of *Hansenula, Rhodotorula, Aspergillus, Alternaria*, and *Fusarium*. The NO_3^--reducing obligate anaerobe *Clostridium* KDHS2 did not produce N_2O. Production of N_2O occurred only in stationary phase. The nitrate-respiring bacteria produced much more N_2O than the other organisms, with yields of N_2O ranging from 3 to 36% of 3.5 mM NO_3^-. Production of N_2O was apparently not regulated by ammonium and was not restricted to aerobic or anaerobic conditions. Plants do not appear to produce N_2O, although N_2O was found to arise from some damaged plant tops, probably due to microbial growth. Concentrations of N_2O above the ambient level in the atmosphere were found in human breath and appeared to increase after a meal of high-nitrate food.

Interest in sources of nitrous oxide was stimulated by recognition that an increase in the concentration of this trace gas in our atmosphere could result in enhanced destruction of the ozone layer (7, 18) or an increased temperature of the planet due to the "greenhouse" effect of N_2O, or both (29). Analysis of recent data, however, suggests that the effect of N_2O on the ozone concentration is likely to be much less important than originally thought (8).

Denitrifying and nitrifying bacteria (1, 9) are generally recognized as producers of N_2O, but the capacity of other organisms to produce N_2O has not been widely investigated. Yoshida and Alexander (30) and Bollag and Tung (2) had noted earlier that a few nondenitrifying heterotrophic bacteria and fungi could produce N_2O, but the significance of these observations was not apparent at that time, and the phenomenon was not further investigated. More recently, when we were evaluating most-probable-number methods for denitrifiers by using N_2O as direct evidence for these organisms, we noted that nondenitrifiers also produced N_2O. This report is a follow-up on our preliminary reports on this finding (N. V. Caskey, M. S. Smith, W. H. Caskey, and J. M. Tiedje, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, N47, p. 170; J. M. Tiedje, N. V. Caskey, M. S. Smith, B. H. Bleakley, and R. B. Firestone, Agron. Abstr. 1979, p. 165) and indicates the extent of N_2O production by several groups of organisms.

Recently, Smith and Zimmerman (25) examined 214 of the numerically dominant nitrate reducers in soil and found that 98% produced N_2O. These organisms could not reduce N_2O but could produce NH_4^+ by a dissimilatory mechanism (not repressed by NH_4^+). Two of these isolates, a species of *Bacillus* and of *Citrobacter*, when added to autoclaved soil, produced more N_2O than denitrifiers; this demonstrated that they can be a potential N_2O source in their habitat. Even though the organisms studied by Smith and Zimmerman and the ones we report on here produce N_2O from NO_3^-, we have not termed them denitrifiers to avoid confusion with the process that microbiologists generally recognize as denitrification. Conventional denitrifiers have distinctive enzymes that reduce the N oxides usually to N_2 and couple this reduction to electron transport phosphorylation (20, 21). Denitrifiers also typically convert most of the N

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anion to gas and cannot ferment, and the denitrification process is inhibited by oxygen. These do not appear to be characteristics of these additional, diverse, N_2O-producing organisms.

MATERIALS AND METHODS

Microorganisms studied. The following organisms were obtained from the Michigan State University Department of Microbiology culture collection: *Escherichia coli* K-12, *Serratia marcescens*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Bacillus subtilis*, and *Acinetobacter* sp. *Erwinia carotovora*, a plant pathogen, was provided by the laboratory of A. Saettler. *Clostridium* KDHS2 was isolated from soil by W. H. Caskey (5). H. Sadoff provided cultures of *Azotobacter vinelandii* strain 12837 and *Azotobacter macrocytogenes* strains 8700 and 9129. The following fungi and yeasts were obtained from 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 in 300-ml Erlenmeyer flasks which contained 250 ml of 3% tryptic soy broth without glucose (TSB) and 3.5 mM KNO_3. The flasks were sealed with butyl rubber stoppers. The culture shifted from aerobic to anaerobic conditions during growth, which was at 30°C on a rotary shaker. After 15 h, stationary-phase cells were harvested by centrifugation, washed in 0.05 M Tris-maleate buffer (pH 7.0), and resuspended to a 10-ml volume. Cell suspensions of 0.5 ml were injected by syringe into serum vials containing 4% TSB without nitrate, under a helium headspace, with 2×10^{-4} M Ti (III) citrate to establish a low redox potential. Autoclaved cells were prepared in a similar manner and served as a sterile control. To initiate the experiment, $^{13}NO_3^-$-$^{13}NO_2^-$ (ca. 1 mCi) produced at the Michigan State University cyclotron (28) 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 (28). Each vial was then opened, and the medium was clarified by filtration through a 0.22-μm filter. The medium was analyzed for ^{13}N ions by radio-high-pressure liquid chromatography (28). All ^{13}N data were corrected for half-life, efficiency, and background, and the detectors were referenced against each other by using the sulfamic acid converison of $^{13}NO_2^-$ to $^{13}N_2$ (27).

Growth and N_2O production by microorganisms. Pure cultures of bacteria, yeasts, and fungi were grown in 26-ml anaerobic culture tubes (Bellco Glass, Inc., Vineland, N.J.), which contained 5 ml of the respective media. Experiments to define growth phase versus N_2O production were done in sidearm flasks with 40 ml of medium. Media were amended with 5 mM KNO_3, unless stated otherwise.

The nitrate-respiring bacteria were grown in 1.5% TSB. Potato dextrose broth was used to culture all yeasts and fungi. Selected yeasts and fungi were also grown in a defined NH_4^+-free mineral salts medium (26) on 1% glucose, amended with 10 ml/liter each of stock vitamin and trace mineral solutions (26); the pH was adjusted to 5.1 with 1 N HCl.

The *Azotobacter* strains were grown in Burk medium (19), with KNO_3 substituted for NH_4NO_3. *Acinetobacter* sp. was grown on a medium of (grams per liter): sodium 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% (vol/vol) of the same trace mineral solution as above. *Clostridium* KDHS2 was grown in the medium of Caldwell and Bryant (4), except that soluble starch and cellobiose were omitted and KNO_3 was added.

The fungi cultures, *Azotobacter* and *Acinetobacter*, were grown under air. The nitrate-respiring bacteria and the obligate anaerobe were grown under O_2-free argon, achieved by evacuating and flushing each tube three times. The yeasts were grown under both aerobic and anaerobic conditions. Inoculated tubes were positioned horizontally and shaken at 100 rpm on a rotary shaker at 25°C in the dark. Culture tubes of fungi were kept stationary, except before gas analysis, when they were shaken to ensure gaseous equilibrium.

Growth of bacteria and yeasts was monitored by measuring optical density at 640 nm. Gas samples (0.25 ml) were taken by syringe periodically during exponential and stationary phases for N_2 analysis. All data are means of five replicates, except for the nitrate-respiring organisms, which were from three replicates.

Resting cells were grown and harvested under conditions similar to those described for the ^{13}N experiment, except that the cells were washed and resuspended in fresh growth media without KNO_3 but with 200 μg of chloramphenicol per ml. *Hansenula* sp. was grown aerobically on potato dextrose broth. Flasks of resting cells were connected to the gas assay system described by Kaspar and Tiedje (12), which continuously recirculated headspace gas from the flasks through the gas chromatographic sampling loop. 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, 5 mM $NaNO_2$ was added to each culture to initiate the experiment. Headspace gas was analyzed every 20 min. At the termination of each experiment, the cells were saved for protein analysis by the Lowry method, with bovine serum albumin as the standard.

Plant studies. Seedlings of the following plants were uprooted in the field and brought to the laboratory: *Amaranthus retroflexus* (redroot pigweed), *Capsella bursa-pastoris* (Shepherd's purse), *Rumex* sp. (dock), *Plantago* sp. (plantain), *Stellaria media* (chickweed), and *Acer negundo* (box elder). The seedlings were rinsed to remove as much soil adhering to foliage as possible. Plant tops were removed and placed into 70-ml bottles, which were sealed with butyl rubber septa. Air and argon were used as headspace gases for aerobic or anaerobic conditions, respectively.

For the diced-leaf experiments, fresh spinach was purchased at local markets, rinsed under cold tap water to remove soil, and then blotted on paper towels. Leaves were then placed flat on plastic trays, covered with clear plastic wrap, and incubated in a growth chamber at 5 to 10°C under incandescent lights for 4 to 12 h (6). Selected leaves were cut into 1-cm^2 pieces, with four pieces put into each bottle. Ten milliliters of 5 mM KNO_3 was pipetted into each bottle; chloramphenicol, when included in this solu-

tion, was at 200 μg/ml. Bottles were sealed and then evacuated and flushed three times with argon to afford infiltration of the nitrogen solutions into the leaf tissue (14). The bottles were opened to pour off the aqueous phase and then resealed under room air. Dark treatment bottles were covered with aluminum foil. Incubation was 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 the N_2O content of breath before and after eating. Samples of breath were obtained by having subjects hold their breath for 15 to 20 s and then exhaling into the plastic inlets of 1-liter Saran bags (Markson Scientific Inc., Del Mar, Calif.). 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 2 and 1 h 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; therefore, the two values were averaged and equated to one. The data reported are the changes in N_2O at each postmeal sampling, referenced to the premeal 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 to 541 ppm of NO_3^- on a fresh weight basis (17), and bacon 20 to 50 ppm of NO_2^- (I. Gray, personal communication). Each individual ate approximately 100 g of spinach.

Analytic methods. Nitrous oxide was measured with 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. The carrier gas was 5% CH_4–95% Ar, with a flow rate of 15 ml/min. Peak areas were determined with computing integrators. The lower level detection limits for N_2O and NO on this gas chromatograph were 0.1 and 1.0 ng of N per ml of gas, respectively (12). The CO_2 peak was well separated from N_2O and did not interfere with N_2O quantitation.

Because of the much higher N_2O production by the nitrate-respiring bacteria, a Carle 8500 gas chromatograph with a microthermister detector was used. The carrier gas was helium, with a flow rate of 15 ml/min. The detection limit for N_2O was 500 ng of N per ml of gas. All N_2O production data include the N_2O in solution, which was obtained by calculation (26).

The presence of NO_3^--NO_2^- in cultures was determined by spot tests with diphenylamine reagent (22). Detection limits for this reagent were 100 μM NO_3^- and 10 μM NO_2^-.

RESULTS AND DISCUSSION

Which microbes produce N_2O? Representatives of most genera of nitrate-respiring bacteria were examined for their ability to produce N_2O, and all were found to produce this gas (Table 1). Particularly surprising is the high quantity produced, up to one-third of the 3.5 mM NO_3^- being converted to N_2O-N under these conditions. When $^{13}NO_3^-$ and $^{13}NO_2^-$ were added to resting cells of these organisms, most of the label was recovered as NH_4^+, but all organisms also produced labeled N_2O (Table 2), confirming that the origin of both nitrogen products was the N anions. The absence of ^{13}N gas production by autoclaved cells indicates that the reaction was catalyzed by active organisms. Neither in the ^{13}N experiments (Table 2) nor in experiments assayed by gas chromatography was N_2O reduced to N_2, as would be the case for most conventional denitrifiers. Nitric oxide was also not found to be produced in significant amounts. Since TSB has sufficient organic nitrogen plus NH_4^+ (more than 10 mM produced during incubation) to repress assimilatory nitrate reduction, both the ammonium and nitrous oxide production must occur by a dissimilatory mechanism.

The difference in the percentages of N_2O formed in the experiments in Tables 1 and 2 illustrate the range we have noted under various conditions. This variation is likely due to two competing pathways for NO_2^-, one producing N_2O and the other NH_4^+, with environmental factors affecting the partitioning between the

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

Organism	N_2O produced (μmol · tube^{-1})	% NO_3^--N recovered as N_2O-N
E. coli	3.15	36
K. pneumoniae	2.62	30
E. carotovora	1.66	19
S. marcescens	1.05	12
E. aerogenes	0.53	6
B. subtilis	0.26	3

[a] Bacteria were grown for 2.5 days in 3% tryptic soy broth with 3.5 mM KNO_3 in 5 ml of medium; autoclaved cells in the same media with NO_3^- or NO_2^- did not produce N_2O.

TABLE 2. ^{13}N-labeled products found after incubation of cells with $^{13}NO_2^-$ + $^{13}NO_2^-$[a]

Sample	% of total ^{13}N recovered				
	N_2 + NO[b]	N_2O	NH_4^+	NO_2^-	NO_3^-
^{13}N substrate	0	0	0	20	80
Escherichia autoclaved	0	0	0	22	78
Enterobacter autoclaved	0	0	0	18	82
Escherichia	0	5	95	0	0
Enterobacter	0	2	98	0	0
Serratia	0	3	97	0	0
Erwinia	0	0.05	85	15	0
Bacillus	0	4	96	0	0

[a] Stationary-phase cells were concentrated 25-fold and incubated anaerobically for 20 min with 10 μM KNO_3 + $^{13}NO_3^-$ and $^{13}NO_2^-$ in 4% tryptic soy broth.

[b] N_2 and NO, if present, would have been analyzed together, since they were not separated by the column used.

two. For example, we found that increasing concentrations of glucose added to TSB inversely affected the amount of N_2O produced (data not shown). Smith and Zimmerman (25) also noted that glucose added to TSB suppressed N_2O production but enhanced apparent NH_4^+ production in a species of *Citrobacter* and *Bacillus*. In our studies, the rate of growth and the rate of nitrate depletion were much more rapid with the added glucose, which apparently favored fermentative metabolism and NH_4^+ production. Furthermore, Smith (24) has noted that the NH_4^+-producing pathway has a much lower apparent $K_m(NO_2^-)$ than the N_2O-yielding reaction (<40 versus 900 μM, respectively). In growth experiments such as that shown in Table 1, the NO_3^- concentration was much higher than in the resting cell experiments shown in Table 2; thus, the N_2O-producing reaction may have been favored under growth conditions.

Growth studies with two enteric bacteria, *E. coli* and *Serratia* sp. (Fig. 1), showed that they produced N_2O only after reaching the stationary phase and that this production continued at a nearly constant rate for at least 36 h. Continuous N_2O production only in the stationary phase was also found by Smith and Zimmerman (25) for the *Bacillus* and *Citrobacter* strains they studied, indicating that these characteristics must be common for this mechanism of N_2O production.

Nitrous oxide production (or lack of it) by other groups of nitrate-reducing microorganisms is summarized in Table 3, in which we show representative N_2O production at one time period in stationary phase (12 days) and the maximum percentage of NO_3^--N converted to N_2O-N at the end of the incubation. Whereas the nitrate-respiring bacteria produced micromole quantities of N_2O, which started a few hours after the onset of stationary phase, these organisms (Table 3) produced only nanomole quantities, which did not appear until a few days after growth ceased. The time course for N_2O production for the yeast is illustrated for *Rhodotorula* sp. in Fig. 2. Nitrous oxide production did not begin until stationary phase, and it continued at an approximately constant rate for 12 days. The yeasts were grown both aerobically and anaerobically, but N_2O production was only observed under aerobic conditions. This finding is in contrast to the results with nitrate-respiring bacteria, which produce much more N_2O under anaerobic conditions (23, 25), but similar to those with fungi, which produce more N_2O under initially aerobic conditions (2). To verify that the N_2O production was catalyzed by active cells, we autoclaved cultures of *Rhodotorula* sp. in early stationary phase and found no N_2O production.

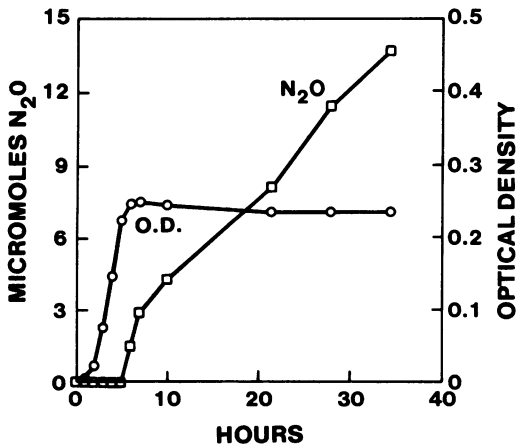

FIG. 1. Relation between N_2O production and phase of growth in *S. marcescens*.

The fungi had the weakest N_2O-generating ability of any group. These were also the only organisms that failed to continue N_2O production over a long period. Bollag and Tung (2) found much higher amounts of N_2O formed in the fungus they studied (*Fusarium oxysporum*), but only when nitrite was the substrate. No N_2O was detected from nitrate, although they used the less sensitive thermal conductivity detector.

When grown in potato dextrose broth, every N_2O-producing organism still had NO_3^- or NO_2^- left at the termination of the assay. But when *Hansenula*, *Aspergillus*, and *Alternaria* sp. were grown in an NH_4^+-free synthetic medium, NO_3^- and NO_2^- were completely consumed, and no N_2O was formed. Only after *Hansenula* sp. received additional NO_2^- did N_2O production start. The potato dextrose broth apparently supplied enough reduced nitrogen compounds to support growth, whereas in the NH_4^+-free medium, the demand for NO_3^--N for growth was so great that it apparently prevented N_2O production from nitrate.

For the nitrate-assimilating bacteria, three *A. vinelandii* strains produced N_2O in stationary phase (Table 3), whereas two *A. macrocytogenes* strains and one *Acinetobacter* strain produced none. All the cultures of *Azotobacter* had NO_3^- or NO_2^- remaining at the end of the experiment.

Since the complex medium contained reduced nitrogen sufficient to repress assimilatory nitrate reduction, we also tested for N_2O production in NH_4^+-free media. There was no marked change in N_2O yields, and in some cases it decreased (Table 3). Lack of NH_4^+ repression of N_2O production was also noted in the other recent studies with nitrate-respiring bacteria (23, 25) and also seems to be the case for these additional groups of organisms.

TABLE 3. N_2O produced in stationary phase by a variety of nitrate-reducing organisms

Organism	N_2O produced (nmol · $tube^{-1}$)[a]		Maximum conversion[b] of NO_3^--N to N_2O-N (%)
	Complex media	Defined NH_4^+-free medium	
Yeasts (aerobic growth)			
Hansenuela	6.0 ± 3.0 (0)[c]	7.4 ± 2.6	0.178
Rhodotorula	5.7 ± 1.8 (0)		0.060
Candida	0 (0)	0	0
Fungi (aerobic growth)			
Aspergillus	2.8 ± 1.6	0	0.022
Alternaria	1.8 ± 0.6	0	0.030
Fusarium	3.9 ± 0.7		0.032
Helminthosporium	0		0
Actinomucor	0		0
Penicillum	0		0
Bacteria (aerobic growth)			
Azotobacter vinelandii		12 ± 8.3	0.28
A. macrocytogenes		0	0
Acinetobacter		0	0
Anaerobes (anaerobic growth)			
Clostridium KDHS2	0		0

[a] Amount of N_2O produced (± standard deviation) from 5 ml of media after 12 days in stationary phase, except for the last four fungi, which were assayed at 90 days. Data not shown for organisms that failed to grow in NH_4^+-free media.

[b] Produced from 5.0 mM NO_3^-.

[c] Amount of N_2O produced under anaerobic growth is shown in parentheses.

The only obligate anaerobe tested, a *Clostridium* sp., did not produce N_2O (Table 3). It had previously been shown to dissimilate NO_3^- to NH_4^+ (5).

Resting stationary-phase cells of three nitrate-respiring bacteria and one yeast were examined to compare and quantify their rates of N_2O production from NO_2^-. The rates, expressed as nanomoles of N_2O per minute per milligram of protein, are as follows: *E. coli*, 0.28; *K. pneumonia*, 0.14; *E. aerogenes*, 0.11; and *Hansenula* sp., 0.04. When tested as resting cells with nitrite, the yeast produced N_2O at rates more nearly equivalent to the bacteria than found during growth with nitrate.

Do plants produce N_2O? We examined plant tops sealed in bottles and diced spinach leaves for evidence of N_2O production. We found none within the first few hours of incubation, regardless of whether the plant material was incubated in the light or dark or aerobically or anaerobically. Thus, we find no evidence that plants produce N_2O, at least under the conditions tested. Plants still might emit N_2O in the field if N_2O were transported through the plant from a soil solution enriched in N_2O.

We did note that plant tops in bottles often began to produce N_2O after 6 to 10 h of incubation, with the rate increasing exponentially. This production was eliminated by the addition of chloramphenicol. The plant tissue was often bruised or lacerated during introduction into the bottle. We suspect that bacterial growth, perhaps stimulated due to tissue damage, resulted in the N_2O production. Since significant N_2O production occurred in the light and under an air atmosphere, it is possible that plant tissue dam-

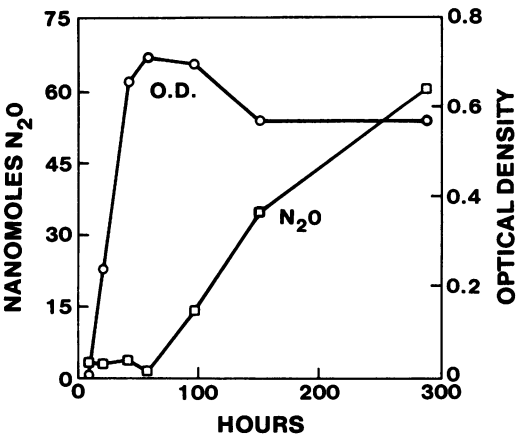

FIG. 2. Relation between N_2O production and phase of growth in *Rhodotorula* sp.

TABLE 4. Changes in N_2O in human breath before and after a meal containing NO_3^- and NO_2^-

Time (h)	Relative N_2O concn in breath[a]
Before eating	
−2	1 ± 0.06
−1	1 ± 0.06
After eating	
1	1.30 ± 0.25[b]
2	1.37 ± 0.46[c]
3	1.32 ± 0.28[c]

[a] N_2O content ± standard deviation was normalized to the before-eating breath concentration of each individual, and all individuals were equated to 1. Differences were evaluated by a two-tailed test.

[b] Significantly different from before eating at the 90% level of significance.

[c] Significantly different from before eating at the 80% level of significance.

aged naturally by wind, insects, or pathogens might result in some N_2O emissions. *Serratia* sp., shown here to be an N_2O producer, is commonly isolated from plant tissue (10). There are recent reports of NO_x (principally NO and perhaps NO_2) emissions from soybean leaves under certain assay conditions (11, 15), but there is no indication that N_2O was produced.

Do animals emit N_2O? Since our laboratory had noted that N_2O was produced but not reduced in the rumen (13), we reasoned that animals might emit N_2O. Random sampling of human breath frequently showed N_2O in concentrations in excess of that in ambient air. The experiment reported in Table 4 was done to see whether NO_3^- and NO_2^- in the diet could be one factor enhancing N_2O levels in breath. An increase in exhaled N_2O was noted after the spinach-bacon lunch. The greater variability in breath N_2O after eating was due in part to different temporal patterns of response among the subjects. Although we have no evidence of the mechanism of this N_2O production, one explanation is that intestinal or oral bacteria were responsible, particularly since the enteric bacteria were substantial producers of N_2O. Additional work on this interesting source of N_2O is needed to determine typical quantities emitted, its extent among animals, factors controlling the production, and the mechanism responsible.

Conclusions. Two questions remain for consideration. (i) What is the mechanism of N_2O production? (ii) Is this source of N_2O of any significance? With regard to mechanism, we suspected that N_2O could either be arising from the reaction of NO_2^- and NH_2OH (or the decomposition of NH_2OH), which produces N_2O (3), or from the incomplete turnover of assimilatory nitrite reductase, especially when the electron donor was limiting. The hydroxylamine mechanism appears unlikely, since we were not able to detect any of this compound by the 8-hydroxyquinoline colorimetric method (16) in N_2O-producing cells of *E. coli*. Furthermore, Smith (24) found that only when rather high concentrations of hydroxylamine (0.1 mM) were added to cell-free extracts did the rate of N_2O production reach the rate found in whole cells. Nitrite reductase also appears not to be the N_2O source, since very recent results of Smith (24) and Satoh et al. (23) show that mutants lacking the assimilatory nitrite reductase activity still produce N_2O at normal rates. In this study, we used only known nitrate-assimilating organisms, but it may be that the activity is unrelated to this property. The production of N_2O only in stationary phase suggests that the responsible enzyme may be one of secondary metabolism.

The question of whether this source of N_2O is significant cannot be answered, since there is no way known to specifically inhibit this mechanism of N_2O production, unlike the nitrifying and denitrifying sources. From the data of Smith and Zimmerman, it appears that most nitrate-reducing bacteria in soil can produce N_2O and that these organisms are more numerous than nitrifiers or denitrifiers (25). Furthermore, we have shown that prevalent soil organisms other than bacteria can also produce N_2O. However, the apparent low level of N_2O production by important organisms like fungi and the poor competitiveness of this process for low concentrations of N anions argue against its general importance. If it is important, there are two environments in which it might be of significance. One is the digestive tract of animals, in which both the high N_2O-yielding enteric bacteria typically reside and NO_3^- is often present in the diet. The other is acid forest soils, in which nitrifiers and denitrifiers are severely restricted by the acidity, and fungi and yeasts predominate.

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