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Steady and Nonsteady State Gas Exchange Characteristics of Soybean Nodules in Relation to the Oxygen Diffusion Barrier[1]

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ABSTRACT

An open gas exchange system was used to monitor the nonsteady state and steady state changes in nitrogenase activity (H_2 evolution in N_2:O_2 and Ar:O_2) and respiration (CO_2 evolution) in attached, excised, and sliced nodules of soybean (*Glycine max* L. Merr.) exposed to external pO_2 of 5 to 100%. In attached nodules, increases in external pO_2 in steps of 10 or 20% resulted in sharp declines in the rates of H_2 and CO_2 evolution. Recovery of these rates to values equal to or greater than their initial rates occurred within 10 to 60 minutes of exposure to the higher pO_2. Recovery was more rapid at higher initial pO_2 and in Ar:O_2 compared to N_2:O_2. Sequential 10% increments in pO_2 to 100% O_2 resulted in rates of H_2 evolution which were 1.4 to 1.7 times the steady state rate at 20% O_2 in Ar. This was attributed to a relief at high pO_2 from the 40% decline in nitrogenase activity that was induced by Ar at a pO_2 of 20%. Changes in nodule respiration rate could not account for the nodules' ability to adjust to high external pO_2, supporting the hypothesis that soybean nodules have a variable barrier to O_2 diffusion which responds slowly (within minutes) to changes in pO_2. Nodule excision and slicing resulted in 45 and 78% declines, respectively, in total specific nitrogenase activity at 20% O_2. In contrast with the result obtained with intact nodules, subsequent 10% increases in pO_2 in Ar:O_2 did not result in transient declines in H_2 evolution rates, but in the rapid attainment of new steady state rates. Also, distinct optima in nitrogenase activity were observed at about 60% O_2. These results were consistent with an increase in the diffusive resistance of the nodule cortex following nodule excision or nodule slicing. This work also shows the importance of using intact plants and continuous measurements of gas exchange in studies of O_2 diffusion and nitrogenase activity in legume nodules.

N_2 fixation in legume nodules depends on an enzyme complex that is O_2 labile yet requires a large amount of ATP derived from oxidative phosphorylation (17). The nodule must therefore exercise stringent control over its internal pO_2 to maintain optimal conditions for both respiration and nitrogenase activity. Studies with soybean nodules have shown that the concentration of free O_2 within the infected zone under atmospheric conditions is approximately 10 nM (1, 2). The high respiration rate of nodules in support of N_2 fixation may play a significant role in maintaining this low internal pO_2, but it has been suggested that O_2 regulation cannot be accounted for by respiration alone, and the presence of a variable barrier to gas diffusion in the nodule has been proposed (18). Evidence for a distinct barrier to gas diffusion in the nodule cortex has been obtained experimentally by direct measurements of pO_2 in the outer and central tissues of soybean nodules (21).

The exact nature of the diffusion barrier and the manner in which it is regulated are unknown, but it has been suggested that the nodules of many symbiotic associations increase their diffusion resistance to O_2 entry when they are exposed to an atmosphere containing 10% C_2H_2, or one in which N_2 is replaced by Ar (25). This increase in diffusion resistance is apparent as a decline in nodule respiration rate, with a concomitant decline in C_2H_2 reduction or H_2 production rate, during the first 30 min of exposure to C_2H_2 or Ar. The presence or absence of an C_2H_2- or Ar-induced decline has been correlated with the optimum pO_2 for N_2 fixation within a specific legume-*Rhizobium* association and, by inference, with the speed with which the diffusion barrier is regulated in that association (25). It has also been suggested that the regulation of the diffusion resistance of the nodule requires physical changes in the diffusion barrier (4), and this may limit the speed with which the nodule responds to changes in its gaseous environment.

The aims of this study were (a) to determine whether the gas exchange characteristics of soybean nodules following changes in external pO_2 are consistent with the presence of a variable diffusion barrier in the nodule cortex, and (b) if so, to identify experimental conditions which could be used to vary this diffusion barrier. An open circuit gas exchange system was used to monitor continuously changes in respiration (CO_2 evolution) and nitrogenase activity (H_2 evolution in N_2:O_2 and Ar:O_2) as the pO_2 surrounding the nodules was varied. Nonsteady state measurements of CO_2 production and H_2 evolution were used to determine the speed with which the nodules adjust to changes in rhizosphere O_2 concentration. Steady state measurements were used to determine the optimum O_2 concentration for nitrogenase activity and to assess the role of respiratory protection in maintaining this activity at high O_2 concentrations. For comparative purposes experiments were performed with intact nodulated roots, detached nodules, and detached nodules in which the diffusion barrier was disrupted by slicing.

MATERIALS AND METHODS

Plant Culture and Experimental Conditions. Seeds of soybean (*Glycine max* L. Merr.) cv Harosoy 63 were surface sterilized with a 0.3% (v/v) solution of sodium hypochlorite for 10 min, rinsed, and planted in sterile silica sand in plastic pots modified to permit sealing for gas exchange analysis (23). The plants were inoculated at sowing with a peat-based inoculum (Urbana Laboratories, Urbana, IL) containing the Hup$^-$ strain *Bradyrhizobium japonicum* USDA 16 (Dr. H. H. Keyser, USDA-ARS, Beltsville, MD). The absence of uptake hydrogenase activity in this symbiosis has been confirmed by the lack of H_2 consumption in the presence of C_2H_2 (11), background rates of $^3H_2$ uptake

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(11), and the inability of bacteroids to reduce methylene blue (10) (data not shown). The pots were watered daily with a N-free nutrient solution, and the seedlings were grown in a greenhouse (25–31°C, February to June, Kingston, Canada) with supplemental lighting (800 μmol quanta m^{-2} s^{-1}; 16 h photoperiod) provided by metal arc lights (Widelite Ltd., London, Ont.). Plants were used in experiments 28 to 35 d after sowing.

All experiments were performed at a temperature of 22 to 25°C with a photon flux density at the leaf surface of 100 μmol quanta physiologically active radiation m^{-2} s^{-1}. Since soybean nodules do not show diurnal fluctuations in N_2 fixation (23), the change in photon flux density between the plant growth and experimental conditions was assumed to have no effect on nitrogenase activity during the time course of the experiments.

Gas Exchange Apparatus. Rates of CO_2 and H_2 production from nodules were measured simultaneously and continuously with an IR gas analyzer (IRGA Series 225, Mark 2, ADC, Hoddesdon, UK) and a H_2 analyzer (12) linked in series. Mass flow controllers (FMA series 100, Omega Engineering Inc., Stamford, CT) were used to mix O_2 with either N_2 or Ar to produce any desired ratio of N_2:O_2 or Ar:O_2 at 1 atmosphere total pressure, and a three-way solenoid valve allowed switching between N_2 and Ar as the balance gas at any time during the experiment. Contaminating CO_2 was removed by a soda-lime column, and the gas mixture was humidified and split into reference and analysis streams. The flow through the reference and analysis streams was controlled and monitored with needle valves and variable area flow meters. The analysis stream passed through a cuvette containing plant material, and both streams were then dried in an ice water bath. A portion of each gas stream was sampled using small pumps (Wisa Ltd. FDR), while the remainder was vented to atmosphere through a flow meter. Before entering the IRGA and H_2 analyzer, the streams were dried further by passage through glass columns (0.6 × 10.0 cm) containing magnesium perchlorate, since the stability and sensitivity of the H_2 analyzer is adversely affected by water vapor (12). The H_2 analyzer was calibrated for pH_2 in N_2:O_2 and Ar:O_2 at each pO_2.

As the H_2 analyzer has at least some sensitivity to all combustible gases (12), evidence was required that nitrogenase-produced H_2 was the only combustible gas evolved from the nodules. This evidence included: (data not shown) (a) the lack of an analyzer response at any pO_2 when gas exchange was measured in nonnodulated or denodulated roots, (b) the lack of an analyzer response when nitrogenase activity in nodulated roots was inhibited by a rapid increase in pO_2 from 20 to 100%, (c) the observation that the peak rate of H_2 production measured by the H_2 analyzer following exposure of the nodulated roots to Ar:O_2 was similar to the peak rate of C_2H_4 production following exposure to 10% C_2H_2, and (d) the observation that the combustible gas produced by nodulated roots at 20% O_2 co-chromatographed with H_2 (12).

The H_2 production rate in Ar:O_2 was used as an indicator of total electron flux through nitrogenase (12). CO_2 production was used as an indicator of O_2 consumption by the nodulated roots, and changes in the respiration rate of nodulated roots with pO_2 were assumed to be associated with nodule activity, since denodulated root respiration did not change with pO_2 (data not shown).

Experiments with Intact Plants in Pots. (a) *Effect of increasing pO_2.* The root systems of intact, nodulated soybean plants were sealed within their growth pots and connected to the gas exchange system as described previously (23). At a gas flow rate of 500 cm^3 min^{-1} one pot volume of gas was exchanged every 36 s. To determine the optimal O_2 concentration for H_2 evolution in N_2, the intact root systems were exposed in turn to 5, 10, and 20% O_2 in N_2, and then to pO_2 in N_2 increasing in 10 or 20% increments up to and including 100% O_2. The optimum pO_2 for total nitrogenase activity was determined in a similar fashion, except that the balance gas was Ar instead of N_2. At each pO_2 in N_2 or Ar, CO_2 and H_2 production rates were monitored until steady state values were attained.

After exposure to 100% O_2, the root systems were reexposed to 20% O_2 in N_2 or Ar to assess the recovery of the initial activities under these conditions. In a separate experiment, steady state measurements of CO_2 and H_2 production from the root systems were made at 20% O_2 in N_2 or Ar, and then the pO_2 was increased directly to 100%. When new steady state rates were attained, the pO_2 in N_2 or Ar was reduced to 20%, and recovery of H_2 production rate and respiratory activity were monitored.

(b) *Effect of Decreasing pO_2 around Nodulated Roots Equilibrated at 100% O_2.* To assess the role of the diffusion barrier in restricting O_2 entry into the nodules, the gas composition in the pot was changed from 20% O_2 in Ar to 100% O_2 in increments of 20%, and when steady state rates of H_2 and CO_2 production were attained under 100% O_2, the concentration was reduced to 5% O_2 in Ar. When initial stable rates of H_2 and CO_2 production were attained (approximately 10 min), the root system was reexposed directly to 100% O_2. With the same plant, the procedure was repeated changing the pO_2 alternately between 100% and 10, 20, 40, 60, 80, and 90%. Steady state rates of H_2 and CO_2 production were measured during each exposure to 100% O_2, and initial stable rates were measured at each lower pO_2 before returning directly to 100% O_2.

(c) *The Argon-induced Decline and Measurements of EAC.*[2] Using intact, nodulated plants, the presence or absence of an Ar-induced decline in nitrogenase activity was determined by measuring steady state rates of H_2 and CO_2 production in N_2:O_2 at a known pO_2 and then switching to Ar:O_2 while maintaining the same pO_2. After steady state rates were attained in Ar:O_2, the balance gas was switched back to N_2 and the recovery of H_2 and CO_2 production rates was measured. With the same plant, this procedure was carried out at increasing pO_2 of 5, 10, 20, 40, 60, 80, and 100%.

The EAC of nitrogenase at each O_2 concentration was calculated according to Edie and Phillips (8):

$$\text{EAC} = 1 - \frac{\text{H}_2 \text{ production rate in } N_2\text{:}O_2}{\text{H}_2 \text{ production rate in Ar:}O_2} \quad (1)$$

At the end of each experiment the root system was removed from the pot, excised, and washed free of silica sand. The nodules were detached, and both the nodules and roots were dried at 80°C for 72 h and weighed.

Experiments with Intact Plants and Detached Nodules in the Cuvette. Since experiments with detached and sliced nodules were performed with a cuvette rather than a growth pot, we assessed the effect of root removal from the pot, washing, and the detachment of the nonnodulated portion of the root. A soybean plant was sealed into its growth pot, and measurements were made of the rates of H_2 and CO_2 evolution from intact, nodulated roots at 20% O_2 in N_2. The plant was then removed from the pot, the roots were washed free of silica sand, the lower nonnodulated roots excised, and the remaining roots sealed into a latex walled cuvette (Fig. 1) containing a scalpel blade. At a flow rate of 500 cm^3 min^{-1}, one cuvette volume of gas was exchanged every 15 s. The portion of the root system enclosed in the cuvette accounted for 95 to 100% of the nodule biomass and displayed a rate of H_2 evolution at 20% O_2 in N_2 similar to that measured in the growth pot. The specific rate of CO_2 evolution from the root system in the cuvette was higher than that in the pot, since nitrogenase-linked respiration comprised a higher proportion of the total root respiration after the excision

[2] Abbreviations: EAC, electron allocation coefficient; DW, dry weight; nod, nodules; NR, nodulated root system.

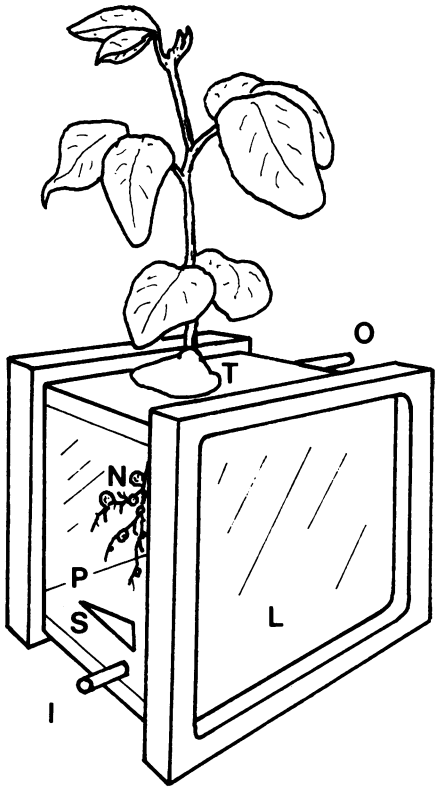

FIG. 1. A cuvette which allowed for continuous, sequential gas exchange measurements in intact attached nodules, detached nodules, and nodule slices from a plant under controlled gaseous environmental conditions. The cuvette consisted of a Plexiglas cube (125 cm^3 internal volume) with a side that could be removed to allow the insertion of an intact plant with the stem protruding through a slit in the top of the cube and sealed with Terostat (T), a flexible sealant. Two opposing sides of the cube consisted of Plexiglas frames supporting latex rubber windows (L) through which a scalpel blade (S) could be manipulated for nodule excision and slicing, without exposing the nodules to the atmosphere. The other four sides (P) were of 6.35 mm Plexiglas, and two of these housed the gas inlet (I) and outlet (O) fittings. To prevent root and nodule desiccation during the course of the experiment, a layer of moist filter paper was placed in the bottom of the cuvette, and the shoot system was sprayed with water and enclosed in a clear plastic bag to reduce transpirational water loss.

of nonnodulated roots.

H_2 evolution in N_2:O_2 and Ar:O_2 in the intact, attached nodules was determined at pO_2 increasing in increments of 10% as described above. The effect of nodule detachment on total nitrogenase activity was investigated by first exposing the nodules to an atmosphere of 5% O_2 in Ar, and then excising the nodules at their point of attachment to the root. The O_2 optimum for nitrogenase activity in the detached nodules was then determined by measuring H_2 and CO_2 production rates in Ar:O_2 at 5 and 10% O_2, and then at pO_2 increasing from 10 to 100% O_2 in 10% increments. This experiment was repeated with nodules that were detached at 5% O_2 in Ar and then sliced in half with the scalpel blade. The effect of a direct increase in pO_2 from 5 to 70% O_2 on H_2 and CO_2 production rates in detached and sliced nodules was also measured.

At the end of each experiment the plant material within the cuvette was removed, and the roots and nodules were dried as above and weighed separately. The root material excised before enclosure in the cuvette was also dried and weighed.

RESULTS

Experiments with Intact Plants in Growth Pots. (a) *Effect of Increasing pO_2.* When the pO_2 surrounding a nodulated root was increased, distinct and highly repeatable transient responses were observed in both H_2 and CO_2 production rates, similar to those shown in Figure 2A. In a N_2:O_2 atmosphere the H_2 and CO_2 production rates declined immediately following an increase in pO_2 and reached minimum rates (t) within 5 min. This was followed by a gradual increase in production, with distinct oscillations, reaching steady state rates (s) within 60 min. In an Ar:O_2 atmosphere an increase in pO_2 also resulted in an immediate trough (t) in H_2 and CO_2 production rates, but these recovered within 10 min to reach peak rates (p) higher than the initial steady state rates (Fig. 2A). The rates then declined to new steady state rates (s) within 60 min. In both N_2:O_2 and Ar:O_2, the trough values in the rate of H_2 and CO_2 production following an increase in pO_2 were O_2 dependent, the rates being lowest when the pO_2 was changed from 10 to 20% and increasing at each higher pO_2 (Fig. 2, B and D).

When the pO_2 was increased from 5 to 10%, and then in 20% increments between 20 and 100%, little change was observed in steady state rates of H_2 and CO_2 production in either N_2:O_2 or Ar:O_2 at pO_2 between 10 and 100% (Fig. 2, B and D). The upward trend in H_2 production rate at O_2 concentrations above 60% was attributed to a decline in EAC associated with low pN_2 at higher pO_2 (Fig. 4C).

When pO_2 was increased in 10% increments, a significant increase was observed in the rate of H_2 production in both N_2 and Ar between 50 and 100% O_2 (Fig. 2C). The transient changes in H_2 and CO_2 production rates at each increase in pO_2 were similar to those illustrated in Figure 2A except that new steady state rates were attained in a shorter time.

After the root system had been exposed to increasing pO_2 in N_2 to 100% O_2, a direct reduction in pO_2 to 20% caused an immediate decline in H_2 production rate to almost zero, with a parallel decline in respiration rate to approximately 60% of its value at 100% O_2 (data not shown). This was followed by a full recovery of H_2 and CO_2 production rates to their previous values at 20% O_2 over a period of 120 to 180 min. A similar response was shown when Ar:O_2 gas mixtures were used rather than N_2:O_2 except that the recovery period was less than 60 min.

In nodulated roots which had fully recovered at 20% O_2 in either N_2 or Ar, H_2 evolution was totally inhibited by subsequent direct exposure to 100% O_2, and CO_2 production declined to a new steady state rate. This inhibition was irreversible in the presence of 100% O_2, but if the roots were exposed to 100% O_2 for only 30 min and then returned to 20% O_2 in N_2 or Ar, there was an initial lag period of 60 min or more and then H_2 and CO_2 production rates recovered slowly to their initial rates over a 13 to 30 h period. A direct increase from 20 to 100% O_2 in either N_2 or Ar without prior exposure to 100% O_2 also caused long-term inhibition of H_2 and CO_2 production.

(b) *Effect of Decreasing pO_2 around Nodulated Roots Equilibrated at 100% O_2.* When the gas composition in the pot was reduced from 100% O_2 to any lower concentration of O_2 in Ar, an immediate decline in H_2 and CO_2 production rates was observed, and trough values were attained within 10 min (Fig. 3A). The magnitude of the inhibition was proportional to the size of the change in pO_2. Therefore, the lowest rates of H_2 and CO_2 evolution were observed following transfer to the lowest pO_2 in Ar (Fig. 3B). Returning the pO_2 to 100% during the duration of the trough resulted in a full recovery of H_2 production (Fig. 3A) and CO_2 production (data not shown) to their initial rates at 100% O_2 within 10 min.

(c) *The Argon-induced Decline and EAC.* Intact attached nodules of soybean showed a distinct Ar-induced decline in H_2 and CO_2 production. Figure 4A illustrates the typical fluctuations

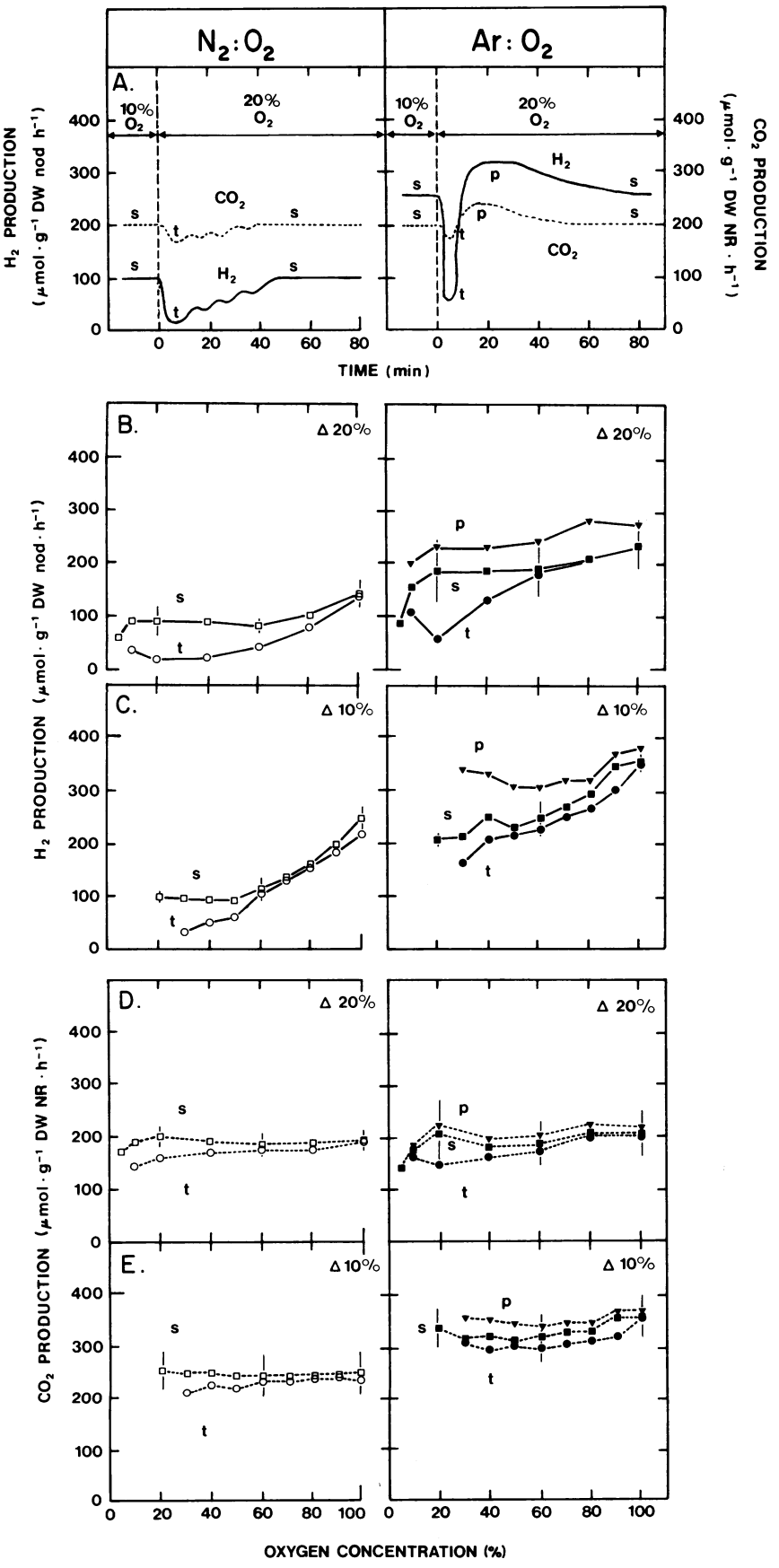

FIG. 2. Steady state and nonsteady state rates of H_2 and CO_2 production in intact, attached nodulated roots of soybean at increasing O_2 concentrations in N_2 or Ar. A, Time course of the transient changes in H_2 and CO_2 production rates on the transfer from 10 to 20% O_2 in N_2 and Ar. B and D, H_2 and CO_2 production rates with O_2 concentration increasing in 20% increments (Δ20%). C and E, H_2 and CO_2 production rates with O_2 concentration increasing in 10% increments (Δ10%). s, Steady state rate of H_2 or CO_2 production; t, trough rate of H_2 or CO_2 production; p, peak rate of H_2 or CO_2 production. Each point is the mean value of 4 to 8 replicate treatments. Representative standard errors are shown.

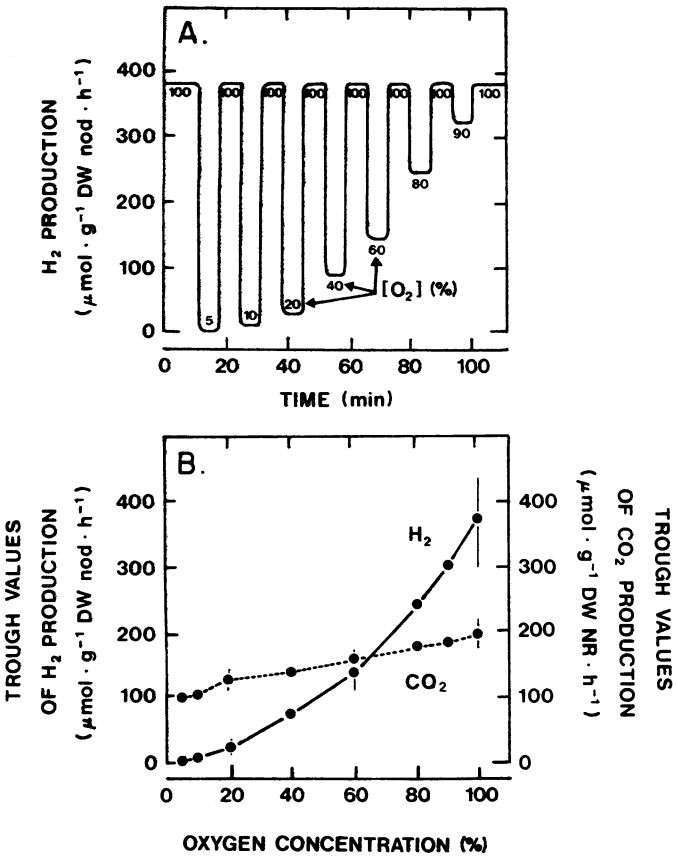

FIG. 3. Steady state rates of H_2 and CO_2 production in intact attached nodulated roots of soybean at 100% O_2, and the minimum rates measured on the transfer to lower O_2 concentrations in Ar. A, Time course response of H_2 production on the transfer from 100% O_2 to lower O_2 concentrations; B, minimum rates of H_2 and CO_2 production following the transfer from 100% O_2 to lower O_2 concentrations. Each point is the mean value of 4 replicate treatments. Representative standard errors are shown.

which were observed in H_2 and CO_2 production following changes from N_2:O_2 to Ar:O_2 and then back to N_2:O_2 at a pO_2 of 20%. On exposure to Ar:O_2, the H_2 production rate increased rapidly as total electron flow through nitrogenase was diverted to H^+ reduction, and a peak value was attained within 5 min. The duration of the peak was usually 3 to 8 min, and it was followed by a decline in total nitrogenase activity that reached a new steady state rate within 60 min. Previous studies in our laboratory have shown that the peak rate of H_2 evolution in Ar:O_2 is similar in magnitude to the peak rate of C_2H_4 production following transfer of a nodulated root to 10% C_2H_2 (data not shown). The CO_2 production rate declined immediately on exposure to Ar:O_2 and a steady state rate was attained simultaneously with that of the H_2 production rate. Reexposure to N_2:O_2 after steady state conditions were attained in Ar:O_2 resulted in a decline in H_2 production rate to a value lower than that measured previously in N_2:O_2, followed by a recovery to the initial rates within 60 min. CO_2 production rate recovered following reexposure to N_2:O_2 and reached its initial value within 60 min.

The magnitude of the Ar-induced declines in total nitrogenase activity and CO_2 production was measured as the percentage declines in H_2 and CO_2 production, respectively, between their maximum and steady state rates in Ar:O_2. Both declines showed a similar dependence on pO_2, being greatest at 20% O_2 and insignificant at 80% O_2 (Fig. 4B).

The EAC of nitrogenase as a function of pO_2 was determined from measurements of H_2 production rate in N_2:O_2 and Ar:O_2

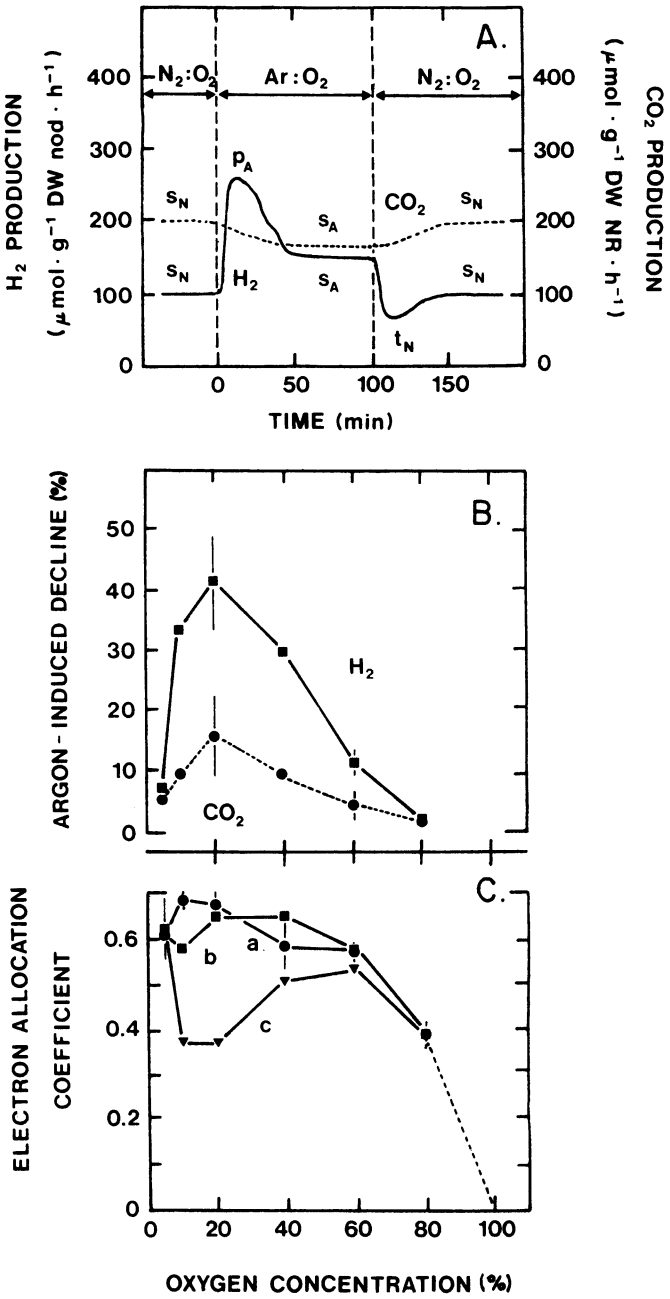

FIG. 4. The Ar-induced decline and EAC as a function of O_2 concentration in intact, attached nodules of soybean. A, Time course of the Ar-induced decline in H_2 and CO_2 production rates at an O_2 concentration of 20%. s_N, steady state rate of H_2 or CO_2 production in N_2:O_2; p_A, peak rate of H_2 production in Ar:O_2; s_A, steady state rate of H_2 or CO_2 production in Ar:O_2; t_N, trough rate of H_2 production in N_2:O_2. B, The Ar-induced decline as a function of O_2 concentration. Percent Ar-induced decline in H_2 production = $((p_A - s_A)/p_A) \times 100$. Percent Ar-induced decline in CO_2 production = $((s_N - s_A)/s_N) \times 100$. C, EAC as a function of O_2 concentration. (a) EAC = $1 - (s_N/p_A)$; (b) EAC = $1 - (t_N/s_A)$; (c) EAC = $1 - (s_N/s_A)$. Each point is the mean of 3 to 6 replicate treatments. Representative standard errors are shown.

before and after the Ar-induced decline. Although all estimates of EAC were calculated according to equation 1, various values for the H_2 production rates in N_2:O_2 and Ar:O_2 were used such that three different estimates were obtained (Fig. 4C). When the steady state rate in N_2:O_2 (s_N), and the peak rate in Ar:O_2 (p_A) were employed, the EAC did not change significantly with pO_2

between 5 and 60%. Similar results were obtained when the trough rate in N_2:O_2 (t_N) following transfer from Ar:O_2 was combined with the steady state rate in Ar:O_2 (s_A). In contrast, when EAC was calculated using the steady state rate in N_2:O_2 (s_N) and the steady state rate in Ar:O_2 (s_A), the EAC declined sharply between 5 and 10% O_2 and then increased between 20 and 60% O_2. All three methods showed that EAC declines at pO_2 above 60%.

Studies with Intact Plants and Detached and Sliced Nodules in the Cuvette. When the nodulated portion of the root system enclosed in the Plexiglas cuvette was exposed to stepwise increases in pO_2 as described for the intact potted plants in Figure 2, the gas exchange characteristics were similar to those observed for the potted plants (data not shown). With 10% increments in pO_2, rates of H_2 evolution in N_2:O_2 and Ar:O_2 increased while rates of CO_2 evolution were relatively stable. At each change in pO_2, fluctuations were observed in H_2 and CO_2 evolution similar to those described previously. Since transfer of plant material to the cuvette had little effect on the gas exchange responses of the nodules, this cuvette was considered suitable for studies of nodule detachment and slicing.

Similar results were obtained for detached nodules and nodules which were detached and sliced in half at 5% O_2 in Ar, although absolute rates of H_2 and CO_2 production from the sliced nodules were lower (Fig. 5B). The results were very different from those obtained with intact nodulated roots in three major respects. First, 10% increases in pO_2 resulted in rapid changes in the rate of H_2 and CO_2 production, and new steady state rates of exchange were observed within 3 min of the change in pO_2 (Fig. 5A). Transient fluctuations (as shown in Fig. 2A) did not occur. Second, the nodules showed a distinct O_2 optimum for nitrogenase activity at about 60% O_2 (Fig. 5B), and the specific rate of H_2 evolution obtained at this pO_2 was equal to, or greater than, the rate of H_2 evolution obtained with intact nodulated roots. The rate of H_2 evolution at 20% O_2 was 45 to 78% lower than that measured in the intact attached nodules at the beginning of the experiment, and unlike that in nodulated roots the rate declined rather than increased at O_2 concentrations between 70 and 100%. Third, in both detached and sliced nodules, an immediate increase in H_2 and CO_2 production to their maximum rates was observed when O_2 concentration was increased directly from 5 to 70%. The response of intact nodulated roots to a similar increase in O_2 concentration was an immediate inhibition of H_2 production and a parallel decline in respiration rate (data not shown).

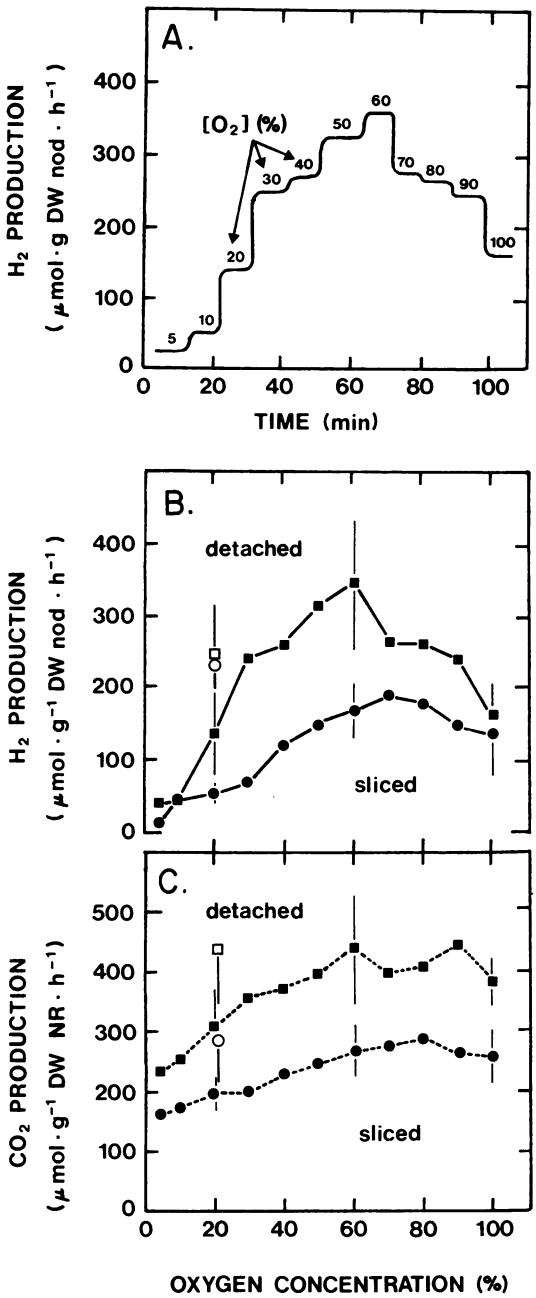

FIG. 5. Rates of H_2 and CO_2 production from detached and sliced nodules of soybean as a function of O_2 concentration in Ar. A, Time course response of H_2 production from detached nodules with increasing O_2 concentration; B, steady state rates of H_2 production from detached (■) and sliced (●) nodules with increasing O_2 concentration; C, steady state rates of CO_2 production with increasing O_2 concentration. Rates of H_2 and CO_2 production at 20% O_2 in the nodulated roots prior to nodule excision (□), and nodule excision and slicing (○) are also shown. Each point is the mean of 3 to 6 replicate treatments. Representative standard errors are shown.

DISCUSSION

Assessment of Methods in the Study of O_2 Effects on *in Vivo* Nitrogenase Activity. In this study, a 10 or 20% increase in the pO_2 surrounding an intact nodulated soybean root resulted in highly reproducible, transient fluctuations in nitrogenase activity and respiration. These observations have not been reported previously, because they can only be made with intact plants in an open gas exchange system which is equipped with flow-through H_2 and CO_2 gas analyzers to monitor continuously nitrogenase and respiratory activity. Many previous studies of O_2 effects on *in vivo* nitrogenase activity in soybean nodules (4, 5, 9, 13, 14, 16, 22) have used closed gas exchange systems in which discrete measurements of acetylene reduction rate at each pO_2 were made over a period of 45 min or less. The results of these studies should be treated with caution because:

(a) The C_2H_2-induced decline that may occur in nitrogenase activity (25) will preclude a steady state rate of acetylene reduction during the course of the assay, and the O_2 dependence of the decline may alter the apparent O_2 optimum for nitrogenase activity.

(b) Following a change in pO_2, steady state rates of H_2 evolution in N_2 or Ar are not usually attained within a 45 min period, and therefore maximum rates would be underestimated.

(c) The pO_2 used in these assays were increased from atmospheric to the experimental values in a single step, and therefore inhibition of nitrogenase activity would be likely at higher pO_2. While an apparent O_2 optimum for nitrogenase activity between 30 and 50% O_2 has been reported in these studies (5, 13, 14, 16, 22), our results demonstrate clearly that high nitrogenase activity

may occur at pO_2 up to 100% (Fig. 2C).

Regulation of the pO_2 in the Infected Cells. The high rates of H_2 production in N_2:O_2 and Ar:O_2 observed in intact nodulated roots of soybean plants as the rhizosphere pO_2 was increased in 20% increments to 100% O_2 (Fig. 2B) indicate that the pO_2 in the infected cells was being regulated to maintain a stable rate of nitrogenase activity. Three methods by which the nodule may maintain a constant pO_2 in the infected cells as pO_2 in the rhizosphere is increased are (a) by increasing respiration rate to consume the additional O_2 (b) by actively excluding O_2 from the infected cells, and (c) by increasing the resistance of the nodule to O_2 diffusion.

For O_2 consumption to account for the nodule's ability to adjust to an increase in pO_2 from 20 to 100%, the rate of O_2 consumption would have to increase by 5-fold. Our results show that CO_2 evolution was either unaffected by pO_2 or rose only slightly as pO_2 was increased (Fig. 2, D and E; Fig. 5C). Although Winship and Tjepkema (24) have reported a decline in respiratory quotient from 1.2 at 20% O_2 to 1.0 at 80% O_2, the calculated increase in O_2 consumption resulting from this decline would not contribute significantly towards the additional O_2 protection required by nodules at 100% O_2. Also, since no active O_2 pump has been reported in any plant or bacterial system, it is likely that the nodule regulates its internal pO_2 by varying its resistance to O_2 diffusion. This conclusion is in agreement with other recent studies (18, 25).

The presence of a variable barrier to gas diffusion is also supported by the results of Figure 3. When nodules were equilibrated at 100% O_2, the diffusion barrier was probably at the maximum resistance it attained during this study, since a change to any lower pO_2 resulted in an immediate decline in nitrogenase activity (Fig. 3A). Subsequent reexposure to 100% O_2 within 5 to 10 min resulted in an immediate recovery of the initial activity at 100% O_2 (Fig. 3A). However, if nodules equilibrated at 100% O_2 were exposed to 20% O_2 for an extended period, full recovery of their initial rate of H_2 evolution at 20% O_2 was attained within 60 to 180 min, suggesting a slow relaxation of the diffusion barrier. Also, following this relaxation of the diffusion barrier and the equilibration of the nodules at 20% O_2 in either N_2 or Ar, a direct increase in pO_2 to 100% caused total inhibition of nitrogenase. Full recovery of activity on subsequent reexposure to 20% O_2 was achieved after 13 to 30 h, suggesting that extensive damage to nitrogenase occurred during the rapid increase in pO_2.

The O_2 Optimum for Steady State Rates of H_2 Evolution in Ar:O_2. With increases in pO_2, H_2 evolution in Ar:O_2 should provide a measure of total electron flux through nitrogenase. However, in a N_2:O_2 atmosphere, the pN_2 will decline with increasing pO_2 and thereby stimulate the rate of H_2 evolution at the expense of N_2 fixation. Consequently, measurements of O_2 optima using H_2 evolution must be done in an atmosphere of Ar:O_2. The effects of pO_2 on the EAC of nitrogenase will be discussed in a later section.

In the experiment involving 20% incremental increases in pO_2 (Fig. 2B), the rates of H_2 evolution in N_2:O_2 and Ar:O_2 at pO_2 above 60% were generally lower than those in the experiment in which pO_2 was increased in 10% increments (Fig. 2C). This result indicates that at least some nitrogenase inhibition may have occurred with the 20% incremental increases in pO_2.

In the Ar:O_2 treatment in which pO_2 was increased in 10% increments, an increase was observed in the steady state rates of H_2 evolution with pO_2 (Fig. 2C). This result suggests that total nitrogenase activity may be O_2 limited at pO_2 up to and including 100%. The results of Figure 3B support this proposal, since the trough values of H_2 production did not plateau with O_2 concentration. If a pO_2 of 100% was not limiting H_2 evolution, treatment with 90% O_2 should have had relatively little effect on H_2 evolution. Rather, this treatment inhibited H_2 evolution more than the inhibition observed between any other two pO_2 separated by 10% O_2 (Fig. 3B). The question of whether nitrogenase activity *in situ* is limited by pO_2 is complicated by the fact that treatment with Ar:O_2 results in a decline in the steady state rate of H_2 evolution, and the decline is more pronounced at lower pO_2 (20–40% O_2) than at higher pO_2 (>60% O_2) (Fig. 4B). Increases in nitrogenase activity at pO_2 between 60 and 100% may therefore represent a reduction in the magnitude of the Ar-induced decline rather than a direct stimulation of nitrogenase activity by O_2. The same problem exists with previous studies that have attempted to use the C_2H_2 reduction assay to measure the O_2 optimum for nitrogenase activity (25).

It was interesting to note that CO_2 production rate did not increase in a similar manner to H_2 production rate as pO_2 in Ar:O_2 was increased in 10% increments (Fig. 2E). The reason why these responses should be O_2 independent is not known.

Transient Responses of H_2 and CO_2 Production to Increases in pO_2 in N_2 and Argon. The cause of the rapid transient declines in H_2 and CO_2 production rate that occur immediately following an incremental increase in the external pO_2 (Fig. 3A) cannot be determined from the results of this investigation. However, three hypotheses may be advanced:

(a) The initial declines may be due to O_2 inhibition of nitrogenase and nitrogenase-linked respiration. If so, the recovery period would represent the time required for *de novo* nitrogenase synthesis following regulation of the infected cell pO_2 by changes in nodule diffusion resistance. This hypothesis is unlikely since the time period for such *de novo* synthesis (10–60 min) is unrealistic (15).

(b) The sudden increase in external pO_2 may cause a rapid increase in nodule diffusive resistance sufficient to result in an O_2 limitation of nitrogenase activity. If so, the slow recovery of H_2 and CO_2 production rate that occurs in a N_2:O_2 atmosphere (about 60 min) may be associated with a slow relaxation of the diffusion barrier. Also, the swifter recovery in an Ar:O_2 atmosphere (about 10 min) would imply that, in the absence of N_2 fixation, the diffusion barrier is under tighter control. Large direct increases in O_2 concentration may be able to overcome the increased resistance of the diffusion barrier and cause irreversible nitrogenase inhibition.

If this hypothesis is correct, nitrogenase activity should be less O_2-sensitive in the trough which follows the increase in pO_2 than in the subsequent recovery period. However, preliminary experiments have suggested that the O_2 sensitivity of nitrogenase is similar in both of these periods. Also, since previous studies suggest that the diffusion barrier is slow to respond to changes in pO_2 (*e.g.* 25), this second hypothesis seems unlikely.

(c) If the diffusion barrier were to respond slowly to an increase in the external pO_2, the internal O_2 concentration would rise sharply, causing a rise in the concentration of free O_2 and oxyleghemoglobin in the infected cells. The increased pO_2 would inhibit nitrogenase activity, causing a subsequent decline in nitrogenase-linked respiration. As the diffusion barrier closed, the infected cell concentrations of O_2 and oxyleghemoglobin would begin to decline, and nitrogenase activity would recover slowly as observed in Figure 3A. This hypothesis would be feasible only if the infected cell pO_2 was high enough to inhibit nitrogenase activity, but not high enough to cause irreversible deactivation of the enzyme. While no such mechanism has been described for *Rhizobium*, in *Azotobacter* high pO_2 has been reported to cause a conformational change in nitrogenase thereby deactivating the enzyme while preventing irreversible O_2 inhibition (17). While this hypothesis is the most likely of the three proposed, we have no experimental evidence in its support.

The Argon-Induced Decline. It was reported in a previous study (25) that the rate of C_2H_4 production from nodules of various legumes declines with length of exposure to C_2H_2, and

evidence was provided that this C_2H_2-induced decline was the result of an increase in the nodules' resistance to O_2 diffusion. It was also reported that the C_2H_2-induced decline was smaller at higher pO_2. Our results show that a decline in H_2 evolution from soybean nodules occurs with length of exposure to Ar:O_2 (Fig. 4A). As with the C_2H_2-induced decline, the magnitude of the Ar-induced decline decreases with an increase in pO_2 between 20 and 80%. It was interesting to note that the Ar-induced declines were also lower at pO_2 below 20%.

Preliminary experiments have been performed to determine whether the Ar-induced decline may be the result of an increase in the resistance of the diffusion barrier. It has been shown that, during the peak rate of H_2 production following a switch from N_2:O_2 to Ar:O_2 at 20% O_2 (p_A, Fig. 4A), exposure to 80% O_2 inhibits H_2 production. However, exposure to 80% O_2 immediately after the Ar-induced decline at 20% O_2 (s_A, Fig. 4A) stimulates H_2 production (data not shown). This result suggests that the Ar-induced decline is brought about by an increase in the resistance of the diffusion barrier with consequent O_2 limitation of nitrogenase.

The EAC of Nitrogenase. Of the three methods used to estimate EAC during measurements of the Ar-induced decline (Fig. 4C), methods (a) and (b) were thought to produce the most reliable results. Both of these methods used rates of H_2 production that were measured just before and just after the change in rhizosphere gas composition, and presumably the nodule would have had a similar resistance to O_2 diffusion in the two atmospheres. Both methods produced similar results, suggesting that EAC does not change significantly with O_2 concentrations between 10 and 60% (Fig. 4C).

In contrast, method (c) used H_2 production rates in N_2:O_2 that were measured before the Ar-induced decline, and H_2 production rates in Ar:O_2 that were measured after the Ar-induced decline. Therefore, it was likely that the resistance of the diffusion barrier would have been greater in the presence of Ar:O_2 than in N_2:O_2. Method (c) suggested that there was a 33% increase in EAC between 20 and 40% O_2. This result is consistent with that of Drevon *et al.* (7) who measured the EAC of nitrogenase in soybean nodules using closed circuit measurements of C_2H_2 reduction and H_2 evolution. However, assuming the presence of a C_2H_2-induced decline in soybean nodule nitrogenase activity, the closed circuit method of measuring EAC would necessarily involve measuring rates of H_2 evolution and C_2H_2 reduction before and after the C_2H_2-induced increase in the resistance of the diffusion barrier. Therefore, although the results from method (c) and those of Drevon *et al.* (7) are similar, they are unlikely to reflect the true response of EAC to pO_2. The problems associated with the measurement of EAC in closed gas exchange systems further emphasize the necessity for continuous open circuit measurements in the study of nodule gas exchange characteristics.

Studies with Detached and Sliced Nodules. The detached and sliced nodules showed very similar responses in H_2 and CO_2 exchange following changes in external pO_2 (Fig. 5). However, these results were very different from those obtained with attached nodules either in pots (Fig. 2) or in the cuvette. Why should the gas exchange characteristics of the nodules change to such an extent following excision or excision and slicing?

It has been proposed that intact soybean nodules have continuous air spaces in the central zone (3), and the presence of such air spaces has been shown by modeling studies to be necessary to maintain aerobic conditions within the infected cells (19). Slicing the nodules may have the effect of filling these air spaces with disrupted cell contents and thereby creating a nonregulated aqueous barrier to O_2 diffusion with consequent O_2 limitation of nitrogenase activity at lower pO_2 (5–50%). Nodule excision, however, may result in a rapid increase in the nodule's resistance to O_2 diffusion and a loss in its ability to control the diffusion barrier. These hypotheses are entirely consistent with our experimental results:

(a) The 45% reduction in H_2 evolution in Ar:O_2 (80:20) in detached nodules relative to attached nodules indicates that the detached nodules may be O_2 limited. This is supported by the full recovery of nitrogenase activity in the detached nodules at a pO_2 of 60%.

(b) The absence of transient fluctuations in H_2 and CO_2 evolution following changes in pO_2 (*cf* Figs. 2A and 5A) suggests that excised and sliced nodules are unable to adjust to changes in pO_2.

(c) The apparent optimum pO_2 of 60% in the detached and sliced nodules and the marked inhibition of nitrogenase activity at 100% O_2 (Fig. 5A) indicate that the nodules cannot adjust to changes in pO_2, and therefore the infected cell pO_2 varies from a suboptimal to a supraoptimal level with respect to nitrogenase activity.

(d) The ability of the detached and sliced nodules to withstand instantaneous changes in pO_2 from 5 to 70% suggests that these nodules have a high resistance to O_2 diffusion similar to that found in attached nodules adapted to 100% O_2 (Fig. 4A).

Although the physical nature of the diffusion barrier cannot be determined from the results of this investigation, a possible mechanism by which the nodule may regulate its internal O_2 concentration is to alter the number, size, or nature of the intercellular spaces that are continuous between the external atmosphere and the infected zone. Since the diffusion rate of a gas through an aqueous phase is several orders of magnitude slower than through a gaseous phase (6, 19, 20), the O_2 concentration in the infected zone of the nodule may be maintained constant while the external concentration is varied, by controlling the amount of gas reaching this zone through continuous air spaces relative to that which must at some point diffuse through an aqueous barrier. A previous study (19) has suggested that an aqueous shell 45 μm wide surrounding the nodule central zone is sufficient to maintain a low pO_2 in the infected cells under atmospheric conditions. Such a shell may be represented by a single layer of cells lacking gas-filled intercellular spaces between the inner and outer cortex of the nodule.

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