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Dissimilatory Reduction of Nitrate and Nitrite in the Bovine Rumen: Nitrous Oxide Production and Effect of Acetylene†

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^{15}N tracer methods and gas chromatography coupled to an electron capture detector were used to investigate dissimilatory reduction of nitrate and nitrite by the rumen microbiota of a fistulated cow. Ammonium was the only ^{15}N-labeled end product of quantitative significance. Only traces of nitrous oxide were detected as a product of nitrate reduction; but in experiments with nitrite, up to 0.3% of the added nitrogen accumulated as nitrous oxide, but it was not further reduced. Furthermore, when $^{13}NO_3^-$ was incubated with rumen microbiota virtually no $[^{13}N]N_2$ was produced. Acetylene partially inhibited the reduction of nitrite to ammonium as well as the formation of nitrous oxide. It is suggested that in the rumen ecosystem nitrous oxide is a byproduct of dissimilatory nitrite reduction to ammonium rather than a product of denitrification and that the latter process is absent from the rumen habitat.

Nitrate poisoning of cattle has long been known (11), but little is known about the mechanisms and diversity of microbial transformations of nitrogen oxides in the rumen ecosystem. Lewis (10) first determined ammonium to be the principal terminal product of nitrate reduction in the rumen. Jones (5) noted the accumulation of small amounts of nitrous oxide during 3-day incubations of enrichment cultures started with a heavy inoculum of rumen fluid; he interpreted this as evidence of denitrification. It is now clear that a number of non-denitrifying organisms also can produce N_2O during nitrate reduction (15; J. M. Tiedje et al., Agron. Abstr., p. 165, 1979), and thus the interpretation that denitrification occurs in the rumen may not be correct.

In the present study we report on the quantitative importance and the mechanism of gaseous nitrogen production during dissimilatory nitrogen metabolism in the rumen ecosystem. The effect of acetylene on dissimilatory nitrite reduction to ammonium and nitrous oxide is also shown.

MATERIALS AND METHODS

Materials. Rumen contents were withdrawn before morning feeding from a fistulated Holstein cow fed 5.5 kg of grain and 1.8 kg of hay. The contents were strained through cheesecloth into a bottle which was capped to exclude air and immediately brought to the laboratory. Sodium nitrate (56.75 atom% ^{15}N) and sodium nitrite (96.60 atom% ^{15}N) were obtained from Mound Laboratories, Miamisburg, Ohio and Prochem, Summit, N.J., respectively. All gases used had at least 99.9% purity (Matheson, Joliet, Ill.).

Experiments. Freshly obtained rumen liquor (60 ml) was transferred to a 125-ml Erlenmeyer flask with a septum-capped sidearm. The flask was then connected to an assay system (6) which allowed a thorough exchange between gaseous and liquid phases as well as frequent gas and liquid sampling under anaerobic conditions. Briefly, the apparatus consisted of a gas pump, a gas chromatograph with sampling loop, and a flowmeter, so that the headspace gas could be continuously circulated through the rumen liquor and the gas sampling loop. The rumen liquor was further agitated by a magnetic stirrer. Foaming was prevented by adding 0.1 ml of antifoam solution whenever necessary (antifoam A, 1:500 plus one drop of Tween 80 per 25 ml). After connection of the sidearm flask with the assay system, the gas space was sparged through a vent valve with 95% Ar/5% CH_4 until all air was removed as verified by gas chromatograph analysis for O_2 (6). The vent valve was closed, and for the following 5 min the system was allowed to equilibrate. Then a gas sample (0.1 ml) was taken, and after another 5 min 1 ml of nitrate or nitrite solution was added by means of a syringe through the sidearm septum. Immediately afterwards, two liquid samples (1 ml) were withdrawn by syringe, transferred to test tubes, and frozen in liquid nitrogen. Gaseous and liquid samples were taken every 10 to 15 min until termination of the experiment. The liquid samples were stored in the freezer until analyzed. The experiments were performed at 22°C and atmospheric pressure. The pH of the rumen liquor was 6.9 before and 7.2 after the experiments. The ammonium content of the fresh fluid was 8.8 mM. Samples were pasteurized or sterilized by exposing them to 70 and 120°C, respectively, for 30 min.

Analysis. (i) Nitrous oxide. Gas samples were taken by means of a 0.1-ml sampling loop and injected

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on a Porapak Q column (1.8 m by ⅛ in. [ca. 3.1 mm] outside diameter) of a gas chromatograph (Perkin Elmer, model 910, Norwalk, Conn.). The separated components were detected by a pulse-modulated electron capture detector, and the peak areas were electronically integrated. The detection limit for N_2O was 5×10^{-3} Pa. For further details on operating conditions see our previous publication (6). Corrections were made for dissolved nitrous oxide.

(ii) Nitrite and ammonium. The frozen rumen liquor was thawed and centrifuged for 5 min at 12,000 × *g*, and the nitrite content of the supernatant fluid was assayed by the Griess-Ilosvay method (1). For ammonium analysis, the thawed rumen liquor samples were transferred quantitatively from test tubes to distillation flasks and the ammonium was collected by steam distillation according to the method of Bremner (1). In intervals of eight samples, the distillation apparatus was cleaned by an ethanol distillation. A portion of the distillate was used for a colorimetric ammonium assay (13).

(iii) ^{15}N mass spectrometry. The above distillate was acidified with 1 N HCl and 3 mg of N as NH_4Cl (natural ^{15}N abundance) added to assure enough N was present for mass spectrometry. The solution was then evaporated to dryness on a hot plate. The residue was dissolved in 1 ml of 1 N HCl and transferred to a small disposable glass vial where it was brought to dryness again. The glass vial was attached to the N conversion unit (12) of the mass spectrometer and evacuated. By dripping alkaline lithium hypobromite into the vial, the ammonium was converted to N_2, and the $^{15}N/^{14}N$ ratio was determined with an isotope ratio mass spectrometer (vg Micromass model 622, Winsford, England). To check for label cross-contamination, we measured a standard in intervals of five samples. Possible contamination by air was checked for every sample by measuring the $^{16}O_2$ peak (*m/e*, 32). The amount of ammonium produced from nitrate or nitrite was determined from the ammonium concentration and the atom% of ^{15}N.

All experiments were performed in triplicate, and the results shown are the mean values.

RESULTS

Preliminary experiments. Since N_2O can be produced by microorganisms other than denitrifiers, a better measure of denitrification is the production of N_2 from NO_3^- or NO_2^-. Because of the extreme sensitivity and direct detection of N_2 allowed by the ^{13}N technique (14), we incubated $^{13}NO_3^-$ with the rumen microbiota in an anerobic culture tube. Only traces of $[^{13}N]N_2O$ and $[^{13}N]N_2$ were observed by gas chromatography-proportional counting. The very limited N_2O production from NO_3^- was confirmed by gas chromatography since no N_2O was detected by the very sensitive electron capture detector. In contrast, substantial amounts of N_2O were produced from nitrite under the same conditions. Rumen liquor which was anaerobically incubated at 37°C on a rotary shaker could not metabolize N_2O at various concentrations in 48 h. Methanogenesis was transiently inhibited at initial nitrate concentrations higher than 10 μM (results not shown).

Nitrite reduction and effect of acetylene. Figures 1 and 2 show the reduction of nitrite and simultaneous accumulation of nitrous oxide and ammonium at two different initial nitrite concentrations (83 and 250 μM). The reduction was completed after 20 and 60 min, respectively. The ^{15}N data show that besides ammonium no quantitatively significant products could have been formed. However, gas chromatographic data revealed the production of N_2O, which amounted to 0.10 and 0.26% of the added nitrite-N, respectively. Samples which had been pasteurized 24 h before the experiment and freshly autoclaved samples did not metabolize nitrite to a measureable extent (Fig. 2), nor did they produce any N_2O.

The effect of acetylene on dissimilatory nitrite reduction to ammonium and nitrous oxide is

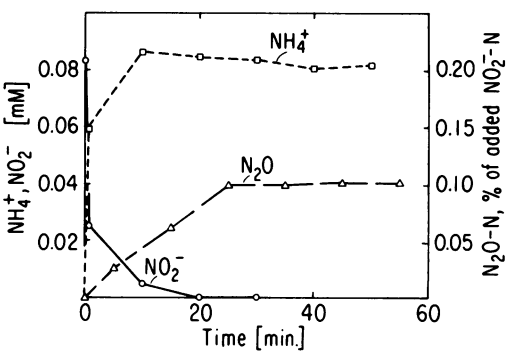

FIG. 1. *Disappearance of nitrite (83 μM initial concentration) with concomitant production of ammonium and nitrous oxide in rumen liquor.*

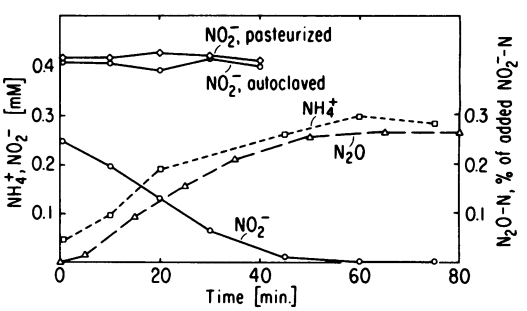

FIG. 2. *Disappearance of nitrite (250 μM initial concentration) with concomitant production of ammonium and nitrous oxide in rumen liquor. Pasteurized and autoclaved samples (420 μM nitrite) did not show any ammonium or N_2O production.*

shown by a comparison of Fig. 2 and 3. Acetylene was added by syringe to comprise 5% of the gases present (Fig. 3). With an initial nitrite concentration of 350 μM, the reaction was not completed after 80 min of incubation, and the rates of nitrite reduction, as well as formation of ammonium and nitrous oxide, were about half of the rates obtained in the experiment without acetylene (Fig. 2). The effect of acetylene on nitrous oxide production was further examined at various acetylene concentrations (Fig. 4). Acetylene at 5, 10, and 50% partial pressure reduced the rate of N_2O production by 35% compared to the rate in absence of acetylene.

Rate of ammonium production from nitrate versus nitrite. Rumen liquor was incubated with 420 μM nitrate. No nitrite could be detected during the 80-min incubation, and nitrous oxide accumulated to only 0.01% of the added nitrogen. ^{15}N data indicated that only 20 to 30% of the added nitrate was reduced to ammonium during the 80-min incubation, suggesting a slower reduction of nitrate than of nitrite. Thus, a closer examination of the maximum reduction rates for both substrates was done in a further experiment but at 1 mM initial N-oxide concentrations. The rate of $^{15}NH_4^+$ formation was 56.8 ± 11.0 ng of N ml^{-1} min^{-1} from NO_2^- but only 12.3 ± 3.8 ng of N ml^{-1} min^{-1} from NO_3^- (±95% confidence interval). Thus, the rate of nitrite reduction to ammonium was about fivefold higher than the rate of ammonium formation from nitrate. No nitrite was detected when nitrate was the substrate, which is consistent with the rate information. The faster rate of nitrite reduction also correlates with much greater N_2O production from nitrite and little or no N_2O from the slower nitrate reduction.

DISCUSSION

Our results demonstrate that nitrous oxide is a product of dissimilatory nitrite reduction by the rumen microbiota. Jones (5) reported N_2O accumulation in cultures which were heavily seeded with rumen microbes and incubated for 3 days and concluded that the rumen microbiota is capable of denitrification. However, due to the nature of his experiments (enrichment cultures), he could not draw any conclusions with regard to the magnitude of a constitutive denitrification capacity in the rumen ecosystem. Our results show that during nitrite reduction in rumen liquor, nitrous oxide production paralleled ammonium production but was only a few thousandths of the rate of ammonium formation.

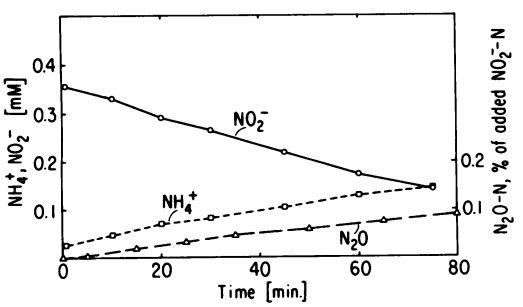

FIG. 3. *Effect of acetylene (ca. 5 kPa) on nitrite reduction and concomitant production of ammonium and nitrous oxide by rumen liquor (control: Fig. 2).*

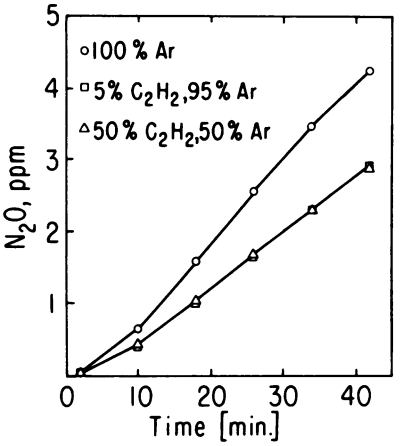

FIG. 4. *Effect of acetylene at various concentrations on N_2O production by rumen liquor.*

The mechanism responsible for N_2O production does not appear to be denitrification, however. Acetylene, which inhibits denitrification at the level of N_2O reduction (17), should cause either no change or usually an increase in the N_2O production rate if the source of N_2O is denitrification. However, in our experiments (Fig. 2 and 3) a marked decrease of the N_2O production rate parallel to a decrease of dissimilatory reduction of nitrite to ammonium was observed. Furthermore, the rumen microbiota was not able to reduce nitrous oxide, even if incubated for 48 h. Therefore, we conclude that nitrous oxide produced in our experiments was not a product of biological denitrification in a strict sense (i.e., reduction of ionic nitrogen oxides to N_2O or N_2 coupled with electron transport phosphorylation). The results strongly suggest that nitrous oxide was a byproduct of dissimilatory nitrate reduction to ammonium and could not be further metabolized by the indigenous rumen microflora. Recent work by Yoshinari (16) showed that *Vibrio succinogenes*, a rumen inhabitant (3), was able to reduce nitrous oxide to N_2 and that acetylene inhibited this reaction. The absence of significant N_2O reduc-

tion suggests that this strain or other species with the same partial denitrification capability were not prevalent in the rumen liquor we studied.

The only evidence of denitrification by the rumen microbiota of this cow was the minute amount of $[^{13}N]N_2$ produced from $^{13}NO_3^-$ that was detected by the extremely sensitive ^{13}N technique. Therefore, we conclude that the rumen ecosystem, at least under the conditions of this study, has virtually no constitutive denitrification activity. This is perhaps surprising since denitrifiers continually enter the rumen on soil or forage and nitrate and degradable carbon are in good supply. Moreover, denitrifiers capable of fermentation appear to be more common than previously thought (H. F. Kaspar, unpublished data). In view of this it appears that denitrifiers do not compete well in this ecosystem.

Ammonium was the only quantitatively significant product of nitrate and nitrite reduction in the rumen liquor we used (Fig. 1 to 3). Earlier work (Kaspar and Tiedje, in preparation) showed that in a digested sludge and a sediment of a eutrophic lake, ammonium accounted for 60 to 70% and about 10% of the nitrate reduced, respectively. In marine sediments, up to 70% of the nitrate was reduced to ammonium (8, 9), whereas only a few percent of the nitrate was reduced to ammonium in soil (2). Both dissimilatory nitrate reduction to ammonium and denitrification are mechanisms of electron disposal in bacterial energy metabolism, but due to the volatile and diatomic-N nature of its products (N_2O and N_2), denitrification acts to remove combined nitrogen and therefore causes economic loss in agriculture. Knowledge of the ecological factors governing the relative importance in various environments of the two nitrate reduction pathways might eventually lead to strategies to minimize soil denitrification.

The capacity of the rumen microbiota from this non-nitrate-adapted cow to produce ammonium from nitrite was about five times as high as from nitrate, and no nitrite accumulated from nitrate. Nitrous oxide production seemed to increase with nitrite concentration (0.1 to 0.26% N_2O for a threefold increase in nitrite concentration). The lower N_2O production from nitrate, therefore, is probably due to the very small nitrite pool formed during nitrate reduction. In nitrate-adapted animals and non-nitrate-adapted animals fed a high nitrate diet, substantial pools of nitrite exist (4, 7) and in the latter case often kill the animal via methemoglobinemia. In these cases larger amounts of N_2O would be expected if the above conditions hold. It is not known, however, whether denitrifiers might be more successful under the high nitrate conditions of a nitrate-adapted animal. If so, this probably would result in a reduction of the N_2O to N_2.

The effect of acetylene on N_2O production that we noted may be of significance to interpretation of denitrification research. Besides being formed by nitrifiers (15), nitrous oxide appears to be a byproduct of many organisms capable of dissimilatory nitrate reduction to ammonium (J. M. Tiedje et al., Agron. Abstr., p. 165, 1979). Long-term anaerobic environments exhibit greater potential for nitrate reduction to ammonium; use of the acetylene inhibition technique for denitrification measurements in such environments (e.g., sediments, mudflats) could lead to errors if a significant portion of the N_2O produced were a byproduct of dissimilatory nitrate reduction to ammonium. Furthermore, an error in denitrification estimates could also occur from the inhibitory effect of acetylene on the nitrate to ammonium reduction, such as was noted here (Fig. 4), if an increase in the proportion of nitrate going to the denitrification pathway was a consequence of this treatment.

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