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Studies on Soybean Nodule Senescence[1]

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ABSTRACT

Soybean *Glycine max.* L. Merr. nodule senescence was studied using the loss of acetylene reduction by intact tap root nodules as its indication. Tap root nodules from two varieties (Calland and Beeson) of field-grown soybeans were used. The specific activities of nitrogenase (micromoles/minute·gram fresh weight of nodules) as measured by the acetylene reduction assay decreased abruptly between 58 to 65 and 68 to 75 days after planting the Beeson and Calland soybeans, respectively. Major changes were not detected in dry weight, total nitrogen, and leghemoglobin levels during the period when *in vivo* nitrogenase activity declined. Ammonium levels in the cytosol of nodules and poly-β-hydroxybutyrate increased moderately just prior to or coincidental with the loss of nitrogenase activity. Neither enzymes that have been postulated to be involved in ammonium assimilation nor NADP⁺-specific isocitrate dehydrogenase exhibited any large changes in specific activities during the initial period when nitrogenase activity declined.

Relatively little information is available concerning the physiological and biochemical events associated with soybean root nodule senescence. This is partially a result of the difficulties involved in assaying large numbers of samples for nitrogenase activity, but the recently discovered acetylene reduction assay alleviates this problem (15). Some data are available on changes in respiration (6), leghemoglobin (14), poly-β-hydroxybutyrate (28), nucleic acids (6), and vitamins (25), but generally the data were not concerned specifically with nodule senescence. Products of photosynthesis are important for nitrogen fixation (29). Recent evidence suggests that symbiotic nitrogen fixation declines during pod-filling as the result of an inadequate supply of photosynthetic products to nodules (22) and CO_2-enrichment studies suggest that photosynthate is a major limiting factor for N_2 fixation (16). Diurnal fluctuations in nitrogen fixation are observed during part of the growing season (17, 23) but the events that lead to loss of N_2 fixation in aging nodules appear to be irreversible. The loss of nitrogen fixation can be delayed or slowed (16, 22), but after the activity decreases, it is not recovered unless new nodules form (22).

Soybean nodules result from a complex but orderly interaction between legume root cells and *Rhizobium japonicum*. The interaction commences when rhizobia penetrate the epidermal cells of root hairs and terminate when remnants of the nodules are sloughed from the roots. Because the amount of nitrogen fixed by nodules is a function of the central tissue volume and the active life of this tissue (27), the factors that determine the longevity of this tissue are extremely important. Many noticeable internal and external changes are observed as nodules age (1) but the sequence of these changes is not known.

The research presented in this paper is concerned specifically with certain physiological and biochemical events associated with nodule senescence. Nodulated tap roots from two varieties of field-grown soybeans were assayed for nitrogenase activity during part of the growing season and samples of nodules were stored for subsequent analysis of nodule components. Although a number of parameters could have been examined, this paper specifically concerns changes in dry weight, total nitrogen, ammonia, leghemoglobin, nitrogen-assimilating enzymes, poly-β-hydroxybutyrate, and NADP⁺-specific isocitrate dehydrogenase during the period when aging nodules lose the capacity to fix nitrogen.

MATERIALS AND METHODS

Plant Materials. Seeds of soybeans (*Glycine max* L. Merr.) varieties Calland (maturity group III) and Beeson (maturity group II) were inoculated with a commercial inoculum (The Nitragin Co., Milwaukee, Wis.) and machine planted on June 5 and June 19, 1973, respectively. The research plot was part of the research area of the Department of Agronomy, University of Nebraska, Lincoln. These fields had been used previously to grow soybeans which were well nodulated. Thus, rhizobia were present in the soil. Ages reported in this paper were computed from the planting date. Plots of each soybean variety consisted of 15 rows that were spaced 76 cm apart and were 30.5 m in length. Rows were divided into 125-cm sections and 10 sections were selected randomly for harvesting at each sampling period. An average section contained about 16 plants. Border rows and ends of rows were not used in this study. Weed control was achieved by applying a pre-emergence herbicide (AMIBEN, Amchem Products, Inc.) 1 day after planting and then the plots were hoed manually during the growing season. Nodules were apparent on both varieties of soybeans within 2 weeks after the planting date. Flowers were evident about 44 and 50 days after planting the Beeson and Calland soybeans, respectively. Plant lodging was not evident with either variety until after the observed decrease in acetylene reduction.

Plants were sampled by removing the tap roots from the soil and transporting the intact plants to the laboratory. The lateral roots were excluded intentionally to yield a more homogenous population of nodules with regard to nodule age (6). The results reported in this paper, therefore, are not total activities of all the root nodules. Plants were randomly selected for nitrogenase assays and the nodules from the remaining plants were excised, weighed, frozen with dry ice, and stored in sealed containers at −20 C for subsequent tests. Harvesting of plants was started at 10:00 AM and was finished, including the acetylene reduction tests, within 2 hr.

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Assays. Acetylene reduction to ethylene was used to measure nitrogen fixation (15) and the procedures were similar to those described by Fishbeck *et al.* (13). Randomly selected plants were divided into two groups of five and one group of 10. After the upper portion of the plants were excised, the groups were placed into 11.5-liter clear Plexiglas cylinders fitted with three serum-stoppered sampling ports and a container for CaC_2. CaC_2 was used to generate acetylene *in situ* and methane was injected into the chamber to serve as an internal standard. Duplicate 0.5-ml gas samples were taken periodically and each sample was injected into a gas chromatograph as described previously (20). Quantitation of ethylene and acetylene was done by integrating the peaks using methane as an internal standard and correcting for differences in detector sensitivity for the three gases. Because of variable lag periods, time courses were run for all groups and rates were obtained from the linear portion of the curves. Acetylene pressure was about 0.06 atm for most tests but because of the variability in generating the same amounts of acetylene each time, acetylene was measured for each sample. The acetylene reduction activities reported in this paper are maximum velocities as computed from the data of Fishbeck *et al.* (13). After the assay, nodules were excised from the roots, counted, and weighed. Although other criteria could be used, all values are reported on a fresh weight basis except for nodule weights and counts.

Dry weights were determined on duplicate 1 g fresh weight of nodules dried to a constant weight in an oven at 80 C. Total nitrogen in nodules was done on duplicate 1 g fresh weight samples using the Kjeldahl method.

Ammonia levels were determined in quadruplet using samples of 1 g fresh weight. Nodules were separated into the bacteroids and cytosol as described by Pankhurst *et al.* (24). Bacteroids were suspended in 2 ml of 40 mM tris-Cl (pH 7.5), sonicated for 5 min, and then 1.8 ml of this solution was used for microdiffusion of ammonia. The cytosol was diluted to 12 ml and a 1-ml sample was subjected to microdiffusion. Microdiffusion was done as described by Burris (10) except that 2 ml of saturated K_2CO_3 solution was used and 90 min were allowed for diffusion. Nessler's reagent was used for determination of the ammonia. Tests showed that virtually 100% of the ammonia diffused during this time and that less than 3% of the glutamine was deamidated.

Poly-β-hydroxybutyrate and leghemoglobin levels were determined using bacteroids and cytosol, respectively. The bacteroids and cytosol were separated as described by Bergersen and Goodchild (7). Leghemoglobin levels were measured using pyridine hemochromogen assays (9) and poly-β-hydroxybutyrate levels were determined as described by Herbert *et al.* (19). All assays were done in triplicate.

Enzymes were assayed in cytosol and bacteroid extracts prepared from nodules by the following method. All procedures were done at 0 to 4 C. Approximately 3 g fresh weight of nodules were placed in a 50-ml centrifuge tube containing 0.6 g of acid-washed, insoluble PVP and 10 ml of 50 mM TES (pH 7.5) and 160 mM sodium ascorbate. Nodules were homogenized for 2 × 45 sec at a 50% setting on a Sorvall Omni-Mixer. The homogenate was filtered through two layers of cheesecloth and the filter cake was suspended in 10 ml of 50 mM TES (pH 7.5), subjected to a second homogenization as described above, and refiltered through the cheesecloth. The filtrates were combined and centrifuged at 6000*g* for 10 min. The supernatant fluid was called the cytosol and the pellet was called the bacteroids. Bacteroids were washed with 10 ml of 50 mM TES (pH 7.5) and suspended in 7.5 ml of 200 mM TES (pH 7.5) and 2 mM dithiothreitol. After two passes through a French Pressure cell at 25,000 p.s.i., the broken bacteroids were centrifuged at 27,000*g* for 40 min and the supernatant fluid was designated the bacteroid extract. All bacteroid extracts contained about 11 mg of protein (biuret assay)/g of nodules (fresh weight). Alanine dehydrogenase (EC 1.4.1.1), glutamate dehydrogenase (EC 1.4.1.2), glutamine synthetase (EC 6.3.1.2), and glutamine amide-α-ketoglutarate aminotransferase oxidoreductase (EC 1.4.1.X) were assayed as described by Dunn and Klucas (12) except that TES (pH 8.0) was used in the assay for alanine dehydrogenase and 25 C was the assay temperature used for glutamine synthetase. Isocitrate dehydrogenase (EC 1.1.1.42) activity was assayed spectrophotometrically at 25 C by the measurement of initial rates of reduction of $NADP^+$ at 340 nm. In addition to the bacteroid extract or cytosol, the assay mixture contained 125 mM HEPES (pH 8.0), 0.35 mM $NADP^+$, 0.5 mM isocitrate, and 1 mM MnCl. Aspartate aminotransferase (EC 2.6.1.1) activities were measured in the cytosol and bacteroid extracts with the use of a reaction mixture of 50 mM HEPES (pH 8.0), 2.5 mM L-aspartate, 2.5 mM α-ketoglutarate and 0.2 mM NADH. An excess of endogenous malate dehydrogenase activity was present to reduce the oxaloacetate formed in the transamination and thus the reaction was followed by measuring the change in absorbance at 340 nm. Alanine aminotransferase (EC 2.6.1.2) activity was assayed by using the reaction mixture of 50 mM TES (pH 7.5), 20 mM DL-alanine, 25 mM α-ketoglutarate, 4 units of lactate dehydrogenase (EC 1.1.1.27), and 0.2 mM NADH. Endogenous NADH oxidation in the absence of added amino acid or α-keto acid was subtracted from initial velocities. One unit of activity was defined as 1 μmole of NADH oxidized or $NADP^+$ reduced per min for all the enzymes except glutamine synthetase. One unit of glutamine synthetase was defined as 1 μmole of γ-glutamylhydroxamate formed per min.

RESULTS

Acetylene reduction to ethylene was used to monitor nitrogen fixation by nodulated tap roots during the latter part of the growing seasons of Calland (Fig. 1) and Beeson (Fig. 2) varieties of soybeans. Neither variety exhibited any significant changes in specific activity, with one exception, until a highly significant decrease occurred. The unexplained exception was a high value observed at 54 days with the Calland soybeans.

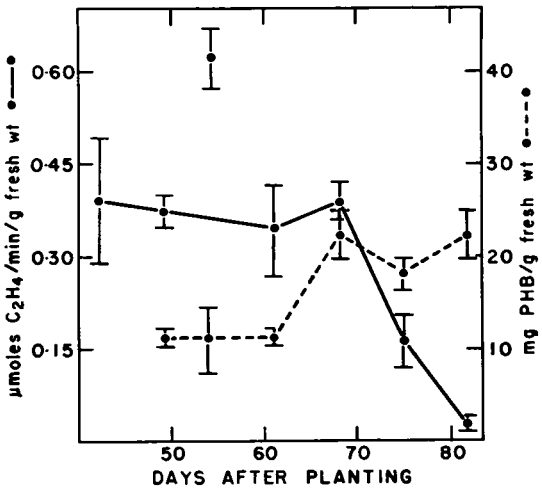

FIG. 1. Nitrogen-fixing activity and poly-β-hydroxybutyrate (PHB) levels in tap root nodules from Calland soybeans of various ages. Acetylene reduction was used to measure nitrogen fixation as described in "Materials and Methods." Each point represents the mean and standard deviation of three replicates.

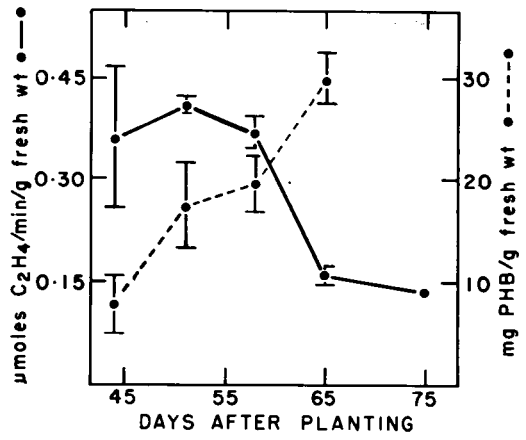

FIG. 2. Nitrogen-fixing activity and poly-β-hydroxybutyrate (PHB) levels in tap root nodules from Beeson soybeans of various ages. Acetylene reduction was used to measure nitrogen fixation as described in "Materials and Methods." Each point represents the mean and standard deviation of three replicates as described in "Materials and Methods" except that the 75-day sample for nitrogenase activity consisted of a single group of nine plants.

Table I. *Weight and Number of Tap Root Nodules on Aging Soybeans*

Weight per nodule and nodules per plant were determined on each of three samples except that the value for 75-day-old Beeson nodules represents a single sample in which the interiors of most of the nodules were badly degenerated. Values are means of three replicates. For a variety, values in the same vertical row followed by the same letter are not significantly different ($P \leq 0.05$) using the Duncan's new multiple range test. Comparisons were not done between Calland and Beeson varieties

Soybean Variety	Age	Wt per Nodule	Nodules per Plant
	days	*mg*	
Calland	54	13.9 A	55 A
	61	11.0 B	86 AB
	68	11.9 C	139 CD
	75	10.5 B	154 D
	82	12.0 C	111 BC
Beeson	44	17.1 A	24 A
	51	14.7 A	44 B
	58	17.6 A	41 B
	65	18.0 A	38 B
	75	20.6	28

Acetylene-reducing activity decreased from about 0.3 to 0.16 µmole of C_2H_2 reduced/min·g fresh nodule weight between 68 and 75 days for Calland soybeans and 58 and 65 days for Beeson soybeans. Although some variations were observed, the mean nodule weight as determined for each group of plants did not exhibit any major changes with either variety of soybeans (Table I). The number of nodules on the tap root increased until 68 and 51 days with the Calland and Beeson plants, respectively. The nodule population was reasonably stable in regard to average number and size when nitrogenase activity declined. Calland tap roots possessed many more nodules than the Beeson roots but the average Beeson nodule weighed more than the average Calland nodule.

Ammonium levels in Calland soybean nodules increased between 54 and 61 days old and 68 and 75 days old (Table II) and poly-β-hydroxybutyrate increased 1 week prior to loss of nitrogen fixation (Fig. 1). With Beeson soybean nodules, ammonium levels increased significantly each week between 51 and 65 days after planting (Table II) and poly-β-hydroxybutyrate increased between 44 and 51 days and 58 and 65 days (Fig. 2). About 90% of the ammonium was in the cytosol of the nodules as compared to the bacteroids (Table II). Although increases occurred in bacteroids from Calland nodules, the greatest changes occurred in the cytosol ammonium, but because of the possible leakage of the bacteroids during isolation, this distribution may not reflect the true distribution in intact nodules.

Dry weight and total nitrogen were measured in nodules of various ages (Table III). A general increase in dry weight and total nitrogen was observed starting at 49 days after the Calland soybeans were planted. The weekly increases were not great enough to be significant but the increases were significant over longer periods. Total nitrogen in Beeson nodules was greatest at 65 days but a uniform increase on a weekly basis was not observed. Beeson nodules did show a significant increase in dry weight between 51 and 58 days. From 4 to 5.5% of the

Table II. *Distribution of Ammonium between the Cytosol and Bacteroids in Tap Root Nodules from Soybeans of Various Ages*

Cytosol and bacteroids were obtained from Calland and Beeson nodules as described in the Materials and Methods section. Values represent means of four replicates. For a variety, values in the same vertical row followed by the same letter are not significantly different ($P \leq 0.05$) using Duncan's new multiple range test. Comparisons were not done between Calland and Beeson varieties.

Soybean Variety	Age	Ammonia	
		Cytosol	Bacteroids
	days	*µmoles/g fresh wt*	
Calland	54	7.0 A	0.81 A
	61	14.9 B	1.44 B
	68	13.8 B	1.25 C
	75	19.2 C	1.71 D
Beeson	44	10.4 A	1.31 A
	51	9.8 A	1.37 A
	58	15.4 B	1.32 A
	65	18.4 C	1.45 A

Table III. *Total Nitrogen, Dry Weight, and Leghemoglobin in Tap Root Nodules from Soybeans of Various Ages*

Values represent means of duplicates for the total nitrogen and dry weight and triplicates for the leghemoglobin. For a variety, values in the same vertical row followed by identical letters are not significantly different ($P \leq 0.05$) using Duncan's new multiple range test. Comparisons were not done between Calland and Beeson varieties.

Soybean Variety	Age	Total N	Dry Wt	Leghemoglobin
	days	*mg/g fresh wt*	*mg/g fresh wt*	*nmoles/g fresh wt*
Calland	42	15.3 ABC	352 A	118 A
	49	10.9 D	270 B	89 B
	54	12.3 CD	253 B	101 BC
	61	13.8 BCD	316 C	109 AC
	68	14.7 ABC	296 C	122 A
	75	15.8 AB	348 A	109 AC
	82	17.7 A	362 A	90 B
Beeson	44	12.9 A	254 A	84 A
	51	13.8 B	252 A	100 B
	58	12.8 A	304 B	88 A
	65	14.6 C	300 B	124 C

dry weight was accountable as nitrogen. Table III also shows levels of leghemoglobin in tap root nodules from aging plants. Although significant changes were detected in leghemoglobin, no general trends nor extreme changes occurred. Comparable levels of total leghemoglobin were present in nodules before and just after the decline in nitrogenase activity with either variety of soybeans.

Activities of enzymes possibly involved in ammonium assimilation (12) were measured in both the cytosol and bacteroids (Tables IV and V). Glutamine synthetase activity was abundant both in the cytosol and in bacteroid extracts with about 85 to 89% of the total activity in the cytosol. Although fluctuations are evident at the four plant ages, high activities were detected throughout the period. Glutamate dehydrogenase activities appeared to be lower in bacteroid extracts from older plants but the weekly changes for the two varieties were not uniform (Table IV). Although differences were observed in glutamate dehydrogenase activities with various cytosols, these differences were not consistent between the two varieties (Table V). Most of the glutamate dehydrogenase activity (74–92%) was detected in the cytosol. GOGAT[2] activity was not detected in the cytosol but was found in the bacteroids (Table IV). Bacteroid extracts from Calland nodules exhibited increases of GOGAT activity in nodules from older plants but similar increases were not observed with Beeson nodules. For the periods shown in Table IV, aspartate and alanine aminotransferases were consistently high with both varieties of soybean nodules. The aspartate aminotransferase activity in bacteroid extracts varied between 1.5 and 2.1 units/g fresh weight and in the cytosol between 5.6 and 8.6. Bacteroid extracts and cytosols exhibited alanine aminotransferase activity that varied between 1.2 and 1.4 and between 5.6 and 6.9 units/g fresh weight, respectively. Alanine dehydrogenase activity was high in the bacteroids and no significant changes were observed among the bacteroid extracts (Table IV).

Table IV. *Activities of GOGAT, Glutamate Dehydrogenase, Glutamine Synthetase, and Alanine Dehydrogenase from Bacteroids of Tap Root Nodules from Soybeans of Various Ages*

The bacteroid extracts were prepared as described in Materials and Methods. Except for glutamine synthetase, all values represent means of duplicates. Glutamine synthetase values represent single samples. For a variety, values in the same vertical row followed by identical letters were not significantly different ($P \leq 0.05$) using Duncan's new multiple range test. Comparisons were not done between varieties.

Soybean Variety	Age	GOGAT	Glutamate Dehydrogenase	Glutamine Synthetase	Alanine Dehydrogenase
	days	*units/g fresh wt*			
Calland	54	0.20 A	0.19 AB	7.9	1.23 A
	61	0.31 B	0.16 AB	8.7	1.57 A
	68	0.30 B	0.21 A	8.0	1.39 A
	75	0.39 C	0.10 B	9.2	1.32 A
Beeson	44	0.34 A	0.18 A	9.7	1.55 A
	51	0.49 B	0.12 AB	8.8	2.03 A
	58	0.37 A	0.03 C	8.3	1.48 A
	65	0.43 C	0.07 BC	8.4	1.35 A

Table V. *Activities of Glutamate Dehydrogenase and Glutamine Synthetase in the Cytosols of Tap Root Nodules from Soybeans of Various Ages*

The cytosol fractions were prepared as described in Materials and Methods. The values for glutamate dehydrogenase represent means of duplicates and for glutamine synthetase represent single samples. For a variety, values followed by the same letter for a variety are not significantly different ($P \leq 0.05$) using the Duncan's new multiple range test. Comparisons were not done between Calland and Beeson varieties.

Soybean Variety	Age	Glutamate Dehydrogenase	Glutamine Synthetase
	days	*units/g fresh wt*	
Calland	54	0.82 AB	51
	61	0.64 B	71
	68	1.16 A	55
	75	0.72 B	62
Beeson	44	0.94 A	54
	51	0.34 B	68
	58	0.45 B	63
	65	0.46 B	70

Table VI. *NADP$^+$-specific Isocitrate Dehydrogenase Activity in the Bacteroids and Cytosol of Tap Root Nodules from Soybeans of Various Ages*

Separation and assay procedures are given in the Materials and Methods section.

Soybean Variety	Age	Bacteroid	Cytosol
	days	*units/g fresh wt*	
Calland	54	1.5	1.8
	61	3.0	1.9
	68	1.5	2.3
	75	1.8	2.0
Beeson	44	2.1	2.4
	51	3.9	1.6
	58	2.9	1.6
	65	3.2	1.5

Because of recent suggestions for possible involvement in nitrogen fixation (5), NADP$^+$-specific isocitrate dehydrogenase activity was determined in bacteroid extracts and cytosols from nodules of various ages (Table VI). High activities were detected, and in contrast to other enzyme activities measured in this study bacteroid extracts had approximately the same amount of activity as the cytosols. Although replicates were not done on these samples, it appeared that isocitrate dehydrogenase activity was not lower in nodules which had decreased in nitrogen-fixing activity. However, an increase in activity appeared to occur with bacteroid extracts from both varieties of soybeans 2 weeks before the detectable decline in nitrogenase activity.

DISCUSSION

Tap root nodules exhibited a 60% decrease in their ability to fix N_2 between 68 and 75 days after the Calland soybeans were planted and between 58 and 65 days after the Beeson soybeans were planted. The reported values are V_{max} values calculated by using an apparent Km value for C_2H_2 of 0.05 atm (13). If another apparent Km value such as 0.007 atm as reported by Hardy *et al.* (17) was used, the V_{max} values are 0.2 before and 0.08 after the decline in activities but the profiles of the curves are unchanged. The declines in activities were not transient in that subsequent samples also had low nitrogenase activity. These initial periods of nitrogenase activity loss are

[2] Abbreviation: GOGAT: glutamine amide-α-ketoglutarate aminotransferase oxidoreductase.

useful reference periods to correlate with other metabolic events that may be involved in the loss of nitrogenase activity. Although total nodule senescence undoubtedly is a long term process, the permanent loss of nitrogenase activity is regarded as nodule senescence in this paper because it is the loss of the primary function of nodules.

Major changes in dry weight or total nitrogen are not evident during the period of nodule senescence, but a general increase in dry weight and total nitrogen occurred as the nodule population aged. The cause(s) for the increases in ammonium levels are not known but could result from increases or decreases of ammonium translocation in or out of nodules, decreases in assimilation, increase in hydrolytic processes, and others. The increases in ammonium were too small to be detected by changes in total nitrogen but both showed the same general trends. Most of the ammonium was in the cytosol and the percentage in the bacteroids decreased with nodule age although this interpretation of the data can be questioned because of the leakage from bacteroids.

The path of nitrogen within soybean nodules is resolved only partially because neither all the enzymes nor the site(s) of ammonium assimilation is known. It is known that nitrogen fixation occurs in the bacteroids (8, 21), that glutamate is an early product (4), and that amino acids, especially asparagine, are translocated from the nodules (26, 28). Enzymes that may be involved in ammonium assimilation (12) such as GOGAT, glutamine synthetase, glutamate dehydrogenase, alanine dehydrogenase, aspartate aminotransferase, and alanine aminotransferase did not decrease in activity when nitrogenase activity was lost. Although the reported conversion ratio of C_2H_2/N_2 reduced is not a constant (15), soybean nodules under conditions described in this paper generally exhibit ratios of approximately 2–4 (15, 23) or a ratio of 1–2 for C_2H_2 reduced/NH_3 formed. If this ratio is applied, most enzymatic activities measured *in vitro* except bacteroid glutamate dehydrogenase are sufficient to account for assimilation of the ammonium that is calculated to be formed.

Leghemoglobin has been implicated as an essential for nitrogen fixation by soybean nodules for many years. Recently, evidence has accumulated to suggest that leghemoglobin supplies O_2 to the bacteroids (9, 30) and therefore alterations in leghemoglobin could be detrimental. Although correlations have been reported between nitrogen fixation and leghemoglobin during part of the growing season (7, 14), the data in this paper suggest that the correlation breaks down for a period when tap root nodules lose nitrogenase activity. Total leghemoglobin did not decrease during the initial decline in nitrogen fixation, but other undetected changes such as in spin states (2) or binding with natural ligands (3) could result in nonfunctional leghemoglobin molecules.

In agreement with other workers (18, 28), poly-β-hydroxybutyrate accumulated in nodules from both soybean varieties as the plants aged and reached levels of 10% of the total dry weight of the nodule. The polymer increased significantly just prior to or coincidental with the decrease in nitrogenase activity. It is interesting that poly-β-hydroxybutyrate accumulated during a period when photosynthate translocation to the roots was or became a limiting factor for nitrogen fixation (16, 22). The function of the polymer in bacteroids is unknown; however, the certain nitrogen-fixing bacteria such as *Azotobacter*, accumulation of poly-β-hydroxybutyrate is known to occur under O_2-limiting conditions (11). Accumulation of the polymer, therefore, could reflect changes in internal p_{O_2} which is certainly influenced by the leghemoglobin in the nodule.

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