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Nicotinate, Nicotinamide, and the Reactivity of Leghemoglobin in Soybean Root Nodules[1]

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ABSTRACT

Nicotinate has been postulated to interfere with the binding of O_2 to ferrous leghemoglobin in soybean (*Glycine max*) root nodules. For such a function, the levels of nicotinate in nodules must be sufficiently high to bind a significant amount of leghemoglobin. We have measured levels of nicotinate, nicotinamide, and leghemoglobin in soybean nodules from plants 34 to 73 days after planting in a glasshouse. On a per gram nodule fresh weight basis, levels between 10.4 and 21 nanomoles for nicotinate, 19.2 and 37.8 nanomoles for nicotinamide, and 170 to 280 nanomoles for leghemoglobin were measured. Even if all the nicotinate were bound to ferrous leghemoglobin, only 11% or less of the total leghemoglobin would be unavailable for binding O_2. Using the measured levels of nicotinate and a pH of 6.8 in the cytosol of presenescent soybean nodules, we estimate that the proportion of ferrous leghemoglobin bound to nicotinate in such nodules would be less than 1%. These levels of nicotinate are too low to interfere with the reaction between ferrous leghemoglobin and O_2 in soybean root nodules.

Lb[2] is a monomeric hemoglobin formed in the symbiotic tissue of legume root nodules where it transports oxygen to the respiring, nitrogen-fixing *Rhizobium* bacteroids (2). The nicotinate (pyridine 3-carboxylate) anion is known to bind with high affinity to the heme iron of ferric or ferrous Lb from soybean (6, 16) and lupin (7, 17), and these reactions are proton dependent (6, 8).

The ligation of exogenous nicotinate to pure ferrous Lb (6) or to ferric Lb in the nodule (14) interferes reversibly with its oxygen-binding reaction. Because of reports that pea nodules (9) and bean nodules (18) have high contents of nicotinic acid, and because a large portion of the Lb in aqueous extracts of soybean nodules is ligated by 'endogenous' nicotinate (= ligand X) (1, 5), it was proposed (6) that changes in nodule pH could, via endogenous nicotinate, control the oxygen reactivity of Lb *in vivo*. Also, it was suggested that, in senescing nodules, a lowered pH might favor the autoxidation of ferrous Lb and LbO_2 to ferric Lb and that the very high affinity binding of nicotinate to ferric Lb might precede the irreversible breakdown of this pigment (13).

For the pursuit of these hypotheses, it is critical to know whether the nicotinate in aqueous extracts of soybean nodules (1, 5, 6) is an endogenous, natural ligand of Lb or merely an artifact of the aqueous extraction procedure. For example, the enzyme nicotinamide deamidase is known to be present in the cytosol and bacteroids of homogenized nodules (13), and it is possible that this and other hydrolytic enzymes could have been responsible for the production of nicotinate from nicotinamide or pyridine nucleotide coenzymes. Accordingly, we have measured the levels of nicotinate and nicotinamide in extracts made by the homogenization of nodules in hot 70% aqueous ethanol, a procedure expected to rapidly inactivate hydrolytic enzymes. Both nicotinate and nicotinamide are soluble in 70% ethanol, and at high temperature (75°C) this solvent denatures Lb nicotinate and liberates nicotinate from it (CA Appleby, unpublished observations).

METHODS

Nodules

Seeds of soybean (*Glycine max* L. Merr.) cv Lincoln were inoculated with *Bradyrhizobium japonicum* strain CC705 and grown in a glasshouse as described elsewhere (10). Seeds of soybean cv Beeson were inoculated with a commercial inoculum (Nitragin, Milwaukee, WI) and grown under field conditions (12). Ages of plants are given as days after planting the seeds.

Nitrogenase

C_2H_2-reducing activity was measured in triplicate on soybean roots as described elsewhere (12). Protein concentration was estimated by the method of Lowry (15).

Leghemoglobin

Homogeneous, nicotinate-free, ferric Lba was isolated by anion exchange chromatography of soybean root nodule extracts on DEAE-cellulose (4), concentrated by pressure filtration over Amicon YM10 membranes to 6 mM in 10 mM potassium phosphate (pH 7.0), and stored in small volumes

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[2] Abbreviations: Lb, the mixture of all leghemoglobin components found in a legume nodule; Lba, the pure component a isolated from soybean Lb by an ion exchange chromatography; ferric Lb, ferric leghemoglobin, which is unable to combine with oxygen; ferrous Lb (or deoxyferrous Lb), the reactive species that combines reversibly with oxygen to give LbO_2 (oxyferrous Lb).

in liquid nitrogen. The affinity of nicotinic acid for ferric Lba was measured in 50 mM sodium succinate buffer (pH 5.0) at 20°C, by procedures described elsewhere (6). The dissociation constant (K_{diss}) of the binding reaction to ferric Lba was 0.65 μM nicotinate.

Total Lb was estimated by preparing pyridine hemochromes of nodule extracts and measuring the absorbance of the dithionite-reduced *versus* ferricyanide-oxidized pyridine hemochrome (556 nm minus 539 nm, $\Delta\varepsilon$ mM = 23.4) (3).

Extraction of Nicotinate and Nicotinamide from Nodules

Within 5 min after removal from vigorously photosynthesizing plants, nodules were washed with water at 0°C, dried of excess water, weighed, and either extracted immediately or frozen immediately in liquid N_2 for subsequent extraction. Duplicate 6-g samples of nodules were immersed in 250 mL of ethanol at 75°C containing 2 mL of 0.5 mM Tris (pH 9.0), and homogenized for 5 min with an Omni-Mixer (Sorvall) while maintaining a temperature of 75°C. One duplicate was supplemented with 4 nmol of [carboxyl-^{14}C]nicotinic acid (60 mCi/mmol) (Amersham), and the other was supplemented with 5 nmol of [carbonyl-^{14}C]nicotinamide (52 mCi/mmol) (Amersham), which served as internal standards for estimating recoveries and identification of nicotinate and nicotinamide. After centrifugation, the supernatant was evaporated to dryness at 75°C under a stream of N_2. The dried material was suspended in ethanol at 75°C; the suspension was centrifuged, and the resulting supernatant taken to dryness under N_2 at 75°C. The dried material was reextracted as described above except 1 mL of glass distilled water replaced the ethanol. The suspension was centrifuged, and the supernatant, designated solution A, was assayed for nicotinate and nicotinamide.

Identification and Recoveries of Nicotinate and Nicotinamide

Nicotinate and nicotinamide in solution A were separated and identified by TLC (13). Standards of nicotinate and nicotinamide as well as samples of solution A supplemented with 5 μmol of nonradioactive carrier nicotinate and nicotinamide were spotted on silica gel thin-layer sheets (Eastman 13179). Sheets were developed in *n*-butanol saturated with 15% (v/v) ammonium hydroxide or chloroform:acetone: diethylamine (5:4:1; v:v), dried, and sprayed with 0.2% (w/v) solution of 2,7-dichlorofluorescin in propanol, which makes nicotinate and nicotinamide visible under ultraviolet light. Spots corresponding to nicotinate and nicotinamide were scraped from the sheets into plastic scintillation vials, and the silica gel was solubilized with hydrofluoric acid. Radioactivity was measured by liquid scintillation spectrometry by the internal standard method to determine counting efficiencies.

Measurement of Nicotinate and Nicotinamide

Nicotinate and nicotinamide in the ethanol extract from nodules (solution A) were quantified from spectral changes associated with binding of nicotinate to ferric Lb (6). Each extract was divided into two samples with one sample used to measure nicotinate and the second used to measure nicotinate plus nicotinamide. Samples were adjusted to pH 5.0 and centrifuged to remove precipitates, and aliquots of the supernatant were added to cuvettes containing ferric Lba and succinate buffer (pH 5.0), final concentrations of 50 μM and 50 mM, respectively. Difference spectra were measured with ferric Lba in succinate buffer as a reference. The wavelength pair corresponding to an absorption peak (558 nm) and trough (626 nm) with a $\Delta\ \varepsilon$ mM = 7.7 was used to calculate the concentration of nicotinate. Nicotinate plus nicotinamide were measured by reacting the second sample at pH 7.0 for 60 min at 37°C with an excess amount (2 mg of protein) of partially purified nicotinamidase (EC 3.5.19) prepared from baker's yeast (11) and then measuring nicotinate as described above. Nicotinamide levels were calculated by difference in levels of nicotinate before and after nicotinamidase treatment. Appropriate blanks without Lba were used to correct absorbance caused by the yellow color in solution A.

RESULTS

The high affinity of ferric Lba for nicotinate and low affinity for nicotinamide at acid pH values and the relatively high extinction coefficient of the resulting hemochrome (6) were exploited for the design of an assay for nicotinate in the presence of nicotinamide. From the difference spectrum of ferric Lba nicotinate *versus* ferric Lba (Fig. 1 A), a wavelength pair of 558 and 626 nm was selected for the assay. A standard curve for nicotinate complexing to 50 μM ferric Lba in 50 mM succinate (anion concentration) at pH 5.0 is shown in Figure 2. The value of 7.7 for $\Delta\ \varepsilon$ mM (558 nm minus 626 nm) is valid up to 30 μM nicotinate. Various organic acids including malate and citrate which are known to be present in nodules (19) did not interfere with formation of the ferric Lba nicotinate complex nor change the absorption spectrum of ferric Lba.

Typical difference spectra, observed upon addition of solution A to ferric Lba in succinate buffer at pH 5.0, are shown in Figure 1B for nodules from 33-d-old plants and in Figure 1C for nodules from 73-d-old plants. Nodule extracts, especially those from older nodules, were yellow and absorbed light (dotted lines, Fig. 1, B and C). The nonspecific absorbance by extracts from older nodules significantly distorted the ferric Lb difference spectra, *e.g.* dashed line of Figure 1C. However, corrected difference spectra illustrated by the solid lines of Figure 1, B and C, in which absorbance of the extract in succinate buffer was subtracted from absorbance of the extract plus 50 μM ferric Lba in 50 mM succinate, are very similar to difference spectrum of ferric Lba plus nicotinate *versus* ferric Lba as shown in Figure 1A.

The extraction of nicotinate and nicotinamide from nodules by hot ethanol was evaluated by determining the recovery of ^{14}C-nicotinate or ^{14}C-nicotinamide. When nodules and ^{14}C-nicotinate were homogenized and extracted together, 92 to 97% of the radioactivity in the final extract cochromatographed on thin-layer sheets with authentic nicotinate and none with nicotinamide. When ^{14}C-nicotinamide replaced ^{14}C-nicotinate in the initial homogenization, 86 to 91% of the radioactivity cochromatographed with authentic nicotinamide and 6 to 10% with nicotinate. Some nicotinamide was deaminated to nicotinate during the extraction procedure, but

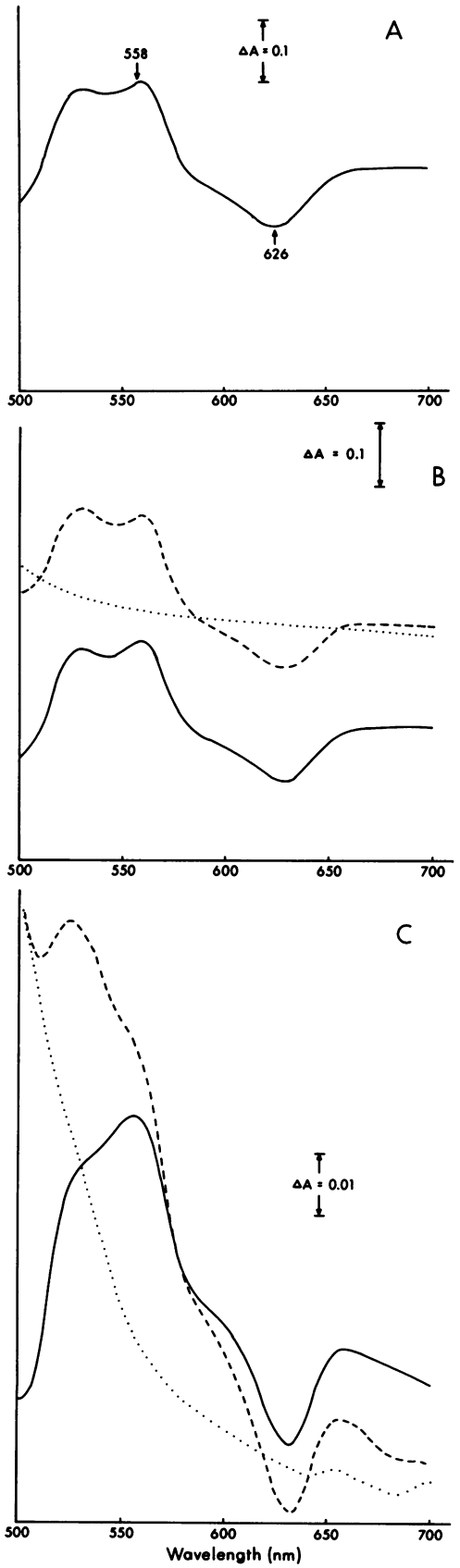

Figure 1. Difference spectra of 50 μM ferric leghemoglobin a (Lb) in 50 mM sodium succinate; succinic acid buffer (pH 5.0), 20°C with and without samples containing nicotinate. A, Ferric Lb with 30 μM nicotinic acid minus ferric Lb; B, dashed line is ferric Lb containing 0.2 mL of extract 'A' from 33-d-old nodules minus ferric Lb, dotted line is 0.2 mL of extract 'A' from 33-d-old nodules minus water; solid line is dashed line minus dotted line; C, dashed line is ferric Lb containing 0.1 mL of extract 'A' from 73-d-old nodules minus ferric Lb; dotted line is 0.1 mL of extract 'A' from 73-d-old nodules minus water; solid line is dashed line minus dotted line. Final volume in all cuvettes is 1.0 mL.

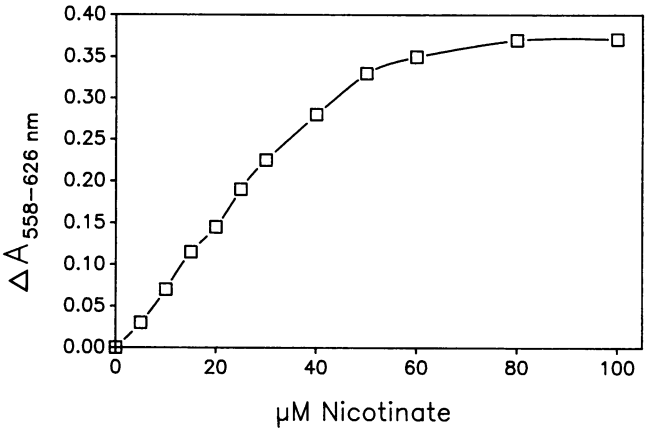

Figure 2. Standard curve for binding of nicotinate to 50 μM ferric leghemoglobin a in 50 mM succinate (pH 5.0). ΔA is the difference in absorbance at 558 and 626 nm.

the amount was quite low. Thus, the procedure in which nodules were homogenized quickly in hot ethanol was quantitative for extraction of nicotinate and nicotinamide from soybean nodules. Inclusion of ^{14}C-nicotinate or ^{14}C-nicotinamide during extraction of nodules permitted routine estimation of recoveries which varied between 55 and 73%. Values reported for nicotinate and nicotinamide were corrected for losses that occurred during the extensive extraction procedure and for additions of radioactive standards.

Levels of nicotinate, nicotinamide, and Lb in soybean nodules, and the relative proportions of nicotinate to Lb, were measured as a function of plant age (Table I). Nodules appeared on the roots of soybean cultivar Lincoln approximately 10 d after planting. Flowers were evident on plants between 34 and 46 d, and pods were evident by 40 d after planting. Plants were still green and nodules intact at 73 d after planting. Nodules were harvested at intervals of 6 to 7 d for 39 d starting at 34 d after planting and assayed for nicotinate, nicotinamide, and Lb. Levels of nicotinate ranged from 10.4 to 21 nmol/g nodule fresh weight, whereas nicotinamide levels varied from 19.2 to 37.8 nmol/g nodules fresh weight. Maximum levels of both were observed at 46 d after planting, which coincided with the termination of flowering. On a nodule fresh weight basis, Lb increased from 170 nmol/g at 34 d after planting to a maximum of 280 nmol/g at d 66.

Nicotinamide, nicotinate, and Lb were also measured in nodules from the soybean cultivar Beeson inoculated with a commercial inoculum and grown under field conditions. Nodules from plants (112 d after planting) that were senescing,

Table I. *Nicotinate and Nicotinamide Levels in Soybean Root Nodules*

The unbracketed values for nicotinate, nicotinamide, and nicotinate plus nicotinamide, expressed as nmol/g nodule fresh weight, represent the means of duplicates. All values are corrected for recoveries, which ranged from 55 to 73%. The values for leghemoglobin, also expressed as nmol/g nodules fresh weight were made by the pyridine hemochrome procedure (3). The bracketed values represent the relative content of nicotinate to Lb at each plant age.

Days After Planting	Nicotinate	Nicotinamide	Nicotinate plus Nicotinamide	Leghemoglobin
34	19.3 (0.114)	27.8	47.1	170
41	14.0 (0.074)	32.8	46.8	190
46	21.0 (0.085)	37.8	58.8	246
52	10.4 (0.042)	30.7	41.1	250
59	15.6 (0.060)	22.4	38.0	260
66	15.6 (0.056)	19.2	34.8	280
73	10.8 (0.051)	30.1	40.9	210
LSD (0.05)	8.3	17.2		

as indicated by fully developed pods, yellowing leaves, and low nitrogenase activity (0.10 ± 0.04 nmol C_2H_4 formed/min/g nodule fresh weight) possessed 29.1 ± 6.1, 24.0 ± 8.6, and 140 nmol/g fresh weight of nicotinate, nicotinamide, and Lb, respectively.

DISCUSSION

Our results show quite clearly that, in physiologically active soybean nodules, the content of free nicotinate is too low to have any significant effect on the ability of oxygen to bind to Lb. From Table I it is seen that the concentration of nicotinate varies from 21 nmol/g fresh weight in the nodules of 46-d-old glasshouse-grown plants at the peak of their nitrogen-fixing activity, to a minimum of 10.8 nmol/g fresh weight in the nodules of 73-d-old plants approaching senescence. For the nodules from 34-d-old plants, the proportion of total nicotinate to total Lb was 0.114, the highest recorded in this experiment (Table I). Even if all of this nicotinate were bound to ferrous Lb, only 11.4% of total Lb would be unavailable for binding to oxygen. In fact, the proportion of ferrous Lb expected to be formed in the nodule would be much less than this.

If one assumes an average nicotinate content in the nodule of 15 nmol/g fresh weight (see Table I), an average Lb content of 229 nmol/g fresh weight (see Table I), and a nodule cytosol of pH of 5.3, then, knowing that K_{diss} = 33 μM for the binding of nicotinate to ferrous Lb at this pH (see Table II of ref. 6), iterative application of the Hill equation [$Y/(1 - Y) = x/K_{diss}$, where Y is the fractional saturation of Lb with nicotinate and x is nicotinate concentration] allows one to calculate that Y = 0.057; *i.e.* 5.7% of the ferrous Lb would be nicotinate bound. If one assumes instead a nodule pH of 6.8 (the only other pH at which nicotinate affinity for ferrous Lb has been measured), then K_{diss} = 1800 μM (6) and Y can be calculated as 0.007; only 0.7% of the nodule ferrous Lb would be nicotinate bound. As the cytosol of presenescent soybean nodules is close to pH 6.5 (13), we estimate that the proportion of ferrous Lb bound to nicotinate in such nodules would be less than 1%. A similar conclusion had been previously reached by the direct spectrophotometric examination of intact clover nodules still attached to the host plant (14). The Lb of such nodules in air or nitrogen is present almost entirely as deoxyferrous Lb. Only when the root systems of such nodulated plants were immersed in 10 mM nicotinate could significant formation of ferrous Lb nicotinate be demonstrated.

In 112-d-old, senescent, field grown soybean (see "Results"), we did observe a significant increase in nodule nicotinate to 29 nmol/g fresh weight as the Lb content fell to 140 nmol/g nodule fresh weight. In such nodules, much of the Lb may be present as ferric Lb, which has a higher affinity for nicotinate and so might be expected to be present as ferric Lb nicotinate. Unfortunately, these older, intact soybean nodules are not amenable to direct spectrophotometry, and, therefore, they cannot be examined directly for the presence of Lb nicotinate *in vivo*.

The question remains as to the origin of the higher concentrations of nicotinate found in aqueous extracts of legume nodules. For such extracts from pea nodules, nicotinate has been reported at a concentration of 1300 nmol/g dry weight (9). If we assume that 0.22 g dry weight is equivalent to 1 g nodule fresh weight as found by F. J. Bergersen (personal communication) for soybean nodules, then this reported value is equivalent to about 286 nmol of nicotinate/g nodule fresh weight. Similarly, for aqueous extracts of bean (*Phaseolus vulgaris*) nodules (18), the content of nicotinate can be recalculated as 271 nmol/g nodule fresh weight. For aqueous extracts of soybean nodules, a direct analysis of nicotinate has not been recorded, although one of us (1, 5, 6) has frequently observed that a large part of the total Lb, typically 300 nmol/g nodule wet weight, is present as ferric Lb nicotinate. We suppose that the lengthy aqueous extraction and centrifugation of such nodule preparations at 0 to 10°C had allowed endogenous nicotinamide deamidase to hydrolyze all nicotinamide to nicotinate. However, inspection of Table I shows that, even in nodules from 46-d-old soybean plants extracted with hot ethanol, the total content of nicotinate plus nicotinamide is only 58.8 nmol/g fresh weight. We conclude, therefore, that the much higher content of nicotinate in aqueous extracts of pea, bean, and soybean nodules may be due to the hydrolysis of pyridine nucleotide coenzymes to nicotinic acid via nicotinamide.

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LITERATURE CITED

1. **Appleby CA** (1969) The separation and properties of low-spin (haemochrome) and native, high-spin forms of leghaemoglobin from soybean root nodule extracts. Biochim Biophys Acta **189:** 267–279
2. **Appleby CA** (1984) Leghemoglobin and *Rhizobium* respiration. Annu Rev Plant Physiol **35:** 443–478
3. **Appleby CA, Bergersen FJ** (1980) Preparation and experimental use of leghaemoglobin. *In* F Bergersen, ed, Methods for Evaluating Biological Nitrogen Fixation. John Wiley & Sons, Chichester, pp 315–335
4. **Appleby CA, Nicola NA, Hurrell JGR, Leach SJ** (1975) Characterization and improved separation of soybean leghemoglobins. Biochemistry **14:** 4444–4450
5. **Appleby CA, Wittenberg BA, Wittenberg JB** (1973) Leghemoglobin II. Changes in conformation and chemical reactivity limited to reaction with a dissociable low molecular weight ligand, X. J Biol Chem **248:** 3183–3187
6. **Appleby CA, Wittenberg BA, Wittenberg JB** (1973) Nicotinic acid as a ligand affecting leghemoglobin structure and oxygen reactivity. Proc Natl Acad Sci USA **70:** 564–568
7. **Arutyunyan EG, Kuranova IP, Tovbis AB, Grebenko AI, Voronova AA, Nekrasov YuV, Vainstein BK** (1980) X-ray structural investigation of leghemoglobin VI. Determination of a structure of complex of ferrileghemoglobin with nicotinic acid at a resolution of 2.8 Å. Sov Phys Crystrallogr **25:** 302–306 (in English)
8. **Atanasov BP, Dimitrova EA, Kudryavtseva NN, Zhiznevskaya GYa, Appleby CA** (1989) The proton dependence of nicotinate binding to soybean, kidney bean and lupin ferric leghemoglobins. Biochim Biophys Acta **998:** 80–84
9. **Begishvili TsK** (1972) Formation of some B-group vitamins in strain-inoculated nodules and roots of leguminous plants. Soobshch Akad Nauk Gruz SSR **66:** 461–463
10. **Fuchsman WH, Appleby CA** (1979) Separation and determination of relative concentrations of the homogeneous components of soybean leghemoglobin by isoelectric focusing. Biochim Biophys Acta **579:** 314–324
11. **Fuller L, Dietrich LS** (1971) Comments of the determination of nicotinamide. Anal Biochem **39:** 538–539
12. **Klucas RV** (1974) Studies on soybean nodule senescence. Plant Physiol **54:** 612–616
13. **Klucas RV, Arp D** (1977) Physiological and biochemical studies on senescing tap root nodules of soybeans. Can J Microbiol **23:** 1426–1432
14. **Klucas RV, Lee KK, Saari L, Erickson BK** (1985) Factors affecting leghemoglobin in legume nodules. *In* PW Ludden, JE Burris, eds, Nitrogen Fixation and CO_2 Metabolism Elsevier, New York, pp 13–20
15. **Layne E** (1957) Spectrophotometric and turbidometric methods for measuring proteins. Methods Enzymol **3:** 447–454
16. **Ollis DL, Appleby CA, Colman PM, Cutten AE, Guss JM, Venkatappa MP, Freeman HC** (1983) Crystal structure of soybean ferric leghaemoglobin a nicotinate at a resolution of 3.3 Å. Aust J Chem **36:** 451–468
17. **Peive YaV, Atanasov BP, Zhiznevskaya GYa, Krasnobaeva NN** (1972) Absorption spectra of leghemoglobin derivatives from lupin (*Lupinus luteus* L.) nodules. Doklady Biochem **202:** 48–51
18. **Shemakhanova NM, Bunko IP** (1968) The content of vitamins of the group B and glutathione in the nodules and roots of bean plants. Selkokohz Biol **3:** 859–863
19. **Stumpf DK, Burris RH** (1979) A micromethod of the purification and quantification of organic acids of the tricarboxylic acid cycle in plant tissues. Anal Biochem **95:** 311–315