Regulation of Oxygen Permeability in Indeterminate Root Nodules

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY
ZURICH

for the degree of
DOCTOR OF NATURAL SCIENCES

presented by

CARINA WEISBACH
dipl. Biol. Universität Hohenheim, Germany
born December 17, 1964
citizen of Germany, Plauen

accepted on the recommendation of

PROF. DR. J. NÖSBERGER
examiner

PROF. DR. N. AMRHEIN and DR. U.A. HARTWIG
(co-examiners)

1996
<table>
<thead>
<tr>
<th>PART 1: Involvement of carbon metabolites in the regulation of oxygen permeability in white clover (Trifolium repens L.) nodules</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>16</td>
</tr>
<tr>
<td>Introduction</td>
<td>17</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>18</td>
</tr>
<tr>
<td>Results</td>
<td>21</td>
</tr>
<tr>
<td>Discussion</td>
<td>28</td>
</tr>
<tr>
<td>Conclusions</td>
<td>31</td>
</tr>
<tr>
<td>PART 2: The oxygen permeability is not regulated through the occlusion of intercellular spaces in the inner cortex of lucerne (Medicago sativa L.) nodules</td>
<td>32</td>
</tr>
<tr>
<td>Abstract</td>
<td>32</td>
</tr>
<tr>
<td>Introduction</td>
<td>33</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>34</td>
</tr>
<tr>
<td>Results</td>
<td>41</td>
</tr>
<tr>
<td>Discussion</td>
<td>65</td>
</tr>
<tr>
<td>Conclusions</td>
<td>69</td>
</tr>
<tr>
<td>GENERAL DISCUSSION</td>
<td>70</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>83</td>
</tr>
<tr>
<td>CURRICULUM VITAE</td>
<td>92</td>
</tr>
<tr>
<td>DANK</td>
<td></td>
</tr>
</tbody>
</table>
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANA</td>
<td>Apparent Nitrogenase Activity</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosintriphosphate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamid adenin dinucleotid phosphate</td>
</tr>
<tr>
<td>Lb</td>
<td>Leghemoglobin</td>
</tr>
<tr>
<td>LbO₂</td>
<td>Oxygenated Leghemoglobin</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PEPC</td>
<td>Phosphoenolpyruvate Carboxylase</td>
</tr>
<tr>
<td>PNA</td>
<td>Potential Nitrogenase Activity</td>
</tr>
<tr>
<td>pO₂</td>
<td>Oxygen Partial Pressure</td>
</tr>
<tr>
<td>pOₑ</td>
<td>Rhizospheric Oxygen Partial Pressure</td>
</tr>
<tr>
<td>cryo-SEM</td>
<td>cryo-Scanning Electron Microscopy</td>
</tr>
<tr>
<td>SS</td>
<td>Sucrose Synthase</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TNA</td>
<td>Total Nitrogenase Activity</td>
</tr>
</tbody>
</table>
SUMMARY

Legumes, especially white clover (*Trifolium repens* L.), play an important role in meadows and pastures of temperate climates. Frequent cutting or grazing of these legumes reduces the nitrogen-fixing capacity significantly. Meanwhile, it is known that the reduced supply of photosynthates from shoot to nodules due to loss of leaf material is not responsible for the decline of nitrogenase activity. In contrast, reduction of nitrogenase activity operates by reducing the oxygen availability in the central zone of nodules where nitrogen is fixed. The mechanism responsible for the reduction of oxygen availability in the central zone is poorly understood.

Several studies indicate a possible involvement of carbon metabolites in the short-term regulation of nitrogenase activity. This possibility was tested in the first part of the present study. For this purpose, nodules of white clover plants were exposed to two different forms of stress, which are known to cause a significant reduction in oxygen permeability in root nodules. These two forms of stress were defoliation and an increased oxygen partial pressure to 40 kPa in the rhizosphere. Although, the concentration of sucrose decreased after defoliation from 0.077 to 0.045 mmol/g dry weight, it remained unchanged under increased oxygen partial pressure. Similarly to the response of sucrose, total concentration of the other carbon metabolites (glucose, fructose, D-pinitol, starch and organic acids) were affected differently by the two treatments. As a result, an involvement of whole nodule carbon metabolite pools in triggering oxygen permeability seems to be unlikely. Nevertheless, an involvement of osmotically active compounds such as sucrose can not be excluded. This is due to possible alterations in the balance of solutes between symplast and apoplast in the inner cortex where import and export of these solutes takes place.

The filling of intercellular spaces with aqueous solutions due to alterations in the balance of solutes between symplast and apoplast was examined in the second part of this study with the help of cryo-fixation techniques. Immersion of nodules in liquid ethane allowed maximum preservation of the actual physiological and morphological states, especially the water content in the various tissues. The interpretation of cryo-scanning electron microscope pictures obtained by the
subsequent examinations of the frozen samples allowed the detection of possible changes in the number of liquid-filled intercellular spaces induced by treatments known to affect oxygen permeability. Neither defoliation nor an increased oxygen partial pressure affected the number of liquid-filled intercellular spaces in lucerne nodules which remained constant at 30%. As expected from the missing response of nodules to the two forms of stress applied, the removal of oxygen stress did not alter the amount of liquid-filled intercellular spaces. This clearly illustrates that nodule oxygen permeability is not regulated through the filling of intercellular spaces with aqueous solutions.

Investigations on lupin show a possible involvement of glycoprotein occlusions in the regulation of oxygen permeability. Such a mechanism seemed not to be involved in the regulation of oxygen permeability in the inner cortex of lucerne nodules. Due to the similar distribution of glycoprotein and liquid-filled intercellular spaces in the inner cortex of lucerne nodules, it is suggested that glycoproteins found in intercellular spaces form a net in which aqueous solutions are incorporated. This suggestion is supported by the hydrophilic character of the glycoproteins.

The results of the present study strongly support the hypothesis of a permanent diffusion barrier localized in the inner cortex of nodules. This barrier, however, is most likely not involved in the short-term regulation of oxygen permeability. It is suggested that the fine regulation of nitrogenase activity occurs in the central zone of the nodule. This may happen either through morphological alterations to restrict oxygen diffusion or through physiological mechanisms apparently limiting oxygen supply.
ZUSAMMENFASSUNG


Das Anfüllen der Interzellularräume mit wässriger Lösung durch Veränderungen des Metabolitengleichgewichts zwischen Symplast und Apoplast, wurde mit Hilfe der "cryo"-Gefriertechnik im zweiten Teil dieser Arbeit untersucht. Das Eintauchen von Knöllchenproben in Flüssig-Ethan ermöglichte die maximale

Untersuchungen über eine mögliche Regulation über die Verstopfung der Interzellularräume durch Glykoproteine wie sie bei Lupine beschrieben wurde, zeigten, dass im Gegensatz zu Lupine, ein solcher Mechanismus nicht für die Regulation der Sauerstoffpermeabilität in Luzerneknöllchen verantwortlich ist. Da glykoproteingefüllte und leere Interzellularräume gleichmäßig im Innencortex verteilt sind, kann vermutet werden, dass Glykoproteine ein Gerüst bilden, in welches sich die Wassermoleküle einlagern. Diese Vermutung wird durch den hydrophilen Charakter der Glykoproteine unterstützt.

Die Ergebnisse der vorliegenden Arbeit unterstützen die Hypothese einer permanenten Diffusionbarriere die im Innencortex der Knöllchen lokalisiert, aber höchstwahrscheinlich nicht an der Regulation der Sauerstoffpermeabilität beteiligt ist. Es wird vermutet, dass die Feinregulation der Nitrogenaseaktivität in der Zentralzone stattfindet. Dies kann entweder durch morphologische Veränderungen die sich negativ auf die Sauerstoffdiffusion auswirken geschehen oder durch physiologische Mechanismen, die scheinbar die Sauerstoffverfügbarkeit in der Zentralzone reduzieren.
GENERAL INTRODUCTION

Forage legumes play an important role in permanent grasslands of temperate climate zones. One of the major aspects in pastures is the ability of legumes to fix atmospheric nitrogen symbiotically in the root nodules. In white clover (*Trifolium repens* L.), the formation of root nodules is induced by the symbiotic association with the single cell eubacteria *Rhizobium leguminosarum* biovar *trifolii*. This symbiosis makes the large atmospheric nitrogen source available to the plants and thus assures independence of external mineral nitrogen (N) supply. Therefore, the input of N fertilizers to mixed grass/legume pastures can be kept significantly lower when compared to grass monocultures.

One major problem in managing these mixed swards is that the nitrogen-fixation (*N₂*-fixation) activity of legumes responds sensitively to alterations of growth conditions. Loss of leaf material by cutting or grazing, drought or N fertilization are recognized as stress factors and cause a significant decrease in *N₂*-fixation. As a result, the survival and establishment of legumes as well as the maintenance of optimal *N₂*-fixation are dependent on the ability of the plants and the symbiotic association to withstand periods of physiological stress.

The response of *N₂*-fixation activity in legume root nodules to stress can not only be observed in white clover nodules but in all nodule systems. The vast majority of root nodules can be classified into two main groups:
1) indeterminate nodules (Fig. 1A) formed by temperate plants (e.g. pea, lucerne, white clover, broad bean, lentil) exporting fixed nitrogen from the root nodules in the form of amides (asparagine, glutamine). Lupine nodules are also indeterminate nodules but do not develop the typical nodule structure (Fig. 1A). They are closely attached to the roots and are called therefore collar typ nodules.
2) determinate nodules (Fig. 1B) formed by plants of tropical origin (e.g. soybean, cowpea, pigeon pea, french bean) exporting fixed nitrogen in form of ureides (allantoin, allantoic acid).
Figure 1. Model of the two nodule types described. A) indeterminate nodule, M: meristematic zone, I: apical zone, II: prefixing zone, III/NF: actively N₂-fixing zone, IV: zone of senescence; B) determinate nodule, NF: actively N₂-fixing zone

Indeterminate nodules (Fig 1A) have a more oval structure in comparison to determinate nodules. This is due to an active meristem at the nodule apex, which continuously adds cells to the cortex and central zone by cell proliferation. Therefore, indeterminate nodules can be divided in the following histological zones (Soupéne et al., 1995): At the top is the apical zone with the meristem (M) (Fig. 1A, Zone I). This cell zone does not contain any bacteria. Zone I changes into the prefixing zone (Zone II) which is characterized by the presence of infection threads through which the cells are invaded by bacteria. After invasion of the cells, bacteria transform into bacteroids and form the actively N₂-fixing zone (Zone III/NF). At the bottom of the nodule is the zone of senescence (Zone IV). Here, the lysis of bacteroids takes place along with a partial degeneration of
the plant cells. Determinate nodules (Fig. 1B) have a more spherical structure due to the loss of activity in the meristematic zone. Both nodule types are surrounded by a cortical zone.

For proper functioning of root nodules, the N₂-fixing enzyme nitrogenase must be protected from contact to molecular oxygen (O₂) to avoid irreversible damage. On the other hand, O₂ is essential to maintain aerobic respiration in order to provide the highly energy demanding process of N₂-fixation with adequate amounts of energy in form of ATP. Therefore, a constant and strong flux of O₂ is required, while maintaining very low concentrations of free O₂ in the N₂-fixing zone. This so called “oxygen dilemma” is solved by the following adaptive features:
1) Strict compartmentation of the nodule structures and development of a gas diffusion barrier to maintain low concentrations of free O₂ in the central zone of nodules
2) Synthesis of the O₂ binding protein leghemoglobin to facilitate O₂ diffusion to bacteroids and plant mitochondria within the infected zone
3) Development of a terminal oxidase with high O₂ affinity in the bacteroid membrane for effective ATP formation under micro-aerobic conditions
These three features are discussed in detail below:
1) Compartmentation and structure of indeterminate nodules and the gas diffusion barrier

a) Compartmentation and structure of indeterminate nodules

Figure 2. Schematic cross section through the actively $N_2$-fixing part of an indeterminate nodule. Sizes of the compartments shown do not represent the actual proportions.
Figure 3. Image obtained by cyro-SEM of a freeze fractured white clover nodule. Partial cross section through the actively N₂-fixing part. Overview from right to left: the outer cortex, the double layer and the inner cortex where vascular bundles are embedded. The central zone (left side) can clearly be identified by the large size of the infected cells.
The outer cortex (Figs. 2 and 3) is composed of large, loosely packed cells which surround the entire nodule. The cells are interlaced by large intercellular spaces.

The inner cortex is clearly distinguished from all other zones in the nodules by the small size of the cells and the intercellular spaces. The vascular bundles are embedded in this zone and nodules are supplied with photosynthates from the shoot via the phloem and N assimilates can be exported from the nodule via the xylem.

The central zone consists of large infected and smaller, uninfected interstitial cells. In the infected cells, the Rhizobium bacteria, transformed to bacteroids, and the O₂ binding protein leghemoglobin are located. The leghemoglobin is only present in the actively N₂-fixing part of the nodule and gives it a reddish colour. This colouring is a clear indication for properly developed and active nodules.

b) The gas diffusion barrier

Oxygen micro-electrode measurements indicate a significant drop in O₂ concentration from 260 mM in the outer cortex to 10-40 nM in the central zone of the nodule at external ambient atmospheric oxygen pressure (Tjepkema and Yocum, 1974; Masepohl et al., 1993). The 10⁴-10⁵-fold decrease of inside relative to outside O₂ concentration can not be achieved only by the high O₂ consumption of the nodules (nodules typically respire at about four times the rate of a root of equal biomass). This observation has led to the hypothesis that the site of restriction of O₂ diffusion is located in the inner cortex (Witty et al., 1984; 1987). Mathematical models of gas diffusion (Sheehy et al., 1987; Hunt et al., 1988; Denison et al., 1992) and the presence of a distinct cell layer (boundary layer) in the inner cortex zone of soybean nodules which could act as such a barrier (Witty et al., 1987; Dakora and Atkins, 1990; Parsons and Day, 1990), support this hypothesis.

The salient characteristic of the O₂ diffusion barrier is its ability to respond to alterations of physiological and environmental conditions. Defoliation, external
N supply, drought or an increase of the O₂ partial pressure in the rhizosphere cause a significant restriction of gas diffusion. Several experiments have shown that a slow increase in external O₂ partial pressure (up to 80 kPa) does not damage the O₂ sensitive nitrogenase despite little if any increase in nodule respiration takes place (Witty et al., 1984; 1987). This indicates that the O₂ flux does not increase. According to Fick's law, O₂ diffusion is dependent on the external O₂ concentration and the resistance along the path of gas diffusion. Therefore, if the consumption remained constant while the external O₂ concentration increased, the resistance to O₂ diffusion must have been enhanced. The diffusion barrier is not only able to down regulate the diffusion of O₂, it can also adapt to O₂ partial pressure below 20 kPa by enhancing O₂ diffusion (Hunt et al., 1987; Atkins et al., 1993; Weisbach et al., 1996). Furthermore, the increase and decrease of O₂ permeability due to changes in the pO₂ in the rhizosphere, is fully reversible as soon as the initial O₂ conditions are restored (Hunt et al., 1989; Hartwig et al., 1994; Weisbach et al., 1996).

2) Leghemoglobin (Lb)

The O₂ binding protein leghemoglobin (Lb) is a monomeric haem protein and binds reversibly to O₂. It is a specific adaptation to the micro-aerobic conditions in the central zone of nodules. Through the presence of the highly O₂ affine Lb, the transport of O₂ to the bacteroids is facilitated. This is essential to allow adequate respiration in the infected cells since the strongly restricted diffusion into the nodule causes a shallow concentration gradient of O₂ in the central zone. Furthermore, the solubility of O₂ in aqueous solutions is very low, therefore, the diffusion of O₂ in the infected cells from cell wall to the bacteroids would not be sufficient. The concentration of leghemoglobin has been estimated to be about 3 nM in the infected cells; 20-40% are oxygenated under ambient atmospheric conditions (King et al., 1988; Appleby, 1984). After passage through the diffusion barrier, O₂ diffuses rapidly through the intercellular spaces to the surface of the infected cells. Deoxygenated Lb molecules inside the cells bind the free O₂ which has entered the cells and diffuse as oxygenated Lb (LbO₂) down.
their concentration gradient to the bacteroids. After release of $O_2$ to the bacteroids, the deoxygenated Lb diffuses back to the cell surface. Approximately 99% of the $O_2$ provided to bacteroids is transported by Lb (Bergersen and Turner, 1975).

3) High $O_2$ affinity terminal oxidase

Bacteroids have developed a branched respiratory chain with a terminal oxidase that is specifically adapted to micro aerobiosis (Hennecke, 1993). The existence of such an oxidase has been postulated for a long time (Appleby, 1984; O'Brian and Maier, 1987). Now, a terminal oxidase complex has been identified in *Bradyrhizobium japonicum* (Bott et al., 1992; Thony-Meyer et al., 1994). It consists of four different subunits encoded by FixN, FixO, FixQ and FixP. FixN encodes an integrated membrane protein. FixO,Q,P encode proteins, which are most probably anchored to the membrane by a transmembrane helical structure. Gene expression only takes place under micro-aerobic conditions and lack of this terminal oxidase reduces nitrogenase activity significantly. The high affinity to $O_2$ is reflected by a $K_m$ in the range of the free $O_2$ concentration that prevails in nodules (10-20 nM; Witty and Minchin, 1990). The location of the oxidase in the bacteroid membrane allows a high efficiency in respiration, despite the low $O_2$ concentration in the infected cells, in order to provide sufficient ATP to drive the energy demanding process of $N_2$-fixation.

**Regulation of $O_2$ permeability**

Nitrogenase activity in root nodules decreases rapidly due to treatments such as application of nitrate, increased rhizospheric $O_2$ partial pressure, defoliation, stem-girdling or extended darkening (Vessey *et al*., 1988; Durand *et al*., 1987; Hartwig *et al*., 1987; 1990). Previous experiments have shown that nitrogenase activity is down regulated through an apparently reduced $O_2$ permeability in the central zone of nodules (Hartwig *et al*., 1987; Layzell and Hunt, 1990; Sung *et al*., 1991; Denison and Layzell, 1991). The assumption of a regulation of nitrogenase activity through the $O_2$ availability in the central zone is
based on the fact that a decreased nitrogenase activity due to stress can be overcome by the external supply of O₂. It has been suggested that the nodule cortical permeability enforces this primary limitation of O₂ supply to the central zone (Witty et al., 1984; 1987; Hunt et al., 1987; Weisz and Sinclair, 1987). The mechanism by which the cortical O₂ permeability could be regulated is still not clear. Several authors have proposed that gas diffusion through pathways in the cortex zone is restricted by cell expansion (James et al., 1991; Iannetta et al., 1993a) or due to aqueous solutions infiltrating the diffusion pathways (Denison et al., 1992; Hunt and Layzell, 1993). Both effects could be achieved by osmotical processes due to variations in the balance of soluble sugar content in the symplast and nitrogenous solute content in the apoplast (Streeter and Salminen, 1993; Purcell and Sinclair, 1994; Walsh, 1995). Another hypothesis is the restriction of O₂ diffusion through occlusions of intercellular spaces in the inner cortex with glycoproteins combined with cell expansion (James et al., 1991; Iannetta et al., 1993a; DeLorenzo et al., 1993). This is supported by recent studies on lupin nodules, which showed a correlation between the occurrence and the accumulation of glycoproteins in the entire nodule and an increased restriction of O₂ diffusion.

The objectives of this study

The aim of this study was to examine the mechanisms involved in the regulation of O₂ diffusion into the central zone of root nodules. Until now, very little is known about this, but as pointed out above, the O₂ diffusion may be regulated by osmotical processes such as cell expansion or infiltration of the O₂ diffusion pathways with aqueous solutions. There is some indication that carbon metabolites might be involved in this regulation since it has been reported that nodules exposed to extended darkening lose their ability to regulate O₂ diffusion (Minchin et al., 1985). Furthermore, defoliation and nitrate fertilization cause a severe decrease in sucrose concentration in nodules which is very much correlated with the decrease of nitrogenase activity and O₂ diffusion. Besides sucrose, other carbon metabolites can also be envisaged as osmotically active
molecules, especially malate. Detailed studies on the possible involvement of carbon metabolites, especially sucrose and L-malate, in the regulation of O₂ permeability have not been reported before and were tested in the first part of the present study. Furthermore, it was evaluated, if the regulation of nitrogenase activity after defoliation continues to operate through an O₂ limitation throughout the entire regrowth period.

Most studies on O₂ diffusion have been undertaken with determinate nodules, especially with soybean nodules. Results of these studies were transferred to indeterminate nodules such as lucerne (Medicago sativa L.) despite of the differences in nodule structure and metabolism. In the second part of the present study it was tested, if the mathematical models and results obtained for soybean and lupin nodules are valid for lucerne as well. Therefore, examinations were focused on the visible, stress induced, filling of intercellular spaces in the inner cortex of lucerne nodules. In one experiment, it was examined if the intercellular spaces in the cortex zone will be filled by aqueous solutions. Structure preserving cryo-fixation techniques combined with cryo-scanning electron microscopy (cryo-SEM) offered a good method for these investigations. In the following experiment, the accumulation of glycoproteins in the intercellular spaces of lucerne nodules in response to stress was investigated by immuno electron microscopy.
THE INVOLVEMENT OF CARBON METABOLITES IN THE REGULATION OF
THE OXYGEN PERMEABILITY IN WHITE CLOVER (TRIFOLIUM REPENS L.)
NODULES

ABSTRACT

The decrease in nitrogenase activity in legume nodules after plant
defoliation is associated with a reduced nodule oxygen permeability. Although
carbon metabolites from current photosynthesis or from reserves have been dis¬
pproved to have a metabolic function in the short-term down regulation of
nitrogenase activity, a decrease in nodule sucrose concentration after defoliation
could indicate that carbon metabolites are indirectly involved in triggering the
oxygen permeability in nodules through osmotic processes. To test this hy¬
pothesis, nodule oxygen permeability was manipulated either by defoliation or by
varying rhizospheric oxygen partial pressure. In contrast to defoliation, a 50%
reduction of the nodule oxygen permeability, due to adapting nodules to 40 kPa
oxygen, had no effect on nodule sucrose concentration. Likewise, total
concentrations of other carbon metabolites such as D-pinitol, fructose, glucose,
starch, L-malate or succinate tended to be affected differently by the two
treatments. To test whether the availability of carbon metabolites is never a
limiting factor for nitrogenase activity, even during a several day growth period
after a 100% defoliation, total and potential nitrogenase activity were monitored.
During the entire regrowth period, nitrogenase activity could be increased at any
time by elevating rhizospheric oxygen partial pressure. Thus, during the entire
growing cycle nitrogenase activity seems primarily oxygen limited. Changes in
whole nodule sucrose pools after defoliation have to be viewed as secondary
effects not necessarily linked to nodule activity. Whole-nodule carbon metabolites
appear not to be determinants of nodule activity, neither through direct metabolic
involvement nor through indirect effects such as triggering oxygen permeability.
INTRODUCTION

Several experiments have demonstrated that nitrogenase activity is very sensitive to various forms of stress, such as stem-girdling, drought, defoliation or extended darkening (Vessey et al., 1988; Durand et al., 1987; Hartwig et al., 1987; 1990). Initially, the decline in nitrogenase activity after defoliation was assumed to be directly induced by an interruption in the supply of photosynthates due to the removal of photosynthetically active tissue. Further investigations gave clear evidence that the decrease in nitrogenase activity after defoliation cannot be explained by a shortage of photosynthates from reserves or from current photosynthesis (Hartwig et al., 1990, 1994). Nitrogenase activity, however, appears to be regulated through the availability of oxygen ($O_2$) in the infected cell zone of nodules (Hartwig et al., 1987; Sung et al., 1991; Denison and Layzell, 1991; Denison et al., 1992; Diaz del Castillo et al., 1992). It has been proposed that a variable $O_2$ permeability regulates the $O_2$ supply that supports nitrogenase activity (Witty et al., 1984; 1987; Hunt et al., 1987; Weisz and Sinclair, 1987). Mechanisms involved in the short-term regulation of $O_2$ permeability in nodules are poorly understood. Previous studies have shown that the nodule $O_2$ permeability may be related to osmotically induced changes in size or shape of cells or intercellular spaces in the cortex and in the infected zone (van Cauwenberghe et al., 1994; Serraj et al., 1995). Such a concept seems plausible, since sucrose concentration was reported to decrease after defoliation (Gordon et al., 1986; Streeter and Salminen, 1993) and after nitrate fertilization (Minchin et al., 1986), treatments that result in changes in nodule $O_2$ diffusion. Other carbon metabolites that could also be envisaged as osmotically active molecules, such as L-malate or succinate, were not investigated in these studies. Since Streeter and Salminen (1993) found that a defoliation-induced decline in whole-nodule sucrose concentration is representative also for the nodule cortex, the present study was performed with whole nodules.

After the nearly instantaneous decrease in nitrogenase activity upon defoliation, enzymatic activity recovers as the plant regrows. During this period,
the regrowing shoot is a strong sink for carbon metabolites and thus nodule activity could suffer from carbon shortage. It is not known whether the import of carbon metabolites to nodules is sufficient during this period and hence nitrogenase activity would still be O₂ limited.

To test the hypothesis that sucrose or other osmotically active carbon metabolites could be involved in regulating the O₂ permeability in white clover nodules, nodule O₂ permeability was manipulated either by defoliation or by adapting the nodulated root system to various pO₂ values in the rhizosphere. Moreover, the possibility of a carbon limitation of nitrogenase activity during several days of regrowth was monitored.

MATERIALS AND METHODS

Plant material, growth conditions and defoliation treatments

White clover (Trifolium repens L. cv. Milkanova) from a single clone was grown in growth chambers (PGR-15, Conviron Instruments Co. Winnipeg, Canada) at 18/13°C day/night temperatures and 90% RH, in a 16-h photoperiod and a photon flux density of 500 μmol quanta PAR m⁻² s⁻¹ (Fluorescent: [Cool White 160 W] and incandescent [138L 100W], Sylvania GTE SA, Geneva, Switzerland). For all experiments, plants were established by transplanting stolon tips, including the first five to six internodes, into gas-tight, sealable pots with a volume of 250 ml, filled with silica sand (0.8-1.2 mm). Plants were watered for 8 days with nutrient solution similar to that of Hammer et al. (1978), supplemented with 7.5 mM nitrate. From day 9 to 14 the nitrate concentration was 1 mM and an N-free nutrient solution was given during the rest of the experiment. We inoculated with Rhizobium trifolii, strain RBL 5020 (generously provided by Dr. H.P. Spaink, Leiden University, NL) at days 14 and 19 after planting. Experiments were conducted 6 to 8 weeks after planting.

Plants were completely defoliated by removing 100% of the leaf area as described by Hartwig et al. (1994).
Measurement of total nitrogenase activity (TNA), potential nitrogenase activity (PNA), apparent nitrogenase activity (ANA) and determination of O₂ limitation of nitrogenase activity

The gas exchange system used to measure nitrogenase activity in all experiments was similar to that described by Minchin et al. (1983); all details are described by Heim et al. (1993). To assess O₂ permeability, O₂ limitation of nitrogenase activity was measured as the pO₂ required for highest nitrogenase activity; all details are described by Heim et al. (1993). TNA, PNA and ANA were measured in a manner similar to that of Diaz del Castillo et al. (1992). For measuring PNA, the initial external O₂ partial pressure of 20 kPa in Argon was increased at a rate of 2 kPa min⁻¹ until nitrogenase activity showed no further increase.

Adaptation of nodules to different O₂ partial pressures

In these experiments, the same gas exchange system as described before was used. For the process of adaptation, plants were exposed to a gas stream of synthetic air (N₂:O₂ = 80:20). When steady state of ANA was reached, O₂ partial pressure was changed at a rate of 1 kPa min⁻¹ to 10 or 40 kPa O₂ respectively. Treatments lasted until ANA reached a new steady state (see also Figs. 4 and 5). Upon this treatment, nodule O₂ permeability was either decreased (40 kPa O₂) or increased (10 kPa O₂) according to Fick's Law (Weisz and Sinclair, 1987; Davey and Simpson, 1989). To assure that the 40 kPa O₂ treatment did not damage nitrogenase, pO₂ was readjusted to 20 kPa O₂ and ANA was checked again.

Preparation of plant extracts for all analyses

Plants used for analysis were rapidly removed from their pots, washed in cold water, frozen in liquid N₂ and stored until freeze drying at -80°C. Freeze-dried material was separated into roots and nodules. Aliquots of ground material (about 100 mg) were extracted at room temperature in 1 ml ethanol using mortar and pestle. To resolve all water soluble substances the pellet from a 15 min centrifugation at 3000 g was re-suspended for 2 times in 65% ethanol and centrifuged again. The pellet was used for starch analysis and collected
supernatants were dried in vacuo and re-suspended in 0.5 ml water for analysis of soluble sugars and organic acids.

**Analysis of sugars (sucrose, glucose, fructose), cyclitols (D-pinitol, myo-inositol), starch and organic acids**

For analysis of sugars and cyclitols, extracts were divided into neutral and ionic fractions using modified ion-exchange columns (Bachmann et al., 1994). Columns were filled with 100 µl of Dowex 1-×8 (HCO₃⁻), 200-400 mesh and Dowex 50-×8 (H⁺), 200-400 mesh omitting the polyvinyl-pyrrolidine layer. The filled columns were washed with 1 ml water and centrifuged for 20 min at 3000 g before use. Fifty µl of the extract was filtered through the column and rinsed with 50 µl water. This procedure was repeated once. While the ionic fraction was discarded, sugars and cyclitols from the neutral fraction were separated by HPLC (RI-Detector, ERC-7512, Erma, Tokyo, Japan), using a SS-100, Pb²⁺ carbohydrate column (7.8 X 300 mm, Sierpa, Separations, Inc., CA, USA) in an isocratic solvent system with bi-distilled water as mobile phase. Column temperature was 80°C. Software from PyramidTM (Axxiom Chromatography, Inc., Moorpark, CA, USA) was used for recording and integration of chromatograms.

The amount of starch was determined in the pellet which was dissolved in 0.6 ml water and heated to 90°C before the addition of 150 µl of a solution containing Termamyl (0.5 ml Termamyl 120L in 10 ml H₂O, Novo Nordisk Fermant Ltd., Dittingen, Switzerland). The mixture was boiled for 15 min in a heating block and immediately chilled on ice. After addition of 0.5 ml of Na-acetate-buffer (pH 4.3) and 120 µl of a solution of amylglucosidase (10 mg amylglucosidase 60U, Boehringer, Mannheim, Germany; dissolved in 1 ml Na-acetate-buffer pH 4.6) the reaction mixture was incubated at a temperature of 60°C for 30 min and then cooled. All samples were diluted with water to 10 ml. For glucose determination, 0.05 ml aliquots were added to a mixture of 0.6 ml TEA-HCl-buffer (pH 7.6) with ATP and NADP, 0.98 ml water and 0.01 ml hexokinase according to the manufacturer's instruction (Boehringer Mannheim GmbH, Mannheim Germany) and analysed immediately. The amount of D-glucose from starch breakdown was determined spectrophotometrically at 340 nm (LKB Bilchrom, Ultraspec 4050,
Turku, Finland) based on the conversion of NADP to NADPH.

For detection of organic acids by HPLC, an Aminex HPX-87H column (7.8 X 300 mm, Bio-Rad Laboratories AG, Glattbrugg, Switzerland) was used in an isocratic solvent system with 6 mM H₂SO₄ as mobile phase at a flow rate of 500 μl min⁻¹. Column temperature was 15°C. Prior to injection all samples were filtered through a micro-filter ACRO LC 3A, 0.45 μm (Disposable Filter Assembly, Gelman Sciences (LC), Ann Arbor, USA). Chromatograms were recorded and integrated using software of Waters (Series 990 PDA, Millipore Cooperation, Waters Chromatography Division, Milford, USA). Integration of peaks was performed at a wavelength of 210 nm using the photodiode array detector system 991 of Waters.

The amounts of all carbon metabolites in nodules from undefoliated plants were comparable to those described by Davis and Nordin (1983), Minchin et al. (1986) and Gordon et al. (1986) in white clover, and by Streeter (1987) and Streeter and Salminen (1993) in soybean.

RESULTS

The short-term response of carbon metabolites to manipulations of nitrogenase activity and O₂ permeability by a 100% defoliation

Along with a reduction in nitrogenase activity and a decrease in O₂ permeability due to a 100% defoliation (data not shown), sucrose concentration in roots and nodules declined sharply (Fig. 4). Within the first hour, the concentration dropped to about 50% of its initial value. Fructose, glucose, D-pinitol and starch concentrations in roots and nodules were not significantly affected by defoliation (Fig. 4). While D-pinitol and fructose concentrations were always about 5 fold lower in roots than in nodules, glucose concentration in roots was much higher than in nodules where it was near the detection limit. No myo-inositol could be found in roots or in nodules. L-malate and succinate contributed to 70% of the organic acid fraction found in nodules; no response to defoliation was observed (Fig. 4). Concentrations of measured organic acids were generally
substantially higher in nodules than in roots (data not shown; Fig. 4).

**Figure 4.** Concentrations of sucrose, fructose, glucose, D-pinitol, L-malate, succinate and starch in roots (▲) and nodules (●) over a four hour period after complete defoliation (at time 0) of white clover. Means of 7 replicates ± SE are shown.
The short-term response of carbon metabolites to manipulation of O₂ permeability by varying rhizospheric pO₂

To manipulate the nodule O₂ permeability without interrupting the phloem sap supply, the rhizospheric O₂ partial pressure (pO₂) was modulated (Fig. 5 and 6).

Figure 5. Response of apparent nitrogenase activity (ANA) to an increase of external O₂ pressure (pO₂) from 20 to 40 kPa. Samples were taken when ANA reached steady state values (arrow). To assure that nitrogenase was not damaged by the treatment, pO₂ was readjusted to 20 kPa O₂ and ANA checked again.
Figure 6. Response of apparent nitrogenase activity (ANA) to a decrease of external O\textsubscript{2} pressure from 20 to 10 kPa. Samples were taken when ANA reached steady state values (arrow).

In response to an exposure of the root system to a pO\textsubscript{e} of 40 kPa, nitrogenase activity increased initially but was re-established within 1h at a level similar to that observed under 20 kPa O\textsubscript{2} (Fig. 5). According to Fick’s Law, the O\textsubscript{2} permeability must have been decreased to 50% of its initial value. To assure that nitrogenase was not damaged by this treatment, nodules were re-exposed to 20 kPa O\textsubscript{2} after the experiment. Since nitrogenase activity recovered completely
to its initial value, it is evident that no nitrogenase damage had occurred. A decrease of pO$_e$ from 20 to 10 kPa caused an initial decline in nitrogenase activity which was partially readjusted after one hour (Fig. 6). Since pO$_e$ was decreased by 50% and nitrogenase activity showed 80% of its initial value, the O$_2$ permeability must have been increased to 160% of its initial level (Fick's Law).

Under conditions of undisturbed phloem sap supply but decreased O$_2$ permeability at a pO$_e$ of 40 kPa, sucrose concentration in nodules showed no response (Table I). Under an increased O$_2$ permeability and reduced nitrogenase activity at a pO$_e$ of 10 kPa, sucrose concentration was 30% higher than under 20 kPa O$_2$ (Table I). The same responses to altered pO$_e$ shown by sucrose was observed with fructose concentration but not with D-pinitol and starch concentrations. Glucose concentration was below the detection limit in this experiment. Both L-malate and succinate were not significantly affected by either of the O$_2$ treatments.

### Table I. Concentrations of carbon metabolites in white clover nodules after adaptation of nodules to various pO$_e$. Means of 7 replicates ± SE are shown.

<table>
<thead>
<tr>
<th>Carbon metabolite</th>
<th>pO$_e$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 kPa</td>
</tr>
<tr>
<td>Starch$^1$</td>
<td>55.4 ± 13.2$^c$</td>
</tr>
<tr>
<td>Sucrose$^2$</td>
<td>0.163 ± 0.006$^d$</td>
</tr>
<tr>
<td>Fructose$^2$</td>
<td>0.134 ± 0.013$^d$</td>
</tr>
<tr>
<td>D-pinitol$^2$</td>
<td>0.075 ± 0.003$^c$</td>
</tr>
<tr>
<td>L-malate$^2$</td>
<td>0.064 ± 0.009$^c$</td>
</tr>
<tr>
<td>Succinate$^2$</td>
<td>0.286 ± 0.022$^c$</td>
</tr>
</tbody>
</table>

$^1$(mg g$^{-1}$ nodule dry weight)$^{-1}$, $^2$(mmol g$^{-1}$ nodule dry weight)$^{-1}$

$^c,d$ Values with dissimilar letters differ significantly from each other (p < 0.05)
The time course of total nitrogenase activity (TNA) and potential nitrogenase activity (PNA) during regrowth after the removal of 100% of the leaf area

In order to examine whether the sucrose pool in nodules can represent a limiting factor for nitrogenase activity after defoliation and during regrowth, PNA was monitored (Fig. 7). Nitrogenase activity could always be significantly increased by elevating $pO_2$ in the rhizosphere.

Figure 7. Changes in total nitrogenase activity (TNA) and potential nitrogenase activity (PNA) after complete defoliation (at time 0) of white clover. Means of at least 3 replicates ± SE are shown. PNA differs significantly from TNA ($p=0.0067$).
DISCUSSION

Correlation between nodule carbon metabolites and \(O_2\) permeability

In the first experiment, \(O_2\) permeability was manipulated by defoliation, a treatment known to cause a decline in nitrogenase activity and a concurrent decrease in nodule \(O_2\) permeability (Hartwig et al., 1987; Sung et al., 1991; Denison and Layzell, 1991; Denison et al., 1992; Diaz del Castillo et al., 1992). Within the first hour after 100% defoliation, sucrose concentrations declined to 50% of initial values both in roots and nodules (Fig. 4). This finding is consistent with the results of Gordon et al. (1986). Unexpectedly, the concentration of other metabolites in roots and nodules, especially those of fructose and glucose, were not affected by removal of 100% of the leaf area (Fig. 4). Nodule glucose concentration was very low and near the limit of detection as was also found by Davis and Nordin (1983). Since Gordon et al. (1990) provided evidence for the presence of sucrose synthase (SS) in white clover nodules, this result may indicate that sucrose in white clover nodules is mainly cleaved by SS because UDP-glucose, the product resulting from this cleavage, can not be detected by the method used in this study. The substantial level of glucose found in soybean nodules by Streeter and Salminen (1993) may suggest that alkaline invertase plays a more significant role in sucrose cleavage in soybean as compared to white clover nodules.

Of all organic acids analysed, only results for L-malate and succinate are shown because these two metabolites are recognized as the main organic acids both in quantity and in support of bacteroid function (Day and Copeland, 1991). Their concentrations were found to be significantly higher in effective nodules than in ineffective ones (Rosendahl et al., 1990; Vance and Gantt, 1992). Furthermore, L-malate and succinate concentrations as those found in nodules may develop a high osmotic activity and could therefore be involved in the regulation of the nodule \(O_2\) permeability. However, neither L-malate nor succinate concentrations in nodules or roots were significantly affected by defoliation (Fig. 4), indicating the lack of a relationship on a whole nodule basis between their concentrations and nodule \(O_2\) permeability. Thus, sucrose is the only whole
nodule metabolite analysed which potentially could influence O\textsubscript{2} permeability.

In a second experiment, O\textsubscript{2} permeability was manipulated without interrupting phloem sap supply by altering rhizosphere pO\textsubscript{2} (pO\textsubscript{e}). For this purpose, nodulated roots were adapted to either 40 or 10 kPa O\textsubscript{2} (Figs. 5 and 6). In contrast to a defoliation-induced decrease in nodule O\textsubscript{2} permeability, the reduction in O\textsubscript{2} permeability induced by 40 kPa pO\textsubscript{e} had no effect on the concentrations of sucrose or other carbohydrates (Table I). The decreased concentrations in L-malate and succinate to 40 kPa O\textsubscript{2}, although not statistically significant (Table I), could indicate a depletion of these two metabolites due to slightly increased nitrogenase activity under 40 kPa O\textsubscript{2} (Fig. 5).

These findings do not support the hypothesis that total overall concentrations of sucrose or other carbon metabolites are involved in triggering nodule O\textsubscript{2} permeability. This would be consistent with the fact that sucrose concentrations in roots and nodules drop in a similar manner after defoliation (Fig. 4) which makes it unlikely that nodule sucrose concentrations are related specifically to nodule gas permeability. This holds true on the basis that possible localized changes in carbon metabolite concentrations are not masked by large amounts from the whole nodules.

**Indication that the import of sucrose into nodules is not controlled by nodule sink activity**

Complete defoliation resulted in similar responses in root and nodule carbon metabolites (Fig. 4). This suggests that export of sucrose from shoot to root is governed by the shoot's supply (source) and would imply that under conditions tested in the present study, variations in the sucrose supply to nodules are not determinants for nodule activity. Experiments by Heim et al. (1993) support this hypothesis and give evidence for ongoing nodule activity even under a significantly reduced sucrose availability. Further evidence for the hypothesis that nodule activity is not affected by the supply of sucrose is given in the present study by the fact that sucrose concentration increased rather than declined after ANA was reduced to 70% under a pO\textsubscript{e} of 10 kPa (Table I, Fig. 6). Under this treatment, import of sucrose into nodules may have continued at high rates in
relation to the reduced nodule activity and hence this sugar accumulated. These data strongly suggest that sucrose supply to the nodules is not governed by the sink activity.

Results from these experiments led us to ask, whether nodule function is never limited by carbon supply, even during periods of severe stress such as a complete defoliation. This, indeed, seems not to be the case since the level of nitrogenase activity could always be significantly increased by increasing the $pO_2$ (Fig. 7). This observation is consistent with that of Denison et al. (1992), who reported that after defoliation of lucerne the decrease in nodule $O_2$ permeability preceded the decrease in the $O_2$ saturated nodule respiration which may mainly depend on nodule carbohydrate availability. It is possible that carbon resources for nodule activity become depleted and hence limiting if excised nodules are studied as reported by Sung et al. (1991). It could also be feasible that plants severely depleted of carbohydrates before defoliation, or with a very limited capacity to store carbon metabolites, cannot support adequate nodule activity during periods of stress like defoliation. However, white clover, grown under favourable conditions with stolons that are not affected by defoliation and in which large amounts of starch can be stored, seems to supply nodules with sucrose in excess. In fact, $N_2$-fixation in white clover after defoliation was proven to be adequate to supply sufficient nitrogen for regrowth (Hartwig et al., 1994).

Although a regulatory function of carbon metabolites in the short-term regulation of the nodule $O_2$ permeability and thus of the nitrogenase activity cannot be supported based on the present data, the presence of a variable $O_2$ permeability is unquestioned. Alternative mechanisms such as the rapid and reversible depolarization of membranes in nodules accompanying decreased $O_2$ permeability (Denison and Kinraide, 1995) and/or the involvement of glycoproteins in the regulation of a nodule $O_2$ barrier (Iannetta et al., 1993a; b; DeLorenzo et al., 1993) may help to explain rapid changes in the $O_2$ permeability.
CONCLUSIONS

This study provides evidence that after defoliation and during regrowth, carbon metabolites at the whole nodule level appear not to be determinants of nodule activity, neither through a direct metabolic involvement nor through indirect effects like triggering the $O_2$ permeability. Even under the severe stress of a complete defoliation, nitrogenase activity seems primarily $O_2$ limited; changes in whole nodule sucrose pool after defoliation have to be viewed as secondary effects not necessarily linked to nodule activity.

Published in Plant Physiol. (1996) 110: 539-545
PART 2

OXYGEN PERMEABILITY IS NOT REGULATED THROUGH THE OCCLUSION OF INTERCELLULAR SPACES IN THE INNER CORTEX OF LUCERNE (MEDICAGO SATIVA L.) NODULES

ABSTRACT

It is still unclear to which extent to which the oxygen diffusion pathways through the inner cortex to the central zone are filled with liquid. Also it is not known whether a continuous aqueous diffusion barrier exists in the inner cortex restricting gas diffusion in indeterminate nodules. Likewise, the possible involvement of such structures in the operation of a variable oxygen permeability to adjust nitrogenase activity is not clear. In the present study the water content in the intercellular spaces and the morphology of indeterminate nodules of lucerne (Medicago sativa L.) and white clover (Trifolium repens L.) was examined in relation to oxygen permeability. For these investigations, the samples were fast frozen and investigated with cryo-scanning electron microscopy (cryo-SEM). This technique allows for direct observation of the frozen water in the intercellular spaces. Thirty per cent of all intercellular spaces in the inner cortex of lucerne nodules were found to be liquid-filled. Because of their random distribution and the presence of structures indicating air-filled interconnections between intercellular spaces, the hypothesis of a continuous aqueous diffusion barrier in the inner cortex cannot be supported. Decreasing the nodule oxygen permeability by total defoliation of the plant or by increasing the rhizospheric oxygen partial pressure to 80 kPa had no effect on the above mentioned structures. The abundance of glycoproteins in the intercellular spaces of the inner cortex was investigated with immuno labelling in the transmission electron microscope. It did not alter due to defoliation or after increase of the rhizospheric oxygen concentrations. Therefore, the results presented in this study do not support the hypothesis of a regulation of oxygen permeability by a blockage of diffusion pathways in the inner cortex of lucerne nodules.
INTRODUCTION

The activity of the nitrogen-fixing (N₂-fixing) enzyme nitrogenase in root nodules of legumes responds quickly and sensitively to various forms of stress or changes in environmental conditions such as defoliation of the plant, drought, N fertilization or altering rhizospheric oxygen (O₂) concentrations in the soil. It is known that the response of nitrogenase activity is regulated through varying the O₂ permeability (Hartwig and Nösberger, 1994). Distinct environmental conditions during nodule development were shown to have an effect on their morphology presumably resulting in appropriate O₂ permeability conditions in the nodules.

It could be shown that nodules of soybean plants, grown at supra-ambient rhizospheric O₂ partial pressure (80 kPa) for several weeks, developed a cortex zone with more tightly packed cells and a smaller volume of intercellular spaces in the inner cortex. This resulted in a very low gas permeability. In contrast, nodules of plants, which were subjected to a sub-ambient rhizospheric O₂ partial pressure (≤10 kPa) for the same period showed the opposite trend (Parsons and Day, 1990; Dakora and Atkins, 1991; Arrese-Igor et al. 1993; Atkins et al., 1993). The influence of environmental conditions on nodule development and its irreversibility due to structural alterations has therefore to be separated from the reversible “long-term” (Weisbach et al., 1996) and “short-term” response of nitrogenase activity through limited O₂ availability (Hunt et al., 1987; Denison and Layzell, 1991; King and Layzell, 1991; Weisbach et al., 1996). The association of persistent morphological features and gas permeability in nodules implicates that reversible changes of gaseous permeability in nodule could also be regulated through morphological adaptations.

Mathematical models predict the existence of a variable short-term O₂ diffusion barrier in the inner cortex. In such a model, reduced nitrogenase activity is suggested to be associated with changes in the amount of liquid-filled intercellular spaces of the inner cortex due to osmotical processes. Under such circumstances an increasing blockage of diffusion pathways would render gaseous diffusion more difficult. It is therefore thought to provide control over O₂ diffusion into the nodule (Sheehy et al., 1985; 1987; Hunt et al., 1988; Parsons
and Day, 1990; Sheehy and Webb, 1991; Denison et al., 1992). Another current hypothesis, based on research on lupin, suggests a regulation of O₂ permeability through an increased accumulation of an occluding glycoprotein in the intercellular spaces of the inner cortex combined with a decrease in size of intercellular spaces through cell expansion (James et al., 1991; DeLorenzo et al., 1993; Iannetta et al., 1993a, b; Minchin et al., 1994). However, the reversibility of these phenomena has not been tested in these studies.

Most of the previous studies have been carried out on annual plants such as soybean (determinate nodule) or lupin (indeterminate, collar type nodule). Therefore, the aim of this study was to examine typical indeterminate, amide transporting nodules of the perennial species lucerne and white clover. Thus, the structure of lucerne and white clover nodules was studied in relation to gas diffusion pathways. Highly structure-preserving cryo-techniques were used before the nodules were analysed by cryo-scanning electron microscopy (cryo-SEM). Possible glycoprotein occlusions were checked using immuno electron microscopy.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of lucerne (*Medicago sativa* L. cv. Resis) were surface sterilized in 65% ethanol for 3 min and planted into gas-tight, sealable pots (volume of 250 ml) filled with silica sand (ø 0.8 to 1.2 mm). The plants were grown in growth chambers (PGR-15, Conviron Instruments Co. Winnipeg, Canada) at 18/13°C day/night temperatures and 90% relative humidity, in a 16 h photoperiod and a photon flux density of 500μmol quanta PAR m⁻² s⁻¹ (Fluorescent: Cool White 160 W; Incandescent: 138L 100W, Sylvania GTE SA, Geneva, Switzerland). Plants were watered with a N-free nutrient solution similar to that of Hammer et al. (1978). Lucerne seedlings were inoculated with *Rhizobium meliloti* strain 102F28 for 3 times, i.e. at day 4, 11 and 18 after planting. The treatments were undertaken 5 weeks after planting. White clover plants (*Trifolium repens* L. cv.
Milkanova) were grown under condition as described in Part 1.

Treatments

In the defoliation experiment, plants were completely defoliated and harvested 7 h after defoliation. As control, undefoliated plants were harvested at the same time. In the oxygen experiment, plants were either subjected to a rhizospheric O₂ partial pressure (pO₂) of 20 kPa (control) or to 80 kPa using the flow through gas mixing unit as described by Heim et al. (1993). To apply the O₂ stress, plants were first adapted to a constant gas-stream of N₂:O₂ (80:20) which was then increased by 1 kPa min⁻¹ to 80 kPa O₂ for cryo-SEM and 40 kPa O₂ for immuno electron microscopy, respectively. O₂ concentration was maintained until nitrogenase activity was constant and plants were harvested. To check the reversibility of this adaptive process, plants were again subjected to 20 kPa O₂ and harvested when nitrogenase activity had re-adjusted to the initial level.

Tissue preparation for cryo-scanning electron microscopy (cryo-SEM)

The plant roots were prepared under a water saturated N₂-stream immediately after harvesting. The upper part of one undetached nodule (including the nitrogen fixing zone and the meristem) was cut off, mounted with gum arabicum (0.2g/ml H₂O) on an aluminum platelet (diameter 3 mm) and plunged into liquid ethane. Time from harvest to freezing of the root nodules was approximately 10 s. The specimens were stored in liquid N₂ for further cryo-SEM preparation.

For examination, the frozen samples were fractured in a freeze etching device (BAF 300, BAL-TEC AG, Princ. of Liechtenstein) with a cryo-microtome at a vacuum of 1 x 10⁻⁷ mbar and a temperature of -110° C and etched for 2 min at the same temperature. Thereafter, the samples were coated by electron beam evaporation with 3.5 nm of platinum-carbon (unidirectional at an angle of 45°) and 5 to 10 nm of carbon (perpendicularly) as described by Walther et al. (1995). After coating, the cold samples were transferred into liquid N₂ and mounted on a Gatan cryo-holder (Gatan, Inc., Pleasanten, CA, USA) and cryo-transferred into the SEM (S-900, in-lens field emission SEM, Hitachi, Tokyo, Japan). During
transfer, the specimen was shielded by the shutter of the Gatan holder.

Images obtained with the back scattered electron signal at a primary accelerating voltage \( V_0 \) of 10 kV were recorded digitally with a Gatan DigiScan 688 (Digital Micrograph 2.0, Gatan, Inc., Okeasabteb, CA, USA) connected to an Apple Quadra 950. The image format was 1024 x 1024 pixels with an integration time of 58 s per pixel. Pictures were prepared and analysed with Adobe Photo Shop (Adobe Systems Incorporated, Mountain View, CA, USA) and Aldus Page Maker (Aldus Co., Edinburgh, Scotland).

**Preparation of plant material for immuno labelling**

A whole nodule per plant was detached and infiltrated with 1-hexadecane (Fluka, Buchs, Switzerland) by application of a mild vacuum in a water stream pump for high-pressure freezing (Moor, 1987). Thereafter, the nodules were dissected into 200-300 \( \mu \)m thin vertical slices with a scalpel in 1-hexadecane (Studer et al., 1989). For cryo-immobilization in the high-pressure freezer (HPM 010; BAL-TEC, Princ. of Liechtenstein), samples were sandwiched between 2 cylindrical aluminum platelets (diameter of cavity 2 mm; depth 0.2 mm, exterior diameter 3 mm) and immediately transferred to liquid \( N_2 \) and stored until freeze substitution.

The freeze substitution was carried out as described by Humbel and Müller (1986) with 0.5% uranyl acetate (Fluka) in anhydrous ethanol using a freeze substitution unit (Balzers FSU 010). After fracturing of the sandwiches, samples were immersed in the medium at -90°C for 8 h. Temperature was raised thereafter to -60°C for 8 h and to -30°C for another 4 h. After this procedure, samples were washed in dry ethanol and embedded in 30% LR gold resin (Sigma) over night at a temperature of -30°C, then in 60% LR gold for 8 h and in 100% LR gold for polymerization under UV light for 2 days. Ultra-thin sections (95 nm) were prepared on a Reichert Jung Ultracut E microtome equipped with a diamond knife (Diatom, Biel, Switzerland).

For immuno-labelling, sections were collected onto carbon coated copper grids and incubated for 10 min on 1 drop of PBG (0.2 % Gelatine (Merck) solved in 300 mOsmol PBS, after cooling, 5 % BSA (Sigma)) to prevent non-specific
binding of antibodies. Grids were then transferred onto drops of the primary antibody MAC 236 (provided by Dr. N.J. Brewin, Norwich, UK) and incubated for 1 h. After washing with PBG, grids were placed on drops of the secondary antibody (goat anti-rat antibodies), conjugated to 10 nm gold particles obtained from Janssen Life Science Products (dilution 1:10 with PBS) for 1h and washed with PBG and distilled water. For examination in a transmission electron microscope (TEM) (H-600, Hitachi, Tokyo, Japan), samples were stained for 3 min in uranyl acetate and lead citrate (Reynolds, 1963). The entire labelling procedure was carried out at room temperature.

Analysis of cryo-SEM pictures

A minimum distance of 25% of the total circumference per nodule was analysed. The zones of interest were the double layer and the inner cortex (Fig. 10). The visible intercellular spaces in this area were defined as “filled” or “empty” (Figs. 8A, B and 13A, B), marked and counted later.

Statistical Analysis

Data were subjected to analysis of variances using SAS (Statistical Analyses System Institute, Cary, NC).
Figure 8. Images obtained by cryo-SEM of a freeze fractured lucerne nodule. Section of the inner cortex. A) Intercellular spaces are empty (air-filled). In the lower intercellular space, a cell wall residue covers most of the space (arrow). B) The intercellular space is filled with an aqueous solution and shows the same segregation pattern as the cytoplasm.
RESULTS

Morphology of indeterminate nodules

In a cross section through the actively N$_2$-fixing zone of lucerne and white clover nodules, the cortex can be divided into three distinct zones: 1) the outer cortex, 2) the "double layer" and 3) the inner cortex (Figs. 9 and 10).

1) The outer cortex is composed of large, rounded, loosely packed cells, interlaced with large intercellular spaces (Fig. 10).

2) The double layer with two regularly arranged cell layers separates the outer from the inner cortex (Fig. 10). The cells of this layer are tightly packed with very small intercellular spaces which appear to be interconnected to each other and to the inner cortex (Fig. 10).

3) The thickness of the inner cortex zone can vary from 2 to more cell layers (Fig. 10). The size of cells varies widely and most of the cells contain starch grains (Figs. 10 and 11). The very small intercellular spaces (size 3-5 μm) in this zone appear to form a tunnel system interconnecting intercellular spaces. This is valid for both lucerne and white clover nodules (Figs. 10 and 13A, B).

The segregation patterns formed by the cytoplasm due to the freezing process is uniform to that formed by intercellular spaces which are filled with an aqueous solution. This allows the differentiation between empty (air-filled) intercellular spaces and those filled with an aqueous solution (Figs. 8 and 13). 30% of the intercellular spaces are filled with an aqueous solution (Figs. 8A, B; 11; 12A, B). The cortex surrounds the entire central zone which contains the infected and uninfected interstitial cells. The central zone can be recognized by the large size of the infected cells, which are 5 times larger in volume than the cells of the inner cortex (Figs. 9 and 10).

The distribution of glycoproteins in intercellular spaces of the inner cortex of lucerne nodules was investigated by immuno electron microscopy using the antibody MAC236, specific to a carbohydrate epitope of a glycoprotein found in pea nodules (Brewin et al., 1988). MAC236 cross reacted with an antigenic determinant found in intercellular spaces of the outer cortex, the inner cortex and in the infected zone of lucerne nodules (Figs. 14-18). Interaction of the goat anti
rat antibody, carrying the gold particles, were highly specific; no labelling without pre-incubation with the antibody MAC236 occurred (data not shown).

Fully labelled intercellular spaces, in which gold particles were distributed over the entire intercellular spaces, as well as intercellular spaces without any label (empty intercellular spaces) were observed in all nodules investigated (Figs. 14-18).

Effect of defoliation and increased pO₂ on the percentage of liquid- versus air-filled intercellular spaces in the inner cortex

After defoliation, no significant difference in the proportion of liquid-filled compared to air-filled intercellular spaces was observed in the inner cortex zone of lucerne nodules. In the control, 30% ± 9.5 of the intercellular spaces were liquid-filled, compared to 34% ± 4.2 in nodules of defoliated plants (Fig. 12A). In nodules of undefoliated plants (control) as well as in nodules of defoliated plants, liquid-filled intercellular spaces were distributed over the entire inner cortex zone.

When the rhizospheric O₂ partial pressure (pO₂) was increased from 20 to 80 kPa at a rate of 1 kPa min⁻¹, the nitrogenase activity did not change (Davey and Simpson, 1989; Hunt et al., 1988; Weisbach et al., 1996). According to Fick’s law, the O₂ permeability must have decreased to 25% of its initial value. Despite of this significant decline of O₂ permeability, no difference in the number of liquid-versus air-filled intercellular spaces was detected (Fig. 12B).

Upon re-setting nodule O₂ permeability due to a re-adjustment of nodules to 20 kPa O₂ (Weisbach et al., 1996), the percentage of liquid- versus air-filled intercellular spaces remained stable at 32% ± 8.9 (Fig. 12B).

Effect of defoliation and an increased rhizospheric O₂ partial pressure on the occurrence of glycoproteins in the intercellular spaces of the inner cortex

Defoliation of plants or exposure of nodules to 40 kPa O₂ did not affect the distribution of labelled intercellular spaces in the area examined (Figs. 14-17). Nodules of defoliated plants, or those exposed to an increased rhizospheric O₂ partial pressure (40 kPa) could not be distinguished from the control nodules.
Likewise, nodules initially exposed to 40 kPa O$_2$ followed by a decrease of pO$_2$ to 20 kPa, showed the same labelling patterns (Fig. 18).
Figure 9. Image obtained by cryo-SEM of a freeze fractured lucerne nodule. Overview of the cortex zone from the actively N2-fixing zone of the nodule. The cortex zone is composed of the outer cortex (I), the double layer and the inner cortex (from top downwards) (II). The cortex encloses the central zone with the infected and uninfected interstitial cells (III). The infected cells can be recognized by their large size (lower part). Definition of zones, see Fig. 10.
Figure 10. Image obtained by cryo-SEM of a freeze fractured lucerne nodule. Section of Fig. 9. Definition of zones: O: outer cortex; X: double layer; cells of the inner cortex are not marked; Z: central zone. One example of an interconnected intercellular space is encircled. Starch grains are marked with white arrows.
Figure 11. Image obtained by cryo-SEM of a freeze fractured lucerne nodule. Section of the inner cortex close to the central zone. Cells show large starch grains (black arrows, center of picture). Intercellular spaces are liquid-filled (white arrows).
Figure 12. Amount of intercellular spaces in the inner cortex of lucerne nodules in % of total spaces, obtained by analysis of the cryo-SEM pictures. A) Amount of liquid-filled intercellular spaces in the inner cortex before (control) and 7 h after complete defoliation (cut). B) Amount of liquid-filled intercellular spaces of nodules exposed to 20 kPa O₂ (control) and of nodules exposed to an increased O₂ partial pressure to 80 kPa. To test the reversibility, nodules were re-adjusted to 20 kPa. Means of 5 replicates ± SE are shown.
Figure 13. Images obtained by cryo-SEM of freeze fractured lucerne nodules. Section of the inner cortex. A) Interconnected intercellular space liquid-filled; B) interconnected intercellular space air-filled.
Figure 14. TEM images of cells in the inner cortex of the actively N₂-fixing part of lucerne nodules. Immunogold localization of the MAC236 antigen in nodules, harvested 7 h after a complete defoliation of the plant. A) A typical occluded intercellular space; B) a typical unoccluded intercellular space.
Figure 14A

Figure 14B
Figure 15. TEM images of cells in the inner cortex of the actively N2-fixing part of lucerne nodules. Immunogold localization of the MAC236 antigen in nodules, not exposed to O2 (control). A) A typical occluded intercellular space; B) a typical unoccluded intercellular space.
Figure 16. TEM images of cells in the inner cortex of the actively N₂-fixing part of lucerne nodules. Immunogold localization of the MAC236 antigen in nodules of the control (exposed to 20 kPa O₂). A) A typical occluded intercellular space; B) a typical unoccluded intercellular space.
Figure 17. TEM images of cells in the inner cortex of the actively N₂-fixing part of lucerne nodules. Immunogold localization of the MAC236 antigen in nodules exposed to an increased O₂ partial pressure of 40 kPa. A) A typical occluded intercellular space; B) a typical unoccluded intercellular space.
Figure 17A

Figure 17B
Figure 18. TEM images of cells in the inner cortex of the actively N2-fixing part of lucerne nodules. Immunogold localization of the MAC236 antigen in nodules re-exposed to 20 kPa O2 after an increased O2 partial pressure of 40 kPa in the rhizosphere to test the reversibility of possible glycoprotein occlusions. A) A typical occluded intercellular space; B) a typical unoccluded intercellular space.
DISCUSSION

Nodule morphology of indeterminate nodules versus determinate nodules in relation to O₂ diffusion

Many investigations on nodule structure in relation to the gas diffusion properties were conducted on determinate (soybean) or indeterminate, collar type nodules (lupin) of annual plants. However, studies have not been carried out with indeterminate nodules as formed by perennial plants such as white clover or lucerne.

Photographs of white clover and lucerne nodules presented here (Figs. 3 and 9) show distinct morphological differences relevant to O₂ diffusion as described for soybean and lupin nodules. While the outer cortex and the inner cortex of lucerne nodules are separated by a double layer (Figs. 9 and 10), the outer cortex of soybean and lupin nodules is separated from the inner cortex by a scleroid layer. The latter is irregularly arranged and possesses typical thickened cell walls (Parsons and Day, 1990; James et al., 1991; van Cauwenberghe et al., 1993), while the double layer is composed of cells without any special cell wall features in a parallel arrangement. The inner cortex of lucerne nodules is composed of cells which vary strongly in size and amount of starch grains. These amyloplast rich cells can be found in the inner cortex bordering the central zone. Furthermore, the inner cortex of lucerne nodules is not differentiated in a boundary layer and a distributing zone observed in soybean nodules (Figs. 9 and 19, p. 77) and thus has a more uniform structure. For soybean and lupin nodules, the boundary layer is described as a continuous cell layer with very small cells and only few (if any) intercellular spaces (Parsons and Day, 1990; Iannetta et al., 1993a). Based on this fact, the boundary layer in soybean nodules is supposed to act as the diffusion barrier (James et al., 1991). For lucerne nodules, this function could probably be attributed to the double layer because of the tight packing of the cells and the only very few and very small intercellular spaces (Fig. 10).

Nevertheless, the photographs indicate the presence of interconnected air-filled pathways from the outer cortex to the central zone throughout the double layer and entire inner cortex of lucerne and white clover (Figs. 10 and 13B). In
contrast, the interconnected intercellular space system in soybean nodules seems to be restricted to the distributing zone which is located between the boundary layer and the central zone (James et al., 1991; Dakora and Atkins, 1991).

Recent mathematical models for gas diffusion in soybean nodules predict that gas diffusion pathways are partly liquid-filled and thus seem to form a continuous diffusion barrier, interlaced by only few air pathways (Sheehy et al., 1985; 1987; Hunt et al., 1988; Parsons and Day, 1990; Denison et al., 1992). However, no evidence for the validity of this model has been presented for lucerne nodules. Optimal preservation of nodule structures and of the water content in tissues is a pre-requisite for such an investigation. Thus, samples were frozen in liquid ethane which has an advantage over freezing in liquid N$_2$, since no freeze-retarding gas phase around the sample is formed. Results of this study clearly indicate that more than 30% of all intercellular spaces in the inner cortex are filled with an aqueous solution. The random distribution of liquid-filled intercellular spaces does not support the hypothesis of the existence of an continuous diffusion barrier with liquid-filled intercellular spaces as suggested for soybean (Hunt et al., 1988; Parsons and Day, 1990; Denison et al., 1992).

**Glycoproteins as a structural component in lucerne nodules**

The antibody MAC236, originally produced against a glycoprotein in pea nodules (Brewin et al., 1988), interacted also with an antigenic determinant in lucerne nodules. The interaction between MAC236 and an antigenic determinant was also observed in soybean and lupine nodules (Brewin et al., 1988; VandenBosch et al., 1989; James et al., 1991). Localization of the MAC236 antigen in lucerne nodules was restricted to the intercellular spaces and cell walls. Labelling of cytoplasm or any other structures inside the cell hardly occurred in the present study. This does not support the findings of labelled globules in the cytoplasm of lupin nodules (DeLorenzo et al., 1993).

The TEM images in the present study show that apparent glycoprotein occlusions of intercellular spaces occur all over the inner cortex and the central zone of lucerne nodules. The distribution of fully labelled (presumably
liquid-filled) and unlabelled (presumably air-filled) intercellular spaces over the entire inner cortex zone is irregular and not restricted to any definable zone. It often occurred that labelled and unlabelled intercellular spaces were located adjacently (image not shown). The distribution of fully labelled intercellular spaces corresponds to that of liquid-filled intercellular spaces found in the cryo-SEM pictures. This indicates a possible coexistence between glycoproteins and aqueous solutions in the intercellular spaces. This suggestion is supported by the fact that glycoproteins are hydrophilic and viscous substances which most likely exist in a water associated form like a gel (MacFall et al., 1992). However, direct evidence for a coexistence of glycoprotein occlusions and aqueous plugs could not be obtained as after sample preparation for immunolabelling, the monitoring of liquid-filled versus labelled intercellular spaces is no longer possible.

Intercellular space occlusions with regard to short-term regulation of nodule $O_2$ permeability

1) Investigation of the number of liquid-filled intercellular spaces in the inner cortex of lucerne nodules:

Osmotical processes are often proposed as regulative mechanisms for altering $O_2$ permeability in nodules. For example, the efflux of aqueous solutions into intercellular spaces due to alterations of the osmotical balance between symplast and apoplast was hypothesized to block gas diffusion into the central zone (Streeter and Salminen, 1993; Purcell and Sinclair, 1994; Walsh, 1995). Results from infiltrating the apoplast of the nodule cortex of soybean with osmotic solutions indicated that the increase in intercellular water content decreases $O_2$ permeability and thus nitrogenase activity (Purcell and Sinclair, 1994). The response of nitrogenase activity to infiltration with non removable substances (polyethylene glycol) supported the concept that recovery to initial permeability is dependent upon removal of the solute from the apoplast (Purcell and Sinclair, 1994).

The results from the present study, conducted on nodules of defoliated and $O_2$ stressed (exposure to 80 kPa $O_2$) lucerne plants, did not provide any evidence that the short-term response to both forms of stress consists of an
increased number of liquid-filled intercellular spaces in the inner cortex (Figs. 12A, B). In both treatments, 34% ± 4.2 (defoliation) and 36% ± 10.9 (80 kPa O₂), respectively, of all intercellular spaces in the inner cortex of nodules were liquid-filled and therefore did not differ from the control. These results are consistent with data from van Cauwenberghe et al. (1993) on soybean nodules in which about 37% of all intercellular spaces in the middle and inner cortex were liquid-filled, both in control and O₂ stressed nodules (exposure to 80 kPa O₂).

To examine the reversibility of a possible regulative mechanism, plants were re-exposed to 20 kPa following 80 kPa O₂ in the rhizosphere. As expected from the missing response to application of 80 kPa O₂, the amount of liquid-filled intercellular spaces did not change (Fig. 12B). This provides further evidence that the decrease in O₂ availability in the central zone is not related to the amount of liquid-filled intercellular spaces in the inner cortex.

2) Investigation of glycoprotein accumulation in the inner cortex of lucerne nodules due to stress:

The fully labelled (filled) and unlabelled (empty) intercellular spaces are irregularly distributed throughout the entire inner cortex in nodules of stressed lucerne plants. Moreover, the percentage of fully labelled versus unlabelled intercellular spaces appears not to change due to the treatment (data not shown). These results correspond to the results of the investigations on the influence of defoliation and excess rhizospheric O₂ on the amount of liquid- versus air-filled intercellular spaces using the cryo-SEM technique. They do not support the hypothesis of a short-term regulation of the diffusion barrier through the blockage of intercellular spaces by glycoproteins as proposed for lupin nodules (James et al., 1991; Iannetta et al., 1993a; b; DeLorenzo et al., 1993; Minchin, 1994).

It is suggested that the variable O₂ permeability operates by removal of the glycoprotein-associated water back into the cells, or as a result of a rapid degradation of glycoproteins in the intercellular spaces (MacFall et al., 1992). However, the operation of such a mechanism is not supported by the results of the present study since the percentage of liquid-filled intercellular spaces in lucerne nodules appear to be unchanged by the treatment tested in this study.
Furthermore, the MAC236 labelling pattern in intercellular spaces of lucerne nodules re-exposed to 20 kPa O$_2$ after exposing to 40 kPa, clearly indicates the lack of a positive correlation between glycoprotein occlusions and the alteration of permeability. Thus, glycoproteins are most probably not involved in the regulation of O$_2$ permeability in lucerne nodules.

Results from this study do not support the hypothesis of a regulation of O$_2$ permeability in the inner cortex. It is suggested that a permanent diffusion barrier exists in the inner cortex but the fine regulation of O$_2$ permeability is regulated elsewhere or through alternative mechanisms. Morphological changes in the central zone of nodules as proposed by van Cauwenberghe et al. (1993) or physiological mechanisms need to be considered in further investigations.

CONCLUSION

The results of the present study show that the structures of indeterminate nodules of lucerne and white clover differ markedly of those of soybean or lupin. The intercellular spaces in lucerne nodules form an interconnected tunnel system from the outer cortex through the entire inner cortex to the central zone through which it is supplied with O$_2$. Furthermore, the present results leads to the assumption that a permanent O$_2$ diffusion barrier exists in the double layer in order to maintain a low free O$_2$ concentration in the infected zone. However, reversible changes in the O$_2$ permeability in lucerne nodules do not appear to be associated with alterations in the number of intercellular spaces filled with an aqueous solution and glycoproteins.
GENERAL DISCUSSION

In numerous studies it has been established that in nodules of all symbiotic systems tested, nitrogenase activity is regulated through the availability of oxygen (O\textsubscript{2}) in the central zone (Hartwig et al., 1987; Denison and Layzell, 1991; Sung et al., 1991; Denison et al., 1992; Diaz del Castillo et al., 1992; Heim et al., 1993). This conclusion is based on the fact that a decrease in nitrogenase activity due to stress can be overcome by an external extra supply of O\textsubscript{2} until the system has adapted again to the higher rhizospheric O\textsubscript{2} partial pressure (Sung et al., 1991, Denison et al., 1992, Diaz del Castillo et al., 1992; Hartwig et al., 1987, 1990, 1994). Interestingly, this regulative mechanism seems to react to environmental alterations that nodules are naturally confronted with such as defoliation, altering O\textsubscript{2} partial pressure in the soil in a range of 5 to 20 kPa or N fertilization. Similarly, nodules also respond to artificial perturbations such as exposure to argon, acetylene or to an increased O\textsubscript{2} partial pressure above 20 kPa. The uniformity of the response to the various forms of perturbations led to the assumption that only a single regulative mechanism operates in determinate as well as in indeterminate nodules.

Several studies support the hypothesis of an O\textsubscript{2} diffusion barrier located in the inner cortex of nodules. The steepest decrease in O\textsubscript{2} concentration occurs between the outer cortex and the central zone of soybean (Tjepkema and Yocum, 1974), which has also been confirmed in pea (Witty et al., 1987). This corresponds to the presence of a special cell zone in the inner cortex of soybean nodules which is interlaced by only very few intercellular spaces through which gas diffusion to the central zone could take place (Dakora and Atkins, 1989; Day and Copeland, 1991; Parsons and Day, 1990). All further hypotheses and mathematical models developed to understand the mechanism of the regulation of O\textsubscript{2} permeability proceeded from this assumption obtained for soybean nodules. However, differences in nodule structure between determinate and indeterminate nodules (Figs. 1, 9 and 19) were not taken into account. A regulation of O\textsubscript{2} availability in the central zone itself was not considered for a long time. The rejection of this alternative based on the fact that the O\textsubscript{2} sensitive nitrogenase...
does not seem to be damaged due to exposure to an increased $O_2$ partial pressure since nitrogenase activity fully recovers after relief of even severe $O_2$ stress (Weisbach et al., 1996). This was taken as sufficient evidence for the fact that the variable $O_2$ diffusion barrier is able to fully adjust to increased $O_2$ partial pressure. It was hypothesized that in case of a regulation of $O_2$ permeability in the central zone, damage to the nitrogenase would occur in the outer layers of the central zone (Lannetta et al., 1995). No data is available to support this assumption. It is known that nitrogenase activity is always $O_2$ limited and therefore functions below its potential capacity (Sheehy et al., 1985; Hunt et al., 1989). This indicates that the damage of some nitrogenase molecules due to an enhanced $pO_2$ could be compensated by an increased activity of other, not damaged nitrogenase molecules (Herdina and Silsbury, 1990). Therefore, rapid re-establishment of full nitrogenase activity does not necessarily indicate the survival of all nitrogenase molecules present in the nodule. This re-introduces a regulation of nitrogenase activity and $O_2$ availability in the central zone into the discussion.

$O_2$ permeability in the inner cortex is not regulated through the amount of liquid-filled intercellular spaces

Various studies indicate that carbon metabolites might be involved in the regulation of $O_2$ permeability. For example nodules of plants exposed to darkness for 24 h lost the ability to adjust $O_2$ permeability to an increased $pO_e$ (Minchin et al., 1985). Furthermore, after defoliation, sucrose concentration declined significantly, which strongly corresponded to the decrease of $O_2$ permeability (Gordon et al., 1986; Streeter and Salminen, 1993; Hartwig et al., 1994). However, while the two different forms of stress, defoliation and an increased $O_2$ partial pressure in the rhizosphere, cause both a significant decrease in nodule $O_2$ permeability, carbon metabolite concentrations declined after defoliation but remained unchanged under increased $pO_2$ (Weisbach et al., 1996). As a result, an involvement of whole carbon metabolite pools in triggering nodule $O_2$ permeability appears unlikely. Nevertheless, an involvement of sucrose in regulating nodule $O_2$ permeability cannot be fully excluded, since alterations in
the osmotical balance between symplast and apoplast in the inner cortex could cause an efflux of water into the intercellular spaces. The filling of diffusion pathways with aqueous solutions could effectively restrict diffusion of O\(_2\) into the central zone, since the diffusion coefficient of O\(_2\) in water is much lower than in air.

Various authors have emphasized a regulation of O\(_2\) permeability through osmotical processes but suggested different mechanisms inducing the proposed osmotical process. Based on experiments with indeterminate nodules such as those of white clover (Trifolium repens L.) and lucerne (Medicago sativa L.) but also with determinate nodules from soybean (Glycine max), Denison and Kinraide (1995) reported an O\(_2\) induced membrane depolarization. The exposure of nodules to a rhizospheric O\(_2\) partial pressure of 100 kPa induced rapid and reversible membrane depolarization in cells of all nodule zones. It is suggested that O\(_2\) reduces the activity of a proton pump in the cell membrane which maintains the electrochemical gradient. The deactivation may cause the opening of an anion channel, allowing efflux of Cl\(^{-}\). The resulting depolarization would change the electrochemical gradient for K\(^{+}\), causing efflux through a K\(^{+}\) channel out of the cells with a consequent efflux of water into the intercellular spaces. The reverse process is thought to be induced by the reactivation of the proton pump and the concomitant re-establishment of the electrochemical gradient.

Another hypothesis relates to the regulation of O\(_2\) permeability through the import and export of solutes via the vascular bundles (Walsh et al., 1989; Walsh, 1995). It is hypothesized that a disturbance in phloem supply to the nodule may affect the water relations of the nodule cortex by altering the solute (e.g. sucrose, amides or ureides) concentrations or by affecting the availability of phloem supplied water in the cortex. It was assumed that N compounds in the nodule apoplasst are in osmotic balance to sucrose within the symplast of unstressed nodules (Walsh et al., 1987). In consequence, as nodule sucrose concentration decreases in response to defoliation or stem-girdling, water may leave the cortical cells thereby filling the intercellular spaces. In case of an increased O\(_2\) partial pressure, the increased nitrogenase activity may cause an enhanced export of N compounds which would also cause an unbalance between symplast
and apoplast solute concentrations with a concomitant water efflux.

Results of the present study obtained by analysis of cryo-SEM pictures clearly indicate that the total number of liquid-filled intercellular spaces in the inner cortex of lucerne nodules did not change in response to different treatments known to affect $O_2$ permeability (Fig. 12). In addition to the evaluation of the total number of liquid-filled intercellular spaces, the intercellular spaces in the area of vascular bundles were counted separately. In the area of vascular bundles, the number of liquid-filled intercellular spaces was indeed higher than in the other parts of the inner cortex zone (data not shown) but had no influence on the total number of liquid-filled intercellular spaces found in the inner cortex. The number of liquid-filled intercellular spaces in nodules varied strongly not only from nodule to nodule within one treatment, but also between the treatments. While some nodules had a lower number of filled intercellular spaces on an average, others had a much higher number of filled intercellular spaces. A clear tendency could not be observed. The large standard deviation between the data of the replicates indicates that the response of nodules to stress is not correlated to the amount of liquid-filled intercellular spaces. Therefore, the hypothesis about a regulation of the $O_2$ permeability through variations in the number of liquid-filled intercellular spaces can not be supported. Also for soybean, a determinate nodule type, a regulation of $O_2$ permeability through changes in the amount of liquid-filled intercellular spaces does not seem plausible (van Cauwenberghe et al., 1994). A further indication that the osmotical balance between symplast and apoplast might not be involved in the regulation is given by the experiments of Streeter and Salminen (1993). Although the ureide content of the whole nodule responded to the different treatments known to affect gas diffusion, the apoplastic ureide content in the inner cortex did not alter significantly. Thus suggested that the apoplastic ureide content is most probably not involved in the regulation of gas diffusion resistance.

$O_2$ permeability in the inner cortex is not regulated through cell expansion

Besides causing an efflux of water into the intercellular spaces, alterations in the osmotical balance could also cause cell expansion. This conclusion is not
supported by van Cauwenberghe et al. (1994) who carried out exact measurements of changes in the size of the intercellular spaces due to stress. These results do not provide any evidence for a significant constriction of the intercellular spaces in the inner cortex of soybean nodules. Therefore, it does not support the hypothesis of an involvement of cell expansion in the regulation of O₂ permeability in the inner cortex.

Open questions about the role of glycoproteins in the regulation of O₂ permeability in the inner cortex

Glycoproteins accumulate in the intercellular spaces of the inner cortex of soybean nodules grown under 40 kPa O₂ (James et al., 1991). In contrast, less glycoprotein was observed in nodules grown under 10 kPa O₂. It was suggested that these glycoproteins are structural components of the diffusion resistance in the cortex of legume nodules. Further studies on lupin nodules indicated an involvement of glycoproteins in the short-term regulation of O₂ permeability as well. The results obtained in these studies strongly indicate a connection between the occurrence of glycoproteins and a decreasing O₂ permeability. Nevertheless, very important questions still remain open:

1) The down regulation of O₂ permeability due to stress is a reversible process. How can accumulated glycoproteins quickly be removed from the intercellular spaces? MacFall et al. (1992) suggested an enzymatic breakdown but no data is available to support such a mechanism.

2) Do processed glycoproteins exist and are they stored in vesicles which would correspond to the rapid response of nodules to application of stress, since a de novo synthesis is most unlikely to take place in the time frame of less than one hour? As reported by DeLorenzo et al. (1993), some labelled globular structures are found in the cytoplasm close to the cell walls, but this had never been observed for lucerne nodules in the present study. Therefore, there is no evidence that glycoproteins are stored in vesicles of lucerne nodules in a form, which would interact with the MAC236 antibody.

3) Labelling of lucerne nodules carried out in this study showed that glycoproteins also occurred in the intercellular spaces of the central zone (picture not shown).
This was not explicitly checked in soybean and lupin nodules. Therefore, the results of the ELISA tests, which were conducted in the studies of Iannetta et al. (1993a; b) with the whole nodule material, are difficult to interpret with regard to the operation of the O₂ diffusion barrier in the inner cortex. Probably, the reported increase of glycoproteins in lupin nodules could be attributed to a large extent to an accumulation in the intercellular spaces of the central zone which makes up more than 70% of the whole nodule tissue.

4) How could one explain that in comparison to lupin, lucerne nodules tested in the present study do not accumulate glycoproteins in the intercellular spaces of the inner cortex due to stress (see Part 2) despite of their existence and the measurable decline of O₂ permeability?

Nevertheless, examinations of nodules under elevated rhizospheric O₂ partial pressure by ³¹P-Nuclear Magnetic Resonance (NMR) spectroscopy indicated that a "modification of the cortical cell chemistry" must have occurred (Pfeffer et al., 1992). It is suggested that the viscosity of the cell wall increased under elevated pO₂ probably due to glycoproteins. This indicates that glycoproteins play a certain role in the response of nodules to rhizospheric O₂ concentration. The occurrence of glycoproteins in all nodule types (e.g. soybean, pea, lupin, lucerne) supports its importance. However, the exact function with regard to the questions raised above awaits further elucidation.

Hypothesis of a permanent, non variable O₂ diffusion barrier in the inner cortex

The influence of the O₂ partial pressure in the rhizosphere on growth and morphology regarding to oxygen diffusion properties of soybean nodules was investigated by many (Criswell et al., 1976; Parsons and Day, 1990; Dakora and Atkins, 1990; 1991; Arrese-Igor et al., 1993; Atkins et al., 1993). A range of structural alterations in nodules were identified. Nodules grown in pO₂ below 10 kPa produced more lenticels in the outer cortex, had smaller nodule sizes, a lower volume of whole cortex relative to the infected central tissue of the nodules, as well as an increase in the size and frequency of intercellular spaces in all tissues with the boundary layer also reduced from four to one or two cell layers.
Nodules grown under elevated pO₂ showed the opposite tendency (Dakora and Atkins, 1991). Measurements of the short-term response indicated that nodules adapted to function effectively at sub- or supra-ambient pO₂ retain their capacity for adjustment to short-term changes in oxygen supply (Atkins et al., 1993). These results support the hypothesis of a permanent, morphologically determined diffusion barrier (Denison et al., 1992; Atkins et al., 1993; Thumfort et al., 1994).

Is the nitrogenase activity fine-regulated in the central zone?

The unchanged number of filled intercellular spaces in the inner cortex of lucerne nodules measured in the present study corresponds to the results of van Cauwenberghe et al. (1994) on soybean. Moreover, a simulation model developed by Thumfort et al. (1994) favours the following hypothesis: "The short-term regulation of nitrogenase activity and therefore oxygen availability does not take place in the inner cortex but in the central zone itself. This would explain why determinate and indeterminate nodules respond in the same way to external perturbations despite of their difference in morphology (Figs. 1, 9 and 19). It has to be emphasized that the composition and the thickness of the cortex zone between outer cortex and central zone is very important, considering the restriction of oxygen diffusion. In soybean and lupin nodules (James et al., 1991; Iannetta et al., 1993; b), the middle and inner cortex are composed of several cell layers including the scleroid layer and is much thicker than the inner cortex of lucerne nodules (Fig. 19).
Furthermore, soybean and lupin nodules both possess a boundary layer which is located in the inner cortex and seems to act as the diffusion barrier. A boundary layer does not exist in lucerne nodules but a comparable zone, the double layer, seems to take over this function (Fig. 10). The double layer in lucerne nodules is located directly underneath the outer cortex and is not covered by a "middle cortex". Therefore, the diffusion pathways in lucerne nodule are significantly shorter. Hence, a regulation of oxygen diffusion through the occlusion of intercellular spaces can be imagined to be much less effective than in soybean or lupin nodules. Nevertheless, both nodule types exhibit the same
response to stress (Hartwig et al., 1994). It is considered that the mechanism or the combination of different mechanisms, involved in the regulation, must be of a similar type in determinate and indeterminate nodules. Furthermore, the most important characteristic of this mechanism is, that it can be compensated by an external extra supply of oxygen since a reduced nitrogenase activity can be recovered by a short-term increase of rhizospheric $O_2$ partial pressure (Hartwig et al., 1994). It is suggested that the short-term response of nodules can either consist of morphological alterations such as changes in the shape of intercellular spaces as proposed from van Cauwenberghe et al. (1994) or/and through physiologically controlled mechanisms. Changes in the affinity of leghemoglobin to $O_2$, and the activity of key enzymes in nodular metabolism are considered to be possible mechanisms.

Possible role of leghemoglobin (Lb) in the regulation of nitrogenase activity

Leghemoglobin (Lb) is a monomeric molecule with a higher affinity to $O_2$ than that of other globins (Powell and Gannon, 1988) and might play a crucial role in the regulation of nitrogenase activity. As emphasized by Thumfort et al. (1994) alterations in the affinity of the leghemoglobin for $O_2$ might regulate the supply of $O_2$ to the bacteroids. The affinity to $O_2$ depends on the ambient pH in the cytoplasm and determines therefore the association and dissociation of $O_2$. Between pH 4 and pH 7, affinity increases 5 fold (Appleby et al., 1983). Therefore, only a small increase in the cytoplasmatic pH would greatly affect the availability of $O_2$ to the respiratory system of the bacteroids due to a decreased dissociation of $O_2$ from the leghemoglobin. The decreased $O_2$ supply negatively affects the production of ATP required to drive nitrogenase activity. To date, data on a positive correlation of pH changes in the cytoplasm and the application of stress to nodules are not available.

Possible regulation of cytoplasmatic pH in relation to the regulation of nitrogenase activity

Regulation of cytoplasmatic pH could be achieved by a balance between carboxylation of phosphoenolpyruvate (PEP) by the phosphoenolpyruvate
carboxylase (PEPC) and decarboxylation of malate by the NADP*-malic enzyme (Davis, 1986). The PEPC may play a major role in this process and is considered to be one of the key enzymes in nodular metabolism (Deroche and Carrayol, 1988). PEPC makes up more than 1-2% of the soluble protein content in lucerne root nodules (Vance and Stade, 1984) and catalyses the reaction: phosphoenolpyruvate + HCO₃⁻ = oxaloacetate + Pᵢ.

Some of the oxaloacetate is converted by malate dehydrogenase to malate. This is taken up by bacteroids and used as a primary carbon and energy source. Malate also enters plant mitochondria as a substrate for production of ATP, reducing equivalents and carbon intermediates through the Krebs cycle, but it can also be decarboxylated by the NADP*-malic enzyme to form pyruvate. In amide transporting nodules such as lucerne or white clover, oxaloacetate provides the carbon skeleton for the synthesis of aspartate and asparagine which are the main export products of the N₂-fixation.

Is PEPC involved in the regulation of the cytoplasm pH, how might the PEPC itself be regulated? It is known that the PEPC in crassulacean acid metabolism (CAM) plants is post-translationally regulated through phosphorylation by a protein kinase (Rajagopalan et al., 1994). It was recently shown that the PEPC of soybean nodules is also regulated through phosphorylation similar to CAM plants (Pathirana et al., 1992; Zhang et al., 1995). In these experiments it could also be shown that the phosphorylation state of nodule PEPC decreased following stem girdling which is a treatment comparable to defoliation. It did not respond to short-term darkness. This response corresponds well to the response of the nitrogenase activity to the same treatments (Hartwig et al., 1987), thus providing further evidence for the strong positive correlation of PEPC activity and nitrogenase activity as previously shown by Deroche and Carrayol (1988).
Ca^{2+} and calmodulin, the key to explain the regulation of PEPC activity?

The regulation of enzyme activities by a reversible phosphorylation through a protein kinase is a general mechanism in the regulation of the cellular metabolism (see Marschner, 1995). It is known that the protein kinase itself is affected by polyamines, calmodulin, and free Ca^{2+}. Calmodulin is an ubiquitous protein which is also involved in signal transduction in animal cells and works through the same mechanism (see Eckert and Randall, 1986). Calmodulin is activated by free Ca^{2+} which enters the cells through a special Ca^{2+} channel (Schroeder and Thuleau, 1991). Beside a possible activation of calmodulin by Ca^{2+} and the concomitant activation of a protein kinase, Ca^{2+} may also activate protein kinases directly (Roberts and Harmon, 1992). Only recently it could be shown that Ca^{2+} is involved in the phosphorylation process of the PEPC since depletion of this ion blocks PEPC phosphorylation in situ in mesophyll cell protoplasts of *Sorghum* (Pierre et al., 1992) and it is suggested that a Ca^{2+}-dependent step is involved in the PEPC phosphorylation pathway (Giglioli-Guivarch et al., 1996). It would therefore be most challenging to investigate the occurrence and function of calmodulin in nodule cells as well as the function of Ca^{2+} in the regulation of the protein kinase which is responsible for PEPC activation in nodules.

The N-feed back hypothesis

In addition to a possible regulation of nitrogenase activity through alteration of the cytoplasmatic pH, a regulation through a N-feed back mechanism is also being discussed. The N-feed back hypothesis represents a very different approach. There is, in fact, evidence for an involvement of the N metabolism in the short-term regulation of nitrogenase activity since several environmental manipulations of the plant N-sink activity have been reported to affect nitrogenase activity (reviewed by Hartwig and Nösberger, 1994). For instance, NO$_3^-$ is known to cause a decrease in nitrogenase activity, accompanied by a decrease in O$_2$ permeability in nodules within 48 h after its addition (Carroll et al., 1987; Minchin et al., 1986; Vessey et al., 1988). The effect of NO$_3^-$ on nitrogenase activity seems to be connected to the presence of nitrate reductase.
(Jacobsen, 1984; Shelp et al., 1991). It was suggested that nitrogenase activity may be regulated by its product ammonia or by other current N2-fixation products such as amino acids or ureides. However, in case of defoliation and exposure of the plants to an argon atmosphere for 6 h, the NH\textsubscript{4}\textsuperscript{+} content in the nodules was not affected despite of a significant reduction in nitrogenase activity (Ohyama and Harper, 1991; Heim et al., 1993). Thus, ammonia does not seem to be involved in the regulation of nitrogenase activity. Similarly, the amino and ureide content is reported to remain constant during 2 h after decapitation of soybean plants (Ohyama and Harper, 1991). Nevertheless, application of amino acids (e.g. asparagine, glutamine) to the root or to the shoot system reduced nitrogenase activity significantly (Silsbury, 1987; Oti-Boateng and Silsbury, 1993). This would support the idea of a feed back control and the concept was further developed by Parsons et al. (1993). They suggested that the concentration of N compounds entering the nodules through the phloem may elicit a change in nodule permeability, most probably through alterations in the balance of solutes between symplast and apoplast. As discussed in Part 2, the restriction of O\textsubscript{2} diffusion is not controlled by such processes since the amount of air-filled intercellular spaces through which gaseous exchange takes place remained constant throughout all treatments tested. Furthermore, it must be mentioned that the points previously discussed strongly support a regulation of nitrogenase activity in the central zone. Therefore, if a N-feed back mechanism should be realized, key enzymes involved in energy production and the assimilation of NH\textsubscript{4}\textsuperscript{+} should be examined in relation to end-product repression.
Outlook

As outlined in the previous chapters there is strong indication that nitrogenase activity is not regulated through mechanisms in the inner cortex but rather in the central zone itself. Therefore, further studies would need to concentrate on mechanisms located in the cells of the central zone, especially in the infected cells. Moreover, in explaining the possibilities to regulate N₂-fixation, it has to be considered that the down regulation of nitrogenase activity in case of stress can be overcome by an external supply of O₂. In this context it could be useful to re-evaluate the importance and involvement of O₂ in the regulation of nitrogenase activity.

A first approach to solve the question about the involvement of O₂ availability in the central zone for the regulation of nitrogenase activity would be to evaluate mechanisms possibly involved in the O₂ limitation inside the cells. One possibility would be measuring pH alterations in the cytoplasm due to stress. As previously described, pH in the cytoplasm largely affects the affinity of leghemoglobin for O₂ and hence the O₂ supply to bacteroids. It also affects enzyme activities. Of special interest are key enzymes involved in energy metabolism as well as in the production of carbon substrates for N assimilation.

Another approach worth concentrating on in detail is the impact of N compounds on the regulation of nitrogenase activity. There is some experimental evidence that N compounds might be involved in its regulation, and a N-feedback mechanism is suggested. Therefore experiments should focus on N compounds originating from the shoot as well as on accumulated N metabolites of N₂-fixation in the nodules with regard to their influence on the activity of key enzymes involved in the nodule metabolism.

A long-term perspective would be the evaluation of the importance of Ca²⁺ and calmodulin in the regulation of nitrogenase through the mechanisms described above. In such studies, it would be useful to evaluate, if key enzymes other than PEPC are regulated through phosphorylation.
REFERENCES


MacFall JS, Pfeffer PE, Rolin DB, MacFall JR, Johnson GA (1992) Observations of the oxygen diffusion barrier in soybean (Glycine max) nodules with magnetic resonance microscopy. Plant Physiol 100: 1691-1697


VandenBosch KA, Bradley DJ, Knox J-P, Perotto S, Butcher GW, Brewin NJ (1989) Common components of the infection thread matrix and the intercellular space identified by the immunocytochemical analysis in pea nodules and uninfected roots. EMBO J 8: 335-42


CURRICULUM VITAE

17. December 1964  Born in Stuttgart, Germany

1971-1975  Primary school in Böblingen, Germany

1975-1985  High school in Böblingen, Germany

1986-1992  Student at the Faculty of Biology, University of Hohenheim, Germany

1992  Diploma in Natural Sciences (Masters of Biology)

1992-1996  Ph.D. student at the Institute of Plant Sciences of ETHZ, Division of Crop Sciences
DANK

Zuerst möchte ich Prof. Dr. J. Nösberger für die Aufnahme in der "Gruppe Nö" sowie für seine fachliche und menschliche Unterstützung danken.

Prof. Dr. N. Amrhein danke ich für die Übernahme des Koreferates, die großzügige Hilfe und das Interesse für meine Arbeit.

Dr. U.A. Hartwig möchte ich für die enthusiastischen Diskussionen danken. Sein Einsatz bei der Fertigstellung der Publikationen war mir eine grosse Hilfe.


Herzlichen Dank auch an Dr. M. Frehner für die großzügige Hilfe bei den "Zucker"-Analysen, an Dr. A.-B. von der Crone-Kopp für die ersten Abklärungen für die Analysen der organischen Säuren und an Dr. A. Lüscher für seine Hilfe bei den statistischen Auswertungen.

Vielen Dank an Dr. Dorothee Staiger aus der Gruppe Apel für ihre Hilfe bei der Einbettung von Knöllchenproben sowie den Diskussionen über Knöllchen und die Welt.

Herzlichen Dank auch an Dr. B. Frey und Dr. Ch. Scheidegger vom WSL, Birmensdorf für ihre großzügige Hilfe und die Möglichkeit Ionenverteilung in Knöllchengewebe zu studieren. Leider konnte dieser Versuch aufgrund methodischer Schwierigkeiten nicht in diese Arbeit integriert werden. Trotzdem vielen herzlichen Dank!

Ebenso herzlichen Dank an Dr. Ravi Sangakkara und Dr. Dariusz Malinowski für die vielen fruchtbaren Diskussionen und Ermuterungen und auch an Jeanette Habenczius für die Frauen-Aperos und die netten Gespräche zwischen "Tür und Angel".

Und nicht zu vergessen meinen "mental support" Karen Isensee, Silvia Zanetti, Carmen Thönnissen, Renate Braun und Kaspar Ruegg. Ebenfalls Dank an alle KollegInnen aus der Gruppe Nö und den anderen Gruppen, die mir bei meiner Arbeit mit Rat und Tat zur Seite standen.

Ende gut, alles gut!

Diese Arbeit wurde durch einen Forschungskredit der ETH ermöglicht.