Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 3811–3815, June 1986
Botany

Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules

(nitrogen fixation/oxygen toxicity/antioxidant)

DAVID A. DALTON*, STERLING A. RUSSELL*, F. J. HANUS*, GARY A. PASCOE†, AND HAROLD J. EVANS*

*Laboratory for Nitrogen Fixation Research and †Environmental Health Sciences Center, Oregon State University, Corvallis, OR 97331

Contributed by Harold J. Evans, January 27, 1986

ABSTRACT **The critical problem of oxygen toxicity for nitrogen-fixing organisms may be related to damage caused by oxygen radicals and peroxides. An enzymatic mechanism is described for removal of peroxides in root nodules of soybean (*Glycine max*). The system utilizes ascorbate as an antioxidant and glutathione as a reductant to regenerate ascorbate. The enzymes involved are ascorbate peroxidase (ascorbate:hydrogen-peroxide oxidoreductase, EC 1.11.1.7), dehydroascorbate reductase (glutathione:dehydroascorbate oxidoreductase, EC 1.8.5.1), and glutathione reductase (NADPH:oxidized-glutathione oxidoreductase, EC 1.6.4.2). The reactions are essentially the same as those involving scavenging of H_2O_2 in chloroplasts. Glutathione peroxidase (glutathione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9) was not detected. During the course of early nodule development, ascorbate peroxidase and dehydroascorbate reductase activities and total glutathione contents of nodule extracts increased strikingly and were positively correlated with acetylene reduction rates and nodule hemoglobin contents. The evidence indicates an important role of glutathione, ascorbate, ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase as components of a peroxide-scavenging mechanism in soybean root nodules.**

All aerobic organisms are subject to oxygen toxicity that results from the formation of reactive intermediates such as hydrogen peroxide (H_2O_2), superoxide radical ($O_2^{\cdot-}$), and hydroxyl radical ($OH^{\cdot}$). One of the main defensive mechanisms against these reactive intermediates is superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1), an enzyme that is ubiquitous in aerobic organisms including both eukaryotes and prokaryotes (1, 2). Superoxide dismutase catalyzes the conversion of $O_2^{\cdot-}$ to H_2O_2. The H_2O_2 may then be decomposed in the presence of catalase, which is widespread in plants and animals (1). In addition, many higher animals contain the selenoenzyme glutathione peroxidase (glutathione:hydrogen-peroxide oxidoreductase, EC 1.11.1.9) which catalyzes the peroxidation of reduced glutathione (GSH), forming the oxidized disulfide form of glutathione (GSSG) as a product (3). The supply of GSH is regenerated in an NADPH-dependent reaction catalyzed by glutathione reductase (NADPH:oxidized-glutathione oxidoreductase, EC 1.6.4.2.; Fig. 1). In mammals, this system is critical in the protection of hemoglobin and some other proteins from peroxide damage.

Another type of peroxidase system (Fig. 1) has been identified in chloroplasts (4). The initial peroxidase reaction of this system utilizes ascorbate as an antioxidant and produces dehydroascorbate. Ascorbate is then regenerated in a GSH-dependent reaction catalyzed by dehydroascorbate reductase. Finally, the GSSG is reduced back to GSH in a reaction, essentially the same as that which occurs in the mammalian system described above, that involves glutathione reductase and NADPH. The enzymes involved are ascorbate peroxidase (ascorbate:hydrogen-peroxide oxidoreductase, EC 1.11.1.7), dehydroascorbate reductase (glutathione:dehydroascorbate oxidoreductase, EC 1.8.5.1, also called glutathione dehydrogenase), and glutathione reductase. The initial product of the ascorbate peroxidation reaction is actually monodehydroascorbate, which spontaneously forms dehydroascorbate. Also present in spinach chloroplasts is an enzyme that catalyzes the direct conversion of monodehydroascorbate back to ascorbate (5). At least some of these enzymes are also present in cyanobacteria (6, 7).

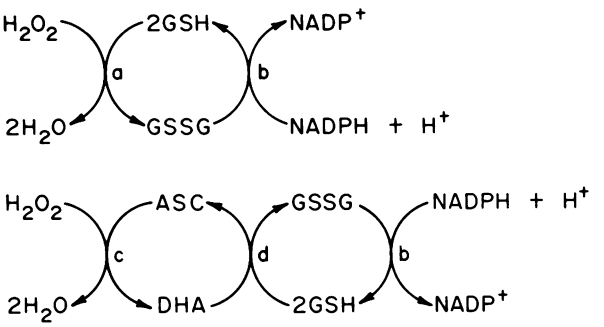

FIG. 1. Oxidation-reduction pathways involved in scavenging of H_2O_2. The upper scheme occurs in mammalian cells. The lower scheme occurs in chloroplasts, cyanobacteria, and soybean nodules. Enzymes: a, glutathione peroxidase; b, glutathione reductase; c, ascorbate peroxidase; d, dehydroascorbate reductase. ASC, ascorbate; DHA, dehydroascorbate.

Nitrogen-fixing organisms are especially vulnerable to O_2 toxicity because of the extreme sensitivity of the nitrogenase proteins to O_2 damage. Consequently, nitrogen-fixing organisms have evolved various strategies to protect nitrogenase from O_2 damage. These adaptations include respiratory protection, physical protection (slime production, thick cell walls, cortical diffusion barriers, etc.), an O_2-buffering system such as leghemoglobin, and temporal or spatial separation of photosynthesis and nitrogen fixation [as in many cyanobacteria (8)].

Part of the O_2 sensitivity of these organisms may be explained by the production of reactive oxygen intermediates by reactions associated with nitrogen fixation. For example, reactions involving ferredoxin, hemoglobin, and hydrogenase can generate $O_2^{\cdot-}$ (2, 9). H_2O_2 is produced by the reaction catalyzed by superoxide dismutase, which is found in both free-living *Rhizobium* and bacteroids (10, 11). Some legume nodules also produce H_2O_2 via the urate oxidase reaction, which converts urate to allantoin (12). This is a key step in the production of ureides, which are the major form in which nitrogen is transported out of nodules in legumes such as

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione.

soybean, snap bean, and cowpea. Although catalase may be present in nodules (13), this enzyme alone may not be sufficient to eliminate all the H_2O_2, because catalase has a high K_m for H_2O_2 and tends to be restricted largely to peroxisomes (3). Further, catalase also seems poorly suited for disposal of H_2O_2 in nitrogen-fixing organisms because the catalase reaction produces free O_2.

In this report, we present evidence for the presence of a H_2O_2-scavenging system in soybean nodules which is similar to that identified in chloroplasts.

MATERIALS AND METHODS

Growth of Plants. Soybean seeds [*Glycine max* (L.) Merr. cv. Williams] were planted in 20-cm plastic pots containing perlite and were inoculated with *Bradyrhizobium japonicum* USDA 122 DES (14). Plants were grown in a greenhouse with a 16-hr photoperiod and a light intensity of 190 microeinsteins per m^2 per sec. The day temperature was 27°C and the night temperature was 21°C. Plants were provided daily with a nitrogen-free nutrient solution consisting of 1.58 mM K_2SO_4, 2 mM $MgSO_4$, 0.57 mM KH_2PO_4, 0.86 mM K_2HPO_4, 12.0 mM $CaSO_4$, 1.0 mM $CaCl_2$, 3.7 μM H_3BO_3, 0.72 μM $MnSO_4$, 0.76 μM $ZnSO_4$, 0.31 μM $CuSO_4$, 0.10 μM Na_2MoO_4, 0.17 μM $CoCl_2$, 0.17 μM $NiCl_2$, and 40.4 μM sodium ferric ethylenediaminedi(*o*-hydroxyphenyl)acetate. Excess nutrient solution drained through holes in the bottom of each pot. Pots were flushed with water twice each week to prevent accumulation of minerals. After germination, the plants were thinned to five per pot. Samples of nodules were collected at each sampling date from each of three replicate groups of plants. Each replicate group consisted of three pots of plants. Nodules were collected only from the upper 5 cm of the main tap root.

Extraction Procedures. Nodules were harvested at various ages as indicated in Figs. 2–5 and ground at 4°C by mortar and pestle with polyvinylpolypyrrolidone (25% by weight) and 4 volumes of buffer consisting of 50 mM KH_2PO_4/K_2HPO_4 and 0.1 mM EDTA at pH 7.0. The macerate was filtered through 100-mesh bolting cloth and then centrifuged at 8000 × *g* for 20 min to remove cell debris and bacteroids. The supernatant (henceforth called extract) was analyzed for leghemoglobin concentration by measuring the absorbance values of the oxidized-versus-reduced difference spectrum of pyridine hemochrome (15) and for total protein by Coomassie blue G-250 binding (16). Bovine serum albumin was used as the protein standard. Standardization with myoglobin gave similar results. Extracts for ascorbate determination were made separately by grinding nodules in 3 volumes of 5% metaphosphoric acid (HPO_3), centrifuging at 15,000 × *g* for 15 min, and filtering the supernatant through a 0.22-μm Millipore filter. Extracts for ascorbate determination were stored at −80°C for later analysis.

Enzyme Assays. Acetylene reduction was determined on excised nodules as described (17). Glutathione peroxidase activity was assayed by coupling to NADPH oxidation in the presence of excess glutathione reductase (18). Purified bovine glutathione peroxidase was purchased from Sigma for use as a positive control. Ascorbate peroxidase was measured by a modified spectrophotometric procedure based on the rate of decrease in absorbance of ascorbate at 265 nm during ascorbate peroxidation (19). The assay was performed in a 1.5-ml quartz cuvette containing 0.25 mM ascorbate, 50 mM KH_2PO_4/K_2HPO_4 at pH 7.0, 1.0 mM H_2O_2, and 37.5 μl of extract. Corrections were made for the low rates of ascorbate disappearance due to nonenzymatic and H_2O_2-independent oxidation.

Preliminary assays of ascorbate peroxidase were complicated by initial low rates of ascorbate disappearance that gradually increased to a much higher constant rate after 4–5 min. This problem was found to be due to the inclusion of 0.1 mM EDTA in the reaction buffers. When buffers without EDTA were used, the lag period was reduced to about 20 sec. Rates were then determined during the linear phase of the reaction from 20 to 60 sec. The persistence of this 20-sec lag period was probably due to the small amount of EDTA (3.8 nmol in 1.5-ml final volume) introduced into the reaction mixture along with the crude extract.

Dehydroascorbate reductase was measured by the rate of increase in absorbance at 265 nm due to ascorbate formation (19). The assay mixture contained 50 mM KH_2PO_4/K_2HPO_4 at pH 7.0, 0.2 mM dehydroascorbate (freshly prepared in N_2-saturated buffer), 0.1 mM EDTA, 2.5 mM GSH, and 37.5 μl of extract in a final volume of 1.5 ml. Correction was made for the nonenzymatic reduction rate. The GSH-independent rate was negligible. The absorbance of GSSG was corrected for by multiplying by a factor of 0.98 (19). Glutathione reductase was assayed by measuring the decrease in absorbance at 340 nm due to NADPH oxidization (20). Reaction mixtures consisted of 0.25 mM GSSG, 0.125 mM NADPH, 50 mM Tricine (pH 7.8), 0.5 mM EDTA, and 50 μl of extract in a final volume of 2.0 ml. Rates were corrected for GSSG-independent NADPH oxidation. The nonenzymatic rate was negligible.

All reported values for enzyme activities are the means of determinations on three separate extracts for each sampling date. Each extract was prepared from the nodules of three pots of plants.

Other Assays. GSH and GSSG contents of cytosol extracts were determined by HPLC analysis of the 2,4-dinitrophenyl derivatives of 3-carboxymethyl-GSH and -GSSG (21). Ascorbate content of nodule extracts was determined by HPLC analysis using a Beckman Gradient LC model 334 with guard and analytical columns of 5-μm RP-18 (Brownlee Laboratories, Santa Clara, CA; ref. 22). Separate extractions were made for glutathione and ascorbate at each sampling date from nodule samples from each of three groups of plants. Triplicate analyses were performed on each extract.

RESULTS

Initial Experiments. Initial assays were designed to check for glutathione peroxidase activity by coupling the peroxidase reaction to NADPH oxidation in the presence of excess glutathione reductase. This essentially duplicates the *in vivo* series of reactions that occurs in mammalian cells (upper scheme of Fig. 1) while providing a convenient means of detecting activity. The preliminary results with crude cytosol extracts suggested low rates of glutathione peroxidase activity. This activity averaged 26.5 nmol of NADPH per mg of protein per min, which was only marginally above the background (peroxide-independent) rate. This rate was only slightly affected by 0.05 mM mercaptosuccinate, a potent inhibitor of mammalian glutathione peroxidase (23). Assays with purified bovine glutathione peroxidase were completely inhibited by this concentration of mercaptosuccinate. Subsequent partial purification of this crude nodule extract by $(NH_4)_2SO_4$ precipitation reduced the apparent glutathione peroxidase activity to a level indistinguishable from the background level. The addition of ascorbate to the partially purified extract resulted in slightly elevated rates of NADPH oxidation. Substantially higher rates of NADPH oxidation (150–210 nmol per mg of protein per min) were obtained when dehydroascorbate was added and peroxide was omitted. These observations are consistent with the conclusion that the nodule extracts contained a H_2O_2-scavenging system in which ascorbate peroxidase and dehydroascorbate reductase were essential (lower scheme of Fig. 1). Glutathione peroxidase is not a component of this system. The initial observations which implied a role of glutathione peroxidase

probably were the result of ascorbate peroxidase and endogenous ascorbate in the crude extract. After proteins were separated from ascorbate by $(NH_4)_2SO_4$ precipitation, no evidence for the presence of glutathione peroxidase activity was observed.

Enzyme Activities During Nodule Development. The activities of the three enzymes of the peroxidase system (lower scheme of Fig. 1) were monitored throughout nodule development. Ascorbate peroxidase and dehydroascorbate reductase activities were low in extracts of 11- and 13-day-old nodules, averaging about 43 nmol of ascorbate oxidized per mg of protein per min, for ascorbate peroxidase, and 16 nmol of ascorbate formed per mg of protein per min, for dehydroascorbate reductase. These activities increased sharply in extracts of 15-day-old nodules (Fig. 2). Activities remained at these higher levels through the final harvest at 61 days. The average activity for all nodules harvested between 15 and 61 days after planting was 283.7 nmol of ascorbate consumed per mg of protein per min, for ascorbate peroxidase, and 54.2 nmol of ascorbate produced per mg of protein per min, for dehydroascorbate reductase. In contrast with ascorbate peroxidase and dehydroascorbate reductase activities, glutathione reductase activity did not increase substantially as nodules matured (Fig. 3). The average activity for glutathione reductase in nodules of all ages (11–61 days) was 15.6 nmol of NADPH oxidized per mg of protein per min.

The increases in activities of ascorbate peroxidase and dehydroascorbate reductase coincided roughly with increases in leghemoglobin concentration and acetylene reduction (nitrogenase) activity (Fig. 4). A low concentration of leghemoglobin was first detected at 15 days after planting. The concentration increased to a high of 22.0 nmol per mg of protein (285 nmol per g of fresh weight) at 27 days and then gradually declined. Acetylene reduction activity was initially detected at low levels in 13-day-old nodules. Acetylene reduction rates reached a maximum at 27 days (31.0 μmol per g per hr) and then slowly decreased.

Location of Enzymes. Ascorbate peroxidase and dehydroascorbate reductase activities were found only in the cytosol fraction of nodule extracts. These enzymes were not detected in pellets (containing bacteroids, cell debris, and probably some intact mitochondria) produced by centrifugation of crude extracts. These enzymes also were not detected in extracts obtained after passage of resuspended pellets twice through a French pressure cell. The total protein concentration of these preparations was approximately equal to that of cytosol extracts in which high activities were observed. We conclude therefore that ascorbate peroxidase and dehydroascorbate reductase are present only in the nodule cytosol and not present in bacteroids. In contrast, glutathione reductase was detected in the pellet fraction from crude nodule extracts. After passage twice through the French pressure cell, the specific activities of glutathione reductase in these extracts were comparable in magnitude to those measured in cytosol extracts.

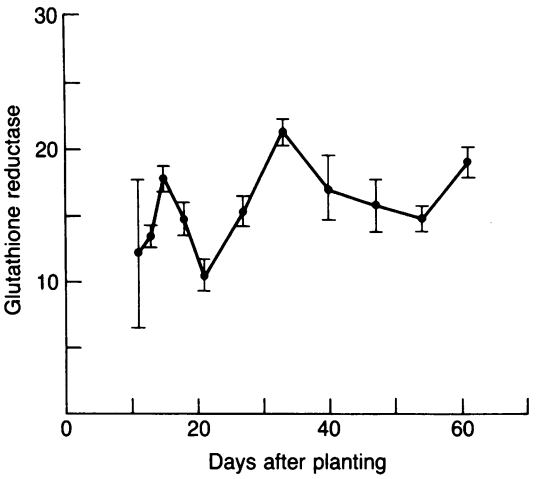

FIG. 3. Glutathione reductase activity in cytosol extracts of soybean nodules during plant development. Rates (nmol of NADPH oxidized per mg of protein per min) were determined spectrophotometrically as described in *Materials and Methods*. Each point represents the mean (±SEM) of three replicates.

GSH, GSSG, and Ascorbate Concentrations During Nodule Development. Glutathione concentrations increased sharply during nodule development (Fig. 5). The concentration of GSH increased from 2.2 nmol per g of fresh weight (0.15 nmol per mg of protein) in 11-day-old nodules to a high of 140.8 nmol per g of fresh weight (9.6 nmol per mg of protein) in 33-day-old nodules. GSSG concentrations increased from 3.2

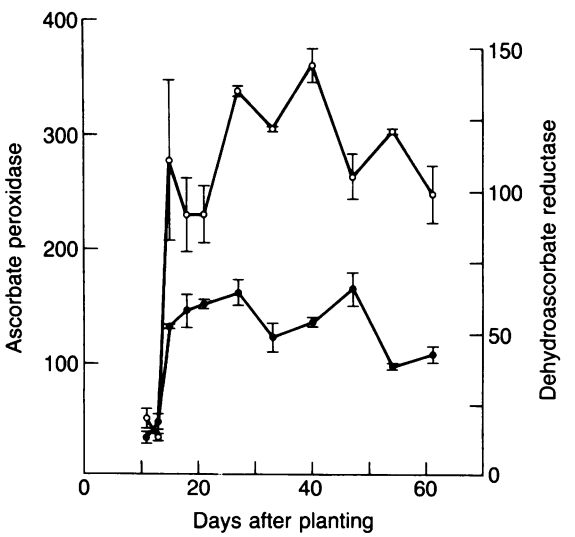

FIG. 2. Ascorbate peroxidase (○) and dehydroascorbate reductase (●) activities in cytosol extracts of soybean nodules during plant development. Rates [nmol of ascorbate consumed (○) or produced (●) per mg of protein per min] were determined by spectrophotometric measurement of ascorbate (19). Each point represents the mean (±SEM) of three replicates.

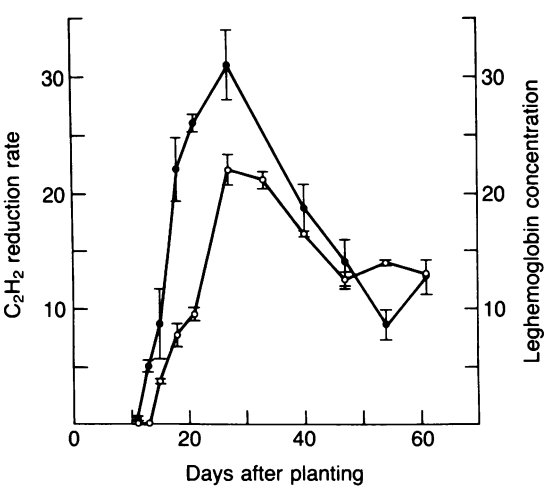

FIG. 4. Acetylene reduction rates (●) and leghemoglobin concentrations (○) of soybean nodules. Acetylene reduction (μmol per g of fresh weight per hr) was determined with excised nodules in sealed vials containing 10% (vol/vol) acetylene. Leghemoglobin concentration (nmol per mg of protein) of nodule extracts was determined by measuring the difference spectrum of pyridine hemochrome. Each point represents the mean (±SEM) of three replicates.

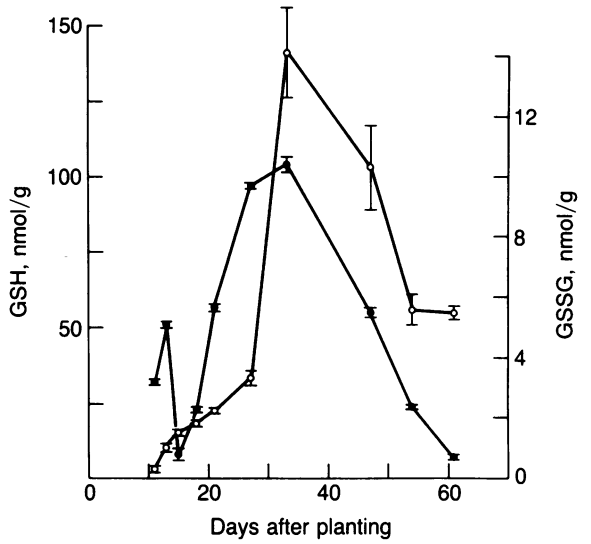

FIG. 5. Concentrations (nmol per g of fresh weight) of reduced (GSH, ○) and oxidized (GSSG, ●) glutathione in soybean nodules. Determinations were by HPLC analysis of derivatized thiols (see *Materials and Methods*). Each point represents the mean (±SEM) of three replicates.

nmol per g of fresh weight (0.3 nmol per mg of protein) in 11-day-old nodules to a high of 10.4 nmol per g of fresh weight (0.7 nmol per mg of protein) in 33-day-old nodules. Both GSH and GSSG concentrations declined after reaching maximal values at 33 days. In contrast, ascorbate concentrations remained roughly constant throughout nodule development. The average concentration from nodules of all ages was 1.69 μmol (range 0.94–1.86 μmol) per g of fresh weight.

DISCUSSION

The data in this investigation have provided no evidence for the occurrence of an active glutathione peroxidase in soybean nodules. However, GSH oxidation proceeds rapidly via a system involving peroxidation of ascorbate, in the presence of ascorbate peroxidase, followed by GSH-dependent reduction of dehydroascorbate to ascorbate.

Correlation between nitrogenase activity and activities of other enzymes has been used as evidence of association between nitrogen fixation and other processes (24). The concurrent increases in concentrations of leghemoglobin and glutathione and activities of nitrogenase, ascorbate peroxidase, and dehydroascorbate reductase during early nodule development indicate that increased capacity for H_2O_2 scavenging is associated with the development of the process of nitrogen fixation. This suggests that these enzymes may be plant proteins that are produced in response to infection (i.e., "nodulins"), but further investigations are required to verify this. The correlation coefficients for acetylene reduction rates versus glutathione concentrations, ascorbate peroxidase activities, and dehydroascorbate reductase activities indicate a strong positive correlation (r = 0.94 to 0.79, Table 1). In addition, significant positive correlations (r = 0.98 to 0.79) between leghemoglobin content of nodules and the same parameters of H_2O_2 scavenging are also apparent. Glutathione reductase activity was not correlated with nitrogen fixation parameters.

The reversible inhibition by EDTA of ascorbate peroxidase from spinach chloroplasts (19) and nodule extracts suggests the involvement of a metal. A heme prosthetic group, which is known to be a constituent of many peroxidases, may be involved. Further research obviously is necessary to elucidate possible metal requirements and other characteristics of ascorbate peroxidase and associated enzymes in legume nodules.

The occurrence of ascorbate peroxidase and dehydroascorbate reductase in nodule cytosol fractions and the absence of these two enzymes in bacteroid extracts strongly suggest that these enzymes are of plant origin. The glutathione reductase in the pellet fraction of nodule extracts probably originated from bacteroids, but some may have been derived from intact mitochondria which were not ruptured in the extraction procedure. Glutathione reductase is known to occur in a wide variety of plants, animals, and microorganisms (25). In plants, the enzyme has been reported to be present in chloroplasts, mitochondria, and cytoplasm, but some of these reports are contradictory (19, 26).

The concentration of glutathione found in nodules is comparable to levels reported for other plant sources. Glutathione concentrations average about 100 μM for most plant tissues, but chloroplast concentrations are substantially higher, ranging from 1.0 to 3.5 mM (27). If it is assumed that glutathione is evenly distributed throughout nodules, the apparent concentration of GSH plus GSSG ranged from 6.1 μM in 11-day-old nodules to 191 μM in 33-day-old nodules. Although glutathione is generally considered to be ubiquitous in living cells, other low molecular weight thiols are present in some plants. A glutathione homolog ("homoglutathione") has been detected in leaves of some legumes, including soybean (28, 29). This compound differs from glutathione by the substitution of alanine for the terminal glycine. In the present study, GSH and GSSG were identified by coelution with commercial standards. Standards for homoglutathione were not available. Chromatograms also contained other peaks, from unidentified thiol compounds. Therefore, the possibility must be considered that nodules may contain glutathione homologs in addition to or instead of glutathione *per se* and that these compounds may be involved in the peroxidase system.

The application of exogenous ascorbate to roots of nitrogen-fixing plants has been reported to increase the weight and number of nodules, leghemoglobin content, growth, yield, and nitrogen content of tissues (30). Also, it has been observed that the endogenous ascorbate content of functional

Table 1. Correlation coefficients for parameters of nitrogen fixation (acetylene reduction rate and leghemoglobin concentration) and parameters associated with H_2O_2 scavenging during early developmental stages (11–27 days) of soybean nodules

	Correlation coefficient	
	Acetylene reduction rates	Leghemoglobin concentrations
Acetylene reduction rates	—	0.90 (0.02)
Leghemoglobin concentrations	0.90 (0.02)	—
Ascorbate peroxidase activities	0.79 (0.06)	0.83 (0.04)
Dehydroascorbate reductase activities	0.79 (0.06)	0.79 (0.06)
Glutathione reductase activities	−0.01 (NS)	0.21 (NS)
Glutathione (GSH + GSSG) concentration	0.94 (0.005)	0.98 (0.001)

Values in parentheses are significance levels; NS, not significant.

nodules is much higher than that of green, senescent nodules (30). Although the reasons for the beneficial effects of ascorbate on symbiotic performance have been unclear, the present work supports a role of ascorbate in a system for the prevention of peroxide damage. The data reported here indicate an approximate ascorbate concentration in nodules of 2.1 mM (assuming uniform distribution throughout nodules). This relatively high concentration compares favorably with the high concentrations of ascorbate (2.5–3.5 mM; ref. 4) associated with peroxide scavenging in chloroplasts.

This work was supported in part by National Science Foundation Grants PCM 81-18148 and DMB 85-13846 and by the Oregon Agricultural Experiment Station, from which this is paper no. 7759. Ascorbate analyses were performed by Virgilio Esquillo and Randall Boone.

1. Fridovich, I. (1976) in *Free Radicals in Biology*, ed. Pryor, W. A. (Academic, New York), pp. 239–277.
2. Fridovich, I. (1979) in *Advances in Inorganic Biochemistry*, eds. Eichhorn, G. L. & Marzilli, L. G. (Elsevier/North Holland, New York), Vol. 1, pp. 67–90.
3. Halliwell, B. (1974) *New Phytol.* **73,** 1075–1086.
4. Nakano, Y. & Asada, K. (1981) *Plant Cell Physiol.* **22,** 867–880.
5. Hossain, M. A., Nakano, Y. & Asada, K. (1984) *Plant Cell Physiol.* **25,** 385–395.
6. Tozum, S. R. D. & Gallon, J. R. (1979) *J. Gen. Microbiol.* **111,** 313–326.
7. Tel-Or, E., Huflejt, M. & Packer, L. (1985) *Biochem. Biophys. Res. Commun.* **132,** 533–539.
8. Postgate, J. R. (1982) *The Fundamentals of Nitrogen Fixation* (Cambridge University Press, Cambridge, England), pp. 60–82.
9. Schneider, K. & Schlegel, H. G. (1981) *Biochem. J.* **193,** 99–107.
10. Dimitrijevic, L., Puppo, A. & Rigaud, J. (1984) *Arch. Microbiol.* **139,** 174–178.
11. Stowers, M. D. & Elkan, G. H. (1981) *Can. J. Microbiol.* **27,** 1202–1208.
12. Reynolds, P. H. S., Boland, M. J., Blevins, D. G., Randall, D. D. & Schubert, K. R. (1982) *Trends Biochem. Sci.* **7,** 366–368.
13. Hanks, J. F., Schubert, K. & Tolbert, N. E. (1983) *Plant Physiol.* **71,** 869–873.
14. Ruiz-Argueso, T., Emerich, D. W. & Evans, H. J. (1979) *Arch. Microbiol.* **121,** 199–206.
15. Bergersen, F. J. (1980) *Methods for Evaluating Biological Nitrogen Fixation* (Wiley, New York), pp. 322–325.
16. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
17. Drevon, J. J., Frazier, L., Russell, S. A. & Evans, H. J. (1982) *Plant Physiol.* **70,** 1341–1346.
18. Tappel, A. L. (1978) *Methods Enzymol.* **52C,** 506–513.
19. Asada, K. (1984) *Methods Enzymol.* **105,** 422–429.
20. Kalt-Torres, W., Burke, J. J. & Anderson, J. M. (1984) *Physiol. Plant.* **61,** 271–278.
21. Reed, D. J., Babson, J. R., Beatty, P. W., Brodie, A. E., Ellis, W. W. & Potter, D. W. (1980) *Anal. Biochem.* **106,** 55–62.
22. Shaw, P. E. & Wilson, C. W. (1982) *J. Agric. Food Chem.* **30,** 394–396.
23. Tappel, A. L. (1984) *Curr. Top. Cell. Regul.* **24,** 87–97.
24. Stripf, R. & Werner, D. (1978) *Z. Naturforsch* **33,** 373–381.
25. Flohe, L. & Gunzler, W. A. (1976) in *Glutathione: Metabolism and Function*, eds. Arias, I. M. & Jakoby, W. B. (Raven, New York), pp. 17–34.
26. Foyer, C. H. & Halliwell, B. (1976) *Planta* **133,** 21–25.
27. Rennenberg, H. (1982) *Phytochemistry* **21,** 2771–2781.
28. Carnegie, P. R. (1963) *Biochem. J.* **89,** 459–471.
29. Price, C. A. (1957) *Nature (London)* **180,** 148–149.
30. Chinoy, J. J. (1984) *The Role of Ascorbic Acid in Growth, Differentiation, and Metabolism of Plants* (Nijhoff/Junk, The Hague, Netherlands).