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Some Processes Related to Nitrogen Fixation in Nodulated Legumes [and Discussion]

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Some processes related to nitrogen fixation in nodulated legumes

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We have summarized information in four areas of the broad topic of legume–*Rhizobium* symbiosis. These include: carbon substrates provided to nodule bacteroids by the host, assimilation of fixed nitrogen by the host, O_2 metabolism in legume nodules and involvement of H_2 in nodule metabolism. Although nodules contain a variety of carbon substrates, both biochemical and genetic evidence indicate that C4 dicarboxylates are the major carbon substrates that support N_2 fixation in nodules. The biochemical pathways for utilization of products of N_2 fixation are fairly well understood but relatively little is known about the regulation of the assimilation of fixed nitrogenous compounds at the gene level. Ureides are primary nitrogenous compounds exported from nodules of the tropical legumes. Because the catabolism of these products may involve the hydrolysis of urea by nickel-dependent urease, the possible importance of nickel as a trace element in the nutrition of legumes is raised. The O_2 supply to nodule bacteroids is regulated by a barrier to free-O_2 diffusion and by leghaemoglobin. Progress has been made in understanding of the molecular genetics and biochemistry of leghaemoglobin but little is known about the mechanisms that control the physical barrier to O_2 diffusion. Legume nodules contain mechanisms for the disposition of peroxide and free radicals of oxygen. The importance of these systems as protective mechanisms for the O_2-labile nitrogenase is discussed. Some strains of *Rhizobium* form nodules which recycle the H_2 produced as a byproduct of N_2 fixation. The genes necessary for H_2 oxidation have been cloned and transferred within and among species of *Rhizobium*. The advantages and disadvantages of H_2 recycling in legume nodules are discussed.

1. Introduction

The interaction of *Rhizobium* with the roots of leguminous plants resulting in the formation of an efficient N_2-fixing symbiotic association is a complex process involving many genes and physiological processes in both participants in the symbiosis. For comprehensive discussions the reader is referred to reviews by Sprent (1984), Verma & Long (1983), Verma & Nadler (1984), Dazzo & Gardiol (1984), Dilworth & Glenn (1984) and Halverson & Stacey (1986).

In summary, exudates from roots of some legumes are reported to stimulate the multiplication of *Rhizobium* in the rhizosphere. Recognition and attachment of the rhizobia to epidermal cells of legume roots may involve a cross-reaction between molecules in the two organisms, such as lectins produced by the roots of the host and polysaccharides of the *Rhizobium* endophyte. Hormones and enzymes, some produced by the plant and others by *Rhizobium*, are reported to be involved in nodule initiation and development. Compounds, such as specific flavones, produced by the host plant are essential for the activation of the nodulation genes of a compatible strain of *Rhizobium*. In response to infection by *Rhizobium*, the host cells of the root synthesize cell-wall materials which are incorporated into an infection thread that allows the endophyte to penetrate into the root tissue. As a consequence of infection, cortical cells of the

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root are induced to divide and differentiate into nodule tissue. After the release from the infection threads, the rhizobial endophyte in the cytoplasm of host cells differentiates into bacteroids that acquire a capability to synthesize the nitrogenase components that catalyse the reduction of atmospheric N_2 to ammonia. Proteins such as leghaemoglobin and other nodulins are synthesized as a consequence of the initiation of infection and nodule formation. The nodule not only provides a physical structure that separates the endophyte from competing soil microorganisms, but also the nodular vascular system furnishes a means whereby minerals, water and carbon substrates from the host legume are made available to support N_2 fixation and other metabolic processes of the endophyte. In return, the *Rhizobium* bacteroids within nodules export NH_4^+ into the cells of the legume host where this product of N_2 fixation is processed and eventually transported via the xylem to various parts of the plant. Furthermore, a functional N_2-fixing legume nodule involves the uptake or release of gases including H_2, CO_2, N_2 and O_2. Regulation of the metabolism of these gases again involves the interaction of genes and systems produced by the two partners of the symbiosis.

This paper summarizes information concerning four aspects of the legume–*Rhizobium* symbiosis. These include: carbon substrates provided by the host, assimilation of nitrogen by the host, O_2 metabolism in nodules, and involvement of H_2. With the exception of the last topic we emphasize information relevant to the host legume. For discussion of *nif*-related bacterial genes and systems, see Johnston *et al.* (this symposium).

2. Carbon substrate utilization by the host

The nitrogenase reaction, which catalyses the reduction of N_2 and H^+ to NH_3 and H_2, consumes about sixteen moles of ATP per mole of N_2 reduced. In addition, eight electrons are required in the reduction of N_2 and H^+ during the reaction (Yates 1980). The energy required for N_2 fixation *per se* and for associated processes in legume nodules must be provided by utilization of carbon substrates transported from the leaves of the host legume to the bacteroids of the legume nodules. Also, carbon compounds from the shoot provide carbon skeletons for transport and incorporation of newly fixed nitrogen into various plant parts. Recently, Rainbird *et al.* (1984) have estimated that the consumption of carbohydrate per gram of N fixed, associated with different aspects of the N_2 fixation process, is as follows: N_2 reduction and H_2 evolution, 7.29 g; nodule maintenance, 2.68 g; assimilation and transport of NH_4^+, 1.86 g; growth, 0.26 g. A similar estimate was obtained by Heytler & Hardy (1984) using a different method. Up to 22% of the plant net photosynthate may be utilized for support of the N_2-fixation system during the period of maximum N_2 fixation (Pate & Atkins 1983).

Although legume nodules contain a variety of carbohydrates, sucrose is the major compound transported from shoot to nodules (Streeter & Salminen 1985; Stowers 1985). In addition, nodules of many legumes contain one or more cyclotols, including myoinositol, D-chiroinositol, D-pinitol, ononitol and *o*-methylscylloinositol (Streeter & Salminen 1985). Although some of these compounds accumulate in nodules at relatively high concentrations, they are not readily labelled and are relatively inert when plants are exposed to $^{14}CO_2$ (Streeter & Salminen 1985). Trehalose is also present in nodules; the extent of accumulation depends on the *Rhizobium* strain used for inoculation. This carbohydrate is synthesized by the nodule bacteroids rather than by host-plant cells (Reibach & Streeter 1983) and is postulated to be important in the free-living portion of the *Rhizobium* life cycle rather than being a significant carbon substrate for the support

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of the N_2-fixing bacteroid. Malonate is also a major carbon compound in legume organs, including nodules (Stumpf & Burris 1981) but its importance as an energy source for bacteroids is in doubt (Streeter & Salminen 1985).

When ^{14}C-labelled carbon substrates are provided by the plant to nodules, ^{14}C rapidly appears in organic acids, primarily malate, and subsequently in amino acids (Streeter & Salminen 1985). Although the available data indicate that dicarboxylic acids are the principal carbon substrates for nodule bacteroids, other carbon substrates may be important at specific stages in the development of bacteroids (Dilworth & Glenn 1984).

A recent summary of the enzymes involved in the carbon metabolism of legume nodules has been provided by Streeter & Salminen (1985). Of particular interest is phosphoenolpyruvate carboxylase, which is widely distributed in the cytosol fraction of legume nodules (Vance *et al.* 1983). This enzyme is reported to play an important role in replenishing carbon removed from the nodule by bacteroids. The rapid increase in the specific activity of this enzyme during nodule development indicates a specific and important role in the N_2-fixation process (Christeller *et al.* 1977; Lawrie & Wheeler 1975).

The use of mutants of *Rhizobium* with defects in pathways for utilization of carbon substrates has provided useful information about carbohydrate catabolic pathways that are important in symbiotic N_2 fixation (see Ronson & Astwood 1985). Mutants defective in several different steps in sugar utilization have been obtained from several species of *Rhizobium*. Those lacking activities of glucokinase, fructokinase and glucose-6-phosphate dehydrogenase formed effective N_2-fixing symbioses; this result suggests that sugars are not essential for N_2 fixation by bacteroids (Streeter & Salminen 1985; Ronson & Astwood 1985). In contrast, mutants with defects in the tricarboxylic acid utilization pathway formed ineffective nodules, indicating that the TCA cycle is essential for bacteroid N_2 fixation. More convincing evidence for an indispensable role of the C4 dicarboxylates in bacteroid N_2 fixation was provided by the performance of mutants of *R. trifolii* and *R. leguminosarum* with impaired capacities to transport C4 dicarboxylates (see Ronson & Astwood 1985). Such mutants formed ineffective nodules but the nodule host cells contained what appeared to be normal bacteroids (Ronson *et al.* 1981).

Considerable progress has been made in cloning and characterizing dicarboxylate transport genes. These results provide a better understanding of the genes required for utilization of dicarboxylates provided by the host and utilized by bacteroid for the support of N_2 fixation. The molecular biology of transport and metabolism of carbon substrates in legume host cells, including peribacteroid membranes, remains for future investigation.

3. Assimilation of nitrogen by the host

Effects of fixed nitrogen

An excessive supply of fixed nitrogen in culture media depresses nodulation and nitrogenase activity (Sprent 1984). Although the mechanism whereby nodulation and N_2 fixation are repressed by fixed nitrogen has not been defined, it is clear that the host legume plays an important regulatory role in the process (see Miflin & Cullimore 1984).

By the use of ethylmethane sulphonate as a mutagen on soybean cultivar 'Bragg', Gresshoff *et al.* (1985) have isolated a large number of soybean mutants altered in nitrate reductase activities and in sensitivity of nodule initiation and development to exogenous nitrate. Fifteen

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independent mutants showing the nitrate-tolerant nodulation phenotype were selected for their ability to nodulate in presence of 5 mM nitrate. In five of these mutants, this characteristic was shown to be inherited as a monogenic recessive trait. Some supernodulation mutants were characterized by extensive nodulation in presence or absence of 5 mM nitrate. This response was observed with five different strains of *Bradyrhizobium japonicum*, indicating that this was a genetic characteristic of the legume host rather than the inoculant. Grafting of shoots of representative supernodulation mutants on to roots of plants lacking the supernodulation characteristic resulted in expression of supernodulation, but reciprocal grafts did not express this phenotype. Gresshoff *et al.* (1985) have concluded that excessive nodulation appears to be counter-productive in terms of plant growth in general and root development specifically. Although these investigations have not, so far, produced a commercially acceptable cultivar of soybeans that nodulate in presence of NO_3^-, this research had advanced our understanding of the role of the host in regulation of nodulation. The methods developed and the mutant lines that have been produced will undoubtedly contribute towards the construction of commercially acceptable cultivars with improved nodulation characteristics.

The glutamine synthetase–glutamate synthase cycle

As discussed by Miflin & Cullimore (1984), NH_4^+ produced by N_2-fixing bacteroids is initially assimilated in the legume nodule cytoplasm by a pathway involving coupled reactions of glutamine synthetase and glutamate synthase. Time course experiments with isotopically labelled N_2 showed that the amido-N of glutamine and glutamate were the initial products of NH_4^+ assimilation in soybean nodules (Meeks *et al.* 1978; Ohyama & Kumazawa 1978). Results of inhibition of either glutamine synthetase or glutamate synthase provided additional support for the coupled glutamine synthetase and glutamate synthase reactions as essential steps in the assimilation of NH_4^+ by soybean nodules (Ohyama & Kumazawa 1980).

Evidence from several sources shows that the activity of glutamine synthetase in nodules of *Phaseolus*, soybean and lupin is many-fold higher than that in roots of legumes (Cullimore *et al.* 1982; Robertson *et al.* 1975). In soybean nodules, glutamine synthetase was estimated to constitute 2% of the nodule soluble proteins (McParland *et al.* 1976). In *Phaseolus*, the increase in glutamine synthetase activity during nodule development was associated with the production of a nodule specific form of the enzyme referred to as GSn1 (Lara *et al.* 1983). This component may therefore be classified as a nodulin. Glutamine synthetase has been purified and characterized from nodules of several legumes (see Miflin & Cullimore 1984). The enzyme from soybean-nodule cytosol has a relative molecular mass (M_r) of about 380000 and is composed of eight subunits arranged in two sets of planar tetramers (McParland *et al.* 1976). On the basis of the high affinity of glutamine synthetase for NH_4^+ and other evidence summarized above, this enzyme clearly plays a key role in the initial step of NH_4^+ utilization by the host legume.

NADH glutamate synthase has been detected in nodule extracts from several legumes (see Miflin & Cullimore 1984) and is likely to be associated with the plastids of nodules (Awonaike *et al.* 1981). Investigations with lupin and soybean have shown that activity of nodule extracts increased up to 10-fold during nodule development; this result suggests an important biological role in the N_2-fixation process (Robertson *et al.* 1975; Reynolds *et al.* 1982*b*). Boland & Benny (1977) have purified the NADH glutamate synthase from nodules of lupin and report that the homogenous enzyme had an M_r of 235000 and contained two molecules of bound flavin per molecule of enzyme.

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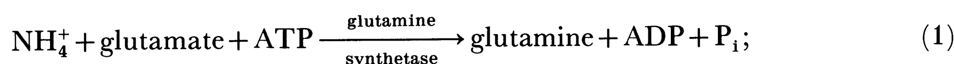
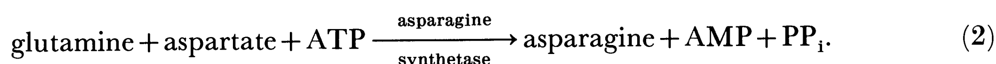
Synthesis and export of amides

Pate & Atkins (1983) and Schubert (1986) have compiled lists of primary nitrogenous compounds that are exported from a large group of legumes. Most legumes from temperate regions, of which *Lupinus* and *Pisum* are typical, export asparagine and glutamine as major products. The glutamine synthetase reaction may be coupled with the asparagine synthetase reaction producing asparagine as a product:



$$NH_4^+ + \text{glutamate} + \text{ATP} \xrightarrow[\text{synthetase}]{\text{glutamine}} \text{glutamine} + \text{ADP} + P_i; \quad (1)$$



$$\text{glutamine} + \text{aspartate} + \text{ATP} \xrightarrow[\text{synthetase}]{\text{asparagine}} \text{asparagine} + \text{AMP} + PP_i. \quad (2)$$

Aspartate, which is a substrate for asparagine synthesis, may be supplied by the aspartate aminotransaminase reaction, in which oxalacetate and glutamate react yielding aspartate and α-ketoglutarate. A scheme showing the interaction of the glutamine synthetase–glutamate synthase cycle with the glutamine synthetase–asparagine synthetase-coupled reactions has been presented by Scott *et al.* (1976).

Relatively little is known about the molecular biology of the reactions of the host legume that are involved in the assimilation of NH_4^+ (Miflin & Cullimore 1984). Although GSn1 of *Phaseolus*, aspartate aminotransferase, uricase and xanthine dehydrogenase have been identified as nodulins (Verma & Nadler 1984), little is known about the regulation of the synthesis of these enzymes at the gene level. Cullimore & Miflin (1983) have shown that the GS mRNA content of *Phaseolus* nodules is higher than that of roots at the period when the rise in GSn1 activity was greatest. This is interpreted to mean that the change in activity of the GSn1 is due to changes at the transcriptional level.

Export and assimilation of ureides

Many legumes of tropical and subtropical origin, such as *Glycine*, *Phaseolus* and *Vigna*, transport a major part of their fixed nitrogen from nodules to other plant parts as the ureides allantoin and allantoic acid (see Thomas & Schrader 1981; Schubert 1986). The apparent advantage in transporting ureides is that these compounds have a higher nitrogen : carbon ratio (4:4) than amides such as asparagine (2:4) or glutamine (2:5) and, as a consequence, use the carbon resources available in the nodule (Sprent 1984) more efficiently.

The synthesis of ureides proceeds in nodules via *de novo* synthesis and aerobic degradation of purines (Reynolds *et al.* 1982*a*) (table 1). This pathway has been extensively investigated and many of the enzymes involved have been purified and characterized (see Miflin & Cullimore 1984). The enzymes uricase and xanthine dehydrogenase are not generally present in roots and are good examples of nodulins (Verma & Nadler 1984).

Ureides are transported in the xylem from the nodules to leaves and are subsequently degraded, releasing nitrogenous components that may be incorporated into proteins (table 1). Until recently, the evidence suggested that the first reaction of ureide degradation involved allantoicase. This reaction eventually leads to the formation of urea and glyoxylic acid. An alternative pathway has been advocated in which allantoate is degraded by allantoate amidohydrolase (Winkler *et al.* 1985). After further reactions, this also leads to the formation of urea and glyoxylic acid. However, two of the four nitrogen atoms originally present in each

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Table 1. A simplified pathway for nitrogen metabolism in legumes that transport ureides

(Details of ureide metabolism are discussed by Schubert (1986).)

location	chemical form	process	enzymes(s)
	N_2		
nodule bacteroids	↓	nitrogen fixation	nitrogenase
	NH_4^+		
nodule plant cells	↓	purine synthesis	many
	xanthine		
nodule plant cells	↓	purine degradation	xanthine dehydrogenase
	uric acid		
nodule plant cells	↓	purine degradation	uricase
	allantoin and allantoic acid		
xylem	↓	transport	
	allantoin and allantoic acid		
leaves	↓	ureide degradation	(see text)
	urea + glyoxylate[1]		
leaves	↓	urea hydrolysis	urease
	$NH_4^+ + CO_2$		

[1] NH_4^+ and CO_2 also may be formed as direct products of allantoic acid degradation (Winkler *et al.* 1985).

allantoin molecule are released directly as NH_4^+. Details of these pathways have been discussed by Schubert (1986).

The assimilation of urea derived from ureides requires urease for hydrolysis to NH_4^+ and CO_2. This reaction has recently received attention because nickel is essential for urease activity. Leaves of nickel-deficient soybean plants have greatly reduced urease activity (Klucas *et al.* 1983) and may develop necrotic lesions owing to the accumulation of toxic concentration of urea (Eskew *et al.* 1983). Recent evidence indicates that nickel may have a practical role in soil fertility. Soils differ considerably in their indigenous nickel content and some may provide insufficient nickel to meet biological demands. Soils with pH values near or above neutrality may be susceptible to nickel deficiency because the availability of nickel decreases strikingly as pH values increase above 6.0 (Dalton *et al.* 1985). The addition of nickel salts to soils with low nickel contents has resulted in increased activities of urease in soybean leaves and of soil microorganisms (Dalton *et al.* 1985). The precise metabolic roles of urea and urease in plants are not clear, but are likely to involve the catabolism of proteins and ureides, the products of which are reutilized for new protein synthesis.

4. Oxygen metabolism in nodules

The utilization of O_2 in N_2-fixing organisms must be accurately regulated because O_2 is required for ATP synthesis by bacteroids and plant host cells, yet nitrogenase is rapidly inactivated by O_2. Nitrogen fixation and respiration in detached legume nodules can be markedly increased by O_2 partial pressures above atmospheric levels (Bergersen 1982). Maximum nitrogenase activity occurs at O_2 concentrations of 40–50%; higher concentrations lead to inactivation of nitrogenase. This suggests that N_2 fixation in nodules may be limited by O_2, but this interpretation has been questioned recently (see Minchin *et al.* 1985). O_2 access

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to the interior of nodules is controlled by a variable diffusion barrier in the nodule periphery. This barrier is sensitive to environmental changes and may limit the penetration of gases into tissues, resulting in an O_2 stress. The increases in nitrogenase activity in detached nodules exposed to high concentrations of O_2 may be a consequence of increased diffusion resistance in response to detachment. Arguments against O_2 limitations have been provided by experiments in which soybean and pea plants were grown at O_2 concentrations of 10, 21 and 30 %. Total dry mass and total amounts of N_2 fixed were not affected by experimental variations of the O_2 content of the atmosphere (Minchin *et al.* 1985). At present, knowledge of the possible O_2 limitation of N_2 fixation in nodules is limited and further research is needed.

Role of leghaemoglobin

Leghaemoglobin has a vital function in nodule oxygen relations. This protein has a high affinity for O_2 and facilitates the diffusion of bound O_2 to the respiring bacteroids, where the free-O_2 tension is maintained at a level that does not inactivate nitrogenase (see Appleby 1984; Bergersen 1982). The synthesis of leghaemoglobin represents a remarkable degree of specialization in the symbiotic association. The protohaem moiety has been reported to be synthesized by the *Rhizobium* symbiont and the apoprotein by the plant host. However, Dilworth & Glenn (1984) state that the 'evidence that heme synthesis is uniquely a bacteroid property is not unequivocal'. The soybean leghaemoglobin genes are encoded in the plant genome as a small family of four genes that are responsible for the four major species of leghaemoglobin (see Verma & Nadler 1984). These genes have been characterized and found to have remarkable structural similarity to animal globin genes. Infection by *Rhizobium* leads to activation of these genes and a dramatic increase in their transcription (see Appleby 1984; Bojsen *et al.* 1985).

Protection from H_2O_2 *and oxygen free radicals*

All aerobic organisms are exposed to products of O_2 metabolism including H_2O_2, superoxide radical ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$) (Fridovich 1976). Diazotrophs may be particularly vulnerable to these reactive components because the strong reducing conditions that are required for N_2 fixation may also lead to the formation of free radicals. $O_2^{\cdot-}$ is generated by reactions involving ferredoxin, haemoglobin and hydrogenase (Fridovich 1979; Schneider & Schlegel 1981), all of which occur in legume nodules. The usual defence against $O_2^{\cdot-}$ involves superoxide dismutase, a ubiquitous enzyme in aerobic organisms, including nodules, which catalyses the conversion of $O_2^{\cdot-}$ to H_2O_2. The H_2O_2 from this reaction may also be damaging and must be removed from tissues by reactions catalysed by either peroxidase or catalase (Puppo *et al.* 1982). The low affinity of the latter enzyme for H_2O_2 raises doubts about its physiological role in peroxide disposition (Dalton *et al.* 1986).

In nodules, H_2O_2 is converted to H_2O in a series of coupled oxidation–reduction reactions in which ascorbate and glutathione are major reactants (Dalton *et al.* 1986) (scheme 1). The dehydroascorbate resulting from peroxidation is reduced back to ascorbate by glutathione and oxidized glutathione is recycled to reduced glutathione in a reaction with NADPH. The enzymes involved are ascorbate peroxidase, dehydroascorbate reductase, and glutathione reductase. A similar system for peroxide scavenging also occurs in chloroplasts (Nakana & Asada 1981) and cyanobacteria (Tozum & Gallon 1979). During the time course of early nodule development, ascorbate peroxidase and dehydroascorbate reductase activities, and

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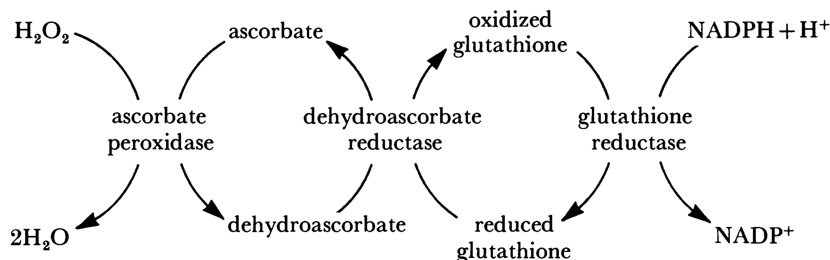
total glutathione contents of nodules, rise strikingly. These increases are significantly correlated with increases in acetylene reduction rates and nodule leghaemoglobin contents, suggesting that rises in N_2 fixation rates may require an increased capacity for peroxide scavenging.



SCHEME 1. Coupled reactions involved in H_2O_2 scavenging in nodules.

The enzymes for removal of peroxides have been detected in nodules from several legumes and red alder (table 2). Research in progress in our laboratory has shown that the ascorbate peroxidase from nodules of soybean is a haemprotein with a relative molecular mass (M_r) of 30000 as measured by SDS gel electrophoresis. The enzyme may be the same as, or similar to, the peroxidase described by Puppo *et al.* (1980). The soybean nodule peroxidase exhibits activity with non-physiological reductants, such as pyrogallol and guaiacol, but is inactive with electron donors such as NADPH, NADH and glutathione. Enzyme activity is strongly inhibited by C_2H_2. Because soybean nodules contain about 2.1 mM ascorbate (Dalton *et al.* 1986), it seems highly probable that this compound is the natural substrate for the soybean nodule peroxidase.

TABLE 2. ACTIVITIES OF ENZYMES INVOLVED WITH PEROXIDE SCAVENGING IN ROOT NODULES[1]

plant species	enzyme specific activities[2] nmol $min^{-1} mg^{-1}$ protein: ascorbate peroxidase	dehydroascorbate reductase
legumes		
Glycine max	284 ± 46	92 ± 14
Arachis hypogea	366 ± 25	68 ± 9
Pisum sativum	155 ± 35	54 ± 7
Vigna unguiculata	258 ± 27	39 ± 6
Medicago sativa	110 ± 11	79 ± 5
Vicia sariva	198 ± 6	64 ± 12
actinorhizal plants		
Alnus rubra	4390 ± 460	132 ± 5

[1] The data presented are from Dalton *et al.* (1987).
[2] Data presented are means of assays of extracts from three replicate nodule samples ± 1 s.e.m. Activity units are based on the change in A_{265} due to ascorbate oxidation (ascorbate peroxidase) or to production of ascorbate from dehydroascorbate (dehydroascorbate reductase).

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5. Involvement of dihydrogen

Dihydrogen evolution and recycling

Dihydrogen evolution is an indispensable part of the nitrogenase-catalysed reaction (see Eisbrenner & Evans 1983). Direct evidence that H_2 evolution during N_2 fixation was catalysed by nitrogenase was obtained by Bulen & LeCompte (1966). When N_2 was placed over reactions, about 25% of the nitrogenase electron flux was utilized in the evolution of H_2. Similar results were obtained from a reaction containing nitrogenase from nodule bacteroids (Koch *et al.* 1967). Fifty atmospheres† of N_2 over a nitrogenase reaction failed to prevent H_2 evolution (Simpson & Burris 1984).

Some strains of several species of *Rhizobium* form nodules on hosts that do not evolve H_2 into the atmosphere, but surveys of nodules from a large number of legumes indicate that the majority of them evolve H_2 during N_2 fixation (Schubert & Evans 1976). Nodules that do not evolve H_2 recycle H_2 generated by the nitrogenase system by a process first explained by Dixon (1972). *Rhizobium* bacteroids with H_2-recycling capacity synthesize enzymes necessary for activation of H_2 and transfer of electrons to O_2 via the bacteroid electron-transport chain (see Eisbrenner & Evans 1983). Homogeneous preparations of hydrogenase from *B. japonicum* are composed of two subunits with relative molecular masses of about 60000 and 30000 (Harker *et al.* 1984; Arp 1985). The purified enzyme contains nickel (Harker *et al.* 1984; Stults *et al.* 1984; Arp 1985), a fact that explains the demonstration by Klucas *et al.* (1983) that nickel is essential for chemolithotrophic growth of and hydrogenase expression by *B. japonicum*. The proximal electron acceptor for *Rhizobium* hydrogenase has still not been identified.

Proposed advantages and disadvantages of H_2 *recycling*

Potential advantages of efficient H_2 recycling to N_2-fixing organisms were listed by Dixon (1972) and have been discussed more recently by Eisbrenner & Evans (1983) and Brewin (1984). Possible disadvantages also need to be considered. H_2 may serve as a respiratory substrate for support of the synthesis of ATP for use in N_2 fixation and other processes. Convincing evidence that H_2, at least in several strains of *Rhizobium*, may function in this capacity has been obtained by Emerich *et al.* (1979) and Nelson & Salminen (1982). Although provision of H_2 or other substrates to isolated bacteroids depleted dissolved O_2 and protected nitrogenase from O_2 inactivation (Emerich *et al.* 1979), extension of this proposed advantage to intact nodules perhaps may not be justified if it is accepted that N_2 fixation in legume nodules in short-term experiments is limited by O_2 (Drevon *et al.* 1982; Minchin *et al.* 1985). Drevon *et al.* (1985) proposed that the H_2 uptake capability may be a disadvantage for legumes grown in solution culture because O_2 may limit N_2 fixation. Furthermore, the P:O ratio during utilization of H_2 may be less than the ratio when succinate is utilized as the carbon substrate (Stam *et al.* 1984). However, increasing the partial pressure of O_2 over legumes in long-term trials has not resulted in increased N_2 fixation and growth. The ratio of fixed N to CO_2 respired in nodules has been shown to be higher in Hup^+ strains, indicating a conservation of carbon substrates associated with efficient H_2 utilization (Drevon *et al.* 1982; Rainbird *et al.* 1983). This may explain why root masses of *Vigna unguiculata* inoculated with a Hup^+ strain of *Rhizobium* were greater than comparable masses of plants inoculated with a Hup^- strain (Rainbird *et al.*

† 1 atm = 101325 Pa.

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1983). Furthermore, provision of H_2 to *Rhizobium* ORS571 growing in a succinate-limited medium increased, by 27–35 %, the molar growth yield based on succinate (Stam *et al.* 1984).

An additional proposed advantage provided by H_2 recycling is that the removal of H_2 from the site of N_2 fixation might prevent H_2 inhibition of nitrogenase. Data supporting this argument have been presented by Dixon & Blunden (1983) but this hypothesis is apparently no longer acceptable to R. O. D. Dixon (personal communication, 1986). Minamisawa *et al.* (1983) have observed that the rate of transport of products of N_2 fixation from nodules to the host xylem was considerably greater in plants nodulated by Hup^+ strains than those inoculated with Hup^- strains, but a plausible explanation for these effects is not apparent. The capacity of several strains of *B. japonicum* to grow chemolithotrophically using H_2 and CO_2 as sources of energy and carbon, respectively, may be advantageous for growth and survival of *Rhizobium* in soil (Lambert *et al.* 1985*b*). Although there is considerable evidence indicating advantages of H_2 recycling, the symbiotic association is so complex that the effects of H_2 recycling must be evaluated in legume growth trials where inoculants with and without H_2-recycling capacity may be compared in carefully designed long-term experiments.

Results of growth trials

A summary (Eisbrenner & Evans 1983) of experiments in which Hup^+ and Hup^- strains of *Rhizobium* were compared as inoculants for legumes revealed that 9 of 13 trials produced significant increases in the total N content of plants from use of Hup^+ inoculants. In most of these experiments, Leonard jars, or similar relatively small culture vessels, were used for growth of the inoculated legumes. More recently Cunningham *et al.* (1985) have compared a Hup^+ inoculant strain of *R. leguminosarum* with a Hup^- mutant of this species that was obtained by Tn5–mob mutagenesis. In these tests *Pisum sativum* and *Vicia bengalensis* were grown in Leonard-jar assemblies under several environmental conditions. It was concluded that the H_2-recycling capacity of the nodules resulted in no significant effect on plant growth. However, the strain of *R. leguminosarum* from which the *hup* determinants were originally derived in these experiments had been reported to couple H_2 oxidation inefficiently to ATP synthesis. Behki *et al.* (1985) also reported that the transfer of a plasmid, pIJ1008, carrying determinants for Hup expression, into *R. meliloti* resulted in increased H_2 uptake rates of *Medicago sativa* nodules but had no effect on the yield or N content of this legume. In these experiments the acquisition of the determinants for Hup by the *R. meliloti* inoculant produced nodule bacteroids which failed to recycle a major part of the H_2 produced during N_2 fixation. We have argued (Lambert *et al.* 1985*b*) that these and several other experiments have not given sufficient attention to such factors as: (i) comparison of stable inoculant strains that are isogenic with exception of the Hup characteristic; (ii) use of a Hup^+ strain which forms nodules that recycle essentially all H_2 produced from N_2 fixation and which are known to couple H_2 oxidation efficiently to ATP synthesis; and (iii) use of cultural conditions that will allow the legume to grow to maturity, thus providing the opportunity for compounding any advantage of H_2 recycling during logarithmic phase of growth.

We have conducted an experiment in which Hup^+ and Hup^- *B. japonicum* strains, which were isogenic with exception of Hup, were compared as soybean inoculants in large tiles filled with a *B. japonicum*-free mixture of sand and peat (Evans *et al.* 1985). After 90 days of growth nodules formed by the Hup^+ inoculant recycled essentially all H_2 from the N_2 fixing process. In contrast

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little or no H_2-recycling capacity of nodules formed by the Hup^- inoculant could be demonstrated. At harvest, after 141 days of growth, the mean percentage increases in N content of plant material from ten replicate plots receiving the Hup^+ inoculant when compared with those receiving the Hup^- inoculant were: seed, 8.6; leaves, 27; total plants, 11. These differences were statistically significant at $p \leqslant 0.03$. We believe that this experiment provides a realistic evaluation of the advantage of H_2 recycling to symbiotically grown legumes. Obviously, more experiments need to be conducted in which additional strains of *Rhizobium* and species of legume are evaluated.

6. Genetics of H_2 recycling system

The majority of evidence supports the view that H_2-recycling capability is beneficial to the legume–*Rhizobium* symbiosis. Consequently, investigations have been directed toward the isolation of H_2 uptake (*hup*) genes from a strain with an efficient H_2-recycling system. It is hoped that transfer of *hup* genes into Hup^- rhizobia may contribute toward the improvement of the efficiency of energy utilization and yields of nodulated legumes.

Isolation of genes for the uptake hydrogenase system

The genetic determinants for H_2 oxidation are plasmid-encoded in *R. leguminosarum* 128C53. However, the cotransfer of Hup activity and nodulation ability between strains could be accomplished only after recombination between the Hup/Nod plasmid, pRL6JI, of *R. leguminosarum* 128C53 and a transmissible plasmid derived from another strain of *R. leguminosarum* (Brewin *et al.* 1980). Subsequently, Behki *et al.* (1985) have transferred the recombinant nodulation plasmid, pIJ1008, of *R. leguminosarum* (Brewin *et al.* 1982) into *R. meliloti*. When the *R. meliloti* recipients were used as inoculants they conferred increased H_2 uptake activity on alfalfa nodules. Plasmid location of *hup* genes in five Hup^+ *R. leguminosarum* strains (Ruiz-Argueso *et al.* 1985*a*) and the mobilization of such plasmids modified by Tn5–mob insertion between strains (Ruiz-Argueso *et al.* 1985*b*) also have been reported. Preliminary evidence is available concerning the cloning of *hup* genes from *R. leguminosarum* B10 (Tichy *et al.* 1985). Contrary to the plasmid location of *hup* genes in the fast-growing *Rhizobium* species, several Hup^+ strains of *B. japonicum* showed no detectable plasmid DNA (Cantrell *et al.* 1982).

Cantrell *et al.* (1983) constructed a gene bank of the *B. japonicum* 122DES DNA in a cosmid vector, pLAFR1, and identified eleven Hup-complementing cosmids by transconjugating the gene bank *en masse* into the Hup^- *B. japonicum* mutant PJ17 and subsequent screening for Hup^+ transconjugants by the rapid colony-screening procedure of Haugland *et al.* (1983). Further characterization of one of the Hup-complementing cosmids, pHU1, was accomplished by generating transposon Tn5 insertions into the *B. japonicum* insert DNA (Haugland *et al.* 1984). Phenotypes conferred by these insertion mutations were scored after marker exchange of the individual Tn5 insertions into the *B. japonicum* USDA 122DES chromosome. It was concluded that the Hup-complementing sequences were located on a 16 kb insert DNA of pHU1 and were clustered into at least two transcriptional units.

The plasmid pHU1, however, did not confer Hup activity on all Hup^- mutants (Haugland *et al.* 1984). Furthermore, transfer of pHU1 into Hup^- wild-type *B. japonicum* and *R. meliloti* did not confer Hup activity in the free-living state, suggesting that additional *hup* specific DNA

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remained to be isolated. Consequently, Lambert *et al.* (1985*a*) isolated an additional Hup-complementing cosmid, pHU52, from the gene bank by using one of the Tn5 generated Hup$^-$ mutants (Tn5 insertion mutant 2) as the recipient (scheme 1). The *B. japonicum* insert DNA in pHU52 was similar to that in pHU1, but contained an additional 5.5 kb *Eco*RI fragment at the right-hand end (figure 1). Interestingly, the cosmid pHU52 conferred Hup activity on all Hup$^-$ *B. japonicum* mutants (except one dominant mutant) and also on Hup$^-$ wild-type strains of *B. japonicum*, *R. meliloti*, *R. leguminosarum* and *R. trifolii* when cultured under free-living conditions. Acquisition of pHU52 in addition enabled these recipient strains to grow chemoautotrophically on H_2 and CO_2; this result provides strong evidence for coupling of H_2 oxidation to ATP formation.

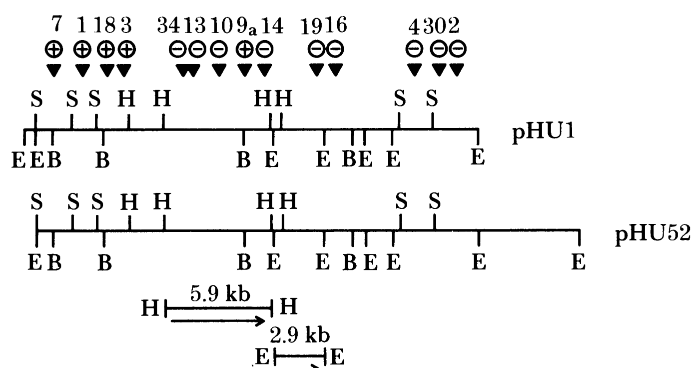

FIGURE 1. Restriction maps of pHU1 and pHU52. The trangles indicate positions of Tn5 insertions. Positions of insertions are numbered, and the + or − symbols below each number indicate whether that insertion resulted in a Hup$^+$ or Hup$^-$ phenotype in a complementation test (see Haugland *et al.* 1984). B, E, S and H are the restriction sites recognized by the enzymes *Bgl*II, *Eco*RI, *Sma*I and *Hin*dIII, respectively. The 5.9 kb *Hin*dIII and 2.9 kb *Eco*RI fragments contain the structural genes for the 60 kDa and 30 kDa hydrogenase polypeptide components, respectively (Zuber *et al.* 1986). Arrows indicate the orientation of respective genes. The Hup activity of the mutant resulting from transposon insertion 9 was weak.

Recently, recombinant cosmids which complement the Nif–Hup mutants for both nitrogenase and hydrogenase activities have been isolated from a *B. japonicum I*-110 gene bank (Hom *et al.* 1985). Because these cosmids also complement some Hup$^-$ mutants, the authors conclude that they contain a *nif/hup* gene and at least one other *hup* gene. The predominant *Eco*RI restriction pattern of these cosmids is similar to that of pHU1 (Cantrell *et al.* 1983) although confirmation of genetic similarity requires DNA hybridization between the two sets of clones.

Identification of uptake hydrogenase structural genes

Despite the isolation of a cosmid pHU52, apparently encoding all essential Hup determinants, direct evidence for the presence of uptake hydrogenase structural genes on pHU52 has been lacking. A recent finding that conjugal transfer of pHU52 into Hup$^-$ rhizobia conferred the ability to synthesize both the 60 kDa and 30 kDa polypeptide components of the uptake hydrogenase (Harker *et al.* 1985) suggested that the determinants for hydrogenase subunit synthesis were in pHU52. Recently, Zuber *et al.* (1986) have constructed subclones from the insert DNA of pHU52 in a plasmid expression vector, pMZ545. Two subclones were identified, which directed the synthesis of 60 kDa and 30 kDa proteins, respectively, in *Escherichia coli*

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'maxicells'. Furthermore, the DNA inserts from these subclones expressed immunologically cross-reactive hydrogenase polypeptides in *E. coli* when they were transcribed from the lambda P_L promoter. These results have provided direct evidence for the presence of the structural genes for uptake hydrogenase on pHU52.

Symbiotic expression of hup genes

Although the cosmid pHU52 confers Hup activity and autotrophic growth capability on Hup⁻ wild-type *Rhizobium* species as determined in the free-living state (Lambert *et al.* 1985*a*), the most important question from the viewpoint of agricultural application is whether the transferred genes are expressed in nodules. The levels of Hup activity also are known to vary with the plant host (Keyser *et al.* 1982; Bedmar *et al.* 1983). It was necessary, therefore, to determine whether Hup activity encoded by pHU52 could be expressed in nodules of soybean, alfalfa and clover. Lambert *et al.* (1985*b*) detected low levels of Hup activity in nodules formed by *B. japonicum* USDA 138 (pHU52), *R. meliloti* 102F28 (pHU52) and *R. trifolii* SU794 (pHU52). These low activities were consistent with the relative instability of the pHU52 in nodule bacteroids. When *B. japonicum* and *R. meliloti* that had acquired pHU1 were used as inoculants for soybean and alfalfa, respectively, Hup activity was detected in nodule bacteroids. However, pHU1 failed to confer Hup activity on *B. japonicum* or *R. meliloti* in the free-living state. Based on the physical maps of pHU1 and pHU52, G. R. Lambert *et al.* (unpublished results, 1986) have suggested that a 5.5 kb *Eco*RI fragment unique to pHU52 (figure 1) contains a gene or part of a gene required for Hup activity in free-living bacteria but not in nodules. Although *hup* genes may be transferred within and among species of *Rhizobium* by use of cosmids pHU1 and pHU52, in the absence of selection pressure these cosmids are unstable. Further research is necessary, therefore, to incorporate cosmid-borne genetic determinants for Hup into the bacterial chromosome.

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Discussion

H. Bothe (*Botanisches Institut, Universität Köln, F.R.G.*). If the peroxide-scavenging oxidative mechanism has a role in nodule tissue, ascorbate must be present in substrate levels of concentration. What is the *in vivo* concentration of ascorbate in nodule tissue?

H. J. Evans. About 2 mm.

J. Döbereiner (*EMBRAPA-UAPNBS, Seropédica, Rio de Janeiro, Brazil*). Has Professor Evans ever considered the possibility of a link between the requirements for nickel of the uptake hydrogenase system and of the urease in leaves, to which he referred in his paper? We have obtained highly significant correlations between the relative efficiencies (Hup activity) in soybean and bean nodules and the relative ureide concentrations (ureide nitrogen as a percentage of the total nitrogen) in the bleeding sap of these legumes.

H. J. Evans. These are very interesting observations but I am unable to comment further.

M. G. Yates. (*AFRC Unit of Nitrogen Fixation, University of Sussex, U.K.*). Could Professor Evans or someone else comment on the nature of the effective limitation or limitations placed on nitrogen fixation rates in legume nodules?

F. R. Minchin (*Animal and Grassland Research Institute, Hurley, Maidenhead, U.K.*). There is no definitive evidence which allows determination of whether legume nodules are limited by availability of carbon substrates or of oxygen. The buildup of carbohydrates in bacteroids suggests that oxygen is limiting, at least for some of the time.

Janet I. Sprent (*Department of Biological Sciences, Dundee University, U.K.*). Does the NADPH used in disposal of peroxide use a significant amount of a nodule's energy supply?

H. J. Evans. To answer this question, one would need to know what portion of respiratory O_2 uptake by nodules is supported by reduced pyridine nucleotides under *in vivo* conditions. Also, one would need to know the rates of peroxide-dependent reduced pyridine nucleotide oxidation in nodules under *in vivo* conditions. We have no estimates of these parameters and, therefore, have no way of determining whether peroxide-dependent oxidation of reduced pyridine nucleotides is a significant portion of the nodule's energy supply.