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Amperometric Method for Determining Nitrous Oxide in Denitrification and in Nitrogenase-catalyzed Nitrous Oxide Reduction

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Abstract. A conventional Clark-type O_2 probe was used to determine N_2O concentrations in suspensions. At a polarizing voltage of −0.95 V versus the reference Ag/AgCl electrode, the probe is almost half as sensitive for N_2O as for O_2, and the detection limit is less than 1 μM N_2O. The probe can also be used to determine NO for which the suitable polarizing voltage is −0.7 V. The method was successfully applied for continuously recording dissimilatory formation or utilization of N_2O by intact *Azospirillum brasilense* Sp 7, NO production by extracts from this bacterium, and N_2O reduction catalyzed by nitrogenase in intact *Klebsiella pneumoniae*. It is concluded that the probe is useful for measuring N_2O or NO contents in bacterial suspensions when the O_2 level is zero or kept constant during the assays.

Generally, the methods to measure nitrogen fixation and denitrification activities require sampling at intervals and subsequent analyses. They are often time consuming and expensive. An exception to the rule is the amperometric method to measure H_2 evolution formed by nitrogenase, which can be performed continuously [5]. Otherwise, there are few and only insensitive methods for determining the continuous formation of products or utilization of substrates in nitrogen fixation [1] or denitrification [4]. During the course of an investigation on nitrogen fixation and denitrification by *Azospirillum* [12, 16], we noticed that nitrous oxide could be determined quantitatively by means of a probe that was routinely used for measuring O_2 concentrations. The present communication describes the method of determining nitric oxide and nitrous oxide amperometrically. In addition, examples are presented where the method can successfully be applied for measuring denitrification and nitrogen fixation activities by microorganisms.

Materials and Methods

Probe used. A Clark-type probe commonly used for O_2 determinations served for the measurements in the present investigation. We used a type EO 12 probe from Wissenschaftlich-Technische Werkstätten (WTW GmbH, D-8120 Weilheim i. OB), containing a gold cathode, an Ag/AgCl anode as the reference, a KCl gel electrolyte of pH 13, and a Teflon membrane impermeable to H_2O and ions but permeable to O_2, NO, and N_2O. Any other Clark-type O_2 probe may be used, but the optimal polarizing voltage may differ slightly. In the current investigation, the electrolyte gel had a KCl concentration of 0.7 M, exhibiting an Ag/AgCl reference potential at the anode of +0.23 V versus the normal hydrogen electrode. Polarizing voltages of −0.95 V for N_2O determinations and of −0.70 V for NO measurements were applied between the cathode and anode by conventional 1.5-V batteries supplemented with a voltage divider. Thus, the effective polarizing voltages were −0.47 and −0.72 V versus the normal hydrogen electrode, respectively. Throughout the text, all the polarizing voltages are given versus the Ag/AgCl electrode (with a concentration of $Cl^- = 0.7$ M) and not versus the normal hydrogen electrode. The current signal from the cathode was amplified and recorded by a standard two-channel recorder (type 2210 from LKB, Bromma, Sweden; for the circuit arrangement of the amplifier see Oehme and Schuler [11], p. 70). The gas measurements were performed in a 1.8-ml jacketed cuvette, similar to the method described by Wang et al. [15]. The solution in the cuvette was stirred by a magnetic bar and the temperature was held at 30°C by circulating water. The cuvette allowed the insertion of a second probe for simultaneous O_2 determinations.

Gas calibrations. In order to calibrate the electrode, the concentrations of the gases O_2, NO, and N_2O had to be determined independently in gas chromatographs equipped with thermal conductivity detectors. A Varian model 920 with a molecular sieve 5-Å column at 50°C and with N_2 as the carrier gas served for O_2 determinations, and a Perkin-Elmer Sigma 3B with a Porapak Q column (1.8 m × ⅛ inches) at 50°C and with He as carrier gas using a flow rate of 40 ml/min was used for NO and N_2O measurements. Under the latter conditions, NO (retention time,

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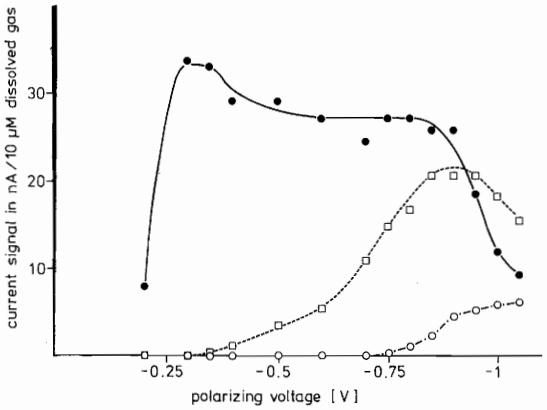

Fig. 1. The current signal for dissolved N_2O, NO, and O_2 in dependence on the polarizing voltage: ●——●, for 10 μM dissolved O_2; □--------□, for 10 μM dissolved NO; and ○–·–·–○ for 10 μM dissolved N_2O.

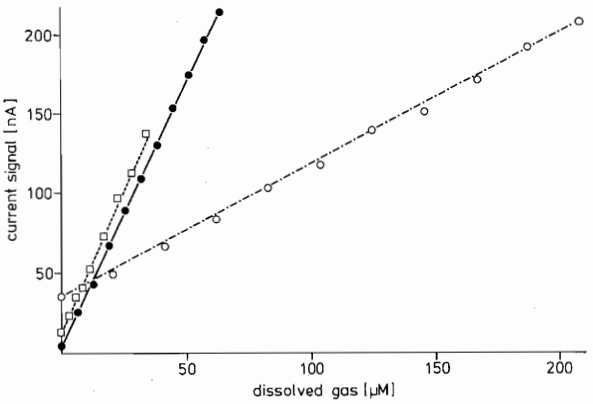

Fig. 2. The current signal in dependence on the concentration of dissolved gases: ○–·–·–·–○, N_2O, polarizing voltage −0.95 V; ●——●, O_2, polarizing voltage −0.3 V; and □--------□, NO, polarizing voltage −0.7 V.

21 s) and N_2O (70 s) could easily be separated from each other and be determined quantitatively. N_2O and O_2 were purchased from Linde (Höllriegelskreuth near München) and NO was generated from $NaNO_2$ and H_2SO_4 under anaerobic conditions [14].

Organisms and experimental conditions. *Azospirillum brasilense* Sp 7 (ATCC 29145) was grown in continuous culture anaerobically with nitrite as respiratory electron acceptor as described previously [16]. For the N_2O-formation assay, the cells were centrifuged (16,000 *g*, 10 min) and suspended in the malate medium [2] in which all combined nitrogen was omitted. All handlings were performed anaerobically. The cell suspension was supplemented with C_2H_2 (final concentration, 2 m*M*) and 1.8 ml cells were transferred into the cuvette, which had previously been flushed with argon. After the electrode had become stable (which was achieved within 10 min), the reaction was started by adding 50 µl of an anaerobic solution of $NaNO_2$ (final concentration in the cuvette = 0.5 m*M* $NaNO_2$), and N_2O formation and O_2 content were monitored by the Clark probes. For measuring N_2O utilization by *Azospirillum brasilense* Sp 7, the cells were grown anaerobically with N_2O as the respiratory electron acceptor under batch culture conditions [12]. The medium was supplemented with 20 m*M* NH_4Cl, which was a better nitrogen source than N_2 and, therefore, enhanced growth. When grown for 24 h, the cells were centrifuged, suspended in fresh medium, and 1.8 ml (optical density of 560 nm = 0.6) were transferred to the cuvette. All steps were also done under anaerobic conditions. The reaction was started by adding N_2O (final concentration, 0.1 m*M*), and the utilization of the gas was monitored as described above.

For NO formation, *A. brasilense* Sp 7 was used that had been grown anaerobically in continuous culture with nitrite to an optical density of 560 nm = 0.2 [16]. The cells were concentrated 50-fold by centrifugation, broken twice in a French press at 140,000 kPa, and centrifuged (16,000 *g*, 10 min). The supernatant was assayed for NO production in the cuvette, which contained: phenazine methosulfate, 0.4 m*M*; Na–ascorbate, 27 m*M*; phosphate buffer, pH 7.5, 50 m*M*; and supernatant with 1.8 mg protein in the cuvette. The reaction was started by adding nitrite (final concentration, 11 m*M*), and NO formation was recorded amperometrically as described above.

For measurements of N_2O reduction by nitrogenase, *Klebsiella pneumoniae* K11 was grown in Hino and Wilson's medium [3] under N_2-fixing conditions for 24 h. The cells were diluted to an optical density of 0.4 by fresh medium under anaerobic conditions, and directly assayed for N_2O reduction in the cuvette under argon. The reaction was started by adding N_2O (final concentration, 0.12 m*M*).

Results

The current signal of the probe for 10 μM O_2, NO, or N_2O dissolved in H_2O was dependent on the polarizing voltage (Fig. 1). When the polarizing potential applied to the cathode was −0.3 V with respect to the Ag/AgCl electrode, the probe measured O_2 as usual, but was insensitive to NO or N_2O. The sensitivity of the probe to O_2 decreased at polarizing voltages between −0.5 and −0.7 V, but then it responded to NO (Fig. 1). At the more negative potentials of −0.9 to −0.95 V, all three gases could be determined by the probe. The sensitivity for N_2O was nearly half as much as that for O_2 at −0.95 V, but was clearly detectable (Fig. 1). When the probe was preconditioned by incubation with N_2O overnight at a polarizing voltage of −0.95 V, the sensitivity increased approximately tenfold as compared with an untreated probe. Such an effect has also been described for the H_2 probe [5, 15]. In contrast to the H_2 probe, the stability of the N_2O probe did not change for several days, even when a different polarizing voltage was applied. The currents for zero N_2O, NO, or O_2 concentrations were negligible at all polarizing voltages up to −1.0 V (see Fig. 2), which could easily be compensated for by the am-

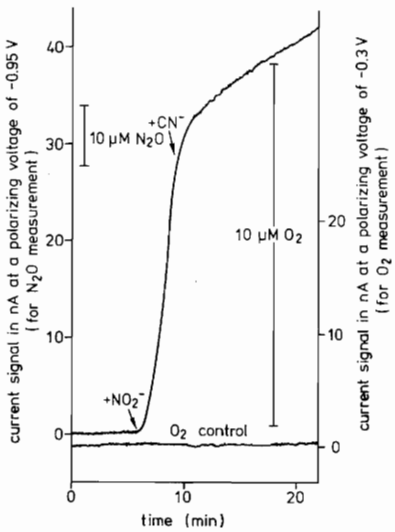

Fig. 3. N_2O formation catalyzed by dissimilatory nitrite reductase in intact *Azospirillum brasilense* Sp 7. Polarizing voltage −0.95 V for N_2O recordings. *Azospirillum brasilense* grown in continuous culture with nitrite as respiratory electron acceptor was used for this experiment. The experimental conditions are described in *Materials and Methods*. KCN concentration, 10^{-5} *M* (when added). O_2 control: simultaneous measurement of the O_2 concentration in the cuvette performed by a second probe at −0.3 V.

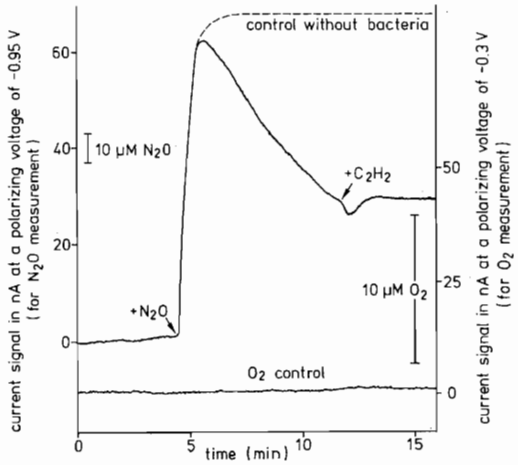

Fig. 4. N_2O utilization by intact *Azospirillum brasilense* Sp 7. The experimental conditions are described in *Materials and Methods*. Polarizing voltage for the N_2O probe: −0.95 V. C_2H_2 concentration: 2.5 m*M* (when added). O_2 control: for the explanation, see the legend of Fig. 3.

plifier of the recorder. The zero current drastically increased above −1.0 V.

The current signal of the probe was strictly dependent on the concentrations of the three gases (Fig. 2). The probe was similarly sensitive to O_2 and NO, but was approximately fivefold less for N_2O. Despite this, the sensitivity of the probe was high enough to determine N_2O concentrations accurately with less than 1 μM being the lower limit of detection. The curve for the current signal versus the N_2O concentration was linear up to at least 1 m*M* (not documented).

Figures 3–6 present examples where the amperometric method of measuring N_2O and NO production or utilization was successfully applied with microorganisms. *Azospirillum brasilense* Sp 7 utilizes nitrite as a respiratory electron acceptor under anaerobic conditions and normally produces N_2 from nitrite. However, when the assays are supplemented with C_2H_2, nitrite is reduced to N_2O, because C_2H_2 specifically blocks the conversion of N_2O to N_2 in dissimilatory nitrite reduction (see Zimmer et al. [16]). This N_2O formation in the presence of C_2H_2 is successfully monitored by the O_2 probe at a polarizing voltage of −0.95 V (Fig. 3). N_2O formation is blocked by adding 10^{-5} *M* KCN in agreement with other previous measurements [16]. The control probe in the cuvette showed that the O_2 concentration was practically zero in the beginning and did not change during the experiment.

Azospirillum brasilense can grow anaerobically with N_2O as the sole respiratory electron acceptor [12]. N_2O utilization in this assay can also be followed by the Clark probe (Fig. 4). The consumption is blocked by C_2H_2 in agreement with previous results [12].

As will be published elsewhere, cell-free preparations from *A. brasilense* catalyze a formation of NO from NO_2^- under anaerobic conditions (Fig. 5). This gas production is strictly dependent on the presence of phenazine methosulfate, Na–ascorbate, and nitrite and is associated with the soluble fraction from this organism. NO formation is probably an artifact of cell-free preparations in *Azospirillum* as in other bacteria [8]. The gas formed was, indeed, nitric oxide, which was verified by gas chromatography and by comparison with standards as well as by oxidation of the NO formed to NO_2, which was then determined by gas chromatography. Figure 5 shows that NO formation by the preparation from *Azospirillum* could easily be monitored by the Clark probe in such assays where no O_2 was present.

N_2O is competitive inhibitor of N_2 fixation and can be reduced to N_2 by nitrogenase. This was shown with intact cells of *Azotobacter vinelandii* [9], with soybean nodules [7], and with cell-free

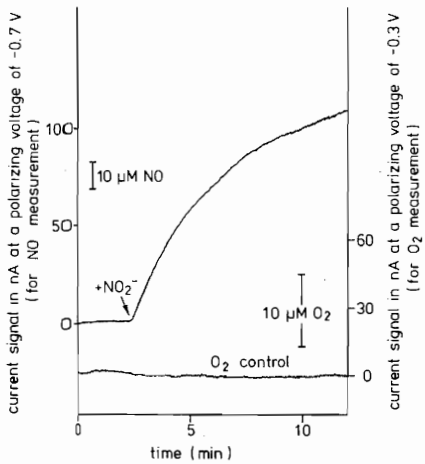

Fig. 5. NO formation catalyzed by dissimilatory nitrite reductase of a cell-free preparation from *Azospirillum brasilense* Sp 7. Polarizing voltage, −0.7 V. For experimental conditions, see *Materials and Methods*.

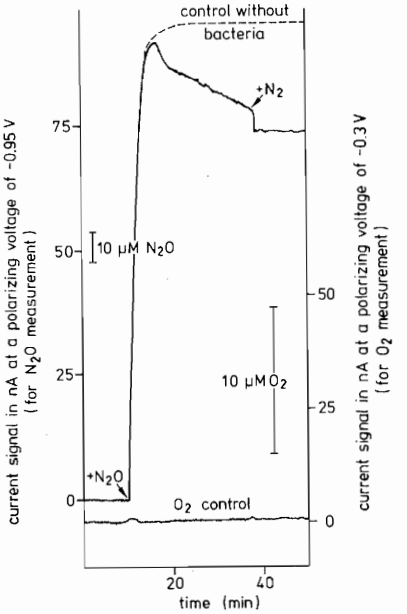

Fig. 6. N_2O reduction catalyzed by nitrogenase in intact *Klebsiella pneumoniae*. Polarizing voltage: −0.95 V. N_2 concentration: 0.1 m*M* (when added).

preparations from *Azotobacter vinelandii* and *Clostridium pasteurianum* [6]. N_2O reduction by N_2 fixing intact *Klebsiella pneumoniae* can be recorded by the Clark probe in assays performed under argon (Fig. 6). The decrease in N_2O content was affected by adding N_2 to the cuvette (Fig. 6), indicating that N_2O reduction was, indeed, due to nitrogenase in this experiment.

Discussion

The present investigation shows that the formation of N_2O and NO and the utilization of N_2O can be monitored successfully by the use of a Clark-type probe when an appropriate polarizing voltage is applied to the cathode. Reliable data are obtained when the experiments with suspension cultures are performed under anaerobic conditions or when the concentration of O_2 is kept constant during the assay. When the O_2 content changes during the experiment (e.g., by respiratory O_2 uptake of the microorganisms), it is difficult to subtract O_2 consumption from N_2O changes. Where the probe responds to N_2O it is also sensitive to O_2, and O_2 consumption by microorganisms normally exceeds by far N_2O (or NO) formation or utilization. The method is, therefore, likely applicable to determine denitrification activities also by other microorganisms and to measure N_2O reduction catalyzed by nitrogenase in bacteria performing N_2 fixation under anaerobic conditions. Difficulties will arise with N_2-fixing microorganisms, which meet their energy requirement by a high respiratory activity, e.g., *Azotobacter vinelandii*. The methods will also hardly be successful with N_2O production by nitrifying bacteria and with the utilization of the gas by photosynthetic N_2-fixing microorganisms. We tried to measure N_2O utilization of the gas by heterocysts of *Anabaena cylindrica* without success in the present investigation. The experiment had to be performed in the light in this case [10], and it turned out to be difficult to compensate the probe for the temperature changes caused by the light at such a high sensitivity required to determine N_2O disappearance.

Although the probe is only one-fifth as sensitive for N_2O as for O_2 both measured at their optimal polarizing voltages, sensitivity should normally not be a problem for following the N_2O content in suspensions of microorganisms, because the detection limit for N_2O by the probe is less than 1 μM. It is worth mentioning in this context that N_2O is about 20-fold more soluble in H_2O than in O_2 [13]. Therefore, the disappearance of N_2O from the gas phase above a bacterial suspension can easily be calculated by measuring the N_2O content in the suspension. It should be possible with the probe to measure and regulate the N_2O content in a culture of *Azospirillum* or other denitrifying bacteria. This should allow the growth of bacteria in continuous culture with N_2O as the sole respiratory electron acceptor, which has not yet been achieved for any of the denitrifying bacteria.

It was not within the scope of the present investigation to elucidate the chemical reaction taking place with N_2O at the cathode. The reaction of the Clark probe with O_2 yields H_2O_2 as intermediate, and the formation of the products is strongly dependent on the pH value in the electrolyte [11]. In the case of N_2O, we imagine the following reaction to proceed at the cathode: $2e^- + N_2O + H_2O \rightarrow 2\,OH^- + N_2$. Such an assumption has not yet been verified experimentally.

Note added in proof. After submission and acceptance of the manuscript, it came to our attention that a similar method to measure N_2O was described by Albery et al. [17]. This method was used by Alefounder and Ferguson [18] to monitor N_2O utilization or production by *Paracoccus denitrificans*.

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Literature Cited

1. Bergersen FJ (1980) Measurement of nitrogen fixation by direct means. In: Bergersen FJ (ed) Methods for evaluating biological nitrogen fixation. New York: John Wiley, pp 65–110
2. Bothe H, Klein B, Stephan MP, Döbereiner J (1981) Transformations of inorganic nitrogen by *Azospirillum* spp. Arch Microbiol 130:96–100
3. Dalton H (1980) The cultivation of diazotrophic microorganisms. In: Bergersen FJ (ed) Methods for evaluating biological nitrogen fixation. New York: John Wiley, pp 13–64
4. Delwiche CC (1981) Denitrification, nitrification and atmospheric nitrous oxide. New York: John Wiley, pp 1–286
5. Hanus FJ, Carter KR, Evans HJ (1980) Techniques for measurement of hydrogen evolution by nodules. Methods Enzymol 69:731–739
6. Hardy RWF, Knight E Jr (1966) Reduction of N_2O by biological N_2-fixing systems. Biochem Biophys Res Commun 23:409–414
7. Hoch GE, Schneider KC, Burris RH (1960) Hydrogen evolution and exchange, and conversion of N_2O to N_2 by soybean root nodules. Biochim Biophys Acta 37:273–279
8. Kim C-H, Hollocher TC (1983) ^{15}N-tracer studies on the reduction of nitrite by purified dissimilatory nitrite reductase of *Pseudomonas aeruginosa*. J Biol Chem 258:4861–4863
9. Mozen MM, Burris RH (1954) The incorporation of ^{15}N-labelled nitrous oxide by nitrogen fixing agents. Biochim Biophys Acta 14:577–578
10. Neuer G, Bothe H (1982) The pyruvate : ferredoxin oxidoreductase in heterocysts of the cyanobacterium *Anabaena cylindrica*. Biochim Biophys Acta 716:358–365
11. Oehme F, Schuler P (1983) Gelöst–Sauerstoff–Messung. Heidelberg: A Hütting, pp 1–146
12. Penteado Stephan M, Zimmer W, Bothe H (1984) Denitrification by *Azospirillum brasilense* Sp 7. II. Growth with nitrous oxide as respiratory electron acceptor. Arch Microbiol 138:212–216
13. Stephen H, Stephen T (1963) Solubility of inorganic and organic compounds, vol 1. Binary systems, parts 1 and 2. Oxford: Pergamon, pp 1–1933
14. Steudel R, Schenk PW (1975) Stickstoffmonoxid NO. In: Bauer G (ed) Handbuch der präparativen anorganischen Chemie, vol 1. Stuttgart: Enke, pp 470–471
15. Wang R, Healey FR, Myers J (1971) Amperometric measurement of hydrogen evolution in *Chlamydomonas*. Plant Physiol 48:108–110
16. Zimmer W, Penteado Stephan M, Bothe H (1984) Denitrification by *Azospirillum brasilense* Sp 7. I. Growth with nitrate as respiratory electron acceptor. Arch Microbiol 138:206–211
17. Albery WJ, Brooks WN, Gibson SP, Heslop MW (1979) An electroanalytical method for the determination of N_2O. Electrochim Acta 24:107–108
18. Alefounder PR, Ferguson SJ (1982) Electron transport linked nitrous oxide synthesis and reduction by *Paracoccus denitrificans* monitored with an electrode. Biochem Biophys Res Commun 104:1149–1155