Plant Physiol. (1991) 96, 137–143
0032-0889/91/96/0137/07/$01.00/0

Received for publication September 17, 1990
Accepted January 17, 1991

Measurement of Legume Nodule Respiration and O_2 Permeability by Noninvasive Spectrophotometry of Leghemoglobin

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ABSTRACT

Physiological regulation of nodule gas permeability has a central role in the response of legumes to such diverse factors as drought, defoliation, and soil nitrate. A new method for quantifying nodule respiration and O_2 permeability, based on noninvasive spectrophotometry of leghemoglobin, was evaluated using intact, attached nodules of *Lotus corniculatus*. First, the relationship between nodule respiration (O_2 consumption) rate and internal O_2 concentration was determined from the rate of decrease in fractional oxygenation of leghemoglobin (FOL) under N_2. The rate of increase of FOL under 100% O_2 was then used to calculate nodule O_2 permeability, after correcting for respiration. Inactivation of nitrogenase by exposure to 100% O_2 for 15 minutes led to decreases in both permeability and O_2-saturated respiration (V_{max}), but the brief (<15 seconds) exposures to 100% O_2 required by the assay itself had little effect on either parameter. A gradual increase in external O_2 concentration from 20 to 40% resulted in a reversible decrease in permeability, but no change in V_{max}. The new method is likely to be useful for research on nodule physiology and might also be applicable to agronomic research and crop improvement programs.

The gas permeability of legume nodules is of considerable current interest because nodule responses to drought, nitrate, and photosynthate supply all apparently involve changes in nodule O_2 permeability (6, 7, 11, 14, 16, 20, 22). Similar permeability changes occur in response to acetylene exposure (12) or with increased or decreased external O_2 concentration (23, 27). Studies with microelectrodes (19, 27) and theoretical considerations (18) suggest that nodule permeability is limited mainly by one or more layers of tightly packed cells in the nodule cortex.

Weisz and Sinclair (25) reviewed four methods for estimating nodule gas permeability. One method was rejected as inconsistent with nodule anatomy. The other three methods have given useful and consistent results, but each method has significant limitations. The steady-state acetylene method (4, 26) is somewhat inaccurate and too slow to monitor rapid changes in nodule permeability. It is, however, the only method that has been used under field conditions (5, 22) or with an individual nodule (26). Permeability estimates based on an entire nodulated root represent an average for a number of nodules. The steady-state oxygen method (17) makes two key assumptions: (a) that O_2 consumption rate in the nodule interior can be calculated from CO_2 production by a nodulated root, and (b) that infected cell dissolved O_2 concentration (O_i) is always zero. These assumptions may not be true under all conditions; nevertheless, this method has been widely used. The unsteady-state acetylene method (24) has not been seriously criticized, but the procedure is tedious, requiring chromatographic analysis of many gas samples for each permeability measurement.

FOL,[1] determined by spectrophotometry in total darkness, has been used to estimate O_i in cut nodules (1), detached nodules (13), and nodules flattened during growth (9, 10). An improved, pulse-modulated method (11) allowed measurement of FOL in intact, attached nodules under ambient light. This method revealed that several treatments which inhibit nitrogen fixation also decrease O_i, under both laboratory (11) and field (6) conditions. It was concluded that lower O_i in inhibited nodules resulted from a decrease in nodule O_2 permeability.

The rate of increase of FOL, after switching the atmosphere around a nodule from nitrogen to oxygen, was greater for control nodules than for inhibited nodules. The rate of change of FOL represents a balance between O_2 diffusion through the cortex and O_2 consumption in the nodule interior. Therefore, it was suggested that a rapid increase in FOL could indicate relatively high O_2 permeability (6, 11). We have subsequently developed a new method to quantify nodule O_2 permeability, based on the rate of change of FOL after a step-change in external O_2 concentration. The method also estimates O_2 consumption rate in the infected cells of legume nodules as a function of infected cell O_2 concentration. This paper explains this unsteady-state oxygen method and illustrates its application.

MATERIALS AND METHODS

Plant Culture and Experimental Conditions

Birdsfoot trefoil (*Lotus corniculatus* L. cv Fergus) seedlings were grown under controlled conditions. Seeds were surface-sterilized in 0.5% sodium hypochlorite for 5 min and then germinated on moist paper towels. Germinated seeds were transferred to growth pouches (Vaughan's Seed Co., Downers

[1] Abbreviations: FOL, fractional oxygenation of Lb; O_i, concentration of dissolved O_2 in the nodule interior; Lb, leghemoglobin.

Grove, IL) and inoculated with *Rhizobium loti* strain 95C11 (Nitragin, Milwaukee, WI). Plants were grown with a 12-h photoperiod and a PPFD of 500 μE m^{-2} s^{-1} at plant height, 24°C day and 18°C night. A nitrogen-free nutrient solution (21) in the growth pouches was drained and replaced twice weekly.

Measurements of FOL were made 55 to 70 d after germination, on attached nodules of plants transferred to the laboratory at least 1 h previously. Root and shoot dry weights per plant averaged 33 ± 13 and 31 ± 13 mg (mean ± SE, $n = 4$), respectively, for 69-d-old plants. A combination of sodium and metal halide lamps provided a PPFD of 500 μE m^{-2} s^{-1} at plant height during the experiments. Lamps were covered during actual assays (typically <2 min out of 20) to improve the signal:noise ratio of the spectrophotometry. All measurements were made at room temperature (22°C).

Measurement of Lb Oxygenation

Lb oxygenation was measured noninvasively with the device shown in Figure 1. The principle of operation is somewhat similar to that of Layzell *et al.* (11), but an instrumentation microcomputer (SPCL-0004-X22, New Micros, Dallas, TX) replaced most of the bulky and expensive electronics. In addition, a second wavelength was added to correct for optical changes unrelated to Lb oxygenation. The device measured nodule transmittance by internal reflection (hereafter referred to as 'transmittance') of red (660 nm) radiation, which varied with FOL (9), and infrared (820 nm) radiation, which was almost unaffected by FOL. The red and infrared radiation were generated by light-emitting diodes (LEDs; Motorola (MFOE76 and MFOE71) coupled to a 1 mm o.d. fiber optic tree (Aster Corp., Milford, MA). A small fraction of the radiation passing through the nodule returned through a second optical fiber to a photodetector (OP598C, TRW, Carrollton, TX). Output from the photodetector was amplified and low-pass filtered, then digitized and recorded by the instrumentation microcomputer. The microcomputer also controlled the LEDs. Readings of photodetector output with the nodule illuminated successively by the red LED, the infrared LED, or ambient light only were made at intervals of approximately 4 ms. The average reading for each mode of illumination was recorded every 0.5 s.

Various concentrations of O_2 in N_2 were used in these experiments. Mass flow controllers (Fig. 1) allowed computer control of gas composition around the nodule. The gas stream was humidified by passing through wet glass wool.

Fractional Lb oxygenation and O_i were calculated from the ratio of red:infrared transmittance, after subtracting ambient light. This ratio was used because of the assumption that transmittance at each wavelength could be approximated by a two-component version of the Beer-Lambert Law:

$$I_{660} = I_0 \cdot e^{-[f(t) + g(\mathrm{FOL})]} \quad (1)$$

$$I_{820} = I_0 \cdot e^{-[f(t) + k]} \quad (2)$$

where I_{660} and I_{820} are the intensities of transmitted light at the two wavelengths, I_0 is the incident intensity for each wavelength, t is time, and k is a constant which reflects possible differences (independent of Lb oxygenation) in absorbance with wavelength. The functions $f(t)$ and g(FOL) represent the assumptions that both wavelengths may be affected by some optical changes with time (*e.g.* slight movements of the probe or surface drying of the nodule), whereas only red light is affected by changes in Lb oxygenation. The ratio of red:infrared should eliminate the time dependence.

$$\frac{I_{660}}{I_{820}} = e^{-[g(\mathrm{FOL}) - k]} \quad (3)$$

Although use of the red:infrared ratio improved stability, successive readings at FOL = 0 still varied slightly over time for many nodules. Therefore, a linear correction for drift was also included (6). Calculation of Lb oxygenation and infected cell O_2 concentration was consistent with previous work (11).

$$\mathrm{FOL} = \frac{\ln(R_t) - \ln(R_0)}{\ln(R_{100}) - \ln(R_0)} \quad (4)$$

$$O_i = \frac{\mathrm{FOL} \cdot k_2/k_1}{1 - \mathrm{FOL}} \quad (5)$$

where R_t, R_0, and R_{100} are the red:infrared ratios at any time, or the steady-state values obtained under 100% N_2 or 100%

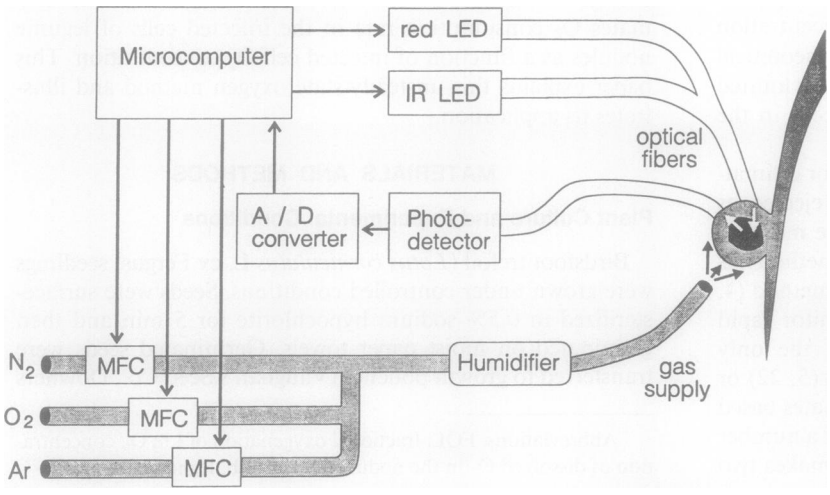

Figure 1. Apparatus used to measure fractional oxygenation of Lb and mass flow controllers (MFC) used to control gas composition around nodule.

O_2, respectively (see below), and k_2/k_1 (ratio of reaction coefficients for Lb) was assumed equal to 37 nM (2). Steady-state values under N_2 and O_2 were assumed to correspond to full deoxygenation and full oxygenation of Lb, respectively.

Standard Assay

In the standard assay, a nodule was exposed first to 100% N_2 (about 40 mol m^{-3}), then to 100% O_2, and finally to 100% N_2. Each gas exposure was maintained until FOL reached a steady state. Between standard assays, the nodule was exposed to 20% (v/v) O_2 in N_2, except as noted. Gas flow rate was always 1.0 L min^{-1}.

Calculation of Respiration Rate and Permeability

Infected-zone respiration (O_2 consumption) rate and nodule O_2 permeability were calculated from the rate of change of FOL after a change in O_2 concentration around the nodule. It was assumed that any change in FOL resulted from an imbalance between inward diffusion of O_2 and O_2 consumption in the nodule interior. Quantitatively,

$$\frac{d\mathrm{LbO_2}}{dt} = P \cdot A \cdot (\mathrm{O_e} - [\mathrm{O_i}/\alpha]) - \frac{V_{max} \cdot \mathrm{O_i}}{K_m + \mathrm{O_i}} \qquad (6)$$

where $d\mathrm{LbO_2}/dt$ is the rate of change in oxygenated Lb (mol m^{-3} s^{-1}), P is the nodule permeability (m/s), A is the surface area of the nodule diffusion barrier (m^2), O_e is the gas-phase O_2 concentration external to the diffusion barrier (mol m^{-3}), O_i is the dissolved O_2 concentration in the infected cells (mol m^{-3}), α is the O_2 solubility in cytoplasm (assumed to be 0.03), V_{max} is the maximum O_2 consumption rate in the nodule interior (mol m^{-3} s^{-1}), and K_m is the value of O_i for which O_2 consumption rate equals one-half of V_{max}. The first term on the right-hand side of the equation represents inward O_2 diffusion (Fick's Law in one dimension), whereas the second term represents O_2 consumption in the nodule, assuming that the dependence of respiration rate on O_i can be described by the Michaelis-Menten equation. Use of the solubility coefficient, α, assumes O_2 equilibrium between the cytoplasm of infected cells and the intercellular air spaces. Although this assumption would not be strictly true under changing conditions, the difference $O_e - (O_i/\alpha)$ is quite insensitive to the value of α, because O_i is approximately zero. All calculations were implemented using MathCAD (MathSoft, Cambridge, MA).

The surface area of the diffusion barrier was calculated from the radius of the infected zone, measured with an ocular micrometer, based on the assumption that the infected zone was spherical. This is a reasonable approximation for the determinate nodules of birdsfoot trefoil. The concentration of oxygenated Lb was calculated from FOL by assuming a total Lb concentration in the infected zone of 0.68 mol m^{-3} (2).

Respiration rate was calculated, as FOL decreased under 100% N_2, from the rate of decrease of FOL. Outward diffusion of O_2 into the external 100% N_2 atmosphere was assumed to be zero, because the gradient driving outward diffusion ($[O_i/\alpha]$ – zero) was negligible relative to that which drives inward diffusion under air ($O_e - [O_i/\alpha]$). The K_m and V_{max} for O_2 consumption were calculated from the relationship between respiration rate and O_i, using the nonlinear curve-fitting capabilities of MathCAD. Oxygen permeability was then calculated from the rate of increase of FOL under 100% O_2, using Equation 6, after correcting for respiration using the previously calculated K_m and V_{max}.

Experimental Treatments

The procedure described above required full oxygenation of Lb for a few seconds per assay. The value of O_i corresponding to full oxygenation is undefined (Eq. 5) but probably exceeds 5 μM. Because nitrogenase can be inactivated by O_2, the effects of even brief exposure to elevated O_i were of concern. Therefore, a series of experiments were designed to evaluate the effects of elevated O_2 concentration on nodule respiration and permeability. Each experiment was repeated three times.

The first experiment evaluated the effects of a single standard assay on nodule respiration. Leghemoglobin oxygenation was continuously monitored as the gas around a nodule was changed from air, to 100% N_2, to 50% O_2 in N_2, back to 100% N_2, to 100% O_2, and finally to 100% N_2. Each gas treatment was maintained until FOL reached a steady state, except that the 50% O_2 treatment was switched to 100% N_2 as soon as Lb oxygenation reached about 50%. Respiration rate, as a function of O_i, was compared as FOL decreased under 100% N_2 following air, 50% O_2, and 100% O_2.

The second experiment compared respiration and permeability before and after a treatment which was intended to inactivate nitrogenase. Standard assays were performed on the same nodule at intervals of 20 min. Between assays, the nodule was exposed to humidified 'air' (20% O_2 in N_2) at the standard flow rate (1.0 L min^{-1}). After four assays, the nodule was exposed to 100% O_2 for 15 min. The nodule was then maintained in flowing air for about 10 min before standard assays resumed at 20-min intervals for a period of at least 4 h. Nodule O_2 permeability and the respiration parameters, V_{max} and K_m, were calculated for each assay, as described above. As a control, the same experiment was repeated without the 15-min exposure to 100% O_2.

A third experiment assessed the ability of birdsfoot trefoil nodules to acclimate to elevated external O_2 concentration. After four standard assays, the mass flow controllers were programmed to increase O_e from 20 to 40% over a 40-min period. Standard assays were then resumed at 20-min intervals, with O_e maintained at 40% between assays. After seven assays under 40% O_2, O_e was returned to 20% (over 40 min) and then four additional assays were performed.

Acetylene Reduction

In a separate experiment (with 69-d-old birdsfoot trefoil plants grown as described above), the rate of acetylene reduction was measured for control nodules (never exposed to elevated O_2), nodules which had been assayed once, and nodules exposed to a 30-min 100% O_2 treatment. A short segment of root with the nodule attached was incubated at room temperature for 15 min in a 13 mL test tube containing 1.0 mL of acetylene. Ethylene production was measured by gas chromatography. The nodule was then detached from the root segment for measurement of fresh weight.

RESULTS

Effects of Standard Assay

Lb oxygenation under air, prior to any treatment, averaged 20.0 ± 4.4% (mean ± SE, $n = 9$), equivalent to a dissolved O_2 concentration of 9.2 nM. Lb oxygenation decreased under 100% N_2 and increased under 50% or 100% O_2 (Fig. 2).

The respiration rate depended on O_i (Fig. 2, inset). At a given O_i, differences in respiration rate due to pretreatment (air, 50% O_2, or 100% O_2) were small enough that a single Michaelis-Menten equation fit the data for all three pretreatments. For the typical example shown in Figure 2, V_{max} was 151 pmol O_2 s^{-1} $nodule^{-1}$ and K_m was 15.4 nM.

O_2 Inactivation of Nitrogenase

The effects of a 15-min exposure to 100% O_2 were apparent both in the Lb oxygenation data and in calculated values for respiration rate and permeability. The results shown in Figure 3 are typical. Relative to the control, FOL under air was lower after treatment (Fig. 3A). The rate of increase of FOL under 100% O_2 and the rate of decrease under 100% N_2 also declined (*cf.* spacing of data points). Respiration data for each treatment generally fit the Michaelis-Menten equation well. However, one or two data points corresponding to the highest O_i values often fell substantially below the fitted curve and were excluded from the curve-fitting procedure (Fig. 3B).

Exposure to 100% O_2 for 15 min resulted in lower V_{max} and K_m. For the example shown, V_{max} decreased from 175 to 112 pmol s^{-1} $nodule^{-1}$, and K_m decreased from 11.4 to 7.5 nM. For all nodules in this treatment, the average V_{max} following 15-min O_2 exposure was 71.5 ± 2.0% ($n = 3$) of the control, whereas K_m was 64.3 ± 4.6% of control.

Nodule permeability, calculated as O_i increased under 100% O_2, varied somewhat within a single assay. In Figure 3C, typical results were plotted against O_i to allow comparison with Figure 3B. Time might also be an appropriate independent variable. For each treatment, there was an initial increase in apparent permeability with O_i or time, followed by a decline. Peak permeabilities of O_2-inhibited nodules averaged 58.9 ± 21.9% ($n = 3$) of the pretreatment values.

The negative effects of the 15-min O_2 treatment on V_{max} and permeability persisted for at least 270 min (Fig. 4). Peak permeabilities and maximum respiration rates of control nodules typically declined gradually with time, although increases with time were occasionally observed (data not shown). The calculated K_m showed more unexplained variability over time than either permeability or V_{max}.

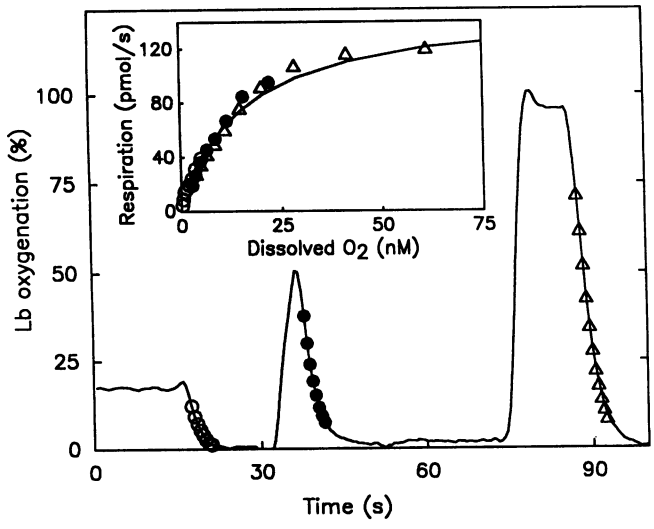

Figure 2. Effects on Lb oxygenation of successive exposures to external O_2 concentrations of 20, 0, 50, 0, 100, and 0%. Inset: respiration rate as a function of O_i after exposure to 20 (○), 50 (●), and 100% O_2 (△).

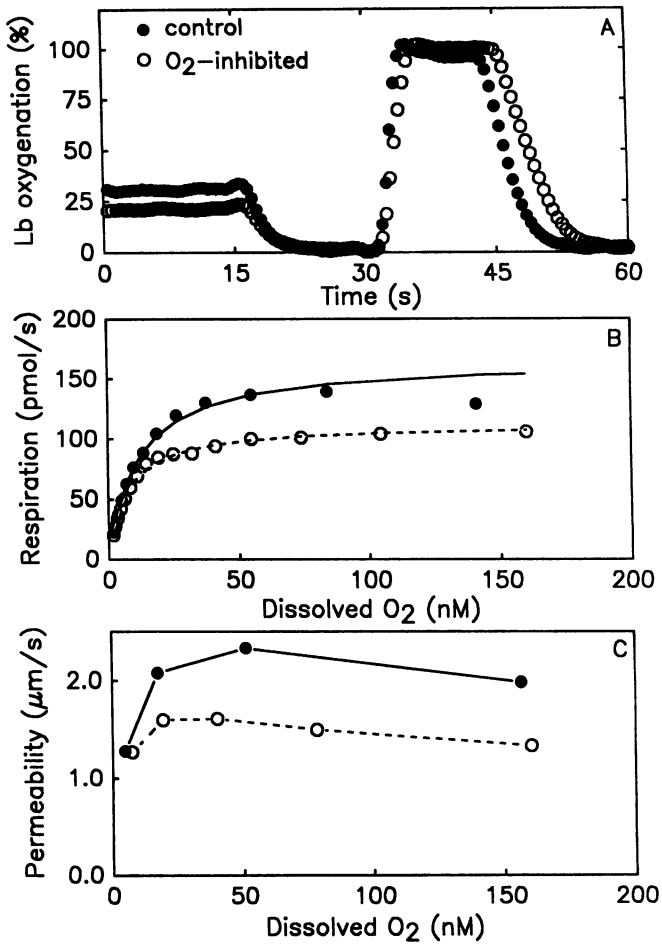

Figure 3. Effects of a 15 min exposure to 100 O_2 on (A) dynamics of Lb oxygenation in response to external O_2 concentration, (B) nodule respiration rate as a function of O_i, and (C) calculated nodule O_2 permeability as a function of O_i.

Effects of a Gradual Increase in External O_2 Concentration

A gradual increase in external O_2 concentration to 40% resulted in lower permeability (Fig. 4A) but did not affect V_{max} or K_m (Fig. 4, B and C). Permeability under 40% O_2 averaged 55.3 ± 1.3% ($n = 3$) of the previous value under

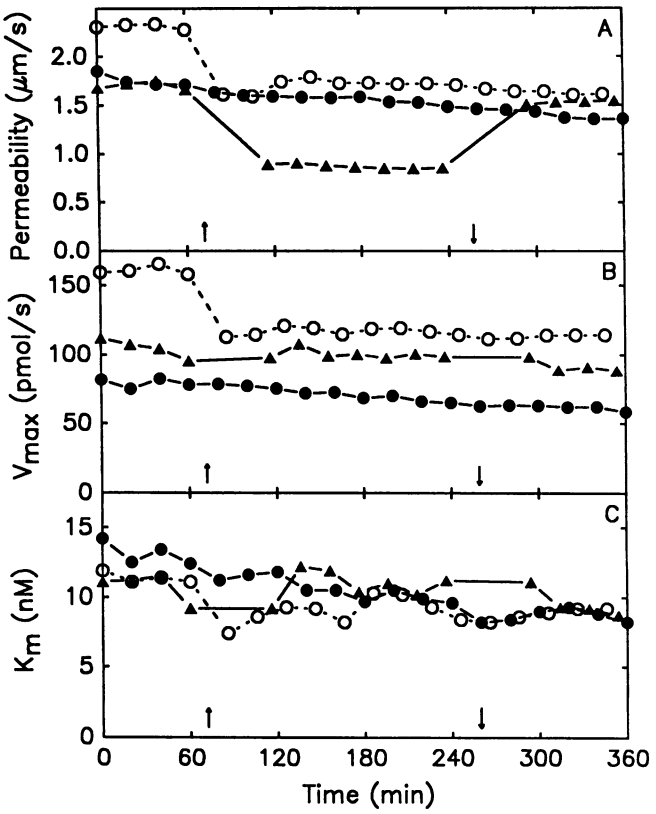

Figure 4. Repeated measurements of (A) nodule O_2 permeability, (B) maximum respiration rate, and (C) K_m for O_2, calculated as described in the text. Treatments were control (●), exposure to 15 min of 100% O_2 (○), or a gradual increase in external O_2 concentration to 40% followed by a return to 20% (▲). Up arrows indicate the time of the 15 min exposure to 100% O_2, or the O_2 ramp up to 40%. Down arrows indicate the O_2 ramp back down to 20%.

20% O_2. The lower permeability was maintained for 2 h under 40% O_2 and increased to nearly the initial value after O_e returned to 20%.

Acetylene Reduction

Acetylene reduction rates for control and assayed nodules averaged 24.3 ± 4.0 and 20.4 ± 4.1 μmol C_2H_4 g^{-1} fresh weight h^{-1} (n = 4), respectively. The difference was not significant. Rates would be about fourfold higher on a dry weight basis. Acetylene reduction by nodules exposed to 100% O_2 for 30 min was undetectable for all three replicates.

DISCUSSION

Overall Assessment of the Method

There were two major conclusions from these experiments. First, noninvasive spectrophotometry of Lb previously used to measure O_i, can also measure respiration parameters (V_{max} and K_m) and O_2 permeability of single attached nodules. Second, the assay itself does not appear to cause serious disruption of nodule function, at least in birdsfoot trefoil.

There remain some unanswered questions, discussed below, which may affect the quantitative accuracy of the method. Nevertheless, it is clear that the unsteady-state oxygen method provides new opportunities for nitrogen fixation research.

Effects of the Assay on Nodule Function

A single assay using 100% O_2 had little immediate effect on respiration (Fig. 2). This suggests that the assay caused little or no inactivation of nitrogenase. Partial inactivation of nitrogenase might occur without a change in respiration. However, the decreased permeability and V_{max} following a 15 min exposure to 100% O_2 indicate that major O_2 damage would have been detected, had it occurred. The acetylene reduction data also showed that a single exposure to the assay did not result in significant damage to nitrogenase.

With repeated assays (Fig. 4), control nodules often showed gradual declines in permeability, maximum respiration, and K_m. Increases in these variables were observed less frequently. Possible explanations for these changes include repeated O_2 exposure, drying of the nodule surface by flowing gas (despite humidification), effects of slight mechanical pressure by the probe, conditions in the laboratory, or physiological changes unrelated to the assay (*e.g.* circadian rhythms). In any case, these changes in control nodules were small relative to treatment effects. We conclude that the repeated brief exposures to high external O_2 concentration required by this assay probably do not cause major disruption of nodule function.

The acetylene reduction assay is often inaccurate because exposure of nodules to acetylene results in decreased nodule gas permeability (12). Nonetheless, it is clear from the acetylene reduction data that prolonged exposure to 100% O_2 essentially eliminated nitrogenase activity, whereas the effect on O_2-saturated respiration was much less severe. The steady-state respiration rate under air would depend on O_2 permeability, which decreased almost 50%, rather than on V_{max} and K_m. It appears that there was still considerable metabolic activity, possibly including resynthesis of nitrogenase, in O_2-treated nodules.

Interpretation of Respiration and Permeability Curves

The good agreement between the respiration data and a standard Michaelis-Menten equation is somewhat surprising because of the multiple sinks for O_2 in nodules. Multiple terminal oxidases differing in K_m (5–40 nM) have been reported for bacteroids from soybean (*Glycine max* [L.] Merr.) and cowpea (*Vigna unguiculata* [L.] Walp.) nodules (3). Oxygen uptake by mitochondria could also be significant. The K_m of Cyt terminal oxidase from cowpea nodule mitochondria is approximately 100 nM (15).

It was observed that a decrease in V_{max} was often accompanied by a decrease in K_m. This could indicate that the low affinity terminal oxidase has higher maximum O_2 uptake. It might be possible to test this hypothesis using specific inhibitors. A positive correlation between apparent K_m and V_{max} can also result from restricted diffusion (4). This is unlikely to be the case with the present method, because both O_2 concentration and O_2 consumption were measured in the nodule interior.

Calculated nodule O_2 permeabilities varied somewhat as

FOL increased under 100% O_2. It seems unlikely that the actual permeability could change so quickly (<10 s). The initial rise and subsequent decline in calculated permeability may indicate a failure of respiration to respond instantaneously to changes in O_i, perhaps because of transient gradients in O_i. A transient overestimate of respiration rate in Equation 6 would result in a transient overestimate of permeability. Therefore, it is possible that later permeability estimates (as FOL approaches 1.0) may be more accurate than peak values.

Relationship between Permeability and V_{max}

The 15-min exposure to 100% O_2 and the gradual increase to 40% O_2 both resulted in decreased permeability, but it is possible that there were differences in mechanism. A decrease in permeability following an increase in O_e has previously been reported (9, 23). This suggests the existence of an O_2 sensor somewhere in the nodule which controls permeability to regulate O_i. A sensor outside the diffusion barrier would detect changes in the external atmosphere but not changes in O_i due to changes in respiration rate. Such an external O_2 sensor could explain the changes in permeability with the gradual increase to 40% O_2 and subsequent return to 20% O_2. However, an external sensor would not explain the failure of permeability to recover after the 15-min exposure to 100% O_2.

Inactivation of nitrogenase by the 15-min 100% O_2 treatment might be expected to decrease maximum respiration rate by decreasing the sink for ATP and reductant. Such a decrease in V_{max} was in fact observed. The decrease in V_{max} could have allowed O_i to rise, which would have triggered a decrease in nodule permeability if there were an O_2 sensor in the nodule interior. Under this hypothesis, the stimulus for permeability decrease in the two different O_2 treatments would be the same. Instead, O_i under air actually decreased following the 15-min 100% O_2 treatment (Fig. 3A). Therefore, neither an internal nor an external O_2 sensor can fully explain the permeability decrease with the 15-min 100% O_2 treatment.

An alternative explanation for the permeability decrease with the 15-min 100% O_2 treatment is that maintenance of high permeability somehow depends on the products of N_2 fixation. This explanation is consistent with previous reports of decreased permeability with acetylene exposure (12) or with replacement of N_2 with argon (8).

Limitations, Assumptions, and Potential of the Method

The unsteady-state O_2 method allows measurements of O_i, respiration rate (including K_m and V_{max}), and O_2 permeability, all noninvasively and on a single nodule. The procedure is faster and easier than previous methods, with the possible exception of the steady-state O_2 method. Because measurements of gas exchange are not required, nodulated roots do not have to be grown or assayed in any type of cuvette. Therefore, the method should be applicable to a wide range of growth conditions, including ordinary field plots. A prototype of an inexpensive portable unit for field use is under development.

As with any new method, there are some areas of uncertainty. The use of assumed rather than measured Lb concentration may somewhat limit the absolute accuracy of this method. Measurement of Lb concentration might be worthwhile in experiments where Lb concentration was expected to vary with treatment. The method, as presently implemented, assumes that exposure to 100% N_2 and 100% O_2 result in fully deoxygenated and fully oxygenated Lb, respectively. The assumption that Lb will eventually become deoxygenated under N_2 is difficult to challenge, but full oxygenation under 100% O_2 might not always be achieved. In a nodule with very low permeability and high V_{max}, a balance between O_2 influx and consumption might occur at less than 100% oxygenation of Lb. In such nodules, FOL would be expected to approach a maximum value (possibly <1) asymptotically. The flat-topped curves in Figure 3A suggest that full oxygenation was achieved in these experiments.

Recent research has highlighted the importance of permeability regulation to maintenance of nodule function. This new technique should facilitate detailed studies of nodule physiology to uncover the mechanisms by which nodules detect and acclimate to stress. Because Lb oxygenation can be measured under field conditions (6), the method may also prove useful for agronomic research and in crop improvement programs.

ACKNOWLEDGMENT

We thank Barry Harter for expert technical assistance.

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