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# *In Vivo* $^{31}$P NMR Spectroscopic Studies of Soybean *Bradyrhizobium* Symbiosis

## I. Optimization of Parameters

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### ABSTRACT

**$^{31}$P NMR spectroscopy was used to study *in vivo* the symbiotic state established between soybean (*Glycine max* [L.] Merr. cv Williams) and *Bradyrhizobium japonicum* (USDA 110 and 138). Different experimental conditions were used to maintain perfused, respiring detached or attached nodules in an NMR magnet. The pH of the perfusion medium affected the cytoplasmic pH and the resolution of the spectra. The internal Pi content and distribution were assessed as a function of nodule age and greenhouse growth conditions and the rate of glucose and 2-deoxyglucose uptake into nodules in split and intact states. The major metabolites (glucose-6-P, fructose-1,6-diP, P-choline, Pi, NTP, UDP-glc, and NAD) were readily identified from $^{31}$P NMR spectra of perchloric acid extracts of nodules with the exception of one unknown phosphorus metabolite. Nodules stressed by glucose deprivation demonstrated movement of Pi between the vacuole and cytoplasmic compartments not previously observed in $^{31}$P NMR studies.**

The majority of biologically fixed nitrogen available for agriculture is formed by *Rhizobia* in symbiotic association with legumes. In the past 20 years, this association between prokaryote and eukaryote cells has been the subject of intense research (19, 28). Nodule metabolism and structure are extremely complex (5) because they encompass the basic metabolic process of each symbiotic partner as well as the unusual metabolic interactions between the host cells and endophyte.

$^{31}$P NMR spectroscopy has proved to be a useful technique for examining plant tissue *in vivo* (10, 20). The spectra can be used directly for monitoring intracellular changes particularly those involving compartmentation (1, 26) as well as identifying and determining the level of mobile phosphorus compounds (9, 16, 21, 27). Recently, reports of $^{31}$P NMR studies of various plant tissue (8, 14), seeds (13), algae (4), leaves (31), bacteria (9), yeast (3), mycorrhizal fungi (12), and plant cells (11) have appeared. These studies have clearly demonstrated that this technique can be exploited to measure energy status (ATP/ADP ratio) (6), changes in intracellular pH induced by uncouplers of oxidative phosphorylation (7), phosphorylation of different monosaccharides (6), effects of hypoxia on pH regulation (22), effects of sodium on phosphate uptake (24), uptake of toxic metals (16, 18), and the rate of mitochondrial synthesis of ATP under different conditions via saturation transfer (25). Presently, the literature contains only one preliminary $^{31}$P NMR study of intact root nodules (14). There has been no serious attempt to optimize the various parameters associated with the viability of these eukaryote-prokaryote assemblies and the quality of their corresponding spectra.

The first part of our ongoing studies investigated the methodology for examining the symbiotic state between soybean and *Bradyrhizobium japonicum* by *in vivo* $^{31}$P NMR. Different experimental conditions were used to maintain perfused, respiring detached or attached nodules in an NMR magnet. The effects of the perfusion medium pH and the contribution of glucose, 2-deoxyglucose, or glucose deprivation in split and intact nodules were investigated. Identification of the major phosphorus metabolites detected by *in vitro* $^{31}$P NMR of a perchloric acid extract of nodules is also reported.

## MATERIALS AND METHODS

### Plant Material

Soybeans (*Glycine* max [L.] Merr. cv Williams) were germinated 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 grown in a greenhouse were supplemented with 2500 Lux from metal halide lamps for a photoperiod of 15 h. Humidity and temperature were not controlled. Temperature varied from 16°C at night during the winter to 38°C during the day in the summer. The environmental conditions were controlled for plants grown in the growth chamber. These plants received 6000 Lux from fluorescent and incandescent lamps for a photoperiod of 15 h, day/night temperatures of 25 to 19°C, and humidity of 80 and 95%, respectively. Plants were irrigated with a nutrient solution containing 1 mM $NO_3$ (15). Nodules were harvested 35 to 55 d after inoculation.

### *In vivo* NMR Experiments

For spectra of attached nodules, one plant with an average of 10 nodules was removed from vermiculite, rinsed in distilled water, and transferred to a 10 mm NMR tube containing 3 mL of distilled water. For spectra of detached nodules, approximately 2 to 3 g of nodules were detached from the roots and washed with distilled water. Detached nodules were either left intact or split in half with a razor blade and transferred to a 10 mm NMR tube equipped with perfusion system similar to that described earlier by Pfeffer *et al.* (18). The perfusion medium (1,000 mL) 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 $CaSO_4$. Oxygen 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 $^{31}$P NMR spectra (obtained with a 54 mm narrow bore magnet) were accumulated at 22°C ± 1 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 36° pulse (12 μs) with low power complete proton decoupling. Each experiment was repeated at least two 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 HMPA[1,2] 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% $H_3PO_4$. All chemical shifts were referenced relative to 85% $H_3PO_4$, which was assigned a value of 0 ppm. Estimates of intracellular pH were made using the standard reference curve of Pi suggested by Roberts *et al.* (26).

### Perchloric Acid Extract and *in Vitro* NMR Experiments

For the perchloric acid extraction, 10 to 40 g of nodules were quickly frozen in liquid nitrogen to avoid hydrolysis of ATP and estermonophosphates and were ground in a mortar in the presence of perchloric acid (0.8 M final concentration). The frozen powder was stored at −10°C. The thick homogenate was centrifuged at 3,000*g* for 10 min to remove particulate matter. The supernatant was titrated with 2 M $KHCO_3$ to pH 6.5 and centrifuged again at 3,000*g* for 10 min to remove $KClO_4$. The resulting supernatant was lyophilized and stored at −80°C. For NMR spectra, the freeze-dried material was redissolved in 3.5 mL of 40 mM Mops buffer (pH 7.8) containing 40 mM CDTA and 20% $D_2O$. The deuterium resonance of $D_2O$ was used as a lock signal. The $^{31}$P NMR spectra were accumulated at 22°C ± 1 over a 16,000 Hz frequency range with 16,000 points. Each spectrum required 1,000 scans with a repetition rate of 2.082 s (total accumulation time of 35 min) utilizing approximately a 56° pulse (20 μs) with low power complete proton decoupling. An exponential multiplication (1 Hz linewidth) was used to increase signal to noise.

### Determination of ATP and ADP

Fresh nodules (0.75 g) were homogenized in 10 mL of ice-cold 50 mM $KH_2PO_4$ buffer (pH 7.6) containing 150 mM NaCl with a Sorvall Omni Mixer at high speed for 30 s. Seventy-five μL of homogenate were placed into 10 mL of boiling Hepes (20 mM)/EDTA (2 mM) buffer (pH 7.5). After 3 min, the extract was cooled in an ice bath and then frozen. ATP was determined for 300 μL aliquots with an Aminco Chem-Glo photometer and 100 μL of reconstituted (as per directions of supplier) luciferin-luciferase (Sigma FLE-50) added to 40 μL reaction buffer (250 mM Hepes [pH 7.5], 250 mM $MgSO_4$ 7 $H_2O$) and 70 μL water. Concentrations of ATP and ADP were determined by incubating 0.5 mL of the extract in the following mixtures for 90 min at 30°C prior to assay: For ATP, 150 μL Hepes (0.2 M [pH 7.4]), 25 μL $MgCl_2$, (0.15 M), and 325 μL water; for ATP plus ADP, 150 μL Hepes, 25 μL $MgCl_2$, 25 μL PEP (10 mM), 25 μL pyruvate kinase (2 mg at 405 units/mg in 0.5 mL, 1 mM phosphate buffer [pH 7.4]) and 275 μL water.

### Acetylene Reduction Assay

Nodules (about 1 g) were placed in a cheesecloth bag. Another set of nodules was split in half before wrapping in cheesecloth. Whole and split nodules were placed separately in a circulating oxygenated perfusion buffer containing 10 mM Mes (pH 7.5), 50 mM glucose, and 0.1 mM $CaSO_4$. After 0, 0.5, 1, 2, and 4 h, nodules were removed from the perfusion buffer and each placed in a 490 mL jar for 10 min for acetylene reduction as described by Van Berkum and Sloger (30).

## RESULTS

### Effects of the State of the Nodules and Perfusion Conditions on the *in Vivo* $^{31}$P NMR Spectra

A representative 161.7 MHz $^{31}$P NMR spectrum of attached soybean nodules (not perfused) is presented in Figure 1A. Three major peaks are visible, 2 Pi peaks (d, e) and a broad unresolved peak representing phosphomonoesters. For the estimation of internal pH from the chemical shift value of the Pi signal, a pH titration curve of Pi was made using the standard reference curve of pH *versus* $^{31}$P chemical shift in ppm (16, 26). The Pi signals at 1.33 ppm (e) represented a compartment with a pH of 6.1. The other peak (d) at 2.64 ppm corresponded to a pH of 7.3. We tentatively assigned those two resonances as Pi in the vacuolar and cytoplasmic

[1] Abbreviations: HMPA, hexamethylphosphoramide; CDTA, trans, 1-2 diaminocyclohexane *N,N,N',N'*-tetraacetic acid; UDP-glc, uridine 5'-diphosphate glucose; UDP-2dglc, uridine 5'-diphosphate 2-deoxyglucose.

[2] Caution should be exercised in handling this chemical since it is a known carcinogen.

compartments. The chemical shifts of nucleotides (upfield resonance −4 to −20 ppm) were not visible in this spectrum after the accumulation of 38,000 free induction decays (scans).

The spectrum in Figure 1B taken from the work of Mitsumori *et al.* (14) shows the spectrum of intact detached nodules obtained from plants that were Pi starved for 2 weeks. The spectra were recorded under anaerobic conditions. The authors (14) commented that $O_2$ bubbling was not employed in this experiment because it did not have a significant effect on the $^{31}P$ NMR spectrum. Although this spectrum shows the presence of Pi, NTP, NAD, UDP-glc, and one peak for the phosphomonoesters, detailed resolution of discrete resonances is relatively poor. The vacuolar and cytoplasmic pHs were estimated to be 5.9 and 7.1, respectively. Long-term stability was not examined.

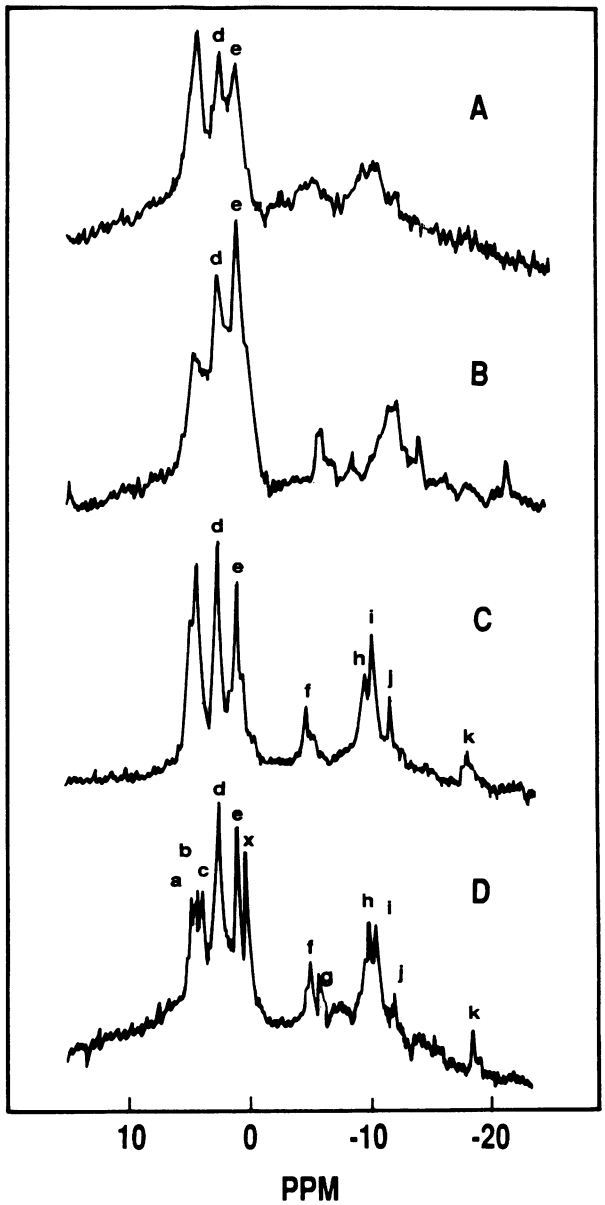


**Figure 1.** *In vivo* $^{31}P$ NMR spectra (161.7 MHz) of soybean root nodules. 38,000 (A) or 20,000 (B, C, D) free induction decays were accumulated with a recycling time of 0.162 s (total time of accumulation 54 min) (A, C, D) and 0.12 s (B). A, Roots of intact plant with 10 attached nodules were introduced into the 10-mm NMR tube with distilled water (not perfused). B, Intact nodules (obtained from plants which were Pi starved for 2 weeks) were introduced directly into the NMR tube. This spectrum is taken from the work of Mitsumori *et al.* (1985). C, Detached nodules were directly introduced into the NMR tube and perfused with a buffer solution containing 50 mM glucose, 0.1 mM $CaSO_4$, 10 mM Mops (pH 7.5). $O_2$ was bubbled into the reservoir. D, Detached nodules were split and perfused as in C. The assignments of resonances are: G-6-P, a; F-1, 6-diP, b: P-choline, c; cytoplasmic Pi, d; vacuolar Pi, e; unknown compound, x; γ-NTP, f; β-NDP, g; α-NTP, h; UDP-glc and NAD nucleotides, i, j; β-NTP, k.

In the present study (Fig. 1C), intact detached nodules perfused with an $O_2$-saturated buffered medium (pH 7.5) improved resolution of the different peaks. The phosphorus resonances of different phosphomonoesters and the vacuolar Pi peak (e) could be resolved from a new phosphorus resonance shoulder noted at 0.37 ppm (Fig. 1C). From this spectrum the vacuolar and cytoplasmic pHs were estimated to be <5.5 and 7.1, respectively. The best resolution of the root nodule spectrum was found when detached nodules were split and transferred into the 10 mm NMR tube (Fig. 1D). With split nodules perfused with an $O_2$-saturated buffered medium (pH 7.5) containing 50 mM glucose and 0.1 mM $CaSO_4$, the resolution of the spectrum improved significantly, especially in the region of the phosphomonoesters resonances. Three resonances in the phosphomonoester region (3–6 ppm) are well defined as is the resonance of an unidentified compound at 0.37 ppm, the frequency region of phosphodiesters. The origin of each resonance (see Fig. 1 legend) was assigned from the $^{31}P$ NMR spectrum of a neutralized (pH 7.8) perchloric acid extract of the nodules as described below. The spectra of the split nodules showed a cytoplasmic Pi resonance whose position corresponded to pH 7.4, whereas the pH of the acidic compartment was about 5.5. In general, the spectra of the split nodules were quite stable over periods up to 24 h. There was little change in the total $^{31}P$ resonance area, indicating no significant leakage of phosphate metabolites.

### $^{31}P$ NMR of Neutralized Perchloric Acid Extracts

Peaks in the $^{31}P$ NMR spectrum were identified using a proton-decoupled $^{31}P$ NMR spectrum of a perchloric acid extract of soybean root nodules at a final pH of 7.8 in the presence of 40 mM CDTA (Fig. 2). Samples prepared in such a manner had well resolved resonances which made the identification of each component straightforward from the literature data (18, 27) and by addition of known compounds to the extract. The following peaks were identified: glucose-6-P, fructose 1-6 P, choline-P, Pi, γ-α-β NTP, β-α NDP, UDP-glc, and NAD. An unidentified peak was also observed with a $^{31}P$ resonance at 0.37 ppm. Results from the addition of F-1, 6-diP to the extracts indicated that another compound, *e.g.* fructose-6-P or ribose-5-P, may be present under the high field C-6 resonance.

A low NTP/NDP ratio (2/1) was observed *in vivo* (Fig. 1D). A slightly lower NTP/NDP was observed in the spectrum of the perchloric acid extracts of the nodules (Fig. 2). The small increase in NDP after extraction may be a result of hydrolysis of NTP during the extraction procedure. However, in both *in vivo* and *in vitro* spectra, the NTP/NDP ratio is unusually

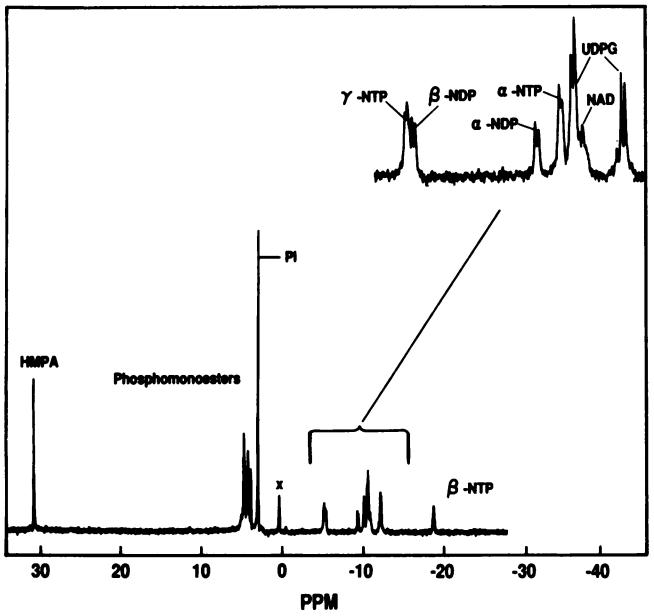


**Figure 2.** Proton decoupled $^{31}P$ NMR spectrum of perchloric acid extract of root nodules of soybean. Perchloric acid extracts were prepared from root nodules infected by *B. japonicum* USDA 110 (24 g fresh weight). Freeze-dried extracts prepared as described under "Materials and Methods" were solubilized in a solution containing CDTA (40 mM). The sample (3.5 mL) containing 700 μL of $D_2O$ was put in a 10 mm NMR tube. The $^{31}P$ NMR spectra were recorded at 161.7 MHz over a period of 2 h with a 56° pulse angle (20 μs), a sweep width of 16,000 Hz, and a recycling time of 2.082 s. Free induction decays (3,600) were accumulated using 16,000 data points. A 1 Hz line broadening was applied. Low power proton decoupling was employed. The nucleotide region is shown in the expanded scale. Peak assignments (from low to high field): HMPA, external reference at 30.73 ppm; phosphomonesters; Pi; x, unidentified peak; NTP; NDP; UDP-glc; NAD.

**Table I.** *ATP and ADP Content of Intact and Split Soybean Nodules before and after perfusion in a Buffer*

| Nodule Treatment[a] | Incubation Time | ATP | ADP | ATP/ADP |
|---|---|---|---|---|
| | *h* | *nmol/g fr wt nodules* | | |
| Whole | 0 | 39.4 ± 10.6[b] | 97.6 ± 13.6[b] | 0.40 |
| | 2 | 30.2 ± 2.7 | 51.0 ± 1.4 | 0.59 |
| | 4 | 27.8 ± 1.1 | 32.9 ± 1.0 | 0.85 |
| Split | 2 | 30.1 ± 1.7 | 93.1 ± 12.5 | 0.32 |
| | 4 | 33.1 ± 4.2 | 44.1 ± 9.9 | 0.75 |

[a] Whole or split nodules infected by *B. japonicum* USDA 138 were perfused with a buffer solution (10 mM Mes [pH 7.5]) containing 50 mM glucose and 0.1 mM $CaSO_4$. [b] The results represent the average of three assays ± SD.

low and appears to be a feature unique to nodule metabolism. The low ratio of ATP/ADP was confirmed by chemical analyses (Table I). Initially in the soybean nodules, the ADP level was 2.5 times larger than the ATP level. Four h after starting perfusion with an $O_2$-saturated buffer, the ATP content decreased 30% while the ADP content decreased 67%. For the split nodules, the diminution of the ATP level was only 16% for the same time period, half that of the intact nodules. The ADP content stayed relatively constant during the first 2 h and decreased 54% after 4 h of perfusion. In general, extremely high NTP/NDP ratios (25/1) have been observed previously by $^{31}P$ NMR spectroscopy of homogeneous plant cells (27) and in plant tissues (16, 23). Therefore, the low ATP/ADP ratio observed with soybean nodules is quite unusual.

In addition, we examined whether splitting of nodules causes a significant perturbation of their metabolism by comparing acetylene reduction activity for whole and split nodules exposed to perfusion media (Table II). A comparison between both split and nonsplit nodules showed a lower acetylene reductase activity for the split nodules. This was presumably due to the extreme sensitivity of nitrogenase to oxygen. Acetylene reduction activity of both whole and split nodules dropped very rapidly after 30 min of perfusion treatment. However, the drop in acetylene reduction activity for split nodules over 4 h is less compared to whole nodules.

### Effect of Greenhouse Growth Conditions and Nodule Age

The spectra of nodules from plants grown in a greenhouse were compared to spectra of nodules from plants grown in a growth chamber (Fig. 3). Forty percent of Pi present in the nodules from the growth chamber plants accumulated in the acidic compartment (pH < 5.5), which is assumed to be the vacuole (14) and the remaining presumably in the cytoplasm (Fig. 3A). Nodules that were grown during the summer in the greenhouse without strict environmental controls displayed a different distribution of phosphate in their spectrum (Fig. 3B). Figure 3B is shown in a reduced form to indicate the true size of the vacuolar Pi resonance. A similar spectrum was obtained for nodules from 3-month-old plants (not shown). The enlarged version of spectrum 3B is shown in Figure 3C. Under these conditions, there was a large increase of Pi in the vacuoles that comprised 96% of the mobile Pi in the nodules. The cytoplasmic Pi remained relatively constant. Indeed, a comparison of the soluble P compounds (Pi, phosphomonoesters, NTP, UDP-glc, NAD) in the cytoplasmic compartment of plants grown under both conditions (Fig. 3, A and C) showed similar intensities. Both cytoplasmic and vacuolar pHs were comparably maintained in the plant nodules derived from both growing conditions.

**Table II.** *Acetylene Reduction Activity of Split and Intact Nodules before and during the Perfusion*

The perfusion medium containing 50 mM glucose and 0.1 mM $CaSO_4$ was buffered with 10 mM Mops at pH 7.5 and saturated with $O_2$.

| Time of Perfusion | Intact Nodules[a] | Split Nodules[a] |
|---|---|---|
| *h* | *μmol $C_2H_2$/g fr wt nodules/h* | |
| 0 | 2.96 ± 0.94[b] | 1.83 ± 0.34 |
| 0.5 | 0.15 ± 0.02 | 0.28 ± 0.05 |
| 1 | 0.09 ± 0.02 | 0.26 ± 0.01 |
| 2 | 0.03 ± 0.02 | 0.15 ± 0.01 |
| 4 | 0.01 ± 0.01 | 0.050 ± 0.00 |

[a] The nodules infected by *B. japonicum*. USDA 138 were 58 d old. [b] The results represent the average of three assays ± SD.

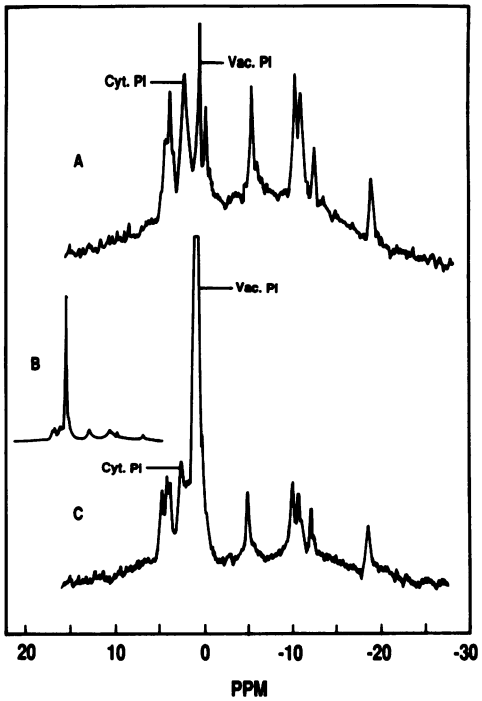


**Figure 3.** Comparison of $^{31}P$ spectra of soybean root nodules between plants grown (A) in a growth chamber under strict control of temperature, humidity, and photoperiod and (B, C) in a greenhouse (see "Materials and Methods"). B, Reduced spectrum of soybean root nodules (to emphasize the relative size of the vacuolar Pi) after their development in a greenhouse without strict control of temperature (16–35°C) and humidity during the summer. C, Normal presentation of spectrum B. Each spectrum represents the accumulation of 20,000 free induction decays. The nodules were split and perfused with a buffered solution containing 50 mM glucose, 0.1 mM $CaSO_4$, and 10 mM Mops (pH 7.5). $O_2$ was bubbled into the reservoir. Cyt.Pi, cytoplasmic inorganic phosphate; Vac.Pi, vacuolar inorganic phosphate.

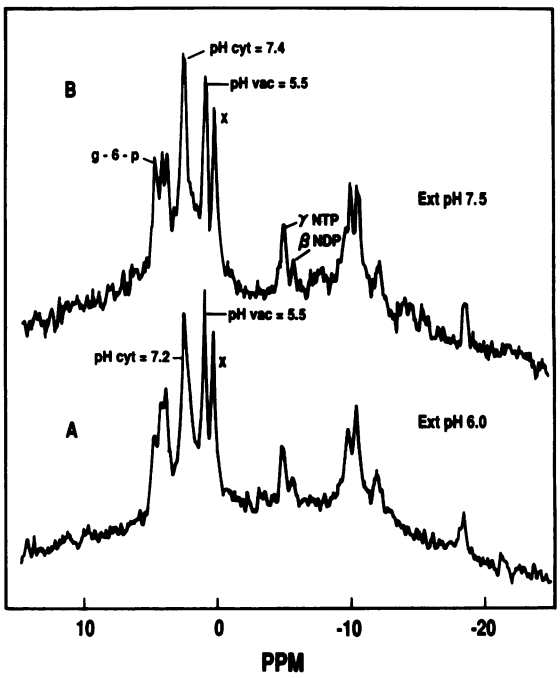


**Figure 4.** *In vivo* $^{31}P$ NMR spectra (20,000 transients) of root nodules infected by *B. japonicum*. The nodules were split and perfused with a $O_2$-saturated buffer solution containing 50 mM glucose, 0.1 mM $CaSO_4$ at pH 7.5 with 10 mM Mops (A) and at pH 6.0 with 1.0 mM Mes (B). Peak assignments as in Figure 2.

### Effect of External pH

In this experiment, root nodules infected by *B. japonicum* USDA 138 were transferred intact to an NMR tube and perfused with an $O_2$-saturated buffered solution containing 50 mM glucose and 0.1 mM $CaSO_4$ (pH 6.0). The spectrum of these nodules (Fig. 4A) is nearly identical to that observed from nodules infected with *B. japonicum* USDA 110. The pHs of the cytoplasm and the vacuole (Pi shift) corresponding to a chemical shift of 2.70 and 1.00 ppm is 7.2 and 5.5, respectively, when perfused with a pH 6.0 buffer. Also, with the pH 6.0 medium, the solution of the three phosphomonoester resonances (3–6 ppm region) was poor (Fig. 4A). Raising the external pH to 7.5 with KOH caused an increase in the cytoplasmic pH from 7.2 to 7.4. In addition, the resonance of glucose-6-P moved downfield to a shift position corresponding to the cytoplasmic pH of 7.4. In general, better resolution of the phosphomonoester resonance was observed. Resonances of vacuolar Pi and of compound X showed no measurable change over the external pH range of 6.0 to 7.5. The observed pH-independence of the chemical shift assigned to compound X could either be due to the absence of compound X from the pH-sensitive cytoplasm or due to a pKa outside the range of 6.0 to 7.5. The better separation of the different phosphomonoesters is probably associated with the diversity of the pKas of these compounds. An increase in glucose-6-P was also observed for the spectrum of the nodules subjected to the higher pH medium.

### Effect of Glucose and 2-Deoxyglucose Supply

Figure 5A illustrates the typical spectrum of soybean nodules observed 90 min after the start of perfusion. After 15 h of perfusion, a significant amount of glucose had penetrated the cells, favoring an increase in G-6-P, a concomitant decrease in vacuolar Pi, and readjustment of the F-1, 6-diP and P-choline levels (see Fig. 5, A and B). The maintenance of a constant level of P-choline, as previously reported by Roby *et al.* (27), suggests that the integrity of the nodule cell membranes has been preserved. After 15 h of perfusion with 50 mM glucose, the system was flushed with a solution containing 5 mM 2-deoxyglucose. After 2 h and 40 min of perfusion with 2-deoxyglucose, there were no significant changes in nucleotides and compound X levels. A similar result was found previously (17) with excised maize root tips treated with 2-deoxyglucose. The resonances corresponding to UDP-glc slowly disappeared and were replaced with the newly formed 2-deoxyglucose metabolite (UDP-2-dglc). Figure 5C depicts an intermediate stage of 2-deoxyglucose incorporation in which there exists a mixture of both UDP-glc and UDP-2-dglc, whereby weak $^{31}P$ shifts are observed for the upfield $\beta$-$^{31}P$ resonances of these derivatives. The lower field resonance is almost a superposition for both derivatives, and consequently its intensity is not significantly affected by the equil-

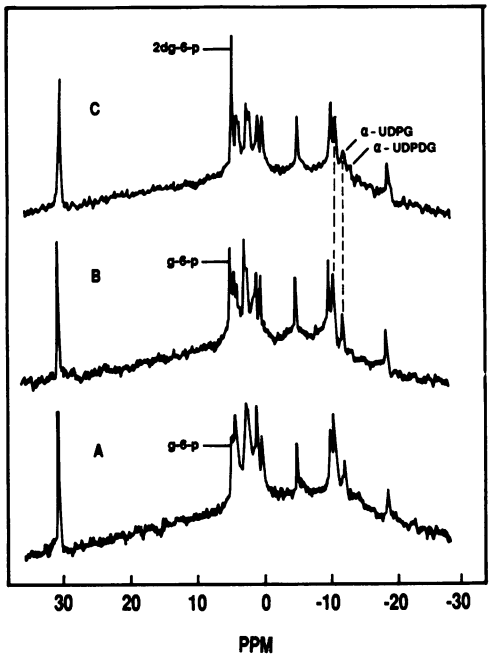


**Figure 5.** A, *In vivo* $^{31}$P NMR spectrum (20,000 transients) of split nodules after 90 min of perfusion with an $O_2$-saturated buffer solution at pH 7.5 (10 mM Mops, 0.1 mM $CaSO_4$, and 50 mM glucose). B, Same as A after 15 h of perfusion. C, Same as B after 160 min following the addition of 5 mM 2-deoxyglucose. 2dg-6-P, 2-deoxyglucose-6-phosphate.

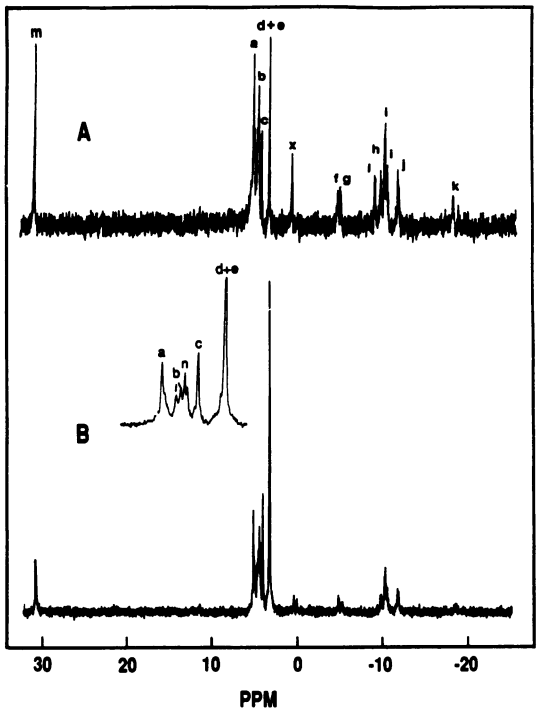


**Figure 6.** Proton decoupled $^{31}$P NMR spectra of perchloric extracts of nodules. A, Nodules were detached, split, and perfused with an $O_2$-saturated buffer solution containing 10 mM Mops (pH 7.5), 0.1 mM $CaSO_4$, and with 50 mM glucose after 4 h, or B, without glucose after 17 h. The perchloric acid extract is described under "Materials and Methods." Spectrum A represents 1000 transients and spectrum B 2000 transients with a recycle time of 2.082 s (total time of accumulation 34 min (A) and 68 min (B). A 1 Hz linebroadening was applied. Peak assignments are as in Figure 1; α-NDP, 1; HMPA, m; NMP, n.

ibration of the two derivatives. The large peak corresponding to 2-deoxyglucose-6-P at 4.95 ppm, which is not resolvable from the resonance of G-6-P, has grown at the expense of the cytoplasmic Pi. Presumably, the movement of vacuolar Pi is not rapid enough to maintain the cytoplasmic Pi level under these conditions. Clearly, glucose or 2-deoxyglucose penetrate the split nodules, are phosphorylated and accumulated rapidly. The small amount of G-6-P in split nodules, after 15 h of perfusion with glucose, indicates that this sugar is also phosphorylated, but is readily consumed to maintain the high level of nucleotides.

Evidence that the cortex of the nodules provides a significant barrier to nutrient penetration is demonstrated in an experiment carried out with intact nodules treated with 2-deoxyglucose. In such a study, buildup of 2-deoxyglucose-6-P occurred only after 11 h of perfusion (spectrum not shown).

In parallel with the *in vivo* study of glucose uptake, a perchloric acid extract of split nodules was evaluated after the nodules were in the glucose medium for 4 h. The spectrum (Fig. 6A; see legend of Fig. 1 for shift assignments) shows high levels of G-6-P, F-1, 6-diP, P-choline, and nucleotides relative to Pi. However, there is no significant change in the relative concentrations of the phosphomonoesters compared to the spectrum in Figure 2. Evidently, endogenous Pi was depleted for the production of the different phosphomonoesters and nucleotides. Under these experimental conditions, the changes in the distribution of the phosphomonoesters *in vivo* occurred only after 4 h of perfusion.

### Effects of Glucose Starvation

The effect of glucose omission was studied with split nodules over a 17 h period (Fig. 7). The spectrum recorded 90 min after the start of the perfusion appeared to have reduced levels of compound X and nucleotides (Fig. 5A). After 6 h (Fig. 7B), the intensity of the P resonance of nucleotides, UDP-glc and NAD were not changed. The phosphomonoesters and cytoplasmic Pi resonances underwent a 23% loss in intensity, whereas the vacuolar Pi resonances had dropped by 50%. After 17 h (Fig. 7C), the nodules continued to maintain the same levels of intracellular nucleotides except for the level of NAD. After 17 h, the G-6-P had increased slightly. Compound X was maintained at its initial intracellular level. After 24 h of perfusion, the total intensity of the $^{31}$P nodule spectrum declined uniformly (spectrum not shown). In parallel with the *in vivo* study of glucose starvation, a perchloric acid extract of nodules, which were perfused for 17 h with a $O_2$-saturated, buffered medium (pH 7.5) containing 0.1 mM $CaSO_4$, was evaluated. The spectrum (Fig. 6B, see legend of Fig. 1 for shift assignment) shows a high level of Pi compared to the nucleotide peaks (between −5 and −20 ppm). In addition, NTP and NDP were scarcely detectable. NMP, G-6-P, F-1, 6-diP, and P-choline were observed (see Fig. 6B enlargement insert of the low field area).

## DISCUSSION

Our $^{31}$P NMR experiments with soybean root nodules have established the optimum growth conditions, perfusion con-

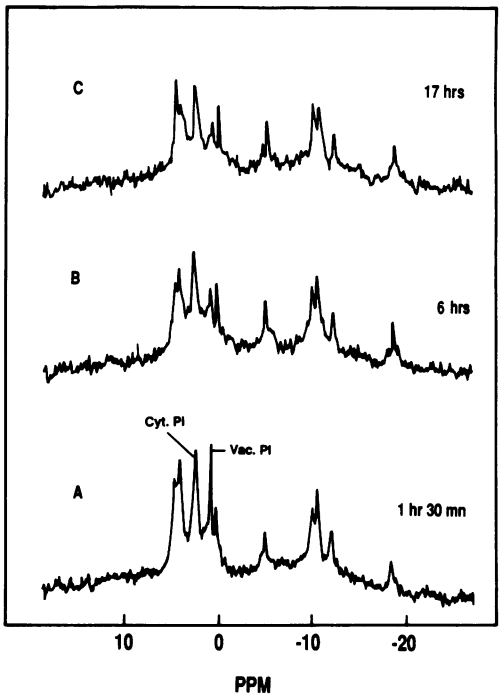


**Figure 7.** Representative *in vivo* $^{31}P$ NMR spectra of carbohydrate starved root nodules. Each spectrum recorded at 23°C represents the time average of 20,000 free induction decays with a recycle time of 0.162 s (total time of accumulation 54 min). Split nodules were perfused with an $O_2$-saturated buffer solution containing 10 mm Mops (pH 7.5) and 0.1 mM $CaSO_4$. Spectrum records A, 1 h 30 min; B, 6 h; C, 17 h after the start of the experiment.

ditions, and spectroscopic parameters necessary for obtaining the best quality, most reproducible *in vivo* spectra possible. The composition of the soluble P compounds was identified by both *in vivo* and *in vitro* NMR. Also, the *in vivo* methodology allowed us to establish that glucose was readily metabolized in the symbiotic system. The P content in the nodules and, in particular, the accumulation of Pi in the vacuole appeared to be very sensitive to the growth conditions. We found that the soybeans growing in a greenhouse without strict control of temperature and humidity were subject to temperature stress and/or water stress and produced nodules that accumulated large amounts of vacuolar Pi. The Pi was stored in the nodule plant cell vacuoles instead of being transported to the shoots because the stomates were closed and evapotranspiration was reduced. Similar results were found by Mitsumori *et al.* (14). These observations suggest one important function of vacuolar Pi is to buffer Pi content of the cytoplasm (8, 21).

Although attached nodules represent the best physiological condition for studying their metabolism by $^{31}P$ *in vivo* NMR, instrument restrictions, inability to perfuse, and the limited quantity of nodule tissue within the probe (10 mm tube) do not allow for acceptable signal/noise and resolution to study metabolism involving UDP-glc, NAD, and other nucleotides.

Although more detached nodules could be located within the probe coils to improve the signal/noise of the spectrum, it was not possible to obtain enough resolution to distinguish the resonances of different phosphomonoesters and nucleotides without perfusion. In our work, the utilization of a perfusion system with intact detached nodules significantly improved the quality of the spectra. Supplying a carbon source and $O_2$ during the recording of the spectrum maintained the viability of intact nodules for more than 41 h (data not shown). Spectral resolution was further improved when the nodules were split and perfused with a pH 7.5 medium. Split nodules provided a compromise for studying *in vivo* processes by $^{31}P$ NMR spectroscopy. Nodules consist of a heterogenous population of cells, that is, cortical cells on the surface and both infected and uninfected cells in the inner sphere. In this intact state, it was difficult to study nodule metabolism since, the penetration time of substrates such as glucose or 2-deoxyglucose was relatively slow. The fast penetration of 2-deoxyglucose in split nodules (2 h and 40 min) was in sharp contrast to the slow uptake (11 h) observed for the nonsplit nodules.

Also, we found that split nodules maintained higher levels of acetylene reductase activity (Table II). These results indicate that the nitrogenase and presumably the metabolism of split nodules was more stable than in intact nodules.

Long-term perfusion experiments with split nodules could not be conducted for more than 24 h, presumably because of phosphate leakage, whereas intact nodules remained viable up to 41 h. In medium buffered at pH 7.5, the NMR spectra of split nodules had better resolution with good separation of the different phosphomonoester resonances. This enhanced separation is probably a result of the increased pH observed for the cytoplasmic compartment and the divergent pH/chemical shift profiles (pKa titration curves) for each of the observable phosphomonoesters, respectively. Similarly, good resolution was also obtained with whole nodules only after 10 h of perfusion, at which time the pH of the cytoplasm had increased.

New knowledge about symbiosis has been provided by this *in vivo* study. The composition of sugar phosphates was determined in spectra of perchloric acid extracts. The two main sugar-phosphates participating in nodule metabolism were G-6-P and F-1, 6-diP. The lack of accumulation of G-6-P under the same conditions when the nodules were perfused with glucose seemed to indicate that glucose is phosphorylated and subsequently metabolized by the nodules. However, it is not known whether glucose is being utilized by the plant cells and/or by the bacteroids. At the same time, a relatively high level of G-6-P was found to be maintained *in vivo* following glucose starvation. This observation suggests the presence of an endogenous source of carbohydrate which is capable of providing the energy necessary to maintain the adenylate energy charge and carbon source. Such compounds could be either sucrose or starch stored in the vacuole of uninfected plant cells and/or trehalose in the bacteroids (29).

The relatively high levels of nucleotides observed during the experiments when the nodules (split or unsplit) were perfused with an $O_2$-saturated medium containing 50 mM glucose suggested that oxidative phosphorylation was highly active. Such activity is not characteristic of the energetic metabolism that was observed in attached nodules (Fig. 1A). At the beginning of each perfusion experiment, the relatively low NTP/NDP ratio observed by $^{31}P$ NMR seemed to confirm

the hypothesis that the energetic charge was initially low in nodules. After more than an hour of perfusion, the NDP resonance disappeared completely (Figs. 5–7). The independent determination of ATP and ADP by an *in vitro* luciferin-luciferase technique showed a big decrease of ADP but without complete disappearance (Table I). The increase in the NTP/NDP ratio is probably related to the stimulation of oxidative phosphorylation and inhibition of nitrogenase by oxygen (a major consumer of NTP).

Electron microscopic studies of nodules (2) show three distinct types of cells within the enclosed structure: (a) the cortical cells which provide protection as a barrier to gas diffusion, (b) the uninfected plant cells containing a vacuole, and (c) the infected cells containing many thousands of bacteroids and no vacuole. On the basis of this description, one might expect to observe three distinct Pi resonances corresponding to the PHs of (a) the relatively alkaline cytoplasms of the cortical and both infected and uninfected plant cells, (b) their respective acidic vacuoles, and (c) the somewhat more neutral pH, cytoplasmic compartments of the prokaryote bacteroid cells. However, as we have seen in the spectra presented herein, only two Pi resonances are observed in the spectra of the nodules (split, unsplit, attached, or detached). It seems evident from the behavior of the resonance at approximately 1.0 ppm (see above) and its correspondence to a relatively low pH (5.5) that it represents the vacuole of the uninfected plant cells as suggested earlier (14) and possibly Pi contained in the peribacteroid space. The somewhat broader resonance corresponding to a pH from 7.2 to 7.4 presumably represents Pi contained in the cytoplasms of both plant and bacteroid cells. Although the cytoplasmic pH of the prokaryote cells are in general slightly more acidic (6.8–7.1) (14) than those of plant cells (7.1–7.4) (16–20), the cytoplasmic Pi resonance of the former was not resolved in these nodule spectra. The lack of resolution of the resonances representing these two compartmental cytoplasmic Pi pools is probably related to the overlap of two lines in a broadened baseline in this region of the spectrum. Alternatively, if there is rapid exchange (<msec) of Pi between the cytoplasmic compartment and peribacteroid space, for example, only one resonance would be visible. In addition, the bacteroids may only contribute a relatively small amount of Pi intensity to the resulting signal in this area. Under certain conditions, we have observed a splitting of the cytoplasmic Pi resonance, suggesting that distinct compartments exist (*e.g.* see Fig. 5). However, this resolution is not always reproducible and may reflect only transitory pH changes in sections of cells receiving different amounts of penetrating $O_2$.

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