

Nitrate Inhibition of Legume Nodule Growth and Activity¹

II. SHORT TERM STUDIES WITH HIGH NITRATE SUPPLY

Received for publication July 20, 1984 and in revised form October 15, 1984

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ABSTRACT

Soybean plants (*Glycine max* [L.] Merr) were grown in sand culture with 2 millimolar nitrate for 37 days and then supplied with 15 millimolar nitrate for 7 days. Control plants received 2 millimolar nitrate and 13 millimolar chloride and, after the 7-day treatment period, all plants were supplied with nil nitrate. The temporary treatment with high nitrate inhibited nitrogenase (acetylene reduction) activity by 80% whether or not *Rhizobium japonicum* bacteroids had nitrate reductase (NR) activity. The pattern of nitrite accumulation in nodules formed by NR⁺ rhizobia was inversely related to the decrease and recovery of nitrogenase activity. However, nitrite concentration in nodules formed by NR⁻ rhizobia appeared to be too low to explain the inhibition of nitrogenase. Carbohydrate composition was similar in control nodules and nodules receiving 15 millimolar nitrate suggesting that the inhibition of nitrogenase by nitrate was not related to the availability of carbohydrate.

Nodules on plants treated with 15 millimolar nitrate contained higher concentrations of amino N and, especially, ureide N than control nodules and, after withdrawal of nitrate, reduced N content of treated and control nodules returned to similar levels. The accumulation of N₂ fixation products in nodules in response to high nitrate treatment was observed with three *R. japonicum* strains, two NR⁺ and one NR⁻. The high nitrate treatment did not affect the allantoate/allantoin ratio or the proportion of amino N or ureide N in bacteroids (4%) and cytosol (96%).

When legume plants are supplied with nitrate, nodule growth (nodule weight/plant), and N₂-fixing activity may both be inhibited. While the negative effect of nitrate on nodule growth can best be examined by supplying moderate concentrations of nitrate over a period of several weeks (10–12), the effect of nitrate on N₂ fixation (acetylene reduction) can best be studied by supplying high concentrations of nitrate (15–20 mM) to well-nodulated plants for a period of several days (3, 6, 9, 10). With this approach there is little effect of nitrate on nodule weight, but a marked depression of nitrogenase activity occurs.

Nitrite is a potent inhibitor of nitrogenase *in vitro* with 50% inhibition at 100 μM NO₂⁻ and detectable inhibition at [NO₂⁻] as low as 25 μM (14). Nitrite accumulated in cowpea, lupine (6), and soybean (9) nodules when nodules were formed by NR²⁺-positive rhizobia. However, when nodules were formed by NR⁻

rhizobia, nitrite was not detected and acetylene reduction activity of these nodules was still inhibited by nitrate (6, 9).

Clear evidence for the accumulation of nitrite in nodules formed by NR⁻ rhizobia is required for acceptance of the idea that nitrite plays a role in the inhibition of N₂ fixation by nitrate. Results reported here indicate that nitrite accumulates in *Rhizobium*-NR⁻ soybean nodules but that [NO₂⁻] is extremely small. In contrast, there were large accumulations of export products (amino acids, ureides) of N₂ fixation in soybean nodules supplied with 15 mM nitrate for 7 d.

MATERIALS AND METHODS

Soybean plants (*Glycine max* (L.) Merr, cv 'Beeson 80') were grown in washed, autoclaved silica sand in a greenhouse. Seeds were inoculated at planting with liquid cultures of *R. japonicum* strains which were described elsewhere (13). Plants were grown with nutrient solutions (11) containing 2 mM nitrate, a concentration which stimulates plant growth but which does not inhibit the growth or nitrogenase activity of nodules under the experimental conditions employed (10–12).

After 37 to 56 d (late vegetative to flowering stage), nitrate concentration supplied to half of the pots was increased to 15 mM for 7 or 8 d after which all pots were thoroughly flushed with deionized H₂O and supplied with nutrient solution containing nil nitrate. During the high nitrate treatment period, 13 mM Cl⁻ was supplied to control plants.

Where NR⁻ *R. japonicum* was used, the possibility of nodule formation by contaminant rhizobia was assessed by monitoring bacteroid NR (11). NR was never detected in bacteroids from these nodules. Acetylene reduction activity was measured by incubating four to six nodulated roots in a 450-ml jar with 10% (v/v) acetylene. Gas samples obtained after 5, 10, and 15 min incubation were analyzed for [ethylene] using GC and the 5- to 15-min period was used for the rate calculation. Ammonium was extracted from 0.5 g samples of nodules as described previously (11) and was analyzed with an ammonium electrode. Nitrite concentration was determined using 1 g samples of fresh nodules (11). Another sample of nodules was thoroughly extracted with ethanol and, after removal of ethanol, this extract was used for the analysis of nitrate, ureides, and α-amino N, using alanine as a standard for the latter analysis (11). Carbohydrate composition of the ethanol extracts was determined by GLC (10).

RESULTS

When 15 mM nitrate was supplied to plants, [NO₂⁻] in nodules formed by NR⁺ *Rhizobium* increased about 10-fold, from 0.2 to 2.0 μg N/g fresh weight (Fig. 1). When nitrate supply was changed from 15 mM to nil nitrate, there was a sharp decline in [NO₂⁻] after a 2-d lag. A decline and subsequent increase in specific

¹ Supported in part by the United States Department of Agriculture under agreement no. 59-2392-1-1-725-0. Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center and The Ohio State University. Journal Article 110-84.

² Abbreviation: NR, nitrate reductase.

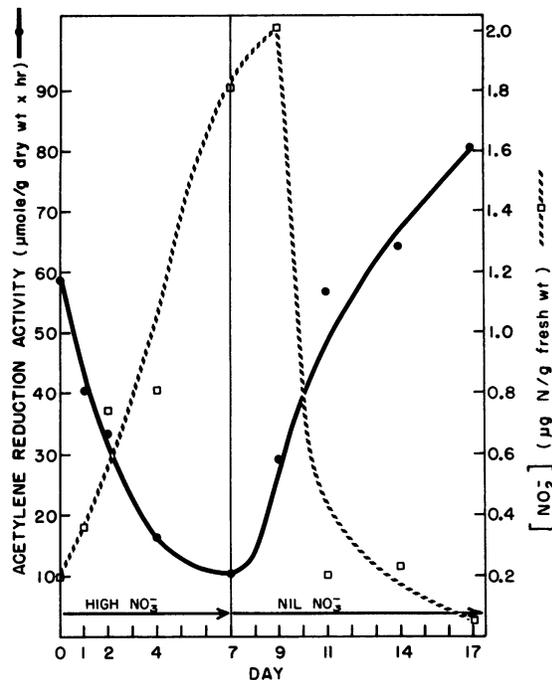


FIG. 1. Effect of nitrate supply on acetylene reduction activity and nitrite concentration in soybean nodules. Nodules were formed by *R. japonicum* USDA 138 and plants were grown for 37 d with 2 mM nitrate (does not inhibit growth or activity of nodules). Half of the pots were then supplied with 15 mM nitrate ("high NO_3^- " in the figure) for 7 d after which all remaining pots received nutrient solution containing nil nitrate. Mean nodule fresh weight (g/plant) across all harvests was 0.99 for the low nitrate treatment and 0.76 for the high nitrate treatment. Data are averages of two reps except for day 0 (four reps). Mean acetylene reduction activity of control nodulated roots (averaged over the entire sampling period) was $76 \mu\text{mol} \cdot \text{g}^{-1} \text{dry wt} \cdot \text{h}^{-1}$ (range, 68–94). The concentration of NO_3^- in nodules is shown in Figure 3.

acetylene reduction activity accompanied the rise and fall of $[\text{NO}_2^-]$ and, with the exception of the 7- to 9-d period, there was a close, negative relationship between $[\text{NO}_2^-]$ in nodules and nitrogenase activity (Fig. 1).

A similar pattern was evident when nodules were formed by NR^- *Rhizobium* with one major exception. Namely, nitrite concentrations were much lower than in *Rhizobium* NR^+ nodules (Fig. 2), the top concentration of about $0.4 \mu\text{g N}$ being 0.2 that in *Rhizobium* NR^+ nodules. Also, the recovery of acetylene reduction activity after withdrawal of nitrate does not appear as great in Figure 2 as in Figure 1, but this may be due to an unusually high value for day 0 in Figure 2 (note acetylene reduction activities of controls given in the figure legends).

Other samples of these nodules were used for the analysis of reduced N composition. Reduced N concentrations in USDA 138 nodules was not greatly influenced by the 15 mM nitrate treatment until after 4 d, when there was a striking increase in ureide concentration in nodules receiving high nitrate (Fig. 3). The positive effect of high nitrate supply on ureide concentration in 138CR1 nodules was evident after only 1 or 2 d and was more consistent across time (Fig. 4). The reason for the difference between the two types of nodules is not known but may be due largely to error variation. Despite differences between USDA 138 and 138CR1 nodules, a consideration of all of the harvests illustrated in Figures 3 and 4 clearly indicates that treatment with 15 mM nitrate caused a significant increase in ureide concentration and a smaller increase in amino N concentration. Ammonium concentration may also have been higher where high nitrate was supplied but the effect, if present, was not large. The accu-

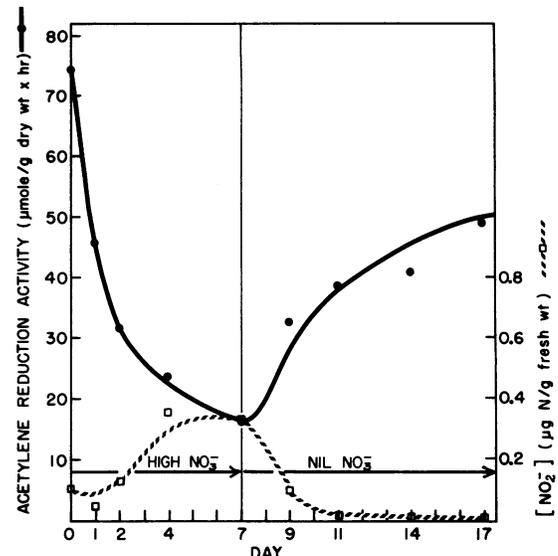


FIG. 2. Effect of nitrate supply on acetylene reduction activity and nitrite concentration in soybean nodules. Conditions were the same as those in Figure 1 except that a *R. japonicum* mutant (138CR1) lacking NR was used to form nodules. Mean nodule fresh weight (g/plant) across all harvests was 0.93 for the low nitrate treatment and 0.74 for the high nitrate treatment. Mean acetylene reduction activity of control nodulated roots (averaged over the entire sampling period) was $65 \mu\text{mol} \cdot \text{g}^{-1} \text{dry wt} \cdot \text{h}^{-1}$ (range, 51–101). The concentration of NO_3^- in nodules is shown in Figure 4.

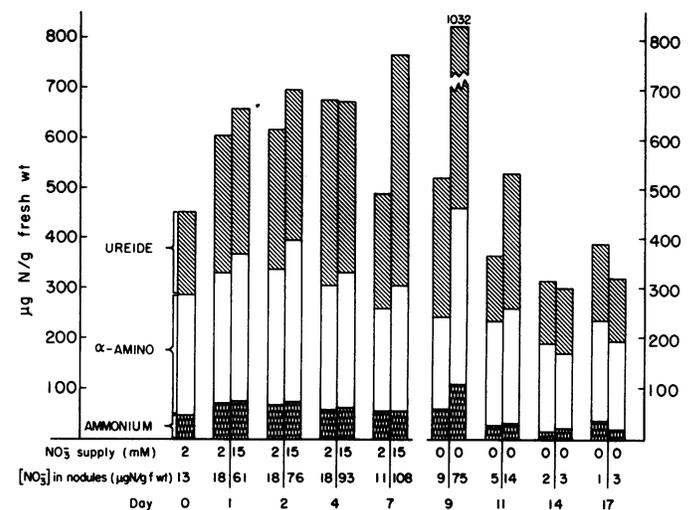


FIG. 3. Effect of nitrate supply on the accumulation of ammonium, amino N, and ureide N in soybean nodules formed by *R. japonicum* USDA 138. The high nitrate treatment was initiated immediately after the harvest on day 0. The experimental approach is described in the legend for Figure 1.

mulation of amino N and ureide N appeared to follow $[\text{NO}_3^-]$ in nodules, with a lag of several days (Figs. 3 and 4). The composition of the ureide fraction was not altered by the high nitrate treatment, with allantoin comprising 40% (USDA 138) or about 46% (138CR1) of total ureide (Table I).

The increase in amino N and ureide N concentrations in nodules supplied with 15 mM nitrate was confirmed using a different *R. japonicum* strain (Table II). These plants were older than those in the main experiment and allantoin as per cent of total ureide was higher, but the proportion was not influenced by the high nitrate treatment. Cytosol and bacteroids were sep-

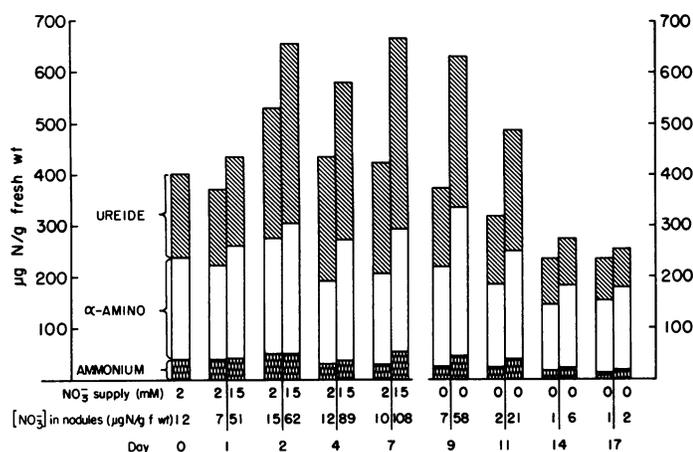


FIG. 4. Effect of nitrate supply on the accumulation of ammonium, amino N, and ureide N in soybean nodules formed by *R. japonicum* 138CR1 (NR⁻ mutant of USDA 138). The high nitrate treatment was initiated immediately after the harvest on day 0. The experimental approach is described in the legend for Figure 1.

Table I. Sugar and Allantoate Concentrations in Soybean Nodules Supplied with Low → High → Nil Nitrate over a 17-Day Period

Data represent means across eight harvests and two replicates and are from the same experiment as that illustrated in Figures 1 to 4.

<i>R. japonicum</i> Strain	Nitrate Treatment	Sugar Conc ^a	Allantoate Conc ^a	Allantoate as % of Total Ureide
	<i>mM</i>	<i>mg/g fresh wt</i>	<i>µg N/g fresh wt</i>	
USDA 138	2	1.24	95	39.1
	15	1.06	127	40.3
138CR1 ^b	2	1.42	80	44.1
	15	1.43	117	48.4

^a Sum of glucose, fructose, maltose, trehalose, and sucrose.

^b NR-deficient mutant of USDA 138.

Table II. Nitrogen Composition of Soybean Nodules on Plants Supplied with 15 mM Nitrate for 8 Days Relative to Nodules on Plants Supplied with 2 mM Nitrate

Nodules were formed by *R. japonicum* USDA 110. Plants were grown for 56 d with 2 mM nitrate and then half of the plants received 15 mM nitrate for 8 d before a single harvest at 64 d after planting. The mean ± SE for four whole nodule samples is shown.

Nitrate Treatment	Amino N Conc ^a	Ureide N Conc ^b	Allantoate Conc ^a	Allantoate as % of Total Ureide
<i>mM</i>	<i>µg N/g fresh wt</i>			
2	361 ± 5	184 ± 9	95 ± 5	52 ± 2
15	444 ± 44	357 ± 36	198 ± 16	56 ± 2

^a Includes ammonium.

^b Total ureide, allantoin basis.

arated and analyzed for amino N and ureide N (Table III). While [amino N] in bacteroids was unaffected by the high nitrate treatment, [ureide] in bacteroids was higher where 15 mM nitrate was supplied. However, the proportions of N compounds in bacteroids was not influenced by the high nitrate treatment, with 96% of amino N and 96% of ureide N being recovered in the cytosol regardless of nitrate supply.

Table III. Relative Concentration of Amino N and Ureide N in Bacteroids and Cytosol from Soybean Nodules Supplied with 2 mM or 15 mM Nitrate

The experimental approach is described in Table II. Nodules were chilled and ground in water at 1°C and were filtered through four layers of cheesecloth. Extracts were kept at 1°C for 0 to 63 min before centrifugation at 48,000g (1°C) for 5 min. Particulate (bacteroid) and supernatant (cytosol) fractions were immediately mixed with hot (75°C) 95% (v/v) ethanol. Twelve samples were obtained with a total elapsed time of 7.0 to 69.2 min between grinding of nodules and mixing of fractions with hot ethanol. There was no significant variation in the distribution of compounds as a function of time, and the mean ± SE of the 12 samples is shown. The mean 'recovery' of amino N and ureide N in cytosol + bacteroid relative to whole nodules (Table II) was 98% and 83%, respectively.

Nitrate Treatment	Amino N ^a		Ureide N ^b	
	Bacteroid	Cytosol	Bacteroid	Cytosol
<i>mM</i>	<i>µg N/g fresh wt</i>			
2	17.5 ± 0.4	354 ± 14	6.9 ± 0.2	148 ± 4
15	16.4 ± 0.5	393 ± 16	11.5 ± 0.4	280 ± 9

^a Includes ammonium.

^b Total ureide, allantoin basis.

DISCUSSION

The patterns of nitrite concentration and acetylene reduction activity shown in Figure 1 provide support for the hypothesis that nitrite is involved in the inhibition of nitrogenase when nodulated plants are supplied with a large amount of nitrate. Furthermore, soybean nodules contain about 82% water (12) and, if the nitrite concentration of 2.0 µg N/g fresh weight (Fig. 1) is uniformly distributed in the nodule water, a nitrite concentration of 175 µM would result. This nitrite concentration, if present in bacteroids, would be high enough to cause >50% inhibition of nitrogenase, based on *in vitro* studies of the inhibition of the enzyme (14). However, bacteroids contain nitrite reductase activity when plants are supplied with nitrate (12, 13), so it is difficult to predict nitrite concentration in bacteroids with any certainty.

The interpretation of Figure 2 is quite different from that of Figure 1. It is highly likely that nitrite concentrations were too low to cause significant inhibition of nitrogenase, based on *in vitro* studies of the enzyme (14). It should be emphasized that nitrite generated in these nodules (Fig. 2) was synthesized in the cytosol because bacteroids lacked NR. Thus, it is likely that the nitrite present in bacteroids of these nodules was essentially nil, and yet acetylene reduction activity was markedly inhibited.

Several workers have suggested that the assimilation of high levels of nitrate requires large quantities of energy and reducing power which may deprive nodules of an adequate supply of carbohydrate (7, 9, and refs. in 10). In the experiment illustrated in Figures 1 to 4, there was virtually no effect of nitrate on the sugar concentration in nodules (Table I). For the 4- and 7-d harvests when the inhibition of nitrogenase was maximal, the sugar concentration (averaged across both *Rhizobium* strains) was 0.89 mg/g fresh weight in low-nitrate nodules and 0.86 mg/g fresh weight in high-nitrate nodules. Sucrose concentration in nodules during the same period was 0.27 mg/g fresh weight in low-nitrate nodules and 0.38 mg/g fresh weight in high-nitrate nodules. Interpretation of reports that sucrose alleviates the negative effect of nitrate on detached nodules (7, 9) is difficult because sugars inhibit the uptake of nitrate (15) and because controls of nonmetabolized sugars have not been included in these experiments. Based on our present and previous results (10), it seems unlikely that carbohydrate deprivation can explain the marked decrease in acetylene reduction which occurs in

response to high nitrate supply.

The results on the accumulation of N_2 fixation products in nodules on plants supplied with very high nitrate suggest that ammonium assimilation, which occurs in the cytosol (4, 5, 8), was not significantly inhibited, because the accumulation of ammonium was not large. Most of the accumulation of reduced N was in the ureide fractions (Figs. 3 and 4; Table II) and the ratio of allantate/allantoin was not influenced by the nitrate treatment (Tables I and II). Thus, ureide synthesis, which also occurs in the cytosol (1, 2, 8), did not appear to be significantly influenced by the high nitrate treatment.

The results suggest an effect of nitrate on the loading of amino acids and ureides into the xylem instead of an effect on biosynthesis. How nitrate might interfere with the export of fixed N from nodules is not clear. Because of the relatively low capacity for nitrate metabolism in *Rhizobium* NR^- nodules, nitrate metabolism in roots may be more important if, in fact, nitrate metabolism is involved at all (3). A moderate nitrate supply (4 mM) to nodulated soybean plants lowered the ureide concentration in soybean stem exudate without much influence on amino-N (11), supporting the idea that there may be an effect of nitrate on the export of ureides from nodules.

It is difficult to say whether the relationship between the accumulation of products of N_2 fixation and the depression of nitrogenase activity is causative or coincidental. It should be noted that the timing of the effects of nitrate on N accumulation and nitrogenase did not coincide perfectly. On d 9 and 11, 2 and 4 d after the withdrawal of nitrate, reduced N content of nitrate-treated nodules remained high relative to controls, but the recovery of nitrogenase activity was already evident (Fig. 1 *versus* 3, Fig. 2 *versus* 4). It should also be noted that, in studies where soybeans have been supplied with moderate amounts of nitrate for relatively long periods of time, accumulations of ureides and amino acids are not always observed (11, 12, unpublished data). Perhaps in these experiments there is a gradual acclimation of nodules which includes adjustments in metabolite concentrations to near normal levels by the time samples are taken.

If the accumulation of fixed N somehow causes the marked decline in nitrogenase, the preliminary evidence in Table III suggests that the effect is indirect. That is, most of the accumulation of amino N and ureide N was in the cytosol and not in the bacteroids. This would be the expected result since these

compounds are synthesized in the cytosol (1, 2, 4, 5, 8). Ureide concentrations in the range from 8 to 12 mM can be estimated in nodules supplied with very high nitrate. Perhaps these concentrations are high enough to affect metabolism in the cytosol which is essential for bacteroid function (e.g. generation of carbon substrates from sugars).

Acknowledgments—I thank Dale Blevins for the chlorate-resistant (NR^-) mutant of *R. japonicum*, and Paul DeVine and Mary Kilpatrick for technical assistance.

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