Denitrification and Anaerobic, Nitrate-dependent Acetylene Reduction in Cowpea Rhizobium

By ROBERT M. ZABLOTOWICZ AND DENNIS D. FOCHT

Department of Soil and Environmental Sciences, University of California,
Riverside, California 92521, U.S.A.

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INTRODUCTION

The dissimilatory reduction of nitrate to nitrous oxide and dinitrogen has recently been discovered in cowpea rhizobia and *Rhizobium japonicum* (Zablotowicz *et al.*, 1978). The general sequence of denitrification, as proposed by Payne (1973), of $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$ is the likely pathway of denitrification in *Rhizobium* in both free-living and bacteroid forms.

The slower growing, alkali-producing 'cowpea-type' rhizobia probably evolved from tropical soils which remained saturated with water for extended periods of time (Norris, 1964). Nodulation in cowpeas is sensitive to short periods of anoxia (Sallee & Smith, 1969), and waterlogging depresses nitrogenase activity by decreasing respiratory activity (Sprent, 1971). Ethanol accumulation induced in soybean nodules may be partially responsible for the subsequent disintegration of nodule integrity (Sprent & Gallacher, 1976).

Minchin & Pate (1975) found that, under waterlogging conditions, peas (*Pisum sativum*) exhibited extreme degradation of nodule tissue in 14 d, while functional bacteroids in cowpeas (*Vigna*) could be observed after 32 d. Though better O_2 transport in cowpeas might be a possible explanation for the differences, as suggested by Minchin & Pate (1975), the possession of a dissimilatory nitrate reductase in the cowpea rhizobia cannot be precluded. Such an enzyme has been found in suspensions of *R. japonicum* bacteroids, though an active dissimilatory nitrite reductase was not detected (Daniel & Appleby, 1972). *Rhizobium japonicum* can use nitrate as an alternative electron acceptor to oxygen to generate ATP for nitrogenase activity, and nitrogenase and nitrate reductase activities remain constant until nitrite accumulates and inhibits nitrogenase activity (Rigaud *et al.*, 1973).

METHODS

Preparation of bacteroids. California no. 5 blackeyes (*Vigna unguiculata* L. Walp.) were grown in sand culture in the greenhouse. Plants were irrigated with half-strength ($12.5 \mu\text{g N ml}^{-1}$) Summerfield solution (Summerfield *et al.*, 1977). The nutrient solution (50 l) was continuously recycled throughout the experiment with no further addition of mineral salts. The plants were inoculated just after seedling emergence with 10 ml of a 5 d culture of strain 176A32 (obtained from J. C. Burton, Nitragin Co., Milwaukee, Wis., U.S.A.) grown on yeast extract/mannitol broth.

The plants were harvested at noon 40 d after emergence. The roots were surface-sterilized by immersion in 70 % (v/v) ethanol. The nodules were removed, rinsed twice in 70 % (v/v) ethanol, then three times in sterile distilled water. They were disrupted under He with a Waring blender in 0.01 M- KH_2PO_4 /0.01 M-sodium ascorbate buffer, pH 7.2, filtered through sterile cheesecloth to remove the coarser tissue particles, and the filtrate was centrifuged for 10 min at 164 g. The supernatant was decanted and centrifuged for 10 min at 12100 g; the resulting pellet, containing the bacteroid fraction, was suspended in phosphate buffer to give $12.8 \text{ mg dry wt cells ml}^{-1}$ and was stored under a He atmosphere.

Incubation. Erlenmeyer flasks (50 ml), containing 10 ml CS7 medium (Pagan *et al.*, 1975) with 30 mM-glucose instead of arabinose, were used to assay acetylene reduction. One set of flasks contained 4 mM-

Fig. 1

Fig. 2

Fig. 1. Nitrogenase activity, as measured by acetylene reduction, in cowpea bacteroid preparations incubated in a He atmosphere in the presence of nitrate (○) or in its absence (●). The results show the mean values from three experiments; bars show the standard deviation.

Fig. 2. Nitrous oxide evolution by cowpea bacteroid preparations incubated in a He atmosphere in the presence of nitrate (●), and nitrous oxide evolved after addition of trichloroacetic acid (○). The results show the mean values from three experiments; bars show the standard deviation.

$K^{15}NO_3$ (enriched with 99.3% ^{15}N) while the other set (control) contained no nitrate. All flasks were autoclaved at 121 °C for 5 min. The sterile flasks were sealed with a sterile serum stopper and flushed four times with He at 1.26×10^5 Pa (1.25 atm); they were then injected with acetylene to give 6.5×10^3 Pa (0.06 atm) and 5.0 ml of the bacteroid preparation were added by syringe. The flasks were placed in a rotary shaker (100 rev. min⁻¹) water bath at 30 °C.

Gas samples (0.05 ml) were removed periodically up to 9 h for acetylene reduction assay and 1.0 ml samples were removed for N_2O analyses between 9 and 25 h. The experiments were terminated after 25 h by adding 5.0 ml 1 M-trichloroacetic acid.

Analytical methods. Ethylene concentrations were determined using a Varian 3700 gas chromatograph with a flame ionization detector and a 2 m $\times$ 1.6 mm column packed with 80 to 100 mesh Poropak N, using He as a carrier gas. Nitrous oxide was determined using the same instrument fitted with a thermal conductivity detector and a Poropak Q column (5.5 m $\times$ 1.6 mm).

A Finnigan 3100C mass spectrometer interfaced with a gas chromatograph was used for isotopic analysis of nitrous oxide and dinitrogen according to Focht (1978).

Nitrate and nitrite were determined in a Technicon Autoanalyzer II using the cadmium reduction method of Strickland & Parsons (1965).

RESULTS

Although nitrate gave an immediate decrease in acetylene reduction in bacteroids, activity steadily increased thereafter (Fig. 1).

Nitrous oxide was first detected at 19 h in the nitrate-enriched treatments of bacteroids. It continued to accumulate until the end of the experiment (Fig. 2). No nitrous oxide was detected at any time in any of the control flasks. Nitrous oxide increased from 15.1 to 31 nmol (mg dry wt cells)⁻¹ when trichloroacetic acid was added to the incubation mixture. This may have been due to disruption of cell membranes and release of 'trapped' nitrous oxide inasmuch as nitrous oxide reductase is membrane-bound (Payne, 1973). The nitrate concentration decreased from 39.2 μ mol per flask at the beginning of the experiment to 19.0 μ mol per flask at the end. Subsequent accumulation of nitrite (less than 0.1 μ mol nitrite detected) did not occur. No $^{15}N_2$ was detected at the end of the experiment and $98.0 \pm 1.0\%$ of the N_2O was of mass 46 (theoretical value 98.6%).

DISCUSSION

We have observed that the anaerobic reduction of acetylene to ethylene in bacteroids was increased by nitrate, which agrees with the results of Rigaud *et al.* (1973) who showed that the dissimilatory reduction of nitrate can support nitrogen fixation in soybean bacteroids. There are, however, three noticeable differences between the two studies. Firstly, we did not observe an immediate reduction of acetylene which may have been due to the induction of the nitrogenous oxide reductases. Secondly, we observed a longer maintenance of acetylene reduction activity, probably because of further reduction of nitrite. Thirdly, Rigaud *et al.* (1973) found an accumulation of nitrite to which they attributed the subsequent cessation of acetylene reduction activity, while we found no accumulation of nitrite.

We have demonstrated that the denitrifying enzyme system, excepting nitrous oxide reductase, is functional in cowpea bacteroids by noting that both atoms of N_2O came from $^{15}NO_3^-$. Failure to observe labelled N_2 during the incubation is most likely due to the inhibition of nitrous oxide reduction since concentrations of acetylene as low as 0.1 % (v/v) reversibly block nitrous oxide reductase (Balderson *et al.*, 1976; Yoshinari & Knowles, 1976). Thus, our results do not preclude the existence of a functional nitrous oxide reductase in bacteroids.

Possession of a denitrification and a nitrogen-fixing pathway in the same organism may appear wasteful in terms of plant nitrogen usage, but, besides helping organisms to survive anaerobic conditions, it might be important to *Rhizobium*-legume symbiosis by maintaining nodule integrity during anoxic conditions in the soil medium. Minchin & Summerfield (1976) described characteristics to be used in a breeding programme for cowpeas to maintain nodule integrity. The presence of a full nitrogen oxide reductase system may be an important criterion in the selection of *Rhizobium* for many irrigated and rain-fed tropical legumes.

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