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Nitrogen fixation and respiration by root nodules of *Alnus rubra* Bong.: Effects of temperature and oxygen concentration*

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Summary Using a root nodule cuvette and a continuous flow gas exchange system, we simultaneously measured the rates of carbon dioxide evolution, oxygen uptake and acetylene reduction by nodules of *Alnus rubra*. This system allowed us to measure the respiration rates of single nodules and to determine the effects of oxygen concentration and temperature on the energy cost of nitrogen fixation. Energy cost was virtually unchanged (2.8–3.5 moles of carbon dioxide or oxygen per mole of ethylene) from 16 to 26°C (pO_2 = 20 kPa) while respiration and nitrogenase activity were highly temperature dependent. At temperatures below 16°C, nitrogenase activity decreased more than did respiration and as a result, energy cost rose sharply. Acetylene reduction ceased below 8°C. Inhibition of nitrogenase activity at low temperatures was rapidly reversed upon return to higher temperatures. At high temperatures (above 30°C) nitrogenase activity declined irreversibly, while respiration and energy cost increased.

Energy cost was nearly unchanged at oxygen partial pressures of 5 to 20 kPa (temperature of 20°C). Respiration and nitrogenase activity were strongly correlated with oxygen tension. Below 5 kPa, acetylene reduction and oxygen uptake decreased sharply while production of carbon dioxide increased, indicating fermentation. Fermentation alone was unable to support nitrogenase activity. Acetylene reduction was independent of oxygen concentration from 15 to 30 kPa. Nitrogenase activity decreased and energy cost rose above 30 kPa until nearly complete inactivation of nitrogenase at 70–80 kPa. Activity declined gradually, such that acetylene reduction at a constant oxygen concentration was stable, but showed further inactivation when oxygen concentration was once again increased. Alder nodules appear to consist of a large number of compartments that differ in the degree to which nitrogenase is protected from excess oxygen.

Introduction

While it is generally accepted that changes in root nodule temperature and in ambient oxygen concentration can affect symbiotic nitrogen fixation, the pattern of these sensitivities remains unclear. The effects of temperature and oxygen on the physiology of actinorhizal root nodules are particularly interesting because of the differences in structure between Rhizobium and *Frankia* induced nodules and the striking diversity of actinorhizal host species. It is important to know of potential limitations or special advantages of the actinorhizal

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symbiosis as a source of reduced nitrogen over a wide range of environmental conditions. The energy requirement for nitrogenase activity is high[8,13] so it is also valuable to know the effects of environmental conditions on the energy cost of fixation. The effects of oxygen concentration and temperature on the energy cost of nitrogen fixation have been investigated in detail in some legume species[6,7,8], but only limited information is available for the *Frankia* symbiosis[14,15,16].

Changes in nitrogen fixation and in nodule respiration caused by shifts in temperature and oxygen can also be used to understand the mechanisms which allow aerobic plant tissue to provide a satisfactory environment for nitrogenase, which is rapidly inactivated by molecular oxygen. There is support for the hypothesis that protection of nitrogenase from excess oxygen in legume and Parasponia nodules is the result of a barrier to gaseous diffusion around the site of fixation combined with the consumption of oxygen by enzymatic reactions inside the barrier[9,11]. While this type of mechanism may also exist in actinorhizal nodules, the location and nature of both the barrier and the uptake system remain unknown. It is known that enzymatic catalysis and oxygen diffusion respond differently to changes in temperature or in external oxygen concentration. A valid model for oxygen protection in actinorhizal nodules must take these biophysical facts into account and successfully predict the pattern of temperature and oxygen effects on individual nodules.

The responses of nitrogen-fixing symbiosis to environmental conditions have most frequently been measured using intact root systems and detached nodules. Such experiments are particularly suitable when defining the interaction between habitat and plant growth (see references in 7 and 8). Previous investigations of temperature and oxygen effects on actinorhizal nitrogen fixation have been done with large samples of detached nodules and with many replicates[1,4,14,15,16].

In contrast, we chose to collect extensive data from single nodules. For the majority of our experiments we took advantage of a technique which allowed us to assay the acetylene reduction and respiration rates of single attached nodules for extended periods of time[17]. In that way, the relationship between nodule respiration and nitrogenase activity was observed directly, the effect of between nodule variation was eliminated, and the nodule environment was tightly controlled.

Materials and methods

Plant material

Seeds of *Alnus rubra* Bong. (Clatsop County, OR) were germinated in sand flats in a growth chamber. When the seedlings were 3–5 cm tall, they were washed free of sand and transferred

to a modified aeroponics box[19] in a greenhouse. Supplemental lighting was provided by 1000 W high-pressure sodium lamps (Sylvania LU-1000). The seedlings were grown on $\frac{1}{4}$-strength Hoagland's solution[5] supplemented with iron (II) phosphate, crushed oyster shells (which maintained the pH between 6.5 and 7.0) and 2 m*M* nitrate. When the plants had reached 10–15 cm tall, they were inoculated with a homogenized suspension of *Frankia* strain ArI3[3] and the nitrate supplement was discontinued. Nodules formed in 2–3 weeks. Nodules used in these experiments were 8 to 12 weeks old and ranged in size from 8 to 15 mm in diameter (0.4 to 1.2 gm, fresh weight).

Assay system

For attached nodules, rates of carbon dioxide and ethylene production and of oxygen uptake were measured using a root-nodule gas-exchange system as previously described[17]. To measure oxygen uptake rates more precisely, some experiments were done on detached nodules enclosed in either a gas-tight syringe or a brass cuvette. Detached nodule assays were limited to the time of constant activity following excission (usually 3 hours, see[13]).

Assay protocols, including the making of gas mixtures, calibration of the gas chromatograph, temperature control and control of the shoot environment were identical to those previously described[13,17] with the following additions. All mixtures contained 10 kPa acetylene, generated from calcium carbide. Each experiment began at 20°C and 20 kPa oxygen. Nodules were inserted into the assay chamber and allowed to equillibrate. When activity had stabilized, the temperature or gas mixture was changed and the new rate determined when activity once again became constant.

A few nodules did not become stable within a few hours. Changes in activity usually followed a pattern of increasing nitrogenase activity and a slowly decreasing respiration rate, perhaps indicating recovery from some form of stress. Since slow changes in respiration and nitrogenase activity made interpretation of changes following shifts in environmental conditions impossible and since such nodules were atypical, they were not used in these experiments.

Temperature curves were made by: 1. lowering nodule temperature in steps, 2. returning to 20°C, 3. waiting until activity became the same as the initial activity, and 4. increasing the temperature in steps. This postponed until last the irreversible changes in activity that often occurred at high temperatures.

Oxygen curves were made by first decreasing the oxygen tension, until the mixture was pure nitrogen and acetylene with a trace of contaminating oxygen. Returning the nodule to higher oxygen concentrations caused almost complete inactivation of nitrogenase. As a result the nodule was allowed to recover (for one or two days) until activity and energy cost returned to the values measured before the oxygen treatment. The same nodule was then re-introduced into the assay cuvette and exposed concentrations above 20 kPa.

Calculations

Gas exchange rates were calculated as described previously[17] and are reported either on a per nodule or a per gram fresh weight basis. Energy cost was estimated as the molar ratio of either carbon dioxide evolution or oxygen uptake to the rate of ethylene production at 10 kPa acetylene. The gas exchnage quotient (GEQ) was calculated as the molar ratio of carbon dioxide evolution to oxygen uptake. Total reductant used during acetylene reduction was calculated as the sum of the electrons used to reduce oxygen and those used to reduce acetylene. We assumed 4 moles of electrons per mole of oxygen reduced and 2 moles of electrons per mole of acetylene reduced. Glucose use per acetylene reduced was calculated either from total reductant, assuming 24 electrons per glucose, or from carbon dioxide evolution, assuming 6 carbon dioxides per glucose.

Note: mixtures of high oxygen concentration and 10 kPa acetylene are extremely flammable and should be made in small quantities, in soft containers, and kept away from any source of spark or flame.

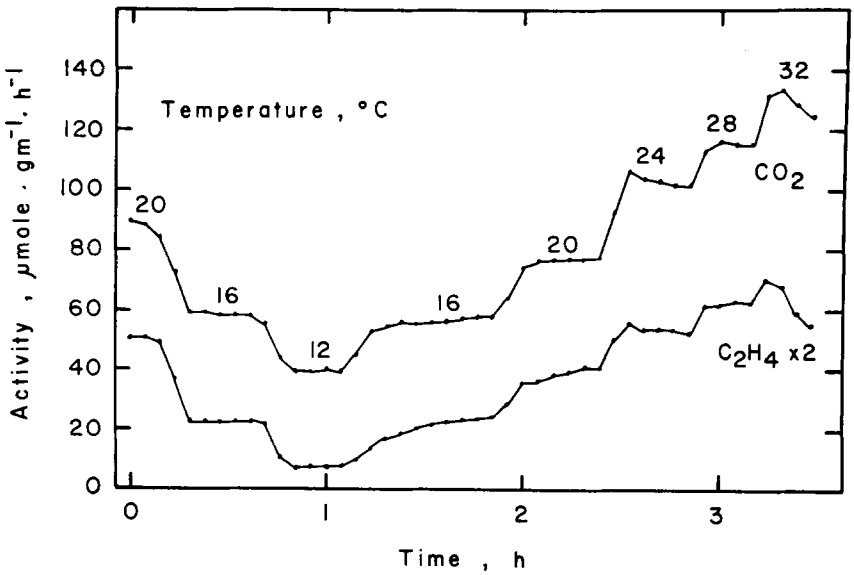

Fig. 1. Time course of temperature response experiment. Gas composition was 20 kPa oxygen, 10 kPa acetylene, balance nitrogen. Each point represents one activity determination, expressed per gram fresh weight. Changes from one temperature to another required about 10 minutes for equillibration.

Results

Individual nodules varied widely in their specific gas exchange rates at 20°C and 20 kPa oxygen. For example, acetylene reduction ranged from 14 to 28 μmole/g fresh weight/hour (mean = 24.5, N = 8). As in previous experiments which used a nodule gas exchange system[17], there was very little variation between repeated measurements of carbon dioxide and ethylene evolution by a single nodule (coefficient of variation of less than 2%). The coefficient of variation of oxygen uptake measurements was 5%.

Although specific respiratory and nitrogenase activity differed between nodules, the ratio of the rate of carbon dioxide evolution to the rate of ethylene production (energy cost) was relatively invariant. After a short period of adaptation to the conditions in the assay growth chamber, energy cost for most nodules became steady at between 3 and 4 moles of carbon dioxide per mole of ethylene produced.

Temperature

The pattern of temperature response was reproducible, nodule to nodule. The same general curve resulted from experiments with individual attached nodules (Fig. 2) and from the averaging of data

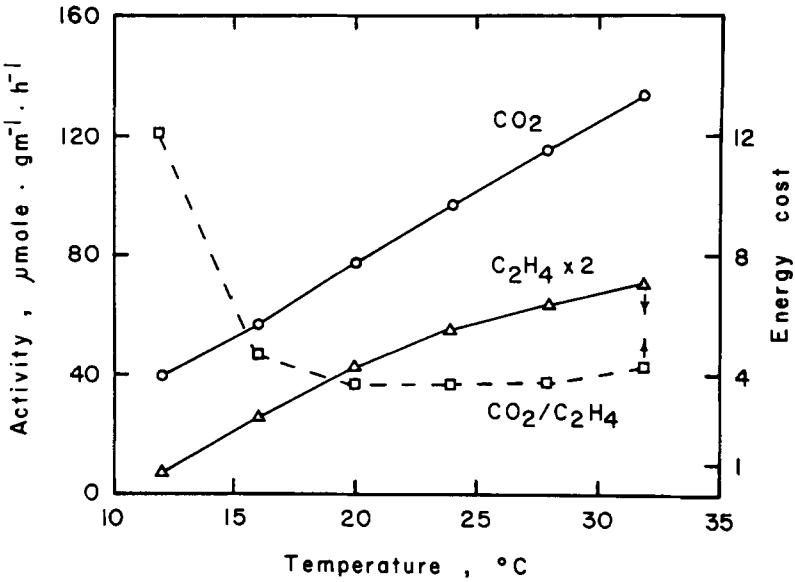

Fig. 2. Typical response curve of an attached nodule to changes in temperature. Each point represents the mean of several activity determinations made under steady state conditions. The data are from the experiment in Figure 1. Energy cost was calculated as the molar ratio of carbon dioxide and ethylene evolution. Since activity at 32°C was unstable, that point represents the higher initial values. Ethylene production progressively declined at this temperature, as indicated by the downward arrow.

from several detached nodules (Fig. 3). Measurements on attached nodules indicated that the response to changes in temperature was rapid (Fig. 1) and activity at any given temperature was constant over the time span of these experiments. As shown in Figure 3, oxygen uptake, measured on detached nodules to ensure precision, closely paralleled carbon dioxide evolution. From 5 to 18°C, oxygen uptake and carbon dioxide evolution were not significantly different. From 20 to 25°C, where acetylene production was greatest, carbon dioxide evolution exceeded oxygen uptake.

The temperature dependence curves can be divided into three regions based upon changes in ethylene and carbon dioxide evolution, oxygen uptake, and in energy cost. While the temperatures bounding these regions varied several degrees between nodules, the regions themselves were evident in every nodule measured.

Region 1. At temperatures below 16–18°C, nitrogenase activity declined more than did respiration (Figs. 2 and 3) and energy cost rose markedly (Figs. 2 and 4). Ethylene production by most nodules

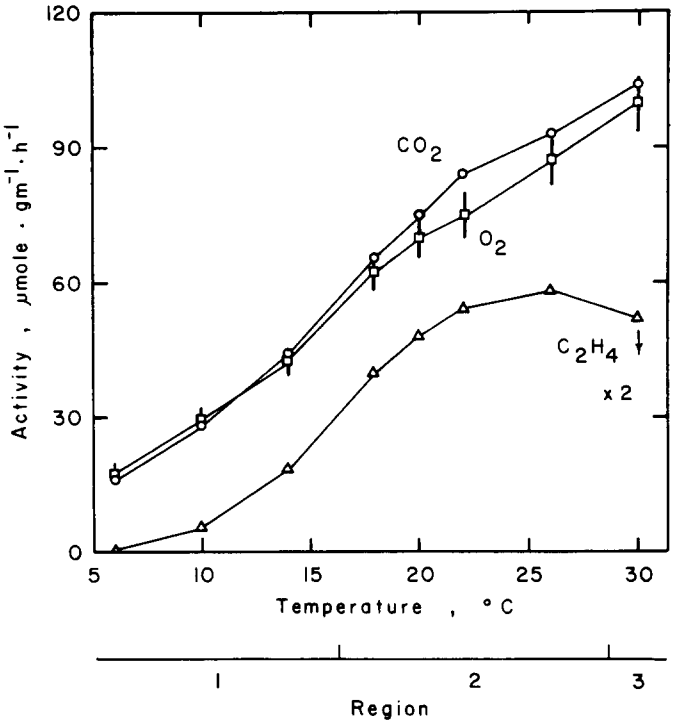

Fig. 3. Average response curve of detached nodules to changes in temperature. Each point represents the mean of 3 to 5 values taken from experiments with different detached nodules, normalized to the mean activity of all nodules at 20°C (fresh weight basis). Error bars on the oxyen points represent ± 1 S.D. Error bars for the ethylene and carbon dioxide data are smaller than the diameter of the symbol. Gas composition was 20 kPa oxygen, 10 kPa acetylene, balance nitrogen. Nitrogenase activity at 30°C was unstable.

ceased altogether below 8°C (Fig. 3). This drop in nitrogenase activity was quickly reversible (Fig. 1). When nodules were exposed to 12°C and then returned to 16°C, carbon dioxide evolution increased at once and acetylene reduction lagged somewhat. The time for complete recovery was short, about 30 minutes.

Region 2. As nodule temperature was increased from 16 to 28°C, acetylene reduction and carbon dioxide evolution increased proportionally, so that the energy cost remained constant (Figs. 2 and 3). For all nodules observed, energy cost was lowest in this region.

Region 3. At or above 30°C, nitrogenase activity became unstable and diminished slowly with time (Fig. 1). Nodules differed in the exact temperature at which this decrease began. Some were stable at

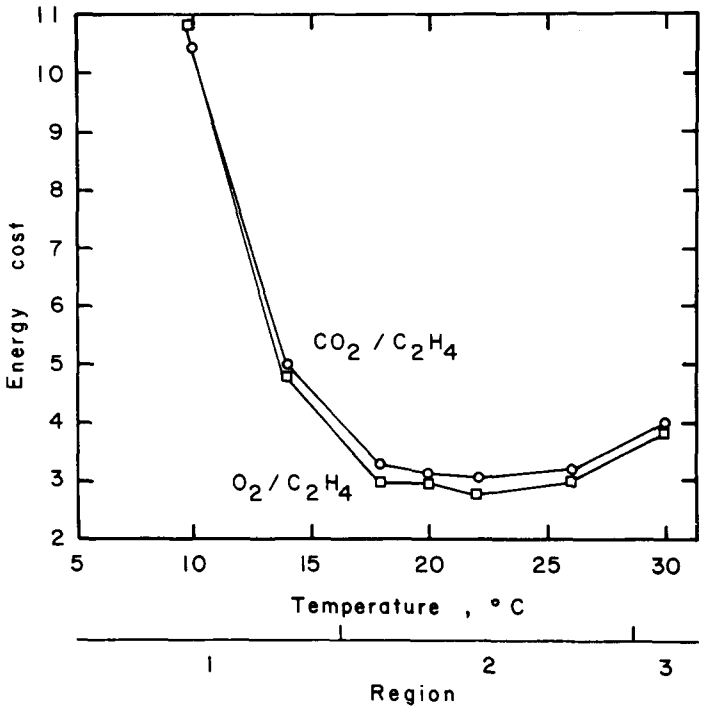

Fig. 4. Calculated energy cost from the data in Figure 3, determined as the molar ratio of either oxygen uptake or carbon dioxide evolution to ethylene production.

temperatures as high as 32°C. Respiration, however, was always more stable than acetylene reduction. Consequently, the energy cost of nitrogen fixation in this region constantly increased. When nodules which had been exposed to temperatures in this region were returned to 20°C, their rates were significantly below those measured initially. Over time (hours), activity did recover.

Oxygen

As with the temperature response curves, the pattern of the effects of different oxygen concentrations on nitrogen fixation and respiration was highly reproducible. Changes in oxygen tension caused distinct changes in the relationship between oxygen and carbon dioxide metabolism and nitrogen fixation, as shown in Table 1.

About 15% of the reducing power produced in nodule tissue during nitrogen fixation at normal oxygen concentrations was used directly by nitrogenase. As a result, the rate of oxygen uptake was significantly lower than the rate of carbon dioxide evolution. The ratio of oxygen consumed to ethylene produced underestimated energy consumption in

Table 1. Reducing equivalents and glucose used (micromoles/nodule, hour) during acetylene reduction by *Alnus rubra* nodules at different oxygen tensions, calculated from the data shown in Fig. 5. (see Materials and Methods for assumptions used)

Region of pO_2 curve	1		2	3	4
pO_2, kPa	0.50	3	10	20	40
Reductant to O_2	3.4	41.0	102.0	147.0	203.0
Reductant to C_2H_4	0.77	8.1	19.0	26.0	8.0
Total reductant used	4.2	49.1	121.0	173.0	211.0
Reductant produced from glucose	35.0	71.0	138.0	180.0	200.0
Glucose/C_2H_4 from reductant used	0.45	0.50	0.52	0.56	2.2
Glucose/C_2H_4 from CO_2 evolution	3.8	0.73	0.59	0.58	2.1

regions of high activity. A better measure of the amount of energy actually used for the activity of nitrogenase was obtained by comparing the glucose used, calculated from both oxygen and acetylene reduction measurements, with the rate of ethylene evolution.

At very low oxygen tensions, carbon dioxide evolution overestimated the direct use of glucose for nitrogenase activity. Again, the consumption of reductant provided a better measure of nitrogenase energy cost. Under such conditions, where metabolism of sugars was not completely aerobic, the conversion from carbon dioxide evolution to the production of reducing equivalents is not valid, but is presented for the purposes of comparison with the reductant used to reduce oxygen and acetylene.

The response curve can be divided into four regions. The values for the usual boundaries of each region are derived from observations at a temperature of 20°C. It is likely that they would differ at other temperatures.

Region 1. At very low oxygen tensions (0–3 kPa) carbon dioxide evolution greatly exceeded oxygen uptake (Fig. 5), resulting in a Gas Exchange Quotient well above 1 (Fig. 6). This indicated a significant amount of fermentative metabolism.

Nitrogenase activity was directly correlated with oxygen concentration and stopped at a concentration just above zero (Fig. 5). Energy cost, based upon carbon dioxide evolution, rose sharply near zero (Fig. 6). Energy cost based upon oxygen uptake continued to decline until both activities became too low for detection. In this region, where nitrogenase activity was oxygen limited, the energy cost based

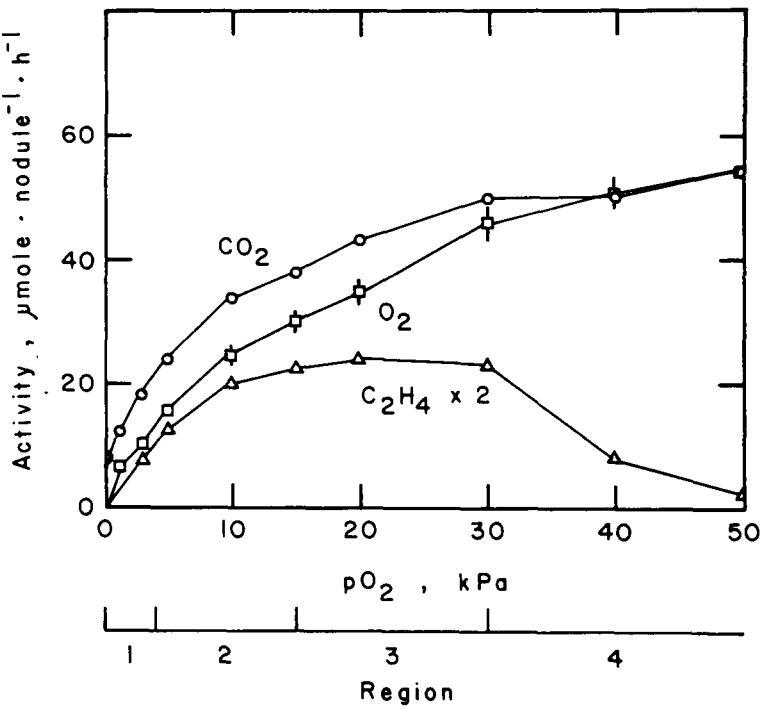

Fig. 5. Typical response of an attached nodule to changes in oxygen concentration – detailed investigation of the lower range. Each point represents the average of several measurements under constant conditions. Error bars are used as in Figure 3. Ethylene production rates were stable over the time span of each determination, even at oxygen concentrations above 30 kPa. Gas mixtures were 10 kPa acetylene, the stated oxygen tension, the balance nitrogen. Nodule temperature was 20°C. Gas flow was 3 ml/min.

upon reductant used was lower than an atmospheric oxygen tensions (Table 1).

Region 2. For most nodules, region 2 extended from 3 to 15 kPa and was characterized by nearly proportional increases in all activities and a relatively constant, low energy cost (Fig. 6). In the experiment shown in Figure 7 the increase in rates appeared to be almost linear, but it was distinctly curved in most experiments, as shown in Figure 5. Oxygen uptake was less than carbon dioxide evolution in this region with the result that the GEQ ranged from 1.6 to 1.2. Total reductant used was also below the rate of carbon dioxide evolution (Table 1), indicating that some fermentation was occurring.

Region 3. At oxygen partial pressures of 15 to 30 kPa, acetylene reduction became independent of oxygen concentration, while

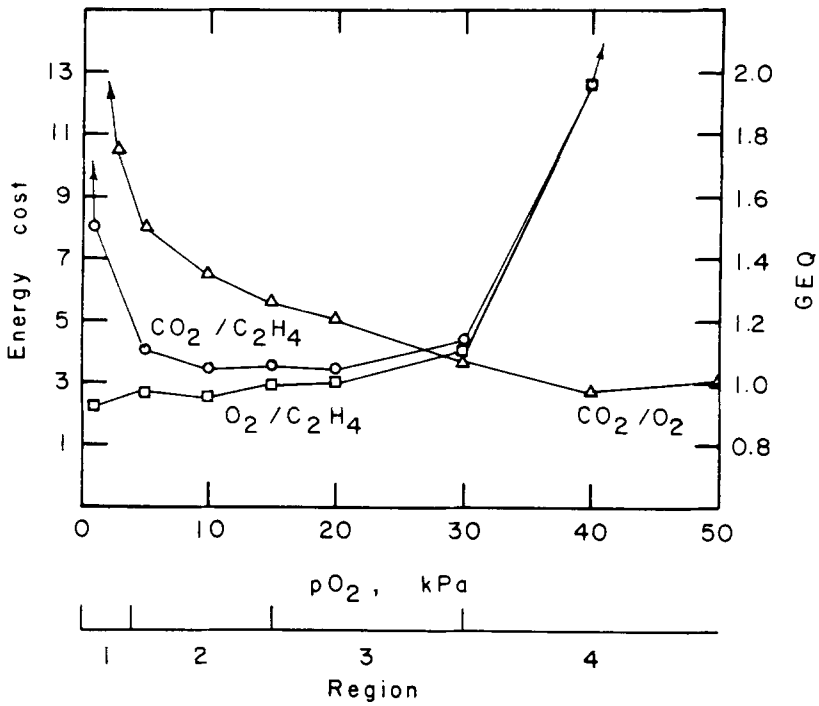

Fig. 6. Energy cost (molar ratio of either oxygen uptake or carbon dioxide evolution to ethylene production) and GEQ (gas exchange quotient, molar ratio of carbon dioxide evolution to oxygen uptake) calculated from the data in Figure 5.

respiration continued to increase (Fig. 5). As a result, energy cost rose somewhat. While the GEQ in this region was significantly above 1 (Fig. 6), there was no evidence of fermentation since total reductant used was equal to that produced (Table 1).

Region 4. Above 30 kPa, ethylene production fell dramatically, in a step-wise fashion (Fig. 7) and energy cost rose accordingly (Fig. 8). At each oxygen concentration, all gas exchange rates were constant and showed no signs of further inhibition or adaptation over the time scale of these experiments (hours). Acetylene reduction by some nodules was still detectable at 80 kPa oxygen (Fig. 7). When nodules were exposed to oxygen concentrations above 30 kPa and then were returned to 20 kPa oxygen, activity was lower than at the start of the experiment. Nitrogenase activity recovered over a period of days. Oxygen uptake and carbon dioxide evolution increased only slightly in this region and were virtually identical in rate (GEQ = 1) (Fig. 8), indicating that respiration was no longer limited by oxygen diffusion.

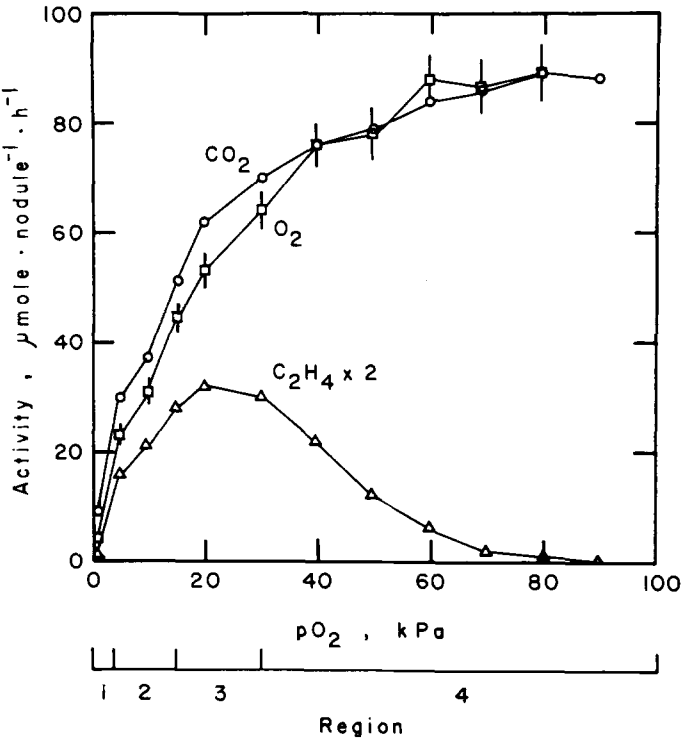

Fig. 7. Typical response of an attached nodule to changes in oxygen concentration up to 80 kPa. Legend as in Figure 5.

Discussion

Our results are in general agreement with previous reports on alders. The complete absence of nitrogenase activity at low temperatures corroborates previous work with detached nodules[14,15]. This loss in activity could be due to an increased concentration of oxygen around the sites of fixation. At lower temperatures, respiration would decrease more rapidly than the rate of diffusion, leading to an increased steady-state concentration of oxygen in the tissue and the inactivation of nitrogenase.

However, this explanation appears to be contradicted by the observation that recovery from low temperatures is much more rapid than from inhibition due to a direct oxygen shock[18]. Adaptation to changes in temperature involves a mechanism much faster than the resynthesis of an enzyme. We conclude, then, that alder nodules must contain a diffusion barrier with a permeability that is affected by temperature.

The constant relationship between respiration and nitrogenase activity (*i.e.*, constant energy cost) and the steady increase in activity

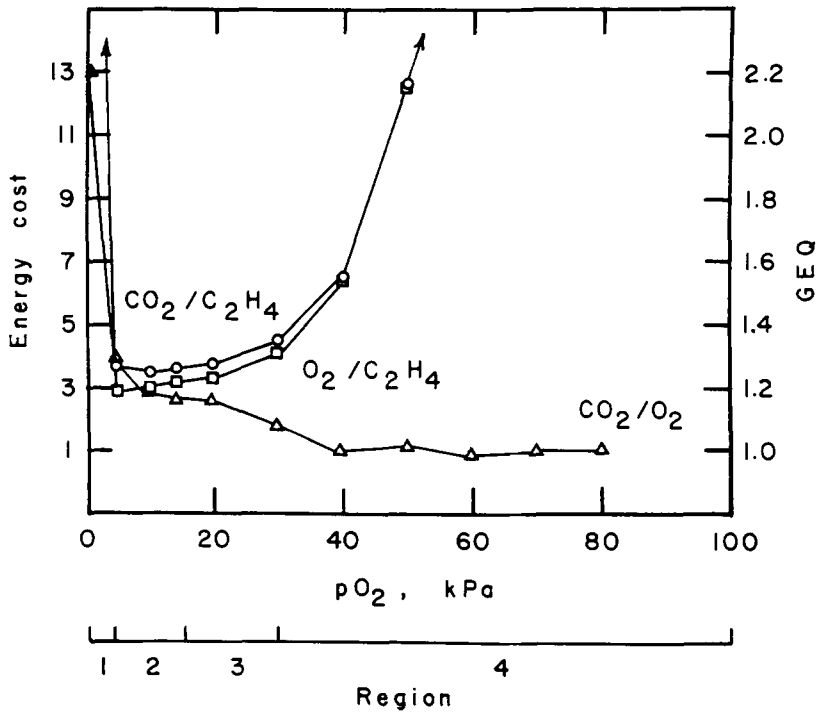

Fig. 8. Energy cost and GEQ (gas exchange quotient) calculated from the data in Figure 7.

from 16 to 26°C also suggest the presence of a diffusion pathway that gradually increases in permeability with increased temperature. Oxidative energy production is limited by the diffusion of oxygen through this barrier, and acetylene reduction is in turn limited by the energy supply. Oxygen tension at the site of fixation remains low.

We have measured the permeability of this diffusion barrier directly (averaged over all active nitrogenase compartments), using low concentrations of acetylene[8]. Average permeability does change over this temperature region, but not as steeply as does nitrogenase activity. We must conclude that the model does not account completely for nodule behavior. Further experimentation is needed.

The irreversible change in nitrogenase activity at high temperatures appears to result from a different mechanism. Since cultures of *Frankia* are routinely grown above 30°C and reduce acetylene readily at high temperatures, the breakdown in the nodule may be due to damage to host tissue. It is also possible that the endobiont in the nodule differs from that in the free-living state.

Our results are also in general agreement with previous measurements

of the effects of oxygen on acetylene reduction[16], ^{15}N uptake[4], and respiration[9] by detached alder nodules. Wheeler *et al.*[16] reported a broad optimum (15–30 kPa) for acetylene reduction at 20°C. Bond[4] found an optimum of 12 kPa.

We conclude that anaerobic energy metabolism cannot support nitrogen fixation in alders. Flooding must represent a serious limiting factor to alder growth, requiring special adaptive responses. For example, nodules of *Alnus glutinosa* were only found on roots above the water table[1].

Since fermentation does not support nitrogenase activity, the energy for fixation must be provided by the remaining oxidative metabolism in the nodule, which can be estimated from total electron flow (Table 1). As activity became increasingly oxygen-limited, the ratio of glucose used per ethylene produced continued to decline in those parts of the nodule still reducing acetylene, but remained well above the theoretical minimum of 0.25 (P/O = 2, 2 ATP/electron, see ref.[8] for complete discussion). This may be a result of biochemical inefficiency or of host respiration not directly linked to nitrogenase. Total carbon loss, however, provides a better measure of the cost to the plant host. Even at an oxygen tension of 3 kPa, this cost was high due to fermentation.

The curvilinear increase in respiratory activity between 3 and 20 kPa of oxygen indicates there must be a series of compartments in the nodule, each differing in the permeability of the diffusion path to them. The Km of the terminal cytochrome oxidase for oxygen is very low, so that in the absence of a diffusion barier, nodule respiration would saturate well below 5 kPa oxygen. Instead, as oxygen concentration is increased, regions of increasingly low permeability begin to take up significant amounts of oxygen and the total uptake increases.

The existence of compartments of different permeabilities also implies that some low permeability sites will remain anaerobic, even at relatively high oxygen tensions. As shown in Table 1, at 10 kPa oxygen, glucose use, measured by carbon loss, exceeded that measured by total electron flow. This is evidence that some parts of the nodule are indeed fermentative.

In the region of highest nitrogenase activity (15–30 kPa) respiratory activity continued to increase. In this region, it appears that more nodule tissue was becoming saturated with oxygen. This implies that more nitrogenase was also becoming inactivated. The plateau may represent a balance between the recruitment of new, low permeability regions into the active pool, and the loss of high permeability regions to oxygen toxicity.

Electron flows from carbohydrate and to reduction of oxygen

and acetylene were approximately equal in this region, indicating either that most nodule tissue was aerobic, or that any fermentative production of carbon dioxide was balanced by carbon dioxide fixation. Since respiration was only 70% of its rate at saturation (Fig. 7) at 20 kPa oxygen, some portions of the nodule remain oxygen-limited, even under atmospheric conditions.

Both Bond[4] and Wheeler *et al.*[16] noted the inactivation of nitrogenase at high oxygen tensions. Our observation that some activity remains and is stable even at 50–70 kPa is further evidence that parts of the nodule differ greatly in their ability to protect nitrogenase from excess oxygen. At tensions above 30 kPa, some compartments have high enough oxidase activity to consume all of the oxygen which diffuses to the site of nitrogenase down the unnaturally high concentration gradient.

Conclusions

The data from this paper are consistent with the following hypothetical model of alder nodule physiology. Nitrogenase is localized in a series of compartments, embedded in host tissue and connected to the rhizosphere by intercellular air spaces. Oxidative metabolism within these compartments, fueled by reduced carbon compounds from the host, produces ATP and reductant for nitrogenase, while using up the oxygen which diffuses in from the air spaces. Each compartment is surrounded by a diffusion barrier, as yet unidentified. The steady state concentration of oxygen in each compartment is determined by the rate of oxygen consumption, by the affinity of the oxygen-consuming enzyme (probably cytochrome oxidase) and the permeability of the diffusion barrier.

The rate of nitrogen fixation in each compartment is limited by the amount of energy produced, which is in turn limited to the amount of oxygen which can diffuse in. Compartments vary in susceptability to oxygen inactivation of nitrogenase, due to variation in the rate of oxidative metabolism and in the properties of the diffusion barrier. While oxidative metabolism remains limited by the diffusion of oxygen into the compartment, oxygen tension within the compartment remains very low and nitrogenase continues to function. When diffusion into a compartment exceeds the rate of oxygen consumption (as during high external oxygen tensikos), the ambient concentration rises and nitrogenase is inactivated. Measurements of whole nodule gas exchange rates represent a summation of these compartments.

Not only do different compartments have different permeabilities, but the net average permeability of nodules changes with temperature.

This change is steep in low temperatures and accounts well for the quantitative changes in activity. At intermediate temperatures, permeability appears to change too slowly to account for the entire change in nitrogenase activity and the constancy of the energy cost. Some other mechanism may be involved.

The existence of compartments is also consistent with the slowly curving increase in respiration and acetylene reduction as ambient oxygen concentration was increased. Some compartments of very high permeability, which would be the only ones active at low oxygen concentrations (in a heavy soil, or under water-logged conditions) or fully active at high temperatures, may become oxygen inactivated at 20 kPa oxygen. As a result, absolute minimum energy cost for nitrogen fixation by alder nodules may occur only at oxygen concentrations around 10 kPa.

Alder nodules are well-adapted to changes in temperature in the mesic range, with the ability to take advantage of increased temperatures and increased activity, with little or no increase in energy cost per nitrogen fixed. The absence of nitrogenase activity below 8°C suggests that growth conditions can modify the range of optimal activity, since alders are known to grow in cold regions. Experiments are needed to establish whether plants and nodules can adapt to other ranges of temperature and oxygen when permitted long periods of time.

Alder nodules also function over a wide range of oxygen conditions. The identity of the oxygen protection compartments remains unknown. Since *Frankia* cultures have shown the ability to form vesicles and fix nitrogen at atmospheric oxygen concentrations[12], the vesicle wall is an obvious candidate for the permeability barrier. Other workers have suggested that the configuration of air spaces surrounding the endobiont might limit oxygen diffusion[16]. Although regions of low oxygen tension in nodules of *Myrica gale* have been found using an oxygen microelectrode[9], it was also evident from experiments with India ink infiltration that regions of oxygen tensions close to those of air exist adjacent to infected cells. Another possible explanation is that host mitochondria, observed to be appressed against and intertwined with *Frankia* vesicles in infected cells (ref.[2] and S. Lancelle, pers. comm.) actively scavenge oxygen from the immediate surroundings of the endobiont.

Clearly, the adaptive mechanism which permits nitrogen fixation in actinorhizal nodules must be a complex combination of host and *Frankia* morphology, membrane composition and oxygen biochemistry. Further work, with intact nodules of diverse cytological types, for example Casuarina, which appears to lack vesicles and Datisca, which

has vesicles located in the center of the infected cells, rather than at the periphery, and with nitrogen-fixing bacterial cultures is needed to test our multiple compartment hypothesis and to discover the identity of the oxygen protection mechanism.

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