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Nitrite Inhibition of Nitrogenase from Soybean Bacteroids

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Abstract. Nitrogenase from soybean bacteroids was purified and used to study NO_2^- effects either as unfractionated enzyme or as reconstituted enzyme from separated nitrogenase components I and II. Partially purified enzyme was strongly inhibited by nitrite at concentrations less than 0.1 mM. This inhibition was typically referred to as competitive with an inhibition constant (K_i) for NO_2^- which was 5.2 mM. Kinetics studies showed an abnormally low apparent constant of association between enzyme and NO_2^- ($k_a = 60\ M^{-1} \cdot s^{-1}$). Nitrite appeared to bind to the MoFe protein, without any effect on Fe component, giving a completely reversible inhibition. Nitrite was found not to be an alternative substrate for nitrogenase.

Key words: *Rhizobium* – Soybean – Bacteroid – Nitrogenase – Nitrite.

Nitrite, added to soybean bacteroid preparations or produced from nitrate, by active bacteroid nitrate reductase, strongly inhibited nitrogen fixation (Rigaud et al., 1973). With crude extracts of nitrogenase, nitrite directly reacted with the enzyme, giving a 50% inhibition at a concentration of 0.1 mM (Kennedy et al., 1975). Low concentration of NO_2^- rapidely suppressed C_2H_2 reduction by root nodules of Alfalfa (Kamberger, 1977). A possible role of nitrite, produced in nodules after feeding with nitrate, was proposed to explain the depressive effect of NO_3^- on nitrogen fixation by root nodules (Rigaud, 1976). However, Gibson and Pagan (1977) proposed that factors other than nitrite were responsible for this inhibition. In this paper, we report experiments undertaken with purified bacteroid nitrogenase to investigate the characteristics of nitrite inhibition on C_2H_2 reduction and to compare with other inhibitors of nitrogenase.

Abbreviations: TES = N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid. PPG = Polypropylene glycol

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Material and Methods

Nodules

Soybeans (*Glycine max* Merr. cv. Altona), inoculated with *Rhizobium japonicum* strain 1809 were grown in a glasshouse and supplied with a nitrogen-free mineral solution as previously described (Rigaud and Puppo, 1975). The nodules harvested 30–35 days after sowing (nodule age 25–26 days), exhibited a C_2H_2 reduction activity in the range of 9–14 nmol C_2H_4/h/mg (fresh weight).

Bacteroid Suspensions

Isolation of bacteroids was routinely carried out with 80 g (fresh weight) of nodules as previously reported (Rigaud et al., 1973). To perform complete nitrogenase purification procedure, 600 g of nodules were generally required.

Nitrogenase Purification

Bacteroids were resuspended in 0.1 M TES buffer pH 8.5, containing 1.2 mM $Na_2S_2O_4$ and disrupted either in a MSE 16 Kc ultrasonicator for two periods of 2 min at 4°C or in a precooled Aminco French pressure cell at 110, 316×10^6 N/m^2. Crude extracts of nitrogenase were obtained by centrifugation at $35{,}000 \times g$ for 30 min at 4°C and stored in liquid N_2 until used. They were pooled before submitting to further purification (protein content: 15–20 mg/ml). The procedure adopted for the nitrogenase purification: PPG P_{400} (Fluka AG, Switzerland) precipitations and DEAE-cellulose chromatography, was conducted as described by Israel et al. (1974), except that heat treatment was omitted. Partially purified enzyme and purified components were stored in liquid nitrogen until used.

Nitrogenase Assays

Activity was measured by acetylene reduction (Hardy et al., 1968) in rubber-cap vials (24 ml) containing in 2 ml (μmol): TES buffer (pH 7.4),

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50; ATP, 5; $MgCl_2$, 5; creatine phosphate, 20 and 100 µg (16.3 units; Sigma Chemical Co., St. Louis, U.S.A.) of creatine kinase. The gas phase was acetylene (5%) in argon. Reaction was initiated by the addition of $Na_2S_2O_4$ (20 µmol) or of nitrogenase extract. The reaction vessels were incubated with shaking (100 rev/min) at 25° C for crude extracts and 30° C for partially purified enzyme. For nitrite inhibition assays, conical flasks (35 ml) stoppered with Suba-Seal, were used and $NaNO_2$ was added, at different concentrations. The vessels were flushed with argon; then C_2H_2 was injected to give final concentration, in the range of 0.025 – 0.2 mM corresponding to 0.6 10^{-3} to 4.8 10^{-3} bar. The flasks were shaken 30 min, at 25° C, to ensure C_2H_2 dissolution before addition of $Na_2S_2O_4$ and enzyme. The reaction was terminated by the injection of 0.5 ml of 2.5 HCl, after 15 min.

Nitrite Separation After Binding

Nitrite, incubated with nitrogenase preparations, was removed by Sephadex filtration. Mixtures were loaded anaerobically onto a G-25 column (1.6 × 10 cm) previously equilibrated with 0.1 M TES buffer (pH 7.4) containing 1.2 mM dithionite and dithiothreitol. The column was eluted with the same buffer, nitrogenase was collected in the 6 – 10 ml fraction and the elution volume for NO_2^- was 14 – 20 ml.

Nitrogenase Activation

After storage in liquid nitrogen, activity of partially purified extracts or Fe protein preparations was restored by addition of a mixture proposed by Bergersen and Turner (1973), containing dithiothreitol, $(NH_4)_2SO_4$, $FeSO_4\ 6H_2O$ and dithionite at a final concentration of 5 mM, for partially purified extracts and 2 mM, for Fe protein preparations.

Quantitative Assays

Protein was measured by the method of Lowry et al. (1951) using the Folin-Ciocalteu reagent, with bovine serum albumin as a standard.

Nitrite was estimated by the colorimetric method of Nicholas and Nason (1957). To avoid interferences with dithionite, incubation mixtures were agitated in the presence of 0.22 mM methyl viologen, in a stream of O_2, to oxidize $Na_2S_2O_4$, before NO_2^- measurement (Kennedy et al., 1975).

Results

Purification of Nitrogenase

The purification procedure, proposed by Israel et al. (1974), was routinely used and gave reproducible results, except that nitrogenase was localized in the 40 – 70% PPG saturation fraction instead of 21 – 41%. Possible variations in the characteristics of PPG, generally purchased as "pactical grade", could be responsible for these differences. Partially purified extract reduced 100 to 120 nmol of C_2H_2 per mg of protein per min. After DEAE-cellulose chromatography, specific activity of the MoFe protein reached 750 – 800 nmol of C_2H_4 formed per min per mg of protein after recombining with an optimal amount of the complementary component. When unfractionated purified nitrogenase or Fe protein preparations were stored in liquid nitrogen the loss of activity was variable, depended on protein content of extracts and needed reactivation treatment before used. In contrast, crude extracts could be kept under the same conditions during several months without any loss of activity.

Nitrite Inhibition of Unfractionated Nitrogenase

When purified extracts of nitrogenase were incubated with $NaNO_2$, C_2H_2 reduction was strongly inhibited. As shown in Fig. 1, 50% inhibition occurred for 0.1 mM NO_2^- concentration. This result was not far from that previously reported with crude extracts of nitrogenase from soybean bacteroids (Kennedy et al., 1975). The inhibition of C_2H_2 reduction, determined after 15 min, increased with increasing NO_2^- levels (Fig. 1, curve b). In contrast, within the first 5 min, no evolution of this inhibition was observed for NO_2^- concentrations over 0.1 mM (Fig. 1, curve a). To determine the characteristics of this inhibition, experiments were performed in the presence of different concentrations of C_2H_2 and $NaNO_2$. The double reciprocal plots representation (Fig. 2) showed that apparent Michaelis constant (K_m) value of nitrogenase for C_2H_2 (0.051 mM) increased with increasing NO_2^- concentration and apparent maximum velocity (V_{max}) value (80 nmol C_2H_4 per min) was unaffected by the presence of NO_2^-. This type of inhibition was referred to as competitive and the apparent inhibition constant (K_i) was 5.2 mM. On the other side, when nitrogenase was incubated with different concentrations of nitrite, in the absence of C_2H_2, no significant disappearance of NO_2^- occurred, pointing out the inability for nitrogenase to use this molecule as substrate.

In further experiments, unfractionated nitrogenase was preincubated for different periods in the presence of $NaNO_2$ at different concentrations. As shown in Fig. 3, in the absence of nitrite, the level of nitrogenase activity was not modified by the preincubation time (K_m = 0.068 mM in average). For lower NO_2^- concentrations (0.025 and 0.05 mM), inhibition of N_2-ase increased with increasing preincubation periods as indicated by higher values of apparent K_m (K_m app 1 = 0.079 to 0.108 mM and K_m app 2 = 0.09 to 0.126 mM). For the highest concentration of NO_2^- (0.1 mM) the slopes of the curves remained constant whatever the time of preincubation was, pointing out no evolution in the inhibition level after 3 min (K_m app 3 = 0.15 mM in average). An evolution of the intercepts of the curves was observed in relation with the preincubation time and Fig. 3c only showed a typical representation of a competitive inhibition. The for-

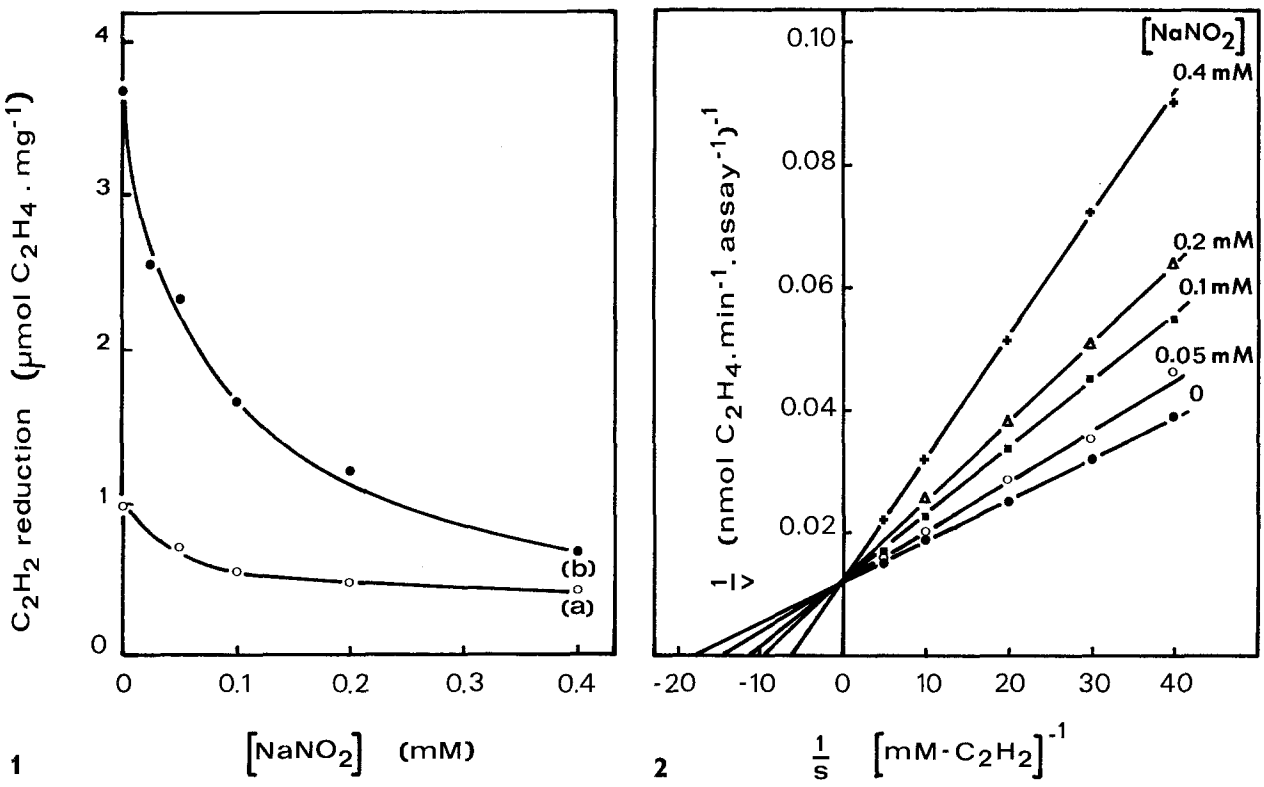

Fig. 1. Effect of nitrite concentration upon C_2H_2 reduction by partially purified nitrogenase. Assays were performed as described in "*Methods*" for: **a** 5 min and **b** 15 min. The shaking rate was 100 rev/min

Fig. 2. Lineweaver-Burk plot for C_2H_2 at several constant concentrations of nitrite. Assays were conducted, at 25° C, for 15 min, with protein content of 3.4 mg

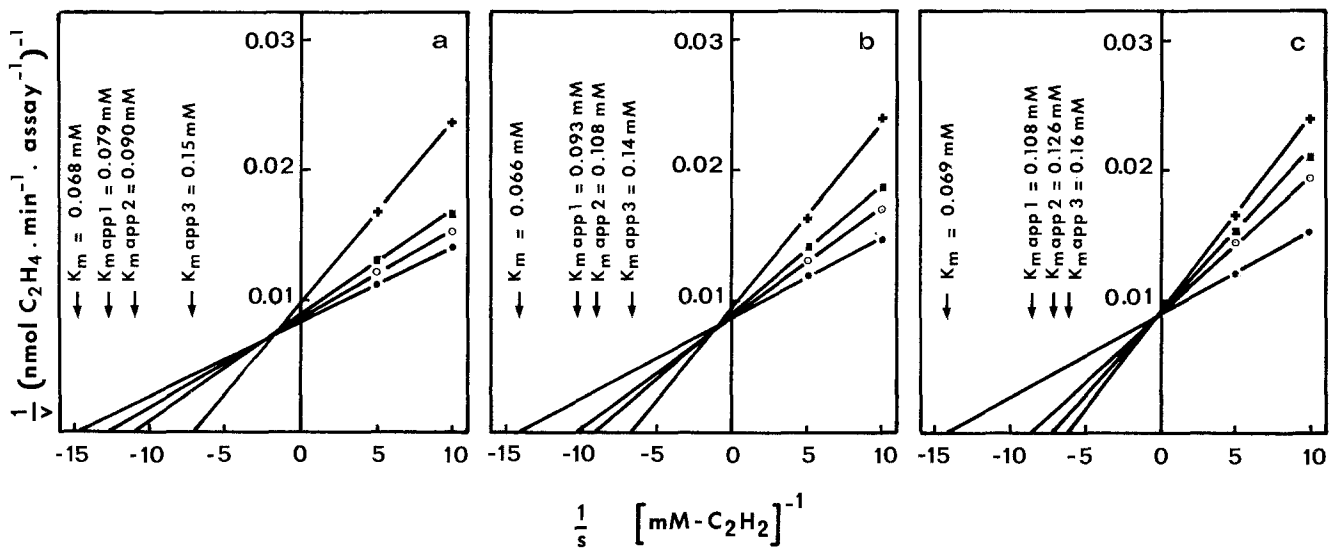

Fig. 3a—c. Double reciprocal plots for C_2H_2 at different $NaNO_2$ concentrations used during the preincubation period. Partially purified nitrogenase (8 mg/ml) was preincubated in 25 mM TES buffer (pH 7.4) at 25° C for: **a** 3 min, **b** 6 min, **c** 12 min, with $NaNO_2$ (○——○ 0.025 mM, ■——■ 0.05 mM, +——+ 0.1 mM) or without $NaNO_2$ (●——●). Samples (0.4 ml) of the incubation mixture were withdrawn, for testing nitrogenase activity; assay mixtures (2 ml) were shaken at 100 rev/min for 15 min

mation of the "enzyme-inhibitor" complex (EI) was faster when the inhibitor concentration was higher. Under our experimental conditions, an abnormally long period of 12 min of preincubation was necessary to reach equilibrium.

The slow formation of the enzyme-inhibitor complex in the presence of 0.025 mM $NaNO_2$ in the preincubation mixture was illustrated in Fig. 4. The value of apparent constant of association (k_a) to form EI was about 60 $M^{-1} \cdot s^{-1}$. The different experiments described above were carried out with nitrogenase preparations freshly purified, as well as with stored reactivated enzyme. With both enzyme preparations similar results were obtained.

Nitrite Effect on Separated Nitrogenase Components

When Fe protein was preincubated in the presence of nitrite (0.1 mM), for 5 min, then recombined with MoFe protein, C_2H_2 reduction rate (60 nmol $C_2H_4 \cdot mg^{-1} \cdot min^{-1}$) was unaffected by this treatment (Fig. 5a). Increasing preincubation time, from 6 to 12 min, with the same concentration of NO_2^- did not modify activity of Fe component after recombination. In contrast, similar experiments carried out with the MoFe component gave 80% inhibition of nitrogenase activity (Fig. 5b), confirming the interaction of NO_2^- with the MoFe component. In other experiments, the MoFe and Fe components together were preincubated with nitrite; as shown in Fig. 5c, inhibition of C_2H_2 reduction was less pronounced (56%) and a 2-fold enhancement of activity was observed in the control, in comparison with that of MoFe or Fe components preincubated separately and in the absence of nitrite (Fig. 5a and b).

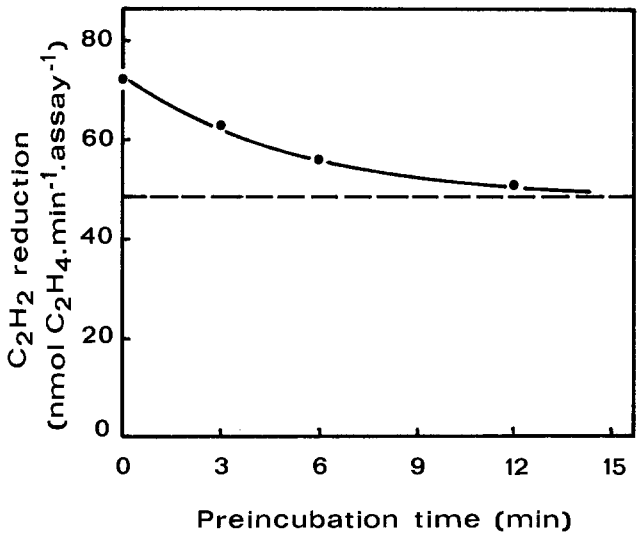

Fig. 4. Effect of preincubation period upon C_2H_2 reduction activity. Experimental conditions are as described in Fig. 3 and C_2H_2 reduction data are plotted for 0.1 mM C_2H_2 and 0.025 mM $NaNO_2$

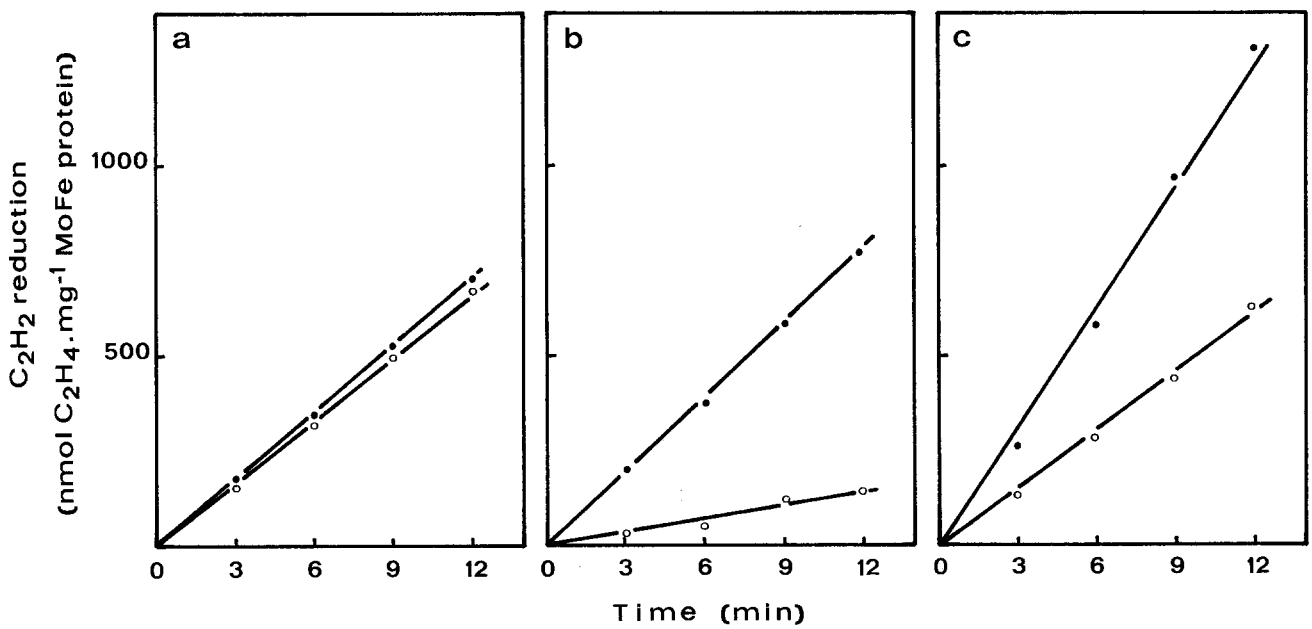

Fig. 5a—c. Time course of C_2H_2 reduction by purified MoFe and Fe components recombined after preincubation for 5 min with 0.1 mM $NaNO_2$ (○——○) or not (●——●). Preincubation of Fe protein alone **a**, MoFe protein alone **b** and MoFe plus Fe proteins **c**. Assays were conducted with 0.15 mg MoFe protein and 0.3 mg Fe protein, at 25° C with shaking (100 rev/min)

Reversibility of Nitrite Inhibition

Unfractionated nitrogenase, preincubated 5 min with 0.1 mM $NaNO_2$ and tested for C_2H_2 reduction activity, reduced 50 nmol $C_2H_2 \cdot mg^{-1} \cdot min^{-1}$ instead of 95 for the control, giving an inhibition level of 48%. Nitrite was removed from preincubation mixture, after 5 min, by Sephadex filtration. Activities of treated nitrogenase and of the control, submitted also to filtration, were about the same: 70 nmol $C_2H_4 \cdot m^{-1} \cdot min^{-1}$. Complete recovery of C_2H_2 reduction activity pointed out a perfectly reversible binding between NO_2^- and nitrogenase. Some differences occurring in enzymatic activities of control before and after gel chromatography could be due to usual losses observed after Sephadex filtration treatment.

Discussion

Nitrite acted as an inhibitor of purified nitrogenase from bacteroids, confirming our previous results obtained with bacteroid crude extracts (Kennedy et al., 1975). This inhibition appeared as typically competitive, with a K_i value of 5.2 mM and exhibited an interesting characteristic: the apparent constant of association to form EI was extremely low ($60\ M^{-1} \cdot s^{-1}$) in comparison with the values usually reported for this type of inhibition (10^8 to $10^9\ M^{-1} \cdot s^{-1}$). This explains the increasing inhibition rate of nitrogenase observed between 5 and 15 min (Fig. 1): 12 min, in average, were required to reach equilibrium in the formation of EI complex.

Substrates of nitrogenase bind to the MoFe component (Burns et al., 1971). The presented preincubation experiments showed that NO_2^- bound with MoFe protein too, giving a 80% inhibition. This is in agreement with the competitive inhibition reported here: substrate and inhibitor binding to the same site. The Fe protein, was not affected when it was preincubated alone with NO_2^-, well known for its strong reactivity. Similar results were reported by Seto and Mortenson (1974) concerning carbamyl phosphate inhibition; this compound bound with MoFe protein from *Clostridium* nitrogenase without any reaction with Fe protein.

A stimulatory effect of C_2H_2 reduction occurred when the two components were preincubated together during 5 min, without nitrite, prior testing their activity (Fig. 5). This result could suggest different changes in the conformation of protein allowing an easier and optimal accessibility of sites for acetylene. Preincubation experiments, in the presence of substrate, inducing conformational changes in nitrogenase have been recently reported with *Anabaena* (Apte et al., 1978). In these same conditions, addition of NO_2^- was responsible for a 56% inhibition of nitrogenase activity instead of the 80% level observed when the MoFe protein was preincubated alone; this result pointed out a possible protective role of MoFe component sites by Fe protein. The slow formation of the EI complex reported above (Figs. 3 and 4) could be in direct relation with this effect.

Acetylene reduction activity of unfractionated nitrogenase, 50% inhibited by nitrite, was restored completely after elimination of NO_2^-, showing a total reversibility. This property could be of great interest, in vivo, if NO_2^- was produced from exogenous nitrate. Although the results obtained by Gibson and Pagan (1977), using nitrate-reductase-deficient mutants of *Rhizobium*, would exclude a physiological role for NO_2^-, Rigaud (1976) reported the presence of NO_2^- in nodules, after NO_3^- treatment, but only when an active nitrate reductase occurred in the bacteroids. The reaction of nitrite with leghaemoglobin (Rigaud and Puppo, 1977) and the possible induction of an NO_2^--reductase in the bacteroid (Rigaud, 1976) could explain the failure to detect free NO_2^- in nodules. In so far as NO_2^- could be involved in the inhibition of bacteroid nitrogenase, our results showed a possible restoration of activity after nitrite disappearance. In this way, Gibson (1976) reported complete recovery of C_2H_2 reduction activity in soybean root nodules within the four days following the end of plant exposure to KNO_3.

In most cases, competitive inhibitors, in the N_2-reducing process, are able to act as alternative substrates for nitrogenase (Schöllhorn and Burris, 1967; Hwang et al., 1973), but nitrite must be considered as an inhibitor. This is a common characteristic with CO, a noncompetitive inhibitor of nitrogenase (Hwang et al., 1973), which also gave a reversible inhibition, as reported by Rivera-Ortiz and Burris (1975).

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