The symbiosis between *Rhizobium leguminosarum* and *Pisum sativum:* regulation of the nitrogenase activity

Promotor: dr. C. Veeger,
hoogleraar in de biochemie
Co-promotor: dr. H. B. C. M. Haaker,
universitair hoofddocent

M. A. Appels

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STELLINGEN

1. Bij de beschrijving van de curve van de nitrogenase activiteit in bacteroïden in afhankelijkheid van de partiële zuurstofspanning als 'bell-shaped' is geen rekening gehouden met de inactivatie van de nitrogenase door hoge zuurstof concentraties.

   - Walker, C.C., Partridge, C.D.P., Yates, M.G.(1981) J. Gen. Microbiol. 124, 317-327
   - Haaker, H., Wassink, H.(1984) Eur. J. Biochem. 142, 37-42

2. Het op de voorhand vaststellen van de functionaliteit van een metabolische route op grond van de aanwezigheid van de betrokken enzymactiviteiten, moet worden afgewezen.

   - Kacser, H., Porteous, J.W.(1987) TIBS 12, 5-12

3. T.a.v. de volgorde van methylering van de 5'-terminale cap GpppA van vesicular stomatitis virus mRNA's hebben en Testa & Banerjee (GpppA - $GpppA^m$ - 7^mGpppA^m) en Moyer (GpppA - 7^mGpppA - 7^mGpppA^m) foute conclusies getrokken.

   - Testa, D., Banerjee, A.K.(1977) J. Virol. 24, 786-793
   - Moyer, S.A.(1981) Virology 112, 157-168

4. De bewering van Bell en Wheeler dat het werkingsmechanisme van het fungicide tricyclazole tegen Pyricularia oryzae berust op remming van de melanine biosynthese en derhalve de vorming van functionele appressoria verhindert, gaat voorbij aan de directe en indirecte effecten van tricyclazole op de waardplant.

   - Bell, A.A., Wheeler, M.H.(1986) Ann. Rev. Phytopathol. 24, 411-451

5. De conclusie van De Wit et al. dat het door hun geïsoleerd eiwit de specifieke elicitor vormt, welke necrose induceert in resistente tomaten cultivars na infectie met incompatibele Cladosporum fulvum fysio's, is onvoldoende gefundeerd.

   - de Wit, P.J.G.M., Hofman, A.E., Velthuis, G.C.M., Kluč, J.A. (1985) Plant Physiol. 77, 642-647

6. Het functioneren van P-glycoproteine in cellulaire transportprocessen zoals waargenomen in hogere eukaryoten, kan de uitscheiding van ergosterol-biosynthese remmers door schimmelspecies als beschreven door De Waard en Van Nistelrooy, verklaren.

   - de Waard, M.A., van Nistelrooy, J.G.M.(1988) Pestic. Sci. 22, 371-382

7. Gezien het feit dat het gewapend handelen van groeperingen als terrorisme danwel als vrijheidsstrijd aangeduid wordt, afhankelijk van de politieke inzichten van de waarnemer, verdient het aanbeveling de woorden terrorisme en vrijheidsstrijd als synoniemen in het woordenboek op te nemen.

8. Oorlogsmisdaden schijnen - per definitie - alleen door de overwonnen partij te zijn gepleegd.

9. De criteria gehanteerd door de Nederlandse regering in 1982 bij het eenzijdig opschorten van het ontwikkelingssamenwerkingsverdrag tussen Nederland en Suriname, worden niet gebruikt bij het toetsen van ontwikkelingssamenwerkingsverdragen met andere landen, hetgeen op politiek en ambtelijk onbehoorlijk bestuur duidt.

10. Wanneer de verkiezingsstrijd in een Westerse democratie vergeleken wordt met de commerciële reclame, kan geconcludeerd worden dat het proces van politieke meningsvorming identiek is met het aan de persoon brengen van goederen.

11. De waarneming dat de mens in de kapitalistische democratie steeds meer tot instrument ten dienste van economische doelen degradeert, maakt een fundamentele reorganisatie noodzakelijk om tot een samenleving te komen waarin menselijke solidariteit, redelijkheid en creativiteit niet afgeremd doch bevorderd worden.

Stellingen behorend bij het proefschrift van Michiel Appels, Wageningen, 17 november 1989.

VOORWOORD

Dit proefschrift beschrijft het onderzoek, dat ik in de periode oktober 1984 tot april 1989 met onderbrekingen heb uitgevoerd bij de vakgroep Biochemie van de Landbouwuniversiteit Wageningen. Het proefschrift omvat alleen de met positief resultaat afgesloten deelonderzoeken.

Bij het onderzoek zijn verscheidene mensen betrokken geweest. Mijn dank wil ik uitspreken aan allen met wie ik heb samengewerkt. Twee mensen wil ik met name noemen, mijn co-promotor de heer Dr. H.B.C.M. Haaker en mijn promotor de heer Professor Dr. C. Veeger. De heer H. Haaker heeft in de afgelopen jaren als mijn leermeester gefunctioneerd. Hij heeft mij bewust gemaakt van de vele aspecten, die verbonden zijn aan het verrichten van onderzoek. Professor Veeger ben ik dankbaar voor de met hem gevoerde stimulerende gesprekken en voor het feit dat hij mij in de gelegenheid heeft gesteld ervaring met onderwijs geven op te doen. Verder dank ik iedereen op het laboratorium voor de sfeervolle tijd die ik daar heb mogen doorbrengen.

Michiel Appels
april 1989

CONTENTS

LIST OF ABBREVIATIONS

ADP	adenosine 5'-diphosphate
Ala	alanine
AMP	adenosine 5'-monophosphate
Asp	aspartic acid
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
Da	Dalton, i.e. one-twelfth of the mass of the pure nuclide ^{12}C
ε	elasticity coefficient
E_m	midpoint redox potential at pH 7.0 and 25 °C
E_h	observed redox potential of a system
EDTA	ethylenediaminetetraacetate
fix	fixation
FPLC	fast protein liquid chromatography
Glu	glutamate
GOT	glutamate oxaloacetate transaminase (EC 2.6.1.1)
hsn	host-specificity nodulation
K_{eq}	equilibrium constant
K_i	dissociation constant of both the enzyme-inhibitor and the enzyme-inhibitor-substrate complexes
K_{ii}	dissociation constant of the enzyme-inhibitor-substrate complex
K_{is}	dissociation constant of the enzyme-inhibitor complex
K_m	Michaelis-Menten constant
ME	malic enzyme (EC 1.1.1.39)
Mes	4-morpholineethanesulfonic acid
MDH	malate dehydrogenase (EC 1.1.1.37)
Mops	4-morpholinepropanesulfonic acid
M_r	relative molecular mass
NAD^+	oxidized nicotinamide-adenine dinucleotide
NADH	reduced nicotinamide-adenine dinucleotide
$NADP^+$	oxidized nicotinamide-adenine dinucleotide phosphate
NADPH	reduced nicotinamide-adenine dinucleotide phosphate
nif	nitrogen fixation
nod	nodulation
PEPC	phosphoenolpyruvate carboxylase (EC 4.1.1.31)
PK	pyruvate kinase (EC 2.7.1.40)
pI	iso-electric point
P_i	inorganic phosphate
Q	$R\text{-}CH_2N^+(CH_3)_3$
*m*RNA	messenger ribonucleic acid
SDS	sodium dodecyl sulfate
sym	symbiotic
Tes	2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino} ethanesulfonic acid
Tricine	N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-glycine
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
v	rate of reaction
V_{max}	rate of enzyme-catalysed reaction at infinite concentration of substrate(s)

1. INTRODUCTION

1.1 *Biological nitrogen fixation in perspective*

Nitrogen is an element of proteins, nucleic acids and many other biological molecules and therefore an indispensable element for life. In combined form, the element nitrogen can exist in different oxidation states varying from +5 (NO_3^-) to -3 (NH_3, NH_4^+). However nitrogen can only be incorporated into an organic compound in the oxidation state -3. In nature, both the oxidation of the compounds NH_4^+, NH_2OH, NO_2^- and the reduction of the compounds NO_3^-, NO_2^- takes place. The four biological processes involved in the reduction and oxidation of nitrogen in the biosphere are shown in Figure 1.

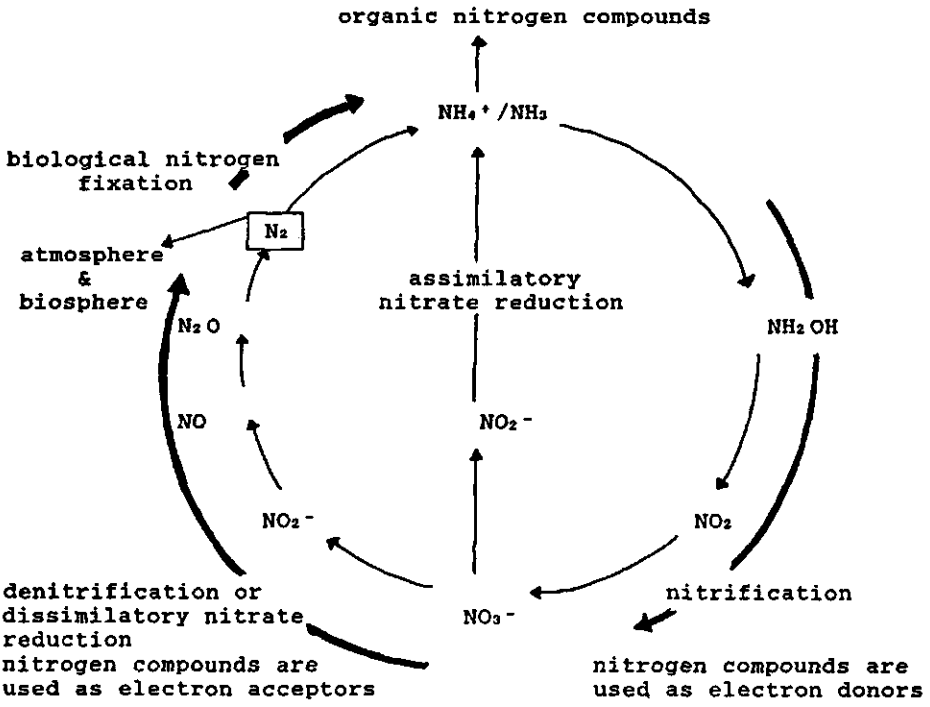

Figure 1. The place of biological nitrogen fixation within the interchange of nitrogen in the biosphere, after Postgate (1) with modification.

The oxidation of NH_3, NH_2OH and NO_2^- is known as nitrification. This common process in the soil is carried out by some obligate aerobic bacteria (*Nitrosomonas, Nitrobacter*). The oxidation results in the release of free energy. The compounds NH_3, NH_2OH and NO_2^- function as electron donors to an energy transducing electron transport chain in these bacteria.

The end product of the nitrification process - NO_3^- - is taken up by the plant roots. In the roots the oxidation state of nitrogen, +5, is changed to the usable state -3 (NH_3) by the action of nitrate reductase (EC 1.6.6.2) and nitrite reductase (EC 1.6.6.4). These enzymes are also found in bacteria. In roots, the necessary reducing power is provided at the expense of the oxidation of photosynthates. In leaves the reducing power can be generated directly by the light reactions of photosynthesis. This process is called the assimilatory nitrate reduction.

Besides the assimilatory nitrate reduction, there is the dissimilatory nitrate reduction or denitrification, which is carried out by many genera of soil bacteria. The more oxidized nitrogen compounds serve as electron sinks and can act as terminal electron acceptors for an anaerobic respiratory chain. The reduction of NO_3^- results ultimately in N_2 with the oxidation state 0. The N_2 formed is spread over the biosphere and the atmosphere.

In the fourth process called biological nitrogen fixation, N_2 is reduced to NH_3. This reduction can only be carried out by a limited number of prokaryotes, not by eukaryotic organisms.

Considering these four processes, biological nitrogen fixation is the most restricting process for life. Nitrogen is not only transferred between the various components of the biosphere, it is also lost to the atmosphere by denitrification as N_2 (Table 1).

Table 1. The contribution of biological nitrogen fixation to the nitrogen cycle of the biosphere. The values are from Burns and Hardy (2).

process	amount of nitrogen involved (10^6 metric tons per annum) output of/input into the biosphere
denitrification	210
combustion	20
volatilization	165
rainfall	200
biological nitrogen fixation	100 - 175
industrial nitrogen fixation	70

Fixation of the N_2 present in the atmosphere takes also place. By the action of lightning and ultraviolet irradiation, N_2 is oxidized to nitrogen oxides in the atmosphere. Rainfall carries the nitrogen back to the biosphere (Table 1). Biological nitrogen fixation is another important process for the input of inorganic nitrogen into the biosphere. It accounts for 30 - 40 % of the total input of nitrogen. On the land, biological nitrogen fixation accounts for about 60 % of the total input of nitrogen. Thus the biological nitrogen fixation forms an indispensable process for the nitrogen cycle on Earth. Without this process no higher state of life on Earth would have been developed.

1.2 *The place of Rhizobium bacteria among the nitrogen-fixing organisms*

The N_2-reducing organisms are generally divided into different groups using three criteria.

The first criterion is the type of relation with other organisms during diazotrophic growth. There are:

A free-living organisms which do not depend on other organisms to reduce N_2,
B organisms which are associated with plants, with little or no morphological adaption by either partner (so-called associative symbiosis),
C organisms which live in symbiosis with plants showing considerable biochemical and morphological adaptions in facilitating the symbiosis.

A second criterion is the carbon source used for growth (autotrophic or heterotrophic) and the energy source (phototrophic or chemotrophic).

A third point differentiating the N_2-reducing organisms is the dependency on O_2. They may be obligate anaerobic, facultative anaerobic (nitrogenase activity only under anaerobic or microaerobic conditions), microaerobic and obligate aerobic. Table 2 (see page 12) provides some examples of each category, showing the great diversity in N_2-reducing organisms.

The category symbiotic microaerobic N_2-reducing micro-organisms consists of three genera of the bacterium family of *Rhizobiaceae*. The three genera - *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* - can live in symbiosis with members of the plant families *Leguminosae* and *Ulmaceae*. The capacity of N_2 reduction by the *Rhizobia* and *Bradyrhizobia* is usually restricted to the symbiotic state. However N_2 reduction under microaerobic conditions by free-living *Rhizobia* has been reported (3). The *Azorhizobia* can grow aerobically diazotrophic. The contribution made by these symbiotic associations to N_2 fixation, is estimated to be a significant part (25-50 %) of the total terres-

trial biological N_2 fixation (2,4).

Table 2. Compendious survey of N_2-reducing organisms, after Postgate (1) with modifications.

category	genus	example
free-living, heterotrophic, anaerobic	*Clostridium*	*C. pasteurianum*
	Desulfovibrio	*D. vulgaris*
	Methanococcus	*M. thermolithotrophicus*
	Methanabacterium	*M. ivanovii*
	Methanosarcina	*M. berkeri*
free-living, photosynthetic, anaerobic	*Rhodospirillum*	*R. rubrum*
	Chromatium	*C. vinosum*
	Chlorobium	*C. limicola*
free-living, heterotrophic, facultative anaerobic/microaerobic	*Enterobacter*	*E. cloaceae*
	Klebsiella	*K. pneumoniae*
	Citrobacter	*C. freundii*
free-living, chemotrophic, facultative anaerobic/microaerobic	*Thiobacillus*	*T. ferroxidans*
	Xanthobacter	*X. autotrophicus*
free-living, photosynsynthetic, facultative anaerobic/microaerobic	*Rhodopseudomonas*	*R. capsulata*
	Chloroflexus	*C. auranticus*
free-living, heterotrophic, aerobic	*Azotobacter*	*A. vinelandii*
	Azotococcus	*A. agilis*
	Derxia	*D. gummosa*
free-living, photosynthetic, aerobic	*Anabaena*	*A. cylindrica*
	Nostoc	*N. muscorum*
	Gloeocapsa	*G. alpicola*
free-living, chemotrophic, aerobic	*Methylosinus*	*M. trichosporum*
	Methylococcus	*M. capsulatus*
associative, phototrophic, aerobic	*Frankia*	*F. alni*
	Nostoc	*N. punctiforme*
	Anabaena	*A. azollae*
associative, phototrophic, anaerobic	*Dermocarpa*	
	Xenococcus	
associative, phototrophic, microaerobic	*Azospirillum*	*A. lipoferum*
	Gloeocapsa	
symbiotic, microaerobic	*Rhizobium*	*R. leguminosarum*
	Bradyrhizobium	*B. japonicum*
	Azorhizobium	*A. caulinodans*

In free-living state the *Rhizobia* are rod-shaped bacteria (0.5 - 0.9 µm x 1.2 - 3.0 µm), having no endospores, being gram-negative, aerobic and motile. The motility is due to the presence of one or up to six flagella. *Rhizobia* are unique that they can induce structures on legumes, which are called root nodules. In these root nodules the bacteria differentiate into so-called bacteroids (see paragraph 1.3). There are two types of nodules, nodules formed on roots and nodules developed on stems.

The distinction made between the genera *Bradyrhizobium* and *Rhizobium* is mainly based upon their growth rates on yeast extracts and their capacity to utilize various carbon compounds. The genus *Rhizobium* consists of species which exhibit high growth rates on yeast extract and which can utilize many sugars, polyols and organic acids. The *Bradyrhizobia* are slow growers, which are more specialized in their requirements and commonly prefer pentoses. The genus *Rhizobium* comprises the four species *R. leguminosarum, R. meliloti, R. loti* and *R. fredii*. *R. leguminosarum* consists of the former three species *R. trifolii, R. phaesoli* and *R. leguminosarum*. The genus *Bradyrhizobium* comprises the species *B. japonicum* and includes all the bacteria referred as slow-growing *Rhizobia* (5,6). The common characteristic of *Azorhizobium* is their ability to form nodules on stems (7). The bacteria are further classified on the basis of host preferences. In Table 3 some examples are given. For details of the classification see (8).

Table 3. Some examples of symbiotic, microaerobic N_2-reducing bacteria and their host preferences.

Species	preferred host
Rhizobium leguminosarum	*Pisum* (Pea), *Vicia* (Vetch)
biovar *viciae*	*Lathyrus* (Vetchling), *Lens* (Lentil)
biovar *trifolii*	*Trifolium* (Clover)
biovar *phaesoli*	*Phaseolus vulgaris* (Beans)
R. meliloti	*Medicago* (Alfalfa), *Melilotus* *Trigonella* (Fenugreek)
R. loti	*Lupinus* (Lupin), *Lotus* (Bird's Foot Trefoil), Anthyllis (Kidney Vetch)
R. fredii	*Glycine max.* (Soybean), *Cowpea*
Bradyrhizobium japonicum	Glycine max (Soybean)
Bradyrhizobium spp.	
B. sp. (Vigna)	*Vigna* (Black-eye Pea)
B. sp. (Lupinus)	*Lupinus, Lotus pedunculatus*
Azorhizobium caulinodans	*Sesbania rostrata*

1.3 *Root nodule development in the legume-Rhizobium symbiosis*

1.3.1 *Morphology and physiology*

The formation of the nodule structure is the result of a number of interactions between the *Rhizobia* bacteria and the host plant. The nodule development can physiologically be distinguished in 4 stages:

- preinfection
- infection process and nodule formation
- nodule function
- nodule senescence.

In the preinfection stage, the free-living *Rhizobia* have to migrate to the rhizosphere of the host plant. The excretion of a variety of compounds by the roots leads to a chemotactic migration of the heterotrophic *Rhizobia* into the rhizosphere. After the migration, the recognition between the host and the bacteria has to take place. The recognition involves the transmission of mutual specific signals for the activation and transcription of genes involved in the symbiosis (9-15). The attachment of the bacteria to the plant is also a specific process which is part of the act of recognition (16,17). The recognition results in the development of meristematic centers in the root cortex and in the curling of the root hairs (18,19). Ultimately the curling of the root hairs causes entrapping of the bacteria.

The actual infection starts with the partial digestion of the root hair cell wall by the *Rhizobia* (20,21). The host cells react by the formation of tubular structures (so-called infection threads) enclosing the dividing bacteria. The infection threads develop into the direction of the induced meristematic centers in the root cortex and form branches. In contact with a meristematic centre, the *Rhizobia* are released into the cytoplasm of the plant cells (22). During the release, the bacteria become surrounded by the so-called peribacteroid membrane, which originates initially from the plant plasmalemma (23,24). The bacteria differentiate into the pleomorphic so-called bacteroids, in which the expression of the nitrogen-reducing enzyme complex is induced.

In Figure 2 (see page 15) a longitudinal representation of a functioning pea-*Rhizobium* root nodule is shown. Five zones can be distinguished, each of the zones represents a distinct stage of development. The most distal zone is the apical meristem. The so-called invasion zone is lying near the meristem and consists of enlarged cells, which may become infected by the *Rhizobia*. In the early symbiotic zone host cells are differentiating into infected and uninfected cells. This early symbiotic zone passes to the late symbiotic zone, in which the dinitrogen reduction

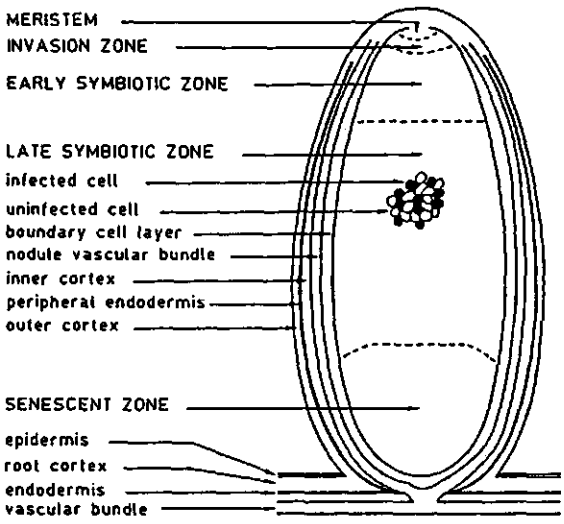

Figure 2. Longituadinal section of an differentiated pea root nodule, after Kijne (25) and Sutton (26).

and the assimilation of reduced nitrogen take place. This zone consist also of both infected and uninfected plant cells. The most proximal region in the nodule is the senescent zone, where degeneration of plant cells and bacteroids occur. The nodule cortex surrounds the central regions of the nodule. The nodule cortex is divided by a peripheral endodermis into an outer and an inner cortex. Vascular bundles are located in the inner cortex. The inner cortex is separated from the central regions of the nodule by a layer of distinct small uninfected cells, the so-called boundary cell layer.

The morphology of a root nodule is under control of both host plant and bacteria (27,28). Physiological and morphological details of nodule development have been reviewed in many papers (27,29-33). Many variations on nodule development exist.

1.3.2 *Genetic regulation*

The morphological and biochemical adaptions observed in the legume-*Rhizobium* symbiosis, are attributed to the genetic and biochemical properties of both organisms.

Bacteria of the genus *Rhizobium* possess chromosomes as well as plasmids. One of these plasmids, the so-called *Sym*-plasmid, carries the majority of genes involved in host-specificity (*hsn*), nodulation (*nod*) and nitrogen fixation (*fix/nif*). Several chromosomal genes and genes on other plasmids than the *Sym*-

plasmid, play a role in symbiosis as well, for instance genes encoding for the synthesis of exopolysaccharides. These genes are involved in the preinfection (14,16,17) and in the stage of infection and nodule development (34-36). Several *hsn* and *nod* genes on the *Sym*-plasmid have been identified (37-42). The organization and the regulation of *hsn* and *nod* genes from one species of *Rhizobium* exhibit great resemblances to counterparts in other species (43-45).

The organization and the regulation of the expression of *nod* genes have been studied in detail. The *nod* genes are not constitutively expressed with the exception of *nodD*. The *NodD* gene product is able to repress its own transcription (50). The expression of the other *nod* genes requires the presence of both a flavonoid excreted by the host plant and the *nodD* gene product (9-13). The composition of flavonoid substances in the rhizosphere and in the roots, differs among the legumes (11-13). There are also small differences in the *nodD* gene product of different *Rhizobium* species (13,46). These differences among legumes may result in a specific induction of *nod* genes (46-49). The induction by the *nodD* gene product and the flavonoid is not restricted to the *nod* genes only; the *hsn* genes seem to be coordinately expressed with the *nod* genes (51).

The rhizobial *nif* and *fix* genes are also specifically expressed during symbiosis. The corresponding gene products are directly involved in the process of N_2 reduction. *Nif* genes exhibit structural or functional analogy with the *nif* genes of *Klebsiella pneumoniae*; this in contrast with the *fix* genes (52-56). The *fix* genes are genes necessary for nitrogen fixation in obligate aerobes but are not present in *K. pneumoniae*. The *nif* genes in *K. pneumoniae* are positively regulated by the *nifA* gene product. In turn, the transcription of *nifA* and therefore the activation of genes by the *nifA* gene product, is regulated by the concentration of O_2, the concentration of reduced nitrogen and by the concentration of Mo (57-59). The organization and regulation of the *nif* genes in *Rhizobia* resembles the system in *K. pneumoniae* (60,61). However differences have been noticed (62-64). The major difference is that the expression of the *nif* and *fix* genes in *Rhizobia* is exclusively controlled by O_2 and not by fixed nitrogen (65,66). Limitation of O_2 is the major regulatory signal for the derepression of *nif* and *fix* genes. The microaerobic environment, which is created by the host plant, is thus necessary for nitrogen fixation.

The genetic properties of the host plant determine also the nodule development. The expression of plant genes in root nodules differs from the expression noticed in uninfected roots. The expression is depending on the stage of nodule development and on the type of cell in question. The plant genes can be distinguished in four groups by their expression, namely:

- genes of which the expression is not influenced at all by the symbiosis
- genes of which the expression is enhanced in the root nodule

(the gene product of such a gene is called nodule-stimulated protein)
- genes of which the transcription is only expressed in the root nodule and not in uninfected roots nor in other parts of the plant (the gene product of such gene is called nodulin)
- genes which are repressed consequent of the bacteria infection.

In the preinfection stage, the *Rhizobia* interact with the plant genome by the release of diffusible growth factor(s) (phytohormones), inducing meristematic centers in the root cortex (14,15). The transcription of plant genes involved in the synthesis and the release of flavonoids may also be regulated in a specific manner during preinfection (67).

In the infection process, the infection threads are formed by the host, indicating the expression of genes and/or enhanced expression of certain genes. The products of the plant genes derepressed in the infection process, are called early nodulins (68). Using *in vitro* translation of nodule *m*RNA, early nodulins have been identified (69-74). Based on their amino acid composition, it has been suggested that some of these early nodulins are cell wall proteins (74). An enhanced expression for at least one gene, has also been established (74).

The depression of plant genes as consequence of the bacteria infection can also be considered at this stage of nodule development. Plants have many possibilities to respond in a defensive manner to pathogens (75-77). The existence of such a defense response by the legumes has been proved using some mutants of *(Brady)Rhizobia* (78-81). The absence of defense reactions during nodule development, indicates the depression of plant genes involved in these reactions. A connection between the reduced expression of some genes in root hairs and a successful infection by *Rhizobia*, have been proposed (74).

In the functioning nodule about twenty plant genes are specifically expressed (72,82-87). The expression of many of these 'late nodulins` starts before the onset of nitrogenase activity. Within the group of 'late nodulins' sub groups of synchronously expressed nodulin genes can be distinguished. Enhanced expression and reduced expression of other plant genes have also been observed in this stage of nodule development.

Nodulins and nodulin-stimulated proteins are present in both infected and uninfected cells (88,89). The nodulin proteins have been localized in the cytoplasm (89-92), in the peroxisomes (88), in the peri-bacteroid membrane (93-95) and in the Golgi apparatus (96).

The biological activity of several nodulins is known. These are leghaemoglobin (97), uricase (EC 1.7.3.3)(88,98), glutamine synthase (EC 6.3.1.2)(99,100) and sucrose synthase (EC 2.4.1.13) (92). The leghaemoglobins are present in all known legume-*Rhizobium* symbioses (97). Of the other functionally identified nodulins, not all are universally present in root nodules. In French bean and Alfalfa, a nodule-specific glutamine synthase has been identified (99,101,102) whereas in pea nodules only a

nodule-stimulated form of the enzyme could be detected. The nodule-specific uricase in soybean is a characteristic enzyme for the ureide biogenesis in legume-*Bradyrhizobium* symbioses and therefore is not be present in the legume-*Rhizobium* symbioses having an amide biogenesis.

The observed coordinated expression of groups of nodulin genes, may be mediated by the interaction of common *trans*-acting factors with conserved sequences in the 5'promotor regions of the genes. The existence of conserved sequences in the 5'promotor regions just as the presence of nodule-specific *trans*-acting factors have been demonstrated (103-106). Studies with mutated (*Brady)Rhizobia* strains give evidence that these nodule-specific *trans*-acting factors are of bacterial origin or are at least induced by the bacteria (107-112).

The transcription of the common *nod* genes (*nodDABC)* is required for the expression of both early and late nodulins (107, 108). The involvement of *nod* genes in the expression of late nodulins is supported by the observation that at least some of the *nod* genes are transcribed in the developed nodule (109). The *nif* and *fix* gene products are also involved by the expression of late nodulins. In root nodules induced by *nif* and *fix* mutants (especially *nifA* mutants), the expression of the late nodulins is low (110-112).

1.3.3 *Physiological regulation*

The physiological conditions within the nodule may also regulate the expression of plant genes. Possible regulators are O_2, haem and NH_4^+.

First of all, a low O_2 concentration is a requirement for the expression of the *nif* and *fix* genes of *Rhizobium* (see above) and a low O_2 concentration is also obligatory for nitrogenase activity. In turn, the expression of *Rhizobium* genes is necessary for the induction of nodulin genes. It has been supposed that a direct connection exists between the low O_2 concentration and the expression of late nodulins, in particular leghaemoglobins (113). However a low O_2 concentration alone, without the presence of bacterial signals, cannot induce the expression of nodulins (114). A low O_2 concentration has also been postulated as regulating factor in the expression of uricase (115).

Haem is also a physiological factor which presence is absolutely necessary for nodule function. Haem is an essential component of the leghaemoglobins. These late nodulins exhibit a high affinity for O_2 and function as O_2 carrier in the root nodule (97). Because the leghaemoglobins bind O_2, the free O_2 concentrations in the root nodule is low, but the O_2 available for respiration is high. In the absence of the leghaemoglobins, the free O_2 concentrations in the root nodule would be high if the same O_2 consumption rate must be reached which is possible with leghaemoglobins.

It has been demonstrated that the concentration of haem can

regulate the expression of plant genes containing the 5'-flanking sequences of one of the soybean leghaemoglobins (116). The 5'-flanking sequences enclose sequences conferring the nodule-specific expression. Thus haem might regulate the expression of at least this specific leghaemoglobin and probably some other nodulins. Free haem has also been mentioned as regulating factor of another nodulin, namely sucrose synthase (92).

The location where haem is synthesized is still questioned (113). The haem moiety is thought to be synthesized by the bacteroid whereas the globin part is plant-genome encoded (117). Consequently the bacteroid genes involved in haem synthesis are defined *fix*. In the absence of haem, the globin synthesis decreases significantly. A mutation in the gene encoding for the first enzyme in haem synthesis (i.e. 5-aminolaevulinic acid synthase) leads to root nodules incapable of reducing N_2. However a *B. japonicum* mutant, mutated in the same gene, induces effective nodules, which can reduce N_2 (119). This implies that the regulation of haem synthesis and coherent the role which haem plays as an effector, is complicated.

NH_3 has been postulated as an important metabolic regulator in root nodules. NH_3 is the product of the action of nitrogenase. The formation of NH_3 is thought to increase the pH (120), which might have consequences for the functioning of enzymes. The altered environment in root nodules -high pH, low O_2 concentration - has been proposed as a reason for the existence of nodule-specific enzymes involved in the carbon metabolism and in the NH_3 assimilation (121). The expression of some isoforms of glutamine synthase is stimulated by NH_4^+ (122). Other effects of NH_4^+ on the expression of root nodule proteins are unknown.

The genetic and biochemical interactions result in a number of changes, which are necessary for the symbiosis. In the cytoplasm of infected plant cells a microaerobic environment is created. This microaerobic environment allows the reduction of N_2 by nitrogenase in the bacteroids. Nodule-specific carbon metabolism and ammonia assimilatory pathways are operating in the plant cells. Carbon metabolism is necessary to supply the bacteroids with usable carbon compounds for the energy metabolism and to deliver carbon skeletons for the ammonia assimilation.

In Table 4, the different stages of root nodule development, the events and the interactions between bacteria and the host plant, are summarized.

In the following paragraphs, attention will be paid to:

- the electron allocation to H^+ and N_2 by nitrogenase
- the generation of ATP and reductant for nitrogenase
- the carbon metabolism of root nodules
- the ammonia assimilation of root nodules
- the O_2 concentration in root nodules.

Table 4. Simplified overview of the stages of nodule development, the corresponding events and the relating (inter)actions between *Rhizobium* and the host plant.

stage of nodule development	events	(inter)actions
Preinfection	migration, attachment, root hair curling, induction of meristem switching-off of the defense system of the plant	specific chemotactic, production of diffusible growth factors,
Infection and nodule formation	infection thread formation, nodule initiation and development, release of bacteria, bacteroid differentiation	flavonoid-*nodD*, O_2-limitation, expression of *nod* and *hsn* genes, expression of early nodulins
Nodule function	nitrogen fixation complementary function persistence of nodule function	O_2-limitation-*nifA* expression of *nif* and *fix* genes, expression of early and late nodulins
Nodule senescence	membrane disintegration cell disintegration nodule breakdown	

1.4 *Electron allocation to H^+ and N_2 by nitrogenase*

Nitrogenase (EC 1.18.6.1) can catalyze the reduction of a number of substrates, for example N_2, N_3^-, N_2O, CN^-, C_2H_2 and H^+. The enzyme complex consists of two O_2-labile proteins namely MoFe protein (component 1) and the Fe protein (component 2). The MoFe protein is an $\alpha_2\beta_2$ tetramer of $M_r \approx 220.000$ Da. This protein contains two FeMo cofactors (FeMoCo), which forms the site of substrate reduction (123-125). The MoFe protein has also the so-called 'P'cluster, which function is unknown. The Fe protein is a dimer of subunits of $M_r \approx 31.000$ Da and contains a Fe_4S_4 cluster, which functions in the electron transfer from the reductant to the MoFe protein. There exists a great homology between the primary structure of the nitrogenase proteins of different N_2-reducing bacteria (126-130). Besides a Mo-containing nitrogenase, a nitrogenase with V and one with only Fe has been described (131, 132).

The requirements for substrate reduction by nitrogenase are a source of reducing equivalents, MgATP, protons and an anaerobic environment. In the nitrogenase reaction, the Fe_4S_4 cluster of the Fe protein is reduced by a low potential electron donor

($E_m \leq -420$ mV), such as ferredoxin, flavodoxin or *in vitro* dithionite (133,134). The Fe protein binds two MgATP. The reduced Fe protein forms a complex with the MoFe protein. The Fe protein reduces the MoFe protein in a reaction in which ATP is hydrolyzed (133,135,136). The role of MgATP hydrolysis in the electron transfer, is not clear. It has been suggested that the free energy of the hydrolysis is used for the formation of thermodynamic unfavorable intermediates (137). After the electron transfer and the MgATP hydrolysis, the MoFe protein and the Fe protein dissociate (138,139). Only one electron is transferred per cycle. Thus in the reduction of a substrate, the cycle has to be repeated.

The reduction of N_2 to NH_3 requires theoretically, six electrons. But it is found that the reduction of N_2 by nitrogenase is accompanied with H^+ reduction. The reduction of H^+ in the presence of N_2 by nitrogenase is variable but a minimum stoichiometry of 1 mol H_2 formed per mol N_2 reduced is found (140-143).

In order to reduce N_2, eight electrons are needed, which indicates that the described cycle must be repeated eight times. Since the transfer of one electron from the Fe protein to the FeMo protein requires - at least - two mol MgATP, a minimum of 16 mol MgATP per mol N_2 reduced are consumed (148). Consequently the overall reaction for the reduction of N_2 is:

$$N_2 + 8\ H^+ + 8e^- + 16\ MgATP \longrightarrow 2\ NH_3 + H_2 + 16\ MgADP + 16\ P_i$$

The obligatory H^+-reduction by nitrogenase means a waste of MgATP and reductant. The more electrons are allocated to H^+ per mol N_2 reduced, the worse the efficiency of nitrogenase reaction becomes.

A model for the N_2-reduction which accounts for the variable and obligatory H^+-reduction, has been proposed (149). In this model (see Figure 3) the symbol E_n is used for the MoFe protein; n refers to the number of electrons by which the MoFe protein has been reduced. In this model three or four one electron reductions leads to E_3H_3 or E_4H_4. These two forms are thought to bind N_2, which in a subsequent reduction can be reduced. Due to the binding of N_2 to the active site of the MoFe protein, H_2 dissociates. This explains that 1 mol H_2 is obligatorily produced per mol reduced N_2. It is a minimum stoichiometry, since H_2 can dissociate from E_2H_2, E_3H_3 and E_4H_4, before the N_2 binds at the active site. It is obvious that the rate of the electron flow to the MoFe protein determines if, for example E_2H_2 is reduced to E_3H_3 (high rate) or is oxidized to E_0 and H_2 (low rate).

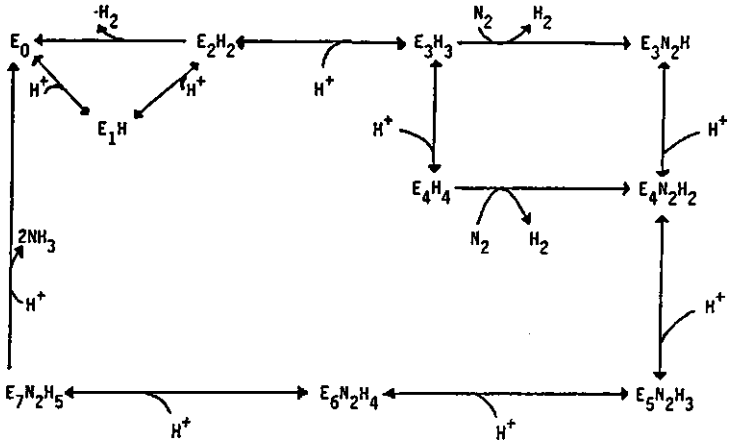

Figure 3. A model for N_2/H^+-reduction by nitrogenase, after Thorneley and Lowe (149). In this model the symbol E_n is used for the MoFe protein; n refers to the number of electrons by which the MoFe protein has been reduced.

It has been shown that the electron flow to the MoFe protein determines indeed the electron allocation to H^+ and N_2 (140). Lowering of the electron flow to the MoFe protein stimulates the electron allocation to H^+. Factors which inhibit the electron transfer from the Fe protein to the MoFe protein are:

- ATP-limitation (150)
- low ATP/ADP ratio (150)
- decline in the ratio Fe protein/MoFe protein (144,151,152)
- shortage of reductant
- lowering of the absolute concentrations of both proteins (153).

The latter observation has been explained with the assumption that the Fe protein associated to the MoFe protein, will block the active site (153), thereby obstructing the oxidation of the intermediates (E_2H_2, E_3H_3 and E_4H_4).

For other substrates than N_2, a less reduce state of the FeMo protein is necessary (123, 143-147).

The electron allocation to H^+ by nitrogenase in isolated bacteroids, has also been studied (154). Inhibition of the electron flow to the MoFe protein by O_2-limitation of the bacteroids, results in an enhanced electron allocation to H^+. The

same effect was observed when a H^+-conducting ionophore was added to the bacteroids. In both cases, the intracellular ATP/ADP ratio decreased. Inhibition of the electron flow to the MoFe protein by O_2-excess or by the addition of K^+ and a K^+-conducting ionophore, was not accompanied by an change in electron allocation. These two ways of inhibition of the electron flow to MoFe protein do not affect the intracellular ATP/ADP ratio. ATP-limitation of nitrogenase in bacteroids which are made permeable, causes no change in the electron allocation. However inhibition of the electron flow by ADP, was associated with an increased electron allocation. It was concluded from these results that the electron allocation of nitrogenase *in situ* is only determined by the intracellular ATP/ADP ratio. By lowering this ratio, the H^+-reduction is favoured above the N_2-reduction.

Concerning the (*Brady*)*Rhizobium*-legume symbioses, several environmental factors have been identified, which affect the electron allocation. These factors are:

- the temperature (155)
- the irradiation and growth cycle (156-158)
- the partial pressure of O_2 (159-162)
- the host plant cultivar (162)
- the strain of bacteria (161,163,164).

The diffusion of O_2 into the nodule is a factor which can be controlled by the plant by means of a variable gas-diffusion barrier (165; see paragraph 1.8). Thus the host plant may regulate the efficiency of the nitrogenase reaction in terms of electron allocation, by means of the variable O_2-diffusion barrier.

The observation that H^+/N_2-reduction by nitrogenase in root nodules depends on the bacterial strain used for infection, is partly connected with the presence or absence of bacterial hydrogenases. Only a few *Rhizobia* strains possess a H_2-oxidizing system. The *Rhizobium* genes encoding for H_2 oxidation (*hup*) are located on the *Sym* plasmid (166). The induction of the *hup* genes is regulated by H_2, Ni and O_2 (167,168). Like the *nif* and *fix* genes, a low O_2 concentration is required for derepression. The presence of CO_2 and a limitations of carbon supply towards the bacteriods, may also induce the expression of the *hup* genes (167).

The H_2-oxidizing system may affect the efficiency of the nitrogenase reaction in symbiosis. Two effects of H_2-uptake hydrogenases on nitrogenase have been mentioned. One effect consists of the removal of O_2 by H_2-oxidation (171,172). Such oxidation will protect the O_2-labile nitrogenase proteins against excess O_2. The second effect, the recycling of H_2, provides the potential for recovering a part of the reductant and ATP wasted by the H^+-reduction. In (*Brady*)*rhizobia* the H_2-oxidation is coupled to the respiratory chain and therefore is used for the ATP synthesis (171-176). The H_2-oxidation coupled

to ATP synthesis, is accompanied with a reduced rate of CO_2 production, indicating a reduced carbon utilization (160,166, 173). This implies that when the carbon supply towards the bacteroid is limited, the presence of H_2-oxidizing system would increase the N_2-reduction by nitrogenase. However no significant increase of the N_2-reduction by nitrogenase in *Rhizobium*-legume symbioses by the presence of an uptake hydrogenase could be demonstrated (173,177).

1.5 *Generation of ATP and reductant for nitrogenase*

1.5.1 *ATP*

ATP is necessary for nitrogenase activity. Theoretically, a minimum of 16 molecules ATP is needed to reduce one molecule N_2. The necessary ATP can be generated by phosphorylation at substrate-level or by oxidative phosphorylation. In microaerobic and aerobic N_2-fixers, oxidative phosphorylation is the main energy transducing system.

Rhizobia possess branched respiratory chains with cytochromes of the type *b,c,a,o* and *d* (178-183). Two cytochrome oxidases with different affinities for O_2, have been identified in *Bradyrhizobia* (180,181) and *Rhizobia* (182). The expression of some cytochrome oxidases is associated with environmental conditions like the partial pressure of O_2 (183). The existence of a branched electron transport chain with two (or three) cytochrome oxidases having different K_m-values for O_2, might imply different efficiencies of oxidative phosphorylation. It is suggested that in *Bradyrhizobium japonicum* the electron transport chain with a low K_m for O_2 (that is high affinity) is more efficient in ATP synthesis than the electron transport chain with a low affinity for O_2 (that is high K_m for O_2) (180,181). The electron transport chain with a low K_m for O_2 functions under conditions favorable for N_2-reduction. The electron transport chain with the high K_m for O_2 is thought to protect nitrogenase against O_2 (180), as has been suggested for *Azotobacter* (184).

In *Rhizobium leguminosarum biovar trifolii* the organization of the respiratory chain is different (182). This bacterium possess also (at least) two different terminal cytochrome oxidases. However the terminal cytochrome oxidase with the lowest K_m for O_2 is associated with a less efficient H^+-translocating pathway than the cytochrome oxidase with a high affinity for O_2.

The extent of the ATP synthesis depends on the number of charges transported across the membrane. A total of three charges transported - in the case of *Rhizobia* - are needed to synthesize 1 molecule ATP (185). In *Rhizobia* three respiratory complexes are present. A fourth respiratory complex could consist of the membrane-bound hydrogenase (if present). The number of H^+ transported across the membrane is different per complex. Complex I transports 3, complex II 4 and complex III

two H^+ across the membrane. Thus, complex I, II and III synthesizes respectively 1, 1.66 and .66 ATP per 1/2 molecule O_2 used.

Depending on the level where the electrons enter the electron transport chain, the amount of ATP synthesized per 1/2 molecule O_2 (the P/O ratio), can have values between 1 and 3. The P/O ratio of vesicles of *Rhizobium leguminosarum* bacteroids depends on the O_2 supplied (186). The maximum value was estimated to be 1.3. The P/O ratio can be estimated indirectly by measuring the number of H^+ transported per O_2 consumed. For *B. japonicum* bacteroids (187) and for *R. leguminosarum* cells (188) a H^+/O ratio of respectively 6.4 and 6.5 were measured. Since three H^+ has to be transported to synthesize one molecule ATP, these H^+/O values imply P/O ratios of about 2.1. Only in *R. leguminosarum* biovar *trifolii* cells (182) higher H^+/O ratios were found namely about 9.4, which indicates the functioning of all the three respiratory complexes. The observed efficiency of the oxidative phosphorylation is common for bacteria.

1.5.2 *Reductant*

A second requirement for the nitrogenase reaction is the generation of reducing equivalents. The redox potential required to reduce the Fe_4S_4 cluster of the Fe protein of nitrogenase, depends on the species in question but has to be low ($\leq$ -500 mV for *Azotobacter vinelandii*, 169). *In vivo*, only two types of electron carriers with sufficiently low redox potential are known, namely ferredoxins and flavodoxins. Flavodoxins have three oxidation states: quinone (oxidized form), semiquinone (e^- reduced) and hydroquinone ($2e^-$ reduced). The redox potential of the hydroquinone/semiquinone couple is sufficiently low to function in nitrogen fixation. It is suggested that only ferredoxins are present in *Rhizobia*, because flavodoxins in these bacteria have not been found (258).

Unlike the N_2-reducing anaerobes *Klebsiella pneumoniae* and *Clostridium pasteurianum*, the *(Brady)Rhizobia* possess neither pyruvate:flavodoxin oxidoreductase nor a pyruvate:ferredoxin oxidoreductase. Electrons needed for the reduction of ferredoxin might be come from NAD(P)H (189,190). However the presence of a NAD(P)H:ferredoxin oxidoreductase has not been demonstrated. There is indirect evidence that the reduction of ferredoxin by NAD(P)H might be driven by the membrane potential (189,191-193). The electron flow can be driven in two ways. ATP hydrolysis can generate a proton motive force, which leads to a reversed electron flow from the pyridine nucleotides to ferredoxin. The flow of electrons through the respiratory chain to O_2, can also generate a proton motive force, which can be used to drive reversed electron transport.

Haaker and Klugkist (189) have postulated a specific distribution of the two electrons from NAD(P)H by a NAD(P)H-dehydrogenase of the respiratory chain of *Azotobacter*. The redox clus-

ter of this complex is specifically oxidized by the transfer of one electron to ubiquinone. By this action, the redox potential of the partially oxidized cluster of the NAD(P)H dehydrogenase complex is lowered ($E_h \simeq -500$ mV). Consequently, the ferredoxins and flavodoxins can be reduced by the cluster. The other electron is transported via ubiquinone to the respiratory cytochrome oxidase and O_2. The reduction of ferredoxin in *(Brady)Rhizobia* might be carried out in the same way.

1.6 *Carbon metabolism*

The N_2-reduction in bacteroids depends on a continuous supply of photosynthate to the nodule, as has been demonstrated for nodulated plants (194-196). The majority of photosynthate is transported in the phloem as sucrose. Sucrose is transported from the shoot to the root nodules (197-198). The carbon requirements of root nodules of different legumes have been estimated. In the *Rhizobium*-pea symbiosis about 32 % of the net photosynthetic carbon is consumed by the nodules (199). About 15 % of the carbon supplied to the pea nodule returns to the shoot as nitrogenous compounds. Nodule respiration, which includes bacteroid respiration, accounts for 12 % of the carbon used. The remaining 5 % is utilized for nodule growth and maintenance. The energy costs for N_2 reduction amounts to 6 - 20 gram carbon used per gram N_2 reduced (200,201).

The utilization of sucrose and other carbohydrates by bacteroids is limited (202-210). Carbohydrates do not stimulate respiration of bacteroids or N_2 reduction. Mutants of Rhizobia which are deficient in the metabolism of sugars or in sugar transport systems, are still able to reduce N_2 at a extent, normal for wild type nodules.

The primary carbon sources used by the bacteroids appear to be C_4-dicarboxylic acids like malate, succinate and fumarate. These C_4-dicarboxylic acids stimulate bacteroid respiration and N_2 reduction (209-211). A specific transport system for the C_4-dicarboxylic acids in bacteroids has been identified (208,212-215). The genes encoding for this transport system in *Rhizobia* are located on the chromosomes and are coordinately expressed with the *nif* genes (216). Mutants in these genes are unable to form N_2-reducing nodules (170,212,214) This indicates that the C_4-dicarboxylic acid transport system is necessary for the symbiosis and that its expression is regulated like the *nif* genes.

The fact that bacteroids only can use C_4-dicarboxylic acids and not carbohydrates, implies that sucrose has to be converted into these organic acids in the cytoplasm of plant nodule cells. Sucrose can be metabolized by alkaline invertase, acid invertase or sucrose synthase. All these three enzymes have been detected in the cytoplasm of root nodule cells. The activity of alkaline invertase and sucrose synthase are much higher than the activity of acid invertase (217,218), which may indicate that the latter

enzyme is less important for the rate of sucrose degradation. The affinity for sucrose of alkaline invertase is higher than that of sucrose synthase (219). On the contrary, sucrose synthase has been identified as nodule-specific (92). In view of this observation, sucrose synthase may be of great importance for the carbon supply towards the bacteroids. The conversion of sucrose to triose-phosphate via the 'sucrose synthase pathway' is also more favorable in energy costs than the conversion of sucrose by the invertase. The first pathway requires the input of three ATP per molecule sucrose converted, whereas the latter pathway demands four ATP per molecule sucrose (220).

The conversion of sucrose by the sucrose synthase pathway as well as by the invertase pathway, yields fructose-1-phosphate as intermediate. Fructose-1-phosphate can be metabolized via the Emden-Meyerhof pathway and subsequently by the tricarboxylic acid cycle. The enzyme phosphofructokinase (EC 2.7.1.11), which is generally characterized as a rate-limiting enzyme of the glycolysis, appears to be nodule-stimulated (221,222). The oxidative pentose phosphate pathway forms an alternative for the glycolysis. Considering label studies and enzymatic studies, this pathway is of minor importance in the cytoplasm of root nodule cells (222-225). These data also argue for an functional glycolysis. However the absolute activity of the glycolytic enzyme aldolase (EC 4.1.2.13) is reduced substantially in cytoplasm of root nodule cells (221). For this reason, it has been suggested that the glycolysis only partly contributes to the energy demands of the bacteroids (221).

The end product of the glycolysis - pyruvate - can be oxidized in the mitochondria in the tricarboxylic acid cycle as acetyl-CoA. The enzymes of the tricarboxylic acid cycle are generally present in plant mitochondria. Furthermore the concentration of mitochondria is higher in infected cells than in uninfected cells (258). However the free O_2 concentration is rather limited in the root nodule. It has been suggested (259) that the O_2 concentration in the cytoplasm of root nodule cells is not sufficient to support significant rates of aerobic respiration. An anaerobic metabolism of pyruvate is possible; enzymes associated with this metabolism (pyruvate decarboxylase, alcohol dehydrogenase and lactate dehydrogenase) have been detected in the cytoplasm of plant nodule cells (226-228).

Regarding the above discussed studies, it has to be concluded that a definite answer about the functionality of main catabolic pathways in the plant nodule cytoplasm is missing.

In legume root nodules, CO_2 can be fixed (229-232). The primary enzyme, which is considered to be responsible for CO_2 fixation, is phosphoenolpyruvate carboxylase (EC 4.1.1.31). Phosphoenolpyruvate carboxylase activity has been detected in a number of legumes (233,234). It has been estimated that 32 % of the respired CO_2 by root nodules, is fixed via this enzyme (235).

The carboxylation by phosphoenolpyruvate carboxylase contributes the energy demand of bacteroids. Oxaloacetate is neces-

sary for the synthesis of malate, catalyzed by malate dehydrogenase (EC 1.1.1.37). Malate being the predominant C_4-dicarboxylic acid in root nodules (227), is used as carbon source by the bacteroids (see above, 209-211). Malate may also be required to balance cation transport (236). Oxaloacetate is thought to deliver the necessary carbon skeletons for ammonia assimilation (251).

The carbon metabolism in bacteroids has not been elucidated in detail. The bacteroids possess the genetic information for the expression of enzymes of the glycolysis, the oxidative pentose phosphate pathway and the Entner-Doudoroff pathway (which metabolizes glucose to equal quantities of glyceraldehyde-3-phosphate and pyruvate). The activity of some of the enzymes involved in these pathways, has been detected (240-241). Although the fermentation pathways are present in the bacteroids (250), their actual functioning remains questionable. As has been stated earlier in this paragraph, the bacteroids can only use C_4-dicarboxylic acids as carbon source. The tricarboxylic acid cycle has been mentioned as the pathway involved in the metabolism of C_4-dicarboxylic acids in the bacteroid (211). It can be assumed that the activity of the tricarboxylic acid cycle in bacteroids is controlled by O_2 limitation.

The dicarboxylic acids are rapidly transported by an active uptake mechanism whereas the carbohydrates like glucose are only slowly, passively absorbed by the bacteroids (237,238). Beside the cytoplasmic membrane of the bacteroids, metabolites have to pass another membrane namely the peri-bacteroid membrane. The peri-bacteroid membrane has a selective permeability (239). Thus, by regulating the transport processes, the bacterial membrane and the peri-bacteroid membrane are possible sites of control of the metabolic activities of the bacteroids.

1.7 *Ammonia assimilation*

The product of the N_2-reduction - NH_3 - can be assimilated in the bacteroids or in the host cells. However the activities of either nitrogen assimilatory enzymes like glutamine synthase (EC 6.3.1.2) and glutamate synthase (EC 2.6.1.53), are low in bacteroids (242,243). In contrast, the activities of these enzymes are high in the host cells (244,245). Even nodule-specific forms of glutamine synthase have been demonstrated in some legumes (99,100). It was also shown that the bacteroids exported NH_3 (246). From these observations, it is concluded that the NH_4^+ is assimilated by means of a glutamine synthase / glutamate synthase pathway in the host cells and not in the bacteroids.

The end products of the NH_4^+ assimilation are excreted to the xylem and translocated to other parts of the plant. Considering the nitrogenous compounds excreted, the legumes can be divided into a group of plants, which export mainly amides (usually asparagine and glutamine) and into a group of plants,

which excretes ureides (usually allantoin and allantoic acids). The NH_4^+ assimilation and the subsequent reactions have been subject of many studies; the results of these studies have been reviewed (247,248,249).

The postulated pathway (after Scott *et al.*, 251) which lead to the export of amides, is given in Table 5. From the pathway, it can be seen that oxaloacetate is the carbon skeleton from which asparagine is ultimately synthesized. Thus, a four-carbon compound is required to export the reduced N_2. Oxaloacetate is also required for the synthesis of malate, which is the principal carbon source for bacteroids. Consequently, oxaloacetate has to be distributed between the carbon supply towards the bacteroids and the NH_4^+ assimilation in the cytoplasm of the nodule cells.

Table 5. Reactions of the glutamine synthase/glutamate synthase pathway and the corresponding subsequently reactions, which lead to the synthesis of asparagine.

<u>NH_3-assimilation</u>
Glutamine synthase (EC 6.3.1.2):
$2\ NH_3 + 2$ glutamate $+ 2$ ATP $\longrightarrow$ 2 glutamine + 2 ADP + 2 P_i
Glutamate synthase (EC 2.6.1.53):
2-oxoglutarate + glutamine + H^+ + NADH $\longrightarrow$ 2 glutamate + NAD^+
Glutamate oxaloacetate transaminase (EC 2.6.11):
glutamate + oxaloacetate $\longrightarrow$ aspartate + 2-oxoglutarate
Asparagine synthase (EC 6.3.5.4):
aspartate + glutamine + ATP $\longrightarrow$ asparagine + glutamate + AMP + PP_i
<u>*overall reaction*</u>
$2\ NH_3$ + 3 ATP + oxaloacetate + NADH + H^+ $\longrightarrow$ AMP + 2 ADP + 2 P_i + NAD^+ asparagine + PP_i

1.8 *Oxygen concentration in root nodules*

A microaerobic environment is necessary to prevent nitrogenase inactivation by O_2 and to facilitate the expression / derepression of genes involved in the symbiosis, like the *nif* and *fix* genes. In theory the protection of the bacteroid against high O_2 concentrations, is possible in four ways namely:

- by respiratory protection
- by H_2-linked O_2 uptake
- by limited diffusion of O_2
- by the presence of leghaemoglobins.

The respiratory protection involves the use of branched respiratory chains. At limited O_2 concentrations, the electron transport chain functions which has a high affinity for O_2 and which has a high H^+-translocating capacity. At higher O_2 concentrations, another electron transport chain becomes more active. This chain exhibits a low affinity for O_2 and has a lower H^+-translocating activity. Thus, at higher O_2 concentrations, the O_2 consumption of the cells is not inhibited due to respiratory control. By this mechanism, cells can respire at different rates and maintain a microaerobic environment.

The second way of O_2 protection requires the presence of an uptake-hydrogenase. The uptake-hydrogenase delivers its electrons to the respiratory chain and thus stimulates respiration in case the supply of electrons is limited. The oxidation of H_2, produced by the reaction catalyzed by nitrogenase, yields less energy than the normal oxidation of NADH since electrons are delivered at the level of coenzyme Q. Thus, no site I H^+-translocation takes place. Consequently H_2 oxidation consume more O_2 per equal amount of H^+ translocated than the oxidation of NADH does. This means that H_2 oxidation can function as a way to spoil O_2. The effect of H_2 oxidation will be limited, because H^+ has to be reduced first by nitrogenase before it can be oxidized.

The diffusion of O_2 from the environment to the bacteroids may be regulated by a variable gas diffusion barrier. The existence of such a barrier has been proved and a model has been proposed (165, 251-254).

The variable O_2 diffusion barrier is probably located in the inner cortex, which tissue has very few air pathways. In the inner cortex, gases has to diffuse through small water-filled channels between the cells. Gas diffuses 10.000 times faster in a gas phase than in H_2O. The length of such channel will determine the rate of the gas diffusion. Changes in turgor pressure could lead to an expansion or shrinkage of the inner cortex cells, which will be associated with alterations in gas diffusion rates.

The variable barrier responds all environmental conditions

which affects the flow of photoassimilates to the root nodules (see paragraph 1.7) (165,254,255). The plant reacts on inhibition or enhancement of the N_2-reduction by increasing respectively decreasing the O_2 resistance. It has been suggested that the O_2 diffusion barrier balances the O_2 input against the carbon supplied towards the bacteroids (165). If this is the case, a direct coupling between N_2-reduction, O_2 demand and carbon supply exists.

The nodule-specific leghaemoglobins protect the host cells against high free O_2 concentrations by binding of O_2. Thus high amounts of O_2 can be present in the host cell, however bound to leghaemoglobin. The second function of leghaemoglobin is to facilitate O_2 diffusion. Consequently, leghaemoglobin prevents restriction of the respiration by very low O_2 concentration. The details of leghaemoglobins functions has been reviewed (97,257).

1.9 *Outline of the thesis*

The experiments reported in thesis are focussed on the biochemical adaptions and factors, which are involved in the nitrogenase activity and the regulation of the electron allocation by nitrogenase in the symbiosis.

Several biochemical factors, which regulate and affect the nitrogenase reaction and the electron allocation by nitrogenase in the symbiosis, have been studied and are described in this thesis:

- In chapter 2, the role of a nodule-stimulated form of malate dehydrogenase for the supply of oxidizable substrates to the bacteroid, has been studied. This nodule-stimulated form catalyzes the formation of malate at a high rate, which in necessary in view of the increased demand for malate in the symbiosis. The concentration of malate in the cytoplasm regulates the rate of catalysis of the nodule stimulated malate dehydrogenase and therefore the supply of oxidizable substrates to the bacteroids.

- In chapter 3 it is described that a malate/aspartate shuttle can operate between the cytoplasm of the infected plant cells and the bacteroid. The involvement of a cytoplasmic nodule-stimulated glutamate oxaloacetate transaminase in this shuttle is proposed. Two physiological functions of this malate/aspartate shuttle are postulated. The shuttle may transfer NADH from the cytoplasm of the nodule plant cell to the bacteroid. A second function of the shuttle may consist of the formation of aspartate in the bacteroid and of the supply of the formed aspartate to the cytoplasm of the nodule cell to enable asparagine biosynthesis.

- The external pH of bacteroids has been identified as an important regulator of nitrogenase activity and of the electron allocation to H^+ and N_2 by nitrogenase. This is described in chapter 4. It is proposed that the cytoplasmic pH in the nodule plant cell determines the rate of respiration of the bacteroid and therefore the nitrogenase activity of the bacteroid without affecting the electron allocation by nitrogenase.

- In chapter 5, the mechanism by which the external pH of bacteroids changes the rate of respiration and the rate of nitrogenase activity is studied. The relationships between the rate of respiration by the bacteroid, the nitrogenase activity and the intracellular ATP/ADP ratio are determined. It is shown that the intracellular ATP/ADP ratio via the P_i potential regulates the rate of respiration. This means that the classical mitochondrial respiratory control also functions in the bacteroid. Two mechanisms are suggested to explain the observed pH effect on respiration.

- In the general discussion (chapter 6) the observed biochemical differentiation in the root nodule is placed in a broader perspective. Four physiological processes in which malate is involved, are illuminated. A mechanism is proposed which accounts for the balance between the supply of photoassimilates by the plant and the supply of O_2 to the bacteroids. The observed pH effect on the rate of respiration of bacteroids is also considered in this mechanism. The regulation of nitrogenase is discussed. Finally bacteroids are compared with mitochondria.

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2.

Identification of cytoplasmic nodule-associated forms of malate dehydrogenase involved in the symbiosis between *Rhizobium leguminosarum* and *Pisum sativum*

Michiel A. APPELS and Huub HAAKER

Department of Biochemistry, Agricultural University, Wageningen

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The malate dehydrogenase activity (EC 1.1.1.37), present in the cytoplasm of *Pisum sativum* root nodules, can be separated by ion-exchange chromatography into four different fractions. Malate dehydrogenase activity present in the cytoplasm of roots elutes mainly as a single peak.

During nodule development an increase in malate dehydrogenase activity per gram of material was observed. This increase occurred concomitantly with the increase in nitrogenase activity.

The kinetic properties of the separated malate dehydrogenases of root nodule cytoplasm and root cytoplasm were studied. The K_m values for malate (2.6 mM), NAD^+ (27 μM), oxaloacetate (18 μM) and NADH (13 μM) of the dominant form of the root nodule cytoplasm are much lower than those of the dominant malate dehydrogenase root form (64 mM, 4.4 mM, 89 μM and 70 μM respectively). Binding of malate by the enzyme-NADH complex from noot nodules results in an abortive complex, thereby blocking the further reduction of oxaloacetate by NADH. The dominant root malate dehydrogenase does not form the abortive complex.

From the kinetic data it is concluded, first, that the root nodule forms of the enzyme are capable of catalysing at a high rate the reduction of oxaloacetate, to meet the demands for malate governed by the bacterioid and the infected plant cell. The second conclusion, drawn from the kinetic data, is that under physiological conditions the conversion of oxaloacetate can be controlled just by the malate concentration.

Consequently the major root nodule forms of malate dehydrogenase are able to allow a high flux of malate production from oxaloacetate but also to establish a sufficient oxaloacetate concentration necessary for the assimilation and transport of fixed nitrogen.

In the absence of an adequate supply of available soil nitrogen, certain leguminous species are capable of forming symbiotic associations with soil bacteria of the genus *Rhizobium*. The intracellular symbiotic association arises in the cortex of legume roots following infection by *Rhizobium*. In the root cortex the bacteria cause formation of meristems. The bacteria penetrate the newly divided host cells through growth and branching of the infection threads. Released bacteria are enclosed by envelopes of host cell plasmalemma and develop into bacteroids. This differentiated form of bacteria is capable of the reduction of N_2 to NH_3 [1]. The continued division of infected cells and the proliferation of bacterial cells result in the formation of root nodules with a specific morphological arrangement (for a review see [2]).

To allow nitrogen fixation to occur in the root nodule there must be an extensive metabolic balance between bacteroids and the different plant cells. Photosynthetic products of the host are used by the plant cells and bacteroids. Dioxygen, transported by leghemoglobin, together with carbon compounds made available to the bacteroids, are used to generate reducing power and ATP for nitrogen fixation. The product of the nitrogenase reaction, NH_3, is assimilated in the cytosol of the plant; the necessary carbon skeletons are supplied by the plant.

Correspondence to H. Haaker, Laboratorium voor Biochemie, Landbouwuniversiteit, De Dreijen 11, NL-6703 BC Wageningen, The Netherlands

Enzymes. Malate dehydrogenase (EC 1.1.1.37); glutamate-oxaloacetate transaminase (EC 2.6.1.1).

Several observations indicate that utilization of carbohydrates in *Rhizobium* bacteroids is limited [3–11]. Nitrogen fixation could be detected only with C_4 dicarboxylic acids, not with the hexoses and disaccharides [3, 4, 12]. Bacteroids possess a transport system for C_4 dicarboxylic acids [11, 13–16] and a functional tricarboxylic acid cycle [12]. The predominant C_4 dicarboxylic acid in root nodules of legumes is malate [17–20], which is synthesized from oxaloacetate and NADH by malate dehydrogenase. Malate and succinate are considered to be the carbon compounds used by the bacteroids.

Oxaloacetate is not only used in the reaction catalyzed by malate dehydrogenase to produce malate but it also acts as a carbon skeleton used for assimilation and transport of fixed nitrogen to other parts of the plant [21, 22]. Oxaloacetate is used in the reaction catalyzed by glutamate-oxaloacetate transaminase to produce aspartate. This enzyme is located in the cytoplasm of the infected plant cells and not in the bacteroids [23, 24]. The K_m value for oxaloacetate has been estimated to be 110 μM for soybean [23] and 100 μM and 20 μM, depending on the type of isoenzyme, for lupin [24]. A sufficient oxaloacetate concentration in the cytoplasm of the infected plant cells is a strict requirement for assimilation and transport of fixed nitrogen.

Under standard conditions the reduction of oxaloacetate by NADH is exergonic ($\Delta G^{\circ\prime} = -2.73 \times 10^4$ J). Under physiological conditions common for plant cytosol (pH 7.0–7.3; $NADH/NAD^+$ 0.2–0.4), the equilibrium of the reaction catalyzed by malate dehydrogenase is completely in favour of malate and NAD^+. If malate dehydrogenase were to catalyse the reaction to equilibrium, then the oxaloacetate concen-

tration would be very low (< 1 μM). Since in the root nodule a significant oxaloacetate concentration is required for assimilation and transport of fixed nitrogen, it is important that the conversion of oxaloacetate by malate dehydrogenase is controlled and limited to some extent.

Since the malate dehydrogenase activity in root nodules is high [17, 18, 25, 26], a very substantial reduction of oxaloacetate to malate under average cytoplasmic physiological conditions is to be expected. Despite the abundant presence of malate dehydrogenase in root nodules, assimilation and transport of fixed nitrogen take place. To solve this problem and to give an answer to the question of the role of malate dehydrogenase in symbiosis, malate dehydrogenase from the cytoplasm of root nodules and of roots was studied.

MATERIALS AND METHODS

Growth conditions of the plants

Seeds of *Pisum sativum* cv. Rondo were surface-sterilized by shaking for 20 min in 3% v/v H_2O_2 to which a minute amount of detergent (Teepol) had been added. The disinfected peas were sown in heat-sterilized grit. Plants were inoculated directly after sowing with 1 ml/seed of a dense suspension of *Rhizobium leguminosarum* strain PRE. The water dose consisted of sterilized medium containing per liter 0.36 g K_2HPO_4, 0.12 g $MgSO_4 \cdot 7H_2O$, 0.25 g $CaSO_4$, 0.03 g $FeCl_3$ and 0.1 ml solution of trace elements. The solution of trace elements contained per liter 200 mg $FeCl_3 \cdot 6H_2O$, 200 mg $MnCl_2 \cdot 4H_2O$, 15 mg $CuCl_2 \cdot H_2O$, 2 mg $CoCl_2 \cdot 6H_2$, 200 mg H_3BO_3, 200 mg $Na_2MoO_4 \cdot 2H_2O$ and 100 mg $ZnCl_2$. The plants were cultured without combined nitrogen in a growth chamber at 18–20 °C and a light intensity of 12000 lx, with a 16-h light/8-h dark period.

Nodules were cut from the main root at 18–19 days after inoculation. Uninfected plants were cultured in the same way as infected plants but with a sufficent nitrogen level, supplied as NH_4NO_3 (0.025 g/liter). The main roots of uninfected plants were harvested; parts containing meristematic tissue were removed. From roots with developing nodules, a 2.5-cm piece of the main roots was cut at the location where normally the nodules appear.

Preparation of extracts

Root nodules were ground in a chilled mortar with isolation buffer (approx. 1 ml/g fresh material). The isolation buffer consisted of 50 ml Tes/KOH, 16% w/v sucrose, 0.9% w/v glucose, 5 mM dithiothreitol and 1 mM EDTA, pH 7.4 at 4 °C. After filtering the homogenate through Miracloth (Calbiochem), bacteroids were removed by centrifugation (20 min at 6000 × *g*). The resulting supernatant contained soluble cytoplasmic plant proteins and cell organelles, mainly mitochondria and microsomes. The soluble cytoplasmic plant proteins were separated from cell organelles by high-speed centrifugation (80 min at 60000 × *g*).

The fraction containing bacteroids was washed five times with 45 ml isolation buffer without dithiothreitol. The soluble proteins of bacteroids were released by sonication using a Soniprep 150 Ultrasonic disintegrator (MSE) four times during 20 s with an amplitude of 26 μm and a frequency of 23 kHz, and subsequently centrifuged at 12000 × *g* for 30 min. Cytoplasmic protein from pea roots was prepared in a similar way using 18–19-day-old main roots of uninoculated plants. These main roots were stripped of their root-caps and adjacer parts. The fractionations were performed at 4 °C.

Free-living *Rhizobium (Rhizobium leguminosarum* PRE were grown axenically in yeast/mannitol medium. The cel were collected by centrifugation. Sonicated extracts were pr pared by sonication four times during 30 s with an amplitud of 26 μm and 23 kHz. The broken cells were centrifuged a 20000 × *g* for 2 h. The resulting supernatant was desalted o a small column of Sephadex G-25 (Pharmacia).

Seeds of *Pisum sativum* were surface-sterilized by shakin for 20 min in 3% v/v H_2O_2 to which a minute amount c detergent had been added. The disinfected peas were laid o moist filter-paper. After three days of incubation under hig humidity at 20 °C, germ root tops (0.75 cm) were collectec These pieces of roots were fractioned in a similar way t uninoculated roots.

Fast protein liquid chromatography

Desalted samples were analyzed using a Mono Q colum (bed volume 1 ml; FPLC system of Pharmacia) equilibrate with 20 mM Tris/HCl, pH 7.4. About 1% of the protein loa capacity of the column was utilized, based on the informatio given by Pharmacia. The proteins were eluted at 0.5 ml/mi with a NaCl gradient. The increase in the NaCl concentratio was 10 mM/ml. Fractions of 0.5 ml were collected an analyzed for malate dehydrogenase activity.

Assay for nitrogenase

Nitrogenase activity of intact nodules was measured b acetylene reduction. Ethylene production was measured wit a Porapac R column in a Packard gas chromatograph mode 433. Pieces of the main roots carrying the root nodules (8 roots) were incubated in a closed serum bottle (100 ml) a room temperature. Acetylene was added to a final concen tration of 10% v/v. Gas samples were taken and assayed fo ethylene production. Ethylene production rates were linea with time up to 40 min.

Assays for malate dehydrogenase

Malate dehydrogenase activity was measured routinely a 25 °C in 25 mM potassium phosphate buffer, 0.2 mM NADH and 0.4 mM oxaloacetate, pH 7.4. Oxaloacetate solution were prepared daily and neutralized to pH 7.4. The rate o disappearance of NADH was monitored at 340 nm befor and after addition of oxaloacetate. The former rate served a a measurement of background NADH oxidation which wa subtracted from the rate of oxaloacetate-dependent activity Initial reaction rates have been shown to be proportional t the concentration of enzyme under the conditions used i these experiments.

The assay system for measuring the oxidation of malat by NAD^+, catalyzed by malate dehydrogenase, involves th reaction of oxaloacetate with L-glutamate in a subsequen reaction catalyzed by glutamate-oxaloacetate transaminase The assay system contained 50 mM Tris/HCl, 40 mM L-glutamate, 0.8 mM NAD^+, 4.0 U/ml glutamate-oxaloace tate transaminase and 100 mM L-malate, pH 8.0. The reac tion rates were measured from the appearance of NADH absorbance at 340 nm. The amount of NADH and oxalo acetate formed in the oxidation of malate was stoichiometri 1:1 with the amount of malate and NAD^+. Initial reactio

rates have been shown to be proportional to the concentration of enzyme under the conditions used.

For the kinetic measurements of malate dehydrogenase conditions were the same as those described for the activity assays, except that substrate concentrations were varied. A range of six suitable concentrations of each substrate was chosen. Results were analyzed graphically as described by Dalziel [27] using linear regression analysis. The substrate concentrations were determined by enzymatic spectrophotometric methods. The substrate coefficients from initial rate data for malate dehydrogenase fractions were estimated at a 99% level of significance.

For pH dependence studies of malate dehydrogenase, buffers used were 0.1 M Mes for pH 5.5, 6.0, 6.5; 0.1 M Mops for pH 6.5, 7.0, 7.5 and 0.1 M Tricine for pH 7.5, 8.0 and 8.5. The concentrations of the substrates were the same as described for the assay of malate dehydrogenase activity. Sufficient glutamate-oxaloacetate transaminase was present for the oxidation of malate by malate dehydrogenase to be rate-limiting.

For studying the effect of pyridine nucleotides on the conversion of oxaloacetate by malate dehydrogenase, both oxaloacetate and NAD^+ were varied in the presence of 50 μM NADH. The NADH/NAD^+ ratio was varied over the range of 0.01 – 0.2. The substrate concentrations were determined by enzymatic spectrophotometric methods. Dependence of rate on oxaloacetate concentration in the presence of varied NAD^+ concentrations with a fixed NADH concentration of 50 μM reveals a linear relationship. In plots of the slopes and intercepts of these lines against NAD^+ concentrations straight lines could be fitted using linear regression analysis. Levels of 99% to 99.9% of significance were achieved.

Inhibition by malate of the conversion of oxaloacetate was analyzed in the same way as described for the determinations of K_m. Three-quarters of the substrate coefficients from initial rate data were estimated at a 99 – 99.9% level of significance; one-quarter of the substrate coefficients were estimated at a 95 – 99% level of significance.

Malate dehydrogenase was visualized in gels using an electron-transfer dye staining method. The method used involved the transfer of electrons via NADH to nitroblue tetrazolium. The enzyme-staining mixture consisted of 0.5 M Tris/HCl, 0.8 mM NAD^+, 0.4 mM nitroblue tetrazolium prepared freshly, 0.1 mg/ml phenazine methosulphate, 50 mM L-malate, pH 8.0. The reaction was carried out in the dark at 25 °C. Checks on the specificity of the staining were included; omission of any one of the substrates resulted in the absence of the blue formazan form.

Electrophoresis

Non-denaturing linear gradient gels, in which the acrylamide concentration increased linearly in the separating gel (from 3% to 30% mass/vol.) were used at an alkaline pH (8.8). The electrophoreses were conducted at 4 °C and were terminated at the moment when the two additions of xylenecyanol to the gels, 1 h apart, were at the same location on the gel. Malate dehydrogenase fractions were made visible by electron-transfer dye staining. Standard proteins used for molecular mass determination were stained with Coomassie brilliant blue. Following staining the malate dehydrogenase activity bands were cut from the gels and subsequently subjected to 15% SDS/polyacrylamide gel electrophoreses as described by Laemmli [28]. Protein bands were stained using silver staining. Molecular mass markers were included in the electrophoreses.

Table 1. *Malate dehydrogenase activity in nodules and uninoculated roots of pea plants*
Extracts of nodules of inoculated and main roots of uninoculated pea plants were separated into fractions by low and high-speed centrifugation as described in Materials and Methods. Fractions containing the soluble cytoplasmic plant proteins and fractions containing bacteroids were obtained. The bacteroid fraction was sonicated and centrifugated to remove membranes and membrane fragments. The malate dehydrogenase activity of the bacteroid fractions were correlated to the activity per gram starting material. Material was harvested 17 – 18 days after sowing

Fraction	Activity (SE)	Specific activity (SE)
	U/g fresh tissue mass	U/mg protein
Cytoplasm of uninoculated main roots	16.8 (1.5)	9.41 (9.95)
Plant cytoplasm of nodules	57.6 (3.7)	24.9 (2.8)
Bacteroids	23.3 (1.5)	0.82 (0.05)

Ultrathin-layer isoelectric focusing was carried out in polyacrylamide gels and agarose gels using ampholytes 4 – 6.5, 3 – 10 (10% w/v, Pharmacia) and 4.5 – 5.0, 5.0 – 5.5, 3 – 10 (5% mass/vol., Serva). pI points were determined by the use of pI marker proteins (Serva protein test mixture 9). After focusing, the malate dehydrogenase fractions were made visible using the described staining method.

The protein concentrations were determinated by the method of Sedmark and Grossberg [29]. Bovine serum albumin was used as the standard.

Chemicals

Oxaloacetate, glutamate-oxaloacetate transaminase, NAD^+ and NADH, were obtained from Boehringer; L-malate and Tricine from Merck and nitroblue tetrazolium, Tes, Mops, Mes and DL-glutamate from Sigma. All other chemicals were of the highest analytical grade available.

RESULTS

Malate dehydrogenase activities present in root and root nodule cells

The total malate dehydrogenase activity of root nodules (per gram tissue) is about five times higher than the activity found in roots (Table 1). About 70% of the malate dehydrogenase activity is found in the cytoplasmic plant fraction of root nodules and about 25% in the bacteroid fraction. The specific malate dehydrogenase activity (U/mg protein extracted) in root nodules was about 2.5 times higher than the cytoplasmic fraction of roots.

Malate dehydrogenase activity in the cytoplasmic fraction of primary and secondary roots of uninfected plants and of the cytoplasmic plant fraction of root nodules was separated by ion-exchange chromatography. The elution profile of malate dehydrogenase in the cytoplasmic fraction of roots showed one main peak at 95 mM NaCl and three peaks of minute activity at higher NaCl concentration (Fig. 1). The first peak of malate dehydrogenase activity of the cytoplasmic plant fraction of root nodules elutes at a slightly higher NaCl

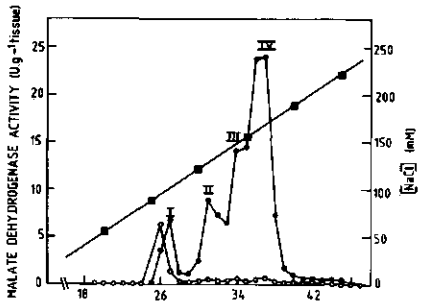

Fig. 1. *Elution profile of malate dehydrogenase activity from Mono Q of the cytoplasmic plant fraction of pea root nodules harvested 19 days after inoculation (●——●) and of the cytoplasmic fraction of uninoculated roots harvested 17 days after sowing (○——○).* The malate dehydrogenase activity was measured by the reduction of oxaloacetate. NaCl concentrations were determined in the fractions (■——■)

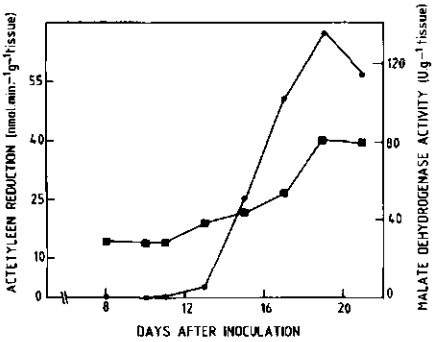

Fig. 2. *Nitrogenase activity (●——●) and malate dehydrogenase activity (■——■) of root fragments with developing nodules harvested at different times following inoculation of* P. sativum *with* R. leguminosarum *PRE.* The root fragments consisted of 2.5-cm pieces of the main roots where nodules normally appear. For nitrogenase activity the reduction of acetylene by the undetached nodules was measured. For malate dehydrogenase activity the root fragments were extracted and fractionated as carried out for nodules. Malate dehydrogenase activity was measured by the reductions of oxaloacetate

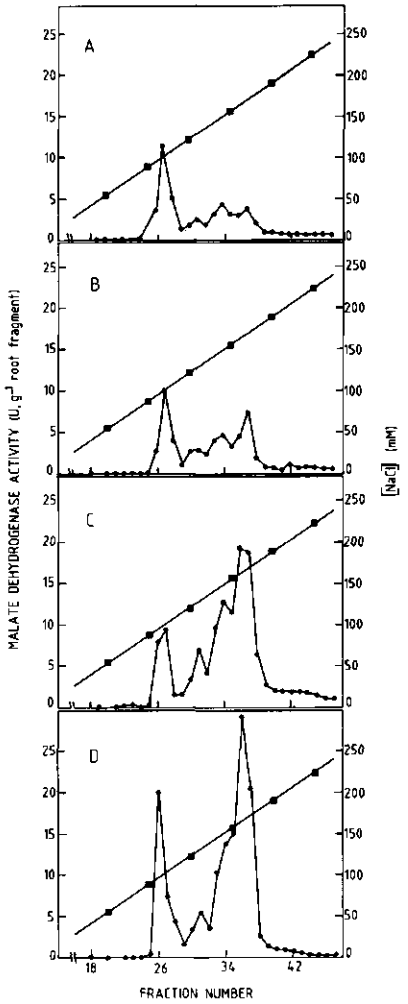

Fig. 3. *Elution profile of malate dehydrogenase activity from Mono Q of root fragments with developing nodules harvested at different times following inoculation of* P. sativum *with* R. leguminosarum *PRE.* (A) Cytoplasmic fraction of root fragments harvested 8 days after inoculation. (B) Cytoplasmic fraction of root fragments harvested 15 days after inoculation. (C) Cytoplasmic fraction of root fragments harvested 19 days after inoculation. (D) Cytoplasmic fraction of root fragments harvested 25 days after inoculation. The root fragments consisted of 2.5-cm pieces of the main roots where nodules normally appear. The root fragments were extracted and fractionated as described for nodules. Malate dehydrogenase activity was measured by the reduction of oxaloacetate. NaCl concentration was determined in the fractions (■——■)

concentration, namely 100 mM. The main malate dehydrogenase activity found in the cytoplasmic plant fraction of root nodules elutes at higher NaCl concentrations: root nodule fraction II at 135 mM NaCl, root nodule fraction III at 145 mM NaCl and root nodule fraction IV at 165 mM NaCl. These fractions: root nodule fractions II, III and IV (Fig. 1), account for more than 90% of the total activity eluted. The minute root fractions, which were eluted at the same concentration interval, corresponded to only 4% of the activity found in root nodule fractions II, III and IV. The major cytoplasmic nodule fraction: root nodule fraction IV, contributed to about 60% of the total activity eluted and the root nodule fractions II and III accounted each for about 15% of the total activity eluted.

The nodule development was also followed. At day 11 nitrogenase activity could be detected. 13 days after inoculation nitrogen fixation activity increased rapidly and reached its maximum 19 days after inoculation, whereupon it started to decrease as a result of the growth conditions of the plants. 13 days after inoculation the total malate dehydrogenase activity estimated in the cytoplasmic fraction of root fragments containing developing nodules, started to increase (Fig. 2). Until that point in nodule development no increase in malate dehydrogenase activity was observed, but already at 8 days

after inoculation root nodule fractions II, III and IV could be registered in infected roots at the location where nodules are developing and where, at day 10, nodules would be macroscopically visible (Fig. 3A). At 8 days after infection structural analysis of infected roots indicates that nodule meristems have been developed and that cell differentiation had already occurred [30]. Our results show that in this stage of nodule development the activity of the three nodule malate dehydrogenase fractions already represented 55% of the total activity. In uninfected roots this is only 4%. The first nodule-like structures visible on the root surface, did not appear until 10 days after inoculation. The activity of root nodule fractions II, III and IV increased over the subsequent days (Fig. 3B, C and D).

Experiments were carried out to ensure that the malate dehydrogenase activity observed in the cytoplasmic fraction of nodules was not caused by the presence of bacterial or bacteroid contamination or was of meristematic origin. A desalted crude extract of *R. leguminosarum* PRE, grown axenically in yeast extract/mannitol medium, was sonicated and subjected to ion-exchange chromatography. The increasing NaCl gradient eluted two peaks of malate dehydrogenase activity at concentrations of 230 mM and 320 mM (Fig. 4B). These NaCl concentrations are much higher when compared with the elution of the cytoplasmic nodule fractions which elute at 100–180 mM NaCl. Malate dehydrogenase activity present in bacteroids elutes as a single fraction from a Mono Q column at 95 mM NaCl (Fig. 4A). The activity elutes at a NaCl concentration nearly identical to that required for the main root fraction activity (see Fig. 1). The elution profile of malate dehydrogenase activity of meristematic tissue (Fig. 4C) was identical to the profile of the cytoplasmic root fraction (see Fig. 1); one main peak elutes at 95 mM NaCl.

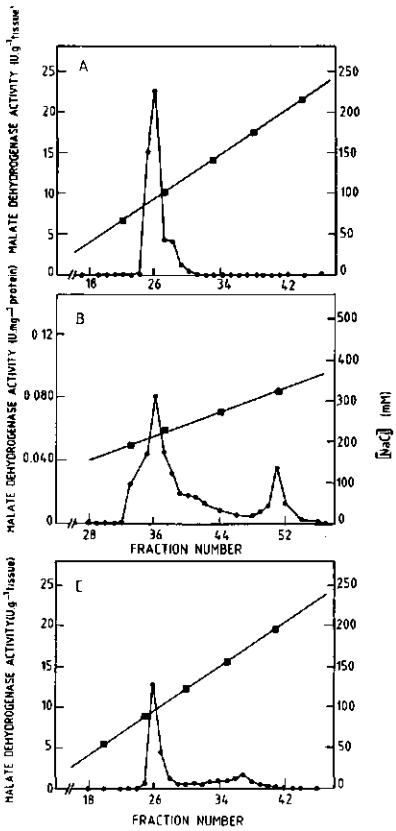

Fig. 4. *Elution profile of malate dehydrogenase activity from Mono Q of various fractions.* (A) Bacteroid fraction obtained after extraction and fractionation of root nodules harvested 19 days after inoculation. (B) Fraction obtained from *R. leguminosarum* PRE bacteria after sonication. (C) Meristematic tissue fraction obtained from germ roots of peas. The malate dehydrogenase activity was measured by the reduction of oxaloacetate. The malate dehydrogenase activity of the bacteroid fraction is correlated to the activity/g nodules which was used in the extraction and fractionation of the bacteroids. Meristematic tissue was extracted and fractionated as described for nodules. NaCl concentration was determined in the fraction eluted from the column

Electrophoresis

Using native gradient polyacrylamide gel electrophoresis the molecular mass of malate dehydrogenase in different fractions was determined. Malate dehydrogenase was made visible using an electron-transfer dye staining method. The native relative molecular masses of malate dehydrogenase of root nodule fraction I, root nodule fraction IV and the major root fraction differ from each other to a small extent; the estimated native molecular masses were respectively 79 kDa, 71 kDa and 74 kDa (±3 kDa). On the gel the fraction containing bacteroid malate dehydrogenase produces two distinct malate dehydrogenase bands, corresponding to molecular masses of approximately 89 kDa and 139 kDa. The subunit determinations of the three plant malate dehydrogenases involved excision of the malate dehydrogenase bands after native gradient polyacrylamide gel electrophoresis, SDS/polyacrylamide gel electrophoresis and silver staining. No significant differences in molecular mass of the subunits of malate dehydrogenase present in root nodule fraction I, root nodule fraction IV and the major root fraction could be detected (39 ± 2 kDa).

Isoelectric focusing

Measurement of isoelectric points was carried out by using ultrathin-layer isoelectric focusing in polyacrylamide and agarose in combination with the electron-transfer dye staining method. Root nodule fraction IV has a different isoelectric point (pI = 4.7) when compared with root nodule fraction I (pI = 4.9) and the major root fraction (pI = 5.0). After focusing, the bacteroid fraction was divided in two malate dehydrogenase bands; one band possesses an isoelectric point comparable to root fraction I and to the main root form (namely pI = 4.9), the second malate dehydrogenase band was detected at a more basic point (pI = 5.5).

Kinetic properties of malate dehydrogenases present in different fractions of root and root nodule cytoplasm

Some kinetic properties of malate dehydrogenase present in the different cytoplasmic fractions have been determined with respect to the regulation of the activity. The results were analyzed as described by Dalziel [27]. The K_m values for all four substrates of malate dehydrogenase activity present in root nodule fraction IV were lower than those of the main root fraction (Table 2). The K_m values of the root nodule

Table 2. *K_m values of malate dehydrogenase fractions*
Fractions were collected after ion-exchange chromatography on Mono Q of the soluble cytoplasmic plant proteins of nodules (root nodule fractions I and IV) and after applying soluble cytoplasmic proteins of uninoculated main roots on Mono Q (main root fraction). In the same way ther meristematic tissue fractions were obtained. Data were analyzed and described by Dalziel [27]. Substrate coefficients were estimated at the 99% level of significance

Fraction	K_m (malate)	K_m (NAD^+)	K_m (oxaloacetate)	K_m (NADH)
	mM		µM	
Root nodule fraction I	9.7	1.4	65	39
Root nodule fraction IV	2.6	0.027	18	13
Main root fraction	64	4.4	89	70
Meristematic	7.8	0.93	94	63

Table 3. *Dissociation constants for the inhibitor complexes malate-dehydrogenase-NAD^+ and malate-dehydrogenase NADH-NAD^+ in the presence of 50 µM NADH of root nodule fractions I, IV and the main root fraction*
The initial rates were measured by varying concentrations of oxaloacetate and NAD^+ in the presence of a fixed concentration NADH. Lineweaver-Burk plots reveal linear relationships. The dissociation constants were obtained by plotting the slopes and intercepts of the Lineweaver-Burk plots against NAD^+ concentration using linear regression analyses. The dissociation constants were estimated at a 99% level of significance

Fraction	K_i (NAD^+)	K_i (NADH, NAD^+)
	mM	
Root nodule fraction I	2.74	1.70
Root nodule fraction IV	3.77	3.91
Main root fraction	1.53	1.54

fraction I were in between the values of the main root fraction and root nodule fraction IV. The K_m values of meristematic tissue are comparable with the K_m values obtained for root nodule fraction I.

The effect of pH variation on malate dehydrogenase activity in both directions was studied. No differences could be detected in initial rate versus pH profile under saturating substrate concentrations between malate dehydrogenase present in root nodule fraction I, IV and the main root fraction (not shown). At physiological pH under saturating substrate concentrations the activity measured as the oxidation of malate of all the fractions amounts to about 5% of the activity measurement as the reduction of oxaloacetate.

The relative proportion of NADH to the total concentration of NADH and NAD^+ can serve as metabolic regulator [31–33]. The effect of the NAD^+ mole fraction has been tested on the reduction of oxaloacetate by malate dehydrogenase present in the different fractions. The NADH/NAD^+ ratio was varied from 0.01 to 0.2 mol/mol with a fixed NADH concentration of 50 µM. The results (Table 3) point to the characteristics of non-competitive inhibitions. The K_i values estimated are in the same order of magnitude.

The possibility was examined that regulation of the reduction of oxaloacetate occurs by formation of an abortive complex of malate dehydrogenase with NADH and malate.

In the case of root nodule fractions I and IV, $\Phi_{oxaloacetate}$ increases at malate concentrations which are regular for root nodules (Fig. 5). This is indicative for the formation of an abortive complex of malate dehydrogenase with NADH and malate as discussed by Dalziel [27]. On the other hand the increase of malate does not find the same response with $\Phi_{oxaloscetate}$ for malate dehydrogenase present in the main root fraction as compared to the other fractions. The other Φ parameters remain constant or increase within the limit of a factor of three. This applies for all the three fractions (not shown).

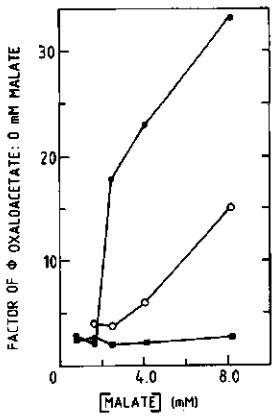

Fig. 5. *Formation of the abortive complex malate-dehydrogenase NADH-malate measured by the Dalziel substrate coefficient $\Phi_{oxaloacetate}$ for root nodule fraction I (●——●), root nodule fraction IV (○——○) and for the main root fraction (■——■).* The substrate coefficient $\omega_{oxaloacetate}$, which is a parameter for the abortive complex mentioned, was determined as described by Dalziel [27] in the presence of different malate concentrations. The substrate coefficient is expressed as factor for the $\Phi_{oxaloacetate}$ in the absence of malate

DISCUSSION

The nodule-specific malate dehydrogenase

The results reported here demonstrate the existence of nodule-specific malate dehydrogenase. Subjecting plant cytoplasm of nodules to ion-exchange chromatography, malate dehydrogenase activity was eluted in four distinct peaks (Fig. 1). Analyses of the cytoplasm of uninoculated roots by the same method gave only one major peak. It is also very unlikely that the root nodule fractions II, III and IV are of bacteroid, bacterial or of meristematic origin. During the extraction and fractionation of the root nodules 0.47 M sucrose was present. At this sucrose concentration the osmotic stability is preserved. Bacteroid malate dehydrogenase can be distinguished from the root nodule fraction by its native molecular mass, its isoelectric point and by the elution characteristics from a Mono Q column. If part of the bacteroids had collapsed during extraction and fractionation of the nodules, the two malate dehydrogenases determined in the bacteroid fraction by native gel electrophoresis and by isoelectric focusing would also be detected in the cytoplasmic plant fraction of root nodules. This is not observed. For the same reasons the possibility can also be excluded that root nodule fractions are of bacterial origin (see Fig. 4B). Root nodule fractions II,

III and IV cannot be of meristematic origin, since the cytoplasm of meristematic tissue contains, like normal root cytoplasm, no or minute amounts of root nodule fractions II, III and IV (Fig. 4C).

The origin of root nodule fraction I is not clear. As discussed above it is very unlikely that rood nodule fraction I is of bacteroid origin nor is it identical to the main cytoplasmic root fraction of malate dehydrogenase; it elutes at a slightly higher salt concentration from a Mono Q column and the estimated K_m values differ. From the kinetic properties it is possible that the major malate dehydrogenase in cytoplasm of meristem is the same protein as root nodule fraction I (see Table 2). During nodule development a considerable part of the nodules is meristematic tissue.

Kinetic properties of the nodule-specific malate dehydrogenases

The results reported in this paper point to the existence of malate dehydrogenase forms within the plant cytoplasm of pea root nodules which are not present at a significant amount in the root cytoplasm of uninfected plants.

Two of these nodule-associated forms: root nodule fractions I and IV, have lower K_m values for NADH and oxaloacetate than the main cytoplasmic root form (Table 2). This holds especially for root nodule fraction IV. Owing to these low K_m values the root nodule forms have the potency to generate a high rate of reduction of oxaloacetate at low NADH and oxaloacetate concentrations. Such a high rate is a requirement to meet the demands for malate of the bacteroid and the infected plant cell.

Owing to the lower K_m values for NADH and oxaloacetate it is possible that malate dehydrogenase will decrease the oxaloacetate concentration in the root nodule cytoplasm dramatically. Without regulation of the conversion of oxaloacetate by malate dehydrogenase, the oxaloacetate concentration will be very low and insufficient for other processes in root nodule cells like assimilation and transport of fixed nitrogen.

There is evidence that pyridine nucleotides can serve as metabolic regulators. However, it is not the single cofactor that is effective; it is the relative proportions of the cofactors expressed as the mole fraction that is important [31–33]. In view of the necessary regulation of the conversion of oxaloacetate by the root nodule malate dehydrogenase, the effect of a varied $NADH/NAD^+$ ratio (0.01–0.2 mol/mol) was studied at the physiological NADH concentration of 50 μM. Simple non-competitive inhibition patterns for root nodule fraction IV and the main root fraction and a mixed non-competitive inhibition pattern for root nodule fraction I were obtained. The relative high K_m values, which are all in the millimolar range, make a physiological function unlikely for the $NADH/NAD^+$ ratio in the regulation of the conversion of oxaloacetate by the enzymes.

The validity of a two-substrate compulsosry-order ternary-complex mechanism has been proved for pig heart mitochondrial malate dehydrogenase [34]. The cofactor, either NADH or NAD^+, acts as compulsory first substrate. It is suggested that the active site of malate dehydrogenase is such that the cofactor itself forms part of the binding site for the other substrate or that binding of the cofactor triggers a conformational change which then exposes the binding site for the other substrate. Thus a binding of malate to the complex of malate dehydrogenase with NADH or a binding of oxaloacetate to the complex of malate dehydrogenase with NAD^+, diverts the enzymes from the reaction pathway, forming an abortive complex. At the same time malate or oxaloacetate may act as product inhibitor, amplifying the formation of the abortive complex. Formation of a complex of malate dehydrogenase with NADH and malate would lead to inhibition and thereby to regulation of the conversion of oxaloacetate by malate dehydrogenase. Analysing the kinetic data as described by Dalziel [27] the formation of such an abortive complex is perceptible in an increase of $\Phi_{oxaloacetate}$, which parameter is contributed by the complex of malate dehydrogenase with NADH. At high malate concentrations the reduction of oxaloacetate by root nodule fractions I and IV was inhibited by the formation of the abortive complex of malate dehydrogenase with NADH and malate; $\Phi_{oxaloacetate}$ increases (Fig. 5). Unlike the root nodule forms, the main root form does not make this abortive complex to a significant extent with malate.

Root nodule fractions I and IV have lower K_m values for malate than the main root fraction (Table 2). This holds especially for root nodule fraction IV which showed a 25-fold lower K_m for malate (2.6 mM) than the main root fraction for these substrates. The estimated K_m values for malate are in accordance with the extent of formation of the abortive complex by the different root nodule fractions and the main root fraction.

A malate concentration of 3–7 mM is thought to be physiological for root nodules [17–20, 35]; for example the concentration of malate within the plant cytoplasm of pea nodules is estimated to be at least 3.4 mM [17]. Therefore the conversion of oxaloacetate by these root nodule forms might be regulated by the malate concentration in the cytoplasm.

Is the nodule-specific malate dehydrogenase a noduline?

Considering all the data described above the role of cytoplasmic malate dehydrogenase in symbiosis can be suggested. The physiological functions of the nodule-specific malate dehydrogenases satisfy the demand for malate as energy source for the bacteroid and the infected plant cell by allowing a high rate of oxaloacetate reduction by the enzyme and at the same time meet the requirement of a sufficient oxaloacetate concentration for assimilation and transport of fixed nitrogen by regulating the conversion of oxaloacetate via an abortive complex of malate dehydrogenase with NADH and malate.

Molecular biological analyses have revealed that there are over 20 different proteins that are specifically associated with the symbiotic process and which are encoded by the plant genome. These nodule-specific proteins are called nodulins [36, 37]. In addition to the nodule-specific proteins there are nodule-stimulated proteins: proteins which are present in both root and nodule but whose frequency in nodules is higher than in roots. Total or partial repression of plant proteins has also been detected. In pea nodules all these phenomena have been established [38, 39]. The nodule-associated malate dehydrogenases, reported here, can be classified as nodule-stimulated proteins. This can be a prejudgement because the infection of plants by fungal, viral or bacterial agents induces non-specific responses. In this view the nodule-associated malate dehydrogenases may only reflect an overall higher biosynthetic activity of the infected plant cell and may not merely be related to the nitrogen fixation process.

The nodule-associated malate dehydrogenases may be the products of separate genes or represent post-translational modifications of a single gene product. Post-transcriptional modification of some nodulin genes have been established [39, 40].

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3. KINETIC PROPERTIES AND FUNCTION OF GLUTAMATE OXALOACETATE TRANSAMINASE IN THE SYMBIOSIS BETWEEN *PISUM SATIVUM* AND *RHIZOBIUM LEGUMINOSARUM*

SUMMARY

The glutamate oxaloacetate transaminase (GOT) activity (EC 2.6.1.1) increases during nodule formation of *Pisum sativum*. Fractionation of root nodule cell extracts demonstrated that the increase in activity was due to the increase of the cytoplasmic forms of GOT and not of the plastid or mitochondrial forms. By using ion-exchange chromatography, the presence of two forms of GOT in the cytoplasm of *Pisum sativum* root nodules cells has been established. The major form of root nodule cells was only present in a small quantity in the cytoplasm of root cells.

The kinetic properties of the different cytoplasmic forms of GOT were studied. The K_m-values for glutamate (27 mM), oxaloacetate (0.14 mM) and aspartate (4.9 mM) of the nodule-stimulated form are much higher than those of the main root form (7.9, 0.019, 0.84 mM respectively). The nodule-stimulated form and the main root form have nearly a similar K_m-value for 2-oxoglutarate (0.23 against 0.21 mM respectively). The K_m-values of the minor root nodule form are comparable with the K_m-values of the main root form. The product inhibition constants of the nodule-stimulated GOT are higher than the constants estimated for the other GOT forms. Malate is identified as an inhibitor of GOT.

Using the determined and calculated kinetic constants, the overall rate equation for GOT was worked out. The initial response of the velocity to infinitesimal changes in substrate concentrations at physiological concentrations were determined. It is demonstrated that the nodule-stimulated form facilitates a high rate of catalysis in both directions of the GOT reaction as compared with the other two GOT forms. This property makes the nodule-stimulated form well-suited for functioning in the increased metabolism of root nodules.

Results are presented which indicate that a malate/aspartate shuttle can operate between the cytoplasm of the plant cells and the bacteroids. It is proposed that the main function of the nodule-stimulated cytoplasmic form of GOT is to participate in this shuttle.

Enzymes

Glutamate oxaloacetate transaminase (EC 2.6.1.1); malate dehydrogenase (EC 1.1.1.37); malic enzyme (EC 1.1.1.39); nitrogenase (EC 1.18.6.1)

INTRODUCTION

The genus *Rhizobium* contains bacteria which are able to induce nodules on roots of members of the *Leguminosae*. Nodule formation results in an arrangement of plant cells containing pleomorphic rhizobial cells termed bacteroids and uninfected plant cells (for a review see reference 1). The bacteroids are enclosed by the so-called peri-bacteroid membrane. The bacteroids are the site of nitrogen fixation. The reduction of dinitrogen by the enzyme nitrogenase requires MgATP and a reductant. Both must be generated by the bacteroids. To achieve this, the bacteroid must be supplied by the plant with oxygen and an oxidizable substrate. Sucrose is thought to be the primary source of carbon for the nodules (2,3). Since the utilization of carbohydrates by *Rhizobium* bacteroids is limited (4-6) and nitrogen fixation by isolated bacteroids is only detected with C_4-dicarboxylic acids (4,7,8), it means that the plant must convert sucrose into C_4-dicarboxylic acids to support nitrogen fixation in the bacteroid. The predominant C_4-dicarboxylic acid in root nodule cells is malate (9,10), which is synthesized by the action of malate dehydrogenase (EC 1.1.1.37) and phosphoenolpyruvate carboxylase (EC 4.1.1.31).

The plant also has the necessary enzymes to assimilate NH_3 formed by the action of nitrogenase. Ammonia is excreted by the bacteroids into the host-cell cytoplasm (11,12). The excreted ammonia is assimilated by the action of glutamine synthetase (EC 6.3.1.2). Glutamine is metabolized and transported as amide or ureide - depending on the type of plant - towards the xylem of the host plant (13). Scott *et al.* (14) and others (for a review see 13) proposed a pathway for amide formation in which oxaloacetate and 2-oxoglutarate function as carbon skeletons for the production of asparagine and glutamine. Studies using ^{15}N-tracer techniques (15,16) and ^{14}C-labels (17,18) support this proposed pathway. The end products of the proposed pathway are asparagine and glutamine. In this proposed pathway oxaloacetate is used in the reaction catalyzed by GOT to produce aspartate, the precursor of the synthesis of asparagine. GOT has been studied in the symbiotic associations *Lupinus angustifolius* with *Rhizobium lupini* (19) and *Glycine max* with *Bradyrhizobium japonicum* (20). In both symbiosis a nodule-specific form of GOT has been identified which is thought to function in the aspartate/asparagine biosynthesis.

However in alfalfa nodules exogenously supplied ^{14}C-aspartate is rapidly metabolized to malate, succinate and fumarate (21). The expected conversion of ^{14}C-aspartate to ^{14}C-asparagine took only place in case GOT was inhibited by the addition of a specific inhibitor of GOT, aminooxyacetate. Other studies (18, 22) confirm these findings. These observations suggest that GOT acts rather in the direction of oxaloacetate than in the formation of aspartate. Another unsolved problem is the low in vitro activity of asparagine synthetase (EC 6.3.1.1) (18,22,23), which makes the proposed pathway for amide formation even more ques-

tionable.

To give an answer to the question of the role of GOT in the symbiosis, particularly the role in the carbon metabolism related with the formation of oxidizable substrates for the bacteroids and the role in the ammonia assimilation, the kinetic properties of the different forms of GOT present in root nodule cells were studied.

MATERIALS AND METHODS

Growth conditions of the plants

Root nodules were produced under controlled conditions on *Pisum sativum* cv. Rondo by inoculation with *Rhizobium leguminosarum* strain PRE as described previously (24).

Preparation of extracts

Fractions containing bacteroid, bacterial, meristematic and cytoplasmatic plant proteins were prepared as described earlier (24).

Fast protein liquid chromatography

Desalted samples were analyzed using a Q-Sepharose column (bed volume 16 ml; FPLC system of Pharmacia) equilibrated with 50 mM Tris/HCl, pH 7.4. About 2% of the protein load capacity of the column was utilized. The proteins were eluted at 2.0 ml/min with a NaCl gradient. The increase in the NaCl concentration was 1 mM/ml. Fractions of 7.0 ml were collected and analyzed for GOT activity. The recovery of GOT activity was 85 $\pm$ 10 %.

Localization studies

Nodules were gently pressed in a chilled mortar with isolation buffer (approximately 2 ml/g fresh material) and filtrated through Miracloth. The isolation buffer contained 50 mM Tes/KOH, 16 % w/v sucrose, 0.9 % w/v glucose, 5 mM dithiothreitol and 1 mM EDTA, final pH 7.4. To remove the bacteroids the homogenate was centrifuged 5 min at 5,000xg. 1 ml Of the supernatant was applied to a continuous sucrose gradient (23-55 % w/v) containing 50 mM Tes/KOH and 5 mM dithiothreitol, pH 7.4. The gradient was layered on a sucrose cushion of 80 % w/v. Gradients were centrifuged 5 h at 100,000xg at 4 °C. 1.5 ml Fractions were collected and sonicated two times 20 s with an amplitude of 26 µm and a frequency of 23 kHz in a Soniprep 150 Ultrasonic disintegrator (MSE). After centrifugation the fractions were examined for GOT, triose-phosphate isomerase (EC 5.3.1.1) and fumarase (EC 4.2.1.1) activity. Triose-phosphate isomerase was considered as marker for the cytoplasm and for the plastids; fumarase was regarded as a marker for the mitochondria. Triose-phosphate isomerase was measured according to Beisenherz (25). Fumarase

was monitored at 240 nm by the formation of fumarate. The assay consisted of 100 mM K_2HPO_4 and 50 mM malate, pH 7.6. The variation in the results among the several experiments was neglectable. Fractions containing transaminase activty were analyzed using an analytical Mono Q column equilibrated with 20 mM Tris/HCl, pH 7.4 (FPLC system of Pharmacia). The proteins were eluted with a NaCl gradient (10 mM/ml). Fractions of 0.5 ml were collected and analyzed for GOT activity.

Assay for nitrogenase

The nitrogenase activity of bacteroids was measured as described earlier (24).

Assay for GOT

The GOT activity was measured routinely in a MDH-linked reaction system. The incubation mixture contained 50 mM K_2HPO_4, 45 mM aspartate, 10 mM 2-oxoglutarate, 0.1 mM NADH, 7.5 U/ml MDH, final pH 7.4 at 25 °C. The rate of disappearance of NADH was monitored at 340 nm before and after addition of substrate. The former rate served as a measurement of background NADH oxidation (if present) which was substracted from the rate of substrate-dependent activity. Initial reaction rates have been shown to be proportional to the concentration of enzyme under the conditions used in these experiments. Additions of the coenzyme pyridoxal-5'-phosphate to the reaction system was not necessary for maximum enzyme activity.

The reaction was also followed spectrophotometrically after Henson and Cleland (26) at 260 nm, which wavelength is the maximum of the difference spectrum of the enol form of oxaloacetate and 2-oxoglutarate. The keto tautomer of oxaloacetate is the enzymatic active form of oxaloacetate. The measured reaction rates showed no initial lag; this observation indicates that the enolization rate exceeded the reaction rates under the conditions used. The reaction mixture for measuring the formation of glutamate and oxaloacetate contained 50 mM K_2HPO_4, 45 mM L-aspartate and 10 mM 2-oxoglutarate, final pH 7.4. For the reverse reaction the incubation mixture consisted of 50 mM K_2HPO_4, 100 mM glutamate and 0.2 mM oxaloacetate, final pH 7.4 at 25 °C. In these assays the initial reaction rates have been shown to be proportional to the concentration of the enzyme. The concentrations of the substrates, particular 2-oxoglutarate and oxaloacetate, were low enough that substrate inhibition was not observed. The amount of oxaloacetate and glutamate formed in the transamination reaction was stoichiometric with the amount of aspartate and 2-oxoglutarate consumed.

For the kinetic measurements of the GOT activity, conditions were the same as described for the activity assays except that the substrate concentrations were varied and/or inhibitors added. The data were fitted to several kinetic models using a computerized nonlinear regression routine called Rosfit (27). The data were weighted by the square of the reciprocal of the calcu-

lated velocity. The weights were normalized. Discrimination between the various kinetic models was accomplished on several goodness-of-fit statistics (the final weighted sum of squares, the residual mean square), the final parameters with their asymptotic standard errors, the 95 % confidence limits for the parameters and the velocity *versus* substrate concentration plots. The preferred model had the smallest residual mean square error, no negative or zero value for the parameters; the 95 % confidence limits did not encompass zero.

For the K_m estimations, a range of suitable concentrations of each substrate was chosen and the rate was measured with all the possible combinations of these substrates. The data obtained were only consistent with a Ping Pong Bi Bi mechanism. The ratio of maximum velocities in forward and reverse direction was determined in a single experiment. The validity of the estimated ratio and the obtained K_m-values were checked by calculating the K_{eq} from the kinetic Haldane relationship for each GOT fraction and considering their mutual equality.

The product inhibition by aspartate and glutamate was determined by measuring the velocities in presence of various concentrations of both substrates and products. The data were treated by construction of double-reciprocal plots, evaluation of the slope and intercepts and making replots of slope and intercepts *versus* inhibitor concentration. Equal variance of the velocities was assumed. The product inhibition constants for the keto acids were calculated from the appropriate Haldane relationships (26), using the estimated K_m-values and the obtained product inhibition constants for glutamate and aspartate.

The inhibition of GOT by aminooxyacetate was studied by varying the aspartate concentration and holding the 2-oxoglutarate concentration saturated in presence of different aminooxyacetate concentrations. The inhibition of GOT by malate was performed in the same way, however the inhibition pattern of malate towards all the four substrates was determined.

The substrate and inhibitor concentrations (except aminooxyacetate) were determined by enzymatic analysis with the appropriate enzymes.

Response of the velocity to changes in substrate concentration

The initial change in the velocity caused by infinitesimal changes in the substrate concentration were calculated by partial differentiation of the complete rate equation with respect to the substrate in question ($\delta v/\delta S$)(46). Using the nomenclature of Cleland (47), the rate equation for GOT in presence of the inhibitor malate (I) which binds only to E, is

$$v = \frac{V_1(AB - \frac{PQ}{K_{eq}})}{K_bA + K_aB(1+\frac{I}{K_{ii}}) + \frac{K_{ia}K_bP}{K_{ip}}(1+\frac{I}{K_{ii}}) + \frac{K_{ia}K_aQ}{K_{iq}} + \frac{K_{ib}K_aPQ}{K_pK_{iq}} + \frac{K_bAP}{K_{ip}} + \frac{K_aBQ}{K_{iq}} + AB}$$

The term $\delta v/\delta S$ was also determined as the tangential slope of the plot of v *versus* S in a chosen value of S, using a computer. No differences between both methods were observed. The concentrations of glutamate, oxaloacetate, 2-oxoglutarate, aspartate and malate were chosen to be respectively 2.9, 0.02, 0.044, 3.2 and 5 mM. The concentrations of glutamate, aspartate and malate are thought to be physiological (9,10,36,37). The activity of the different GOT fractions is expressed as the units present per mg cytoplasmic protein.

The elasticity coefficients being the ratio of the fractional response of the velocity $\delta v/v$ to the causative fractional change in substrate concentration $\delta S/S$, was calculated by the product of $(\delta v/\delta S)$ (S/v), according to Kacser and Porteous (46). The coefficients were determined at the concentrations mentioned for the non-fractional response, but the concentration of the perturbing substrate in question was raised 1 %.

Electrophoresis

Non-denaturing linear gradient electrophoresis were conducted as described earlier (24). GOT fractions were made visible by a staining on oxaloacetate according to Decker and Rau (28). The staining was substrate dependent. Standard proteins used for molecular mass determinations were stained with Coomassie Brilliant Blue.

Malate/aspartate shuttle

The bacteroids were isolated from the nodules as described earlier (29) but the bacteroids were washed twice instead of once. The incubation medium contained 50 mM Tes/KOH, 5 mM $MgSO_4$, 0.2 mM myoglobin, 2.5 % w/v fatty acid free serum albumin, 16 % w/v sucrose, pH=7.4 at 30 °C. Myoglobin was oxygenated as described by Wittenberg *et al.*(30). The substrates concentrations were 5 mM. The changes in the external concentrations of the substrates and the nitrogenase activity were estimated in time during 20-30 min at 30 °C. The incubations were terminated in two ways. The reaction mixture was chilled by placing the assay bottles in ice after which the bacteroids were removed by centrifugation. The other method consisted of quenching the reactions with $HClO_4$. Denatured protein was removed by centrifugation whereupon the supernatant was neutralized. Malate and 2-oxoglutarate were determined with the appropriate enzymatic assay and the amino acids by using a Biotronik LC 6000E analyzer equipped with a Durrun DC 6A ion-exchanger (physiological run). No significant differences between both methods were observed. The changes in concentrations as well as the nitrogenase activity exhibited a linear relationship with the incubation time; the changes were estimated at the 99 % level of significance. The respiration of the bacteroids catabolizing the different substrates was measured by using a Gilson-water jacketed oxygraph equipped with a polarographic Clark electrode.

Malic enzyme

The reaction mixture contained 5 mM malate, 0.5 mM NAD^+ or $NADP^+$, 6 mM $MnCl_2$, 70 mM KCl, 50 mM Tes/NaOH, final pH 7.4 at 25 °C. The reaction was terminated by addition of $HClO_4$, centrifuged, neutralized whereupon the reaction mixture was examined for pyruvate using an enzymatic assay with lactate dehydrogenase. Each velocity was determined by stopping five independent incubations at different times. Each datum point was estimated in duplicate or triplicate. Enzyme activity was linear in time for at least 10 min. Significant substrate inhibition was observed at malate concentrations higher than 10 mM. The rate was proportional to the enzyme concentration. The K_m-value for malate was estimated as described for GOT.

MDH

MDH activity was measured as described earlier (24).

Protein determinations

The protein determinations were determined by the method of Sedmark and Grossberg (31). Bovine serum albumin was used as standard.

Chemicals

L-Aspartate, L-glutamate, 2-oxoglutarate, aminooxyacetate, Fast Blue BB, fatty acid-free Bovine serum albumin and Tes were obtained from Sigma; oxaloacetate, NADH, NAD^+, NADH, $NADP^+$ from Boehringer and L-malate from Merck. All other chemicals were of the highest analytical grade available.

RESULTS

GOT activities present in root and root nodule cells

The total GOT activity of root nodules per g fresh tissue weight was about ten times higher than the activity found in roots (respectively 8.8 ± 0.7 against 0.79 ± 0.09 U/g fresh tissue). The GOT activities present in the cytoplasmic plant fraction of root nodules and in the sonicated bacteroid fraction were in the ratio of 4 to 1; the ratio of the specific GOT activities of the cytoplasmic plant fraction and the bacteroid fraction was 8 to 1 (1.15 ± 0.04 against 0.15 ± 0.02 U/mg protein). Bacteroids were removed from an extract of root nodules cells by a low speed centrifugation. The remaining plant fraction of a root nodule extract was fractionated by a continuous density gradient. Nearly 90 % of the GOT activity was recovered in the cytoplasmic fractions (Table 1). The remaining 10 % was mainly found in the mitochondrial fractions. Nearly the same distribution of GOT activity between mitochondrial fractions and cytoplasmic fractions was found for extracts of uninoculated roots. The specific GOT activity in the cytoplasmic plant fraction of root nodules (1.15 ± 0.04 U/mg protein) was about 2 times higher than the activity found in the cytoplasmic fraction of uninoculated roots (0.65 ± 0.03 U/mg protein).

Table 1. Distribution of GOT activity among mitochondria, plastids and cytoplasm. Root nodule extracts from which the bacteroids were removed by a low speed centrifugation, were subjected to sucrose gradients as described in Materials and Methods.

Fraction	Density	Distribution of the enzyme activity of:		
		triose-phosphate isomerase	fumarase	GOT
	(g/ml)	(%)	(%)	(%)
cytoplasmic fraction	1.10-1.14	74	39	89
mitochondrial fraction	1.20-1.22	10	41	8
plastid fraction	1.23-1.25	16	20	3

GOT in the cytoplasmic fraction of root nodule cells and of the cytoplasmic fraction of cells of primary roots of uninfected plants were separated by ion-exchange chromatography. The elution profile of GOT activity present in the cytoplasm of root nodule cells showed a main peak which eluted at 130 mM NaCl; this peak (root nodule fraction II) accounted for 79 % of the activity eluted (Figure 1). A minor peak eluted at 85 mM NaCl (root nodule fraction I) and accounted for 18 % of the activity recovered. A peak with minute activity was observed at 185 mM NaCl (root nodule fraction III). The elution profile of GOT activity present in root cytoplasm consisted also of three peaks which eluted at 80, 130 and 185 mM NaCl; the peaks correspond to respectively 71, 21 and 8 % of the activity eluted.

The presence of the different forms of GOT in root cells and root nodule cells was followed during nodule development. Structural analysis of infected roots have revealed that 8 days after inoculation nodule meristem has been developed and that cell differentiation already occurred (32). At day 10 nodule-like structures became macroscopically visible. Nitrogen fixation was detectable at day 11. Until 11 days after inoculation no increase in GOT activity was observed in the cytoplasmic fraction of root fragments containing developing nodules (Figure 2).

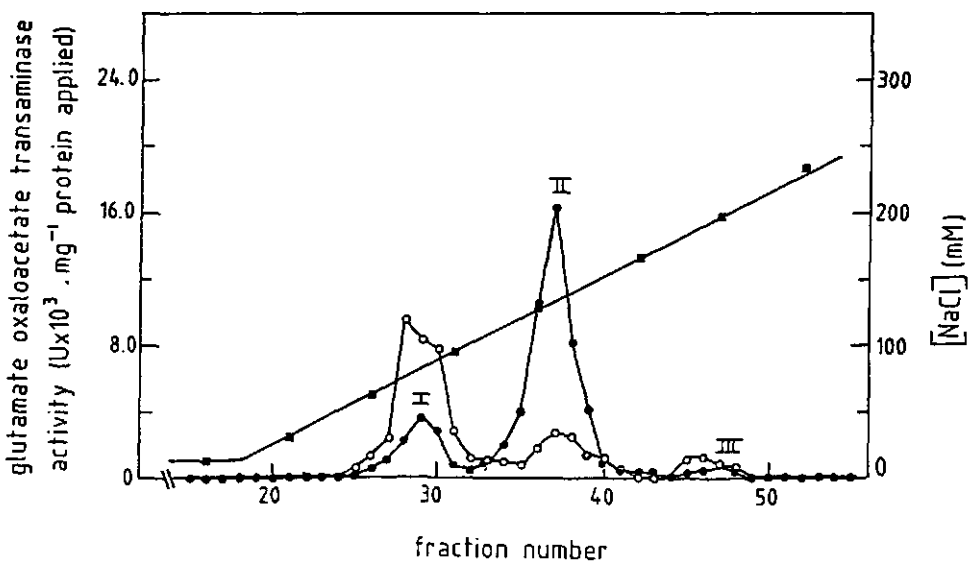


Figure 1.
Elution profile of GOT activity from Q-Sepharose of the cytoplasmic plant fraction of pea root nodules harvested 19 days after inoculation (●-●) and of the cytoplasmic fraction of uninoculated roots harvested 19 days after sowing (o-o). NaCl concentrations were determined in the fractions (▄-▄).

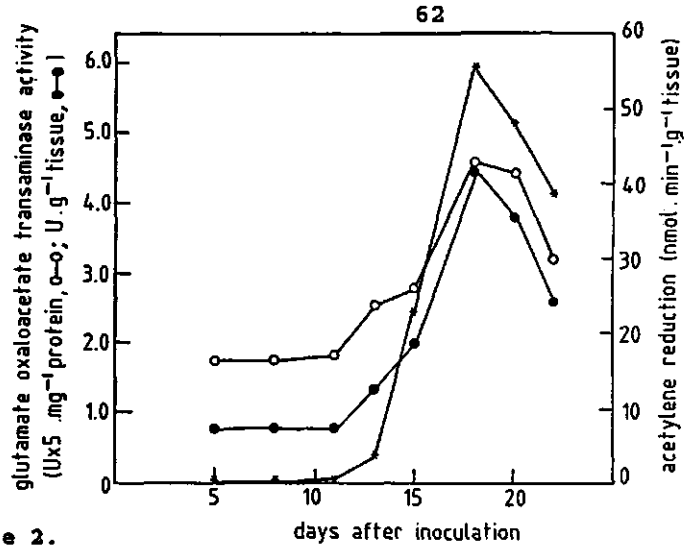
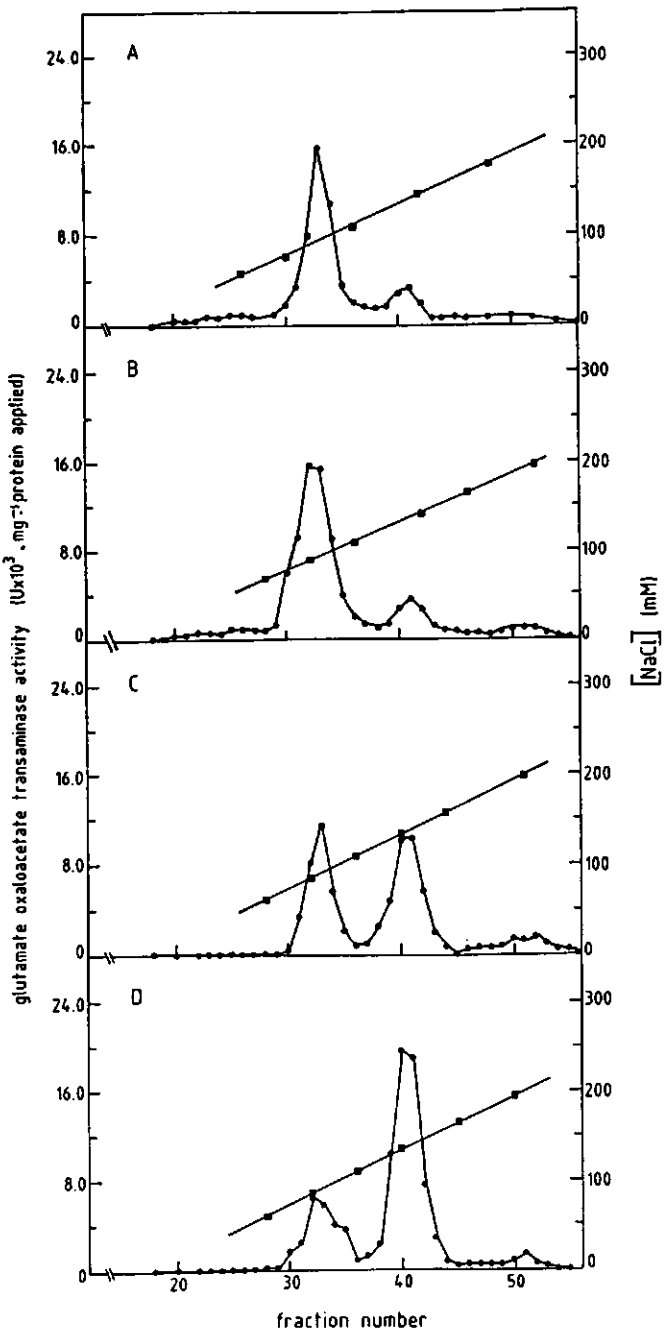


Figure 2.
Nitrogenase activity (*–*) and GOT activity based on mg protein (o–o) and g fresh tissue (●–●) of root fragments with developing nodules harvested at different times following inoculation of *Pisum sativum* with *Rhizobium leguminosarum* PRE. The root fragments consisted of 2.5 cm pieces of the main roots where the nodules normally appear. Nitrogenase activity and GOT activity were measured as described in Materials and Methods.

Thirteen days after inoculation there was a steady increase in nitrogen fixation activity. The nitrogen fixation activity reached its maximum 18 days after inoculation, whereupon it tardily started to decrease due to the growth conditions of the plants. Simultaneously with the increase in nitrogenase activity, the total GOT activity increased. With the decline in nitrogen fixation activity the GOT activity also decreased.

The elution profile of GOT at different stages of nodule development are shown in Figure 3. At day 5 the root pattern is observed (Figure 3A). The same pattern is observed at day 10 (Figure 3B). The shifting in root to root nodule pattern started at day 13 (Figure 3C). At that moment the root nodule fraction covered 49 % of the total activity eluted. The activity of root nodule fraction II increased over the subsequent days (Figure 3D).

Figure 3 (facing page).
Elution profile of GOT activity from Q-Sepharose of root fragments with developing nodules harvested at different times following inoculation. The root fragments were harvested at 5 days (A), 10 days (B), 13 days (C) and 18 days (D) after inoculation. The root fragments consisted of 2.5 cm pieces of the main roots where nodules normally appear. The root fragments were extracted and fractionated as described for nodules. NaCl concentration was determined in the fractions (■–■).

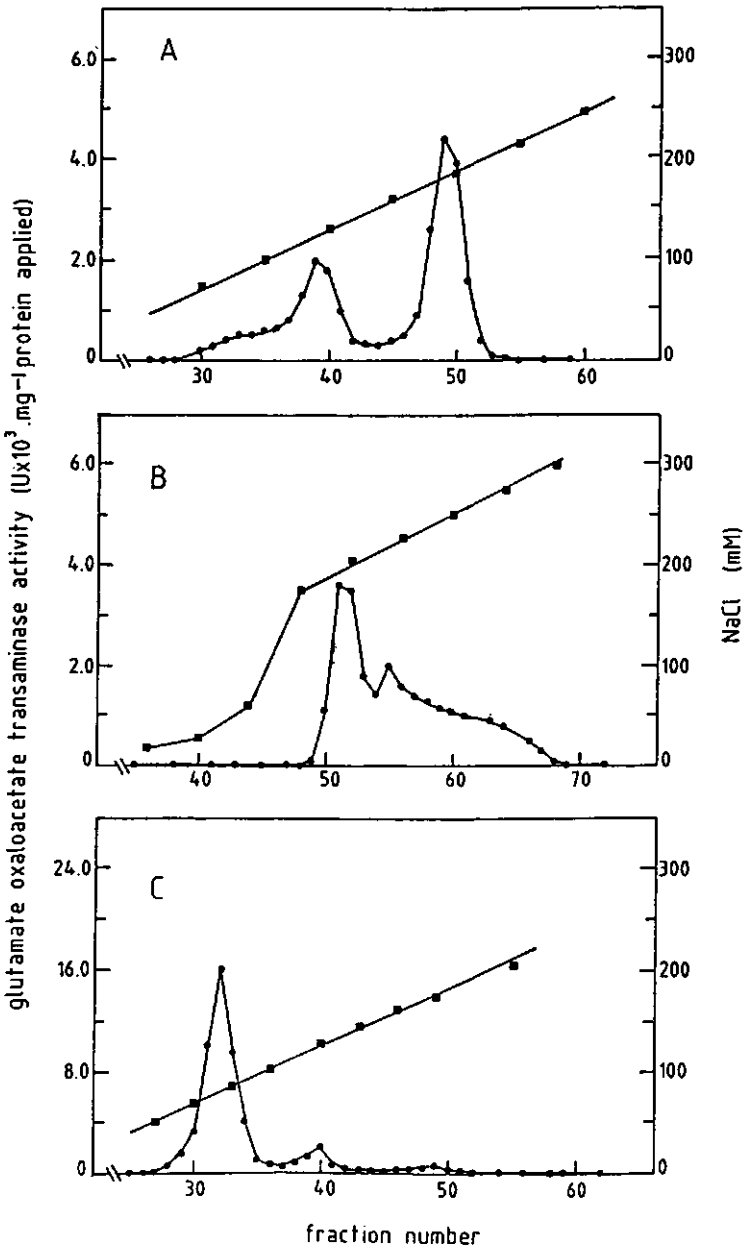

A
B
C
D
24.0
16.0
8.0
0
300
200
100
0
20
30
40
50
fraction number
glutamate oxaloacetate transaminase activity (Ux10^3.mg^{-1}protein applied)
[NaCl] (mM)

Experiments were carried out to exclude the possibility that GOT activity found in the cytoplasmic fraction of root nodule cell extracts was caused by the presence of bacteroid, bacterial or mitochondrial contamination or was of meristematic origin. The cytoplasmic plant fractions of root nodules contained on an average 4.4 ± 0.6 % of the total fumarase activity recovered, in consequence of broken bacteroids, bacteria and mitochondria. GOT present in the bacteroid fraction eluted mainly (70 %) at a NaCl concentration of 185 mM; a small part of the total activity (25 %) was recovered in a peak at 125 mM NaCl (Figure 4A). A desalted crude extract of *Rhizobium leguminosarum* PRE, grown in yeast-mannitol medium, was sonicated and subjected to ion-exchange chromatography. The GOT activity eluted at a broad interval between 190 and 300 mM NaCl. Two peaks could be distinguished at 190 and 220 mM NaCl (Figure 4B). The elution profile of GOT activity of meristematic tissue bears a great resemblance to the elution profile of the cytoplasmic fraction of uninoculated roots. One main peak, accounting for 90 % of the activity recovered, was eluted at 85 mM NaCl (Figure 4C).

Electrophoresis

The native relative molecular mass of GOT of root nodule fraction I, II and root fraction I differed from each other in a small measure; the estimated native relative molecular masses were respectively 128,000 , 125,000 and 122,000 Da (± 6,000 Da). On the gels, the fraction containing bacteroid GOT showed two distinct GOT specific bands, corresponding to relative molecular masses of approximated 78,000 and 99,000 (± 5,000 Da). The main meristematic fraction was similar in native relative molecular mass to root fraction I (122,000 Da ± 6,000 Da).

Figure 4 (facing page).
Elution profile of GOT activity from Q-Sepharose of various fractions:
A. bacteroid fraction obtained after extraction and fractionation of root nodules harvested 19 days after inoculation,
B. fraction obtained from *Rhizobium leguminosarum* PRE bacteria,
C. meristematic tissue fraction obtained from germ roots of peas.
Meristematic tissue was extracted and fractionated as described for nodules. NaCl concentrations was determined in the fractions eluted from the column (▪-▪).


A
B
C
6.0
4.0
2.0
0
24.0
16.0
8.0
300
200
100
30
40
50
60
70
glutamate oxaloacetate transaminase activity (Ux10³.mg⁻¹protein applied)
NaCl (mM)
fraction number

Kinetic properties of GOT present in different fractions of root and root nodule cytoplasm

The initial velocity patterns obtained for the different GOT fractions, matched the pattern to be expected for a Ping Pong Bi Bi mechanism. The K_m-values for oxaloacetate, glutamate and aspartate of GOT present in root nodule fraction II (respectively 0.14, 27, 4.9 mM, Table 2) were higher than those of the main root fraction (respectively 0.019, 7.9 and 0.84 mM). Only the K_m-values for 2-oxoglutarate of the three different fractions were comparable. Comparing the K_m-values of root nodule fraction I with the K_m-values determined for root fraction I, only the K_m-value for oxaloacetate differs significantly.

The equilibrium constant of the reaction catalyzed by the different GOT fractions was calculated from the K_m-values and from the ratio of the V_{max}-values in both directions (Table 2) using the appropriated kinetic Haldane relationship. The calculated K_{eq} for the three fractions was 2.4 ± 0.1 when the reaction is considered as the formation of aspartate and 2-oxoglutarate. This observation indicates the internal consistency between the obtained kinetic constants derived from a number of different experiments.

The product inhibition by glutamate and aspartate was determined in order to work out the rate equation. The results of the inhibition experiments showed that glutamate and aspartate acts as a competitive inhibitor towards each other. Glutamate is a classical noncompetitive inhibitor towards 2-oxoglutarate whereas aspartate functions in the same way towards oxaloacetate. These observations are in agreement with the theoretical predictions of Cleland (33). The dissociation constant of the enzyme-glutamate complex of root nodule fraction II (24 ± 3 mM Table 2) was about three times higher than the values estimated for the root nodule fraction I (8.3 ± 1 mM) and the root fraction (6.9 ± 0.5 mM). Also the dissociation constant of the enzyme-aspartate complex of root nodule fraction II is much higher than the values obtained for the other two fractions (Table 2). The relative low affinity of root nodule fraction II for aspartate, was confirmed using a structure-analogue of aspartate, namely aminooxyacetate (Table 2).

By combining the estimated kinetic properties with the appropriate Haldane relationships, the dissociation constants for the keto acids were calculated. These values are given in Table 2.

Malate, the predominant C_4-dicarboxylic acid in root nodules, inhibits the GOT catalyzed reaction. Analyzing the data using the routine Rosfit (27), the mode of inhibition with respect to glutamate and aspartate was characterized as competitive; towards the keto acids the inhibition was identified as uncompetitive. The dissociation constants obtained (Table 3, page 68) indicate that root nodule fraction I is relatively more inhibited by physiological malate concentrations than root nodule fraction II.

Table 2. The kinetic constants for the different GOT fractions. The K_{is}-values for oxaloacetate and for 2-oxoglutarate as well as the K_{eq} are calculated using the appropriate Haldane relationships. The K_{ii} for malate is obtained from Table 3. The K_{eq} is expressed considering aspartate and 2-oxoglutarate as products. The value for K_{eq} is calculated using the kinetic constants of the GOT fraction in question. V_1 is the maximum velocity for the formation of aspartate and 2-oxoglutarate; V_2 is the maximum velocity in the reverse reaction. NT stands for not tested. The value between the parentheses is the standard error.

kinetic constant(*)	root nodule fraction I	root nodule fraction II	root fraction I
K_m (glutamate)	9.4 (0.6)	27 (3)	7.9 (0.4)
K_m (oxaloacetate)	0.076 (0.003)	0.14 (0.01)	0.019 (0.002)
K_m (aspartate)	1.22 (0.03)	4.9 (0.4)	0.84 (0.03)
K_m (2-oxoglutarate)	0.26 (0.01)	0.23 (0.01)	0.21 (0.01)
K_{is} (glutamate)	8.3 (1)	24 (3)	6.9 (0.5)
K_{is} (oxaloacetate)	0.015	0.066	0.0081
K_{is} (aspartate)	0.54 (0.04)	6.4 (0.4)	0.51 (0.12)
K_{is} (2-oxoglutarate)	0.54	0.55	0.27
K_{ii} (aminooxyacetate)	0.025 (0.004)	0.12 (0.02)	0.021 (0.004)
K_{ii} (malate)	10 (2)	24 (5)	NT
K_{eq}	2.47	2.36	2.46
V_1/V_2	2.33	2.78	1.46
V_1	0.57	2.56	0.67
V_2	0.24	0.91	0.46

(*) The K_m and K_i-values are in mM; V_1 and V_2 are expressed in $\mu mol.min^{-1}.(mg\ cytoplasmic\ protein)^{-1}$

Table 3. The dissociation constants of the enzyme-malate complex (related to competitive inhibition) and of the enzyme-malate-substrate complexes (related to uncompetitive inhibition) of GOT present in root nodule fraction I and II. The data were processed using the routine Rosfit as described in Materials and Methods. The value between the parentheses is the standard error.

	substrate varied			
	oxaloacetate	glutamate	2-oxoglutarate	aspartate
Type of inhibition	uncompetitive	competitive	uncompetitive	competitive
	K_{I1} (mM)	K_{I2} (mM)	K_{I1} (mM)	K_{I2} (mM)
root nodule fraction I	11 (2)	7.9 (0.6)	13 (3)	8.2 (1)
root nodule fraction II	21 (2)	19 (1)	30 (4)	24 (5)

The initial changes in the reaction rate caused by infinitesimal changes in the substrate concentrations have been determined at physiological concentrations under equilibrium conditions. The concentrations used for glutamate (2.9 mM), aspartate (3.2 mM) and malate (5 mM) have been measured in root nodules (9,10,36,37). The physiological oxaloacetate concentration was assumed to be 0.02 mM, which implies that the 2-oxoglutarate concentration has to be 0.044 mM to achieve equilibrium (K_{eq} = 2.4). The quantitative responses of the local reaction rate to the infinitesimal changes in the substrate concentrations i.e. $\delta v/\delta S$, are given in Table 4. The values obtained for root nodule fraction II were - under the given conditions - at least four times higher than the values observed for root nodule fraction I. The reason is that the amount of this enzyme present in a root nodule extract is much higher than that of the other GOT fractions. The response of the reaction rate to changes in the amino acids concentrations is negligible compared with the response to changes in the keto acid concentrations. The responses of the reaction rate on perturbing changes in the substrate concentrates are not significantly influenced by changes in the malate concentration under these physiological conditions.

If the responses of the reaction rate to perturbing changes in the substrate concentrations are taken as fractional changes $(\delta v/v)/(\delta S/S)$ i.e. as elasticity coefficients, no significant differences between the different GOT fractions is noticed under the given conditions (Table 4). Also the elasticity coefficients towards the different substrates of each GOT fraction are nearly the same under the given conditions. Only when the conditions are considerably modified - for instance one of the substrate concentration is bisected - significant differences in elastic-

Table 4. The quantitative responses of the reaction rate to causative changes in the substrate concentrations $\delta v/\delta S$ and the elasticity coefficients (ε) under physiological conditions. The quantitative responses and the elasticity coefficients were calculated as described in Materials and Methods. The quantitative responses were estimated at the following concentrations: glutamate 2.9 mM, oxaloacetate 0.02 mM, aspartate 3.2 mM, 2-oxoglutarate 0.044 mM and malate as indicated. The enzyme activity is expressed as units present per mg cytoplasmic protein of a root nodule extract. The elasticity coefficients were calculated at the indicated concentrations. The concentration of the perturbing substrate was raised by 1%. The elasticity coefficient for malate was determined at the concentrations mentioned except for the glutamate concentration; the glutamate concentration was raised 1%. NT stands for not tested.

Response(*)	[malate] (mM)	Root nodule fraction I	Root nodule fraction II	Root fraction I
$\delta v/\delta$(glutamate)	0	0.0044	0.020	0.0075
	5	0.0042	0.019	NT
$\delta v/\delta$(oxaloacetate)	0	0.63	2.9	1.1
	5	0.60	2.7	NT
$\delta v/\delta$(2-oxoglutarate)	0	0.29	1.3	0.50
	5	0.28	1.2	NT
$\delta v/\delta$(aspartate)	0	0.0039	0.018	0.0067
	5	0.0037	0.017	NT
ε (glutamate)	0	105	105	105
	5	105	105	NT
ε (oxaloacatate)	0	105	105	105
	5	105	105	NT
ε (2-oxoglutarate)	0	105	97	97
	5	105	97	NT
ε (aspartate)	0	96	97	96
	5	97	97	NT
ε (malate)	5	0.044	0.060	ND

(*) $\delta v/\delta S$ is expressed as $10^{-3}.min^{-1}$

ity coefficients are observed (not shown). The elasticity coefficients towards malate of both root nodule fractions are low under the given conditions.

The operation of a malate/aspartate shuttle between bacteroids and plant cytoplasm

In mammalian cells cytoplasmic and mitochondrial GOT function in the malate/aspartate shuttle to transport reducing equivalents from the cytoplasm to the mitochondria. To examine whether a similar shuttle operates between the plant cytoplasm and bacteroids, bacteroids were incubated with different substrates at different O_2 concentrations to test the effect of the substrates on nitrogen fixation. The optimum O_2 concentration required for maximal nitrogenase activity in the assay system, is dependent on the rate of respiration of the bacteroids. The higher the rate of respiration the higher the nitrogenase activity. The rate of respiration thus reflects in the nitrogenase activity. Table 5 presents the results of the effect of different substrates and substrate combinations on the nitrogenase activity of bacteroids. The optimum pO_2 for nitrogenase activity with glutamate was 60 % of that of malate or the combination of malate plus glutamate. The substrates 2-oxoglutarate and aspartate required nearly the same pO_2 for maximal nitrogenase activity. This is about 45 % of the pO_2 for maximal nitrogenase activity with malate as substrate. When the substrate combination 2-oxoglutarate plus aspartate was used the maximal nitrogenase activity was measured at a higher pO_2. The simultaneous increase in nitrogenase activity was somewhat variable from preparation to preparation and varied between 60 % and 90 % of the malate driven nitrogenase activity.

Table 5 also shows the consumption or production of substrates under conditions of nitrogen fixation. The consumption of malate was higher in the presence of glutamate than in the case when only malate was present. The consumption of glutamate increased also in the presence of malate. When malate and glutamate are both present, aspartate and 2-oxoglutarate are formed and this is accompanied with an increase of the alanine concentration in the incubation mixture. The simultaneous addition of 2-oxoglutarate and aspartate to the incubation medium caused an increase of the consumption of both compounds and in the production of glutamate, malate and alanine.

If a malate/aspartate shuttle functions between the cytoplasm of the plant cells and the bacteroids, it is necessary that MDH and GOT are present in both the bacteroids as well as in the cytoplasm of the plant cells. GOT activities have been established in the bacteroids (0.15 ± 0.02 U/mg protein) and in the cytoplasm of root nodule cells (1.15 ± 0.04 U/mg protein). MDH activity was estimated to be 3.8 ± 1.7 U/mg protein in the bacteroids and 31 ± 5 U/mg protein in the cytoplasm of root nodules cells.

Table 5. Changes in the concentration of malate, glutamate, 2-oxoglutarate, aspartate and alanine in the incubation mixture of bacteroids under conditions of nitrogen fixation. The incubation were performed at optimum pO_2 for nitrogenase activity as described in Materials and Methods. Consumption and production are assigned to respectively negative and positive.

Substrate	Nitrogenase activity	Consumption/production of				
		Glu	Asp	malate	2-oxoglutarate	Ala
	($nmol.C_2H_4$ formed.min^{-1}.mg $protein^{-1}$)	($nmol.min^{-1}$.mg $protein^{-1}$)				
malate	54	0	0	-15	0	0
glutamate	36	-3.1	0	0	0	0
malate + glutamate	73	-31	2.0	-22	6.7	11
2-oxoglutarate	17	0	0	0	-9.8	0
aspartate	20	5.3	-1.2	0	0	0
2-oxoglutarate + aspartate	48	58	-29	4.4	-70	3.2

The bacteroids were also analyzed for enzyme activities which catalyze the conversion of malate or oxaloacetate into pyruvate. This activity is necessary for the oxidation of C_4-dicarboxylic acids in the citric acid cycle. Using a standard assay oxaloacetate decarboxylase (EC 4.1.1.3) could not be detected but malic enzyme activity (EC 1.1.1.39) was established. The activity was strongly dependent on Mn^{2+}, K^+ and NAD^+ (Table 6, page 72). The K_m for malate is low namely 0.71 $\pm$ 0.12 mM, which makes a physiological role for this enzyme plausible.

Table 6. The presence of malic enzyme activity in extracts of bacteroids. The assays were performed as described in Materials and Methods. The value between parentheses is the standard error.

Incubation mixture	Malic enzyme activity ($nmol.min^{-1}.mg\ protein^{-1}$)	
complete system (NAD^+)	197	(14)
- malate	<2	
- NAD^+	<2	
- malate, - NAD^+	<2	
- Mn^{2+}	88	(20)
- K^+	52	(19)
complete system ($NADP^+$)	32	(3)
- malate	<2	
- $NADP^+$	<2	
- malate, - $NADP^+$	<2	
- Mn^{2+}	12	(3)
- K^+	8.9	(1.5)

DISCUSSION

The nodule-stimulated GOT

The observations reported here, indicate the existence of a nodule-stimulated GOT. The GOT activity present in the cytoplasm of root nodule cells could be separated by ion-exchange chromatography into a minor peak (18 %, referred to as root nodule fraction I) and a main peak (79 %, referred to as root nodule fraction II, see Figure 1). Analyses of the cytoplasm of uninfected roots by the same method revealed an elution profile with different proportions of the peaks. The first peak eluted corresponded to 71 % of the activity recovered, the second only to 21 %. Elution profiles of GOT activity of root fragments with developing nodules harvested at different times during nodule development, showed an increasing contribution of root nodule fraction II to the total activity recovered (Figure 3). The amount of root nodule fraction II remained low until the onset of the nitrogenase activity (Figure 2), which fact pointed to a possible involvement of this enzyme activity in the ammonia assimilation and not in the general metabolism of the different

types of nodule cells. It is possible that root nodule fraction II is identical with root fraction II. For this reason the classification 'nodule-stimulated'is preferred to 'nodule-specific'.

The possibility that the cytoplasmic root nodule fraction II corresponds to bacterial or bacteroid contamination was excluded. At the sucrose concentration used - 0.47 M - the osmotic stability of bacteroids and bacteria is preserved. Bacterial and bacteroid GOT can be distinguished from the root nodule fraction II by the elution characteristics from the ion-exchange column used (Figure 4). Also the native molecular mass of bacteroid GOT differs from the fractions found in the cytoplasm of the plant cells. Fumarase - abundantly present in bacteroids and mitochondria - could only be determined in minute quantities in the cytoplasmic nodule fraction. It is therefore concluded that no major part of the bacteroids or bacteria has been collapsed during extraction and fractionation of the nodule cells.

Considering the elution profiles of the ion-exchange columns (Figure 4), it can also be excluded that root nodule fraction II is of meristematic origin. If root nodule fraction II originated from meristematic tissue, the nodule-stimulated form should already be expressed at the beginning of nodule development, which is not the case.

In the localization studies, some GOT activity was detected in the mitochondrial fractions (Table 1). The elution characteristics of this activity resembled the elution characteristics of root nodule fraction II. Since fumarase activity was also found in the cytoplasmic fractions of the gradients, the extraction of the nodule cells and the fractionation at the sucrose gradients may have caused some breakage of the mitochondria. If it is assumed that all the fumarase activity found in the cytoplasmic fractions originates from the mitochondria, it can be concluded that 19 % of the total GOT activity recovered in the plant fraction of nodules, originates from the mitochondria (8 % in the cytoplasm, 8 % in the mitochondrial fraction and 3 % in the plastid fraction). This proportion has been estimated for not inoculated pea roots by others (35). When cytoplasmic fractions were prepared by a high speed centrifugation, the fumarase activity never exceeded 5 % of the total fumarase activity detected in nodules. This observation makes a mitochondrial origin of root nodule fraction II doubtful.

A trace of GOT activity (3 %) was detected in fractions containing plastids. The distribution of triose-phosphate isomerase among cytoplasm and plastids obtained in our studies, is not unusual (34,35), therefore a complete collapsing of the plastids during fractionation is not likely. Thus it can be excluded that root nodule fraction II is localized in plastids.

The identity of root nodule fraction I is not clear. As discussed for root nodule fraction II, it is clear that this fraction is not from bacteroid, bacterial, mitochondrial or plastid origin. Root nodule fraction I and root fraction I showed great resemblances in elution characteristics from the ion-exchange columns, native molecular mass and several kinetic properties (Table 2). However both enzymes can be distinguished by the K_m-

value for oxaloacetate and by a different V_{max}-ratio.

Kinetic properties of GOT present in nodule cytoplasm

The product formation catalyzed by GOT depends on the intrinsic kinetic properties of the enzyme and on the enzyme concentration as well as on the concentrations of substrates, products and effectors in question. Comparing the quantitative responses of the reaction rate to causative changes of the substrate concentrations - $\delta v/\delta S$ - under physiological conditions (Table 4), it is clear that root fraction II can facilitate a much higher rate of product formation than the other two fractions. This potential to catalyze at a relative high rate can not be explained by specific kinetic properties. On the contrary, the relatively high K_m-values for glutamate, oxaloacetate and aspartate (Table 2) do not favour catalysis at physiological concentrations, in particular not in the direction of aspartate and 2-oxoglutarate formation. The difference in K_{ii} for malate between root nodule fraction I and II can also not be the explanation because even root nodule fraction I which has the lowest K_{ii} for malate, is not significantly inhibited by malate under the conditions described (Table 4). Consequently only the high enzyme concentration of root nodule fraction II is the cause of the relative great response of the reaction rate to changes in the substrate concentrations.

The elasticity coefficients of the substrates - $(\delta v/v)/(\delta S/S)$ - under the physiological conditions are nearly the same for the different GOT fractions. This indicates that although there are differences in the intrinsic kinetic properties (like K_m-values and K_i-values) between the different GOT fractions, the impact of these differences is limited under the given conditions. This observation confirms that the observed high rate of catalysis by the nodule-stimulated GOT fraction II is caused by the relative high concentration of this fraction.

Regulation of the rate of catalysis by malate was expected based on the observed inhibitor constants. But the low elasticity coefficients of malate implies that malate is not an efficient effector of the GOT catalyzed reaction. The relative high but physiological concentrations of both amino acids underlies the limited effect of malate.

The presence of a malate/aspartate shuttle

The results presented in table 5, indicate that malate, glutamate, aspartate and 2-oxoglutarate - separately - can be taken up and metabolized by the bacteroids. The transport of dicarboxylic acids have been established in the past (38,39). The consumption of aspartate and glutamate confirms the observations made by Salminen and Streeter by soybean bacteroids (40).

Experiments with the substrate combinations demonstrate that a malate/aspartate shuttle functions between the bacteroids and the cytoplasm of root nodule cells. The stoichiometry of the consumption and the formation of the different compounds indi-

cate that uptake and exchange mechanisms have been operative at the same time. The lack of knowledge of the different mechanisms makes it difficult to interpret the results quantitatively. The possibility of the shuttle has been suggested previously for legumes nodules (41) and for *Alnus glutinosa* nodules (42). Streeter and Salminen (43) gave some evidence for the existence of the shuttle in soybean bacteroids.

The alanine formation which is observed by the addition of the substrate combinations to the incubation medium (Table 5) can be explained. Under both conditions - malate/glutamate and aspartate/2-oxoglutarate - the glutamate and malate concentration in the bacteroids will be high. In the last case glutamate and oxaloacetate are formed by the action of GOT and malate by the action of MDH. Pyruvate is formed from malate by the action of malic enzyme, which presence in the bacteroid has been established (Table 6). Glutamate pyruvate transaminase (EC 2.6.1.2), present in the bacteroids (unpublished results), will catalyze the transamination of pyruvate to alanine by glutamate. Alanine may be transported outside the bacteroid.

With an operative shuttle both respiration and nitrogenase activity of the bacteroid were increased (Table 5). Thus the shuttle may fulfil different functions towards the bacteroid. The shuttle can be used to transport compounds into the bacteroid which can be used in the respiratory metabolism of the bacteroid. The transport of malate inside the bacteroid is important for the respiration. The presence of malic enzyme which has a low K_m-value for malate, allows the oxidation of malate to pyruvate and malate can also be oxidized to oxaloacetate by bacteroid MDH. Thus the tricarboxylic acid cycle present in the bacteroid (7), can function. Glutamate might be metabolized in the bacteroid by the action of GOT, glutamate pyruvate transaminase or glutamate dehydrogenase (EC 1.4.1.3).

A second essential function of the shuttle might be the transamination of oxaloacetate to aspartate by the bacteroid GOT. It is shown by Snapp and Vance (21) that ^{14}C-aspartate which is added to root nodules is rapidly converted to C_4-dicarboxylic acids. This experiment indicates that GOT in root nodules catalyzes the formation of oxaloacetate from aspartate and not the transamination of oxaloacetate. However the concentration of aspartate in the cytoplasm of root nodule cells is 3.2 mM (36). This indicates that an other reaction must form aspartate with the same rate as the rapid conversion of aspartate into oxaloacetate . The aspartate producing reaction might be the shuttle. Aspartate can be formed inside the bacteroid and subsequently transported into the cytoplasm of the root nodule cell. In this way the aspartate concentration in the cytoplasm of root nodule cell can be kept high despite the high rate of conversion into oxaloacetate. Aspartate can be withdrawn from the shuttle for asparagine biosynthesis and the carbon lost from the shuttle can be replenished by malate. It is not necessary that asparagine is synthesized in the cytoplasm of the plant cells. Since asparagine synthetase has been detected in bacteroids (18), it can be synthesized in the bacteroid.

The transfer of reducing equivalents into the bacteroid catalyzed by the shuttle might be of importance for the nitrogenase activity. If the shuttle resembles the mammalian mitochondrial malate/aspartate shuttle, the shuttle transfers not only reducing equivalents but as well as protons into the bacteroid by the aspartate-glutamate carrier (44). The exchange is stimulated by the presence of an electrical potential gradient across the barrier and not by pH (44). The activity of the shuttle in the bacteroid would be facilitated by an electrical potential across the barrier. The flow of reducing equivalents to nitrogenase is exclusively regulated by this electrical potential (45). Lowering of the potential reduces the flow of reducing equivalents to nitrogenase. Consequently the nitrogenase switch off is associated with a lowering of the activity of the malate/aspartate shuttle.

It is also possible that the nodule-stimulated GOT participates in a malate/aspartate shuttle between the cytoplasm of root nodule cells and the mitochondria. But evidence for this is lacking.

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4. THE EFFECT OF PH ON NITROGENASE ACTIVITY AND ELECTRON ALLOCATION TO H^+ AND N_2 BY NITROGENASE OF *RHIZOBIUM LEGUMINOSARUM* BACTEROIDS

SUMMARY

The effect of O_2 on the nitrogenase activity and the electron allocation to H^+ and N_2 by nitrogenase of nodulated roots of pea plants and of isolated bacteroids was studied. When the nitrogenase activity of isolated bacteroids was less than 40 % of the maximal activity, due to a limiting O_2 supply, the reduction of H^+ above N_2 was favoured. At an O_2 supply which give 10 % of the maximal nitrogenase activity, 15 % of the electrons flux through nitrogenase is used for N_2 reduction. This was not observed for nodulated roots. At a pO_2 of 0.06 atm at which the nitrogenase activity is 10 % of the maximal activity still 75 % of the reducing equivalents are used for N_2 reduction.

To explain the different response to O_2 of nitrogenase present in intact root nodules and in isolated bacteroids, the effect of respiration on whole cell nitrogenase activity of isolated bacteroids was studied. It is shown that the type of substrate oxidized by the bacteroids determines the rate of respiration and also the maximal nitrogenase activity. But no effect of the type of substrate on electron allocation to H^+ and N_2 by nitrogenase at low nitrogenase activity was found. The pH of the incubation medium influences whole cell nitrogenase activity. From pH 7.0 to 7.9 the nitrogenase activity gradually declines to about 10 % of the maximal activity at pH 7.0, while still 75 % of the reducing equivalents is used for N_2 reduction. The O_2 consumption during nitrogen fixation decreases simultaneously with nitrogenase activity.

Based on our results a proposal for the regulation of the O_2 consumption by the bacteroids, nitrogenase activity and electron allocation to H^+ and N_2 by nitrogenase in pea will be presented.

Enzymes

Nitrogenase (EC 1.18.6.1)

INTRODUCTION

Bacteria of the genera *Rhizobium* and *Bradyrhizobium* are capable of fixing N_2 in symbiosis with legumes. After recognition and infection of root hairs by the bacteria, root nodules are formed. In this process bacteria differentiate into bacteroids. The bacteroids are present in the cytoplasm of the

infected plant cell, enclosed by a plant encoded membrane, the so called peri-bacteroid membrane. The plant supplies the bacteroids with O_2 and oxidizable substrates both necessary for nitrogen fixation. During nitrogen fixation N_2 is reduced to NH_3. NH_3 is not used by the bacteroids but excreted into the cytoplasm of the plant, where it is incorporated into organic molecules, which are used for plant growth (for a review see 1).

The reduction of N_2 to NH_3 is catalyzed by the enzyme nitrogenase, which consists of two proteins called Fe-protein and MoFe-protein (for a review see 2). For nitrogenase activity an anaerobic environment, N_2, MgATP, a source of reducing equivalents (reduced ferredoxin or flavodoxin and an energized cytoplasmic membrane (2)) and protons are required. Besides the reduction of N_2, H^+ is also reduced by nitrogenase. It has been found that at least one molecule of H_2 is formed per molecule N_2 reduced, but higher values are also reported (3-7). The reduction of H^+ by nitrogenase is an unwanted side reaction of the enzyme which consumes ATP and reductant. Especially when nitrogen fixation limits growth, a low rate of H^+ reduction by nitrogenase will have a positive effect on plant growth.

The factors that affect the reduction of H^+ *versus* the reduction of N_2 have been studied for the isolated enzyme, for bacteroids and to a less extent for the symbiotic associations. Studies with the isolated enzyme showed that the activity of MoFe protein of nitrogenase determines the allocation of electrons to N_2 or H^+ (6). Inhibition of the activity of MoFe-protein caused by ATP-limitation (6), shortage of reductant (6), decreasing of the ratio Fe-protein/MoFe-protein (5,6) or lowering of the absolute concentration of both proteins (7), stimulates the electron allocation to H^+. Studies with intact bacteroids (8) have revealed that nitrogenase in its physiological environment, behaves differently compared with isolated enzyme. Inhibition of nitrogenase by O_2-limitation and by the addition of a H^+-conducting uncoupler, both conditions lower the intracellular ATP/ADP ratio, result in an increased electron allocation to H^+. However when nitrogenase is inhibited by O_2-excess or by the addition of K^+ and the K^+-conducting ionophore valinomycin the ATP/ADP ratio and the electron allocation is not influenced. It was therefore suggested that the intracellular ATP/ADP ratio determines the electron allocation by nitrogenase: when the ATP/ADP ratio is low, reduction of H^+ is favoured above reduction of N_2.

Concerning the symbiotic nitrogen fixation several environmental factors have been identified which affect the electron allocation. These factors are the temperature (9), irradiation and growth cycle (10,11), the strain of bacterium used to inoculate the plant (12) and the partial pressure of O_2 (13). From studies with isolated bacteroids, it is clear that O_2 supply for the bacteroids in the central tissue of a root nodule is very important for nitrogenase activity. When the O_2 supply is low, energization of the bacteroids is not enough to support nitrogen fixation efficiently. When too much O_2 diffuses into the central tissue, nitrogenase will be irreversibly inactivated. Thus the

regulation of the O_2 influx into the central tissue of a root nodule is of great importance for nitrogen fixation of a plant. In several experiments the existence of a plant regulated variable O_2-diffusion barrier has been demonstrated (see for a review 14). The variable diffusion barrier responds to several environmental conditions as well as to at least one intrinsic property of nitrogen fixation, which is NH_3 produced by nitrogenase (15). The plant reacts on inhibition or induction of the production of NH_3 by increasing respectively decreasing the O_2 resistance.

In this paper the effect of O_2 on nitrogenase activity and electron allocation to H^+ and N_2 by nitrogenase in root nodules of pea plants and of isolated bacteroids will be described. It will be shown that by changing the pH the O_2 consumption, nitrogenase activity and electron allocation by nitrogenase of isolated bacteroids is regulated. Based on these results it is proposed that a change of O_2 influx into a root nodule is associated with a change of pH. By this way H^+ reduction by nitrogenase can be suppressed at different nitrogenase activities.

MATERIALS AND METHODS

Growth conditions of the plants and isolation procedures

Root nodules were produced under controlled conditions on *Pisum sativum* cv Finale by inoculation with *Rhizobium leguminosarum* strain PRE as described previously (16). The strain PRE does not contain an active hydrogenase. The bacteroids were isolated from the nodules as described earlier (8). The isolation procedure was performed at 4 °C.

Analytical methods

Whole cell nitrogenase activity was measured at 30 °C in a reaction mixture containing 50 mM Tes/NaOH, 5 mM $MgSO_4$, 480 mM sucrose, 0.2 mM myoglobin and 2.5 % w/v fatty acid free serum albumin with a final pH of 7.4, unless indicated. Myoglobin was oxygenated as described by Wittenberg et al.(17). The substrate was added at a concentration of 5 mM. Unless otherwise specified L-malate is used as substrate. The reaction mixture was flushed with argon in a butyl rubber stoppered serum bottle of 7.9 ml. Different amounts of O_2 were added to the gas phase of the serum bottle and the reaction was started by the addition of bacteroids (routinely 0.9 mg protein/ml reaction mixture), final volume 0.7 ml. The assay bottles were incubated in a water bath and shaken reciprocally with a stroke of 2.5 cm and 160 cycles/min. Gas samples were taken and analyzed as described earlier (8). Calibration of the O_2 input rate was performed by comparing the nitrogenase activity of bacteroids at different O_2 concentrations in the gas phase in the shaken assay bottles with the nitrogenase activity of a bacteroid suspension incubated in a

vessel with a Clark type electrode at the bottom as described earlier (18). The O_2 input rate into the oxygraph was calibrated as described earlier (18). A pO_2 of 0.01 in the gas phase of the serum bottles gave an O_2 input rate of 7.1 nmol $O_2 \cdot min^{-1}$. (0.7 ml reaction mixture)$^{-1}$ under the conditions described. The incubations were performed in duplicate and were analyzed for 15 min after the addition of bacteroids. The rate of nitrogenase activity was calculated based on cumulative product formation. At higher O_2 input rates, the nitrogenase activity was sometimes not linear for 15 min. In that case the activity was calculated from the first 10 min of product formation. In all cases product formation was linear within the first 10 min.

The nitrogenase activity of whole plants and nodulated root fragments was measured as H^+ reduction under Ar/O_2 mixtures or as C_2H_2 reduction under $Ar/O_2/C_2H_2$ (15 %) mixtures as described earlier (12). After removal of plants from the rooting medium, the shoots were removed and the measurement of the nitrogenase activity of the nodulated roots was started ≈ 7 min after removal of the plants from the rooting medium. If not indicated otherwise the nitrogenase activity was calculated based on cumulative product formation during the first 10 min of the incubation. For each datum point, the root systems of 20 infected plants were assayed at room temperature. Electron allocation of nitrogenase was calculated from the H^+-reduction under Ar/O_2 and under N_2/O_2 mixtures, based on cumulative product formation during the first 10 min of incubation. The electron allocation is expressed as % of the total electron flux through nitrogenase that is used for N_2 reduction.

In experiments carried out for the determination of the actual nitrogenase activity, the measurement of the nitrogenase activity was immediately (within 1 min) started after removal of the plants from the rooting medium. The actual rate of C_2H_2 reduction was calculated from the increase in the C_2H_4 concentration divided by the time between the two measurements.

The maximal rate of respiration of the bacteroids was measured with air saturated reaction mixture at 30 °C with a Gilson-water jacketed oxygraph equipped with a polarographic Clark electrode.

The intracellular ATP and ADP concentrations were measured after quenching the reaction by the addition of $HClO_4$ (final concentration 7 %). ADP was converted into ATP using pyruvate kinase (EC 2.7.1.40) and phosphoenolpyruvate. AMP was converted into ADP by myokinase (EC 2.7.4.3) and into ATP by pyruvate kinase in an appropriate assay. ATP concentrations were determined using a bioluminescence assay with luciferin and luciferase (EC 1.13.12.7). The intensity of the emitted light which is liberated by the luciferase reaction, was directly proportional to the ATP concentration. An internal standard of ATP was employed to correct for inhibition of luciferase and for emission interference by compounds of the incubations.

The protein concentration was determined by the method of Sedmark and Grossberg (19). Bovine serum albumin was used as standard.

Chemicals

L-aspartate, L-glutamate, 2-oxoglutarate, Tes and myoglobin were obtained from Sigma; myokinase, pyruvate kinase, fatty acid free bovine serum albumin, fumarate, succinate, pyruvate, oxaloacetate, ATP and ATP bioluminescence assay kit from Boehringer and L-malate from Merck. All other chemicals were of the highest analytical grade available. All gases were purchased from Hoek Loos.

RESULTS

Effects of plant disturbance on nitrogenase activity

Minchin *et al* (20) have reported that nitrogenase activity in many legumes declined rapidly after the addition of acetylene. This phenomenon is attributed to an increase in oxygen diffusion resistance of root nodules (21). In order to determine the nitrogenase activity of root nodules accurately, changes in the resistance of the O_2-diffusion barrier during measurements have to be excluded. It was therefore tested if the removal of the plants from the rooting medium has an effect on nitrogenase activity. As can be seen in Figure 1 (page 84), no effect on the nitrogenase activity was observed. The removal of the shoot of the roots caused a loss of nitrogenase activity of about 20 ± 7 % in 15 min (not shown).

The nitrogenase activity of nodulated root fragments showed a continuous loss in nitrogenase activity in the presence of C_2H_2. This decline was linear in time for at least 30 min and was also observed when the nitrogenase activity was measured by H_2 production in the presence of N_2 or Ar. If the activity is calculated based on the cumulative product formation from t= 0 to t= 8 min or from t= 0 to t= 15 min the difference between both measurements is 7 ± 2 % in case of C_2H_2 reduction. The same value was found when the nitrogenase activity was measured by the H_2 production in the presence of N_2 or Ar (not shown). Thus the decline in nitrogenase activity is not induced by the inhibition of the NH_3 formation. It can therefore be concluded that the variable O_2-diffusion barrier in both intact plants and nodulated root fragments is not influenced by the absence of NH_3 formation. This implies that nitrogenase activity and the electron allocation by nitrogenase at different oxygen concentrations can be measured correctly by H_2 evolution under Ar/O_2 and N_2/O_2 mixtures or by acetylene reduction.

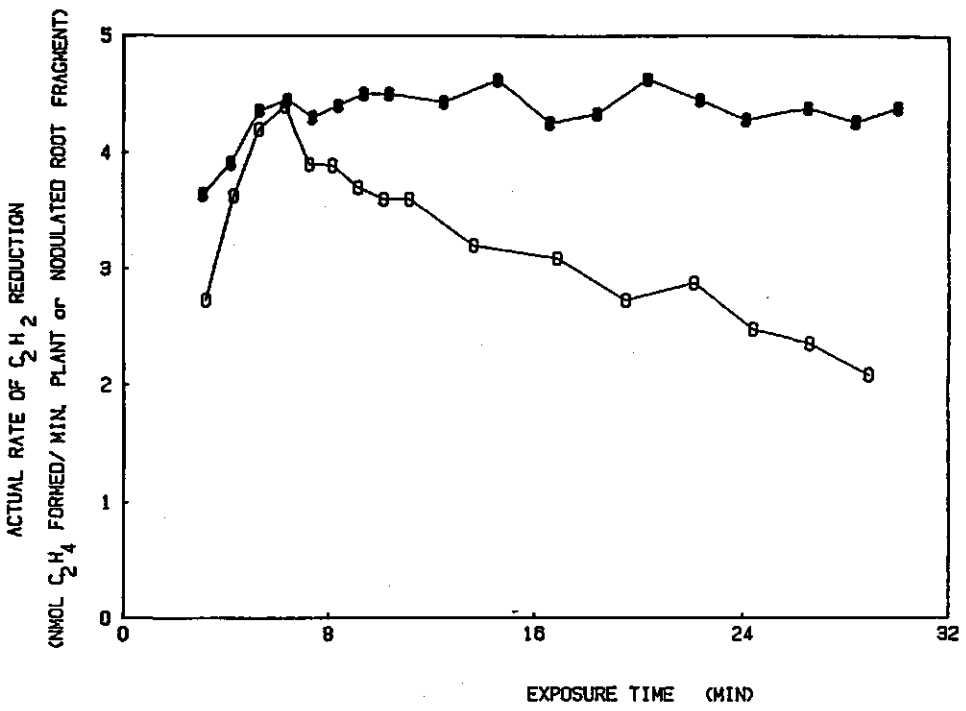

Figure 1.
The actual rates of C_2H_2 reduction of nodulated root fragments (O-O) and of intact plants (●-●). Plants were removed from the rooting medium at t=0 and exposed within 1 min to a mixture of air and 15 % C_2H_2.

Nitrogenase activity and electron allocation in nodulated root fragments

Nitrogenase activity of nodulated root fragments was varied by the O_2 concentration in the gas phase over the range of 5 - 38 %. The H_2 evolution by nitrogenase under Ar increases 10 fold (Figure 2). The same pattern is obtained for H_2 evolution under N_2. Above 40 % O_2, nitrogenase activity declines rapidly and irreversibly. This indicates that nitrogenase is inactivated by O_2. As can be seen in Figure 2, nitrogenase activity increases 10 fold without any significant change in the electron allocation. At all O_2 concentrations tested the reduction of N_2 by nitrogenase was favoured : 69 ± 9 % (n= 6).

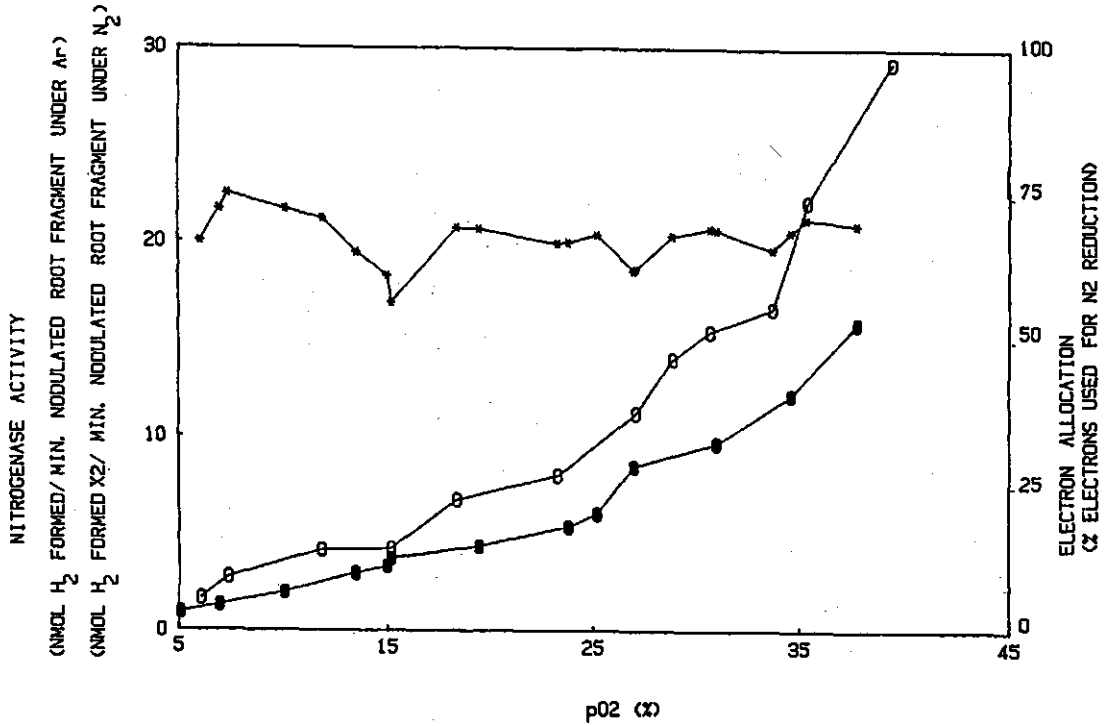

Figure 2.
Nodular nitrogenase activity and electron allocation by nitrogenase of nodulated root fragments at different oxygen concentrations in the gas phase. (O-O) H^+ reduction under Ar, (●-●) H^+ reduction under N_2. (*-*) electron allocation was calculated at each datum point by using the measured activities. If the corresponding activity was not measured at the particular pO_2, a value obtain by interpolation from the presented curves is used.

Nitrogenase activity and electron allocation of isolated bacteroids

When the O_2 input rate into the incubation medium is increased nitrogenase activity increases (Figure 3A). The maximum nitrogenase activity determined at a pH 7.4 was 15 ± 5 nmol C_2H_4 formed. min^{-1}. mg^{-1} bacteroid protein (n= 20). As expected from an obligate aerobic organism, the intracellular ATP/ADP ratio increases with an increased O_2 input. The ATP/ADP ratio does not vary in a regular way with the O_2 input rate. At low O_2 input rates, the ATP/ADP ratio increases linearly with the O_2 input rate, then the increase levels off and when nitrogenase is inhibited by excess O_2, the ATP/ADP ratio increases sharply. In Figure 3B the electron allocation between H^+ and N_2 at different nitrogenase activities is plotted. Nitrogenase activity was modulated by the O_2 input rate. The maximum electron allocation was found at maximal nitrogenase activity and was in favour of the N_2 reduction: 67 ± 10 % (n= 20) of the total electron flux through nitrogenase was used for N_2 reduction. The electron allocation to N_2 declines strongly when nitrogenase activity is less than 40 % of the maximal activity. The difference between the electron allocation by nitrogenase in isolated bacteroids and nodulated root fragments is striking. One expects a similar behaviour of nitrogenase since O_2 limitation will induce in both systems a low intrabacteroid ATP/ADP ratio and a low nitrogenase activity and this will stimulate electron allocation to H^+ (compare Figure 2 and Figure 3B). Since this is not observed for nodulated roots there must be a factor in the plant cell which allows nitrogenase to allocate the maximal amounts of electrons to N_2 despite a sub-optimal O_2 supply.

It was shown earlier (8) that electron allocation to H^+ by nitrogenase is stimulated by a low ATP/ADP ratio and low nitrogenase activity. Both the ATP/ADP ratio and nitrogenase activity depend on the rate of respiration, thus factors that influences whole cell respiration might affect nitrogenase activity and electron allocation.

In *Azotobacter* respiration is decreased when K^+ is omitted from the incubation medium (22). We found no effect of K^+ (0-80 mM added to the incubation mixture) on the rate of respiration, nitrogenase activity and electron allocation in *R. leguminosarum* bacteroids. The possibility of an effect of an ATP-driven K^+ pump present in the peribacteroid membrane on nitrogenase activity and electron allocation was also considered. However no K^+ specific effect in the presence of ATP on respiration and nitrogenase activity and electron allocation was found.

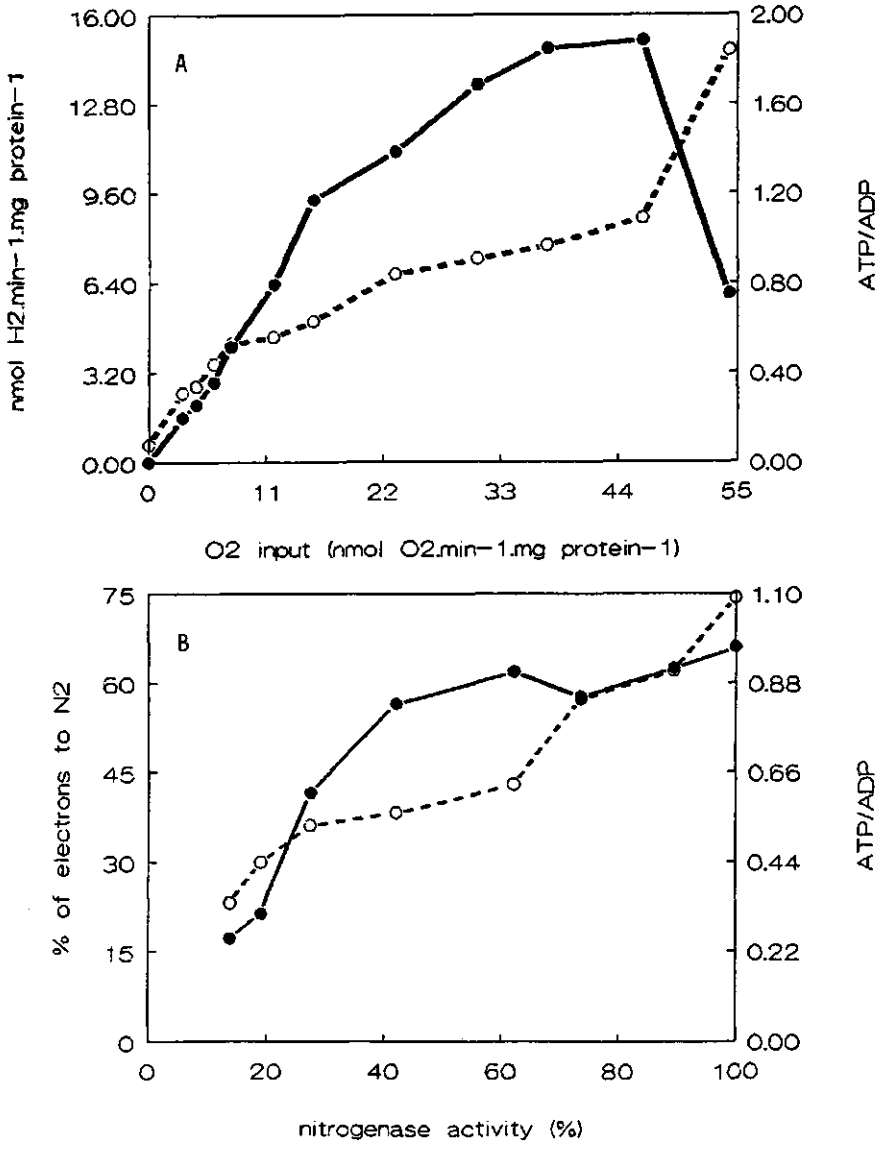

Figure 3.
Nitrogenase activity and electron allocation by nitrogenase in bacteroids at different O_2 input rates. Experiments were performed at pH 7.4 with L-malate as substrate as described in Materials and Methods.
A. ●-●, nitrogenase activity, o-o, intracellular ATP/ADP ratio.
B. ●-●, electron allocation of experiments described in A. o-o, intracellular ATP/ADP ratio. 100 % nitrogenase activity was 15 nmol H_2. min^{-1}. mg $protein^{-1}$. Nitrogenase activity was modulated by O_2 input rate.

The effect of citric acid cycle intermediates on respiration, nitrogenase activity and electron allocation was tested (Table 1). By the addition of substrates, the rate of respiration of *R. leguminosarum* bacteroids can be stimulated 2.6 fold. It is also clear that not only the rate of respiration at saturating O_2 concentrations determines the maximal nitrogenase activity. In case of respiration with endogenous substrates, the O_2 uptake at saturating O_2 concentrations is 40 % of that with succinate but the nitrogenase activity is only 11 % of the activity with succinate. The reason is that cells respiring endogenous substrates can not oxidize fast enough under conditions of nitrogen fixation, thus at low free O_2 concentrations. During nitrogen fixation the O_2 concentration must be kept below 1 µM (18). The conclusion is that substrates stimulate respiration at low and high O_2 concentrations. The stimulation at low free O_2 concentrations is important for nitrogen fixation. It can also be seen in Table 1 that the type of substrate does not influence the electron allocation to H^+ and N_2. This is only determined by the rate of respiration that is possible with a particular substrate.

In Figure 4A (page 90) the effect of the pH of the incubation medium on whole cell nitrogenase activity is presented. As can be seen the nitrogenase activity declines together with the amount of O_2 necessary for maximal nitrogenase activity. A determination of the ATP and ADP concentration in bacteroids at the O_2 input rate for maximal nitrogenase activity showed that up to a pH of 7.5 the ATP/ADP ratio was not lowered despite the lower rate of respiration of the bacteroids. At higher pH values the ATP/ADP ratio declines. But even at a low O_2 consumption rate at pH 7.9 the ATP/ADP ratio was not lower than 0.6. This ratio is still high enough to allow nitrogenase to function as nitrogenase and not as a hydrogenase. The electron allocation to N_2 is still maximal (65-75 %) despite the low nitrogenase activity (Figure 4B).

Table 1. Respiration, nitrogenase activity and electron allocation to N_2 and H^+ by nitrogenase in bacteroids oxidizing different substrates. Activities are measured as described in Materials and Methods. NT stands for not tested. Values in parentheses represent standard error.

substrate	maximal O_2 uptake	O_2 uptake during N_2 fixation	maximal nitrogenase activity	electron allocation
	(nmol $O_2 . min^{-1} . mg^{-1}$ protein)		(nmol C_2H_4 formed. $min^{-1} . mg^{-1}$ protein)	(% electrons used for N_2 reduction)
endogenous substrates	54 (3)	10.3 (2.3)	2 (1)	NT
aspartate	67 (10)	19.4 (4.6)	6 (2)	19 (10)
2-oxoglutarate	70 (11)	17.0 (2.3)	7 (2)	20 (10)
glutamate	74 (3)	22.8 (4.6)	9 (2)	24 (6)
2-oxoglutarate + aspartate	120 (21)	39.9 (10.3)	15 (5)	52 (10)
malate	130 (10)	55.9 (3.8)	17 (1)	63 (4)
malate + glutamate	131 (6)	55.9 (4.6)	18 (2)	59 (4)
succinate	133 (8)	59.2 (3.4)	18 (3)	64 (7)
fumarate	139 (14)	NT	NT	NT

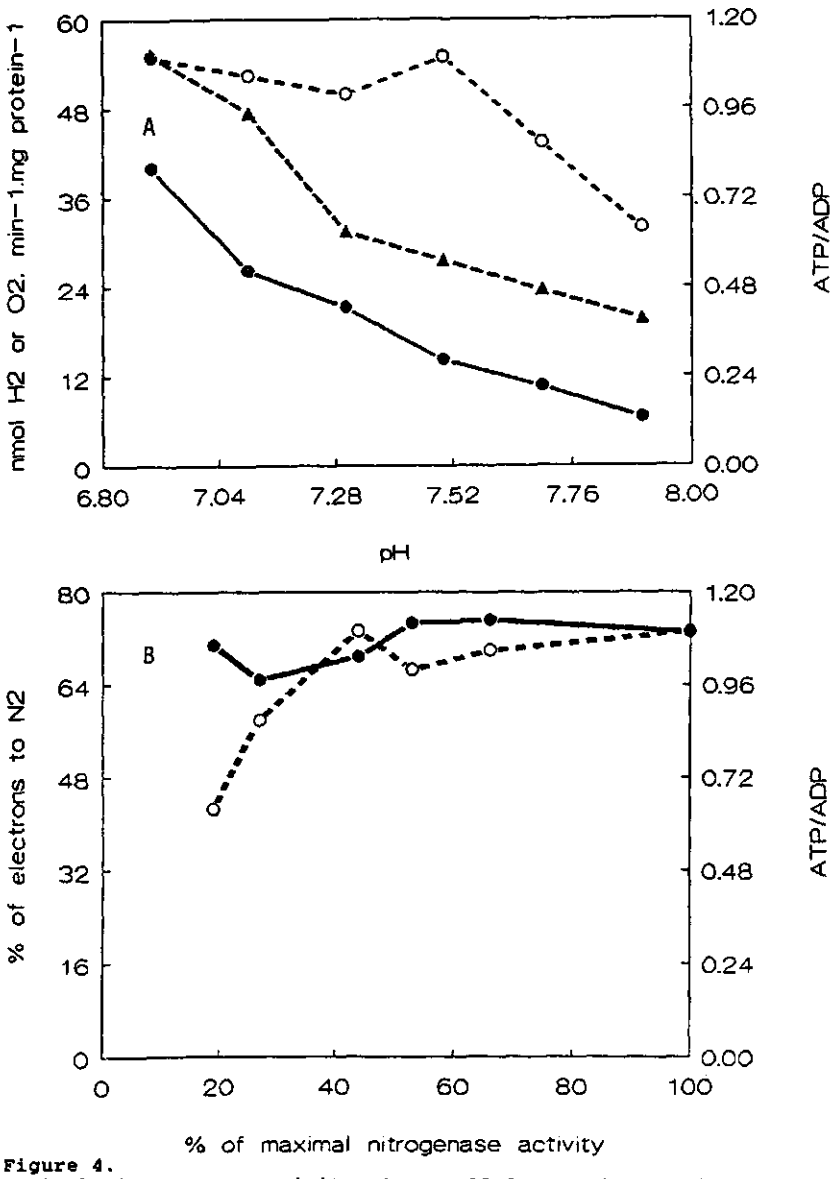

Figure 4.
Maximal nitrogenase activity, intracellular ATP/ADP ratio, O_2 uptake rate and electron allocation of isolated bacteroids at different pH values. Experiments were performed with L-malate as substrate as described in Materials and Methods.

A. ●-●, maximal nitrogenase activity; ▲-▲ O_2 input rate at maximal nitrogenase activity; o-o, intracellular ATP/ADP ratio.

B. ●-●, electron allocation of experiments described in A. 100 % is nitrogenase activity at pH 6.9; o-o, intracellular ATP/ADP ratio.

DISCUSSION

Reliability of the nitrogenase activity measurements

In this study the effect of O_2 on nitrogenase activity and electron allocation to N_2 and H^+ is studied in intact root nodules and isolated bacteroids. Minchin *et al.* (15) have shown that during a nitrogenase activity measurement of intact plants, disturbance of the plant increases the diffusion barrier for a gas of a root nodule and this inhibits the measured nitrogenase activity. We have tested the possibility that disturbance of the plants used, influences the nitrogenase activity measurements but no decline in nitrogenase activity was found when whole plants were removed from the rooting medium for an activity measurement. In addition with the observation that the nitrogenase activity is not maximal in air and can be stimulated by an increase in pO_2, we conclude that there is an O_2 diffusion barrier present which does not change by plant disturbance (21). Detachment of the roots gave a 20 % decrease of nitrogenase activity. This might indicate that the O_2 resistance increases, but during the measurements no specific acetylene- or argon-induced decline in nitrogenase activity was observed, thus the electron allocation can be determined from the experiments described. It has already been noticed that different plant genotypes react differently upon changes of the gas phase changes and removal of the shoot (23).

A plant factor affecting the nodular nitrogenase activity

Measurements of nodular nitrogenase activity showed that a low activity was not accompanied with change of the electron allocation by nitrogenase as was observed with isolated bacteroids. This difference indicates the existence of a plant factor which regulates the metabolism of the bacteroid in such a way that the nitrogenase activity can vary without affecting the electron allocation by the enzyme.

Studies with isolated bacteroids have shown that the intracellular ATP/ADP ratio determines the electron allocation of nitrogenase (8). The results presented in Figure 3 indicate that if the intracellular ATP/ADP ratio drops below 0.5 , electron allocation to H^+ is favoured. This must mean that the proposed plant factor influences metabolism of bacteroids in such a way that despite a low O_2 consumption of the bacteroids the intracellular ATP/ADP ratio remains above 0.5.

As described in Table 1, it was tested if the type of substrate could influence nitrogenase activity and electron allocation. It is clear that not the rate of respiration at saturating O_2 concentrations determines nitrogenase activity, but the capacity to take up O_2 at low free O_2 concentrations. Under these conditions the difference between endogenous respiration and respiration with a well oxidizable substrate become clear. The reason might be that substrates are capable to maintain the re-

dox state of the cells low enough to allow electron transport to nitrogenase together with electron transport to O_2. This might not be possible with endogenous substrates (mainly polyhydroxybutyrate (24)) since these substrates might be metabolized only at low NADH/NAD^+ ratios (25). When a substrate is oxidized, the rate of respiration determines the nitrogenase activity. The higher the rate of respiration the higher the nitrogenase activity. The results also show that the electron allocation is linked to the nitrogenase activity in a similar way as found for O_2 limitation of bacteroids with a particular substrate (Figure 3). Below 50 % of the maximal nitrogenase activity (oxidation of glutamate, aspartate and 2-oxoglutarate) the electron allocation to H^+ is favoured. The conclusion is that the type of substrate determines the rate of respiration at low free O_2 concentrations and the nitrogenase activity of bacteroids, but it does not enhance electron allocation to N_2 at low nitrogenase activities.

As shown in Figure 4, the pH has a significant effect on the maximal nitrogenase activity. At a higher pH value nitrogenase activity is lower but the electron allocation to N_2 is still high despite the lower O_2 consumption rates. Measurements of the intracellular ATP and ADP concentration showed that at higher pH values at the O_2 input rate for maximal nitrogenase activity, the ratio is still above 0.5. This explains why at a lower O_2 consumption rates the electron allocation to N_2 can be maintained high. The mechanism by which the bacteroid maintains the ATP/ADP ratio high at a low O_2 consumption rate is the subject of a accompanying paper.

In this paper we have shown that by changing the pH of the incubation medium, nitrogenase activity can be modulated with maintenance of the maximal electron allocation to N_2. Since we observed a similar behaviour of nitrogenase in nodulated roots, it is therefore possible that the pH in a root nodule changes in response with the O_2 influx/consumption. When the O_2 influx into the central tissue is limited, the plant increases the pH around the bacteroids to maintain the electron allocation by nitrogenase to N_2 high. The opposite must occur when the O_2 input into the nodules is increased. The pH must be lowered to allow the bacteroids to respire faster and this increases the nitrogenase activity. It is also possible that not the pH of the cytoplasm of the plant changes but that the pH of the peribacteroid space is changed. In this respect is worthwhile to mention that the presence of a H^+-translocation ATPase in the peribacteroid membrane has been reported (26,27).

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5. RESPIRATORY CONTROL DETERMINES NITROGENASE ACTIVITY IN *RHIZOBIUM LEGUMINOSARUM* BACTEROIDS

SUMMARY

The nitrogenase activity of *Rhizobium leguminosarum* bacteroids has been studied at pH 6.8 and pH 7.4 . The relationship between whole cell nitrogenase activity, the O_2 input rate, the free O_2 concentration and the intracellular ATP/ADP ratio has been determined at the two pH values. It will be shown that *Rhizobium leguminosarum* bacteroids can have only a stable steady state nitrogenase activity if the free O_2 concentration is kept below 1 µM. The maximal nitrogenase activity at pH 7.4 is 40 % of the maximal activity at pH 6.8. This is caused by a lower rate of respiration at O_2 concentrations below 1 µM.

Experiments with the proton conducting ionophore carbonyl cyanide *m*-chlorophenylhydrazone demonstrate that respiratory control determines the rate of respiration at low O_2 concentrations. It will also be shown that under conditions of nitrogen fixation nitrogenase hydrolyzes about 70 % of the ATP generated by oxidative phosphorylation.

Experimental evidence will be presented that indicates that ATP hydrolysis by nitrogenase is necessary for respiration at low free O_2 concentrations and thus for nitrogen fixation.

Enzymes

Nitrogenase (EC 1.18.6.1)

INTRODUCTION

Nitrogen fixation is essentially an anaerobic process (1). The enzyme nitrogenase that catalyzes the reduction of N_2 to NH_3 consists of two O_2-labile proteins (2). Furthermore the physiological electron donors for nitrogenase, flavodoxins and ferredoxins, are auto-oxidizable (1). Besides an anaerobic environment and a source of reducing equivalents, MgATP is necessary *in vitro* for nitrogenase activity. Dioxygen plays a dualistic role in nitrogen fixation of obligate aerobic diazotrophs, like *Azotobacter* and *Rhizobium*. In these organisms MgATP is generated by oxidative phosphorylation and electron transport to nitrogenase is coupled to electron transport through the respiratory chain to O_2 (3). In *Azotobacter* nitrogen fixation is protected against O_2 inactivation by enhanced respiration (respiratory protection (1)) and a conformational protection (formation of a complex with an Fe-S protein (2,4)). No protective protein has been de-

monstrated in *Rhizobium* and respiratory protection is supposed to be of minor importance compared with *Azotobacter* (2).

In *Rhizobium* bacteria or bacteroids nitrogen fixation can only take place under microaerobic conditions. In symbiosis with legumes the microaerobic condition is created in the root nodule of the legume. The influx of O_2 into the central tissue of a root nodule is regulated (5) and inside the plant cells O_2 transport is facilitated by O_2 binding proteins, the leghaemoglobins (6). Leghaemoglobin by itself does not protect nitrogenase against O_2 inactivation but it allows a high flux of O_2 at low free O_2 concentration (7).

From studies with intact plants and nodulated roots (8,9), it is clear that the O_2 resistance of root nodules can vary in such a way that the O_2 concentration in air limits nodular nitrogenase activity. In an accompanying paper we have shown that nitrogenase activity and O_2 consumption of *Rhizobium leguminosarum* bacteroids is dependent upon the pH of the incubation medium. At pH values above 7.0 the maximal nitrogenase activity and the associated O_2 consumption declines. The effect of the pH is different as compared with a decrease of nitrogenase due to O_2 limitation (10). When whole cell nitrogenase activity is inhibited due to O_2 limitation, the intracellular ATP/ADP ratio declines together with the O_2 limitation. At lower nitrogenase activities the electron allocation to N_2 by nitrogenase declines also. This is not observed for the pH induced nitrogenase activity inhibition. The mechanism by which a change in pH decreases the nitrogenase activity and the O_2 consumption is the subject of this paper.

MATERIALS AND METHODS

Growth conditions of the plants and isolation procedures

Root nodules were produced under controlled conditions on *Pisum sativum* cv. Finale by inoculation with *Rhizobium leguminosarum* strain PRE as described previously (11). The bacteroids were isolated from the nodules as described earlier (10) and respiratory membranes according to Laane *et al.* (12). The isolation procedure was performed at 4 °C.

Analytical methods

Whole cell nitrogenase activity was measured at 30 °C in a reaction mixture containing 50 mM Tes/NaOH, 5 mM $MgSO_4$, 480 mM sucrose, 0.2 mM myoglobin and 2.5 % w/v fatty acid free serum albumin with a final pH of 7.4 unless indicated. Myoglobin was oxygenated as described by Wittenberg *et al.* (13). L-malate (5 mM) was used as substrate. The reaction mixture was flushed with argon in a butyl rubber stoppered serum bottle. 1.95 ml of the reaction mixture was transferred to the argon flushed reaction chamber of an oxygraph (Ranks Brothers Bottisham, Cambridge,

U.K.) as described earlier (14). 0.05 ml bacteroids suspension was added (approximately 0.62 mg protein). Total volume of the system was 5.5 ml. The reaction was started by the addition of different amounts of O_2 to the gas phase of oxygraph. Gas samples were taken and analyzed as described earlier (10). Calibration of the O_2 input rate was performed as described earlier (14). A pO_2 of 0.01 atm in the gas phase of the oxygraph gave an O_2 input rate of 4.3 nmol O_2 . min^{-1} . (2 ml reaction mixture)$^{-1}$ under the conditions described.

The maximal rate of respiration of the bacteroids was measured with an air saturated reaction mixture at 30 °C with a Gilson water jacketed oxygraph equipped with a polarographic Clark electrode.

The intracellular ATP and ADP concentrations were measured after quenching the reaction by the addition of $HClO_4$ (final concentration 7 %). ADP was converted into ATP using pyruvate kinase (EC 2.7.1.40) and phosphoenolpyruvate. AMP was converted into ADP by myokinase (EC 2.7.4.3) and into ATP by pyruvate kinase in an appropriate assay. ATP concentrations were determined using a bioluminescence assay with luciferin and luciferase (EC 1.13.12.7). The intensity of the emitted light which is liberated by the luciferase reaction, was directly proportional to the ATP concentration. An internal standard of ATP was employed to correct for inhibition of luciferase and for emission interference by compounds present in the incubations.

The protein concentration was determined by the method of Sedmark and Grossberg (15). Bovine serum albumin was used as standard.

Chemicals

Tes and myoglobin were obtained from Sigma; myokinase, pyruvate kinase, fatty acid free bovine serum albumin, ATP and ATP bioluminescence assay kit from Boehringer and L-malate from Merck. All other chemicals were of the highest analytical grade available. All gases were purchased from Hoek Loos.

RESULTS

In obligate aerobic diazotrophs O_2 plays a dual role. O_2 is necessary for the generation of the proton motive force but excess O_2 inhibits nitrogen fixation. In Figure 1 (see page 98) the effect of an increasing O_2 input on nitrogenase activity and the intracellular ATP/ADP ratio of *Rhizobium leguminosarum* bacteroids and the free O_2 concentration is shown. As can be seen the ATP/ADP ratio increases only slightly with an increased O_2 input and increasing nitrogenase activity. The situation becomes completely different when nitrogenase activity is inhibited by excess O_2. With a small increase in the O_2 input rate, from 75 to 77 nmol O_2 . min^{-1} . mg protein^{-1}, the ATP/ADP ratio increases from 1.3 to 2.8. A similar observation was made by Trichant *et*

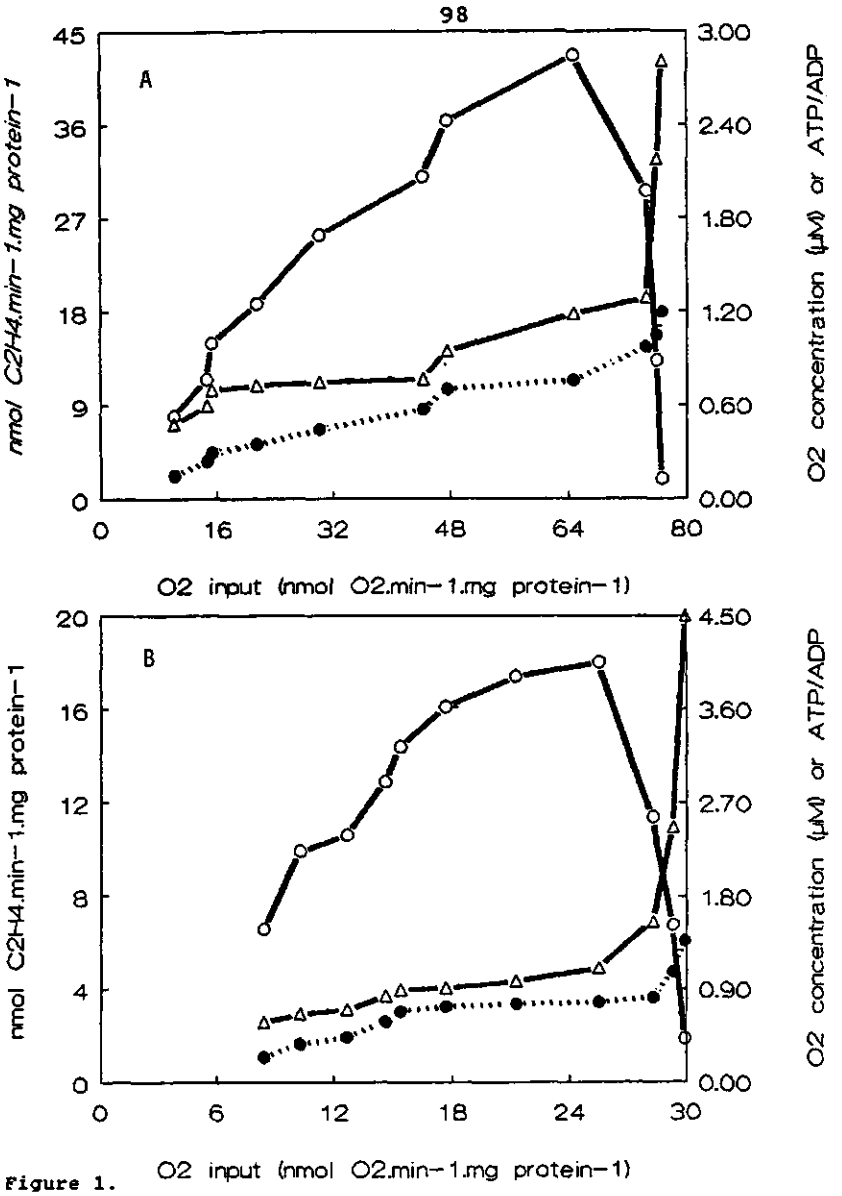


Figure 1.
Free O_2 concentration, nitrogenase activity and intracellular ATP/ADP ratio of *R. leguminosarum* bacteroids at different O_2 input rates. Experiments were performed as described in Materials and Methods.

A. The pH of the incubation mixture was 6.8, o-o, nitrogenase activity, Δ - Δ, intracellular ATP/ADP ratio, •-•, free O_2 concentration.

B. The pH of the incubation mixture was 7.4, o-o, nitrogenase activity, Δ - Δ, intracellular ATP/ADP ratio, •-•, free O_2 concentration.

al. (16) for *R. phaseoli* bacteroids. The observed inhibition of whole cell nitrogenase activity is probably not caused immediately by an irreversible inactivation of nitrogenase since the initial inhibition under these conditions ([O_2] < 1.3 µM and exposure time less then 5 min) is still reversible. It is therefore more likely that the components of the electron transport chain to nitrogenase are oxidized. When the bacteroids are exposed longer than 5 min at O_2 concentrations above 1 µM, the free O_2 concentration will increases steadily at a constant O_2 input which indicates that respiration becomes inhibited. When nitrogenase activity was measured with dithionite and ATP in permeable cells (10), it was found that nitrogenase was irreversibly inactivated (not shown).

In Figure 1B the results of a similar experiment are shown. The experiment is performed at pH 7.4 instead of at pH 6.8. The pattern is similar as found for pH 6.8, but nitrogenase is inhibited at a much lower O_2 input rate than at pH 6.8 (25 and 65 nmol O_2 . min^{-1} . mg $protein^{-1}$ respectively). This difference can not be explained by a lower maximal rate of respiration at the different pH values. In at typical experiment the rates of respiration at high O_2 concentrations (> 200 µM) at pH 6.8 and 7.4 differs not much (121 and 127 nmol O_2 . min^{-1} . mg $protein^{-1}$ respectively). This means that also at pH 7.4 the bacteroids can respire 65 nmol O_2 . min^{-1} . mg $protein^{-1}$ but not at an O_2 concentration at which nitrogenase activity is possible.

In Figure 2 (page 100) the effect of an active nitrogenase and the proton conducting uncoupler CCCP on respiration at low free O_2 concentrations is shown. The concentration of free CCCP is less then 47 µM since fatty acid free bovine serum albumin which is present in the incubation medium will bind a considerable amount of CCCP. At an O_2 input rate of 62 nmol O_2 . min^{-1} . mg $protein^{-1}$, the free O_2 concentration is 0.4 µM, the nitrogenase activity 25 nmol C_2H_4 formed. min^{-1} . mg $protein^{-1}$ and the ATP/ADP ratio 1.1.

When nitrogenase is inactivated by an exposure of the cells for 3 min to 20 µM O_2 at the same O_2 input rate of 62 nmol O_2 . min^{-1} . mg $protein^{-1}$, the free O_2 concentration is now 2.2 µM and the ATP/ADP ratio 2.9. Inactivating nitrogenase and thus inhibiting the ATP hydrolysis by nitrogenase (17) changes the O_2 concentration at which a certain amount of O_2 can be respired.

That the O_2 treatment did not inactivate components of the respiratory system is shown by the experiment where respiration at low free O_2 concentrations is stimulated by the addition of CCCP to the O_2 treated bacteroids. This experiment shows that a low ATP/ADP ratio is obligatory for respiration at low free O_2 concentrations. The experiment also shows that nitrogenase is able to keep the ATP/ADP ratio low.

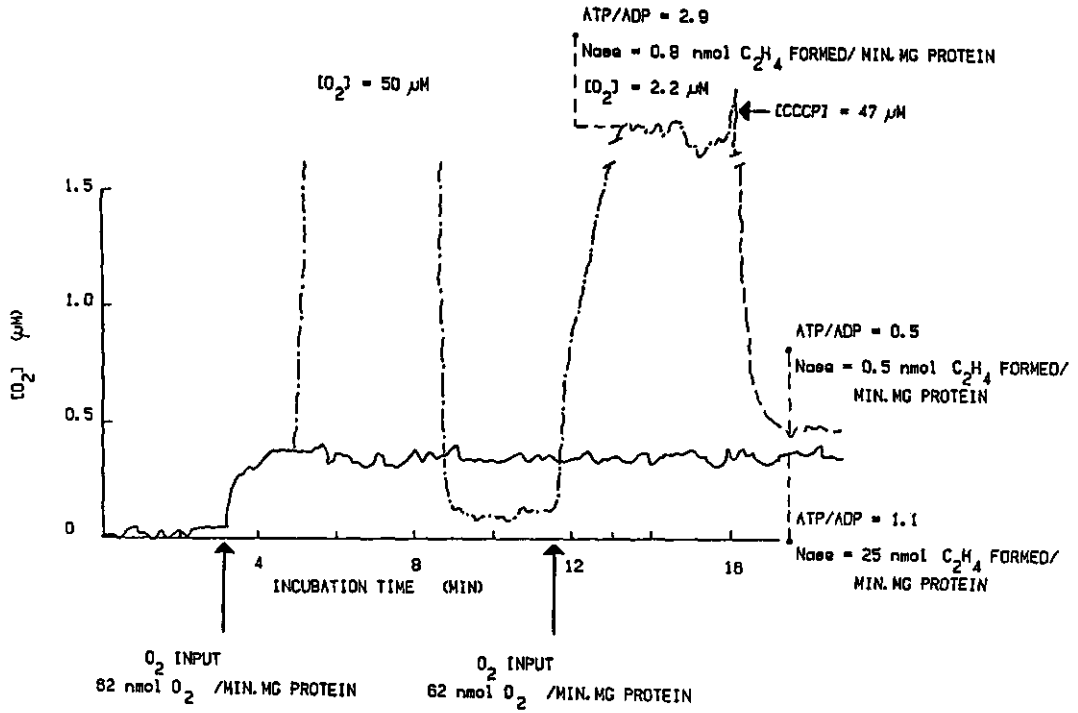


Figure 2.
Time course of the free O_2 concentration of incubations of *R.leguminosarum* bacteroids at different O2 input rates. The incubations were performed as described in Material and Methods. The gas phase was argon and the O_2 input rate was changed by adding O_2 as indicated. ——, cells with active nitrogenase; —•—, cells with an O_2 inactivated nitrogenase; — —, cells with an O_2 inactivated nitrogenase and 47 µM CCCP added. At the end of each experiment the incubation was quenched with $HClO_4$ and ATP and ADP were determined as described in Materials and Methods. The O_2 input rate is expressed in nmol O_2. min^{-1}. mg $protein^{-1}$.

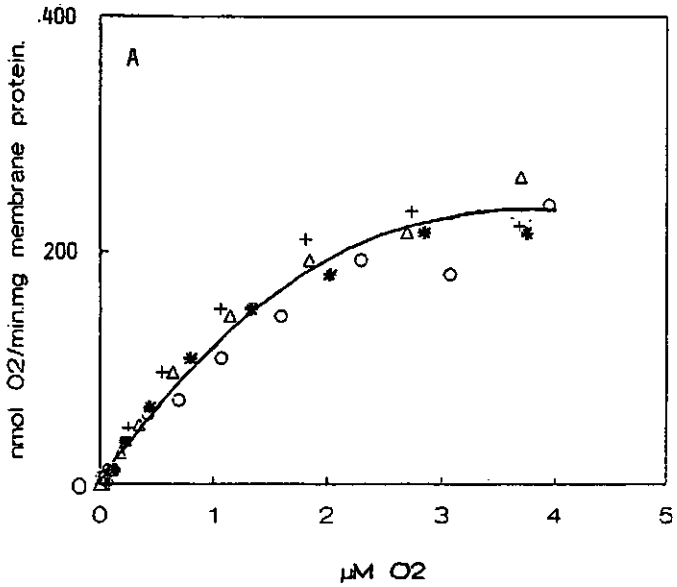
The effect of the pH on respiration and nitrogenase activity as described in Figure 1 might be explained in terms of inhibition of respiration by a high ATP/ADP ratio (the classical mitochondrial respiratory control). At pH 7.4 the respiratory H^+/O ratio might be higher and/or the ATP hydrolysis lower. In Table 1 the results of experiments at pH 6.8 and at pH 7.4 are shown. Nitrogenase activity was measured at two O_2 input rates. At the optimal O_2 input rate for nitrogenase activity at pH 6.8 (62 nmol O_2 . min^{-1} . mg $protein^{-1}$) nitrogenase activity is inhibited when measured at pH 7.4. Inhibition is caused by a low rate of respiration ($[O_2]$ = 20 µM). When at pH 7.4 the proton permeability of the cytoplasmic membranes is increased by the addition of CCCP, respiration is stimulated which results in a lower free O_2 concentration (0.6 µM) at the same O_2 input rate and surprisingly nitrogenase is active. This experiment demonstrates that at pH 7.4 bacteroids can have nitrogenase activity at the same O_2 input rates as at pH 6.8. The only requirement is that the proton motive force must be lowered with as result a lower ATP/ADP ratio. When the CCCP concentration is increased to 50 µM nitrogenase activity at this O_2 input rate is also inhibited (not shown).

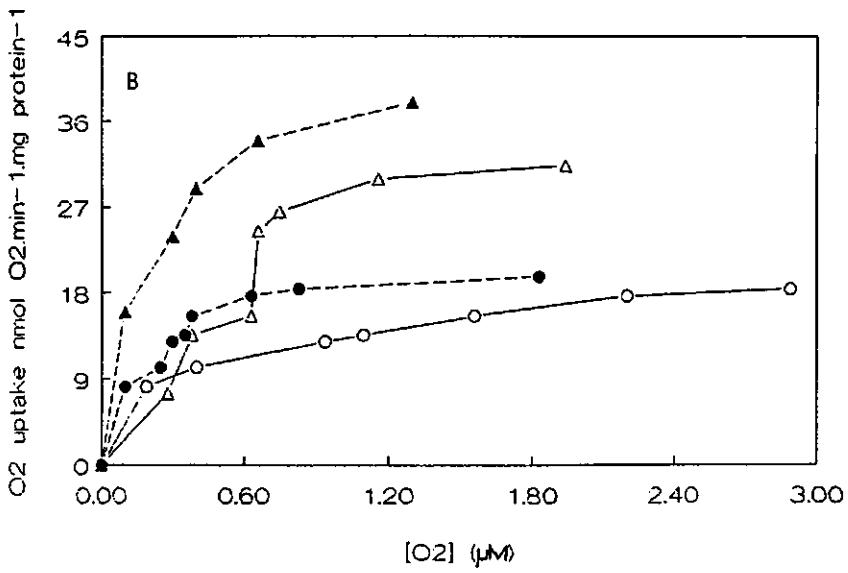
Table 1.

The effect of the pH on respiration and nitrogenase activity of *R. leguminosarum* bacteroids. The experiments were performed as described in Materials and Methods. *, 34 µM CCCP was present.

O_2 input (nmol O_2 . min^{-1} .mg $protein^{-1}$)	pH	$[O_2]$ (µM)	nitrogenase activity (nmol O_2 .min^{-1} . mg $protein^{-1}$)	ATP/ADP ratio
28	6.8	0.3	20	0.4
28	7.4	0.6	18	0.6
62	6.8	0.8	42	0.8
62	7.4	20	0	3.5
*62	7.4	0.6	22	0.4

The results presented in Figure 2 and Table 1 indicate that the ATP/ADP ratio determines the rate of respiration at low free O_2 concentrations. Therefore the effect of the O_2 concentration on respiration of bacteroids was investigated. There are two methods to determine the rate of respiration at different O_2 concentrations. The first method is to determine the first derivative from the decline of the O_2 concentration *versus* time and the second method is to measure the free O_2 concentration at different O_2 input rates.


A
400
200
0
nmol O2/min.mg membrane protein.
0
1
2
3
4
5
µM O2


B
45
36
27
18
9
0
O2 uptake nmol O2.min-1.mg protein-1
0.00
0.60
1.20
1.80
2.40
3.00
[O2] (µM)

For isolated membranes the derivative method was used and for intact cells the steady-state method. (see discussion). The kinetics of the respiration of isolated cytoplasmic membranes are presented in Figure 3A. The data fit to Michaelis-Menten kinetics. The V_m is 297 ± 56 nmol O_2 consumed. min^{-1}. mg $protein^{-1}$ and the apparent K_m value is 1.4 ± 0.4 µM. The results show that the terminal oxidase is almost saturated at 10 µM O_2.

The respiratory properties of two types of bacteroids were investigated: bacteroids with an active nitrogenase and bacteroids with an O_2 inactivated nitrogenase. The results of the steady state experiments are presented in Figure 3B. At low free O_2 concentrations the two types of cells behave almost similarly. But at higher O_2 input rates when nitrogenase becomes more active ($[O_2]>0.6$ µM), the O_2 consumption of cells with an active nitrogenase is stimulated strongly with a slight increase in the free O_2 concentration.

The effect of a possible inhibitory proton motive force on respiration is illustrated by the effect of CCCP. At all O_2 input rates respiration of bacteroids with an active or an O_2 inactivated nitrogenase is enhanced by the addition of CCCP resulting in a lower O_2 concentration in the new steady state. This might indicate that at non-saturating O_2 concentrations the proton motive force inhibits respiration also in cells with an active nitrogenase.

The enhanced respiration of cells with an active nitrogenase compared with O_2-treated cells with CCCP, can not be attributed only to the ATP hydrolysis by nitrogenase. The possibility that oxidation of electron carriers of the electron transport chain to nitrogenase is responsible for an extra O_2 consumption can not be ruled out. The time course of respiration at high O_2 concentration in the presence of CCCP (no respiratory control and nitrogenase activity) indicates that this electron transport chain might be inactivated by O_2 just like nitrogenase. When bacteroids are exposed to high O_2 concentrations in the presence of CCCP the rate of respiration declines about 10-20 nmol O_2. min^{-1}. mg $protein^{-1}$ within 5 min after the start of the incubation (not shown). This decline of respiration might be the inactivation of components of the electron transport chain to nitrogenase.

Figure 3 (facing page).
Kinetics of O_2 consumption by *R. leguminosarum* bacteroids and isolated cytoplasmic membranes.
A. The respiratory activity was calculated from the derivative of the concentration-time relationship. The data were fitted to the Michaelis-Menten equation. + and Δ, respiratiory activity of vesicles of two preparations in the absence of CCCP; o and *, respiratory activity of vesicles of two preparations in the presence of 50 µM CCCP.
B. O_2 uptake under steady-state rates of respiration. The experiments were performed as described in Materials and Methods. o-o, cells with an O_2 inactivated nitrogenase (exposure to pure O_2 for 2 min.); •-•, cells with an O_2 inactivated nitrogenase in the presence of 50 µM CCCP; Δ - Δ, cells with an active nitrogenase; ▲ - ▲, cells with an active nitrogenase in the presence of 50 µM CCCP.

DISCUSSION

At pH 7.4 the maximal nitrogenase activity of *R. leguminosarum* bacteroids is 40 % of the activity at pH 6.8. At all O_2 input rates at which nitrogenase is active, the intracellular ATP/ADP ratio is higher in cells incubated at pH 7.4 compared with cells incubated at pH 6.8 (compare Figures 1A and 1B). Up to an O_2 input rate of 24 nmol O_2. min^{-1}. mg $protein^{-1}$ the nitrogenase activity at the two pH values is nearly the same. The intracellular pH of *R. leguminosarum* bacteroids changes from 7.7 to 7.9 when the extracellular pH changes from 6.7 to 7.4 (18).

The ATP/2e ratio of isolated nitrogenase is constant between pH 6.5 and 7.8 (19). Thus at a similar nitrogenase activity, energy consumption by nitrogenase (MgATP and reductant) can not explain the difference in intracellular ATP/ADP ratio. Therefore the higher ATP/ADP ratio at pH 7.4 must be caused by a higher proton translocation activity of the respiratory chain and/of by a lower ATPase activity of the cells.

The P_i concentration in bacteroids is about 22 mM and the adenine nucleotide pool 0.9 mM (not shown). Therefore the P_i potential will be determined by the ATP/ADP ratio. The experiments presented in Figure 1 indicate that above an ATP/ADP ratio of 1, the P_i potential becomes inhibitory for respiration at low free O_2 concentrations. This is further proven by the experiments presented in Figure 2. When substrate reduction by nitrogenase is inactivated by an O_2 treatment, reductant dependent ATP hydrolysis by nitrogenase is also inhibited. This is 90 % of the ATPase activity of nitrogenase (17).

The inhibition of the ATP hydrolysis by nitrogenase has a significant effect on respiration at non-saturating O_2 concentrations. At the same O_2 input rate, the ATP/ADP ratio in O_2 treated cells is much higher than in non-treated cells (see Figure 2) and the respiration of bacteroids with an inactive nitrogenase is much lower at non-saturating O_2 concentrations than respiration of bacteroids with an active nitrogenase. By the addition of a proton conducting ionophore, the proton motive force is lowered and this stimulates respiration resulting in a new steady state with a much lower free O_2 concentration (0.5 µM). This shows that respiratory control limits respiration.

That the magnitude of the proton motive force is important for the rate of respiration is also shown in Table 1. By lowering the proton motive force partially (addition of a non-saturating CCCP concentration) respiration is stimulated and nitrogenase activity is possible at a high O_2 input rate. This also shows that the pH effect on respiration and nitrogen fixation is an effect of the P_i potential on respiration. Both types of experiments indicate that the ATP hydrolysis by nitrogenase can lower the proton motive force so that respiration at O_2 concentrations below 1 µM is stimulated.

If the ATP hydrolysis by nitrogenase would be lowered by genetic engineering, the possibility can not be ruled out that

cells with the modified enzyme will have no nitrogenase activity because respiration at non-saturation O_2 concentrations might be inhibited by respiratory control.

Our data support the observation of Appleby *et al.* (20) that stimulation of nitrogenase activity also stimulates O_2 consumption. The effect of nitrogenase activity on respiration via the ATP/ADP ratio can be explained by the fact that nitrogenase is a major ATP consumer in bacteroids at low O_2 concentrations. If the P/O of bacteroids is 2 (21), an O_2 consumption of 25 nmol O_2. min^{-1}. mg $protein^{-1}$ (Figure 1B) will give a rate of phosphorylation of 100 nmol ATP. min^{-1}. mg $protein^{-1}$. The nitrogenase activity is 18 nmol C_2H_4 formed. min^{-1}. mg $protein^{-1}$. If the ATP/2e ratio of nitrogenase is 4 (19), nitrogenase will consume 72 nmol ATP. min^{-1}. mg $protein^{-1}$ and this is 72 % of the ATP formed by oxidative phosphorylation.

That nitrogen fixation not only influences respiration via the P_i potential can be concluded from the results shown in Figure 3B. There it is shown that bacteroids with a low proton motive force (the addition of CCCP) have a higher O2 uptake rate than bacteroids actively reducing C_2H_2. The explanation might be that ATP hydrolysis by nitrogenase lowers the P_i potential not enough to prevent inhibition of respiration by respiratory control. It is also possible that in the presence of CCCP due to a low ATP/ADP ratio electron transfer from Fe protein to MoFe protein of nitrogenase is inhibited and the reducing equivalents which are normally used for nitrogen fixation are now used for O_2 reduction.

The lower rate of respiration of O_2 treated bacteroids in the presence of CCCP compared with non O_2-treated bacteroids also in the presence of CCCP, indicates that the O_2 treatment inhibits beside nitrogenase an other O_2 sensitive redox system that can reduce O_2. This might be a component of the electron transport chain to nitrogenase, for instance a ferredoxin which looses its Fe/S cluster upon oxidation by O_2. This is supported by the observation that during a measurement of the rate of respiration of bacteroids in the presence of CCCP at high O_2 concentrations ($[O_2] > 150$ µM) the O_2 uptake rate declines 10-20 nmol O_2. min^{-1}. mg $protein^{-1}$ within 5 min after the start of the experiment.

Bergersen and Turner (22,23) studied the relation between O_2 consumption, intracellular ATP concentration and nitrogen fixation in *Bradyrhizobium japonicum* bacteroids. The rate of respiration was calculated from the time course of O_2 consumption. The reaction was started at 10 µM O_2 and the bacteroids were allowed to deplete O_2 from the reaction mixture. It was found that the intracellular ATP/ADP ratio was low at an O_2 concentration of 10 µM. The ATP/ADP ratio increased during time and at an O_2 concentration of 0.1 µM the ratio was maximal. At lower O_2 concentrations it declined. From these type of experiments it

was concluded that soybeans bacteroids contains two oxidase systems. One is only active in the presence of leghaemoglobin at low O_2 concentrations (<0.2 µM) and has a high P/O ratio. At higher O_2 concentrations the high affinity system has a low activity and in the absence of an O_2 carrier a second oxidase system becomes active at an O_2 concentration of 1 µM and has a low P/O ratio.

With our results we can give an alternative explanation for the observations of Bergersen and Turner. The ATP/ADP ratio in anaerobic stored bacteroids is low (14) and it takes about 1 min before the steady state ratio is reached. At the same time the O_2 concentration is lowered enough to allow nitrogenase activity and nitrogenase will keep the ATP/ADP ratio low. At lower free O_2 concentrations, for instance < 0.1 µM in the presence of oxymyoglobin, respiration declines due to diffusion limitation and thus electron transport to nitrogenase. A lower nitrogenase activity can increase the ATP/ADP ratio if this ATP hydrolyzing system is more inhibited than oxidative phosphorylation. This might explain the observation (22,23) that the maximal nitrogenase activity was measured before the maximal ATP/ADP ratio was reached. We therefore suggest that the observed changes in intracellular ATP/ADP ratios (22,23) are caused by the activity of nitrogenase and not by more or less efficient oxidase systems.

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6. DISCUSSION

The central theme of this thesis deals with the factors which regulate the nitrogenase activity and the electron allocation by nitrogenase in the *Rhizobium*-pea symbiosis. The studied factors will be illuminated and discussed considering the overall functioning of the root nodule. Since malate plays a prominent role in the metabolism of the root nodule, it is chosen as starting-point of this treatise.

Malate is involved in (at least) four physiological processes that take place in the root nodules, namely:

- malate is used as an oxidizable substrate for the root nodule mitochondria
- malate is implicated in the regulation of the pH
- malate is the main carbon source for the bacteroids
- malate is involved in the malate/aspartate shuttle between the cytoplasm of the nodule cells and the bacteroids.

6.1 *The role of the plant mitochondria in the symbiosis*

Plant mitochondria can oxidize malate. They contain specific dicarboxylate carriers (1) and two malate oxidizing enzymes namely malate dehydrogenase and malic enzyme (2). By the combined action of both enzymes and the pyruvate dehydrogenase complex, citrate can be formed, which can be metabolized in the tricarboxylic acid cycle. This cycle fulfills an anaplerotic function towards processes of the cytoplasm.

A second function of the plant mitochondria is to supply the plant cytoplasm with ATP. Plant mitochondria possess two pathways for electron transport namely the so-called alternative pathway and the cytochrome oxidase linked respiratory chain. The alternative pathway is not linked to phosphorylation (3). The coupling between the two respiratory chains takes place at the level of ubiquinone, that is after complex I. The non-phosphorylating alternative pathway facilitates the anaplerotic function of the tricarboxylic acid cycle, by uncoupling the cycle from the regulation by the cytoplasmic phosphorylation potential.

The mitochondria present in the infected nodule cells may fulfill the same functions as the common plant mitochondria. Isolated nodule mitochondria can readily oxidize malate (6,7). However the mitochondria in infected cells are larger and more numerous than those in the uninfected cells (4,5), suggesting a specific role for these mitochondria. Differences between the common plant mitochondria and nodule mitochondria have been noticed. In soybean and cowpea nodule mitochondria, only little malic enzyme activity could be demonstrated (7,8) and these mitochondria lack the alternative pathway (6,8).

It is thought (8) that the lack of malic enzyme activity restricts the ability of nodule mitochondria to fulfill an anaplerotic function because no pyruvate can be formed from a citric acid cycle intermediate. However, the mitochondria can obtained pyruvate from the glycolysis (9,10). Thus nodule mitochondria lacking malic enzyme activity, may still be able to fulfill an anaplerotic function.

It is assumed (7) that the prevailing low O_2 concentrations in nodules would limited the functioning of the nodule mitochondria. The observations that mitochondria are enlarged and more numerous (4) and have increased cytochrome oxidase activity (8), argue for a function in oxidative processes.

In summary the malate oxidation by nodule mitochondria will assist the anaplerotic functioning of the tricarboxylic acid cycle and the ATP provision of the plant cytoplasm. The supply of carbon skeletons to the plant cytoplasm supports the NH_3 assimilation (for instance the export of 2-oxoglutarate supports the glutamate synthase reaction) and the provision of ATP contributes to the energy requirement for NH_3 assimilation and other cell activities.

6.2 *The involvement of malate by the pH regulation in nodules*

Malate being a strong acid (pK_s 3.4 and 5.1) is involved in the regulation of plant cytoplasmic pH (11-13,27). Cytoplasmic pH regulation by malate can occur through:

- intracellular production or consumption of malate
- the transport of malate with or without H^+ across the membranes of the different compartments.

The effect of malate on the pH can be related to a short-term range but it might also be involved in long-lasting pH changes.

According to Davies (11), the intracellular production or consumption of malate fulfills a function in the maintenance of a constant pH in short-term range. Davies proposes that reactions involved in removing and introducing a carboxyl group from malate and which show different pH optima, could regulate the cytoplasmic pH of plant cells (Figure 1). An increase in cytoplasmic pH leads to increased carboxylation by increasing the activity of phosphoenolpyruvate carboxylase and reducing the decarboxylation by malic enzyme. This results in a net gain of carboxyl groups which brings the pH back to its original value. On the other hand, a decrease in cytoplasmic pH increases the activity of malic enzyme and decreases the activity of phosphoenolpyruvate so that pyruvate, CO_2 and NAD(P)H are formed. Consequently the pH returns to its original value. These reactions have been shown to occur *in vivo* (11-13). The sensitivity of this mechanism for pH regulation, depends on the steepness of

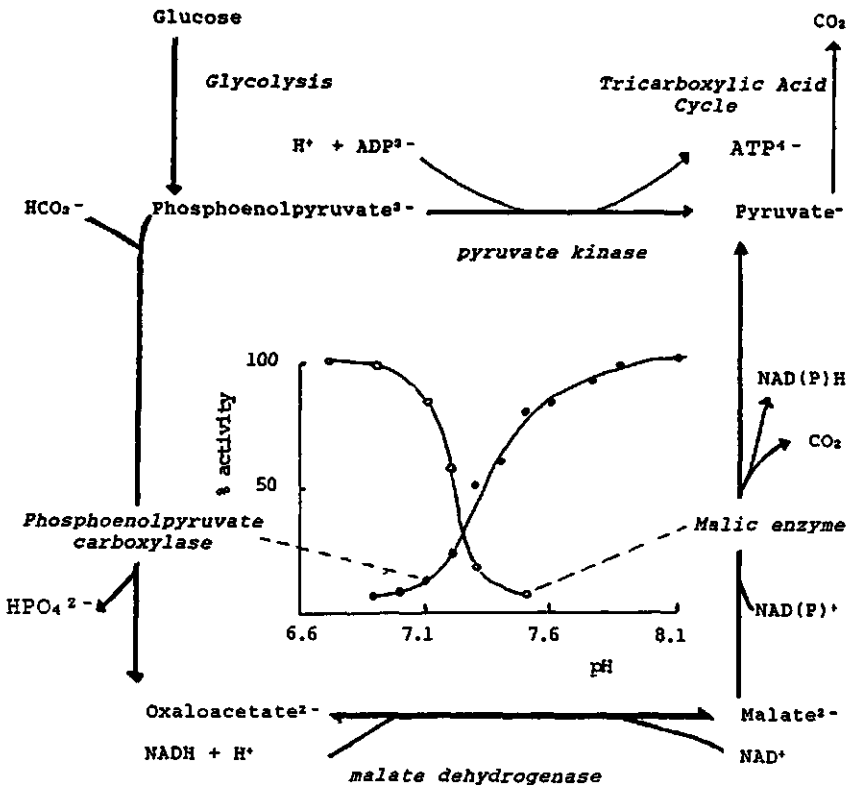

Figure 1.
Reactions involved in a malate-based pH regulation. The pH activity curves of phosphoenolpyruvate carboxylase and malic enzyme are shown in the insert. Figure after Davies (11) with modifications.

the activity *versus* pH curves and is further enhanced by metabolites that act in opposing ways on malic enzyme and phosphoenolpyruvate carboxylase. For instance, malate activates malic enzyme and inhibits phosphoenolpyruvate carboxylase. The capacity of this mechanism for pH regulation depends on the pools of the respective reactants. If the mechanism is considered as a bypass of the reaction catalyzed by pyruvate kinase, the cost for the control of pH is about 8 kcal/mol H^+.

This mechanism for short-term pH regulation might be functioning in the plant cytoplasm of the root nodule because the involved enzymes are present (unpublished results) The pH optimum of malic enzyme (pH = 6.6) and of phosphoenolpyruvate carboxylase (pH = 8.1) and the steepness of the activity *versus* pH curves (unpublished results) are in agreement with the model of Davies. This mechanism will not apply for the bacteroids, because bacteroids do not have phosphoenolpyruvate carboxylase

activity.

When the amount of photosynthates increases in the root nodule cells, malate will be formed. In first instance, malate will be converted to pyruvate$^-$, CO_2 and NAD(P)H by the action of malic enzyme, according to the model of Davies. Consequently, the pH of the cytoplasm of the nodule cells will not change. If the supply of photosynthates by the plant continues, malate is accumulated in the cytoplasm of the root nodules (14,48-50). Ultimately, the accumulation of malate causes a decrease in the cytoplasmic pH. The acidification will approach a new steady state of pH as has been observed in several studies, for instance with root hair cells of *Sinapis alba* (16), *Zea mays* cells (17) and cells of *Neurospora crassa* (18). Changes in the cytoplasmic pH are important for the bacteroid respiration and the nitrogenase activity. In chapter 4, the effect of the external pH of the bacteroids on respiration, nitrogenase activity and the electron allocation by nitrogenase of bacteroids, is described. A small increase in external pH within the physiological pH-range, causes a decline in the rate of bacteroid respiration and a decrease in nitrogenase activity, The observations described in chapter 4 suggest the possible existence of a mechanism which attunes the supply of photosynthates by the plant, the rate of bacteroid respiration and the O_2 supply towards the bacteroids. Ultimately this mechanism couples the nitrogenase activity with the supply of photosynthates. Here, we postulate such a mechanism.

The proposal involves the variable O_2 diffusion barrier in the inner cortex of the root nodule. The barrier is based on the diffusion of O_2 through water-filled channels between the cortex cells (19,20, paragraph 1.8). O_2 has to diffuse through the channels because there are no air pathways in this part of the root nodule. The length of the channels can be varied depending on the turgor pressure of the cells involved. Variations in the length of the channels provide the observed variability of the barrier. The way upon the turgor pressure of the cells changes, may resemble the mechanism which determines the stomatal turgor of guard cells.

In Table 1 a simplified representation of the stomatal turgor mechanism is given. When the pH in the guard cells increases, the pH-sensitive starch phosphorylase catalyzes the formation of glucose-1-phosphate from starch. Subsequently the formed glucose-1-phosphate is converted into malate by the action of the glycolytic enzymes, phosphoenolpyruvate carboxylase and malate dehydrogenase. The ultimately accumulation of malate in the guard cells results in an increased turgor pressure. When the pH decreases, only starch accumulation is observed.

In the inner cortex cells the described mechanism of changing the turgor pressure, might function because the components of the stomatal turgor mechanism are present. Large starch accumulation in the inner cortex cells (21,22) and the activities of starch phosphorylase, phosphoenolpyruvate carboxylase and malate dehydrogenase, have been established for the root nodule cells

Table 1. Simplified representation of the enzymatic reactions involved in the stomatal turgor mechanism.

Starch phosphorylase (EC 2.4.1.1.)

$$\text{Starch} + HPO_4^{2-} \underset{\text{pH = 5}}{\overset{\text{pH = 7}}{\rightleftharpoons}} \text{Starch} + \text{Glucose-1-Phosphate}^{2-}$$

Phosphoglucomutase (EC 2.7.5.1.)

$$\text{Glucose-1-Phosphate}^{2-} \rightleftharpoons \text{Glucose-6-Phosphate}^{2-}$$

Glycolyse

$$\text{Glucose-6-Phosphate}^{2-} + ADP^{3-} + 2\ NAD^{+} + 2\ HPO_4^{2-} \longrightarrow 2\ \text{Phosphoenolpyruvate}^{3-} + ATP^{4-} + 2\ NADH + 3\ H^{+} + 2\ H_2O$$

Phosphoenolpyruvate carboxylase (EC 4.1.1.31)

$$\text{Phosphoenolpyruvate}^{3-} + HCO_3^{-} \longrightarrow \text{Oxaloacetate}^{2-} + HPO_4^{2-}$$

Malate dehydrogenase (EC 1.1.1.37)

$$\text{Oxaloacetate}^{2-} + NADH + H^{+} \rightleftharpoons \text{Malate}^{2-} + NAD^{+}$$

(23, chapter 2 and unpublished results). The presence of the glycolytic enzymes is generally assumed (see paragraph 1.6).

The postulated mechanism can be visualized as follows. When the supply of photosynthates (sucrose) by the plant to the inner parts of the nodule increases, malate accumulates in the infected cells causing ultimately a new, lower cytoplasmic pH. The decline in pH will affect also the pH of the surrounding cells. This 'pH-signal' reaches the inner cortex cells. The decrease in pH causes an increase in the ratio starch formation/starch degradation and the malate concentration in these cells decreases, like in the guard cells of the stomata. Consequently the turgor pressure of the inner cortex cells reduces. The length of the waterchannels between the cells decreases and the O_2-resistance reduces. Ultimately, the O_2 supply towards the bacteroids in the inner parts of the root nodule increases.

The second effect of the shift in pH, caused by the increased

supply and degradation of photosynthates, is the increase in the rate of bacteroid respiration (chapter 4). The increase in respiration of bacteroids in the root nodule is possible, because an increase in the supply of O_2 is accomplished by the lower pH. The incline in the respiration of bacteroids is accompanied with an increase in nitrogenase activity (chapter 4).

When the supply of photosynthates decreases, the cytoplasmic pH will rise. The ratio of starch formation/starch degradation in the inner cortex cells will decreases and the malate concentration increases. The high malate concentration is related to a high turgor pressure and as a result the O_2-resistance increases. Less O_2 will be available for the bacteroids. A higher pH induces a decrease in the respiration of bacteroids and therefore a decrease in nitrogenase activity (chapter 4).

If this suggested mechanism is functional, the O_2 supply to the bacteroids is balanced with the supply of photosynthates by the plant, mediated by pH. The suggestion that the pH shows characteristics of a cellular messenger is not unfounded. There are indications that changes in the cytoplasmic pH play the role of messenger in both animals (24,25) and plants (26,27). Cellular activities in which the pH fulfills a role of messenger are e.g. the endocytosis of transferrin and epidermal growth factors in several cell types (28), the chemotaxis by neutrophils (29), the synthesis of *m*RNAs as a response on elicitors in plant cells (30,31) and the metabolic responses of root cells to hypoxia (32).

Malate may also be involved in changes of the pH of the bacteroid. This might be the case, if the transport of malate from the plant cytoplasm to the bacteroid is coupled to the transport of H^+ and the resulting import of H^+ is not completely compensated by H^+ extrusion. Such a situation will result in a new pH state.

In theory, three different transport mechanisms for malate are possible (see Figure 2). Malate can be transported independently into the bacteroid or in combination with glutamate (chapter 3). The independent transport of malate can occur via both passive and active mechanisms (14).

One passive transport mechanisms for malate is possible. This mechanism consists of a neutral antiporter, which catalyzes the electroneutral exchange of dicarboxylate anions.

The most favoured hypothesis to account for the observed active transport of malate, consists of a symport of malate and H^+, with the pH gradient as driven force. The malate might be transported as $malate^{2-}$, monovalent malate - as has been observed in soybean bacteroids (53) - or even as neutral malate.

If the newly identified malate/aspartate shuttle between the plant cytoplasm and the bacteroid (chapter 3) resembles the mammalian mitochondrial shuttle, it will also transport H^+ into the bacteroid. In mammalian mitochondria, the 2-oxoglutarate carrier catalyzes an electroneutral $malate^{2-}$/2-$oxoglutarate^{2-}$ exchange, whereas the aspartate/glutamate carrier catalyzes an electrogenic transport of $glutamate^- + H^+$ against $aspartate^-$ (15). In our

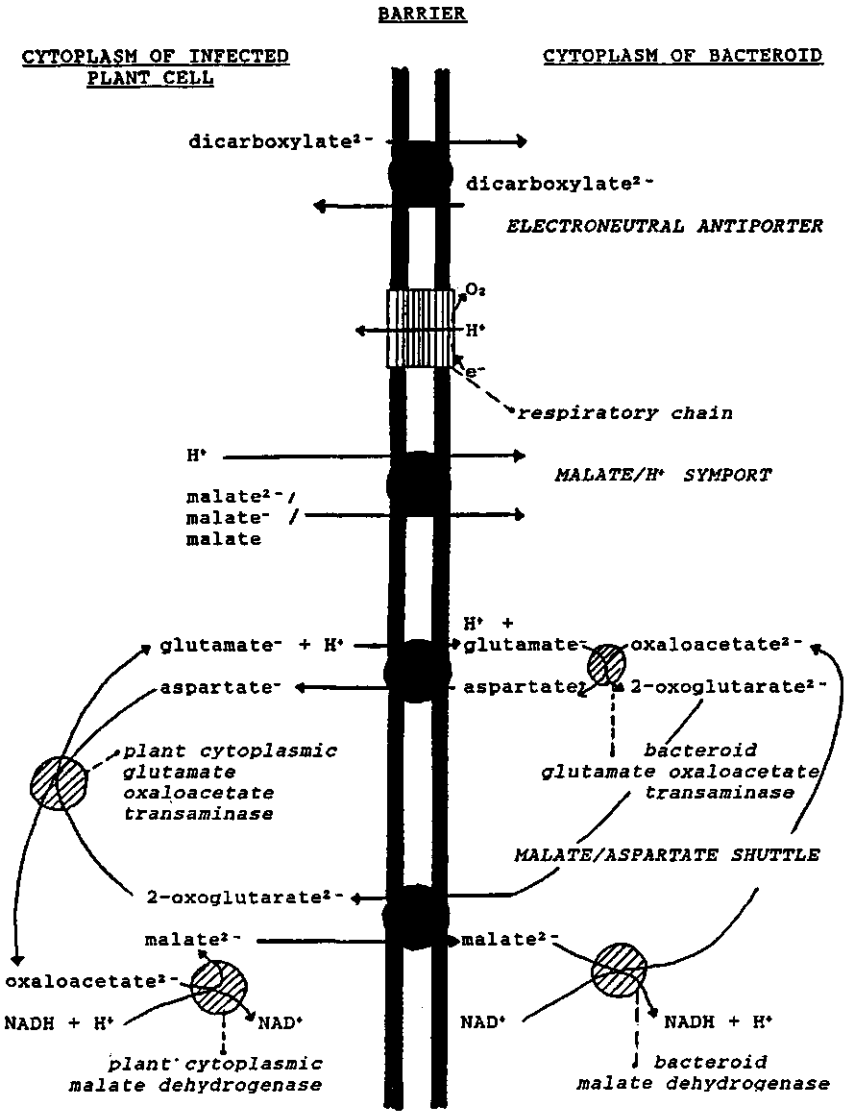

Figure 2.
Schematic representation of the transport mechanisms between the cytoplasm of the infected plant cell and the cytoplasm of the bacteroid.

case, glutamate and malate are transported into the bacteroids (chapter 3). Consequently the exchange will be stimulated by a proton motive force. Because a negative charge is transported across the barrier to the outside of the bacteroid, the membrane potential (negative inside) contributes mostly to the exchange of the compounds. The H^+ taken up by the bacteroid during the exchange, can be pumped out electrogenically by the respiratory chain.

Thus, the transport of malate by means of the malate/aspartate shuttle and the malate/H^+ symport driven by the pH gradient, can both lower the pH of bacteroids, if the net amount of H^+ taken up is not counterbalanced by H^+ extrusion.

6.3 *The oxidation of malate by the bacteroids*

The reduction of N_2 in the bacteroid requires energy in the form of MgATP and reductant (see paragraph 1.4), which are both generated from substrates oxidized by the bacteroid. The oxidizable substrates of the bacteroid are supported by the plant. Whether or not a compound contributes significantly to the energy requirements of the bacteroid depends on:

- the rate at which the compound is transported across the peribacteroid membrane and the bacteroid cytoplasmic membrane
- the capability for oxidation of the compound by the bacteroid.

The results presented in chapter 3 indicate that several compounds besides malate, can be transported into the bacteroid. The rate at which a compound is transported across the membranes is affected by the presence of other compounds. For instance, glutamate is transported at a much higher rate into the bacteroid in the presence of malate than in absence of malate (chapter 3). The same effect can be observed for 2-oxoglutarate and aspartate (chapter 3). Thus, considering the transport of compounds into the bacteroids, one should take account of a symport or an combined exchange of compounds, including small cations like K^+. This is often not done (51,52), which leads to wrong conclusions.

From the results presented in chapter 3 and 4, it can be concluded that several compounds including glutamate, stimulates the respiration of bacteroids. The fact that a compound can be oxidized by bacteroids, do not imply that the compound in question also stimulates nitrogenase activity in the same extent. For instance, the rate of respiration of glutamate, 2-oxoglutarate, aspartate is half of the rate observed for malate. The maximum nitrogenase activity of the three compounds is about one third of the activity found for malate (chapter 4). The rate of respiration at low O_2 concentrations, reflects the maximum rate of nitrogenase activity for a given compound (chapter 3 and 4). When a compound is well oxidized by the bacteroids, the intra-

cellular NADH / NAD^+ ratio will be high and enables electron transport to O_2 through the respiratory chain. This stimulates electron transfer to nitrogenase. A compound which is metabolized at a lower rate, can not maintain a high NADH / NAD^+ ratio. This will result in a more oxidized state of the cell and the nitrogenase activity will be inhibited. Only C_4-dicarboxylic acids can be oxidized rapidly enough at low O_2 concentrations to support the N_2-reduction substantially (chapter 3, 33, 34). Other studies (34,35) indicate that disaccharides, hexoses and amino acids are not respired at a high rate by bacteroids.

Since malate is the predominant C_4-dicarboxylic acid in the root nodule (14,48-50) and malate is well oxidized (chapter 3) and *Rhizobium* mutants missing their ability to transport C_4-dicarboxylic acids are unable to reduce N_2 in the symbiosis (36-38), malate can be considered as the principal contributor to the energy requirements of the bacteroid in the symbiosis. The oxidation of malate by the bacteroids is facilitated by the presence of malate dehydrogenase (chapter 2), malic enzyme (chapter 3) as well as by the presence of the enzymes of the tricarboxylic acid cycle (39).

6.4 *The malate/aspartate shuttle*

Malate is one of the compounds which is involved in the described malate/aspartate shuttle (chapter 3).

The existence of the shuttle has been suggested previously for legumes nodules (40) and for *Alnus glutinosa* nodules (41). Streeter and Salminen (42) gave some evidence for the existence of the shuttle in soybean bacteroids. Our results (chapter 3) support the possible operation of a malate/aspartate shuttle in the symbiosis. The shuttle can perform several functions.

The shuttle does not transport carbon compounds from the cytoplasm to the bacteroid but it transfers NADH from the plant cytoplasm to the bacteroid (Figure 2). Ultimately NADH can be used for the formation of ATP.

If the shuttle resembles the mammalian mitochondrial malate / aspartate shuttle, the shuttle transfers not only reducing equivalents but also H^+ by the aspartate/glutamate carrier (15). This implies that aspartate is effectively pumped out of the bacteroid due to the proton motive force, in particular by the favorable membrane potential. This coupling between membrane potential and the function of the shuttle is useful for the root nodule from a view of regulation of nitrogenase, because also the flow of reducing equivalents to nitrogenase is regulated by the membrane potential (43). Consequently a switch off of nitrogenase by lowering of the membrane potential, is coupled to a decline in supply of cytoplasmic NADH from the plant to the bacteroid. Thus catabolism is coupled with anabolism.

A second function of the shuttle is the transamination of oxaloacetate to aspartate in the bacteroid. Aspartate is necessary for the synthesis of asparagine, which is the main product of the NH_3 assimilation in pea root nodules.

Our results presented in chapter 3, indicate that the predominant nodule-associated glutamate oxaloacetate transaminase (GOT) facilitates a relative high rate of catalysis in both directions. Labelling studies (44,45) have demonstrated that aspartate is rapidly converted into malate in the cytoplasm of nodule plant cells. These observations indicate that GOT in the cytoplasm of root nodule cells, functions rather in the direction of oxaloacetate than in the direction of aspartate, due to the prevailing physiological conditions. This means that no significant formation of aspartate from oxaloacetate will be catalyzed by GOT in the cytoplasm of root nodule cells.

The synthesis of aspartate can be performed by the activity of the malate/aspartate shuttle. The high rates of catalysis of both nodule-stimulated malate dehydrogenase (chapter 2) and GOT (chapter 3) in the plant cytoplasm and the large consumption of reducing equivalents by the N_2-reducing bacteroid, cause an efficient operating shuttle. Aspartate is synthesized inside the bacteroid and subsequently transported into the cytoplasm of the nodule plant cells. Since the shuttle operates at a high rate, the supply of aspartate towards the plant cytoplasm will be large. Thus the high rate of aspartate conversion into oxaloacetate by GOT in the cytoplasm of root nodule cells can be compensated by the supply of aspartate synthesized in the bacteroid. In this way the aspartate concentration is maintained high in the plant cytoplasm, which has indeed been observed (54). If aspartate is withdrawn from the cycle for the asparagine biosynthesis, carbon lost from the cycle by this action can be replaced by malate.

Besides malate, glutamate can be metabolized by the bacteroid. Respiration and nitrogenase activity are stimulated by glutamate (chapter 3). These observations support the hypothesis, that glutamate acts as carbon source for bacteroids in nodules, as has been proposed by Kahn *et al.* (40). This possibility is also supported by the results of Salminen and Streeter (44), who showed uptake, incorporation and respiration of endogenous glutamate by soybean bacteroids. However, it has also been suggested that glutamate will not be transported at all over the peribacteroid membrane (51).

The malate/aspartate shuttle can function in two directions (chapter 3); it can transport aspartate and 2-oxoglutarate from the plant cytoplasm into the bacteroid. In our view, this exchange of aspartate and 2-oxoglutarate from the plant cytoplasm against malate and glutamate in the bacteroid, will serve no useful purpose. The transport of NADH out of the bacteroid will result in a decline in nitrogenase activity.

In summary, it is proposed that the malate/aspartate shuttle fulfills at least two important functions in the symbiosis. It transfers reducing equivalents to the bacteroid which can be used for energy transduction. Secondly, it enables the formation of aspartate, which is an essential step in the NH_3 assimilation of the root nodule.

6.5 *The role of malate dehydrogenase*

The host plant transports sucrose from the shoot to the root nodule. Malate is involved in four physiological processes of the root nodule as mentioned before. This implies that sucrose has to be converted into malate and that the regulation of the malate concentration is of importance.

Phosphoenolpyruvate and pyruvate are the products of the glycolyse in the cytoplasm of root nodule cells from which malate can be synthesized. In theory, there are three ways possible to synthesize malate (Figure 3, page 120):

- in the cytoplasm from phosphoenolpyruvate catalyzed by phosphoenolpyruvate carboxylase (PEPC) and malate dehydrogenase (MDH)
- in the cytoplasm from phosphoenolpyruvate catalyzed by pyruvate kinase (PK) and malic enzyme (ME)
- in the mitochondria from pyruvate catalyzed by mitochondrial pyruvate carboxylase and malate dehydrogenase.

If the formation of malate takes place in the cytoplasm, the synthesis catalyzed by PEPC and MDH is the most likely pathway. Three arguments speaks for this pathway.

The activity of MDH and PEPC are higher than the activities observed for PK and ME. The activities of PEPC, MDH, PK and ME were respectively, about 0.30, 25, 0.10 and 0.15 U. (mg cytoplasmic protein)$^{-1}$. Secondly the enzyme activity of PEPC present in the cytoplasm of nodule plant cells is strongly stimulated - 15 times - compared with the activity measured in the cytoplasm of uninfected roots. This points to a specific role of PEPC in the root nodule. The other three enzymes are also stimulated in root nodule but to a less extent (five times at most). The third argument is that only trace levels of ME activity with pyruvate, CO_2/HCO_3^- and NAD(P)H as substrates are detected (unpublished results). Only ME activity measured as the formation of pyruvate could be demonstrated. The unidirectional functioning of ME have been reported earlier (47), however there are studies which question this (46). The synthesis of malate by the anaplerotic functioning of the mitochondria can not be ruled out. Information is lacking to assess this possibility.

To our opinion, malate is formed in the cytoplasm of nodule plant cells by the catalysis of PEPC and MDH.

Under physiological conditions the reaction catalyzed by MDH can also be considered as unidirectional towards the formation

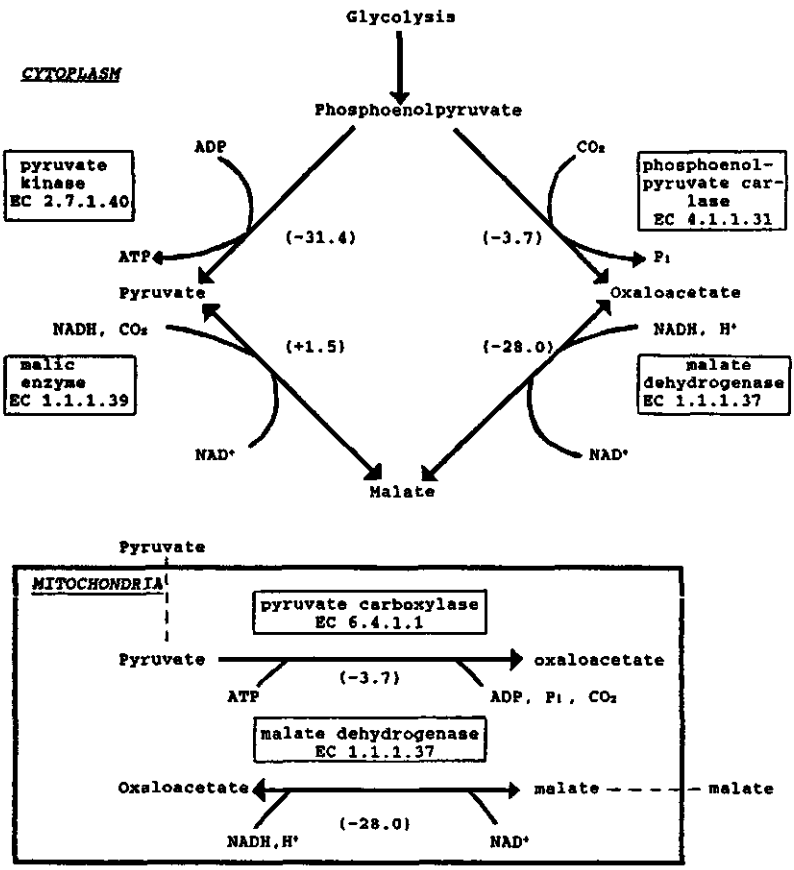

Figure 3.
The enzymes possible involved in the formation of malate from phosphoenolpyruvate. The number between parentheses are $\Delta G_o'$ in kJ.mol^{-1} of the reaction in question with a view to the formation of malate.

of malate. Under standard conditions, the reduction of oxaloacetate by NADH is exergonic (G_o'=-28 kJ.mol^{-1}). Under physiological conditions prevailing in the nodule cell (NADH / NAD$^+$= 0.3, [malate]= 4 mM), the reaction is completely in favour of NAD$^+$ and malate. If the enzyme would catalyze its reaction to equilibrium, the concentration of oxaloacetate would be only 0.2 µM. Therefore, the regulation of the malate formation catalyzed by MDH is essential for the functioning of the root nodule.

Malate can bind to the enzyme-NADH complex, thereby blocking the further reduction of oxaloacetate by NADH. The dominant nodule-stimulated MDH described in chapter 2, forms this abortive complex at physiological malate concentrations. The main MDH present in the cytoplasm of uninfected roots, does not form the abortive complex at low malate concentrations. Consequently the conversion of malate catalyzed by the dominant nodule-stimu-

lated form will be less completed as has been calculated above.

The importance of cytoplasmic MDH for the functioning of the root nodule can be judged from the differences in kinetic constants between nodule-associated MDH and MDH of uninfected root cells. The K_m-values of the substrates of the dominant nodule form are much lower than those estimated for the main root form (chapter 2). These observations suggest that under physiological conditions of the root nodule ([oxaloacetate]$\approx$ 0.02 mM, [malate] $\approx$ 4 mM, [NADH]$\approx$ 0.3 x [NAD^+]$\approx$ 0.01 mM) the nodule-stimulated form exhibits a higher rate of catalysis than the main root form. Thus, the dominant nodule form is more suited to function within the increased metabolic activity of the root nodule cell than the root form.

The observation that cytoplasmic MDH increases during nodule development, points also to the importance of MDH for the nodule. The increase starts already eight days after infection. This indicates that the stimulated MDH forms are not only involved in nodule function but also in nodule development.

In summary, it is proposed that the cytoplasmic MDH is essential for the symbiosis. The cytoplasmic MDH activity is increased and the related enzyme is adopted in two ways to its functioning in nodule cells. Firstly, the formation of malate is catalyzed at a high rate, which is necessary in view of the increased metabolic demand for malate in the root nodule cell. Secondly, the activity of MDH is controlled by malate to prevent the formation of a too high malate concentrations, which would deregulate the processes mentioned.

6.6 *Regulation of nitrogenase activity*

The infected plant cell has different possibilities to regulate the metabolism of the bacteroid and thus nitrogenase activity. Four factors are extremely important for nitrogenase activity of the bacteroid:

- the free O_2 concentration,
- the intracellular ATP/ADP ratio,
- the membrane potential
- the rate of electron transport through the respiratory chain to O_2.

The four factors are determined (at least) by three processes namely:

- the supply of oxidizable substrate,
- the supply of O_2
- the external pH of the bacteroid.

From our studies (chapter 4 and 5), it is concluded that variation of the pH is a very effective way to control nitrogenase activity without affecting the efficiency of the nitrogenase reaction. The external pH of the bacteroid determines the rate of respiration and nitrogenase activity at low free O_2 concentrations, without affecting the intracellular ATP/ADP ratio. The mechanism of pH regulation can be conceived if the relationships between intracellular ATP/ADP ratio (or phosphate potential), the rate of respiration, and nitrogenase are considered.

Our results (chapter 5) show that an increase in intracellular ATP/ADP ratio above 1.2, is accompanied by an decline in the rate of respiration of the bacteroid at low free O_2 concentrations. Ultimately, this results in a higher free O_2 concentration. In turn, higher free O_2 concentration ($\geq$ 1 µM) causes inhibition of nitrogenase and this increases the ATP/ADP ratio.

In this respect, it should be noticed that the nitrogenase reaction is the largest ATP-consuming process in the bacteroid. From the results presented in chapter 5, it has been calculated that about 70 % of the ATP formed by the oxidative phosphorylation, is used by nitrogenase. This implies that inhibition or inactivation of nitrogenase results in a substantial increase in the intracellular ATP/ADP ratio.

Conversely, at high free O_2 concentrations, a decrease in intracellular ATP/ADP ratio will induce an increase in the rate of respiration, as can be concluded from the experiments with the uncoupler CCCP (chapter 5). Consequently, the free O_2 concentration decreases.

These observations indicate that the flow of electrons through the respiratory chain, is inhibited if the ATP/ADP ratio is too high. It is concluded that the bacteroids have a respiratory control. A high intracellular ATP/ADP ratio inhibits nitrogenase activity. The decrease in the rate of respiration results in a higher free O_2 concentration and consequently in inhibition of nitrogenase by O_2.

This means that a low intracellular ATP/ADP ratio ($\leq$1.2) is a requirement for nitrogenase activity in bacteroids. On one side, nitrogenase needs ATP; on the other hand nitrogenase is inhibited by ADP. These contradictory requirements imply that in the bacteroid a compromise has to be accomplished between respiratory control and ADP inhibition of nitrogenase in order to facilitate maximum nitrogenase activity.

The observed effect of the external pH on the rate of respiration by the bacteroid can be explained in two ways:

- An increase in external pH induces a lower ATPase activity and therefore the intracellular ATP/ADP ratio will rise. Consequently the high intracellular ATP/ADP ratio results in a decrease in the rate of respiration, due to a higher proton motive force.

- An increase in external pH induce a higher H^+-translocation activity of the respiratory chain. Thus the H^+-transmitting conformational changes of the energy-conserving sites, which are coupled to the electron flow to O_2, are changed by the pH. More H^+ is transported across the membrane at the same rate of electron flow to O_2. The proton motive force increases and more ATP will be synthesized when the H^+ flows through the ATP synthase. Ultimately, the increase in pH results in a higher intracellular ATP/ADP ratio at the same rate of respiration. Because the intracellular ATP/ADP ratio increases more substantially at a higher pH than at lower pH, the observed respiratory control will occur at a lower rate of respiration.

Conflicting and contradictory reports have been published about ATP synthesis and respiration in bacteroids.

In *Bradyrhizobium japonicum* bacteroids, the ATP/ADP ratio decreases as the O_2 tension is raised (56). Bergersen and Turner (56,57) suggest that the ATP/ADP ratio is controlled by O_2 by means of a branched electron-transport system. The high ATP/ADP ratio is associated with an oxidase with a high affinity for O_2 and the low ATP/ADP ratio is coupled to a branch terminated by an oxidase with a lower affinity for O_2.

In *Rhizobium leguminosarum* (55,58,61, chapter 4 and 5), *R. leguminosarum* bv. *phaseoli* (59) and *R. leguminosarum* bv. *trifolii* (60), the intracellular ATP/ADP ratio increases as the O_2 concentration is increased. Thus, the relation between the intracellular ATP/ADP ratio and the respiration observed for *B. japonicum* bacteroids is different from that of other *Rhizobia*. This difference could be due to oxidases with different affinities for O_2. However, the cytochrome profile of the lupin (62) and clover bacteroids (60) looks very similar to that of soybean bacteroids (63). We found only one K_m for O_2 of the terminal oxidase, which makes the existence of a branched chain in our bacteroids doubtful (chapter 5). It should be noticed that the intracellular ATP/ADP ratio is the result of both the ATP synthesis driven by the proton motive force and of ATP-consuming processes. As has been stated, the N_2-reduction itself is the largest ATP-consuming process (chapter 5). Consequently, one should take care if the efficiency of oxidative phosphorylation is judged by the intracellular ATP/ADP ratio.

In isolated bacteroids, an increase in intracellular ATP/ADP ratio is accompanied with an increased electron allocation to N_2 by nitrogenase (chapter 5). The electron allocation to N_2 can reach its maximum, even when the nitrogenase activity is not maximal. This situation corresponds with an ATP/ADP ratio above 0.6 . From these results, it can be concluded that the electron allocation by nitrogenase depends on the intracellular ATP/ADP ratio, as has been suggested by Haaker and Wassink (61).

In isolated bacteroids, O_2 limitation causes a decrease in

nitrogenase activity and in the intracellular ATP/ADP ratio (chapter 4 and 5). In this situation, the electron allocation by nitrogenase favours the H^+-reduction (chapter 4).

When root nodules are limited in O_2, the nitrogenase activity declines (chapter 4). However the electron allocation by nitrogenase is unaffected by the O_2 limitation.

In chapter 5, it is demonstrated that an increase in external pH, decreases the nitrogenase activity without affecting the electron allocation by nitrogenase. This suggests that the response of the root nodule on O_2 limitation can be explained by an increase in pH of the cytoplasm of the infected cell. This support the suggestion that in the root nodule the supply of O_2 is coupled to the pH of the infected cells.

The infected plant cell might accomplish changes in the external pH of the bacteroid in different ways. One should consider that the term 'external pH' can refer to the pH in the plant cytoplasm but maybe also to the pH in the interstitial peribacteroid space between the bacteroid and the peribacteroid membrane. In the symbiosis, the infected plant cell has the possibility to alter the pH in the peribacteroid space. An H^+-translocating ATPase in the peribacteroid membrane, capable of transporting H^+ from the plant cytoplasm into the peribacteroid space, has been identified (66,67). The presence of an H^+-ATPase in the peribacteroid membrane implies that the ATP/ADP ratio in the plant cytoplasm might determine the pH in the peribacteroid space and therefore might affect the nitrogenase activity. However, this is a suggestion.

The role of the peribacteroid membrane is still questioned. In the past investigators used the term bacteroid for bacteroids without a peribacteroid membrane as well as for bacteroids surrounded with a peribacteroid membrane. The preparations used in our studies consisted of bacteroids enclosed by the peribacteroid membrane (85 %) and of bacteroids without this membrane (15 %). This distribution is based on measurement of the maximum nitrogenase activity (with dithionite and MgATP in permeabilized bacteroids), after fractioning the bacteroids by a sucrose gradient. Using a comparable isolation method, Brewin *et al.* (68) have found the same distribution of bacteroids enclosed with the peribacteroid in their *R. leguminosarum* bacteroid preparations from pea nodules. The undamaged state of the peribacteroid membrane can only be assumed. In principe the bacteroid preparations used in our studies, refer to bacteroids with a peribacteroid membrane.

6.7 *The bacteroids as mitochondria*

Bacteroids can be compared with mitochondria because both 'organelles' share common features.

The function of mitochondria from both plants and animals is the conversion of energy obtained from the respiration to forms, which can be used in the cell and to convert metabolites. The main products of the mitochondrial metabolism are ATP and carbon skeletons. In order to perform their function, transport through the mitochondrial membranes is required. Enzymes that utilize ATP and carbon skeletons are usually outside the mitochondria, whereas the enzymes involved in the ATP synthesis are inside the mitochondria.

Mitochondria are surrounded by two membrane systems that have different biochemical properties (for a review see 69). The membranes enclose two compartments, the intermembrane space and the matrix space. The outermembrane is permeable to most small molecules. The permeability of the innermembrane is restricted. Specialized carrier proteins within the membrane catalyze the transport of specific metabolites. Some of these mitochondrial carriers may function to control metabolism. H^+ is pumped out of the matrix space into the intermembrane space by the respiratory chain. This results in a pH gradient and an electrical potential gradient across the inner membrane, negative inside. The energy of the proton motive force may be used for ATP synthesis by the mitochondrial ATPase. The rate of oxidative phosphorylation is determined by the intracellular ATP/ADP ratio. Transport of anions against the electrical potential, requires energy. A number of anions are transported into the mitochondria in symport with H^+, which neutralizes the unfavorable charge movement and allows the anions to accumulate in the matrix. Anions may be exchanged directly for other anions outside the matrix.

The function of the bacteroid towards the infected plant cell, is the reduction of N_2. In order to reduce N_2 the bacteroid converts energy obtained from its respiration into the formation of NH_3. Like the mitochondria, the bacteroid possesses a operating respiratory system and a functional tricarboxylic acid cycle (39). The bacteroid respiration depends on the supply of oxidizable substrates by the plant cytoplasm (paragraph 1.6). There exists respiratory control, like in the mitochondria. The NH_3 formed in the bacteroid is excreted into the plant cytoplasm, where it is assimilated (paragraph 1.7). The enzyme reducing the N_2 i.e. nitrogenase is inside the bacteroid whereas the enzymes assimilating the formed NH_3 are located outside the bacteroid.

The bacteroid is surrounded by three membrane systems, that have different properties. The inner membrane, the bacteroid membrane originates from the bacteria, whereas the outer membrane - the peribacteroid membrane - is of plant origin. Between the inner and the outer membrane, the bacteroid outer membrane is localized. This membrane is permeable to most small mole-

cules. The membranes together, have specialized transport mechanisms, which restrict the transport of compounds across the membrane For instance, the bacteroid possesses a malate/aspartate shuttle, like the mitochondrium (chapter 3). Malate is transported passively and actively inside the bacteroid (14). The passive transport of malate is likelihood facilitated by an electroneutral antiporter and an electroneutral symport (53). The active transport of malate might be driven by a H^+-gradient, which has to be created. The transport of malate by the malate / aspartate shuttle or by one of the other transport mechanisms, is necessary for the N_2-reduction (36-38). Therefore the transport of malate can be considered as controlling the metabolic activity of the bacteroid.

In summary, the bacteroid resembles the mitochondria in function, in the generating of energy and in transport mechanisms.

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SUMMARY

Bacteria of the genus *Rhizobium* can form a symbiosis with plants of the family *Leguminosae*. Both bacteria and plant show considerable biochemical and morphological changes in order to develop and carry out the symbiosis. The *Rhizobia* induce special structures on the legumes, which are called root nodules. In these root nodules, the differentiated bacteria - so-called bacteroids - are localized. Within the root nodule the bacteroids are able to reduce atmospheric N_2 to NH_3, which - after assimilation - is used by the plant. In turn, the plant supplies the bacteroids with carbon compounds from which the energy required for the N_2-reduction is derived.

The N_2-reduction within the bacteroids is catalyzed by the enzyme nitrogenase. Nitrogenase requires for activity energy in the form of ATP and a low potential electron donor. An anaerobic environment at the site of nitrogen fixation is a requirement for nitrogenase because O_2 inhibits the activity of this enzyme. However O_2 is necessary for the respiration of the bacteroids. Without bacteroid respiration, no ATP is synthesized and no reducing equivalents are generated, which are both required for nitrogenase activity. This means that the O_2 supply to the bacteroids must be strictly regulated.

As a side reaction during N_2-reduction, H^+ is reduced. Consequently, by reducing H^+ ATP and reducing equivalents are consumed. Under optimum condition, about 75 % of the electron flow through the nitrogenase reaction is utilized for the reduction of N_2. The remainder is consumed in the reduction of H^+. The apparent waste of energy through H^+-reduction can be much greater than 25 %. The magnitude of loss is influenced by many factors.

The aim of the experiments described in this thesis, is to identify the plant factors which determine the nitrogenase activity and the electron allocation to N_2 and H^+ by nitrogenase. The experiments were performed with *Rhizobium leguminosarum* strain PRE and the host plants *Pisum sativum* cv. Rondo and *Pisum sativum* cv. Finale. Different physiological aspects underlying the functioning of the root nodule, were studied, namely:

- the role of malate dehydrogenase in the supply of oxidizable substrates to the bacteroids
- the role of glutamate oxaloacetate transaminase in the NH_3 assimilation and the exchange of metabolites between the symbionts in the root nodule
- the influence of the external pH of bacteroids on bacteroid respiration and nitrogen fixation
- the relationships between the bacteroid respiration, the intracellular ATP/ADP ratio and nitrogenase activity.

In chapter 2, the presence of root nodule-stimulated forms of malate dehydrogenase is demonstrated. From a comparison of the kinetic properties of the predominant nodule-stimulated form and the main malate dehydrogenase form from uninfected root

cells, it is concluded that the nodule-stimulated form is capable of catalyzing a high rate of malate formation from oxaloacetate. The second conclusion drawn from the kinetic data is that under physiological conditions the reduction of oxaloacetate to malate catalyzed by the nodule-stimulated form is inhibited at higher malate concentrations. Only the nodule-stimulated form exhibits this kinetic property. This prevents the enzyme from catalyzing the reaction to equilibrium, which would lead to a very low oxaloacetate concentration in the cytoplasm of the root nodule cells. The malate concentration has to be controlled because malate is the main substrate of the bacteroids, it plays a central role in the metabolism of the mitochondria and malate -being a strong acid - affects the pH.

In chapter 3, the action of malate/aspartate shuttle between the cytoplasm of the infected plant cell and the bacteroid has been demonstrated. The involvement of a nodule-stimulated glutamate oxaloacetate transaminase, present in the cytoplasm of root nodule cells, in the shuttle is suggested. The shuttle might have the following functions for nitrogen fixation.

The shuttle can transport NADH from the cytoplasm of the nodule plant cells to the bacteroid, where NADH can be oxidized by the respiratory chain. The second function of the shuttle is the transamination of oxaloacetate to aspartate in the bacteroid. The aspartate formed in the bacteroid, is transported at high rates to the cytoplasm of the nodule. This is important because labelling studies of other investigators with ^{14}C-labelled aspartate have demonstrated that aspartate is rapidly converted to malate in the cytoplasm of nodule plant cells. The aspartate formed in the bacteroid and transported to the plant cytoplasm by the shuttle, can replenish the loss in aspartate in the plant cytoplasm. The presence of a sufficient concentration of aspartate is necessary for the asparagine synthesis, a reaction of the NH_3 assimilation.

In chapter 4, the effect of O_2 on nitrogenase activity and the electron allocation by nitrogenase in the root nodules and in the bacteroids, has been described. Oxygen limitation in bacteroids results in a decreased nitrogenase activity and a decreased electron allocation to N_2 by nitrogenase. In root nodules, the O_2 limitation causes also a decrease in nitrogenase activity, however the electron allocation remains constant. It is shown that the external pH of bacteroids determines the rate of respiration by the bacteroid and consequently the rate of nitrogenase activity, without affecting the electron allocation by nitrogenase. By comparing the electron allocation by nitrogenase in root nodules and that in isolated bacteroids, it is proposed that in the intact root nodule the nitrogenase activity is modulated by the pH.

In chapter 5, the mechanism is studied by which the external pH of bacteroids changes the rate of respiration and the rate of nitrogenase activity at low O_2 concentrations. The relationships between the rate of respiration by the bacteroid, the nitrogenase activity and the intracellular ATP/ADP ratio are determined.

The results demonstrate that a high rate of respiration of the bacteroids at low free O_2 concentrations is associated with an intracellular ATP/ADP ratio which is lower than ≈ 1.2 . A high rate of respiration is necessary to achieve maximum nitrogenase activity. When the intracellular ATP/ADP ratio increases above 1.2 , the respiration of the bacteroids decreases and the free O_2 concentration increases, which ultimately results in an inactivation of nitrogenase. From experiments with a H^+-conducting ionophore, it is concluded that the lower rate of respiration at higher pH is caused by a higher intracellular ATP/ADP ratio. These observations demonstrate that the intracellular ATP/ADP ratio via the P_i potential regulates the rate of respiration. This is similar with the classical mitochondrial respiratory control.

In chapter 5 the ATP consumption by nitrogenase is compared with ATP synthesis by oxidative phosphorylation. The calculation shows that under conditions of nitrogen fixation the N_2-reduction, is a major ATP-consuming process in the bacteroids. About 70 % of the ATP synthesized by oxidative phosphorylation is hydrolyzed by nitrogenase. Thus, nitrogenase by itself keeps the intracellular ATP/ADP ratio low and thereby stimulates the respiration.

In chapter 6, the studied biochemical processes of the root nodule, are placed in a broader perspective. Four physiological processes in which malate is involved, are illuminated. A mechanism is postulated, which accounts for the balance between the supply of photosynthates and the supply of O_2 to the bacteroids. A change of the pH of the root nodule cells induced by changes of the malate concentration is the central theme of the proposal. The pH might influence the rate of respiration of the bacteroids and thus nitrogenase activity, but it also might regulate the O_2 influx into the central tissue of the root nodule. The pH changes are determined by the availability of sucrose for the root nodule cells. Finally the comparison between bacteroids and mitochondria is discussed.

SAMENVATTING

Bacteriën behorende tot het geslacht *Rhizobium* kunnen een symbiose aangaan met planten van de familie van vlinderbloemigen. Zowel de bacterie alsook de plant moeten zich in biochemisch en morfologisch opzicht aanpassen om de symbiontische relatie te kunnen ontwikkelen en in stand te kunnen houden. Na infectie door *Rhizobia* ontwikkelen zich op de wortel zogenaamde wortelknollen. In deze wortelknollen bevinden zich de gedifferentieerde bacteriën, die bacteroïden genoemd worden. De bacteroïden zijn in staat N_2 uit de atmosfeer te reduceren tot NH_3, dat na assimilatie kan worden gebruikt door de plant. Op zijn beurt voorziet de plant de bacteroïden van koolstofbronnen. De bacteroïden verbranden deze koolstofbronnen. De energie die hierbij vrijkomt, wordt voornamelijk aangewend voor het reduceren van N_2.

Het reduceren van N_2 wordt gekatalyseerd door het enzym nitrogenase, dat zich in de bacteroïden bevindt. Om N_2 te kunnen reduceren heeft het nitrogenase energie nodig in de vorm van MgATP en een elektrondonor met een lage redoxpotentiaal. Tevens dient de directe omgeving van nitrogenase vrij te zijn van O_2, omdat de activiteit van nitrogenase geremd wordt door O_2. De bacteroïden hebben echter wel O_2 nodig voor hun ademhalingsketen; dit om ATP en de elektrondonor met de lage redoxpotentiaal te kunnen produceren en deze beide zijn nodig voor de stikstoffixatie. Om deze redenen moet de O_2 voorziening van de bacteroïden nauwkeurig gereguleerd worden.

Tijdens de reductie van N_2 wordt als nevenreactie ook H^+ gereduceerd. Ook deze reactie verbruikt MgATP en elektronen met een lage redoxpotentiaal. Onder optimale omstandigheden wordt 75 % van de elektronen, die door nitrogenase gebruikt worden, gebruikt voor de reductie van N_2. De overige elektronen worden verspild voor de reductie van H^+, hetgeen een verlies aan energie inhoudt. Dit verlies van elektronen en MgATP kan veel meer bedragen dan 25 %. De grootte van het verlies wordt bepaald door vele factoren.

Het doel van de in dit proefschrift beschreven experimenten is plantenfactoren te identificeren, die de nitrogenase activiteit en de elektronen verdeling over N_2-reductie en H^+-reductie door nitrogenase bepalen. De experimenten werden uitgevoerd met de bacteriestam *Rhizobium leguminosarum* PRE met als gastheren *Pisum sativum* cv. Rondo en *Pisum sativum* cv. Finale. Er is onderzoek verricht aan verschillende fysiologische aspecten, welke verbonden zijn aan het functioneren van de symbiose. Te noemen zijn:

- de functie van het enzym malaat dehydrogenase in de koolstofvoorziening van de bacteroïden
- de functie van het enzym glutamaat oxaalazijnzuur transaminase in de NH_3 assimilatie en in de uitwisseling van metabolieten tussen de plant en de bacteroïden

- de invloed van de extracellulaire pH van de bacteroïden op de bacteriële ademhaling en op het functioneren van nitrogenase
- het verband tussen de bacteriële ademhaling, de intracellulaire ATP/ADP ratio en de nitrogenase activiteit.

In hoofdstuk 2 van dit proefschrift, wordt het bestaan van een malaat dehydrogenase aangetoond, welke in verhoogde mate in het cytoplasma van de wortelknol voorkomt. De kinetische eigenschappen van dit enzym zijn vergeleken met de eigenschappen van een malaat dehydrogenase, welke in de niet-geïnfecteerde wortel voorkomt. Het wortelknol-gestimuleerde malaat dehydrogenase is in staat de vorming van malaat vanuit oxaalazijnzuur met een hoge snelheid te katalyseren. Tevens blijkt dat het wortelknol-gestimuleerde malaat dehydrogenase in activiteit geremd wordt door een abortief complex, dat het enzym met malaat vormt. Dit complex wordt al gevormd bij lage malaat concentraties. Het malaat dehydrogenase dat in de niet-geïnfecteerde wortel voorkomt, vormt het genoemde complex alleen bij hogere malaat concentraties. De vorming van het abortieve complex verhindert dat de reactie, die door malaat dehydrogenase gekatalyseerd wordt, tot evenwicht komt. Het evenwicht komt overeen met een zeer lage concentratie oxaalazijnzuur en een zeer hoge concentratie malaat. De malaat concentratie in de wortelknol moet gereguleerd worden, omdat malaat betrokken is in verschillende fysiologische processen van de wortelknol. Malaat fungeert als koolstofbron voor de bacteroïden en het speelt een rol in het metabolisme van mitochondriën. Tevens is malaat, zijnde een sterk zuur, betrokken bij de pH regulatie in de wortelknol.

In hoofdstuk 3 wordt het functioneren van een malaat/aspartaat uitwisselingsmechanisme tussen het cytoplasma van de geïnfecteerde plantecel en de bacteroïd aannemelijk gemaakt. Ook van het enzym glutamaat oxaalazijnzuur transaminase blijkt een gestimuleerde vorm in het cytoplasm van geïnfecteerde cellen voor te komen. Naar alle waarschijnlijkheid is deze vorm betrokken bij het functioneren van het malaat/aspartaat uitwisselingsmechanisme. Het uitwisselingsmechanisme kan verschillende functies in de wortelknol vervullen.

Het uitwisselingsmechanisme kan NADH van het cytoplasma van de geïnfecteerde cel naar de bacteroïd vervoeren. In de bacteroïd kan NADH geoxideerd worden door de ademhalingsketen. De tweede functie die het uitwisselingsmechanisme kan vervullen is het vormen van aspartaat in de bacteroïd. Het in de bacteroïd gevormde aspartaat wordt met een hoge snelheid vanuit de bacteroïd naar het cytoplasma van de geïnfecteerde cel getransporteerd. Dit is belangrijk omdat uit studies gebleken is, dat in het cytoplasma van de wortelknol ^{14}C-gelabeld aspartaat zeer snel omgezet wordt in malaat. Het aspartaat dat getransporteerd wordt van de bacteroïd naar het plant cytoplasm door het uitwisselingsmechanisme, kan het verlies aan aspartaat in het cytoplasma van de geïnfecteerde cel compenseren. Dit resulteert in een aspartaat concentratie, die voldoende is om de asparagine biosynthese te laten verlopen. De asparagine synthese is de

laatste stap uit de NH_3 assimilatie.

In hoofdstuk 4 wordt het effect van O_2 op nitrogenase activiteit en op de elektronenverdeling door nitrogenase, aanwezig in wortelknollen en in geïsoleerde bacteroïden, beschreven. In geïsoleerde bacteroïden veroorzaakt O_2 tekort een afname in de nitrogenase activiteit en een toename in gebruik van elektronen voor H^+-reductie. In wortelknollen leidt O_2 tekort ook tot een afname in nitrogenase maar niet tot een verandering in de elektronenverdeling door nitrogenase. Er is aangetoond dat de extracellulaire pH van de bacteroïden de ademhalingssnelheid en daarmee de nitrogenase activiteit bepaalt. De extracellulaire pH van bacteroïden beïnvloedt niet de elektronenverdeling door nitrogenase. Wanneer men deze waarnemingen in verband brengt met het beschreven effect van O_2, kan men suggeren dat in intacte wortelknollen de nitrogenase activiteit bepaald wordt door de extracellulaire pH.

In hoofdstuk 5 is het hierna genoemde mechanisme bestudeerd: de beïnvloeding van de bacteriële ademhaling en de nitrogenase activiteit bij lage O_2 concentraties door de extracellulaire pH van bacteroïden. Het verband tussen bacteriële ademhaling, nitrogenase activiteit en de intracellulaire ATP/ADP verhouding is onderzocht. De resultaten laten zien dat een hoge bacteriële ademhalingssnelheid bij lage O_2 concentraties gekoppeld is aan een intracellulaire ATP/ADP verhouding welke lager is dan 1.2 . Een hoge bacteriële ademhalingssnelheid is noodzakelijk voor een hoge nitrogenase activiteit. Als de intracellulaire ATP/ADP verhouding boven de waarde 1.2 stijgt, neemt de ademhalingssnelheid af, hetgeen tot een stijging in de vrije O_2 concentratie leidt. Wanneer de vrije O_2 concentratie teveel stijgt, wordt nitrogenase geïnactiveerd. Op grond van experimenten met de H^+ -verplaatsende ionofoor CCCP, kan worden geconcludeerd dat de lagere ademhalinssnelheid bij hogere pH wordt veroorzaakt door een hogere intracellulaire ATP/ADP ratio. Dit duidt op een regulatie van de ademhaling door de intracellulaire ATP/ADP verhouding via de fosfaat potentiaal. Er is dus sprake van een analoge situatie als bij de mitochondriën waar ook ademhalingscontrole voorkomt.

In hoofdstuk 5 wordt ook het ATP verbruik door nitrogenase vergeleken met de ATP productie door de oxidatieve fosforylering. Berekeningen laten zien dat nitrogenase onder omstandigheden waarbij stikstoffixatie optreedt, de grootste consument van ATP in de bacteroïden is. Ongeveer 70 % van het, in de oxidatieve fosforylering gevormde, ATP wordt door nitrogenase gehydrolyseerd. Dit impliceert dat nitrogenase zelf de intracellulaire ATP/ADP verhouding laag houdt en daardoor de ademhaling stimuleert.

In hoofdstuk 6 worden de bestudeerde biochemische processen die in de wortelknol plaatsvinden in een breder perspectief geplaatst. Vier fysiologische processen waarin malaat een rol speelt, worden beschreven. In dit hoofdstuk wordt een mechanisme gepostuleerd, dat de afstemming van de toevoer van O_2 op de toevoer van fotoassimilaten door de plant kan verklaren. In dit mechanisme staat de pH van de wortelknolcellen centraal. De extracellulaire pH bepaalt de ademhalingssnelheid van de bacteroïden

en daardoor de nitrogenase activiteit. Mogelijk bepaalt de pH ook de toevoer van O_2 naar het centrale gedeelte van de wortelknol. Er wordt gesuggereerd dat de pH veranderingen worden bepaald door de sucrosetoevoer naar de wortelknol. De discussie wordt afgesloten met een vergelijking tussen bacteroïden en mitochondriën.

Curriculum vitae

Michiel Alexander Appels werd op 30 augustus 1958 te Breda geboren. In 1977 behaalde hij het diploma Atheneum B aan de Rijksscholengemeenschap Epe. In hetzelfde jaar begon hij zijn studie Planteziektenkunde aan de Landbouwuniversiteit te Wageningen. In januari 1981 werd het kandidaatsexamen behaald en in mei 1984 het doctoraalexamen. In zijn doctoraalfase heeft hij onderzoek verricht op de gebieden Virologie (verzwaard hoofdvak), Fytopathologie (hoofdvak) en Biochemie. In het kader van zijn praktijktijd was hij werkzaam bij de firma Ciba-Geigy AG, Basel, Zwitserland.

Van 1 oktober 1984 tot 1 oktober 1987 was hij werkzaam in de functie van onderzoekmedewerker in een dienstverband met de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.). Hij was verbonden aan het Laboratorium voor Biochemie van de Landbouwuniversiteit Wageningen. In de periode van 1 oktober 1987 tot 5 juli 1988 was hij als toegevoegd docent verbonden aan dit laboratorium. Vanaf 20 november 1989 hoopt hij als biochemicus werkzaam te zijn bij de Faculteit der Medische Wetenschappen van de Anton de Kom Universiteit van Suriname.

...Ihr, die Ihr auftauchen werdet aus der Flut
In der wir untergegangen sind
Gedenkt
Wenn Ihr von unsren Schwächen sprecht
Auch der finsteren Zeit
Der Ihr entronnen seid...

...Ach, wir
Die wir den Boden bereiten wollten für Freundlichkeit
Konnten selber nicht freundlich sein.
Ihr aber, wenn es so weit sein wird
Das der Mensch dem Menschen ein Helfer ist,
Gedenkt unsrer
Mit Nachsicht.

(Bertolt Brecht)

CORRECTIONS

Chapter 3 'Kinetic properties and function of glutamate oxaloacetate transaminase in the symbiosis between *Pisum sativum* and *Rhizobium leguminosarum*'

p. 69 Table 4

for

$\delta v/\delta S$ is expressed as $10^{-3}.min^{-1}$

read

$\delta v/\delta S$ is expressed as $10^{-3}.min^{-1}.mg\ protein^{-1}.l$