Nitrate Inhibition of Legume Nodule Growth and Activity[1]

I. LONG TERM STUDIES WITH A CONTINUOUS SUPPLY OF NITRATE

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ABSTRACT

The synthesis and accumulation of nitrite has been suggested as a causative factor in the inhibition of legume nodules supplied with nitrate. Plants were grown in sand culture with a moderate level of nitrate (2.1 to 6.4 millimolar) supplied continuously from seed germination to 30 to 50 days after planting. In a comparison of nitrate treatments, a highly significant negative correlation between nitrite concentration in soybean (*Glycine max* [L.] Merr.) nodules and nodule fresh weight per shoot dry weight was found even when bacteroids lacked nitrate reductase (NR). However, in a comparison of two *Rhizobium japonicum* strains, there was only 12% as much nitrite in nodules formed by NR⁻ *R. japonicum* as in nodules formed by NR⁺ *R. japonicum*, and growth and acetylene reduction activity of both types of nodules was about equally inhibited. In a comparison of eight other NR⁺ and NR⁻ *R. japonicum* strains, and a comparison of *G. max*, *Phaseolus vulgaris*, and *Pisum sativum*, the concentration of nitrite in nodules was unrelated to nodule weight per plant or to specific acetylene reduction activity. The very small concentration of nitrite found in *P. vulgaris* nodules (0.05 micrograms NO_2^--N per gram fresh weight) was probably below that required for the inhibition of nitrogenase based on published *in vitro* experiments, and yet the specific acetylene reduction activity was inhibited 83% by nitrate. The overall results do not support the idea that nitrite plays a role in the inhibition of nodule growth and nitrogenase activity by nitrate.

Nitrite may play a role in the inhibition of growth and N_2 fixation by legume nodules supplied with nitrate. Support for the inhibition of N_2 fixation comes from demonstrations of the *in vitro* inhibition of nitrogenase by nitrite (6) and the *in vitro* oxidation of leghemoglobin to ferric leghemoglobin by nitrite (13). However, there is relatively little evidence for nitrite accumulation in legume nodules when plants are supplied with nitrate.

I recently reported a significant negative correlation between nitrite concentration in soybean nodules and nodule mass per plant (15). When nodules were formed by *R. japonicum* lacking NR[2], much lower concentrations of nitrite were found in nodules (15). While a relationship between [NO_2^-] and nodule mass was not established for nodules formed by NR⁻ *R. japonicum*, the results suggested that nitrite generated by NR in the nodule cytosol might be sufficient to interfere with nodule growth and function.

In the work reported here, the relationship between nitrite accumulation and nodule growth and activity was explored using additional *R. japonicum* strains and NR-deficient mutants, and two other legume species. In this paper, the accumulation of nitrite in nodules after long-term (5–7 weeks) exposure to moderate concentrations of nitrate is discussed.

MATERIALS AND METHODS

Plant Culture. The soybean (*Glycine max* [L.] Merr.) cv 'Beeson' was used. The pea (*Pisum sativum* L.) cultivar was 'Alaska' and the bean (*Phaseolus vulgaris* L.) cultivar was 'Kentucky Wonder'. The *Rhizobium phaseoli* strains (127K12, 127K24) and the *R. leguminosarum* strains (128C53, 128C78) were described by Manhart and Wong (7). The *R. japonicum* strains were recently described elsewhere (16).

Seeds were planted in large pots containing 15 kg of washed, autoclaved, silica sand and were inoculated with about 10^8 *Rhizobium* per seed on the planting date (15). Sand was moistened with deionized H_2O for 3 to 4 d after which nutrient treatments were initiated. Plants received two irrigations of nutrient solution per day using sufficient solution to cause drainage from a hole in the bottom of each pot. Nitrate concentration was balanced with chloride and the composition of the solution was described previously (14). Nitrate treatments varied from 2.1 to 6.4 mM, and the concentrations used in each experiment are given in the figure and the tables.

After about 10 d, plants were thinned to about 14 (beans, soybeans) or 20 (peas) plants per pot. Plants were grown in a greenhouse. Supplementary light (metal halide, 400 μE m^{-2} s^{-1}, 400–700 nm) and a photoperiod of 15 h was used during the fall and winter months. The experimental design was randomized block or completely random with three to five replicates. Plants were sampled between 30 and 47 d after planting which corresponds to late vegetative to early bloom stages of growth. This time period was chosen to provide large enough samples of nodules for analysis while avoiding nodule senescence.

Analyses. Acetylene reduction activity was determined using four to six nodulated roots incubated in a jar with 10% (v/v) acetylene and periodic sampling and analysis of ethylene (14). Extraction and assay of NR (15) and nitrite reductase (16) were the same as described previously. Nitrite concentration was determined using 1 g samples of freshly picked nodules (15). Nitrate, amino N, and ureide N concentrations were determined using ethanol extracts (15).

RESULTS

In the experiment illustrated in Figure 1 only the 4.3 and 6.4 mM nitrate treatments depressed nodule weight per plant. A nil or small positive effect of low concentrations of nitrate on nodule development has been noted before by several workers (3, 12, 14). The effect of low nitrate concentrations lessens the goodness

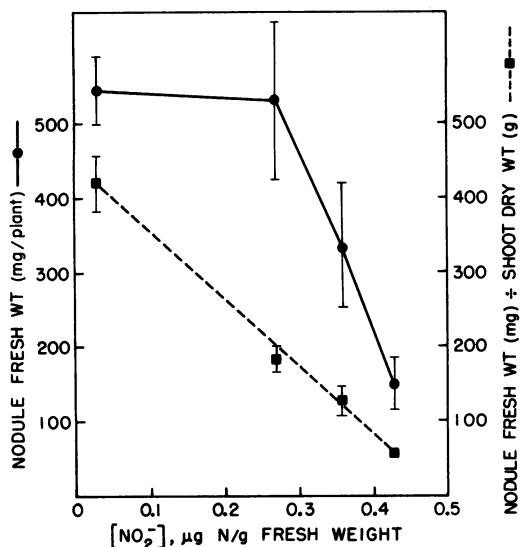[2] Abbreviation: NR, nitrate reductase.

FIG. 1. Relationship between nitrite concentration in soybean nodules and nodule weight. Nodules were formed by 76CR6, a *R. japonicum* strain which lacks NR (16). The nitrate treatments (plotted from left to right) were nil, 2.1, 4.3, and 6.4 mM. Plants were harvested 44 d after planting. The number of nodules per plant was not significantly affected by the nitrate treatments and the average was 32 (range, 25–38). The mean and SE of five replicates was plotted and the R^2 value for the relationship between [NO_2^-] and nodule weight ÷ shoot weight was 0.99.

of fit in the regression of nodule weight on nitrite concentration (15). However, this problem can be overcome by normalizing the nodule weight data against shoot weight (Fig. 1). Since the low nitrate treatment stimulates plant growth, nodule weight as a proportion of shoot weight is reduced and all of the points fall very close to a straight line (Fig. 1). Note that normalizing nodule weight by dividing by shoot weight also substantially lowered error variation. More importantly, the normalized results indicated a very close correlation between [NO_2^-] in nodules and nodule mass when nodules are formed by NR⁻ *R. japonicum*.

A comparison of several NR⁻ and NR⁺ strains of *R. japonicum* is shown in Table I. The 6.4 mM nitrate treatment reduced nodule mass about 70% for both *R. japonicum* 61A76 and 76CR6. Acetylene reduction activity of 76CR6 nodules was greater than that of 61A76 nodules when plants were grown with 6.4 mM nitrate, but the per cent decline in activity due to the nitrate treatment was substantial for both strains. While the sensitivity of 76CR6 (NR⁻) nodules and 61A76 (NR⁺) nodules to nitrate was similar, [NO_2^-] in 61A76 nodules was 8-fold greater than in 76CR6 nodules.

With one exception (110CR1), nitrate inhibited the growth and acetylene reduction activity of nodules formed by all *R. japonicum* strains tested in experiment II (Table I). The inhibition of 110CR2 and 110CR3 nodules was greater than the inhibition of the NR⁺ USDA 110 nodules, and nitrite in the NR⁻ nodules was barely detectable. Relatively high [NO_2^-] was found in nodules formed by some of the NR⁻ mutants of USDA 138 (*e.g.* 138CR3). As was the case with the 110 group of strains, there was no apparent relationship between nitrite accumulated and the depression of either nodule weight or acetylene reduction activity of the 138 group of strains. The [NO_2^-] in nodules formed by USDA 110 and USDA 138 was lower than that in nodules formed by 61A76 because, among the *R. japonicum* strains used in these studies, 61A76 has exceptionally high NR activity (Table I, footnotes).

R. leguminosarum and *R. phaseoli* do not express NR in bacteroids (7). Thus, in the comparison of different legumes (Table II) a NR⁻ *R. japonicum* was used so that all nitrite in nodules would be generated by cytosol NR. In a preliminary experiment, 6.4 mM nitrate reduced nodule weight of pea plants

by 85 to 90%, making it very difficult to obtain samples for analysis. The lower nitrate level (2.9 mM) used in the experiment reported here did not depress nodule weight per plant for peas (Table II). However, nodule weight as a proportion of shoot weights was reduced by 50% and acetylene reduction activity was markedly inhibited with this relatively low nitrate supply.

Among the three legumes, the inhibition of *Phaseolus* nodules was greatest with a 51% decline in nodule weight and an 83% decline in acetylene reduction activity when plants were supplied with 6.4 mM nitrate (Table II). However, nitrite was barely detectable in *Phaseolus* nodules. This may have been due to a relatively low concentration of nitrate in these nodules coupled with low NR activity and high nitrite reductase activity (Table II). NR activity was barely detectable in pea nodules and the relatively high activity reported by Chen and Phillips (2) may have been due to their use of very high nitrate. There was >100-fold more nitrite reductase than NR in pea nodules and the 78% decrease in acetylene reduction activity in response to nitrate was accompanied by very little accumulation of nitrite (Table II). Nitrite concentration in soybean nodules was unusually low in this experiment and the reasons are unknown. In the preliminary experiment, soybean, pea, and bean nodules supplied with 6.4 mM nitrate contained 0.41, 0.10, and 0.01 µg NO_2^--N/g fresh weight, respectively.

DISCUSSION

The involvement of nitrite in the inhibition of nodule growth and function by nitrate is a logical proposition. Nitrite reacts spontaneously with primary and secondary amines and, thus, could disrupt the activity of catalytic proteins. Experimental demonstrations of the reaction of nitrite with nitrogenase and leghemoglobin make the hypothesis more attractive (6, 13). When a correlation between [NO_2^-] in soybean nodules formed by NR⁺ *R. japonicum* and nodule weight per plant was found, it seemed possible that nitrite accumulation might, in fact, be important in the inhibition of nodules by nitrate (15). Results reported in Figure 1 for nodules formed by NR⁻ *R. japonicum* also showed a remarkably close relationship between [NO_2^-] in nodules and nodule weight per shoot weight. This result is important because several workers have shown that nodules formed by NR⁻ *Rhizobium* are still sensitive to nitrate (see 15).

Although [NO_2^-] was correlated with nodule development in comparisons across nitrate treatments within a single *Rhizobium* strain, [NO_2^-] was not correlated with nodule growth or activity in comparisons across *Rhizobium* strains (Table I). Nodules formed by 61A76 contained much higher [NO_2^-] than any other nodules but did not appear to be significantly more sensitive to inhibition by nitrate than nodules formed by several other strains. While there are some anomalous results in Table I (*e.g.* the lack of inhibition of 110CR1 nodules; relatively high [NO_2^-] in 138CR3 nodules), the overall result seems clear. Namely, there was no apparent relationship between the accumulation of nitrite and the negative effect of nitrate on either nodule weight or nitrogenase activity.

There have been several recent reports of the response to nitrate of nodules formed by a wide range of *Rhizobium* strains (4, 9, 10). Some strains form nodules which are less sensitive to nitrate (4, 10) while other studies show no difference among strains (9). In general, where differences have been reported, they were small and the overall conclusion was that the growth and acetylene reduction activity of all nodules was markedly inhibited by nitrate regardless of the *Rhizobium* strain involved.

The results reported in Table II confirm the results of Manhart and Wong (8) that pea and bean nodules are inhibited by nitrate even though *R. phaseoli* and *R. leguminosarum* do not express NR in bacteroids (7). They reported that no nitrite accumulated in pea and bean nodules. In contrast, we detected nitrite in pea and bean nodules (Table II), perhaps because of greater recovery of very small quantities of nitrite (15). However, the quantities

Table I. *Effect of Nitrate on Nodule Growth, Acetylene Reduction Activity, and Nitrite Accumulation in Soybean Nodules Formed by NR^+ and NR^- R. japonicum*

The 'CR' (chlorate resistant) strains are NR^- derivatives of the wild type strains.

R. japonicum Strain	Nitrate Supply	Nodule Fresh Wt	Acetylene Reduction Activity	[NO_2^-] in Nodules
	mM	mg/plant	$\mu mol/g$ fresh wt · h	μg N/g fresh wt
Experiment I[a]				
61A76	0	593 (61)	4.6 (0.6)	0
	6.4	183 (42)	2.1 (0.3)	3.51 (0.86)
	% change	−69	−54	—
76CR6	0	545 (45)	6.5 (2.7)	0
	6.4	149 (35)	3.8 (0.6)	0.43 (0.11)
	% change	−73	−41	—
Experiment II[b]				
USDA 110	0	530 (68)	11.4 (2.4)	0
	5.4	430 (46)	9.4 (1.5)	0.91 (0.12)
	% change	−19	−18	—
110CR1	0	431 (19)	8.9 (2.5)	0
	5.4	553 (67)	8.2 (0.6)	0.15 (0.04)
	% change	+28	−8	—
110CR2	0	618 (61)	6.5 (3.0)	0
	5.4	375 (15)	4.0 (0.6)	0.05 (0.01)
	% change	−39	−38	—
110CR3	0	514 (33)	17.3 (1.9)	0
	5.4	361 (120)	5.0 (1.6)	0.08 (0.02)
	% change	−30	−71	—
USDA 138	0	677 (106)	16.5 (1.3)	0
	5.4	493 (78)	7.2 (1.3)	0.73 (0.11)
	% change	−27	−56	—
138CR1	0	698 (56)	10.0 (0.6)	0
	5.4	499 (116)	3.5 (0.8)	0.41 (0.05)
	% change	−29	−65	—
138CR2	0	1120 (104)	8.9 (0.5)	0
	5.4	520 (68)	5.8 (1.1)	0.15 (0.04)
	% change	−54	−35	—
138CR3	0	879 (178)	11.9 (0.7)	0
	5.4	348 (81)	6.4 (1.6)	0.71 (0.15)
	% change	−60	−46	—

[a] Results are from the same experiment as that illustrated in Figure 1. For the treatments shown here, mean cytosol NR activity (20 observations) was 158 nmol·mg^{-1} protein·h^{-1}. Mean bacteroid NR (61A76 only, $n =$ 10) was 3.2 $\mu mol·mg^{-1}$ protein·h^{-1}. NR was not detected in any sample of 76CR6 bacteroids. Nitrate concentration in nodules supplied with nitrate averaged 128 μg N/g fresh wt.

[b] Plants were harvested 40 d after planting (USDA 110 and derivatives) or 47 d after planting (USDA 138 and derivatives). Data represent the mean ± SE of four replicates. Mean cytosol NR activity (64 observations) was 84 nmol·mg^{-1} protein·h^{-1}. Mean bacteroid NR (USDA 110 and 138 only, $n = 8$) was 75 nmol·mg^{-1} protein·h^{-1}. NR was not detected in any sample of CR mutant bacteroids. Nitrite reductase could be quantitated with confidence only where nitrate was supplied; mean nitrite reductase activity in cytosol and bacteroids was 251 and 129 nmol·mg^{-1} protein·h^{-1}, respectively ($n = 32$). Nitrate concentration in nodules supplied with nitrate ranged from 54 to 71 μg N/g fresh wt; the mean across all *Rhizobium* strains ($n = 32$) was 61 μg N/g fresh wt.

found were extremely small, especially in bean nodules. The very low concentration of nitrite in bean nodules relative to soybean nodules may be due to relatively low levels of nitrate and cytosol NR in bean nodules. Also, the abundance of nitrite reductase, relative to NR, in legume nodules (Refs. 1 and 16; Table II) may be partly responsible for the very low [NO_2^-] in nodules.

The fresh weight/dry weight ratio for soybean nodules was 5.76 ± 0.06 (SE, $n = 70$) and for bean nodules was 7.19 ± 0.06 (SE, $n = 20$) under the conditions used in these experiments. Thus, soybean nodules are about 82% water and bean nodules about 86% water. If we assume 0.84 g water/g fresh weight and equal distribution of nitrite in this water, then the 45 ng NO_2^--

Table II. *Effect of Nitrate in Nutrient Solution on Growth, Acetylene Reduction Activity, N Composition of Nodules, and on Enzymes of Nitrate Metabolism in Root Nodules of Phaseolus vulgaris, Pisum sativum, and Glycine max*

Two strains of *Rhizobium* (see text) were used to form nodules on peas and beans. For most variables results for the two strains were very similar and data represent means across two strains. Nodules were formed on soybeans by 76CR6 (Fig. 1). Soybeans, peas, and beans were harvested 30, 33, and 35 d after planting, respectively. There were four replicates and changes >25% were statistically significant, based on analysis of variance.

Variable	P. vulgaris			P. sativum			G. max		
	0	6.4 mM nitrate	% change	0	2.9 mM nitrate	% change	0	6.4 mM nitrate	% change
Nodule growth									
Nodule fresh wt (mg/plant)	1443	649	−51	239	248	+4	356	204	−43
Nodule fresh wt/shoot dry wt (g/g)	1.20	0.16	−87	0.46	0.23	−50	0.46	0.09	−80
Nodule activity									
Acetylene reduction (μmol/g fresh wt nodule·h)	10.2	1.7	−83	10.5	2.3	−78	13.3	9.0	−32
N composition									
[NO$_3$⁻] (μg N/g fresh wt)	0	23.6	—	0	67.7	—	0	113	—
[NO$_2$⁻] (μg N/g fresh wt)	0	0.045	—	0	0.135	—	0	0.115	—
α-Amino N (μg N/g fresh wt)	482	332	−31	764	505	−34	454	353	−22
Ureide N (μg N/g fresh wt)	64.2	46.7	−27	16.2	20.3	+25	269	292	+9
Enzymes of nitrate metabolism[a]									
Cytosol nitrate reductase (nmol/mg protein·h)	17.3	16.6	−4	3.7	5.0	+35	94	113	+20
Cytosol nitrite reductase (nmol/mg protein·h)	99	767	+675	204	690	+238	20	351	+1650
Bacteroid nitrite reductase (nmol/mg protein·h)	68	306	+350	98	332	+239	124	225	+81

[a] Bacteroids of all *Rhizobium* species lacked nitrate reductase activity (40 samples).

N/g of bean nodules (Table II) is equivalent to a [NO$_2$⁻] of 3.8 μM. The concentration of 115 ng NO$_2$⁻-N/g in soybean nodules would be equivalent to a [NO$_2$⁻] of 9.8 μM. Both of these concentrations, if representative of concentrations in bacteroids, are below that required for inhibition of nitrogenase (17). At the higher concentrations of 0.4 μg NO$_2$⁻-N (Fig. 1) or 0.7 μg NO$_2$⁻-N (Table I) sometimes observed in NR⁻ soybean nodules, [NO$_2$⁻] might be high enough to cause a slight inhibition of nitrogenase. But it is highly unlikely that the [NO$_2$⁻] found in bean nodules can account for the marked decrease in acetylene reduction activity (Table II) unless it is concentrated in bacteroids. This latter possibility is unlikely because bacteroids lacked NR and had nitrite reductase activity (Table II).

The results are consistent with recent conclusions by others that there is very little nitrate assimilation in soybean nodules. Ohyama (11) reported that after a 10 h feeding of ¹⁵NO$_3$⁻ only 0.4% of the recovered ¹⁵N was in nodules relative to 36% in leaves and 36% in roots. Perhaps most of the nitrate absorbed by roots does not pass through nodules and, of that fraction which passes through nodules, little is unloaded. This suggestion is consistent with the finding of several-fold higher nitrate concentrations in soybean roots than in nodules (5, 15). In bean roots, [NO$_3$⁻] was 8- to 10-fold greater than the [NO$_3$⁻] in nodules (data not shown).

In summary, the hypothesis that nitrite plays a role in the inhibition of legume nodules by nitrate was fortified by the finding of a highly significant correlation between [NO$_2$⁻] in nodules and nodule weight/plant. However, this correlation was found only in a comparison of the response of nodules to varying nitrate supply within a particular *Rhizobium* strain. A correlation between [NO$_2$⁻] and nodule weight or acetylene reduction activity was not found in comparisons across *R. japonicum* strains or legumes. The comparison of legumes is especially convincing if the mechanism underlying nitrate inhibition is the same among all legumes. Relative to soybean nodules, pea and bean nodules contained less nitrite and less NR but were more sensitive to inhibition by nitrate.

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