

³¹P Relaxation Responses Associated with N₂/O₂ Diffusion in Soybean Nodule Cortical Cells and Excised Cortical Tissue

Philip E. Pfeffer*, Dominique B. Rolin, Thomas F. Kumosinski, Janet S. MacFall, and Julian H. Schmidt

U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, Pennsylvania 19118 (P.E.P., D.B.R., T.F.K., J.H.S.); and Department of Radiology, Duke University, Durham, North Carolina 27710 (J.S.M.)

ABSTRACT

N₂-fixing *Bradyrhizobium japonicum* nodules and cortical tissue derived from these nodules were examined *in vivo* by ³¹P nuclear magnetic resonance (NMR) spectroscopy. Perfusion of the viable nodules and excised cortical tissue with O₂ followed by N₂ or Ar caused a loss of orthophosphate (Pi) resonance magnetization associated with the major portion of acidic Pi (δ 0.9 ppm, pH 5.5) residing in the cortical cells. Resumption of O₂ perfusion restored approximately 80% of the intensity of this peak. Detailed examination of the nuclear relaxation processes, spin-lattice relaxation time (T₁), and spin-spin relaxation time (T₂), under perfusion with N₂ or Ar as opposed to O₂, indicated that loss of signal was due to T₁ saturation of the acidic Pi signal under the rapid-pulsed NMR recycling conditions. In excised cortical tissue, Pi T₁ values derived from biexponential relaxation processes under perfusing O₂ were 59% 3.72 ± 0.93 s and 41% 0.2 ± 0.08 s, whereas under N₂ these values were 85% 7.07 ± 1.36 s and 15% 0.39 ± 0.07 s. The T₁ relaxation behavior of whole nodule vacuolar Pi showed the same trend, but the overall values were somewhat shorter. T₂ values for cortical tissue were also biexponential but were essentially the same under O₂ (38% 0.066 ± 0.01 s and 63% 0.41 ± 0.08 s) and N₂ (39% 0.07 ± 0.01 s and 61% 0.37 ± 0.01 s) perfusion. Soybean (*Glycine max*) root tissue as well as Pi solutions exhibited single exponential T₁ decay values that were not altered by changes in the perfusing gas. These data indicate that oxygen induces a change in the physical environment of phosphate in the cortical cell tissue. Although under certain conditions oxygen has been observed to act as a paramagnetic relaxation agent, model T₁ experiments demonstrate that O₂ does not significantly influence Pi relaxation in this manner. Alternatively, we suggest that an increase in solution viscosity brought on by the production of an occlusion glycoprotein (under O₂ perfusion) is responsible for the observed relaxation changes.

The reduction of gaseous N₂ by nitrogen-fixing bacteroids within a plant nodule is a highly energetic process requiring at least six electrons and two ATP molecules for each electron transported (16, 33). Rhizobia, once they are encapsulated as bacteroids in the nodule, require high concentrations of oxygen for oxidative ATP formation. Paradoxically, at the same time an excess of oxygen is detrimental to the stability of the nitrogenase enzyme (33). Therefore, the partial pressure of oxygen must be maintained at a very low level in the interior of the nodule. To meet the requirements of this delicate balance, the nodule must be able to modulate the movement

of O₂ according to the respiratory needs of the bacteroids (33).

Soybean nodules contain three cortical cell layers (44% of the nodule volume) (28), an outer cortex, an endodermis, and an inner cortex. The inner cortex functions as a diffusion barrier to balance inward oxygen flux with bacteroid respiratory oxygen demand (15, 20, 31, 32). Tjepkema and Yocum (29) and later Witty et al. (34) verified that the oxygen concentration decreased across the cortical layer until it reached a minimum at the inner cortex, the presumed major barrier to gas diffusion. Numerous investigators (2, 7, 8, 11, 27, 28) have examined the diffusion of O₂ through this barrier and have proposed that the sites of entry are air spaces filled with water the depth of which can be varied through expansion or contraction of the adjacent inner cortex cells. On the other hand, Witty and coworkers (26, 34) have speculated that cell expansion into the intercellular space is responsible for restricting the passage of O₂ into the inner nodule in response to diminished respiration. VandenBosch et al. (30) and Bradley et al. (3) observed a unique glycoprotein in the infection thread matrix and intercellular space of pea nodules. Further investigation of this material in soybean nodules by James and coworkers (9) demonstrated that its elaboration was greatly enhanced in the presence of oxygen. These authors (9) hypothesized that this "occlusion protein" is responsible for increasing O₂ diffusion resistance in the cortical cell layer by blocking the intercellular air spaces.

At present the nature of the oxygen-regulating mechanism(s) associated with the cortical layer is controversial. In this and the accompanying paper (12), we describe relaxation data obtained from ³¹P NMR spectroscopy and ¹H magnetic resonance imaging studies that suggest that increasing viscosity resulting from the production of cortical cell glycoprotein may be associated with enhanced O₂ diffusion resistance in soybean (*Glycine max*) nodules.

MATERIALS AND METHODS

Plant Material

Soybeans (*Glycine max* [L.] Merr cv Williams) were germinated for 72 h in wet vermiculite in the dark at 28°C in a growth chamber. Germinated seeds were transplanted to plastic pots containing vermiculite and inoculated with *Bradyrhizobium japonicum* USDA 110 or 138. Plants were grown in a growth chamber receiving light from fluorescent and incandescent lamps for a photoperiod of 15 h, day/night

temperatures of 25 and 19°C, and humidity of 80 and 95%, respectively. Plants were irrigated with a nutrient solution containing 1 mM NO₃ and 2 mM Pi (22). However, during the last 12 to 14 d of growth, the concentration of Pi was raised to 10 mM. Nodules were harvested 35 to 55 d after inoculation.

In Vivo NMR Experiments

For spectra of nodules, approximately 2 to 3 g of nodules were detached from the roots and washed with distilled water. Detached nodules were split in half with a razor blade and transferred to a 10-mm NMR tube equipped with a perfusion system similar to that described earlier by Pfeffer et al. (19). The cortical layers of the nodules were carefully excised with a scalpel and washed thoroughly to remove any remnants of leghemoglobin or central matrix tissue. Viability of the cortical cells was verified by their ability to phosphorylate perfusing 50 mM glycerol (appearance of glycerol 3-phosphate resonance at 4.82 ppm).

Excised soybean segments (2.5 cm) were cut from 3-d-old soybean seedlings grown in paper-lined trays at 27°C with 0.1 mM CaCl₂, 5.0 mM Pi, pH 6.8. The perfusion medium (1 L) was generally buffered to pH 7.5 with 10 mM Mops buffer or to pH 6.0 with 10 mM Mes buffer. Unless otherwise indicated, each solution contained 50 mM glucose and 0.1 mM CaSO₄. O₂, N₂, or Ar was bubbled into the reservoir containing 600 to 1000 mL of perfusate. To change the perfusion medium during continuous experiments, a second reservoir was interconnected by a three-way stopcock assembly and the intermediate tubing was primed with the new perfusate. Approximately 100 mL of the returning perfusate was discarded to flush the system.

The 161.7 MHz ³¹P NMR spectra (obtained with a 54-mm, 9.3-T narrow bore magnet JEOL GX-400 NMR spectrometer) were accumulated at 22 ± 1°C over a 16,000 Hz frequency range with 2,000 data points zero filled to 16,000. Unless otherwise indicated, each rapidly acquired spectrum required 20,000 transients with a repetition time of 0.162 s (total accumulation time of 54 min) utilizing approximately a 45° pulse (12 μs) with low-power complete proton decoupling. Each experiment was repeated at least three times. Spectra were obtained consecutively and stored sequentially onto the disk memory. After the completion of the experimental time course, each spectrum was normalized (allowances were made for computer scaling) to the initial spectrum for comparison of relative concentrations of mobile phosphorus compounds. A reference capillary containing 120 mM HMPA¹ was used to give a satisfactory size reference peak for each spectrum. The HMPA exhibited a resonance of 30.73 ppm down field from 85% H₃PO₄. All chemical shifts were referenced relative to 85% H₃PO₄, which was assigned a value of 0 ppm. Estimates of intracellular pH were made from the standard reference curve of Pi suggested by Roberts et al. (21).

¹ Abbreviations: HMPA, hexamethylphosphoramide; T₁, spin-lattice relaxation time; T₂, spin-spin (or transverse) relaxation time; FIRFT, fast inversion recovery Fourier transform; S/N, signal to noise ratio.

Relaxation Measurements

T₁ values were determined on the perfused samples by the FIRFT method (5). The FIRFT technique was thoroughly compared with the standard, slower inversion-recovery method (25) on Pi solutions to ensure that identical relaxation values could be obtained with the former. Typically, 10 to 12 τ values were chosen in the range from 0.1 to 10.0 s. The sets of τ values were randomized to guard against systematic errors due to changes in the viability of the tissue with time. Two hundred scans with 2.5-s repetition times were used for each point in the sequence for excised tissue. Whole nodule spectra required 1000 scans for acceptable S/N. Because of the heterogeneity of the samples, the probe was carefully tuned prior to each measurement. Typically, 4.5 h of perfusion with N₂ or Ar preceded relaxation measurements to ensure the establishment of a hypoxic state.

Transverse T₂ values were obtained by the Carr-Purcell-Meiboom-Gill (14) spin echo modification sequence. Two τ was equal to 2 ms; the repetition time was 12 s; and 64 scans were collected for each of the 10 τ values from 2 to 500 ms. Each relaxation measurement was replicated at least three times with different samples of tissue to ensure good experimental precision. For in vitro Pi relaxation measurements, Pi solutions were saturated with O₂ in sealed NMR tubes. Degassed solutions were prepared by bubbling N₂ continually, then freezing and thawing the solutions three times. Each solution contained 25 mM KH₂PO₄, 100 mM KCl, and 10 mM Mes buffer at pH 5.5. Solutions of varying viscosities (6) were prepared from 0, 24, 48, 60, and 68% (w/w) glycerol in D₂O containing 25 mM KH₂PO₄ at pH 5.5. Measurements were made at 25°C. In vitro T₂ measurements were made with the Carr-Purcell-Meiboom-Gill modification sequence, 2τ values of 4 ms were used with multiple loops of 32 to 1500. Areas were used for three-parameter iterative data fits. Pulse delays varied from 5 s for 60% (w/w) glycerol to 45 s for 0% (w/w) glycerol. No attempt was made to remove paramagnetic impurities. From atomic absorption measurements, the phosphate used contained 5.2 ppm Fe³⁺ and 0.8 ppm Mn²⁺. Good precision was difficult to attain because of the sensitivity of T₂ to paramagnetic impurities and difficulties in obtaining good magnet homogeneity and accurate 90° pulses with highly viscous solutions.

Relaxation Calculations

Nonlinear regression analysis of all data was performed using an RS/1 software package (BBN Software Products, Cambridge, MA). Calculations were carried out on a VAX 8350 computer. For the comparisons of the goodness of fit, the *F*-test was used (10) with the following *F* value:

$$F = (SSR_1 - SSR_2)/(df_1 - df_2) \div SSR_2/df$$

where *SRR* refers to the sum of the squares of the residuals and *df* refers to the number of the degrees of freedom (number of data points minus the number of fitting parameters). The subscript 1 refers to the simpler model, the one with the fewer parameters, i.e. three parameters versus five parameters. *F* distribution tables (4) were consulted for (*df*₁ - *df*₂) and *df*₂ degrees of freedom at the 20 and 15% levels of

statistical significance. For relaxation data (T_1) on whole nodules, S/N was poor due to dilution of the Pi with extraneous tissue. Therefore, only a limited number of τ values (five or six) could be obtained to establish the relaxation kinetics. Consequently, only three-parameter, single-relaxation plots could be generated from these data.

RESULTS

^{31}P Assignments for in Vivo Spectra of Soybean Nodules

Soybean root nodules consist of a heterogeneous cell population. The cortex surrounding the central matrix represents between 40 and 50% of the total volume of the nodule. The active, N_2 -fixing tissue is a central core of enlarged host parenchyma cells that are packed with the endosymbiotic bacteria. The cells of the central tissue form a compact mass with the uninfected cells (1). Both the interior uninfected host cells and cortical cells contain a cytosolic and a vacuolar compartment (1). The bacteroids have an intracellular pH of approximately 6.9 as determined from ^{31}P spectra of isolated preparations (23). Figure 1 shows the ^{31}P spectrum of the eukaryote/prokaryote matrix of soybean nodules following 6 weeks of development. The resonance areas do not represent the true ratios of the mobile phosphorus compounds in the tissue because the rapid recycling did not allow for complete relaxation of all nuclei (19). In particular, the area of the Pi resonances were grossly underestimated due to their inherently long T_1 values. In spite of this, the distorted spectra are useful for examining changes in minor phosphorus nucleotide levels under various stress conditions (19). In general, there was little change in the total ^{31}P resonance area of nodule spectra over periods up to 24 h, indicating no significant leakage of mobile phosphorus compounds (22). However, under hypoxic conditions nodule viability was limited to not more than 15 h, and 15 to 20% leakage of mobile phosphorus was noted after 10 h of perfusion. Nodule viability was verified by the detection of glycerol-3-P in the nodule spectra upon the addition of glycerol to the perfusion medium (23).

All resonances have been identified from the ^{31}P NMR spectra of neutralized perchloric acid extracts as described previously (22). A unique feature in the spectrum of soybean nodules is the "x" resonance located at 0.37 ppm. This recently identified phosphodiester resonance corresponds to a cell-associated phosphocholine-substituted β -1,3;1,6 cyclic glucan from the endosymbiotic bacteria (17, 24). The resonances at approximately 2.8 and 0.9 ppm represent the cytosolic Pi at pH 7.4 and Pi in environments with pH values, approximating pH 5.5, respectively. Limitations in resolution prevented the direct observation of compartmentalized Pi (pH 6.9) in the bacteroids. However, we previously demonstrated (23) that approximately 60% of the Pi resonance area observed at 0.9 ppm corresponds to Pi residing in the large cortical cell vacuoles. The remaining 40% of this signal represents vacuolar Pi of the host cells in the matrix.

^{31}P Spectra of Soybean Nodules under Aerobic and Hypoxic Conditions

Figure 1 illustrates the changes that occur in the phosphorus spectra following the shift from an aerobic to a hypoxic

condition. Following aerobic perfusion for 1 h and 30 min (Fig. 1A), Ar was introduced for 2 h and 40 min (Fig. 1B). At this time, we observed the normal consequences of hypoxia, i.e. loss of nucleotide triphosphate and glucose-6-P with a concomitant increase in cytosolic Pi (19). There was also an unusual 46% drop in the intensity of the vacuolar Pi signal. Recycling back to aerobic conditions (Fig. 1C) with 55 min of O_2 perfusion restored approximately 76% of the original acidic Pi resonance. An additional cycle of N_2 hypoxia (Fig. 1D) for 55 min reduced the Pi signal again to 50% of the initial aerobic value. Final aerobic treatment (Fig. 1E) for 55 min returned the acidic Pi resonance to 69% of its original state.

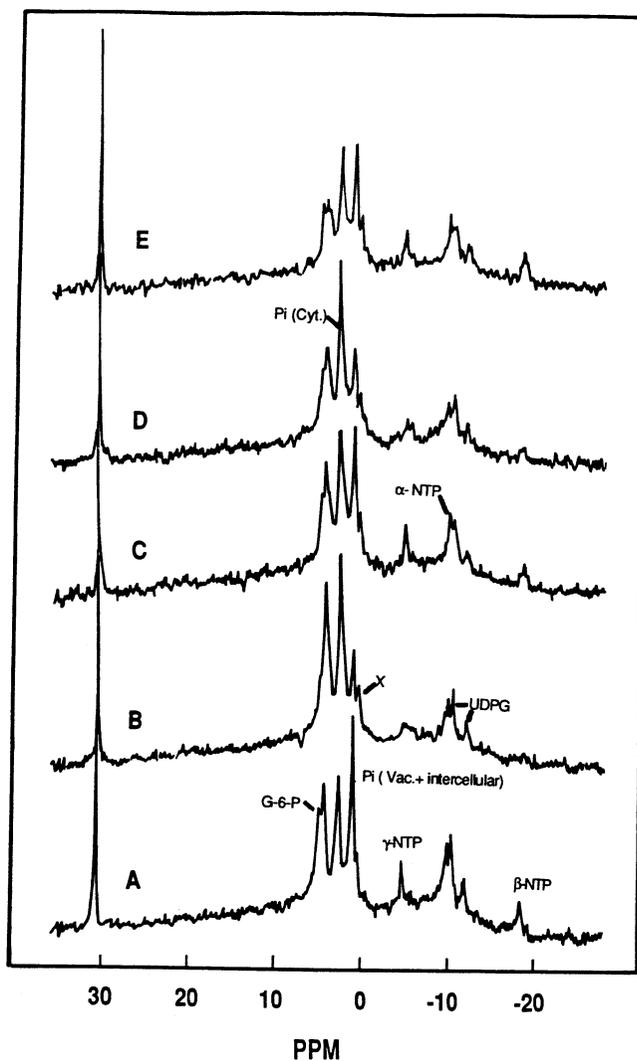


Figure 1. 161.7 MHz in vivo ^{31}P spectra of detached split soybean nodules. The perfusion medium was bubbled with O_2 , N_2 , or Ar for different periods of time as indicated: A, 1 h and 30 min O_2 ; B, same as A with an additional 2 h and 40 min of perfusing Ar; C, same as B with an additional 55 min of perfusing O_2 ; D, same as C with an additional 1 h and 40 min of perfusing O_2 followed by 55 min of N_2 ; E, same as D with an additional 55 min of perfusing O_2 .

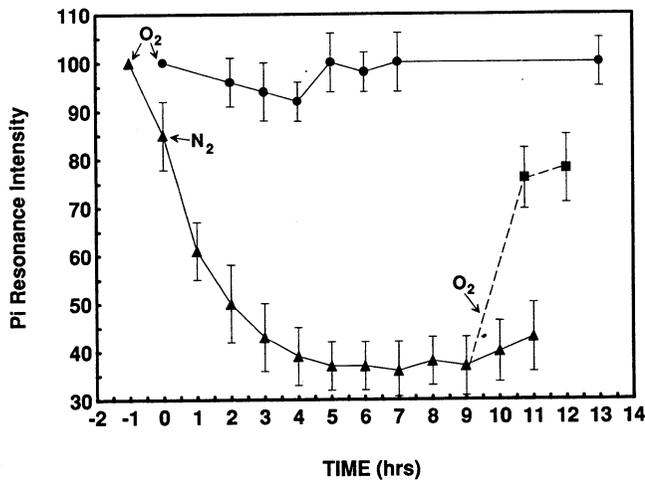


Figure 2. Area of cortical tissue vacuolar Pi resonance as a function of the duration of hypoxia treatment (circulating N_2 -saturated perfusate medium). At zero time, N_2 was introduced into the perfusion medium and O_2 perfusion ceased. Each point represents a replicate of four independent experiments: \blacktriangle , O_2/N_2 experiment; \bullet , control experiment with O_2 alone; \blacksquare , experiment in which O_2 was reintroduced after 9 h of N_2 treatment.

Loss of Cortical Cell Tissue Pi Resonance Signal Area under Hypoxia

To determine the time frame for the loss of Pi signal attributable to the cortical cells, we excised the cortical tissue from the split soybean nodules (23). The corresponding mass of inner matrix cells was also separated at this time to evaluate the effects of hypoxia on the Pi signal. Unfortunately, the separated inner matrix containing leghemoglobin and infected and noninfected host cells was difficult to maintain in a viable state once the mass was physically extracted from the cortical shell of the nodules and placed in a dialysis bag within the perfused NMR tube. In general, the tissue lost mobile phosphorus and deteriorated within a few hours in the circulating perfusion medium (data not shown).

The carefully separated and thoroughly washed cortical hemispheres were perfused with oxygenated perfusion medium in the NMR system for 1 h prior to the switch to N_2 gas. It is rarely possible to observe any cytosolic phosphorus metabolites in cortical cells because they are highly vacuolated. Consequently, to ascertain their viability we perfused the tissue with a perfusate containing 10 mM glycerol. Under aerobic conditions, this tissue exhibited a phosphorylation of glycerol with the appearance of the ^{31}P resonance at 4.82 ppm, corresponding to glycerol 3-P (23). Figure 2 demonstrates that, following the introduction of N_2 , the area of the cortical Pi resonance gradually dropped until it had lost approximately 63% of its intensity after 4 h. This area remained constant for an additional 5 h and increased slightly. A switch-over to O_2 perfusion following 9 h of N_2 treatment restored 76% of the Pi signal within 1.5 h. No change in Pi linewidth (57 Hz) was observed during the transition from the aerobic state to hypoxia. Because the pH of the cortical tissue vacuoles is in the range of 5.5 to 6.0 (flat portion of

the pH versus chemical shift titration curve), it is difficult to assess by a measurement of ^{31}P shift any change in pH that is less than 0.5 pH units (19). No measurable difference in the acidic Pi resonance (pH approximately 5.6) was observed in either nodule or cortical tissue under N_2 or O_2 perfusion. Acidic Pi changed little when the tissue was perfused with O_2 for 13 h (see control, Fig. 2).

The reversibility of the Pi response to changes in O_2/N_2 perfusion in cortical tissue from the soybean root nodules is evident in Figure 3. We note that, following an extended period of N_2 perfusion (9 h), the signal dropped (Fig. 3B) to approximately 40% of its original intensity under O_2 (Fig. 3A). Restoration of the aerobic conditions yielded almost 75% of the original Pi area (Fig. 3C). ^{31}P spectra of cortical tissue (vacuolar Pi) under N_2 or O_2 perfusion examined with

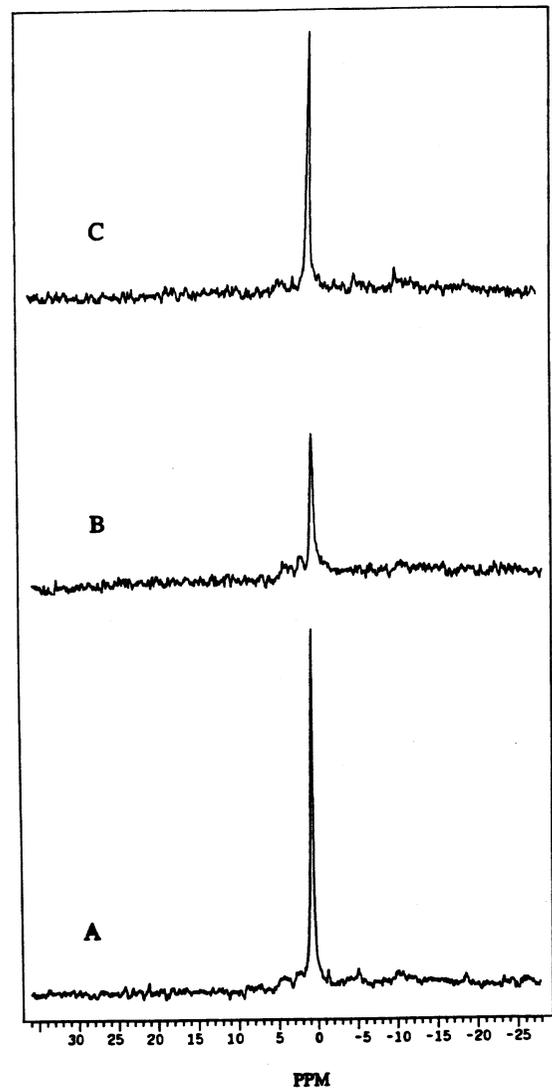


Figure 3. 161.7 MHz *in vivo* ^{31}P spectra of excised cortical tissue acquired as in Figure 1. The perfusion medium was bubbled with O_2 or N_2 for different periods of time as indicated: A, 1 h and 30 min O_2 ; B, same as A with an additional 9 h of perfusing N_2 ; C, same as B with an additional 1 h and 30 min of perfusing O_2 .

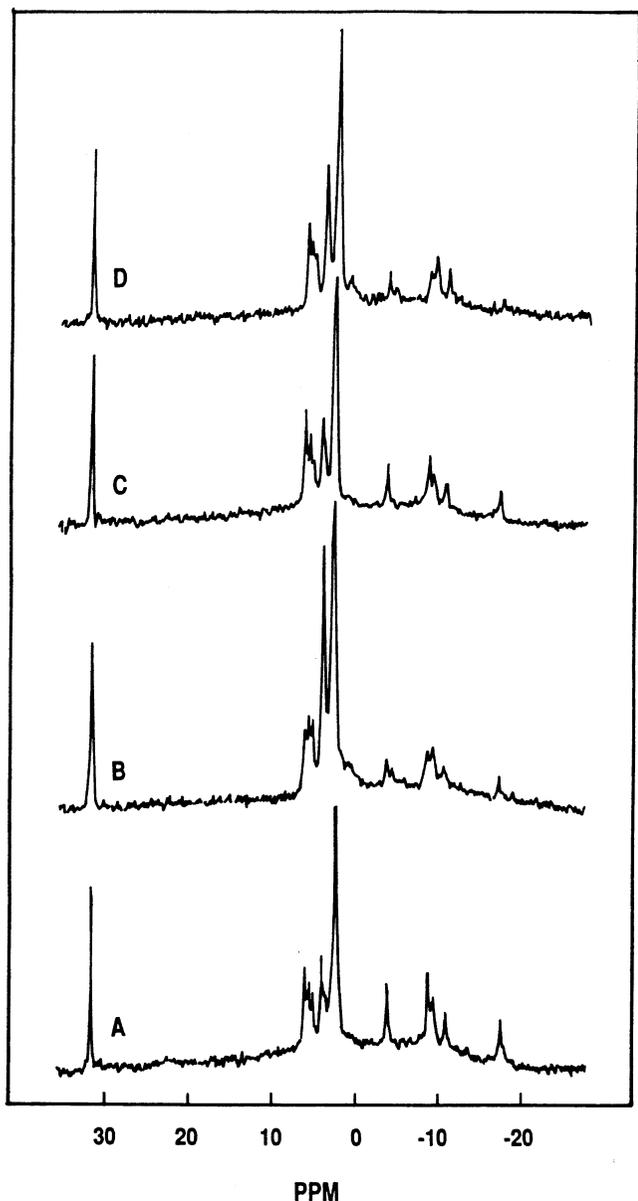


Figure 4. 161.7 MHz *in vivo* ^{31}P spectra of excised soybean root tips acquired as in Figure 1. The perfusion medium was bubbled with O_2 , Ar, or N_2 for different periods of time as indicated: A, 1 h and 30 min O_2 ; B, same as A with an additional 2 h and 45 min of perfusing Ar; C, same as B with an additional 1 h and 50 min of perfusing O_2 ; D, same as C with an additional 55 min of perfusing O_2 followed by 1 h and 50 min of N_2 .

25-s recycling times were identical in intensity within experimental error ($\pm 10\%$) (data not shown).

^{31}P Spectra of Soybean Root Tips under Aerobic and Hypoxic Conditions

Excised soybean root tips (3–5 mm) were perfused in the same manner as soybean nodules described above. Figure 4 illustrates the changes that occur in the ^{31}P spectra of excised

soybean root tips after a shift from aerobic to hypoxic conditions. The typical ^{31}P spectrum of root tips observed 90 min after the start of perfusion with oxygenated medium is observed in Figure 4A, with the characteristic chemical shifts of nucleotides (upfield resonances -4 to -20 ppm), the resonances of the phosphomonoesters between 4 and 5 ppm (glucose-6-P, fructose-6-P, P-choline), and two Pi peaks corresponding to the vacuolar Pi (0.90 ppm) and the cytoplasmic Pi (2.73 ppm). Following 1 h and 30 min of O_2 (Fig. 4A), the tissue was subjected to 2 h and 45 min of Ar (Fig. 4). We note a loss of nucleotides and glucose-6-P, with a significant increase in cytosolic Pi. A change in cytosolic pH from 7.4 to 7.0 (shift of the Pi resonance from 2.73 to 2.26 ppm) was also noted, although it was not discernible from these spectra. Recycling back to aerobic conditions (Fig. 4C) with 140 min of O_2 perfusion, the root cells were able to accumulate glucose-6-P and nucleotides to the detriment of cytoplasmic Pi. In contrast with the nodules, the root tissue showed a minor increase (approximately 10%) in the vacuolar Pi resonance under both N_2 and Ar perfusion conditions (Fig. 4, B and D).

Relaxation Values under Aerobic and Anaerobic Conditions

Table I gives the T_1 and T_2 values for Pi *in vitro* and *in vivo* under aerobic and anaerobic conditions. ^{31}P T_1 and T_2 values of 25 mM Pi solutions at pH 5.5 are not significantly affected by saturation with N_2 or O_2 .

For the whole-nodule spectra, the relaxation times we observe in the single acidic Pi resonance are a weighted average of Pi residing in the cortical (60%) and inner matrix host cells (40%) (23). The cytoplasmic Pi resonance represents almost exclusively the Pi within the interior of the nodule, which includes host cells as well as bacteroids (23). A single exponential value was calculated for these data because only a limited number of data points (five) could be obtained from the lower S/N spectra (see "Materials and Methods"). In fact, there could be more than a single relaxation decay. However, it was not feasible to determine this under the experimental conditions. The acidic Pi showed a significant T_1 lengthening under N_2 perfusion, whereas the cytoplasmic Pi T_1 remained constant.

Excised cortical tissue Pi exhibited a complex T_1 relaxation pattern. At least two independent relaxations for T_1 and T_2 were observed in this tissue. Under aerobic conditions, the average T_1 was 2.3 ± 0.5 s and average T_2 was 0.28 ± 0.02 s. With N_2 perfusion, these values were 6.07 ± 0.7 s and 0.25 ± 0.01 s, respectively. Thus, there is a significant change in the distribution of T_1 values, the longer relaxation becoming more dominant under hypoxia. T_2 appears to remain essentially the same under both perfusion conditions.

Excised soybean root segments (2.5 cm) were used as a control plant tissue. Segments were chosen for the relaxation studies because there is less diversity of cell type in this tissue as opposed to root tips, which contain a very heterogeneous cell population including meristematic cells with a large amount of cytosol (17). Under both aerobic and anaerobic treatments, there was essentially no difference within experimental error in measured single exponential vacuolar Pi T_1

Table I. *T*₁ and *T*₂ Relaxation Values for Pi *In Vitro* and *In Vivo* under Aerobic and Anaerobic Conditions

Source of Pi	Perfusing Gas	<i>T</i> ₁ ^a	<i>T</i> ₂ ^a
Pi solution ^b	O ₂	9.74 ± 0.10	0.157 ± 0.004
	N ₂	9.94 ± 0.08	0.145 ± 0.002
Nodules (Vac. Pi) ^c	O ₂	0.77 ± 0.08	
	O ₂	0.95 ± 0.19	
Nodules (Vac. Pi)	N ₂	3.98 ± 0.69	
	N ₂	0.95 ± 0.20	
Cortical tissue (Pi)	O ₂	3.72 ± 0.93 (59%) ^e	0.41 ± 0.02 (62%) ^f
		0.20 ± 0.08 (41%) ^e	0.07 ± 0.01 (38%) ^f
	N ₂	7.07 ± 1.36 (86%) ^e	0.37 ± 0.01 (61%) ^f
		0.39 ± 0.07 (14%) ^e	0.07 ± 0.01 (39%) ^f
Soybean root tissue (stem segments, Vac. Pi)	O ₂	6.30 ± 0.93	0.87 ± 0.11
	N ₂	5.80 ± 0.65	0.79 ± 0.09

^a Average ± SD of three independent measurements. ^b Contained 25 mM Pi, 100 mM, 10 mM Mes, pH 5.54 no attempt was made to remove paramagnetic contaminants. ^c Vac. Pi, Vacuolar Pi. ^d Cyt. Pi, Cytoplasmic Pi. ^e Mobile Pi. ^f Relatively immobile Pi.

values. The *T*₁ values for the latter were approximately 30% shorter than those observed for the *in vitro* study of Pi solutions mentioned above. The *T*₂ values for vacuolar Pi were essentially the same under N₂ and O₂. However, they were approximately six times the magnitude of those observed in *in vitro* Pi solutions and twice those found in cortical tissue.

In Vitro Relaxation Values as a Function of Viscosity

Figure 5 shows the effects of increasing relative viscosity (6) of water solutions by the addition of glycerol on the relaxation rate (1/*T*₁, s⁻¹) of Pi at pH 5.5. A 10-fold increase in viscosity decreased the value of the Pi *T*₁ by 75%. However, the Pi *T*₂ (Fig. 6) appeared to be relatively insensitive to viscosity change. Over a 20-fold increase in viscosity resulted

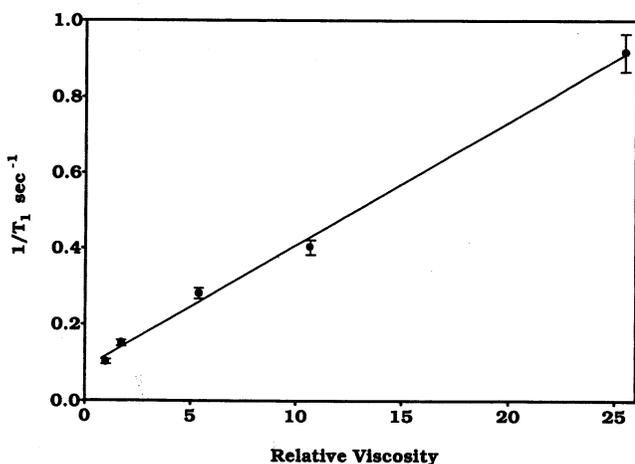


Figure 5. ³¹P spin lattice relaxation rates (1/*T*₁, s⁻¹) as a function of viscosity of water/glycerol solutions containing 25 mM KH₂PO₄, 100 mM KCl, 10 mM Mes at 25° and pH 5.5.

in only a minor (20%) change in *T*₂. The marginal change is probably insignificant and due primarily to instrumental problems in obtaining accurate *T*₂ data in the presence of paramagnetic metal ions and highly viscous solutions (see "Materials and Methods").

DISCUSSION

In a previous *in vivo* ³¹P NMR study of soybean nodules, we observed what appeared to be the loss of vacuolar Pi (particularly cortical cell vacuolar Pi) following the onset of hypoxia (17). Upon careful quantitative evaluation of the ³¹P spectra of the nodules, we were unable to account for vacuolar migrated Pi. Although cytosolic Pi in previously studied plant tissues typically increases under hypoxia from breakdown of nucleotides and phosphomonoesters, vacuolar Pi levels consistently remain the same (18, 19). Soybean tissue is no exception (Fig. 4). In contrast, both nodule and excised cortical tissue derived from the nodule show a loss of an acidic Pi signal under nonaerobic conditions, i.e. N₂ or Ar perfusion (Figs. 1 and 2). In addition, we have demonstrated

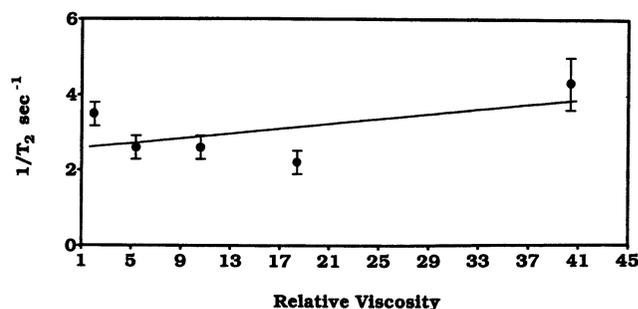


Figure 6. ³¹P spin-spin relaxation rates (1/*T*₂, s⁻¹) as a function of viscosity of D₂O/glycerol solutions containing 25 mM KH₂PO₄, 100 mM KCl, and 10 mM Mes at 25° and pH 5.5.

that this Pi resonance reappears rapidly following the resumption of an aerobic state.

To investigate further the cause of the loss of the Pi signal, we examined the T_1 values of the Pi resonance under different gas-perfusing conditions. T_1 is a measure of the kinetics of nuclear reorientation to a low-energy state in the magnetic field (25). This reorientation can be facilitated by many different mechanisms, which include the presence of a paramagnetic species, decreased temperature, increased solution viscosity, or increased solute concentration (25). For small, fast-moving molecules such as Pi, any phenomena that cause a slowing down of molecular motion generally induce the Pi molecule to relax because its motion approaches the Larmor or nuclear precessional frequency where relaxation is most efficient. Alternatively, electron-nuclear relaxation can be induced by such agents as paramagnetic ions and/or oxygen (25). This interaction is dipolar and its efficiency depends on the square of both the magnetic moment of the electron and that of the nucleus. Because the electron's magnetic moment is approximately 3 orders of magnitude larger than that of the proton, it is a powerful relaxing agent.

T_1 values of acidic Pi in soybean nodules under N_2 or O_2 perfusion were significantly different (Table I) and, in fact, account for the divergent levels of observed spectral magnetization in the two respective states. Under O_2 perfusion, we observed that the average acidic Pi resonance T_1 of the nodule was 0.77 s and in excised cortical tissue this value was 2.3 s, whereas under N_2 these values increased to 3.98 and 6.07 s, respectively (Table I). The unexpected change in T_1 resulting from the switch from O_2 to N_2 manifests itself in a significantly greater degree of Pi T_1 saturation (loss of signal due to too short recycling times when the T_1 of Pi has lengthened) under N_2 perfusion when spectra are obtained with rapid recycling conditions (19). This has the effect of producing a lower level of Pi spectral magnetization. When spectra of excised cortical tissue were obtained with either O_2 or N_2 perfusion under quantitative conditions, i.e. 30-s recycle times, the levels of observed Pi magnetization were comparable within experimental error (data not shown). A mechanism that might account for the observed phenomenon is the interaction of oxygen or an oxygen-generated paramagnetic species with vacuolar Pi in the cortical tissue. However, oxygenated and degassed solutions of Pi did not exhibit any significant differences in T_1 (Table I) (13). Efforts to observe a paramagnetic species in the separated, oxygenated cortical tissue by electron paramagnetic resonance were unsuccessful largely because of the heterogeneity of the frozen sample (only very broad signals were observed).

^{31}P T_2 does not appear to be measurably affected by either O_2 or N_2 in the cortical tissue or Pi solutions (Table I). Unlike T_1 , which is the time constant for an energetic process, T_2 is the time constant for an entropic process dependent on spin-spin exchange between nuclei. In the ^{31}P Pi model system (Pi solutions) as well as the tissue studies, T_1 does not equal T_2 and T_2 is considerably shorter than T_1 . This is probably because T_2 relaxation is strongly dominated by chemical exchange processes with various metal ions that have little effect on T_1 (25). In addition, T_2 relaxation, unlike T_1 , can also be strongly influenced by chemical shift anisotropy, i.e.

molecular motions that produce fluctuations in an asymmetrical environment surrounding the molecule (25).

It is evident from the results of our experiments that the cortical tissue of the nodule undergoes modification in response to O_2/N_2 perfusion that directly affects the solution properties of Pi residing in this environment. In contrast, soybean root tissue does not exhibit T_1 changes with alternating O_2/N_2 treatment, nor has it been established that soybean root tissue can function as an oxygen diffusion barrier (Table I, Fig. 4).

Whatever the mechanism(s), the measured T_1 responses in both cortical Pi of excised nodules and cortical tissue suggest that change in partial pressure of O_2 is directly associated with a modification of cortical cell chemistry. Although the presence of oxygen alone has no measurable paramagnetic effect on Pi T_1 or T_2 values in solution (Table I), viscosity can influence Pi T_1 significantly. Figure 5 demonstrates that increasing viscosity results in a sizable decrease in Pi T_1 . In contrast, T_2 is relatively insensitive to viscosity change even over a range extending to 20 times the viscosity of water (see Fig. 6).

A recent study (9) describes the elaboration of a glycoprotein in cortical tissue of soybean nodules under the influence of O_2 perfusion. This glycoprotein is reported (9) to be localized in the intercellular spaces of the cortical cells in nodules only following their exposure to O_2 . It is presumed that this material is synthesized within the cells in response to O_2 and then secreted into the intercellular space. If this material is present at the same time in a soluble form in the cytoplasm and vacuole, the viscosity of the tissue should increase significantly. The *in vitro* experiments with Pi in which T_1 and T_2 were studied as a function of viscosity very closely model changes taking place in the cortical cell layer. The observed shortening of the acidic Pi T_1 in the cortical cells from an average value of 6.07 s under N_2 to 2.3 s under the influence of O_2 corresponds to a 5-fold increase in viscosity (Fig. 5). We estimate that the average viscosity of the cortical cell environment under N_2 perfusion is approximately 2 times that of pure water, but, under N_2 , the viscosity is probably closer to 10 times that value according to the model (Fig. 5).

It is quite clear that the acidic Pi resonances observed for cortical tissue represent at least two populations of Pi in the cortical tissue (Table I). They are the more mobile (longer T_1) and more immobile (shorter T_1) phosphate populations, respectively. Under the influence of O_2 , there is enhanced secretion of insoluble intercellular occlusion glycoprotein, which is most likely produced within the cortical cells but may also reside in a soluble form in the cortical cells' vacuoles, since the published micrographs (9) of nodules suggest that glycoprotein may also be present in the outer cortical layers. This would account for the observed relaxation behavior of the acidic Pi resonance. Note that we could not monitor the relaxation changes in the cortical cell cytoplasm because of the small volume of its compartment.

Although we were unable to resolve two acidic Pi T_1 s in intact nodules because of poor S/N and heterogeneity of the host cell types, an overall drop in the average T_1 from N_2 to O_2 was comparable to that observed for the isolated cortical tissue (3.25 s in nodules versus 3.7 s in excised cortical tissue).

We also observed that O₂ perfusion had no measurable effect on the T₁ of the cytoplasmic Pi residing in the host cells within the inner matrix (Table I). This is to be expected because oxygen is maintained at low, well-modulated levels in this section of the nodule (33) and no evidence of occlusion protein has been observed in this tissue (9). The shortness of the average cytoplasmic Pi T₁ value (0.95 s) is undoubtedly a consequence of the presence of deoxyhemoglobin and excessive paramagnetic metal ions, e.g. Fe³⁺ and Mn³⁺, in the surrounding tissue (1).

This paper presents some new observations concerning the relaxation effects of O₂ gas in nodules of leguminous plants formed by the symbiotic association of the plant root and nitrogen-fixing bacteria. The specialized cortical cells that are not infected by the endosymbiotic bacteria are genetically derived from the soybean root cells. However, unlike the latter, these cells are characterized by the production of occlusion protein (9) and an ability to modulate the diffusion of O₂. Elaboration of this glycoprotein and its possible occurrence in both the intercellular space and cell vacuoles could account for the observed change in T₁ of Pi in the latter compartment. Our observations concerning the relaxation response of these cell vacuoles mirrors those presented in the accompanying paper (12). Although this study strongly suggests that viscosity could play a major role in the relaxation of Pi, we cannot entirely rule out the effect of O₂ on some mediating species, e.g. a chelating metal ion, to create a paramagnetic center capable of inducing Pi relaxation (12). Further investigations are needed to elucidate the mechanism of how O₂ mediates the production of this glycoprotein in cortical cells as well as how and where the glycoprotein is synthesized and catabolized, and how it acts to regulate the O₂ diffusion process.

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