Nitrate and Nitrite Reduction in Relation to Nitrogenase Activity in Soybean Nodules and \textit{Rhizobium japonicum} Bacteroids

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ABSTRACT

Soybean (\textit{Glycine max} L. cv Williams) seeds were sown in pots containing a 1:1 perlite-vermiculite mixture and grown under greenhouse conditions. Nodules were initiated with a nitrate reductase expressing strain of \textit{Rhizobium japonicum}, USDA 110, or with nitrate reductase nonexpressing mutants (NR\textsuperscript{-}108, NR\textsuperscript{-}303) derived from USDA 110. Nodules initiated with either type of strain were normal in appearance and demonstrated nitrogenase activity (acyetylene reduction). The \textit{in vivo} nitrate reductase activity of \textit{N}\textsubscript{2}-growing nodules initiated with nitrate reductase-negative mutant strains was less than 10\% of the activity shown by nodules initiated with the wild-type strain. Regardless of the bacterial strain used for inoculation, the nodule cytosol and the cell-free extracts of the leaves contained both nitrate reductase and nitrite reductase activities. The wild-type bacteroids contained nitrate reductase but not nitrite reductase activity while the bacteroids of strains NR\textsuperscript{-}108 and NR\textsuperscript{-}303 contained neither nitrate reductase nor nitrite reductase activities.

Addition of 20 millimolar KNO\textsubscript{3} to bacteroids of the wild-type strain caused a decrease in nitrogenase activity by more than 50\%, but the nitrate reductase-negative strains were insensitive to nitrate. The nitrogenase activity of detached nodules initiated with the nitrate reductase-negative mutant strains was less affected by the KNO\textsubscript{3} treatment as compared to the wild-type strain; however, the results were less conclusive than those obtained with the isolated bacteroids.

The addition of either KNO\textsubscript{3} or KNO\textsubscript{2} to detached nodules (wild type) suspended in a semisolid agar nutrient medium caused an inhibition of nitrogenase activity of 50\% and 65\% as compared to the minus N controls, and provided direct evidence for a localized effect of nitrate and nitrite at the nodule level. Addition of 0.1 millimolar sucrose stimulated nitrogenase activity in the presence or absence of nitrate or nitrite. The sucrose treatment also helped to decrease the level of nitrite accumulated within the nodules.

A full understanding of the relationships between NO\textsubscript{3} reduction and N\textsubscript{2} fixation is needed to help maximize the use of both N sources by nodulated legumes. Recent reports (9, 23) have indicated that soybeans can benefit from the complementary operation of both biological nitrogen fixation and leaf nitrate assimilation.

An increase in the availability of NO\textsubscript{3} may result in a larger contribution from NO\textsubscript{3} assimilation, however nodulation and nitrogenase activity may be decreased (4, 5, 16). The primary cause of this inhibitory effect by NO\textsubscript{3} is not clear and information is still lacking with regard to ways to relieve the inhibition of N\textsubscript{2}-ase activity by NO\textsubscript{3}.

Nitrate accumulation within the nodules has been shown in NO\textsubscript{3}-treated plants of several legumes including soybeans (15, 22). NO\textsubscript{3} is a known inhibitor of several aspects of N\textsubscript{2} fixation (12, 19, 21, 24). However, Gibson and Pagan (6) and Manhart and Wong (15) have concluded that NO\textsubscript{3} the product of bacteroid NR, may not play a role in the inhibition of acetylene reduction by NO\textsubscript{3}. These authors based their conclusions on whole plant studies. The use of whole plants may be helpful to obtain an integrated view of the relationships between NO\textsubscript{3} reduction and N\textsubscript{2} fixation but may be complicated by limitations imposed by the processes of NO\textsubscript{3} uptake, mobilization, storage, and reduction in compartments other than the nodules. Studies using isolated parts of the symbiotic system may provide more direct information on the metabolism of NO\textsubscript{3} within the nodules and its relationship to N\textsubscript{2} fixation. The availability of NR-negative mutants of \textit{Rhizobium japonicum} (25) facilitates such studies. Mutants lacking nitrate reductase (NR\textsuperscript{-}), produced from \textit{R. japonicum} strain 61A76, have been shown to produce normal nodules and fix N\textsubscript{2} in symbiosis with soybean plants (25). The objectives of this study were: (a) to characterize the relationships between NO\textsubscript{3} reduction, NO\textsubscript{3} reductase, and N\textsubscript{2}-ase activity using detached soybean nodules and \textit{R. japonicum} bacteroids; (b) to reevaluate the role of the bacteroid NR as a mediator of the NO\textsubscript{3} effect on N\textsubscript{2}-ase activity and; (c) to develop NR-negative mutants of \textit{R. japonicum} tolerant to normally inhibitory levels of NO\textsubscript{3}.

MATERIALS AND METHODS

Plant and Bacteria Culture. The \textit{Rhizobium japonicum} strain (USDA 110) used in this study was obtained from Dr. D. F. Weber (USDA at Beltsville, MD). The bacteria were cultured and mutant strains lacking NRA (NR\textsuperscript{-}) were prepared and tested as previously described (25). In one experiment (Fig. 2), another \textit{R. japonicum} strain, 61A76, (obtained from Dr. J. C. Burton of the Nitragin Company, Milwaukee, WI) was used. Soybean (\textit{Glycine max} L. cv Williams) seeds were provided by Dr. J. Justin (Soils and Crops Department, Rutgers University, NJ). Soybean seeds were surface sterilized by treatment with 70\% ethanol (v/v) for 5 min followed by extensive rinsing with distilled H\textsubscript{2}O prior to inoculation (10\textsuperscript{5}–10\textsuperscript{9} cells/ml). The surface-sterilized seeds were planted in an autoclaved mixture (1:1) of perlite and vermiculite in pots (20.5 cm diameter x 21.5 cm high). Each pot, containing

\textsuperscript{1} Abbreviations: NR, nitrate reductase; NRA, nitrate reductase activity; NiR, nitrite reductase.

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three plants, was supplied daily with 500 ml of a N-free nutrient solution containing: 2 mM CaSO4·2H2O, 5 mM K2SO4, 1.0 mM MgSO4·7H2O, 0.25 mM K2HPO4, 20 μM Fe (EDTA), and minor elements were added as 1 ml stock/L. The stock solution contained in g/L: 3.72 KCl, 1.54 H3BO3, 0.83 MnSO4·H2O, 0.57 ZnSO4·7H2O, 0.125 CuSO4·5H2O, and 0.12 NaMoO4·2H2O. KNO3 (20 mM) was added as indicated in the text. Plants were grown in a greenhouse on a 16/8 h day/night regimen, and illumination was provided by daylight supplemented with incandescent lamps. The light intensity fluctuated throughout the experimental period between 15 and 25 klux (determined at noon at plant height).

Detached Nodules. Plants were carefully uprooted 25 to 35 d after planting, and the roots systems were rinsed thoroughly with distilled H2O. Nodules of similar size and appearance were carefully detached, rinsed with distilled H2O, and pooled. Nodules (100 mg), selected at random from the pooled sample, were placed detachment point down in 25-ml serum bottles containing 1 ml of semisolid nodule culture medium consisting of N-free plant nutrient solution, 0.15% agar, and 50 mM K-phosphate (pH 6.8).

Sucrose (0.1 M), KNO3 (20 mM), and KNO3 (5 mM) were added to the semisolid nodule culture medium as indicated in the text. The nodules remained partially submerged in the semisolid medium allowing diffusion from both the gas phase and the supporting medium. The nodules were vacuum infiltrated for 2 min at 50 cm Hg. The serum bottles were capped with airtight serum stoppers, incubated under 0.85 atm air and 1.5 atm acetylene, and assayed for C2H2 reduction. Nodules initiated by the different Rhizobium strains were processed separately.

Bacteroid Preparation. Nodules were harvested as previously indicated. All subsequent steps were carried out under a N2 atmosphere in an anaerobic glove bag (Instruments for Research and Industry, Cheltenham, PA), and all solutions were thoroughly degassed and flushed with N2. All preparatory procedures were carried out at 4°C. Nodules were homogenized, using a Waring Blender, with 5 volumes of buffer containing 0.3 M sucrose, 100 mM K-phosphate (pH 7.5), 5 mM dithionite, and 2% (w/v) PVP 40. The homogenate was filtered through four layers of cheesecloth and centrifuged for 2 min at 500 g to remove plant debris. The supernatant was then centrifuged for 10 min at 6000 g to pellet the bacteroids. The pellet was washed twice in 50 mM K-phosphate (pH 6.8) containing 0.3 M sucrose. Finally, the bacteroids were resuspended in an incubation medium containing: 0.3 M sucrose, 50 mM K-phosphate (pH 6.8), and 10 mM Na-succinate. Bacteroid enzyme assays were conducted in 25-ml serum bottles containing 2 ml of incubation medium and 1.0 to 1.5 mg bacteroid protein. The bacteroids were assayed by following the number of NO2 gas reduction after addition of 20 mM KNO3. NiR assays were conducted by following the disappearance of NO2 from the media after addition of 0.3 M KNO3 (17). Acetylene reduction assays were conducted in the presence or absence of 20 mM KNO3. All procedures had been conducted under a N2 atmosphere, and 1% O2 was added to the vials before assays. The vials and contents were continuously shaken at 120 rpm on a gyratory shaker (New Brunswick Scientific) at room temperature.

Enzyme Assays. Leaf and nodule homogenates for in vitro NR and NiR were prepared by grinding with 10 volumes of cold extraction medium containing: 25 mM K-phosphate (pH 7.6), 5 mM Na2EDTA, and 5 mM cysteine (18). Leaf and nodule cytosol fractions were obtained after centrifugation of the homogenates at 30,000g at 4°C for 10 min. The pellet was discarded. NR activity was assayed as described by Hageman and Hucklesey (7). In vivo NR assays were conducted in the dark at 30°C as previously described (4). Leaf tissue was cut into small sections (about 1 cm), and nodules were sliced and placed into 15-ml vials containing 5 ml of incubation medium composed of: 50 mM K-phosphate (pH 7.5), 100 mM KNO3, 1% (v/v) propanol, and 0.05% (v/v) Neutro- nyx 600 (Onyx Chemical Co., Jersey City, NJ). The tissues were vacuum infiltrated and subsamples taken after 10 and 70 min for NO2 determinations (17). NiR activity was determined using a dithionate-methylene blue oxidoreductase system and following the disappearance of NO2 from the assay media (11).

Nitrogenase activity was assayed using the C2H2 reduction method (8) in detached nodules or bacteroids prepared as described above, or whole root systems, washed and placed in Mason jars (480 ml) fitted with serum stoppers. After incubation at 30°C, the amount of C2H2 produced was determined using a Perkin-Elmer (Sigma 4) gas chromatograph equipped with a H2/air flame ionization detector and a stainless steel column packed with Porapak N (192 × 0.3 cm). The column was at a 100°C, and N2 was used as the carrier gas (flow rate, 30 ml/min). Protein was determined using the method described by Bradford (1).

Bacteroids were lysed, prior to protein analysis, by boiling in 0.1 N NaOH for 5 min (13). Nitrite accumulation in nodules was determined by grinding 100 mg nodules with 2 ml of an alkaline solution (pH 10) made of 25 mM trisphosphate (K3PO4· H2O) preheated at 80°C. An aliquot from the homogenate was reacted with 2 ml of the color reagent (17), and water was added to complete a total volume of 4 ml. The mixture was centrifuged at 8000 g for 10 min, and the supernatant was used to determine the A at 540 nm using a spectrophotometer.

RESULTS AND DISCUSSION

NR- Mutants of R. japonicum. Strain USDA 110 grown on agar plates containing 10 mM KClO3, and incubated microaerophilically, produced only a few, sparsely distributed colonies. Individual colonies were labeled as nitrate-resistant mutants, and isolates lacking the ability to reduce NO2 to NO were then relabeled as NR- mutants. Two of these isolates, NR- 108 and NR- 303, were selected for the remainder of this work. The symbiotic characterization of the parent (USDA 110) and the two NR- strains (108 and 303) is illustrated in Table I. Both NR- strains produced normal nodules containing leghemoglobin and showing a positive N2-ase activity (nodules assayed attached to the roots). The NR activity of intact nodules initiated with both NR- strains was less than 10% of the NR activity of nodules initiated by the parent strain (Table I). This indicated that the bacteroid NR was probably responsible for more than 90% of the NO2 reduction occurring within the nodules. NO2 accumulation in the nodules of 35-d-old soybean plants treated with 20 mM KNO3 for 48 h was observed only in nodules initiated by the NR- strain (Table I). Thus, NO2 accumulation in soybean nodules is very likely dependent upon the bacteroid NR as proposed for other legumes (6, 15).

Bacteroid NR and Nitrogenase Activities. Only bacteroids of the parent strain (USDA 110) were able to reduce NO2 to NO2 under microaerophilic conditions. Bacteroid preparations from nodules initiated with any of the three strains (USDA 110, NR- 108, NR- 303) were devoid of nitrite reductase activity under both aerobic (Table II) and anaerobic conditions (data not shown). Thus, the rate of NO2 production and accumulation can be taken as an approximation of the level of NR activity by R. japonicum bacteroids. The observed microaerophilic rate of NR activity was 333 nmol h-1 g-1 protein which is about 3- and 1.5-fold larger than the NR activity reported for bacteroids of R. japonicum 61A133 assayed aerobically and anaerobically, respectively (14). The NR assays were made under microaerophilic conditions to facilitate comparisons with N2-ase assays measured at similar O2 tensions. The time course of C2H2 reduction by isolated bacteroids was linear over the 2 h assay time, but linearity was lost and the activity decreased by more than 50% in bacteroids of strain 110 (wild type) assayed with 20 mM KNO3 (Fig. 1). The two NR- strains (108 and 303), however, showed similar rates of N2-ase activity in the presence or absence of KNO3 (Fig. 1). These results have several implications: (a) the inhibition by NO2 of acetylene

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NITRATE REDUCTION AND NITROGENASE ACTIVITY

Table I. NRA, Nitrite Accumulation, Nitrogenase Activity, and Weight of Nodules from 35-Day-Old Soybean Plants Nodulated by NR+ (USDA 110) or NR– (108 and 303) Strains of Rhizobium japonicum

<table>
<thead>
<tr>
<th>Rhizobium Strain</th>
<th>Nodule Fresh Wt</th>
<th>Enzyme Activities</th>
<th>NO2– Accumulation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/plant</td>
<td>µmol h–1 g–1 fresh wt</td>
<td>nmol/g fresh wt</td>
</tr>
<tr>
<td>USDA 110</td>
<td>1.03 ± 0.21</td>
<td>5.45 ± 0.68</td>
<td>12.40 ± 0.71</td>
</tr>
<tr>
<td>NR– 108</td>
<td>1.09 ± 0.17</td>
<td>0.18 ± 0.04</td>
<td>11.39 ± 2.14</td>
</tr>
<tr>
<td>NR– 303</td>
<td>1.06 ± 0.23</td>
<td>0.35 ± 0.02</td>
<td>18.44 ± 3.65</td>
</tr>
</tbody>
</table>

* NO2– was determined 48 h after exposure to 20 mM KNO3.

Table II. NR and NiR activities of Leaves, Nodule Cytosol, and Bacteroids Obtained from Soybean Plants Nodulated with NR+ (USDA 110) or NR– (108) Strains of Rhizobium japonicum

<table>
<thead>
<tr>
<th>Fraction</th>
<th>NR+ (USDA 110)</th>
<th>NR– (108)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
</tr>
<tr>
<td>NR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaves</td>
<td>27 ± 1.1</td>
<td>47 ± 2.1</td>
</tr>
<tr>
<td>Nodule cytosol</td>
<td>28 ± 0.3</td>
<td>118 ± 2.0</td>
</tr>
<tr>
<td>Bacteroids</td>
<td>205 ± 39</td>
<td>206 ± 4.1</td>
</tr>
<tr>
<td>NiR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaves</td>
<td>686 ± 71</td>
<td>875 ± 90</td>
</tr>
<tr>
<td>Nodule cytosol</td>
<td>432 ± 19</td>
<td>652 ± 41</td>
</tr>
<tr>
<td>Bacteroids</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Distribution of NR and NiR Activities. Soybean plants, nodulated with wild-type R. japonicum USDA 110 or NR– 108, were separated into different fractions: leaves, nodule cytosol, and bacteroids. These fractions were then assayed for NR and NiR activities. Regardless of the bacterial strain used for inoculation, the nodule cytosol, and the cell-free extracts of leaves contained both NR and NiR activities. The wild-type bacteroids contained NR but not NiR activity while the NR– 108 bacteroids contained neither NR nor NiR activities (Table II). The occurrence of both NR and NiR in the nodule cytosol of plants inoculated with NR– bacteria, permits the conclusion that both of these enzymes are of host origin. The nodule cytosol NR and NiR activities increased following treatment of N2-grown plants with KNO3 (20 mM) for 48 h; however, the bacteroid NR activity of the parent strain was unaffected (Table II). It is the nodule cytosol NR that most likely accounts for the residual NR activity shown by nodules initiated by the NR– strains in Table I. The occurrence of NR, but not NiR, has previously been shown in nodule cytosols of peas (2) and soybeans (22). The activity of NiR assayed in vitro was several times the corresponding activity of NR (Table II) and, therefore, in principle NR but not NiR should be considered as the rate-limiting step in the sequence of reactions involved in NO3– metabolism in nodules. However, the relationships between the rates of NO3– and NO2– reduction in situ may be very different, since NO2– can accumulate within the nodules (Table I).

Nitrogenase Activity by Detached Nodules. To study the effect of NO3– or other substances on the N2-ase activity associated with detached nodules, the selected compound must be in solution. However, suspension of the nodules in an aqueous nutrient medium results in lower rates and a rapid loss of N2-ase activity (Fig. 2). Nodules suspended in a semisolid agar nutrient medium and vacuum infiltrated gave a continuous and near linear production of C2H4 for at least 4 h (Fig. 2). Furthermore, the rates of N2-ase activity by detached nodules in the semisolid medium were similar to the rates obtained with whole roots assayed in air (Fig. 2). These results indicated that separation of the nodules from the plant did not result in an impairment of their N2-fixing capacity and that the semisolid nutrient medium provided adequate conditions for optimal functioning of the nodules. The supporting media reported here had the following characteristics and advantages. (a) The presence of a number of salts in the media helped to maintain a more natural physiological environment (Fig. 2). (b) By decreasing the agar concentration to 0.15%, thus making the medium semisolid, the nodules will not sink in the medium; however, the nutrients and test compounds may diffuse into the nodules more easily. (c) The detached nodules can no longer depend on the vascular system of the plant for the supply of basic nutrients; therefore, vacuum infiltration of the nodules aids in delivering the nutrients and test compounds from the medium to the interior of the nodule.

Effects of Sucrose, NO2–, and NO3– Additions. Long-term CO2 enrichment studies have been shown to increase nodule activity (total and specific) in soybean plants (10, 20), and the authors

Fig. 1. Tolerance of nitrogenase activity to NO3– in R. japonicum bacteroids isolated from soybean nodules initiated by NR+ strain USDA 110 (○, ■) and two NR– strains: NR– 108 (●, □) and NR– 303 (▲, △). (●, ■, ○, □, ▲), controls without NO3–; (●, □, △), activities in the presence of 20 mM KNO3. Each point in the figure represents the mean of three replicates ± SD.

reduction by bacteroids of strain 110 (wild type) was associated with the accumulation of NO3–; (b) the lack of NR activity in bacteroids of NR– 108 and NR– 303 was the most likely reason for their tolerance or insensitivity to NO3–; and (c) the presence of NO3– but not NO2– inhibited the N2-ase activity of the isolated bacteroids.
Fig. 2. Time course of acetylene reduction by detached soybean nodules initiated with R. juponicum 61A76 and suspended in a liquid (Δ) or a semisolid agar nutrient medium (Ο). The time course of C2H2 reduction by nodulated whole roots (□) is included for comparison. Each point in the figure represents the mean of three replicates ± SD.

Fig. 3. Effects of NO3 and sucrose (A) and NO2 and sucrose (B) on the nitrogenase activity of detached soybean nodules suspended in a semisolid agar nutrient medium. Nodules were initiated by R. japonicum USDA 110. (Ο), No additions; (Δ), sucrose (0.1 m); (□), KNO3 (20 mm); (□), KNO2 (20 mm) + sucrose (0.1 m); (Δ), KNO3 (5 mm); (Δ), KNO2 (5 mm) + sucrose (0.1 m). Each point represents the mean of three replicates ± SD.

Table III. Effects of Nitrate, Nitrite, and Sucrose on the Level of Nitrite Accumulation in Detached Soybean Nodules Initiated by R. japonicum USDA 110

Nitrite levels were determined after incubation for 2 h in the semisolid nodule culture medium. Each value represents the mean of three replicates ± SD.

<table>
<thead>
<tr>
<th>Additions</th>
<th>NO3- Accumulation</th>
<th>nmol/g fresh wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. None</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B. KNO3 (20 mm)</td>
<td>289 ± 10</td>
<td></td>
</tr>
<tr>
<td>C. B + sucrose (0.1 m)</td>
<td>201 ± 4</td>
<td></td>
</tr>
<tr>
<td>D. KNO2 (5 mm)</td>
<td>649 ± 70</td>
<td></td>
</tr>
<tr>
<td>E. D + sucrose (0.1 m)</td>
<td>360 ± 87</td>
<td></td>
</tr>
</tbody>
</table>

suggested that the increased supply of current photosynthate was directly responsible for the observed increase in nitrogenase activity. However, Finn and Brun (3) have recently reported that neither total nor specific nodule activities were increased during exposure to short-term (36 h) CO2 enrichment and concluded that nodule activity was not directly limited by current photosynthesis but rather by the partitioning and utilization of photosynthate in the plant. Other studies have shown that addition of sugars (fructose, glucose, or sucrose) to the rooting media helped to alleviate the inhibitory effects of NO3 on symbiotic N2 fixation by nodulated lentils (26). The author concluded that the added sugars alleviated the inhibitory effects of NO3 not only by increasing the carbohydrate supply so that both N2 fixation and NO3 assimilation could be supported but also by inhibiting the accumulation of NO3 and hence lowering NR activity in the leaves (26). We have obtained more direct evidence for the stimulatory effects of sucrose on nitrogenase activity by using detached soybean nodules (Fig. 3). Addition of sucrose (0.1 mM) stimulated the N2-ase activity in both the presence and absence of NO3 or NO2 (Fig. 3). The concentration of sucrose reported here was found to be optimal in preliminary experiments using a range of concentrations from 0 to 300 mM (data not shown). Addition of 20 mM KNO3 to the minus sucrose treatment caused an inhibition of about 50% after 2 h of exposure (Fig. 3A), and the KNO3 treatment inhibited N2-ase activity by approximately 65% (Fig. 3B). These results provided direct evidence for a localized effect of NO3 and NO2 at the nodule level. Similarly, the stimulatory effect of sucrose indicated that sucrose was being taken up and possibly metabolized by the nodules to provide extra reducing equivalents for the N2-ase reaction. The ability of sucrose (0.1 mM) to provide at least partial relief for the inhibition of N2-ase activity by NO3 and/or NO2 could suggest that competition for reducing equivalents (energy) at the nodule level, between NO3 reduction and N2 fixation, may account, at least partially, for the NO3 effects. In addition, the sucrose treatment was associated with a decrease in the amount of NO3 being accumulated within the nodules (Table III). We observed no excretion of NO2 to the nodule culture medium by nodules supplied with KNO3; therefore, NO3 accumulation within the nodules may be considered to depend on the rates of NO3 reduction and NO2 reduction. Because the bacteroids do not contain NiR, it is likely that the NO3 levels in the nodule may be related to a nodule cytosol NiR of host origin (Table II) which may be aided by reducing equivalents arising from the oxidation of sugars. The data in Table III show that supplying sucrose at relatively high concentrations may be one effective way of decreasing the accumulation of NO3 in the nodules. It can be postulated that, in addition to sucrose, there may be other factors which may increase the rate of NO3 reduction in the nodule cytosol and in turn alleviate the inhibition of N2-ase activity by relatively high levels of NO3 that otherwise would accumulate.

Fig. 4. Effect of NO3 on the nitrogenase activity of detached soybean nodules initiated by two NR- strains of R. japonicum: NR 108 (□, △) and NR 303 (Δ, ▲). (□, △) controls without NO3; (□, △), activities in the presence of 20 mM KNO3. The parent strain USDA 110 (Ο, □) is included for comparison. Each point in the figure represents the mean of three replicates ± SD.
Conversely, factors affecting NiR activity negatively may result in higher levels of NO₂⁻ accumulated within the nodules. It is also possible that the inability of sucrose to completely eliminate NO₂⁻ accumulation resulted in only a partial relief of the inhibition of N₂-ase activity. Nonetheless, we feel that the observations reported here have added a new dimension to the relationships between N₂-ase activity and the processes of NO₃⁻ and NO₂⁻ reduction in soybean nodules. All of these results support the contention that increasing the allocation of sugars to the nodules, followed by the occurrence of efficient oxidative systems, should provide sufficient reducing power for the reduction of N₂, NO₃⁻, and NO₂⁻ with the possibility of maintaining the efficiency of N₂-ase activity without interferences from either NO₃⁻ or NO₂⁻ metabolism. The bacteroid appears to be the major source of NO₂⁻ within the nodules, and the use of NR⁻ mutants may be of major significance in decreasing the actual input of NO₂⁻ to the nodule cytosol (Tables I and II). Although the N₂-ase activity of nodules initiated with NR⁻ 108 or NR⁻ 303 was somewhat less affected by the NO₃⁻ treatment than the N₂-ase activity of nodules initiated with the NR⁺ (wild type) strain (Fig. 4), the results were less conclusive than those obtained with isolated bacteroids (Fig. 1). This may be due to the presence of a nodule cytosol NR in nodules initiated with either NR⁺ or NR⁻ strains (Table II), and, therefore, any practical solution to the observed inhibition of N₂-ase activity by NO₃⁻ will probably have to consider both the nodule cytosol and the bacteroid NR activities.

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LITERATURE CITED