Nitrate Alters the Symbiotic Expression of *Bradyrhizobium japonicum* Bacteroid Enzymes *in planta*

MARK T. SMITH, JAMES K. WATERS, DALE B. KARR, GLENN G. PRESTON, FUMIKO SUZUKI, AND DAVID W. EMERICH
Department of Biochemistry, University of Missouri, 117 Schweitzer Hall, Columbia, MO 65211, USA, Tel. +314-8824252, Fax. + 314-8825635

Abstract

The addition of nitrate to nodulated legumes causes inhibition of nitrogen fixation. The mechanism by which nitrate causes this inhibition is not known. To determine whether nitrate mediates its effect through inhibition of intermediary metabolism of the microbial symbiont, nitrate was added to nodulated soybean (*Glycine max* L. Merr.) plants inoculated with *Bradyrhizobium japonicum* strain 143 at four different times relative to the day on which acetylene reduction activity was first observed (T_{ARA}). Bacteroids were isolated aerobically from nodules and several enzymes of the citric acid cycle, polyhydroxybutyrate cycle, carbohydrate metabolism and amino acid metabolism were measured. In the absence of nitrate, the activity of enzymes of the citric acid cycle increased, but the activity of the enzymes of carbohydrate metabolism and amino acid metabolism decreased. Nitrate additions increased the activity of enzymes of carbohydrate and amino acid metabolism, when it was added at any time relative to the onset of ARA. Nitrate additions had little effect on the citric acid cycle enzymes, except when added three days prior to T_{ARA}, it prevented the increase in the citric acid cycle enzymes. These results suggest that nitrate influences the expression of citric acid cycle and non-citric acid cycle enzymes differently and that the metabolic commitment to the bacteroid state, with regard to the citric acid cycle enzymes, is made near the end of the mitotic phase of nodule development, between 2 and 3 days prior to T_{ARA}. *In planta* nitrogenase activity was more susceptible to nitrate additions than all of the enzymes measured. This suggests that the cause of nitrate mediated inhibition of nitrogen fixation activity is not caused by the repression of bacteroid citric acid cycle enzymes.

Keywords: *Bradyrhizobium*, soybean nodules, carbon metabolism, nitrate.

1. Introduction

The reduction of atmospheric dinitrogen by biological systems requires considerable amounts of energy in the form of ATP and reducing equivalents. The energy for reduction of atmospheric dinitrogen by symbiotic nitrogen-fixing bacteroids is derived from carbohydrates synthesized in the leaves and transported to the root nodules. The carbohydrates are metabolized to dicarboxylic acids, particularly malate, in the plant portion of the nodule which are then transported across the peribacteriod membrane to the bacteroids (Reibach and Streeter, 1984; Yang et al., 1990; Price et al., 1987). The dicarboxylates provide the greatest rates of respiration and of nitrogen fixation by isolated suspensions of bacteroids (Appleby, 1984).

Because the nitrogen fixation process requires tremendous amounts of biological energy, the energy-generating enzymes would be expected to be highly expressed during symbiotic development. The citric acid cycle of the bacteroids is believed to be the primary energy-generating pathway for nitrogenase. Previously, we have shown that several of the enzymes of the citric acid cycle increased with the expression of nitrogenase activity as measured by the acetylene reduction technique (Karr et al., 1984). This type of analysis is correlative in nature, but more thorough analysis of the citric acid cycle enzymes through biochemical and molecular genetic methods has not been readily forthcoming.

Nitrate markedly inhibits symbiotic nitrogen fixation by nodulated leguminous plants. Both the activity of the enzyme that reduces atmospheric dinitrogen to ammonium, nitrogenase, and the growth and development of the plant root nodule, the morphologically unique structure that contains the encapsulated symbiotic bacteria, (Brady)rhizobium, are inhibited (Streeter, 1988). The physiological basis by which nitrate mediates this inhibition of nitrogen fixation has not been determined, although some have been eliminated. Nitrate additions to soybean plants with fully developed nodules was shown to reduce acetylene reduction activity which could be partially restored by increasing oxygen levels (Carroll et al., 1987). This suggested that oxygen limitation my be a factor in reducing nitrogenase activity in nodulated plants subjected to nitrate.

The effects of nitrate on a number of plant metabolic enzymes in nodules have been measured. For example, nitrate had no effect on plant nodule cytoplasmic glutamine synthetase, glutamine oxoglutarate aminotransferase, xanthine dehydrogenase, uricase or allantoinase (Schuller et al., 1986). Sawhney et al. (1988) reported that plant nodule cytosol phosphoenol pyruvate carboxylase, invertase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and malate dehydrogenase were significantly decreased upon treatment of

nodulated pigeonpea with nitrate. Interestingly, the first study focused on nitrogen metabolism, while the second assayed carbon metabolism

Whereas the effects of nitrate on nitrogenase activity are well documented, the effects on enzymes of the citric acid cycle of the bacteroid are not. We sought to determine whether nitrate additions to nodulated soybeans, added just prior to and at the onset of acetylene reduction activity (ARA), had an effect on bacteroid enzymes. During normal symbiotic development enzymes of the citric acid cycle are elevated whereas the enzymes of carbohydrate metabolism are reduced in activity. The rationale was that enzymes that need to be expressed for symbiotic nitrogen fixation would be repressed by the nitrate treatment whereas enzymes that are normally repressed during symbiotic nitrogen fixation may remain expressed in the presence of nitrate. The repression of bacteroid enzymes needed to supply the energy to nitrogenase could be the mechanism by which nitrate mediates the inhibition of symbiotic nitrogen fixation.

2. Materials and Methods

Soybean (cv. Williams 82) seeds were sterilized and were inoculated with *Bradyrhizobium japonicum* strain 143 and grown in modified Leonard jars with equal parts of sand and vermiculite in the greenhouse with supplemental lighting (Karr et al., 1984). Potassium nitrate, at various concentrations, was added to the plant nutrient solutions. The root systems were thoroughly rinsed with 5 volumes or more of the nitrate solutions to effectively replace the nitrogen-free solutions. An equal volume of nitrogen-free nutrient solution was rinsed through the jars of control plants. ARA on nodulated root segments (measured at 2 min intervals for 10–12 minutes), aerobic isolation of bacteroids, preparation of desalted bacteroid extracts and measurement of the following bacteroid enzymes were performed as described previously (Karr and Emerich, 1988; Karr et al., 1984): malate dehydrogenase, fumarase, isocitrate dehydrogenase, hydroxybutyrate dehydrogenase, acetoacetyl-CoA thiolase and pyruvate dehydrogenase. α-ketoglutarate dehydrogenase was measured as described by Reed and Mukherjee (1969). Briefly, the four dehydrogenases were assayed by monitoring the change in absorbance of the pyridine nucleotide at 340 nm. Fumarase was monitored at 240 nm following fumarate production from malate. Acetoacetyl-CoA thiolase was measured by following the disappearance of the Mg^{2+} enol-acetoacetyl-CoA complex at 303 nm. Glutamine synthetase I was detected as described by Darrow and Knotts (1977). General acyl-CoA thiolase I was assayed as described by Suzuki et al. (1987). Aspartate aminotransferase was measured as described by Reed and Hess

(1975). Glucose-6-phosphate dehydrogenase assays contained in 1 ml: 80 mM imidazole buffer, pH 7.5, 5 mM $MgCl_2$, 10 mM glucose-6-phosphate, 1 mM NADP and 50 µl of enzyme extract. Glyceraldehyde-3-phosphate dehydrogenase assays contained in 1 ml: 85 mM triethanolamine, pH 7.6, 1 mM EDTA, 2 mM $MgCl_2$, 0.2 mM ATP, 10 mM DL-glyceraldehyde-3-phosphate, 0.2 mM NADH and 5 units of 3-phosphoglycerate kinase. Leghemoglobin was measured as described by Appleby and Bergersen (1980). Protein was determined by the method of Lowry et al. (1951). Bacteroids were isolated at intervals after the addition of nitrate and crude extracts were desalted before use to eliminate background activity due to the presence of carbon substrate and pyridine nucleotides. The desalting also removed nitrate or nitrite that may have been present in the extracts. To determine whether nitrate or nitrite had an effect on any of these enzymes, control assays were performed with nitrate or nitrite added at 1 mM final concentration to the desalted bacteroid extracts. No consistent or significant effect of these two compounds on any of the enzymes included in this study was observed. Bacteroids were isolated from untreated plants and the desalted crude extracts were used as controls. Quadruplicate enzyme and protein measurements were recorded and the mean and the standard deviations were calculated for each. The experiment was repeated four times. Enzyme specific activities are presented rather than total activities; the form of presentation does not affect the results or their interpretation. Chemicals and reagents were obtained from Sigma Chemical Co., St. Louis, MO, USA and Fisher Scientific, St. Louis, MO, USA and potting medium was obtained from local sources.

3. Results

Potassium nitrate at concentrations of 1, 2, 6, 8, 10, and 12 mM in the plant nutrient solutions (Ahmed and Evans, 1960) were used to replace the nitrogen-free nutrient solutions of 12 to 15-day-old soybean plants grown in modified Leonard jars (Karr et al., 1984). The concentrations of 1 and 2 mM did not significantly affect whole plant acetylene reduction activity (ARA), concentrations of 6 to 8 mM gave partial repression of acetylene reduction activity and 10 and 12 mM nitrate gave 80 to 90% repression (Fig. 1). There was little difference between 10 and 12 mM. A concentration of 10 mM was chosen for all further experiments as it appeared to be the minimum concentration to yield maximal repression of ARA. Leghemoglobin contents, as ηmol/mg protein, declined greater than 50% due to the addition of 10 mM nitrate (data not shown).
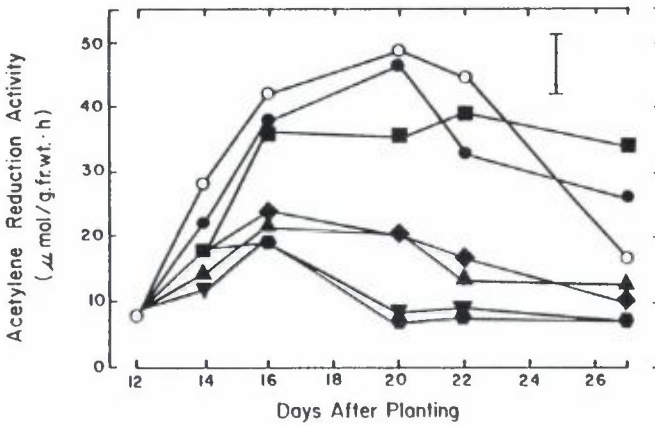
Figure 1. Acetylene reduction activity of nodulated soybean plants in the absence or presence of various levels of nitrate. Nitrate, at concentrations of 0 (O), 1(●), 2 (■), 6 (♦), 8 (▲), 10 (▼), or 12 (⬣) mM, was added to nodulated soybean plants on the first day that acetylene reduction activity was observed. The values represent four separate determinations. The standard deviation for all measurements was <19.4%, which is indicated by the error bar on the top right side of the figure. Including all experiments, the values obtained at 0, 1, and 2 mM were not significantly different from each other, the values obtained at 6 and 8 mM were not significantly different from each other, nor were the values obtained at 10 and 12 mM. The results shown represent one of two separate experiments.

Preliminary experiments suggested that the age of the plant, and hence the period of nodule development, when the nitrate additions were initiated affected the activity of the bacteroid citric acid cycle enzymes chosen for measurement. Thus, to attempt to normalize for the effect of nitrate on different stages of bacteroid metabolism, which cannot be conveniently or accurately determined by non-destructive inspection of the intact soybean plant nodules, it was decided to make the nitrate additions relative to the anticipated onset of ARA. This convention was useful in distinguishing the effects of nitrate on the various metabolic enzymes under study.

The time at which nitrate was added relative to the start of ARA had an effect on the level of ARA observed in the treated plants (Fig. 2). The addition of nitrate on the day at which acetylene reduction was observed (T_{ARA}) resulted in an approximate 50% decline in ARA by the third day after the treatment (Fig. 2A). The addition of nitrate, one, two or three days prior to T_{ARA} caused a more severe reduction of nitrogenase activity of 80, 72 and 91%, respectively, three days after T_{ARA}, but did not prevent its initial expression (Figs. 2B, C, D). The ARA of treated and untreated plants were usually not statistically different when measured on the first day after T_{ARA}. Significant

differences usually were apparent on the second day for all treatments except when nitrate was added on T_{ARA}. The nitrate additions caused a decline of activity on subsequent days in all treatments. Examination of all experiments suggested that full repression of nitrogenase activity required six days after the addition of nitrate regardless of when the addition occurred relative to T_{ARA}. This interpretation appears valid for the additions of nitrate on the day of, or one, to two days prior to T_{ARA}. The low levels of activity observed in the plants treated three days prior to T_{ARA} make it difficult to state with certainty that this group follows the same pattern. For comparison, maximum ARA of the control plants was achieved about six days after T_{ARA}.
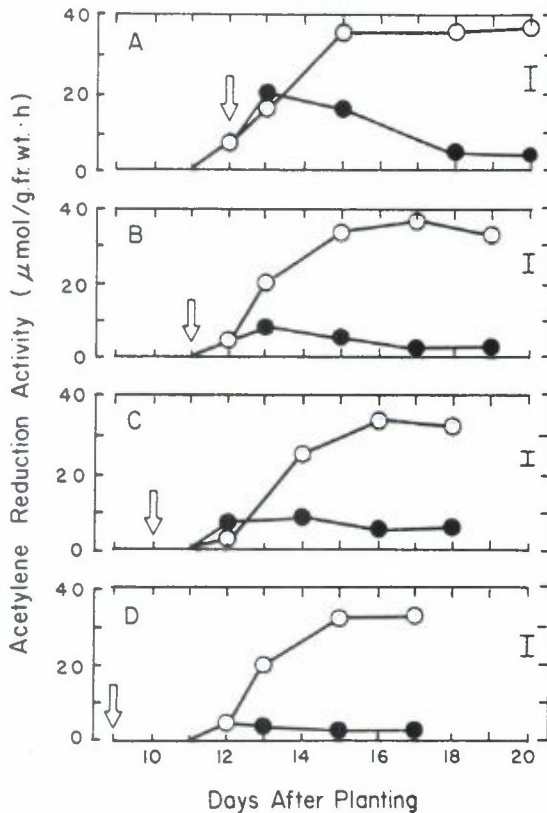

Figure 2.   Acetylene reduction activity of nodulated soybean plants in the absence or presence of 10 mM nitrate added to nodulated soybean plants: A, on the day of; B, one day prior to; C, two days prior to; or, D, three days prior to the initiation of acetylene reduction activity. Open symbols are untreated control plants and closed symbols are nitrate treated plants. The arrow indicates the day on which nitrate was added. The largest standard deviation for each experiment is indicated by the error bars at the right side of each panel. The results represent one of four separate experiments.

The addition of nitrate severely reduced nodule growth. Nitrate, at 10 mM, reduced nodule fresh weight 50 to 67% in all treatments four days after the addition. Typically, the increase in nodule fresh weight in control plants over this period was about 1.5 mg fresh weight per day. Nitrate additions reduced nodule fresh weight accumulations to approximately 0.7 to 1.0 mg fresh weight per day over the first three to four days after the addition and prevented further increase thereafter. The addition of nitrate three days prior to the initiation of ARA was as early as it could be added and still obtain sufficient nodule mass from which to extract adequate quantities of bacteroids for analysis. Although, the numbers of nodules per treatment was not recorded, nitrate appeared to have a greater effect on nodule mass than on nodule number.

The time at which nitrate was added relative to T_{ARA} also had an effect on the protein content of bacteroids isolated from the treated soybean nodules (Fig. 3). The earlier nitrate was added, the lower the bacteroid protein content per gram of nodule fresh weight. If nitrate was added on T_{ARA}, or one day prior to T_{ARA}, both the rate of bacteroid protein accumulation and the final amount of protein were reduced (Figs. 3A, B). The protein contents were reduced by 30–50% in these treatments. The protein content of bacteroids from plants treated with nitrate 2 and 3 days before T_{ARA} of control plants showed no increase in protein content (Figs. 3C, D).

In control plants, the bacteroid enzymes of carbohydrate and amino acid metabolism declined during the course of the experiment. These same enzymes showed greater levels of activity in bacteroid extracts obtained from plants treated with nitrate relative to those untreated. The time at which nitrate was added did not appear to have a significant effect. There was some variability between various experiments but overall the four different times of nitrate additions appeared to restore the enzyme activities to the same final level. The enzymes in this group include: aspartate aminotransferase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase and general acyl-CoA thiolase I.

Fig. 4 shows the developmental profile for glucose-6-phosphate dehydrogenase activity in control plants compared to those treated with nitrate three days prior to T_{ARA}. The results observed for glucose-6-phosphate dehydrogenase is representative of those enzymes in this group. In general, this group of enzymes displayed rapid decreases up to 4 and sometimes up to 6 days in the absence of nitrate. Between 6 to 10 days after nitrate addition, there were either smaller or no significant further decreases in activity. In the presence of nitrate, these enzymes showed no decline or a small increase relative to the initial measurement of activity if nitrate was added three days prior to T_{ARA}. If nitrate was added to older plants in which repression of these
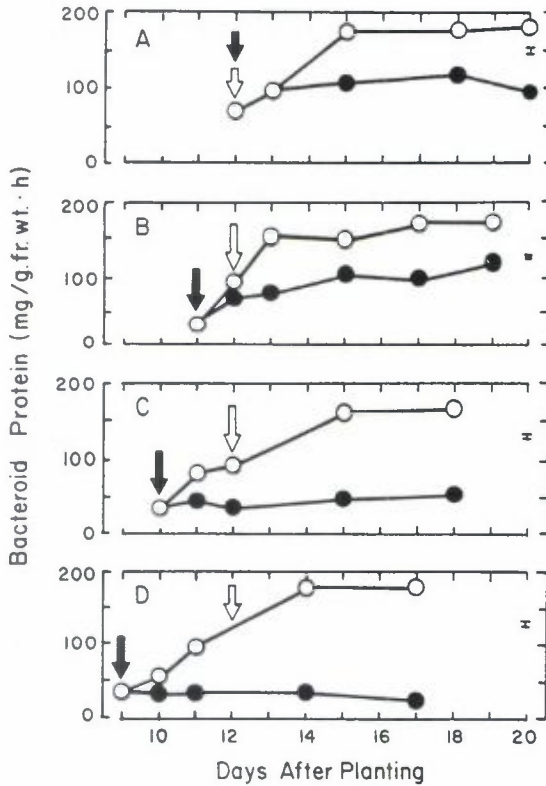
Figure 3. Protein contents of bacteroids isolated from nodulated soybean plants in the presence or absence of nitrate. Nitrate, at 10 mM, was added to nodulated soybean plants: A, on the day of; B, one day prior to; C, two days prior to; or, D, three days prior to the initiation of acetylene reduction activity. Open symbols are untreated control plants and closed symbols are nitrate treated plants. The open arrow indicates the day on which acetylene reduction activity was first observed, the closed arrow indicates the day on which nitrate was added. The largest standard deviation for each experiment is indicated by the error bars at the right side of each panel. The results represent one of four separate experiments.

enzymes had occurred, one or two days were required to restore the level of activity to that observed prior to symbiotic repression.

Table 1 list the change in activity for each enzyme after six days in the absence of nitrate relative to that found after nitrate addition. The values of percent change are presented as positive numbers for this group as the activity of the enzymes in the control, that is no nitrate added, decreased during nodule development and therefore the addition of nitrate resulted in a relative

Table 1. Specific activities of enzymes from bacteroids isolated from soybean nodules six
days after the plants were treated with nitrate and three days after the
initiation of acetylene reduction activity.

| Enzyme | Specific activity[a] (nmol/min/mg protein) | | % Change |
	Control (− Nitrate)	+ Nitrate	
Glyceraldehyde-3-P dehydrogenase	2.4 ± 2.1	35.4 ± 2.8	+47
Glucose-6-P dehydrogenase	78.3 ± 1.9	148.1 ± 3	+89
Aspartate aminotransferase	108.1 ± 11.3	160 ± 14.9	+48
Glutamine synthetase I	28.1 ± 2.2	41.7 ± 3.1	+48
Isocitrate dehydrogenase	464 ± 33	362 ± 32.1	−22
Hydroxybutyrate dehydrogenase	44.9 ± 2.3	34.1 ± 1.4	−24
Malic enzyme	12.5 ± 1.5	9.1 ± 1.1	−27
Malate dehydrogenase	3,984 ± 161	2,271 ± 202	−43
Fumarase	168.2 ± 5.1	59.1 ± 3	−65
α-Ketoglutarate dehydrogenase	24.1 ± 2.3	7 ± 0.9	−71
Pyruvate dehydrogenase	21.4 ± 1.8	23.1 ± 1.9	+8
Acetoacetate-succinyl-CoA transferase	105.5 ± 6	113.9 ± 10.9	+8
Acetoacetyl-CoA thiolase	2,020 ± 153	2,201 ± 164	+9
Acyl-CoA thiolase	203.2 ± 24.1	211.3 ± 13	+4

[a] Mean of 4 measurements ± standard deviation.

increase in activity when the data are compared at day six. The enzymes in
this group showed increases of approximately 50% or greater when compared in
this manner.

In contrast to the enzymes of carbohydrate and amino acid metabolism,
nitrate caused a repression of the citric acid cycle enzymes and two other
enzymes or organic acid metabolism when added prior to T_{ARA}. The enzymes in
this group include malate dehydrogenase, fumarase, isocitrate dehydrogenase,
α-ketoglutarate dehydrogenase, malic enzyme and hydroxybutyrate
dehydrogenase. Although they all showed repression by nitrate, each enzyme
responded differently to nitrate additions. Malate dehydrogenase was highly
expressed even at three days prior to T_{ARA} in the absence of nitrate. The
addition of nitrate three days prior to T_{ARA} resulted in a decline in malate
dehydrogenase activity of approximately 450 ηmol/min/mg protein per day or
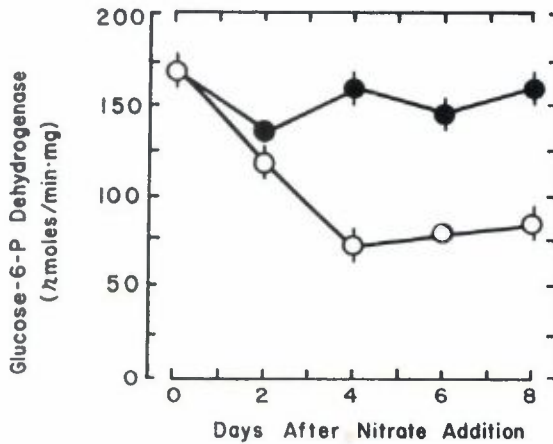
Figure 4.   Activity of bacteroid glucose-6-P dehydrogenase isolated from nodulated
soybean plants in the presence or absence of nitrate. Nitrate, at 10 mM, was
added to nodulated soybean plants three days prior to the initiation of acetylene
reduction activity (closed circles). The open circles represent bacteroid enzyme
activities from untreated control plants The standard deviations are indicated
by the error bars. The results represent one of four separate experiments.

12% per day (Fig. 5). However, there was no decline if nitrate was added two
days prior, one day prior or on T_{ARA}. Fumarase repression occurred if nitrate
additions were made two days prior, or later, to T_{ARA}, but not if nitrate was
added three days prior (Fig. 6). α-ketoglutarate dehydrogenase was repressed
when nitrate was added two days prior, and occasionally one day prior, but not
three days prior to T_{ARA} (data not shown). Isocitrate dehydrogenase showed a
decrease during nodule development, unlike the other citric acid cycle enzymes
(Karr et al., 1984).   Isocitrate dehydrogenase showed a slightly greater
activity if nitrate was added two days or closer to T_{ARA}, but less activity if
nitrate was added at three days prior to T_{ARA} (data not shown).

When nitrate was added at T_{ARA}, it often resulted in elevated expression of
all of these enzymes. Table 1 lists the relative change for each of the enzymes
in this group at six days after nitrate addition to be comparable to those of the
other groups of enzymes. The enzymes in this group showed decreases of
approximately 20% or greater when compared in this manner.

A third group of enzymes showed relatively little if any change in activity
due to the nitrate treatment. These enzymes include pyruvate dehydrogenase,
acetoacetyl-CoA thiolase, acyl-CoA thiolase I and acetoacetate-succinyl-CoA
transferase. The change in activity for this group of enzymes was usually
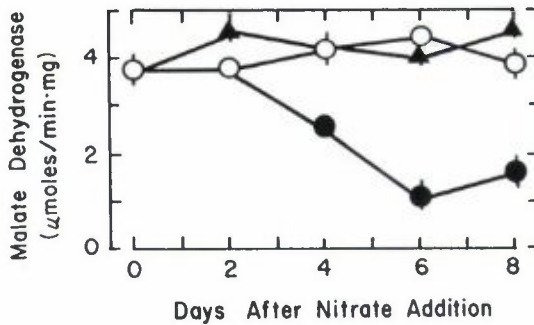within 10% or less of the value for the controls (Table 1).

Figure 5. Activity of bacteroid malate dehydrogenase isolated from nodulated soybean plants in the presence or absence of nitrate. Nitrate, at 10 mM, was added to nodulated soybean plants two days prior (closed triangles) or three days prior to the initiation of acetylene reduction activity. Open circles represent bacteroid malate dehydrogenase activities from untreated control plants. The standard deviations are indicated by the error bars. The results represent one of four separate experiments.
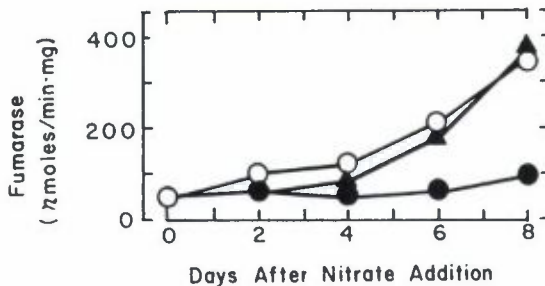

Figure 6. Activity of bacteroid fumarase isolated from nodulated soybean plants in the presence or absence of nitrate. Nitrate, at 10 mM, was added to nodulated soybean plants two days prior (closed triangles) or three days (closed circles) prior to the initiation of acetylene reduction activity. Open circles represent bacteroid fumarase activities from untreated control plants The standard deviations were smaller than the size of the symbols used in the figure. The results represent one of four separate experiments.

4. Discussion

The period of soybean nodule development prior to the appearance of acetylene reduction activity (T_{ARA}) is the mitotic phase (Newcomb, 1981). Anthon and Emerich (1990) reported that this phase occurs until 12 to 15 days after inoculation of *B. japonicum* 143 onto Williams 82 cultivar of soybean.

With this plant and bacterial combination, this phase is characterized by a declining soluble protein content of the plant portion of the nodule, but a slowly rising total protein content per nodule (Anthon and Emerich, 1990). The bacteroid protein content begins to increase during the late mitotic phase (Fig. 2). In this study, three of the four nitrate additions were made during late mitotic phase just prior to the cell expansion phase of nodule development (Newcomb, 1981). The cell expansion phase is characterized, in the nodule plant cells, by a relatively constant soluble protein content (mg protein/g fresh weight) and a more rapidly increasing total protein content (mg protein/nodule). The rapid rise in total protein coincides with the appearance and development of acetylene reduction activity.

The bacteroid protein content rises rapidly during the cell expansion phase (Fig. 2) in parallel to that of the total protein content of the nodule (Anthon and Emerich, 1990). The addition of nitrate causes a decrease in the protein content of bacteroids, the severity of which is dependent upon the time of application. In plants treated with nitrate on the day of, or 1 day prior to T_{ARA} by control plants, the initial rate of protein accumulation and the final protein content are reduced. Bacteroids from plants to which nitrate was added 2 or 3 days prior to T_{ARA} by control plants did not greatly increase their protein content beyond that observed at the time of nitrate addition, thus the specific activity measurements provided a valid comparison between changes of enzyme activities at each time interval.

The citric acid cycle is believed to be the primary metabolic pathway of bacteroids providing the energy for nitrogenase (Glenn et al., 1984; Udvardi et al., 1990). Bergersen and colleagues (1990, 1991) have shown that polyhydroxybutyrate can be mobilized for the support of symbiotic nitrogen fixation when exogenous energy supplies are limiting or unavailable. In general, during nodule development the citric acid cycle enzymes are expressed whereas the non-citric acid cycle enzymes are repressed (Karr et al., 1984; and present data). Nitrate reverses the repression of the non-citric acid cycle enzymes at any stage, but nitrate can only repress the citric acid cycle enzymes if added two or three days prior (depending on the particular enzyme) to T_{ARA}. This decline in the specific activity of citric acid cycle enzymes is not due solely to the low protein contents of the bacteroids resulting from this particular treatment, as other enzymes showed increases or no change in specific activity. This suggests that the enzymes of the citric acid cycle and those of other metabolic pathways are regulated differently, at least under the conditions in which the nitrate additions were imposed here. The results described here imply that the metabolic commitment to the bacteroid state, with regard to the citric acid cycle enzymes, was made between 2 and 3 days prior to T_{ARA}. The symbiotic expression of these enzymes of the citric acid cycle

is initiated late during the mitotic phase and just prior to the expansion phase of soybean nodule development. Nitrate additions to nodulated soybeans during the later stages of the mitotic phase of nodule development may be useful for studying the symbiotic expression of the citric acid cycle enzymes.

In planta nitrogenase activity was more susceptible to nitrate than other bacteroid enzymes. Nitrogenase, as measured by the acetylene reduction assay, was severely inhibited by 10 mM nitrate regardless of when it was added to the plants. As only whole plant acetylene reduction activity was recorded, it is not known whether the bacteroids still possessed activity *ex planta* or alternatively, whether the addition of nitrate specifically inhibited the synthesis of nitrogenase proteins. Schuller et al. (1986) reported that nitrate had no effect on *ex planta* nitrogenase activity of bacteroids isolated two days after the plants were treated with nitrate. Their plants were treated with nitrate only after symbiosis was well established, so their results and ours are not directly comparable. However, it is interesting to note that the nitrate additions take several days to elicit their effect in both types of experimental treatments. Since whole nodule nitrogenase activity was more susceptible to nitrate than were the apparent *in vitro* activities of bacteroid enzymes measured in this study, it suggests that the cause of nitrate inhibition of nitrogen fixation activity is not mediated through repression of bacteroid citric acid cycle enzymes. However, *in situ*, the effects of nitrate on certain bacteroid enzymes may be directly responsible for the observed inhibition of nitrogen fixation.

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