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Effect of Increases in Oxygen Concentration during the Argon-Induced Decline in Nitrogenase Activity in Root Nodules of Soybean[1]

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ABSTRACT

When intact nodulated roots of soybean (*Glycine max* L. Merr. nodulated with *Bradyrhizobium japonicum* strain USDA 16) were exposed to an atmosphere lacking N_2 gas (Ar:O_2 80:20), total nitrogenase activity (measured as H_2 evolution) and respiration (CO_2 evolution) declined with time of exposure. In Ar-inhibited nodules, when the O_2 concentration in the rhizosphere was increased in a linear 'ramp' of 2.7% per minute, 93% of the original H_2 evolution and 99% of the CO_2 evolution could be recovered. The internal nodule O_2 concentration (estimated from leghemoglobin oxygenation) declined to 56% of its initial value after 60 minutes of Ar:O_2 exposure and could be partially recovered by the linear increases in O_2 concentration. Nodule gas permeability, as estimated from the lag in ethylene production following exposure of nodules to acetylene, decreased to 26% of its initial value during the Ar-induced decline. Collectively, the results provide direct evidence that the Ar-induced decline results from decreased nodule gas permeability and indicate that the decline in permeability, rather than being immediate, occurs gradually over the period of Ar:O_2 exposure.

The nodules of various legumes undergo a distinct decline in TNA[3] and CO_2 evolution following exposure to 10% acetylene (2, 3, 12–14, 22, 23). A similar decline occurs when N_2 in the gas phase is replaced by an inert gas such as Ar (5, 13). Both the acetylene- and Ar-induced declines can be largely prevented by carrying out experiments with nodules adapted to higher O_2 concentrations (5, 23). Furthermore, Witty *et al.* (22) showed that nodules could recover from the acetylene-induced decline if the rhizosphere O_2 concentration was increased in stepwise increments.

The O_2 dependence of the acetylene- and Ar-induced declines (5, 23), and the ability of O_2 at least to partially overcome the acetylene-induced decline (22), suggest that both declines are caused by a decrease in the nodule gas permeability (P), which restricts the entry of O_2 for respiration in support of nitrogenase activity (23). However, this suggestion is based on theoretical considerations of the relationship between O_2 concentration gradients, O_2 uptake rates, and P. To date, neither P nor the infected cell O_2 concentration (O_i) have been measured in acetylene- or Ar-inhibited nodules, nor has it been demonstrated whether increases in O_2 concentration can overcome the Ar-induced decline.

In addition, various actinomycete symbioses also exhibit acetylene-induced declines or transient fluctuations of TNA (16, 17, 19), which are superficially similar to those of legumes. However, the anatomy of the nodules formed in these symbioses differs considerably from that of legume nodules, and the main barrier to gas diffusion is thought to be associated with the *Frankia* endosymbiont itself (18) rather than with the uninfected nodule cortex, as appears to be the case in legumes (20, 24). Silvester and Winship (16) have provided evidence that the acetylene- and O_2-induced transient responses in several actinomycete symbioses are associated with the *Frankia* vesicles and are not due to changes in nodule gas permeability. Rather, the authors attribute them to a biochemical 'switch-off' of nitrogenase activity. A similar switch-off has previously been suggested to occur in soybean nodules in response to increases in O_2 concentration (5–7) and could possibly be involved in the response of legumes to acetylene and Ar:O_2 as well.

Therefore, on the basis of the studies to date, it may be premature to attribute either the acetylene- or Ar-induced declines to decreased P, either in whole or in part. In light of this, the aims of the present study were two-fold: to characterize the time course of the Ar-induced decline in respiration and H_2 evolution in intact, attached soybean nodules; and to determine whether or not the decline results from decreased P and consequent O_2 limitation of nitrogenase activity.

MATERIALS AND METHODS

Plant Culture and Gas Exchange Measurements

Seeds of soybean (*Glycine max* L. Merr. cv Harosoy 63 or Maple Arrow) were inoculated with *Bradyrhizobium japonicum* USDA 16, a strain which lacks uptake hydrogenase activity (10), and grown in silica sand culture as described previously (5) in a growth chamber with 16-h photoperiod,

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[3] Abbreviations: TNA, total nitrogenase activity as estimated from H_2 production in Ar:O_2 or from acetylene reduction; NR, nodulated root; nod, nodules; O_i, O_2 concentration in the cytosol of the infected cells of the nodule; P, nodule permeability to gas diffusion; t, time constant for ethylene production.

25°C day and 20°C night. The design of the growth pots (8) permitted sealing for open circuit gas exchange measurements of H_2 and CO_2 evolution from the root systems. The gas exchange system contained computer-controlled mass flow controllers (model FMA-100 series, Omega Engineering, Stamford, CT) which allow linear changes in gas concentrations at a constant total flow rate. CO_2 evolution was measured by infrared gas analyzers (model 225, Mark III, Analytical Development Corp., Hoddeson, UK), O_2 evolution with an O_2 electrode (model CB1D, Hansatech, King's Lynn, UK), and H_2 evolution with custom-built H_2 analyzers (11). The complete system has been described previously (6, 8). At a gas flow rate of 500 mL min^{-1} past the root systems, the time constant for the entire system was approximately 30 s.

Time Course of the Ar-induced Decline and the Effect of Increasing O_2 Concentration

Plants were connected two at a time to the gas exchange system, and H_2 and CO_2 evolution from the root systems were monitored under N_2:O_2 (80:20) until steady state rates were obtained, as described in Hunt *et al.* (5). The balance gas was then switched from N_2 to Ar, and monitoring was continued under Ar:O_2 until new, lower steady-state rates of H_2 and CO_2 evolution were obtained (typically after 60 to 80 min). A linear increase ('ramp') in O_2 concentration from 20 to 100% was then carried out as in Hunt *et al.* (6), over a period of 15, 30, or 60 min, corresponding to rates of increase ('ramp rates') of 5.3, 2.7, and 1.3% O_2 min^{-1}, respectively. The gas exchange rates during the ramps were monitored continuously. At the end of each ramp, when the nodulated roots were exposed to 100% O_2, monitoring was continued until steady-state gas exchange rates were obtained.

As a control, plants were exposed to 30-min ramps from 20 to 100% O_2 (a ramp rate of 2.7% O_2 min^{-1}) in N_2:O_2, and the rates of H_2 and CO_2 evolution were monitored as above.

Measurement of Infected Cell O_2 Concentration

O_i was measured by a noninvasive, pulse-modulated spectrophotometric technique which monitors leghemoglobin oxygenation of individual nodules. The complete method is described elsewhere (9) and in the present study was modified to allow continuous gas exchange measurements to be made during the measurements of O_i. This involved sealing the gas exchange pot with a Plexiglas lid around the base of the shoot and sealing the fiber optic probe into the side of the pot with Terostat sealant (Teroson GmbH, Hiedelberg, Germany) and vacuum grease prior to measurements of O_i.

After steady-state rates of CO_2 and H_2 evolution were obtained under N_2:O_2 (80:20), the nodulated roots were exposed for 45 s to 100% N_2 (5 L min^{-1}) to provide a spectrophotometric measurement of deoxygenated leghemoglobin. When the measurement had stabilized upon return to N_2:O_2, the balance gas was switched to Ar and gas exchange measurements were continued under Ar:O_2 (80:20) until the Ar-induced decline had run its course. A second 45-s anaerobic treatment was then implemented. This was followed by a 15-min ramp to 60% O_2 (*i.e.* a ramp rate of 2.7% min^{-1}, as in the 30-min ramp to 100% O_2 described above). After this treatment, a third and final anaerobic treatment was carried out.

Immediately following the final anaerobic treatment, the root system was reexposed to 100% N_2 for approximately 5 min to ensure that leghemoglobin was completely deoxygenated during the 45-s treatments. Finally, the root system was exposed to 100% O_2 until leghemoglobin was fully oxygenated (about 5 min), and then the plant was harvested as described above.

In a separate experiment, point measurements of O_i were obtained by periodic anaerobic treatments at various times during the Ar:O_2 exposure. For this experiment, concurrent measurements of H_2 and CO_2 evolution were not made.

Measurement of *P*

P was measured for control and Ar:O_2-treated plants by the lag-phase technique of Weisz and Sinclair (21). Plants were connected one at a time to the gas exchange system, and rates of H_2 and CO_2 evolution were monitored under N_2:O_2 at 500 mL min^{-1} until steady-state was obtained. For each control plant, the flow rate was then increased to 3000 mL min^{-1} and the pot was flushed with 10% acetylene. Syringe samples of 1 mL of gas were then withdrawn downstream from the pot at intervals of 10 or 15 s for the next 2 to 3 min. The samples were subsequently analyzed for ethylene by flame ionization gas chromatography.

The experimental plants were exposed to Ar:O_2 (80:20) until steady-state gas exchange rates were obtained following the Ar-induced decline. Acetylene was then introduced, and samples were withdrawn for measurement of ethylene as for the control plants.

P for the control and Ar:O_2 treatments was estimated from *t* using the computer program developed by Weisz and Sinclair (21). Mean nodule radius was determined for each plant, and central zone radius was estimated from nodule radius using the data of Bergersen (1) for central zone volume relative to that of the whole nodule. The lag time of the gas exchange system (about 10 s) was accounted for in the calculations.

All data are expressed as means ± 1 SE. The differences between means were tested for statistical significance at the 5% level using Student's *t* distribution and a two-tailed test.

RESULTS

Time Course of the Ar-Induced Decline

The rates of H_2 and CO_2 evolution during exposure of nodulated roots to Ar:O_2 (80:20) followed a highly reproducible pattern, as shown for three individual plants in Figure 1. The original rate of H_2 evolution in N_2:O_2 is indicated by point a. The initial peak in H_2 production (point b), caused by the diversion of e^- from N_2 reduction to H_2 production, was reached within 4 min of Ar:O_2 exposure and was immediately followed by a steep decline. A trough value (c) was reached at approximately 7 min. The decline was then followed by a partial recovery in the form of a broad peak (d). The duration and shape of this broad peak was variable; frequently it consisted of a relatively sharp initial peak followed by a broad shoulder (*e.g.* Fig. 1C). However, the height of peak d was always lower than that of peak b.

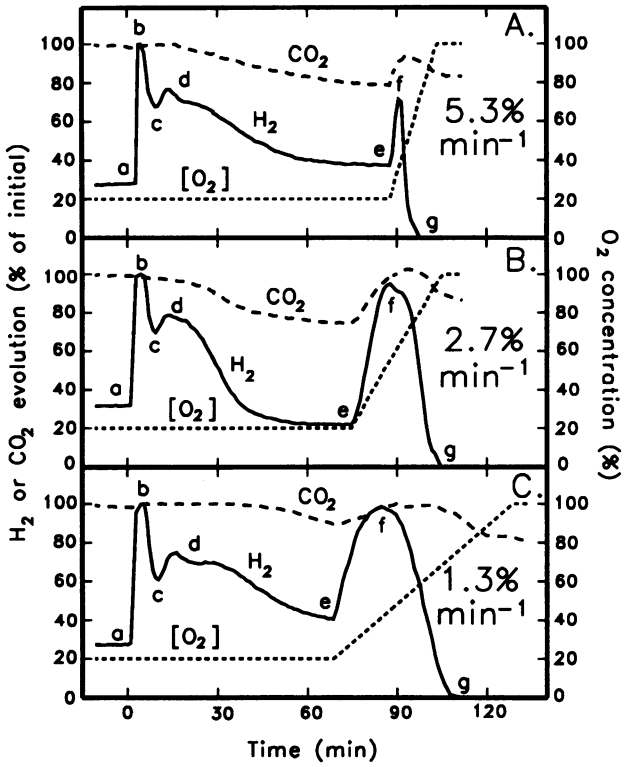

Figure 1. Time course of H_2 (——) and CO_2 (– – –) evolution following the exposure of intact nodulated soybean roots to Ar:O_2 (80:20), and the effect of linear increases in rhizosphere O_2 concentration (- - - - -). The O_2 concentration was increased at rates of 5.3% min^{-1} (A), 2.7% min^{-1} (B), and 1.3% min^{-1} (C). The significance of points a through g is explained in "Results." The mean rates of H_2 and CO_2 evolution and the recovery obtained during the ramps are given in Table I.

Peak d was followed by a second, gradual decline to a new steady-state value (e), which was on average 30% ± 3 (n = 9) of the original rate, and which was obtained 60 to 80 min after the start of Ar:O_2 exposure.

The pattern of CO_2 evolution (Fig. 1) was somewhat different, and the changes were less pronounced, probably due to the presence of a background of root respiration and nodule respiration not associated with nitrogenase activity. The responses of CO_2 evolution could be ascribed to nodule activity only, since denodulated roots showed no response to either Ar:O_2 exposure or to increases in O_2 concentration (data not shown). After exposure to Ar:O_2, there was no rapid inhibition of CO_2 evolution corresponding to trough c for H_2 evolution. Instead, CO_2 evolution began to decline gradually, with the time of onset of the decline varying among individual plants (*cf.* Fig. 1, A and C, for two extremes). The pattern of this decline appeared to reflect that of the second, gradual decline in H_2 evolution following peak d. After 60 to 80 min of Ar:O_2 exposure, the rate of CO_2 evolution approached a new steady-state, which was on average 77% ± 2 (n = 9) of the original rate.

Effect of Increases in External O_2 Concentration on TNA and Respiration

When control plants were exposed to ramped increases in O_2 concentration from 20 to 100% O_2 in N_2 (ramp rate 2.7% min^{-1}), the fluctuations in CO_2 and H_2 evolution were as shown in Figure 2. As O_2 concentration increased, H_2 and CO_2 evolution were initially inhibited, and this was followed by oscillations which were similar to the patterns observed previously for stepwise increases in O_2 concentration of 10 or 20% in N_2 (5) and for ramps at 2% O_2 min^{-1} from 20 to 30% in N_2 (6). At high O_2 concentrations, progressive damage to nitrogenase appeared to occur, so that at about 80% O_2 and above, H_2 evolution had declined to zero, and CO_2 evolution was correspondingly lower. No stimulation of H_2 or CO_2 evolution relative to the initial values was seen at any time, and no distinct O_2 optimum was apparent.

The response to ramped increases in O_2 concentration after 60 to 80 min of Ar:O_2 exposure was very different from that of the control plants under N_2:O_2. Representative examples for the 5.3, 2.7, and 1.3% min^{-1} ramps are shown in Figure 1, panels A through C. All three ramps caused an immediate and pronounced stimulation of H_2 evolution, reaching a peak indicated as f, and a corresponding increase in CO_2 evolution occurred. Following the peak, a decline in H_2 evolution occurred, such that by the time 100% O_2 was reached, H_2 evolution was reduced to zero (g). A concurrent, but less pronounced decline in CO_2 evolution was observed.

The mean values for maximum recovery of TNA and CO_2 evolution during the ramps and the mean O_2 concentration under which the maximum values were obtained are shown in Table I. Both the 2.7 and 1.3% min^{-1} ramp rates caused greater than 90% recovery of TNA. The 2.7% min^{-1} ramps caused a 99% recovery of CO_2 evolution, and the 1.3% min^{-1} ramps, a 107% recovery. The 5.3% min^{-1} ramp rate caused 79% recovery of H_2 evolution, with a sharp O_2 optimum, and 99% recovery of CO_2 evolution.

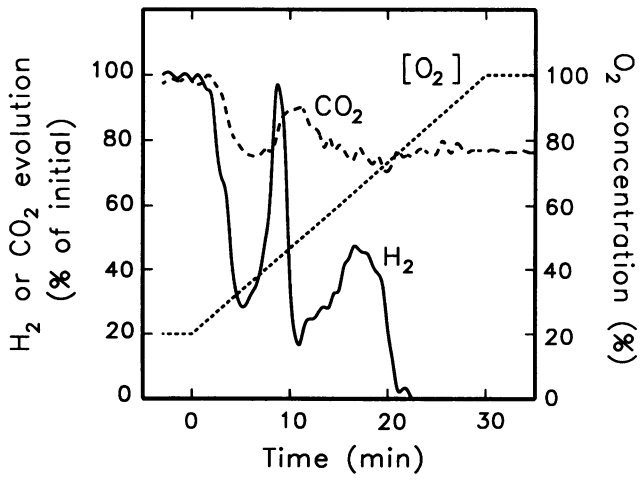

Figure 2. Time course of H_2 (——) and CO_2 (– – –) evolution in intact nodulated soybean roots during linear increases in rhizosphere O_2 concentration (- - - -) from 20 to 100% in N_2.

Table I. *Recovery of H_2 and CO_2 Evolution in Intact Nodulated Roots Exposed to Ar:O_2 (80:20) for 60 to 80 min and then Subjected to a Linear Increase in O_2 Concentration from 20 to 100% over 15, 30, or 60 min (corresponding to ramp rates of 5.3, 2.7, and 1.3% min^{-1}, respectively).*

All activities are expressed as percentages of the original peak rate of H_2 evolution 4 min after exposure to Ar:O_2 (Fig. 1, point b; 276 ± 21 μmol g^{-1} dry weight nod h^{-1}; n = 9) and the accompanying CO_2 evolution rate (241 ± 22 μmol g^{-1} dry weight NR h^{-1}; n = 9). The peak rates of H_2 and CO_2 evolution during the ramps correspond to point f on Figure 1.

Ramp Rate	N	H_2 Evolution in Ar:O_2		CO_2 Evolution	
		O_2 at peak rate	Peak rate	O_2 at peak rate	Peak rate
% O_2 min^{-1}		%	% of initial	%	% of initial
1.3	3	44.8 ± 2.5	96.4 ± 8.0	53.8 ± 5.4	107.5 ± 1.2
2.7	4	51.9 ± 5.6	92.9 ± 4.0	66.3 ± 3.5	99.1 ± 2.0
5.3	2	41.3	79.1	51.8	98.5

Changes in Infected Cell O_2 Concentration during the Ar-Induced Decline

Before the Ar:O_2 exposure (shown for a typical plant in Fig. 3A), the mean O_i under N_2:O_2 (80:20) was 34 nM (Fig. 3B). During the stable phase of gas exchange following the Ar-induced decline (Fig. 3A), O_i declined to 11 nM (Fig. 3B), and after the 2.7% min ramp to 60% O_2 (Fig. 3A), it had recovered to 24 nM (Fig. 3B). The ramp recovered 98% ± 3 of the original H_2 evolution rate and 99% ± 2 of the original CO_2 evolution rate.

When O_i was measured at various points during the Ar-induced decline (Fig. 3C), it was seen to decline gradually, reaching 59% of its initial value within 30 min and 56% of the initial value after 60 min.

Changes in Nodule Gas Permeability during the Ar-Induced Decline

Typical data for ethylene production from the control (N_2:O_2) and Ar:O_2-treated root systems are shown in Figure 4A. For both treatments, the rate of ethylene production increased during the first 30 to 60 s after the start of acetylene exposure, followed by the establishment of a plateau between 60 and 120 s. Logarithmic transformation of the data as described by Weisz and Sinclair (21) yielded straight lines over the first 60 to 90 s from the start of acetylene exposure (Fig. 4B). The t was determined as the reciprocal of the slope of these lines.

For the control (N_2:O_2) plants, P, as determined from t by the procedure of Weisz and Sinclair (21), was 24.0 ± 7.0 μm s^{-1} (n = 3), and for the Ar:O_2 treated plants it was 6.2 ± 1.1 μm s^{-1} (n = 3). The Ar:O_2 treatment, therefore, caused P to decline to 26% of its initial value.

DISCUSSION

O_2 Regulation and the Ar-Induced Decline

The observation that ramped increases in O_2 concentration during the stable phase of the Ar-induced decline cause pronounced recovery of TNA and respiration (Fig. 1; Table I) shows that O_2 limitation of TNA and respiration occur during the Ar-induced decline, most likely due to a decrease in gas permeability in the pathway of O_2 diffusion to the infected cells. This suggestion is supported by the observation that O_i decreases significantly during the Ar-induced decline (Fig. 3, B and C), and that O_i can be recovered by ramps in O_2 concentration (Fig. 3B). Such O_2 limitation appeared not to be present in the control plants under N_2:O_2 (Fig. 2), since for these plants the ramps in O_2 concentration did not cause stimulation of either H_2 or CO_2 evolution, but rather caused pronounced, transient inhibition followed by oscillations,

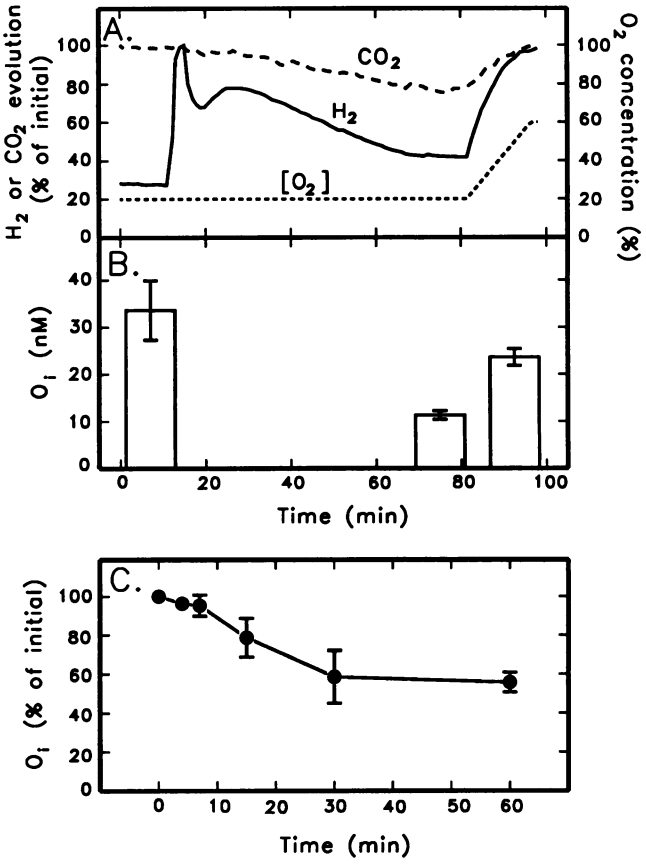

Figure 3. Effect of exposure to Ar:O_2 (80:20) on the infected cell O_2 concentration (O_i) in soybean nodules. A, The response of a typical plant to Ar:O_2 exposure (begun at 12 min) and a subsequent linear increase in O_i concentration to 60%. (- - - -), O_2 concentration; (——), H_2 evolution; (– – –), CO_2 evolution. B, O_i measurements (n = 3, ±1 SE) before and after the Ar-induced decline and after recovery of nitrogenase activity by a subsequent linear increase in the rhizosphere O_2 concentration to 60%, as shown in A. The mean value during the Ar-induced decline was significantly different at the 5% level from both the value before Ar:O_2 exposure and that following the ramp. C, Time course of changes in O_i during the Ar-induced decline. For these plants, H_2 and CO_2 evolution were not measured during the Ar:O_2 exposure. Each point represents the mean of five replicates ±1 SE. Error bars are not shown where they do not exceed the dimensions of the symbol. The difference between times 0 and 30 s and times 0 and 60 s was significant at the 5% level.

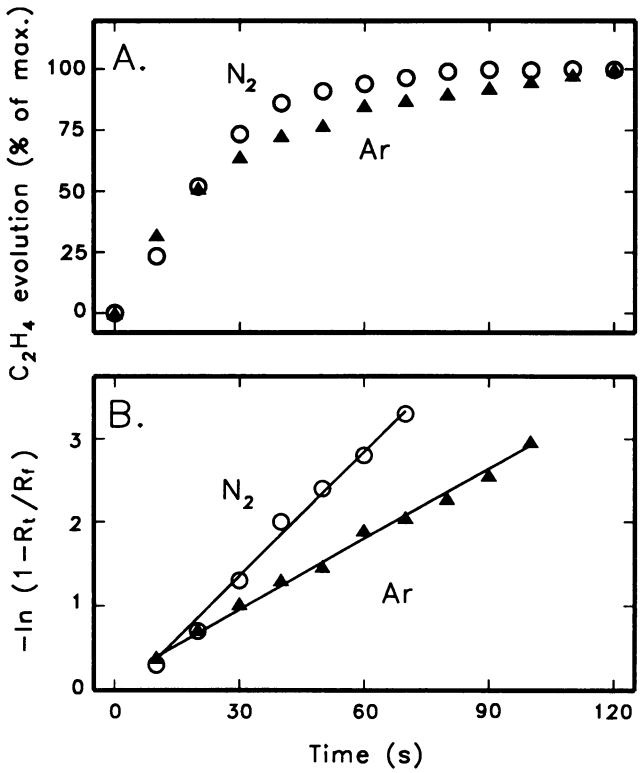

Figure 4. A, Typical time course of ethylene production for control and Ar:O_2-treated plants after introduction of 10% acetylene into the gas stream at 0 s. Between 30 and 90 s, the difference in mean values for the control and Ar:O_2-treated plants was significant at the 5% level. B, Logarithmic transformation of the data in panel A, showing the linear portion of the resultant curves. R_t represents the ethylene production rate at a given time, and R_F the maximum rate. The R^2 values for the lines were 0.996 and 0.978 for the control and Ar:O_2-treated plants, respectively. The *t* given by the reciprocal of the slope were 6.1 and 17.9 s for the control and Ar:O_2-treated plants shown and were significantly different at the 5% level.

similar to the pattern observed by Hunt *et al.* (5, 6). Further confirmation of the hypothesis that the Ar-induced decline is caused by decreased *P* is provided by use of the lag-phase technique of Weisz and Sinclair (21), which showed that *P* declined to 26% of its initial value during the decline (Fig. 4).

Collectively, these experiments constitute the first detailed study of the Ar-induced decline in legume nodules. This study corroborates previous reports on the acetylene-induced decline (13, 22, 23) and provides direct and conclusive evidence that the Ar-induced decline in soybean, like the acetylene-induced decline, is caused by a decrease in *P*, which restricts the entry of O_2 for respiration in support of nitrogenase activity.

Time Course of the Ar-Induced Decline

Given that the overall decline in H_2 and CO_2 evolution results from decreased *P*, the cause of the distinct pattern of transient responses in H_2 evolution during Ar:O_2 exposure can be partially addressed. The pattern of responses is very similar to the 'decline/recovery/decline' pattern seen by Schuller *et al.* (14) for acetylene reduction in soybean. Schuller *et al.* suggested that the initial, steep decline they observed was due to a decrease in *P* caused by the cessation of NH_3 production. The resulting reduction in O_2 availability might then act as a counter-stimulus, triggering an increase in *P*, an increase in O_i, and a resultant recovery of respiration and TNA. They suggested that ultimately, however, the cessation of NH_3 production was the stronger stimulus, leading to a second decrease in *P* and the second, long-term decline in respiration and TNA. Schuller *et al.* provided no evidence in support of this hypothesis and did not suggest a mechanism by which the lack of NH_3 or a product of its assimilation might lead to a decrease in *P*.

The results of the present study appear to contradict this hypothesis in several respects. First, the sharp initial decrease in H_2 production (between points b and c on Fig. 1) was not accompanied by a sharp decline in O_i (Fig. 3C) or in CO_2 evolution (Fig. 1), such as would be expected if a sharp decrease in *P* occurred at this time. Instead, O_i declined gradually throughout the first 30 min of Ar:O_2 exposure. The decline in CO_2 evolution, while variable in its time of onset, was also gradual, occurring over a period of 60 to 80 min.

Second, the recovery of TNA between 10 and 20 min (point d on Fig. 1) was not accompanied by a recovery of O_i or of CO_2 evolution; rather, O_i was declining throughout this phase (Fig. 3C), and CO_2 evolution was either stable or declining (Fig. 1).

In light of these results, we suggest that the observed decreases in *P* and in O_i can account for the long-term decline in TNA and respiration but cannot account for the short-term transient responses. An alternative hypothesis to explain the transient responses is that the observed inhibition and recovery of TNA are due to a reversible inhibition of nitrogenase. Such a biochemical mechanism has been suggested previously as an explanation for the transient responses to increased O_2 concentration in soybean (5–7) and in actinomycete symbioses (15, 19) and for the transient responses to acetylene in actinomycete symbioses (16). The means by which Ar:O_2 exposure or increasing O_2 concentration could cause such reversible inhibition is not clear. Possibly, the effect arises from perturbation of metabolite pools such as ATP and reductant within the nodule, as has been suggested for the transient responses to increased O_2 concentration in soybean (6). If a common biochemical mechanism underlies the transient responses to Ar:O_2, 10% acetylene, and increased O_2 concentration, this could be an important feature of the regulation of nitrogen fixation in both legume and actinomycete symbioses. This possibility warrants detailed investigation.

The changes in *P*, which account for the long-term Ar-induced decline, and which appear not to occur in actinomycete symbioses (16), may be a separate phenomenon from the short-term responses. The mechanism by which Ar:O_2 exposure affects *P* is not clear. It has been suggested that *P* is regulated by osmotic changes in the nodules, possible within a region of the nodule inner cortex (4). Further studies in our laboratory will address the feasibility of this hypothesis.

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