Cover: The investigator by Charles Hammes

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MOLECULAR ASPECTS OF THE NITROGEN FIXING SYSTEM IN PEA ROOT NODULES

Proefschrift

ter verkrijging van de graad
van doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas
hoogleraar in de organische scheikunde
in het openbaar te verdedigen
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van de Landbouwhogeschool te Wageningen.

Werkvoorzieningsschap Nijmegen en Omgeving

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STELLINGEN

1. De veronderstelling van Brun, dat de morfologie van het kinetoplast DNA van *Herpetomonas muscarum* een transformatie ondergaat wanneer het organisme vanuit het insekt in cultuur wordt gebracht, zou pas gerechtvaardigd zijn indien was aangetoond dat het uitgangsmateriaal rein was.

   Brun, R. (1974) Acta Tropica 31, 219-290.

2. Jacobs *et al.* hebben onvoldoende duidelijk gemaakt dat de eerste stap van de processing van de eventuele precursor voor rat calcitonine een proteolytische splitsing is o.i.v. pancreas membranen.

   Jacobs, J.W., Potts, J.T., Bell, N.H. and Habener, J.F. (1979) J. Biol. Chem. 254, 10600-10603.

3. Bij de invoering door Holten van het nieuwe serotype 15 van *Neisseria meningitidis* is het betrokken antigen onvoldoende gekarakteriseerd.

   Holten, E. (1979) J. Clin. Microbiol. 9, 186-188.

4. De conclusie van Ludwig en Signer dat glutamine synthetase een rol speelt bij de regulering van de nitrogenase activiteit in *Rhizobium* berust op de vooronderstelling dat *Rhizobium* in dit opzicht analoog moet zijn aan *Klebsiella pneumoniae*, maar wordt niet voldoende gesteund door hun resultaten.

   Ludwig, R.A. and Signer, E.R. (1977) Nature 267, 245-248.

5. De conclusie die Lepidi *et al.* uit hun resultaten trekken, dat *Rhizobium* bacteriën DNA overdragen aan wortelcellen van de waardplant, is voorbarig.

   Lepidi, A.A., Nuti, M.P., Bernacchi, G. and Neglia, R. (1976) Plant and Soil 45, 555-564.

6. Het opnemen van stellingen bij een proefschrift, die tot doel hebben de promotiecommissie een gemakkelijke oppositie aan te bieden, bergt het gevaar in zich, dat hierdoor niet de "brede kennis" van de promovendus wordt gedemonstreerd, maar de beperkte interesse van de betrokken hoogleraren wordt gemaskeerd.

Wageningen, 11 juni 1980 Ton Bisseling

VOORWOORD

Dit proefschrift is tot stand gekomen dankzij de hulp van velen. Daarvan wil ik er een aantal met name bedanken.

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CONTENTS

SCOPE OF THE INVESTIGATION

This thesis deals with research on symbiotic nitrogen fixation of *Pisum sativum* and *Rhizobium leguminosarum*. Nitrogen fixation takes place in the *Rhizobium* bacteroids which are located within root nodule cells. Two important proteins in nitrogen fixation are nitrogenase and leghemoglobin. Nitrogenase, the enzyme that reduces N_2, is synthesized by *Rhizobium*. Leghemoglobin, which has a function in the O_2 supply of the bacteroids is synthesized by the plant. The molecular biology of symbiotic nitrogen fixation has hardly been the subject of investigation. The regulation of the synthesis in root nodules of e.g. nitrogenase and leghemoglobin is not at all completely clear.

In this thesis some general aspects of nodule formation and the regulation of nitrogenase and leghemoglobin (Lb) synthesis have been studied.

General aspects

The transformation of *Rhizobium* bacteria into nitrogen fixing bacteroids was studied by investigating the DNA content of bacteroid cells by means of cytofluorometry (chapter I). Furthermore the RNA content and the quality of the RNA of *R. leguminosarum* (PRE) bacteroids were followed during pea development (chapter IV) and the protein synthesis in bacteroids and in the plant fraction of pea root nodules during nodule development was studied (chapter IV). The regulation of nitrogenase and leghemoglobin synthesis were studied in more detail.

Regulation of nitrogenase and Lb synthesis

Nitrogenase and Lb synthesis were studied by $^{35}SO_4^=$ labeling of intact pea plants. Intact pea plants are a rather complicated system to study Lb or nitrogenase synthesis, but in more simple systems like e.g. detached root nodules, nitrogenase and Lb synthesis are repressed. We did not succeed in developing a more simple system suitable for studying nitrogenase or Lb

synthesis (chapter VII). The regulation of nitrogenase and Lb synthesis were studied by culturing pea plants under conditions that diminished *in vivo* nitrogenase activity and by determining nitrogenase and Lb synthesis during nodule formation and development. Growth conditions used to diminish *in vivo* nitrogenase activity were: 1. the addition of NH_4^+ to the growth medium (chapter II) and 2. waterlogging (chapter III). The synthesis of the two nitrogenase components and Lb during nodule formation and development was studied in two different ways: 1. the synthesis of these three proteins was followed by $^{35}SO_4^=$ labeling of pea plants of different ages, and analysis of bacteroid and plant proteins by polyacrylamide gel electrophoresis (chapter IV), 2. the sequence of appearance of the two nitrogenase components and Lb during pea nodule formation was determined with specific radioimmunoassays for these three proteins (chapter V).

In principle, turnover of proteins can be an important factor in the regulation of protein composition in the bacteroid or plant fraction of root nodules, e.g. during nodule development or after changes in the environmental conditions. Therefore turnover rates of bacteroid and plant proteins of pea root nodules, especially nitrogenase and Lb, were determined (chapter VI).

INTRODUCTION

The ability to fix nitrogen is a property of some bacteria and blue green algae only. Actual fixation is governed by the nitrogen fixation genes (nif-genes), and nitrogenase is the enzyme that catalyses the reaction. The reduction of N_2 is represented by the equation (1):

$$N_2 + 6e + 6H^+ + nATP \xrightarrow{\text{nitrogenase}} 2NH_3 + nADP + nPi$$

Besides the enzyme nitrogenase, electrons and energy in the form of ATP are required for nitrogen reduction. Nitrogenase is extremely sensitive to molecular oxygen. In anaerobic nitrogen-fixing organisms like *Clostridium* this is obviously not a problem, but in aerobic nitrogen-fixing organisms (e.g. *Azotobacter* and *Rhizobium*) nitrogenase has to be protected against molecular oxygen (2).

In this introduction some aspects of the symbiotic nitrogen fixation of rhizobia and legumes will be described. Also nitrogen fixation of the facultative anaerobically nitrogen fixing *Klebsiella* will be discussed and the genetics of the nif-genes of *Klebsiella* will be considered in some detail.

Symbiotic nitrogen fixation of rhizobia and legumes is located in root nodules. The process of nodule morphogenesis has been extensively reviewed by Dart (3), Libbenga and Bogers (4), and Bergersen (5). The first reaction of the legume to *Rhizobium* in the soil is a curling of the root hairs. This is followed by the formation of an infection thread containing *Rhizobium* bacteria, which grows towards the base of the epidermal cells and then into the cortex. After the infection thread has penetrated some cell layers of the cortex, cell division occurs in small groups of inner cortical cells. It appears that, as a result of dedifferentiation of cortex cells, meristematic tissue is formed. These cells are invaded by rhizobia released from the infection threads by endocytosis (6) and subsequently differentiate into non-dividing cells containing rhizobia. The bacteria multiply after which they

enlarge and deform into y shaped structures, which are called bacteroids, each within a membrane envelope. In root cells invaded by *Rhizobium* bacteria leg-hemoglobin synthesis is induced. This gives the root nodules a red color. Leg-hemoglobin (Lb) is a plant protein only synthesised in root nodules. Nitrogen fixation takes place in the bacteroids where N_2 is reduced to NH_4^+, which is excreted by the bacteroids (7,8). Assimilation of NH_4^+ into amino acids takes place in the plant cytoplasm (7).

Thus both bacterial differentiation and differentiation of plant cells is involved in establishing nitrogen fixing root nodules. This process of morphogenesis is typical for *Pisum sativum* and *Rhizobium leguminosarum*. The nodule formation of other *Rhizobium* and legume species can differ from it in some details.

The formation of effective root nodules is a complex process, since the legume and *Rhizobium* mutually regulate each others differentiation. This can be illustrated with the following phenomena: there are ineffective *Rhizobium* strains which give rise to small white root nodules lacking Lb and which are unable to fix nitrogen. So the ineffective *Rhizobium* strains apparently fail to induce the expression of the Lb genes in the plant cells. The reverse is also possible, whereby the host fails to induce the expression of *Rhizobium* genes. *R. leguminosarum* (PF2) is effective on *P. sativum* (var. Rondo), while ineffective on *Vicia faba* (cv. Minor) (9) and some other pea strains (10), in which infection results in small white root nodules. In these ineffective root nodules *Rhizobium* does not differentiate into y shaped nitrogen fixing bacteroids. The phenotype of these nodules is similar to the root nodules of legumes inoculated with an ineffective *Rhizobium*. The fact that the effective *R. leguminosarum* does not differentiate into nitrogen fixing bacteroids in e.g. *V. faba*, means that the host interferes with the differentiation of *Rhizobium*.

Rhizobia can be divided into two groups: fast and slow-growing rhizobia. They differ in several characteristics (11). For instance, fast-growing rhi-

zobia produce acid on yeast-mannitol agar and have a mean generation time of 2-4 hours, while slow-growing rhizobia have a mean generation time of 6-8 hours and produce alkali on yeast-mannitol agar. The fast-growing rhizobia utilise a wider range of carbon sources than the slow-growing rhizobia and have more glycolitic pathways. The fast-growing rhizobia have laterally arranged flagella with slow-growing rhizobia most of the cells have polar or sub-polar flagella. *R. leguminosarum, R. trifolii, R. phaseoli, R. meliloti* belong to the fast-growing rhizobia and *R. japonicum, R. lupini* and *R.*' cowpea' to the slow-growing rhizobia.

In 1975 three groups reported for the first time that rhizobia were able to fix nitrogen *ex planta* (12,13,14). Since then, these reports have been confirmed by several other groups, but they all concern slow growing rhizobia (15). This means that the capacity of reducing nitrogen *ex planta* confirms that rhizobia can be divided into two groups.

In the next paragraphs I want to describe the genes that are specifically expressed in symbiotic nitrogen fixation after which nitrogen fixation *ex planta* of slow-growing rhizobia will be discussed.

Genes that are involved in symbiotic nitrogen fixation

Rhizobium genes

In effective root nodules the *Rhizobium* nif-genes are expressed which results in the synthesis of nitrogenase. Besides nitrogenase there are immunological indications that some new surface antigenes are formed when bacteria differentiate into bacteroids (16). Bacteroids of *R. japonicum* and *R. lupini* contain the cytochrome P-450, which is not found in air-grown rhizobia (17,18). In bacteroids of *R. leguminosarum* the cytochrome P-450 is not found (19). The cytochromes *a* are missing in N_2 fixing *R. leguminosarum* bacteroids when com-

pared with bacteria. In nitrogen fixing root nodules there is a low O_2 concentration necessary for protection of nitrogenase. However, *R. leguminosarum* bacteria grown at a low O_2 concentration still contain cytochromes *a* (19). Thus the lower O_2 concentration within a root nodule is not the facot responsible for the repression of the cytochrome *a* genes in bacteroids of *R. leguminosarum*.

Nitrogenase is built up of two components, a FeMo protein (component I) and a Fe protein (component II). Component I consists of two subunits with molecular weights of about 50,000 D and 54,000 D (20) and probably the FeMo cluster is present in a small peptide; the FeMo cofactor (21). Component II consists of two subunits with molecular weights of 34,000 D.

Recently it has been demonstrated that rhizobia contain a few large plasmids (22) varying in size from 90 to 350 $\cdot 10^6$ D. Recently the genes for *Klebsiella* nitrogenase have been cloned in *Escherichia coli* K12 by means of the recombinant DNA technique (22a). By molecular hybridisation of *Klebsiella* DNA containing the genes for CII and one of the subunits of CI, it was shown that in *Rhizobium* these genes are localised on one of the large plasmids (23). The DNA carrying the *Klebsiella* gene for the other CI subunit did not hybridise with *Rhizobium* DNA; neither did DNA carrying other *Klebsiella* genes involved in nitrogen fixation. Besides the two nif genes, some of the *Rhizobium* genes involved in the infection process are localised on a plasmid (24). Whether the other genes involved in nitrogen fixation and nodule formation are localised on plasmids or chromosomal DNA is yet unknown.

Some *Rhizobium* genes might be involved in Lb synthesis as discussed in the next paragraph.

Besides the genes that are directly involved in symbiotic nitrogen fixation other gene products undoubtedly have a function, e.g. during the infection and induction of root nodule formation. Regulation of these genes, however, falls beyond the scope of this thesis.

Host genes

In the plant part of the root nodule at least four genes are specifically induced; three different Lb genes (25) and the gene coding for the protein Nodulin-35 (26).

Lb represents about 25% of the total fraction of soluble plant proteins in effective root nodules (27). It consists of a protein part with a molecular weight of about 15,000 D and a prosthetic groups; protoporphyrin IX (28). In soybeans eight different Lb's (a, b, c_1, c_2, c_3, d_1, d_2, d_3) are found with isoelectric focusing (29). They appear to originate from at least three different genes: a, c_1 and c_2. Lb a and Lb b might be biosynthetically related as might be Lb c and Lb d (29,30). For instance Lb b originates from Lb a after the N-terminal amino acid is deleted and the new N-terminus is acetylated (30). Whether the leghemoglobins have different functions in root nodules is unknown.

Lb is a myoglobin like protein which can bind O_2 reversibly. Soybean Lb is half saturated with O_2 at a partial O_2 pressure of about 0.06 mm Hg (28). In soybean root nodules about 20% of total Lb is in the Lb O_2 form. It can be calculated, that the O_2 partial pressure in these root nodules is 0.006 mm Hg (28). Probably Lb functions in root nodules by facilating a high flux of O_2, at a low O_2 tension which is nevertheless adequate for a highly efficient oxidative phosphorylation by the bacteroids. This low free O_2 concentration is necessary to protect nitrogenase from O_2 damage. Pea root nodules contain about $5\text{-}10 \cdot 10^9$ bacteroids per ml nodule implicating that probably O_2 would be rate-limiting in nitrogen fixation in the absence of Lb (31).

Using complementary DNA of Lb mRNA as a probe it was shown, that apo Lb synthesis is specified by plant DNA and that a total of 40 copies of the three Lb genes are present per soybean genome.

Both host and *Rhizobium* possess the genetic information for heme synthesis. To determine whether heme was synthesized in the bacteroid or plant part of the root nodule, Cutting and Schulman (32) and Godfrey (33) made a nodule brei, that was separated by centrifugation into a bacteroid and plant fraction. With ^{3}H-δ amino levulinic acid (ALA), a precursor for heme synthesis, it was shown that the bacteroids were more active in heme synthesis than the plant fraction. Both groups concluded that heme synthesis for Lb was localised in bacteroids. However, the experiment described is unsuitable for drawing this conclusion. This is because the bacteroid fraction consists of intact cells while the plant cells are disrupted. This difference can influence heme synthesis. Moreover, *Rhizobium* bacteria are as active in heme synthesis as bacteroids (33). This fact shows that the observed heme synthesis by bacteroids is not necessarily similar to the heme synthesis for Lb in root nodules. A better approach to determine whether the bacteroids or the host is responsible for heme synthesis of Lb is the quantification of enzyme activities involved in heme synthesis. In soybean and lupin root nodules (32,34,35) γ-amino-levulinic acid synthase (ALAS) in only found in the bacteroid fraction, while γ-amino levulinic acid dehydrase (ALAD) is found as well in the plant as bacteroid fraction. Plant ALAD is found in both effective and ineffective root nodules and falls during nodule formation, while leghemoglobin concentration is increasing. Bacteroid ALAD is only present in effective root nodules and increases during nodule development (35). These results support the suggestion that the bacteroid ALAS and ALAD are involved in the formation of Lb heme. Moreover in *R. japonicum* the cellular heme content increases ten fold and visible amounts of porphyrin were released into the culture medium under micro-aerobic conditions (36). ALAS and ALAD activities were stimulated by these conditions. This suggests that the reduced oxygen tension in root nodules may play a role in inducing heme synthesis by *Rhizobium* necessary for Lb formation.

Leghemoglobin is first detected in root nodules a few days before the onset of nitrogenase activity (33,37,38) suggesting an essential role of Lb in nitrogen fixation. When soybeans were inoculated with an ineffective *R. japonicum* strain with a mutation in the CII gene, the Lb genes are expressed normally (39). This indicates also that active nitrogenase is not necessary for the expression of the Lb genes.

Besides the Lb genes the Nodulin-35 gene is specifically induced in soybean root nodules (26). Nodulin-35 is a protein that represents about 40% of the total fraction of soluble plant proteins in soybean root nodules. With immunological techniques it was demonstrated that this plant protein can only be detected in root nodule tissue. The function of Nodulin-35 is not yet clear.

Regulation of nitrogenase and leghemoglobin synthesis in root nodules

Environmental conditions can influence nitrogenase activity of root nodules. For example Lie (40) reviewed the effects of the composition of soil atmosphere, soil moisture and acidity, combined nitrogen, temperature and light on nitrogenase activity. Nitrogenase activity can be regulated in several ways, e.g. by the supply of reducing equivalents or by the supply of ATP, and the amount of Lb (61) or nitrogenase. Therefore, if nitrogenase activity is reduced by an environmental condition, it is impossible to conclude from such observations whether nitrogenase or Lb synthesis are repressed. Till now nitrogenase and Lb synthesis have hardly been subject of investigation. For this reason it has been impossible to draw conclusions about the regulation of nitrogenase and Lb synthesis at different environmental conditions.

By analogy with *K. pneumoniae* where glutamine synthetase (G.S.) plays a central role in the regulation of nitrogenase synthesis (41), several groups have investigated whether *Rhizobium* G.S. has a similar function.

Genetic evidence supports a role of G.S. in nitrogenase synthesis since G.S.$^-$ mutants fail to fix nitrogen in root nodules (42,43) and *ex planta* (42). G.S. is one of the enzymes involved in NH_4^+ assimilation. In root nodules it has been demonstrated that fixed nitrogen is exported to the host cytoplasm as NH_4^+, where it is assimilated to glutamine and asparagine. Therefore, G.S. concentrations inside the bacteroids are low (44,45). In *Klebsiella*, NH_4^+ increases the adenylylation level of G.S. (see also page 23) and this is an essential step in the regulation of nitrogenase synthesis. However, NH_4^+ does not influence the adenylylation level of bacteroids G.S. in soybean root nodules. These facts contradict a key role of bacteroid G.S. in the regulation of nitrogenase synthesis.

Regulation of nitrogenase synthesis ex planta

Slow-growing rhizobia are able to reduce actylene *ex planta*. However, a repeatable and consistent nitrogenase activity *ex planta* has not been found for fast-growing rhizobia (15). Only 2 reports have described acetylene reduction *ex planta* for fast-growing rhizobia; *R. leguminosarum* (13) and *R. trifolii* (46). Two other reports have described nitrogenase activity *ex planta* of mutants of *R. trifolii* (47) and *R. leguminosarum* (15). O'Gara and Shanmugam made methioninesulfoximine resistant mutants of *R. trifolii* that reduced nitrogen *ex planta*. However, Ludwig *et al.* (48) showed that these nitrogen fixation mutants are *R. japonicum* and are very probably not derived from the putative parent *R. trifolii*. Skotnicki *et al.* made spectinomycin resistant fast-growing rhizobia (*R. trifolii* and *R. leguminosarum*) and several strains had nitrogenase activity (15) *ex planta*. These spectinomycin mutants were still able to nodulate the original host. This was not the case with the "mutant" prepared by O'Gara and Shanmugam.

Conditions that are necessary for nitrogen fixation *ex planta* are: a low

oxygen concentration and a combined nitrogen source. Free-living nitrogen fixation by rhizobia only occurs at the end of the exponential growth and no condition is known in which bacterial growth is dependent on reduction of N_2 (15). So rhizobia *ex planta* and bacteroids do not use fixed nitrogen for their own growth, since nitrogen is fixed by non-growing cells and is excreted as NH_4^+.

The low optimal oxygen concentrations essential for nitrogenase activity *ex planta* are consistent with the low free O_2 concentrations in root nodules. The spectinomycin-resistant mutants of fast-growing rhizobia that reduced acetylene *ex planta*, were found to be rods of about twice the size of aerobically grown cultures (non-fixing) and very few odd-shaped cells were observed (15). This indicates that besides the phenotypic expression of the *nif*-genes, low O_2 concentrations are presumably also important in triggering the morphological differentiation of fast-growing rhizobia during the transition from bacteria into bacteroids.

Whether the growth conditions necessary for nitrogenase activity *ex planta* are really essential for nitrogenase synthesis as well is unclear because nitrogenase synthesis or the presence of nitrogenase proteins has hardly been studied. There has only been one report of nitrogenase CI synthesis in non-nitrogen-fixing *Rhizobium* bacteria (49). But since nitrate reductase might cross-react with antiserum against CI (50) it cannot be excluded that they have measured nitrate reductase instead of CI. This is a good possibility since the highest amounts of cross-reacting protein was observed in bacteria grown in the presence of NO_3^-.

As mentioned above combined nitrogen is not inhibitory but even essential for the expression of the nitrogenase genes of *Rhizobium ex planta*. This is contrary to the regulation of the nif-genes in free-living organisms like *Klebsiella* and *Azotobacter*. In these organisms even low concentrations of combined nitrogen completely repress nitrogenase synthesis (41,51).

In conclusion it seems that the regulation of nitrogenase synthesis in *Rhizobium* is still unclear, simply because it has hardly been studied. In this thesis some experiments to study the regulation of Lb and nitrogenase in root nodules of pea plants will be described. *Klebsiella pneumoniae* is one of the best investigated nitrogen-fixing organisms, especially concerning the regulation of nitrogenase synthesis, and probably parallels may be drawn from this work. Therefore, in the next paragraphs the organisation of the nif-genes and the regulation of nitrogenase synthesis in *K. pneumoniae* will be discussed.

The regulation of nitrogenase synthesis in Klebsiella pneumoniae

The regulation of the nitrogenase genes has been best investigated in *K. pneumoniae*. As *Klebsiella* is closely related to *E. coli* genetic techniques developed for *E. coli* have been used to study this organism.

K. pneumoniae is a facultative anaerobic nitrogen fixing organism and nitrogen fixation is governed by the nif-genes. The nif-genes are not localised on a plasmid but on the chromosomal DNA.

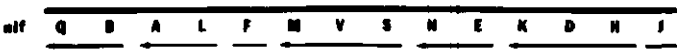

Fig. 1. Nif-genes and mRNA transcripts. The arrows indicate the direction of transcription.

By transduction and conjugation experiments it was shown that the nif-genes map near his (52). The nif-genes were mapped by measuring co-transduction with his (52,53). By complementation analysis the number of nif-genes was defined and polar mutations were used to determine the number of nif transcripts and their direction of transcription (54). The results of this

type of analysis is shown in Fig. 1. Fourteen nif-genes and seven transcription units have been defined in *K. pneumoniae*.

By comparing protein synthesis in different nif$^-$ mutants Roberts *et al.* (55) have tried to characterise the proteins encoded by the nif-genes. They demonstrated that nif K, D and H code for the 2 subunits of CI and the subunit of CII respectively. The subunits of CI and CII have molecular weights of 60,000, 56,000 and 35,000 respectively. Besides the 35,000 subunit of CII a protein with a molecular weight of 39,000 is encoded by nif H. This 39,000 protein is only detectable in cells pulse labeled with radioactive amino acids, so it has a high turnover, and probably is a precursor of CII.

As indicated in Fig. 1, the structural genes for nitrogenase (nif K, D and H) are localised on one operon with *K. pneumoniae*. Whether this organisation is also the case in other nitrogen fixing organisms is unknown. The *Klebsiella* DNA carrying the nif-genes D and H hybridises with DNA from (all tested) nitrogen fixing organisms, while the DNA carrying the other 12 nif-genes has not a high degree of homology in the nucleotide sequence (56). This result indicates that the two structural nif-genes D and H are rather conservative, while the other nif-genes are probably not. Experiments with *Azotobacter* indicate that the organisation of the structural genes in one operon differs from the organisation in *K. pneumoniae*. Ausubel used cloned nif D, H DNA from *K. pneumoniae* as a probe for detecting which restriction fragments of *Azotobacter* DNA contain sequences homologous to *K. pneumoniae* (56). His results indicate that the structural genes of nitrogenase of *Azotobacter* are probably not localised in one operon as is the case in *K. pneumoniae*. However, the result can also be explained by partial digestion of the *Azotobacter* DNA by the restriction enzyme. This possibility cannot be completely excluded.

Nif genes N, E and B play a role in the synthesis of the FeMo-cofactor. Nif N and E code for proteins with molecular weights of 50,000 and 46,000.

Nif A and L are probably involved in the regulation of the nif genes and nif F codes for a 17,000 D protein, functioning in electron transport. Nif M protein is probably necessary for the processing of CII, since in nif M^- strains active CII is only present in low amounts, while there is an accumulation of a 39,000 protein, which is probably the precursor of CII. Like nif Q^-, nif V^- strains have about 50% reduced levels of acetylene reduction. This suggests that the nif Q and V proteins are probably not absolutely essential for the generation of an active CI or CII. Nif J codes for a 120,000 D protein which is only necessary for *in vivo* and not for *in vitro* nitrogenase activity. It probably has a function in the regulation of nif F, the product of which is involved in electron transport. The functions of the nif genes are shown in Fig. 2.

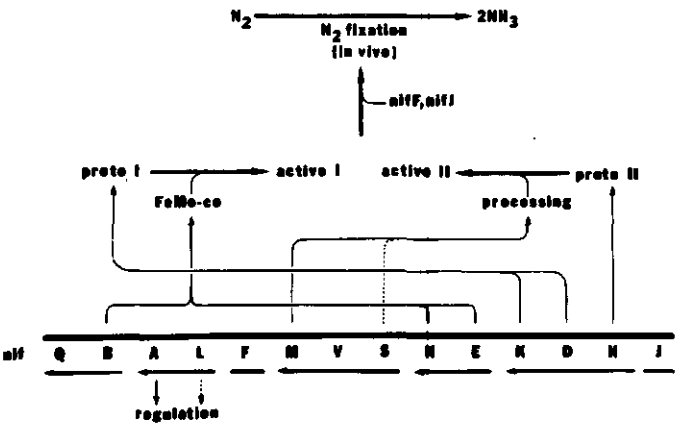

Fig. 2. Functions of nif-gene products (55).

In *K. pneumoniae*, NH_4^+ produced by the nitrogen fixation reaction is assimilated into glutamate by G.S. and glutamate synthase (GOGAT) (57). If combined nitrogen (e.g. NH_4^+) is present nitrogenase is not synthesised. A simple model for the regulation of nitrogenase by NH_4^+ was proposed by Shanmugam *et al.* (41). G.S. plays a key role in this regulation mechanism.

G.S. can be modified into a catalytically inactive form by attachment of 12 adenyl groups to tyrosine residues (41). NH_4^+ triggers this cascade system (Fig. 3). It was proposed that adenylylation of G.S; blocks the binding of G.S. to the nif promotor while deadenylylated G.S. binds and induces transcription of the nif genes (Fig. 3)

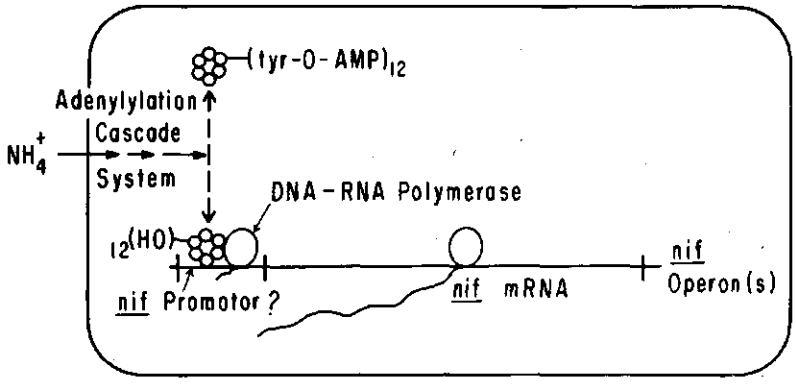

Fig. 3. Working model for the genetic regulation of nif genes by NH_4^+ (41).

This hypothesis is based on genetic research with Gln^- mutants (41). The Gln A^- mutant having no detectable G.S., is unable to synthesize nitrogenase. Nitrogenase synthesis can be restored by introducing into the Gln A^- mutant an *E. coli* episome carrying an intact G.S. gene. Gln C^- mutants have constitutive levels of active G.S. in the presence of NH_4^+, and nitrogenase synthesis is also not repressed by NH_4^+ in that case.

More recent experiments have been described however, which demonstrated that the regulation mechanism shown in Fig. 3 is imcomplete. For instance, mutants without any immunologically detectable G.S. nor enzymatic activity, but able to synthesize nitrogenase were isolated (58). Moreover, mutants that synthesize nitrogenase in the presence of NH_4^+ can still be repressed by some amino acids (59). These results indicate that the nature of other effects of the Gln^- mutants have to be studied before the components which control the

synthesis of nitrogenase can be identified (58).

Oxygen can also repress nitrogenase synthesis in *Klebsiella* (60). Experiments with a NH_4^+ constitutive nitrogenase strain show that O_2 can repress nitrogenase synthesis although the adenylylation level of G.S. is not significantly changed under O_2 (60). This result indicates that O_2 represses nitrogenase synthesis by an other mode of action at the molecular level than NH_4^+.

If the regulation of nitrogenase synthesis in *Klebsiella* is compared with the (poor) information about this regulation in *Rhizobium*, it is clear that there are several differences. For instance combined nitrogen probably does not repress the nif genes in *Rhizobium* while this is the case in *Klebsiella*. Also there are indications from the work with *Azotobacter*, that the organization of *Klebsiella* nif genes is not general to all nitrogen fixing organisms. So although the research on the nif-genes of *Klebsiella* may provide a good working hypothesis, one has to be conscious that the regulation and organisation of the nif-genes in e.g. rhizobia may differ in several aspects.

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CHAPTER I

Cytofluorometrical Determination of the DNA Contents of Bacteroids and Corresponding Broth-cultured Rhizobium Bacteria

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DNA contents of different species of broth-cultured Rhizobium bacteria and bacteroids from root nodules were determined by cytofluorometry. In almost all species bacteroids appeared to contain more DNA than the corresponding bacteria. A correlation was found between the DNA content and size of the bacteroid, the latter being influenced by the host plant.

INTRODUCTION

Rhizobium bacteria are capable of infecting legume roots and inducing the formation of root nodules (Bergersen, 1974). Within these root nodules the bacteria penetrate into the plant cells and most of them are transformed into so-called bacteroids in which the nitrogenase system is expressed. During the transformation into bacteroids, profound physiological and biochemical changes occur in the bacterial cell. The cells increase in size and become incapable of further multiplication. The transformation appears to be irreversible, since transformation of bacteroids into multiplying bacteria has not been observed (Bergersen, 1974). The loss in reproductive capacity might be due to a change in the quantity or quality of the DNA in the bacterial cell.

The DNA content of Rhizobium bacteroids has been a controversial matter in some publications. In work on *Rhizobium japonicum* (Bergersen, 1958), *Rhizobium lupini* (Dilworth & Williams, 1967) and rhizobia associated with *Lotus* species (Sutton, 1974), the same amount or considerably less DNA per cell was found after bacteroid formation. However, Reijnders *et al.* (1975) found an appreciable increase in DNA content per cell in bacteroids of *R. leguminosarum* compared with broth-cultured bacteria. We now report the results of cytofluorometrical measurements on Feulgen-stained preparations of different Rhizobium species in the bacterial and the bacteroid forms. In contrast to other reports (Bergersen, 1958; Dilworth & Williams, 1967; Sutton, 1974), we find that in most species investigated the bacteroids contain more DNA per cell than the corresponding bacteria.

We adopted a cytofluorometrical method (Böhm & Sandritter, 1975; Prenna, Leiva & Mazzini, 1974; Bosman, 1976) for these measurements because the commonly used diphenylamine reaction (Burton, 1968) has several disadvantages. First, counting the bacteroid cells may be prone to errors due to their aggregation and lysis. Second, chemical analysis

dance with the relative frequency of that class in the preparation.

only gives information about the total amount of DNA in the sample, and thus the result may be influenced by contaminating DNA of non-rhizobial origin. Finally, only the mean DNA content per cell can be determined by the diphenylamine reaction and differences in DNA content between individual cells do not become evident. The cytofluorometrical

Electrophoresis was carried out at constant voltage; the first 30 min at 50 V, then 3 h at 100 V. Gels were stained with 0.25% Coomassie Brilliant Blue in 45% ethanol, 9% acetic acid, for 2 h at 60°C and destained with 7.5% acetic acid, 5% ethanol at 60°C. Gels were dried on Whatman 3MM paper and radioautographed on Kodak RP Royal X-Omat film (14). Estimation of ^{35}S label incorporated into component I (CI) and component II (CII) of nitrogenase was performed after cutting the relevant bands from the gel and preparing them for scintillation counting as described above for proteins on GF/C filters. Radioactivity in the remainder of the gel was also determined. The synthesis of CI and CII is given as a percentage of the total ^{35}S label incorporated into soluble bacteroid proteins.

Nitrogenase activity. Nitrogenase activity of intact nodules was measured by the acetylene reduction method (15) on pieces of the main root carrying the root nodules. Ethylene concentrations were measured in a Pye 104 gas chromatograph with a Porapack R column.

Heme concentrations. Heme concentrations in plant nodule protein preparations were measured by the hemochrome method (16). To the protein samples an equal volume of 4.2 M pyridine in 0.2 M NaOH was added. The mixture was divided between two 1-cm cuvettes; one sample was oxidized with a few crystals of $K_3 Fe(CN)_6$ and the other reduced with a few crystals of $Na_2S_2O_4$. The differential absorbance at 556 nm (ΔA_{556nm}) and at 539 nm (ΔA_{539nm}) were recorded and the heme concentration was calculated using $\varepsilon_{(mM)} = A_{556nm} - A_{539nm} = 23.4$.

Apo-leghemoglobin concentrations. Apo-leghemoglobin concentrations were determined by a radioimmunoassay developed for purified leghemoglobin of *Pisum sativum* as will be described in chapter V.

Isolation and purification of nitrogenase components from R. leguminosarum. All manipulations were done under a stream of argon; buffers were flushed with argon for 1 h before use. Bacteroids ($2 \cdot 10^{11}$) were isolated anaerobically

and suspended in 10 ml 25 mM Tris·HCl, pH 7.5, 20 mM $Na_2S_2O_4$. The suspension was sonicated for 10 min with a Branson B30 sonifier (Branson, Danbury, Conn., U.S.A.), equipped with a microtip in a 50 ml plastic tube chilled in ice. The lysate was centrifuged for 1 h at 105,000 x *g* in polyallomer tubes in a Spinco 30 fixed-angle rotor. The supernatant was sucked off in a syringe and loaded unto a 1 x 10 cm DEAE-cellulose column equilibrated with 25 mM Tris·HCl, pH 7.5, 20 mM dithionite. The nitrogenase components were isolated by stepwise elution of the column with portions of the same buffer solution with consecutively no, 0.15, 0.28 and 0.50 M NaCl. The enzymic activity of the two components was assessed by an *in vitro* acetylene reduction assay, essentially as described by Whiting and Dilworth (17). The test mixture contained the following components: 25 mM HEPES, pH 8.0, 1.5 mM ATP, 2 mM magnesium acetate, 10 mM creatine phosphate, 4 units/ml creatine phosphokinase, 1 mM dithiothreitol, 20 mM $Na_2S_2O_4$. Fractions of the DEAE-cellulose column containing CI or CII activity were concentrated anaerobically by ultrafiltration through an Amicon PM10 membrane (Amicon Corp., Mass., U.S.A.). Components I and II were further purified by gel filtration on Ultrogel ACA 34 (LKB) and ACA 44 columns (1.6 x 60 cm), respectively. The columns were eluted with 0.28 M NaCl, 25 mM Tris·HCl, pH 7.5, 5 mM dithionite (flow rate 1.5 ml/h).

RESULTS

Influence of NH_4^+ on the development of nitrogenase activity

The influence of NH_4^+ on the nitrogenase activity was studied with plants cultured in reagent tubes. Such plants showed a similar rate of development of acetylene reduction activity as plants cultured in gravel (Fig. 1). Between 17 and 21 days after sowing the peas there was a rapid increase of the nitrogenase activity (Fig. 1). 17-day-old plants had already formed

visible nodules and fresh nodule weight per plant increased 2-3 fold from day 17 to day 21. For these reasons 17-20-day-old plants appeared most suitable for assessing the influence of NH_4^+,

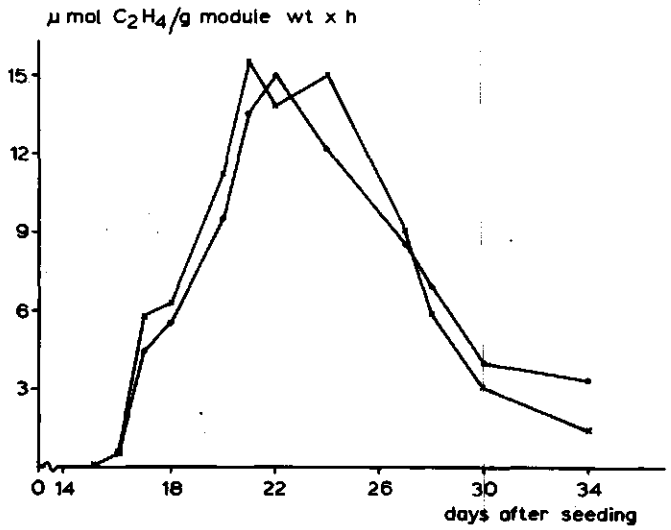

Fig. 1. Acetylene reduction of plants grown on gravel (x——x) and plants transferred to reagent tubes with nutrient medium (●——●). Six plants were harvested at each sampling for acetylene reduction. Reduction was measured on nodules attached to the main root.

Fig. 2 shows the effect of different concentrations of NH_4NO_3, administered at day 17, on nitrogenase activity, measured on day 21. Low concentrations (1 mM) of NH_4NO_3 have no effect or cause a small stimulation of nitrogenase activity, higher concentrations cause a 40-50% decrease of this activity.

$(NH_4)_2SO_4$ and NH_4Cl inhibited nitrogenase activity similarly to NH_4NO_3. The decrease of the acetylene reduction by NH_4^+ varies somehow in different experiments, e.g. 20 mM NH_4^+ can result in a decrease of 50-80% (see also Fig. 5). KNO_3 slightly stimulated or did not affect nitrogenase activity. For these reasons we think that NH_4^+ is responsible for the decrease of the

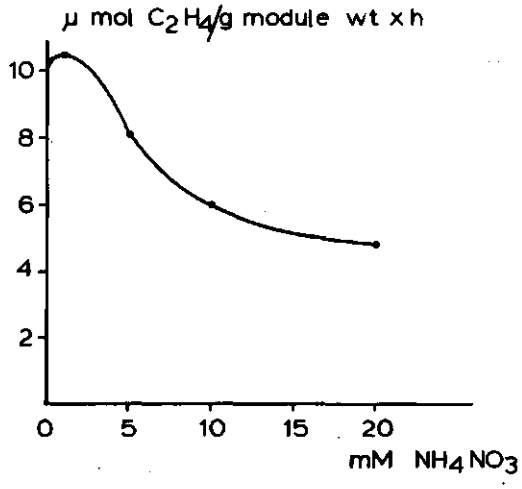

Fig. 2. Acetylene reduction of 21-day-old plants cultured on solutions supplied with different concentrations of NH_4NO_3 at day 17. The plants were grown on NH_4NO_3 medium for 4 days as described in Materials and Methods. For each concentration 10 plants were harvested and acetylene reduction was measured of nodules attached to the main root.

nitrogenase activity when the pea plants are supplied with NH_4NO_3.

In Table I (column 1) it is shown that plants supplied for 1 or 4 days with NH_4NO_3 reduce less acetylene per g nodule weight than control plants. Nodules of plants cultured on NH_4NO_3 contain the same number of bacteroids per g nodule weight (Table I, column 2) as do nodules from control plants and these bacteroids have an equal protein content (Table I, column 3). Thus the reason for the decreased acetylene reduction is a diminution of nitrogenase specific activity and not a decreased amount of bacteroid protein per g nodule weight.

Only plants supplied with 20 mM NH_4NO_3 for 4 days showed a substantial reduction of nodule weight per plant (Table I, column 4). $(NH_4)_2SO_4$ and NH_4Cl had similar effects as NH_4NO_3 on the nodule characteristics shown in Table I.

Regulation of nitrogenase synthesis

To investigate whether the decrease in nitrogenase activity per cell in the presence of NH_4^+ is due to a decreased synthesis of one or both of the nitrogenase components, as in *Azotobacter* (1) and *Klebsiella* (2), we measured the synthesis after the addition of NH_4Cl or NH_4NO_3. This synthesis was determined by labeling of the newly-synthesized proteins with ^{35}S sulfate and estimation of incorporated label into CI and CII after the soluble bacteroid proteins were separated by gel electrophoresis. Protein bands corresponding to CI or CII of nitrogenase were identified taking purified components as a reference (Fig. 3). CI consists of 2 subunits with molecular weights of 56,000 and 53,000 and CII has subunits of 33,000 daltons, as determined on polyacrylamide SDS gels. Omission of sulfate for 4 days, necessary for ^{35}S sulfate labeling (see Materials and Methods), had no effect on the development of the nitrogenase activity.

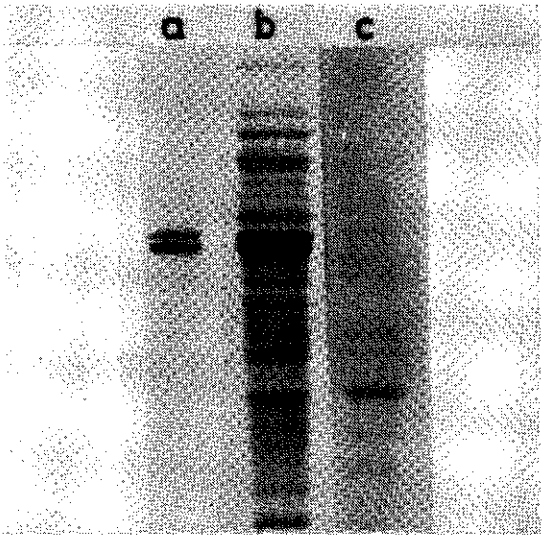

Fig. 3. Polyacrylamide gel electrophoresis of bacteroid proteins: CI (a), soluble bacteroid proteins (b), CII (c). Polarity: anode at bottom.

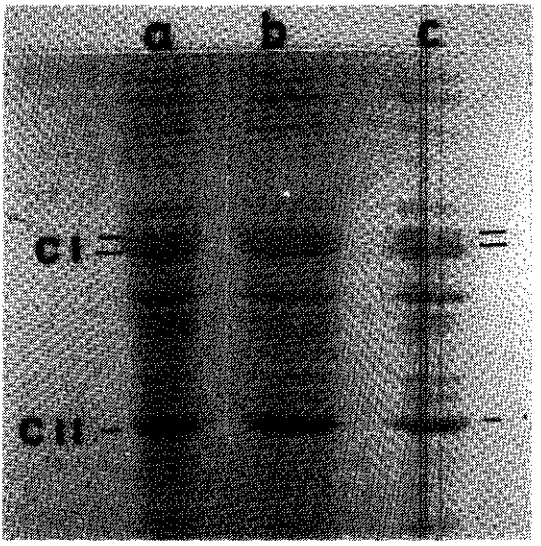

Fig. 4. Autoradiograph of soluble bacteroid proteins, analysed on 10% acrylamide gels. (a) control (no NH_4^+ added). (b) 4 days growth in 10 mM NH_4Cl. (c) 4 days growth in 10 mM NH_4NO_3.

To study the effect of NH_4^+, 3 groups of five plants each were grown in reagent tubes. One group was grown without NH_4^+, and two groups from day 17 to day 21 on 10 mM NH_4Cl or 10 mM NH_4NO_3, respectively (Table II, column 1). All plants were labeled with ^{35}S sulfate from day 20 to day 21. The incorporation of ^{35}S into soluble bacteroid protein was linear with time for 24 h, after a lag phase of approx. 6 h.

The overall protein synthesis measured as specific radioactivity (Table II, column 2) appears not to be influenced by 10 mM NH_4NO_3 or NH_4Cl.

To examine whether NH_4^+ has a specific influence on the synthesis of the two nitrogenase components we looked at the distribution of incorporated label in the different proteins after SDS gel electrophoresis of the soluble bacteroid proteins. An autoradiograph of a gel is shown in Fig. 4. It is clear that ^{35}S label is incorporated into CI and CII, so the genes coding for CI and CII are still expressed after the addition of NH_4^+. Since the labeling pattern is

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not significantly changed by the addition of NH_4^+, the synthesis of the other soluble bacteroid proteins appeared not to be influenced either by the addition of 10 mM NH_4^+.

To quantify the synthesis of CI and CII the amount of label, present in the protein bands corresponding to CI and CII was assessed as described in Materials and Methods. The radioactivity in CI and CII is shown in Table II (columns 3 and 4) and is presented as a percentage of the total ^{35}S label incorporated into the soluble bacteroid proteins recovered from the gel. This relative synthesis is given because the specific radioactivity of the soluble bacteroid proteins is not equal in the different groups of plants (Table II, column 2). The data given in Table II, columns 3 and 4, show that in spite of the fact that the nitrogenase activity is decreased by the addition of 10 mM NH_4^+, nitrogenase synthesis is unaffected. The total incorporation of ^{35}S into bacteroid protein was about 50% reduced when plants were cultured on 20 mM NH_4^+. However, also in the presence of 20 mM NH_4^+ the relative synthesis of CI and CII is not decreased.

Since the half-life of nitrogenase is in the order of 2 days (chapter VI). the breakdown of nitrogenase might significantly influence the amount of nitrogenase in a period of 4 days. Thus, if an NH_4^+ treatment caused enhanced degradation of the nitrogenase components, this could explain the reduction of nitrogenase activity by NH_4^+. However, also when proteins are labeled with ^{35}S sulfate from day 17 to day 21, while the plants were in NH_4NO_3 medium, no difference in incorporation of ^{35}S in the nitrogenase components was detectable (control: CI, 12.3; CII, 7.4; 10 mM NH_4NO_3: CI, 13.5; CII, 7.3). Thus, the breakdown of nitrogenase was at similar rates in NH_4NO_3 treated and control plants.

Leghemoglobin concentrations

Since our results indicate that the reduction of nitrogenase activity is

not caused by a decreased synthesis, we considered another factor which influences nitrogenase activity in root nodules, viz. leghemoglobin (Lb). Several groups found a quantitative relationship between Lb content and nitrogen-fixing efficiency of nodules (18,19).

We determined the effect of NH_4^+ on heme and apo-Lb concentrations. Fig. 5 shows the effect of 20 mM NH_4Cl in time on nitrogenase activity, and the amounts of heme and apo-Lb per mg plant protein. After one day acetylene reduction has already been affected by NH_4^+ and after 4 days only 17% of the original nitrogenase activity is left. The first decrease of heme as well as apo-Lb concentrations was observed on the second day of the NH_4^+ treatment. Apo-Lb concentration decreases a little bit more rapidly than the heme con-

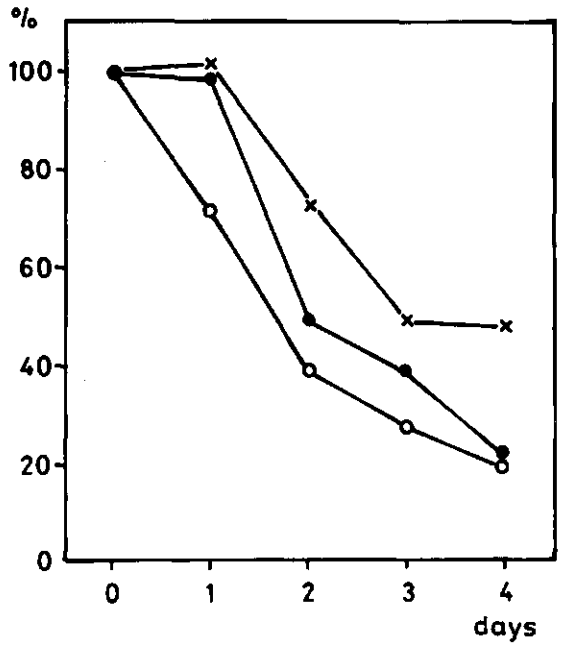

Fig. 5. The effect of 20 mM NH_4Cl in time on acetylene reduction per g nodule wt, and the amount of heme and apo-Lb per mg plant nodule protein. Heme was quantified with the pyridine hemochrome assay and the amount of apo-Lb was determined with a radioimmunoassay. 0——0, acetylene reduction; ●——●, apo-leghemoglobin; x——x, heme.

protein in bacteroid cells (in lupin bacteroids about 6% of the soluble protein is nitrogenase (17) it is very unlikely that co-migrating proteins could seriously influence the amount of ^{35}S label incorporated into protein bands considered to be CI and CII.

Houwaard (27) demonstrated that nitrogenase activities of toluene-treated bacteroids (22) (strain PRE) isolated from 25 mM NH_4Cl-treated and control pea plants (28 days after sowing) were equal although the nitrogenase activity of the whole plants was reduced by NH_4Cl to about 40%. This result confirms that ammonium-treated pea plants contain the same amount of nitrogenase per g of nodule.

The fact that the synthesis of the nitrogenase components is not repressed by NH_4^+ is in contrast to data reported for other N_2-fixing organisms like *Azotobacter* (1) and *Klebsiella* (2). Consistent with the fact that NH_4^+ does not repress the nitrogenase genes in the endosymbiotic form of *R. leguminosarum* are the results published on the regulation of nitrogenase activity in cultured *Rhizobium* sp (32 H1). Indeed, Tubb (23) found a partial inhibition of the nitrogenase activity by NH_4^+, but Scowcroft *et al.* (24) then showed that this inhibition was completely relieved by increasing the O_2 tension. Therefore NH_4^+ did also not repress the nitrogenase genes in *Rhizobium ex planta*. Moreover, rhizobia *ex planta* reduce nitrogen only if the culture medium contains a source of fixed N (e.g. amino acids). Such growth conditions normally repress nitrogenase synthesis in free-living nitrogen-fixing organisms, like *Klebsiella* and *Azotobacter*.

It was demonstrated by Houwaard (28) that the nitrogenase activity of isolated bacteroids is insensitive to the addition of NH_4^+, while nitrogenase activity of detached root nodules declines by the addition of NH_4^+. So, NH_4^+ decreases nitrogenase activity of whole plants and of detached root nodules, but does not decrease the nitrogenase activity of isolated bacteroids. The fact that nitrogenase activity of isolated bacteroids is in-

sensitive to NH_4^+ can be explained by the observation of Laane (29) that NH_4^+ is not taken up by isolated bacteroids. Since NH_4^+ decreases nitrogenase activity of root nodules but not of isolated bacteroids, Houwaard (28) proposed that probably NH_4^+ is assimilated in the plant cytoplasm into an effective inhibitor (e.g. aminoacids) of nitrogenase activity.

By means of a radioimmunoassay and the pyridine hemochrome assay it was shown that the concentrations of apo-Lb and heme respectively, decrease by the addition of NH_4^+. Fig. 5 suggests that apo-Lb has a higher turn-over rate than heme, but this is probably due to the fact that also some of the breakdown products of heme cause absorption in the pyridine hemochrome assay. Fig. 5. indicates that the decrease of nitrogenase activity preceeds the decrease of Lb. This result confirms the experiments of Chen and Philips (30), who demonstrated a similar phenomenon in soybean root nodules.

It was rather surprising that the Lb concentration decreases by the addition of NH_4^+, but apo-Lb synthesis is not affected by NH_4^+. Possible explanations for this phenomenon can be: a) Lb is modified by the addition of NH_4^+, e.g. by removal of heme and the modified Lb is not recognized anymore by the anti-Lb serum used in the RIA, but its Rf value on SDS containing polyacrylamide gels is unchanged; b) NH_4^+ induces an increased degradation of Lb.

The proposed functions of Lb, such as facilitating a high influx of oxygen (25) at a low concentration and an enhancement of the efficiency of the oxidative phosphorylation in the bacteroids (26,27), imply that a reduction of the amount of Lb in the nodule would decrease the nitrogenase activity. Since the decline of nitrogenase activity preceeds the decrease of the amount of Lb, it is very unlikely that the decrease of the amount of Lb is responsible for the initiation of the decline of nitrogenase activity. Therefore the decrease of the Lb concentration will probably reinforce the decrease of the nitrogenase activity.

in free-living nitrogen fixing organisms like e.g. *Azotobacter* there are two effects of NH_4^+ on nitrogen fixation. An immediate effect on the nitrogenase activity by switching off the flow of reducing equivalents to nitrogenase by lowering the $\Delta\Psi$ (29) and secondly a repression of the nitrogenase genes. Possibly also in *Rhizobium* bacteroids *in vivo* there are two effects: a decrease of the amount of Lb, which may be preceded by an other effect of NH_4^+, which is yet unclear.

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CHAPTER III

THE EFFECT OF WATERLOGGING ON THE SYNTHESIS OF THE NITROGENASE COMPONENTS IN BACTEROIDS OF *RHIZOBIUM LEGUMINOSARUM* IN ROOT NODULES OF *PISUM SATIVUM*

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SUMMARY. The effect of waterlogging of root nodules on nitrogenase activity and synthesis was studied in *Pisum sativum* inoculated with *Rhizobium leguminosarum* (strain PRE). It was shown that: 1. nitrogenase activity of intact pea plants was decreased by waterlogging, 2. this decrease was paralleled by a decline of the amount of active nitrogenase determined in toluene EDTA treated bacteroids, 3. SDS-polyacrylamide gel electrophoresis revealed that the amount of nitrogenase component II (CII) decreased by waterlogging while the amount of component I (CI) was not markedly affected, and 4. analysis of bacteroid proteins after $^{35}SO_4^=$ labeling of pea plants showed that CII synthesis was repressed while CI synthesis continued indicating that the synthesis of CI and CII is regulated by independent mechanisms.

INTRODUCTION. In *Klebsiella pneumoniae* the nif-genes can be repressed in at least two different ways. A fixed nitrogen source (e.g. NH_4^+ or amino acids) will repress nitrogenase synthesis; glutamine synthetase (GS) probably plays a role in this repression (1). Secondly, oxygen can also repress nitrogenase synthesis in *Klebsiella*, which is a facultative anaerobic nitrogen fixing organism (2) and GS does not participate in the repression of nitrogenase synthesis by O_2 (2).

Regulation of nitrogenase synthesis in rhizobia probably differs from that in *K. pneumoniae*. In symbiotic as well as nitrogen fixing rhizobia *ex planta*, NH_4^+ decreases nitrogenase activity. In both cases, however, nitrogenase synthesis is probably not repressed as in *K. pneumoniae* and other nitrogen fixing organisms (3,4,5).

Oxygen is probably important in the regulation of nitrogenase synthesis in *Rhizobium*, a micro-aerobic nitrogen fixing organism. In nitrogen fixing *R. japonicum ex planta* 0.16% O_2 is the optimal concentration for nitrogen fix-

ation (6). Higher as well as lower O_2 concentrations cause a decrease in nitrogenase activity. In root nodules of legumes, the O_2 concentration is important for nitrogenase activity. If the oxygen supply is reduced by waterlogging of the root nodules, nitrogenase activity is decreased (6,7). Whether nitrogenase synthesis is also influenced, however, was not determined. In this paper we report on the effect of waterlogging of the root system on nitrogenase activity and on the synthesis of the two nitrogenase components of *Pisum sativum* nodulated with *R. leguminosarum*.

MATERIALS AND METHODS. The growth of pea plants (*P. sativum*, var. Rondo) nodulated with *R. leguminosarum* (PRE), ^{35}S -sulfate labeling of pea plants, polyacrylamide gel electrophoresis and autoradiography were performed as described previously (5,9).

Waterlogging treatment. Plants of 20 days old were used for the waterlogging treatment. Waterlogging was effected by filling the trays, that contained the pea plants, with distilled water, so that all root nodules were submerged in the growth medium.

Preparation of soluble bacteroid proteins. Anaerobic isolation and lysis of bacteroids by osmotic shock after lysozyme treatment, were performed as described before (5,9). The bacteroids were isolated in a glove box flushed with N_2 (9) and buffers contained 20 mM dithionite and 4% polyvinylpyrrolidone.

Acetylene reduction. Nitrogenase activity of intact pea plants was measured by the acetylene reduction assay in 500 ml Erlenmeyer flasks containing 10% acetylene and 90% air. The waterlogged root nodules were blotted with absorbent paper prior to analysis. Acetylene reduction was followed for 15 min, after which the pea plants were placed back in the culture medium. Nitrogenase activity was expressed as nmole acetylene reduced per plant per hour.

Acetylene reduction of anaerobic bacteroid suspensions was measured after the bacteroids were treated with EDTA and toluene as described by Houwaard (4)

RESULTS.

The effect of waterlogging on nitrogenase activity. The effect of waterlogging on nitrogenase activity was studied on whole pea plants. Two groups of 6 nodulated pea plants of 20 days old with about equal acetylene reducing capacity were selected out of 10 groups. One group was placed under waterlogging conditions while the other was used as a control. Nitrogenase ac-

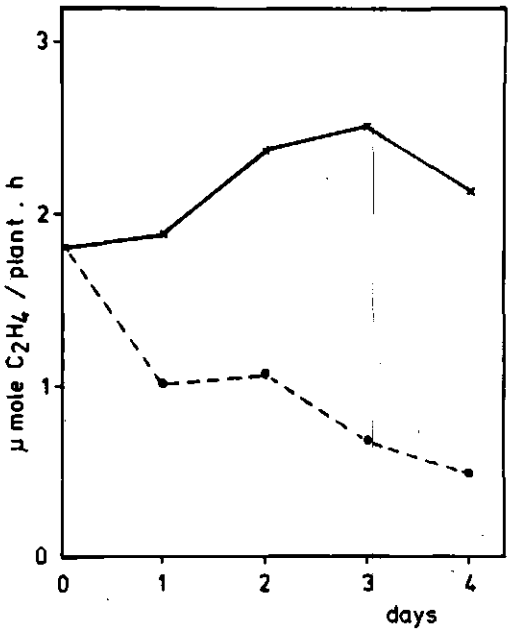

Fig. 1. Acetylene reducing activity of whole pea plants. The same plants were used throughout the experiments. x—x, control; ●--●, waterlogging.

tivity was followed over a period of 4 days. Fig. 1 shows that the activity of the control plants increased slightly during the first 3 days. At the third day nitrogenase activity reached its maximal value after which it decreased slightly at the fourth day. Nitrogenase activity of the waterlogged plants decreased rapidly after the onset of waterlogging. After 4 days of waterlogging nitrogenase activity of the waterlogged plants was 25% of that in control plants.

Nitrogenase activity depends on several factors, e.g. amount of nitrogenase and supply of energy, and reduction equivalents. To determine if the amount of active nitrogenase is limiting in waterlogged root nodules we determined the amount of active nitrogenase in bacteroids isolated from these nodules. Anaerobic nitrogenase activity of bacteroid suspensions, treated with toluene and EDTA, supplied with energy (ATP) and reduction equivalents

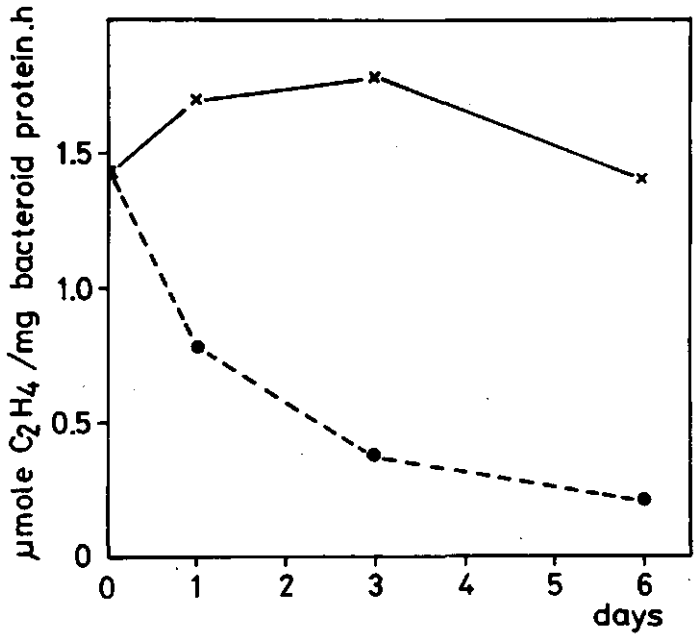

Fig. 2. Acetylene reducing activity of toluene EDTA treated bacteroids, isolated from control and waterlogged root nodules. x—x, control; •--•, waterlogging.

(dithionite) was determined. Nitrogenase activity of waterlogged bacteroids decreased parallel to the activity of whole plants while nitrogenase activity of control bacteroids remained about constant over a period of 5 days (Fig. 2). After 5 days waterlogging, only 15% of the control activity was left. This result strongly indicated that by waterlogging the amount of active nitrogenase in bacteroids decreases, as in toluene EDTA treated bacteroids the amount of active nitrogenase limits activity.

Nitrogenase synthesis during waterlogging. The amounts of the separate nitrogenase components in bacteroids can be estimated by polyacrylamide gel electrophoresis of bacteroid proteins. Fig. 3 shows an electropherogram of proteins stained with Coomassie brilliant blue, prepared from bacteroids isolated from control plants (Fig. 3b) and from pea plants placed for 4 days

under waterlogging conditions (Fig. 3c). The nitrogenase components were identified with purified nitrogenase components (Fig. 3a, d). This figure shows that the amounts of the two subunits of component I (CI) are not markedly affected but the amount of component II (CII) is reduced in com-

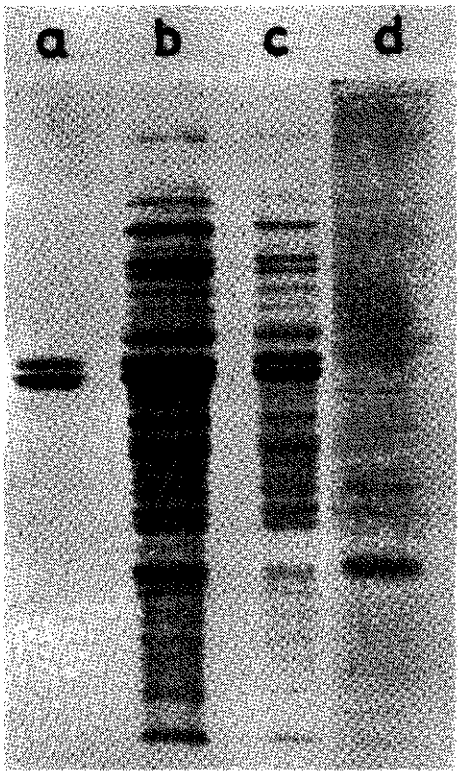

Fig. 3. Polyacrylamide gel electrophoresis of bacteroid proteins isolated from control and waterlogged root nodules. a. Component I, b. Control bacteroid proteins, c. Bacteroid proteins from waterlogged root nodules, d. Component II.

parison to the other protein bands.

To determine whether this decrease of the amount of CII is caused by a repression of the synthesis of CII, bacteroid protein synthesis was followed by $^{35}SO_4^=$ labeling. Two groups of pea plants were labeled with $^{35}SO_4^=$ for 24

h. Bacteroid proteins were analysed by polyacrylamide gel electrophoresis and an autoradiograph was made. Figs. 4a and b show densitograms of autoradiographs of the bacteroid proteins shown in Fig. 3b and c. Total bacteroid protein synthesis is not markedly affected by waterlogging: control, 166 cpm/µg protein and waterlogging 181 cpm/µg protein. Fig. 4 shows that the incorporation of ^{35}S into CII is markedly reduced, since the peak corresponding with CII is reduced in comparison to the other proteins. The incorporation of ^{35}S into the two subunits of CI is hardly affected by waterlogging however. Besides the repression of CII, proteins with molecular

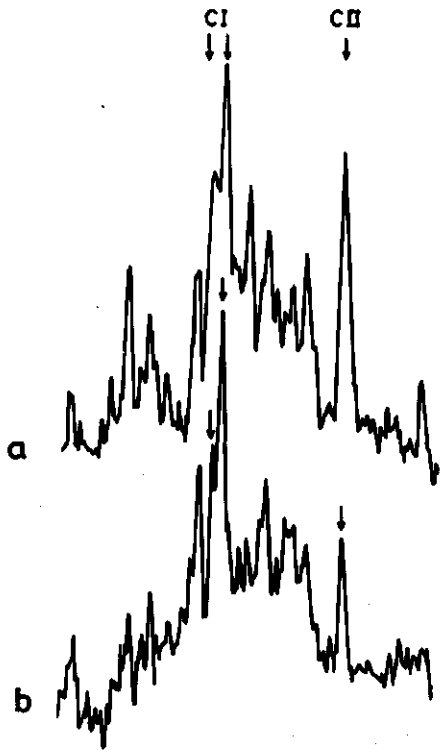

Fig. 4. Densitograms of bacteroid proteins isolated from $^{35}SO_4^=$ labeled control and waterlogged root nodules. A. Control bacteroid proteins, B. Bacteroid proteins of waterlogged root nodules. The arrows indicate the position of the two subunits of CI and CII.

weights of about 80, 48 and 22,000 were also repressed.

The decrease of ^{35}S incorporation into CII can be caused by an increased degradation or a repressed synthesis of CII. Analysis of ^{35}S labeled bacteroid proteins isolated from 1 day waterlogged root nodules, revealed that the amount of CII on polyacrylamide gels was not significantly decreased, while the incorporation of ^{35}S into CII was repressed (result not shown). This result indicates that the decrease of ^{35}S incorporation into CII is caused by a repressed synthesis rather than increased degradation of CII.

DISCUSSION. Sprent (8) proposed that a reduced oxygen supply to the root nodules, causing a decrease in the energy supply to the bacteroids, is responsible for a decreased nitrogenase activity under waterlogging. If only this reduced energy supply to the root nodules was responsible for the decrease of nitrogenase activity, we would expect an immediate decrease of nitrogenase activity at the beginning of the waterlogging treatment after which it should remain constant. Our results confirm those of e.g. Minchin *et al.* (10), who showed that nitrogenase activity decreases continually during the waterlogging treatment.

It was shown in experiments with toluene EDTA treated bacteroids that waterlogging of the root nodules caused a decrease in the amount of nitrogenase enzyme in parallel with the decrease of nitrogenase activity. $^{35}SO_4^=$ labeling showed that CII synthesis was repressed. Therefore, besides the reduced O_2 supply to the root nodules, a decreased amount of active nitrogenase is probably responsible for the continued decrease of nitrogenase activity during waterlogging. It appears that the decrease in the amount of nitrogenase is parallelled by a decreased CII synthesis, while CI synthesis seems rather unaffected.

Since only the synthesis of CII of nitrogenase seems to be repressed by waterlogging, the synthesis of CI and CII of nitrogenase probably can be regulated independently. This is in accordance with results we reported in previous papers on nodule development (9,11).

The mechanism by which waterlogging causes a repression of CII synthesis is yet unclear. We speculate that the decrease of the O_2 supply to the root nodules plays a role in the repression of CII synthesis.

The decrease of nitrogenase activity of toluene EDTA treated bacteroids is caused by a repression of CII synthesis. If nitrogenase activity is proportional to the amount of CII, with CI present in excess, the turnover rate of CII during waterlogging can be estimated. Fig. 2 shows that after 3-5 days of waterlogging acetylene reduction has decreased to 15% of the original value. This indicates that $t_{\frac{1}{2}}$ of CII is between 1 and 2 days. This value is consistent with the turnover rates determined in other ways (Bisseling, unpublished results).

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CHAPTER IV

DEVELOPMENT OF THE NITROGEN-FIXING AND PROTEIN-SYNTHESIZING APPARATUS OF BACTEROIDS IN PEA ROOT NODULES

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Key words: Nitrogenase development; Nitrogen fixation; Leghemoglobin; Bacteroid; (Pea root nodule)

Summary

Some aspects of root nodule development of *Pisum sativum* inoculated with *Rhizobium leguminosarum* were examined.

1. Nitrogenase activity (measured as acetylene reduction) appears to be preceded by leghemoglobin synthesis (measured immunologically).

2. Syntheses of component I and component II of nitrogenase are not strictly coordinated. Synthesis of component I starts before component II.

3. Plant and bacteroid protein synthesis (measured by [^{35}S]sulfate labeling) in root nodules declines rapidly during nodule development. Corresponding with this decline is a decrease in quantity and quality of rRNA.

Introduction

In legume-rhizobium symbiotic nitrogen fixation, several proteins play an important role, of which the most important probably are leghemoglobin and nitrogenase. Leghemoglobin is a myoglobin-like protein synthesized only in effective root nodules of *Leguminosae*. The apoprotein is encoded by plant genes [1] while at least some enzymes necessary for the synthesis of the heme prosthetic group are localized in the bacteroids [2]. Nitrogenase is made up of two component proteins: a molybdenum-iron protein (CI) and an iron-containing protein (CII) [3]. Leghemoglobin and nitrogenase, are newly synthesized during nodule formation, which means that the genes coding for

Abbreviation: SDS, sodium dodecyl sulfate.

Leghemoglobin concentration in soluble plant protein was determined by the radial immunodifussion technique described by Mancini [9] with the modifications proposed by Broughton et al. [10]. The diameter of the precipitated zones was measured on a photograph of the slide taken after the diameter of the zone was constant (after approx. 3 days). Calibration curves of the area of the zone against concentration of antigen were made in each analysis with purified leghemoglobin and every analysis was performed in duplicate. As a blank, soluble plant protein preparations isolated from uninoculated roots were used.

Results

Appearance of leghemoglobin and nitrogenase activity

Fig. 1 shows the development in time of acetylene reducing activity and leghemoglobin content, together with the increase in fresh nodule weight per plant. Nodule formation can be detected at 10—13 days after seeding. Fig. 1 shows that at 14 days, both leghemoglobin and nitrogenase were not detectable. At 15 days leghemoglobin as well as nitrogenase were perceptible, leghemoglobin being 4.8% and nitrogenase just 0.12% of the value on 20 days. In the following days nitrogenase activity increased very rapidly; between days 15 and 17 300 times, while leghemoglobin concentration increased only 10 times in this period.

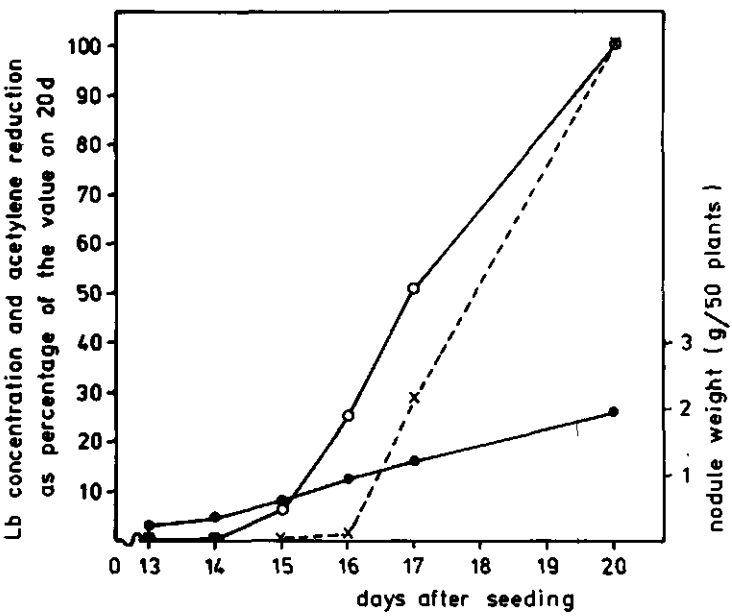

Fig. 1. Acetylene reduction, leghemoglobin content and nodule weight during development of the root system of *P. sativum*. 50 plants were used per day and nodule weight, leghemoglobin content and acetylene reduction were determined on the same plants. ○———○, leghemoglobin content; X———X, acetylene reduction; ●———●, nodule weight (g fresh weight/50 plants). At 20 days, leghemoglobin accounted for 8.7% of the total soluble plant protein, and acetylene reducing activity was 8.3 μmol C_2H_4/g nodule fresh wt. × h.

Also in other experiments, leghemoglobin and nitrogenase activity were first detectable at the same day, but always nitrogenase activity, expressed as percentage of the value at 20 days, was much smaller than the leghemoglobin content.

Nitrogenase synthesis

The rate of synthesis of the two nitrogenase components was studied by labeling of the pea plants with [^{35}S]sulfate and polyacrylamide gel electrophoresis of the soluble bacteroid proteins as described previously [4]. Fig. 2 shows an autoradiograph of soluble bacteroid proteins isolated from pea plants of different ages, labeled for 24 h with 0.2 mCi [^{35}S]sulfate/plant, and separated on a polyacrylamide gel slab. In younger bacteroids more ^{35}S is incorporated into protein than in older ones (see Fig. 3). For this reason the blackening of the radioautograph decreases when older bacteroids were analysed (Fig. 2). Table I shows that in younger bacteroids the ^{35}S incorporated into CII relative to CI is lower than is the case for older bacteroids. Synthesis of the two nitrogenase components is thus not strictly coordinated.

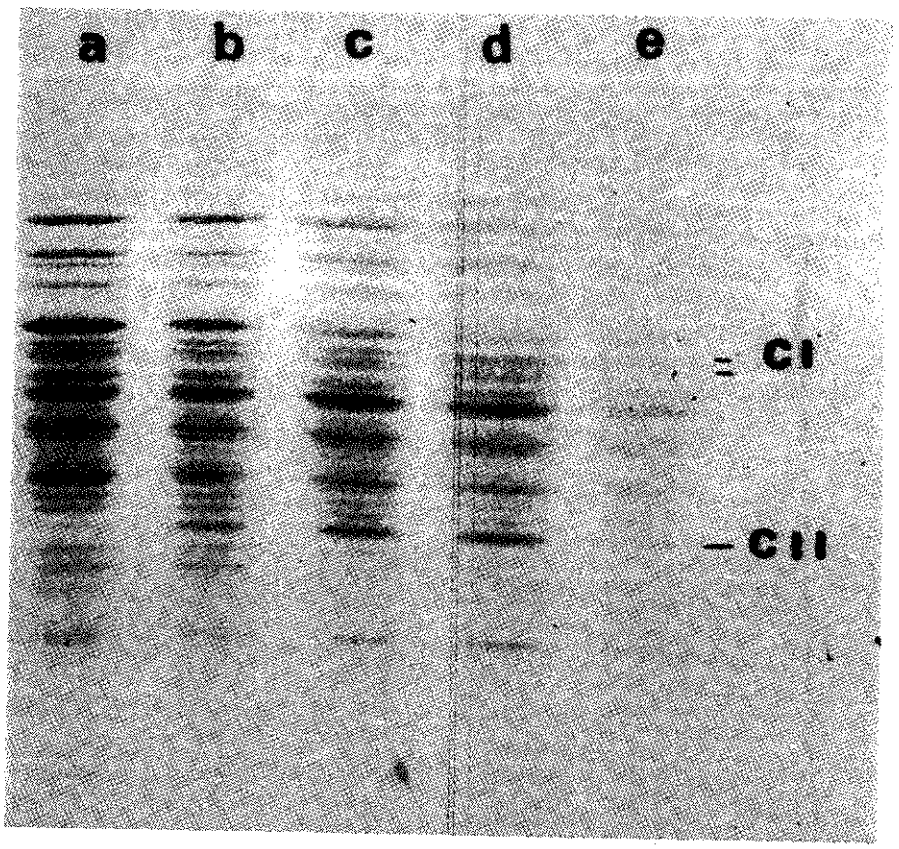

Fig. 2. Radioautograph of soluble bacteroid proteins (15 μg protein in slot a and b and 25 μg in c, d and e) analysed on 10% acrylamide gels. Bacteroids were isolated from 10 plants of different ages: a, 16 days; b, 19 days; c, 22 days; d, 23 days; e, 28 days. The direction of electrophoresis was downward.

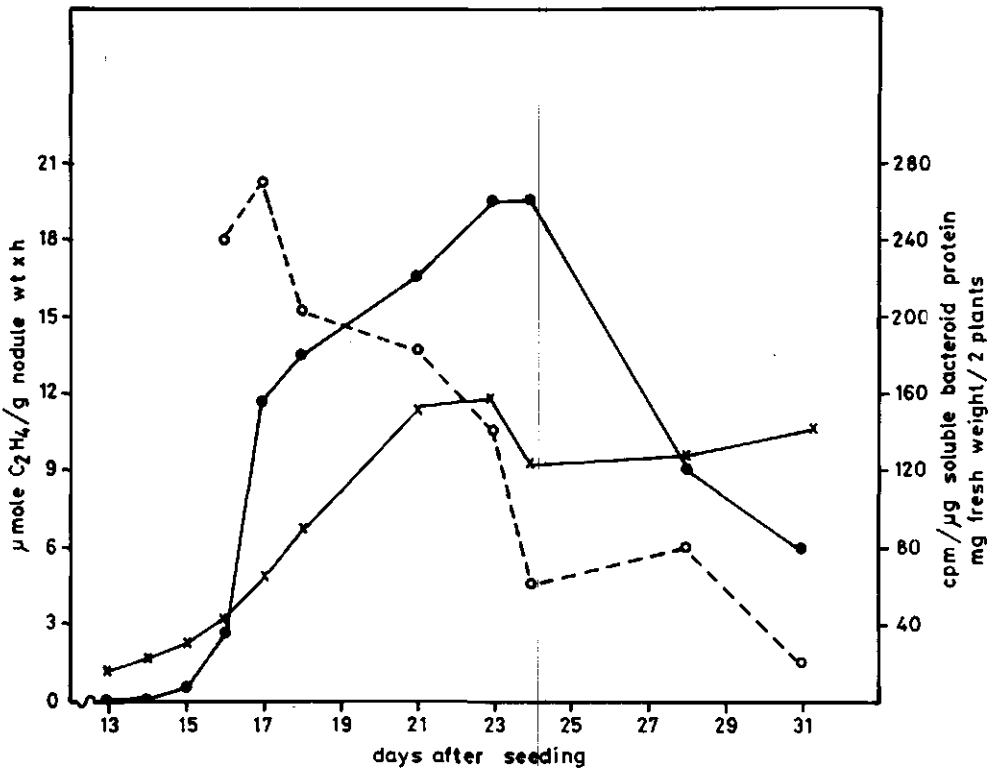

Fig. 3. Nodule weight, acetylene reduction and bacteroid protein synthesis during development of *P. sativum*. Nodule weight and acetylene reduction were determined on 35 plants and protein synthesis in 10 plants. ●———●, acetylene reduction (μmol C_2H_4/g nodule fresh wt. X h); X———X, nodule weight (mg fresh wt./2 plants); ○- - - - -○, protein synthesis (cpm/μg soluble bacteroid protein).

Protein synthesis

Fig. 3 shows the development of nodule weight, nitrogenase activity and in vivo protein synthesis. Nodule weight per plant increased till 23—25 days after seeding, whereupon it remained the same for the rest of the period we examined. At about 5 weeks after seeding, nodules showed signs of senescence and became green. Nitrogenase activity measured as acetylene reduction per gram

TABLE I

RATIO OF ^{35}S INCORPORATED INTO CI AND CII

Ratio of ^{35}S incorporated into CII and CI determined after electrophoresis of bacteroid proteins on a 10% polyacrylamide gel (Fig. 2). ^{35}S incorporated into CI and CII was determined.

Age of the pea plants (days)	$\frac{^{35}S \text{ incorporated into CII}}{^{35}S \text{ incorporated into CI}}$
16	0.29
19	0.69
22	0.87
23	0.95
28	0.85

nodule (fresh weight) increased rapidly after 15—17 days till 23—25 days after seeding, was then constant for a few days after which it decreased rapidly. After the pea plants had grown for 24 h on a medium containing [^{35}S]sulfate the rate of protein synthesis expressed as ^{35}S cpm/μg protein was determined. The rate of protein synthesis in bacteroids and in the plant fraction of the nodule (not shown) ran quite concurrently. As can be seen in Fig. 3 the rate of protein synthesis was maximal in the younger nodules (15—17 days), at a period when increase in acetylene reduction and nodule weight per plant was maximal. After day 17, protein synthesis declines to less than 10% of the maximal value in about 2 weeks (17—31 days).

Amount of RNA per cell in bacteroids of different ages

Growing cells synthesize more protein and therefore contain more rRNA per cell than resting cells. To investigate if the decrease in protein synthesis by bacteroids (Fig. 3) with age is accompanied by a diminished amount of RNA per cell, an estimation of bacteroid RNA content was made.

Bacteroids from nodules picked from plants of 17, 21 and 30 days after inoculation were purified from contaminating plant material and bacteria by sucrose gradient centrifugation [4]. Numbers of bacteroids were determined by counting in a Bürker-Türk counting chamber. RNA and protein content were determined after lysis of the cells with SDS as described under Methods with the results as shown in Table II. The ratio of RNA/protein appears to decrease with the age of the plant while the decrease in the ratio RNA/cell is less pronounced, probably because the cell size and thus the amount of protein per cell increases with age.

Gel electrophoretic analysis of bacteroid ribosomal RNA

The integrity of ribosomal RNA from bacteria and bacteroids was analysed by polyacrylamide gel electrophoresis. The conventional phenol extraction procedure was avoided, to prevent RNA degradation; the ribosomes were resuspended in SDS-sample buffer, which causes dissociation, and were loaded directly on the gel. The absorbance profile (at 260 nm) of the gels is shown in Fig. 4. In panel A the profile of rRNA from logarithmically growing rhizobium cells is seen, in which 23 S and 16 S rRNA are prominent. In panels B and C the profile of rRNA from bacteroids of 21 and 32 days old plants is shown respectively. The position of 16 S rRNA is marked with arrows. The exact position of 16 S rRNA in these profiles was determined in separate experiments in which bacteroid rRNA was mixed with rRNA from bacteria (as in panel A; results not shown). In panels B and C the same amount of rRNA (10.0 μg) was

TABLE II

RNA CONTENT OF BACTEROIDS ISOLATED FROM PEA PLANTS OF DIFFERENT AGES

Age (days)	g RNA/cell ($\times 10^{-15}$)	g RNA/g protein
17	79.5	0.107
21	79.6	0.082
30	61.4	0.062

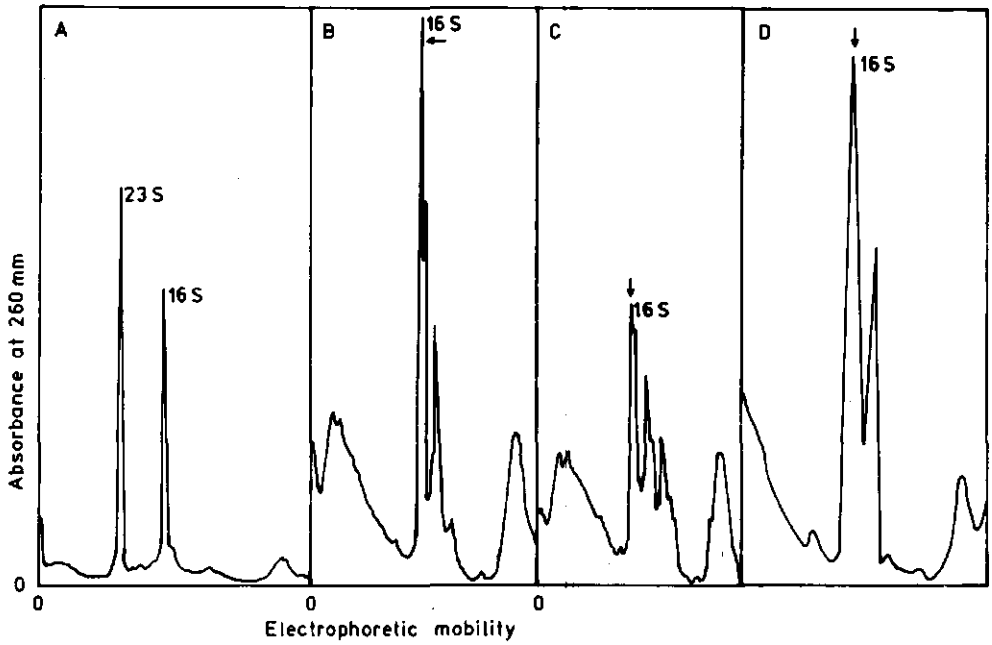

Fig. 4. SDS-polyacrylamide gel electrophoresis of rRNA from *R. leguminosarum* (PRE). Electrophoresis was from left to right. Absorbance and electrophoretic mobility are given in arbitrary units. rRNA was isolated from: logarithmic bacteria (A), bacteroids 21 days (B), bacteroids 32 days (C) and stationary phase bacteria (D).

loaded per gel. The result shows that in bacteroid rRNA no intact 23 S rRNA is present and that the amount of intact 16 S rRNA decreases with the age of the bacteroid. If rRNA from stationary phase bacteria is analysed in the same way (panel D) a result similar to that in panel C is obtained.

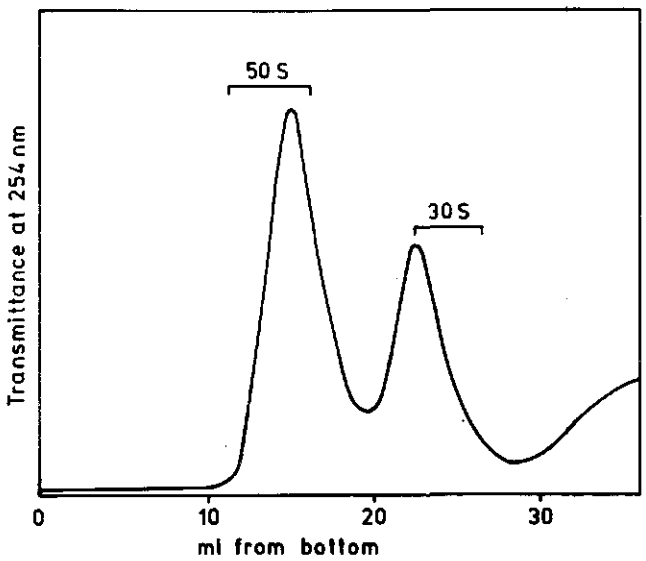

Fig. 5. Sucrose gradient centrifugation of ribosomal subunits isolated from 21 day old bacteroids.

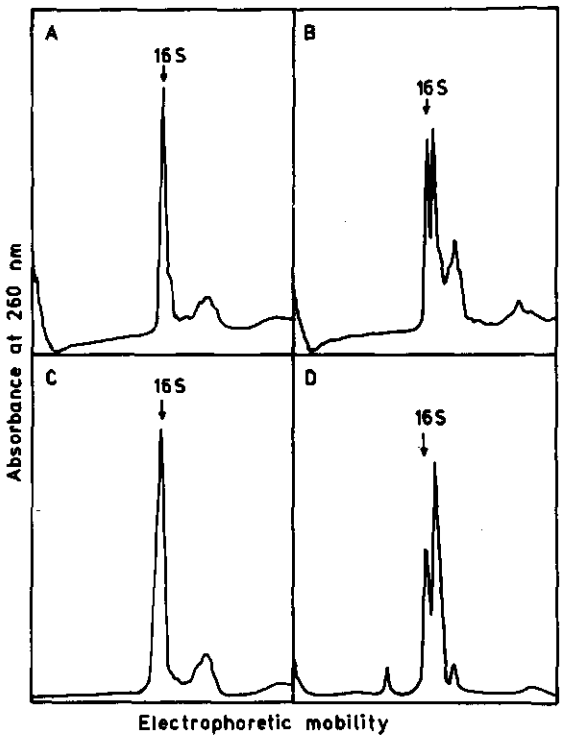

Fig. 6. SDS-polyacrylamide gel electrophoresis of ribosomal subunits. Isolation of subunits is shown in Fig. 5; 3—5 μg of subunits were electrophoresed. A_{260} was measured along the gel. A, 30-S subunits from bacteroids; B, 50-S subunits from bacteroids; C, 30-S subunits from stationary phase bacteria; D, 50-S subunits from stationary phase bacteria.

To investigate if the 16 S RNA indeed derives from 30-S subunits and if the smaller degradation products derive from 50-S subunits, the separate ribosomal subunits were isolated by sucrose gradient centrifugation in low-magnesium buffer (see Methods). The gradient profile obtained by centrifugation of the ribosomal subunits derived from 21 day bacteroids is shown in Fig. 5. The fractions indicated were pooled and the subunits pelleted by centrifugation. The RNA in the subunits was analysed by SDS-gel electrophoresis (as in Fig. 4). The result is shown in Fig. 6. A parallel experiment was carried out with subunits from stationary phase broth-cultured bacteria. The sucrose gradient profile (not shown) was very similar to that in Fig. 5 and the analysis of the RNA is included in Fig. 6. The 30-S subunits of bacteroids as well as stationary phase bacteria contain intact 16 S rRNA, while the 23 S rRNA in the 50-S subunits is degraded.

Discussion

We found that nitrogenase activity was first perceptible at the same day as leghemoglobin; however our interpretation of Fig. 1 is that leghemoglobin is present before nitrogenase activity is detectable. This apparent discrepancy is caused by a difference in sensitivity between the two techniques used. Limiting

sensitivities of the two assays were: 10^{-2} μmol C_2H_4/g nodule wt. × h, which is equal to 0.1% of the acetylene reduction on 20 days (Fig. 1) and 2 μg leghemoglobin/ml, which corresponds to 2.5% of the leghemoglobin content on 20 days (Fig. 1). So the acetylene reduction assay is about 25 times as sensitive as the immunodiffusion technique, used to determine leghemoglobin concentrations. Since leghemoglobin is present before nitrogenase activity is perceptible, this suggests that leghemoglobin is obligatory for nitrogenase activity of pea root nodules. From the experiment shown in Fig. 1 we cannot conclude if nitrogenase synthesis depends on the presence of leghemoglobin or not. The nitrogenase components may have been synthesized before leghemoglobin, in which case leghemoglobin would only be necessary for the final expression of nitrogenase activity in the pea rhizobium symbiosis.

The observation that leghemoglobin synthesis starts before nitrogenase activity was perceptible, is in agreement with data of Godfrey [2] (*R. lupini*), who also determined the apo-leghemoglobin concentration with an immunological technique and of Bergersen and Goodchild [11] (*R. japonicum*), who measured the heme concentration with a hemochrome assay. Also Robertson et al. [12] (*R. lupini*) showed that leghemoglobin was present before acetylene reduction, but they did not describe how leghemoglobin was measured. In contrast to the finding that leghemoglobin is present before acetylene reduction, is the work of Broughton et al. [10] who showed that in *Vigna unguiculata* and *Centrosema pubescens* nitrogenase activity appeared before apo-leghemoglobin, determined with the Mancini immunodiffusion technique, was detectable. Whether the discrepancy between the results is caused by the different species of plant and rhizobium is not clear.

Also Nash and Schulman [13] (*R. japonicum*) argued that acetylene reduction was detectable before leghemoglobin. They only measured heme concentration and acetylene reduction, however, in nodules in which both were already present. They showed that the ratio of nitrogenase activity per unit leghemoglobin declined during nodule development. They extrapolated this result and concluded that leghemoglobin is not causal in the development of nitrogenase activity. However, Bergersen and Goodchild [11] (*R. japonicum*) demonstrated, that notwithstanding a declining ratio of nitrogenase activity per unit of leghemoglobin, after acetylene reduction was detectable, leghemoglobin was perceptible 2 days before acetylene reduction. This indicated that the conclusion of Nash and Schulman based upon this extrapolation is not valid.

Consistent with our conclusion, that leghemoglobin is present before nitrogenase activity, is the fact that in root nodules of legumes leghemoglobin seems to be necessary for nitrogenase activity, because no effective symbiosis in legumes has been described without the presence of leghemoglobin. Only in the symbiosis of rhizobium with *Parasponia* (Trema) [14,15] no leghemoglobin seems to be necessary for nitrogen fixation, but the presence of another mechanism for the regulation of the pO_2 in these root nodules cannot be excluded.

The two components of nitrogenase were not synthesized strictly coordinated; CI was synthesized slightly before CII. Also in bacteroids isolated from pea plants of one age, separated according to their size on a sucrose gradient, we showed recently [4] that the ratio of synthesis of CI and CII varied with the

developmental stage of the bacteroids. This resembles the result of Seto and Mortenson [16] with *Clostridium pasteurianum*. In contrast, for *Azotobacter vinelandii* it has been shown that the synthesis of the two components during repression and derepression of the nif-genes is coordinated [17]. From the biochemical experiments we describe in this paper, it is not possible to get a direct answer to the question how the genes coding for CI and CII are organized, but one of the most probable explanations is, that the genes coding for CI and CII are localized on different operons, and are independently regulated.

We have demonstrated that the rate of protein synthesis, expressed as ^{35}S incorporated per μg protein, per 24 h, in plant as well as in bacteroid tissue decreases rapidly with increasing age of the plant. This rate of protein synthesis is an average value for whole nodules; however, nodules are not homogeneous and contain plant cells and bacteroids of different ages [18]. Most protein synthesis probably is located in the meristematic part of the nodule and contiguous to the plant cells containing the youngest bacteroids [4], which in an absolute sense has a rather constant size, but the relative contribution of which decreases gradually during nodule growth. This may explain why protein synthesis decreases during nodule growth. When bacteroids isolated from nodules of one age were separated on a sucrose gradient, also the smaller bacteroids, which are the youngest, were most active in protein synthesis [4]. The decrease in protein synthesis by bacteroids is accompanied by a decrease in the ratio RNA/protein. In bacterial cultures a correlation was found between growth rate and RNA synthesis [19—21]. A close coupling appeared to exist between protein synthesis and the amount of ribosomes needed to sustain such synthesis. We suggest that the same situation occurs in bacteroids, where the ratio of RNA/protein (Table II) is correlated to the rate of protein synthesis (Fig. 3).

As for the quality of the rRNA, bacteroids appear to be analogous to bacteria in the stationary growth phase. Bacteroids are probably not dividing inside the nodule and therefore protein synthesis must be much lower than in logarithmically growing bacteria. Even in the youngest nodules we could not detect intact 23 S rRNA though strict precautions were taken to avoid ribonuclease action during the isolation procedure. We found that 23 S rRNA is present in a partially degraded state in the 50 S subunit; the molecule is split in at least two fragments which are all smaller than 16 S rRNA. The same was found in stationary phase cultured bacteria. When the nodule grows older also the 16 S rRNA appears to be degraded. Another conclusion must be that 50-S ribosomal subunits can be active in protein synthesis even when their 23 S rRNA is no longer intact.

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CHAPTER V

THE SEQUENCE OF APPEARANCE OF LEGHAEMOGLOBIN AND NITROGENASE COMPONENTS I AND II IN ROOT NODULES OF *PISUM SATIVUM*

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SUMMARY

The sequence of appearance of nitrogenase component I and component II, and leghaemoglobin, in root nodules of pea plants inoculated with *Rhizobium leguminosarum* (PRE) was studied using radioimmunoassays. Leghaemoglobin was detected before nitrogenase activity could be measured. The MoFe component (I) of nitrogenase was detectable at the same time as leghaemoglobin, while the Fe component (II) of nitrogenase and nitrogenase activity appeared 1 and 2 d later respectively.

The order of appearance of nitrogenase activity and leghaemoglobin was also studied in root nodules of cowpea plants inoculated with *Rhizobium* sp. 32H1. Leghaemoglobin was also demonstrable before nitrogenase activity in this system.

INTRODUCTION

In an earlier paper (Bisseling *et al.*, 1979) we demonstrated that leghaemoglobin in pea root nodules induced by *Rhizobium leguminosarum* was synthesized before nitrogenase activity could be measured. We also found that in the early phase of the development of nitrogen fixing activity, the synthesis of the MoFe component (CI) of nitrogenase surpasses the synthesis of the Fe component (CII) (Bisseling *et al.*, 1979). In this paper we report further experiments on the order of synthesis of leghaemoglobin and CI and CII of nitrogenase, which are three essential constituents for nitrogen fixation in pea-rhizobium symbiosis. A radioimmunoassay was developed to quantify leghaemoglobin and CI and CII. This method is a few orders of magnitude more sensitive than the Mancini

immunodiffusion assay (Mancini *et al.*, 1965), which we used previously for the determination of leghaemoglobin; it allows us to extend the measurements towards the beginning of nodule development and to confirm that leghaemoglobin is present before nitrogenase activity is measurable. Moreover, we now find that CI is synthesized before the presence of CII can be demonstrated in bacteroid extracts.

Fast and slow-growing rhizobia differ in many respects (e.g. Paau, 1978). To investigate whether slow-growing rhizobium bacteroids differ from the fast-growing rhizobia (*R. leguminosarum*) in the order of appearance of nitrogenase activity and leghaemoglobin synthesis, we studied the appearance of leghaemoglobin and nitrogenase activity in root nodules of cowpea plants inoculated with *R.* sp. 32H1. Similar results were obtained as with the pea root nodules.

MATERIALS AND METHODS

Growth of plants. Pea plants (*Pisum sativum* var. Rondo) were cultured and nodulated with *Rhizobium leguminosarum* (PRE), as described earlier (Bisseling *et al.*, 1978) at 19 °C to 20 °C with an 8 h dark and 16 h light period. *R. leguminosarum* bacteria were cultured in a yeast mannitol medium (Bisseling *et al.*, 1978). *Vigna unguiculata* Walp (Blackeye Early Ramshorn) was cultured and nodulated with *Rhizobium* sp. 32H1, essentially under the same conditions as the pea plants, but at 25 °C.

Assay of nitrogenase. Nitrogenase activity of intact nodules was measured by the acetylene reduction method on pieces of the main root carrying the root nodules. Ethylene concentrations were measured in a Pye 104 gas chromatograph with a Porapack R column (Bisseling *et al.*, 1978).

Preparation of bacteroid proteins. The isolation of bacteroids and the preparation of soluble bacteroid proteins and plant proteins from root nodules were performed as described before (Bisseling *et al.*, 1979). All manipulations were performed under anaerobic conditions in the presence of argon to prevent precipitation of nitrogenase. Root proteins from non-inoculated plants were prepared from pieces of main root in a similar way to soluble plant nodule proteins.

Preparation of antisera. Specific antisera against CI and CII of *R. leguminosarum* nitrogenase were prepared by injecting rabbits intravenously with 0.5 mg of electrophoretically pure CI or CII (Bisseling *et al.*, 1978) in 0.9% NaCl; 1 month later this was followed by a subcutaneous injection of 2 mg of CI or CII in 0.9% NaCl, mixed with an equal volume of Freunds complete adjuvant (Difco, Detroit, MI). The rabbits were bled through the jugular earvein 7 d after the second injection. Under these conditions the titres allowing immunoprecipitation with 1 mg ml^{-1} CI or CII in an Ouchterlony double diffusion are 1/64 and 1/32 respectively. Pre-immune serum showed no reaction.

Antiserum against leghaemoglobin from pea plants was raised in a rabbit as described previously (Bisseling *et al.*, 1979). Antiserum against leghaemoglobin from cowpea plants was prepared in a goat and was a generous gift from Dr. W.J. Broughton (Broughton *et al.*, 1978).

Iodination of antigens. Electrophoretically pure CI, CII and the leghaemoglobins were each iodinated with $Na^{125}I$ (Amersham). Five µg of protein, in 20 µl of 0.25 M-phosphate buffer, pH 7.5, was labelled with 1 mCi of $Na^{125}I$ (10 µl) by the addition of 30 µg of chloramine-T in 10 µl of 0.05 M-phosphate buffer, pH 7.5. The reaction was stopped by the addition of 400 µg of Na_2SO_3 in 10 µl of 0.05 M-phosphate buffer and 1 ml of 0.05 M-phosphate buffer, pH 7.5 containing 2 mg of bovine serum albumin. Iodinated protein was separated from unreacted iodine by dialysis for 16

h against 5 l of 0.05 M-phosphate buffer, pH 7.5, 0.85% (w/v) NaCl. The specific radioactivity of the proteins was about 10^7 cpm μg^{-1} of protein.

Radioimmunoassays. Radioimmunoassays(RIAs) against CI, CII and the leghaemoglobins were performed essentially as described by Hunter (1967). Iodinated protein (20 ng) and 1 µl of CI, CII or pea leghaemoglobin antiserum or 0.1 µl of cowpea leghaemoglobin antiserum was used in each assay. The final volume was made up to 1 ml with RIA buffer (0.05 M-phosphate pH 7.5, 0.85% (w/v) NaCl, 0.2% (w/v) bovine serum albumin, 0.01% (w/v) thimerosal, 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate) and the assay mixture was incubated overnight at 4 °C. Complexes of CI, CII or leghaemoglobin with antibodies were separated from the unbound molecules by precipitation with protein A sepharose CL-4B (Pharmacia). The precipitate after centrifugation (1 min, 10,000 g) was washed 3 times with RIA buffer. The precipitate was suspended in RIA buffer and radioactivity was quantified with hydroluma (Lumac, Basel) in a liquid scintillation spectrometer (Packard 2450).

RESULTS

Characteristics of the radioimmunoassays

The inhibition of binding of ^{125}I-CI by non-radioactive CI is shown in Fig. 1. The lowest amount of CI that can be detected with this RIA is 20 ng. The lowest amounts of CII, pea leghaemoglobin and cowpea leghaemoglobin that can be detected with the different RIAs are 20, 20 and 10 ng respectively.

To test the specificity of the RIAs for CI and CII, bacterial proteins prepared from free-living *R. leguminosarum* bacteria were used as a control. No inhibition of binding could be detected with 5 mg bacterial proteins.

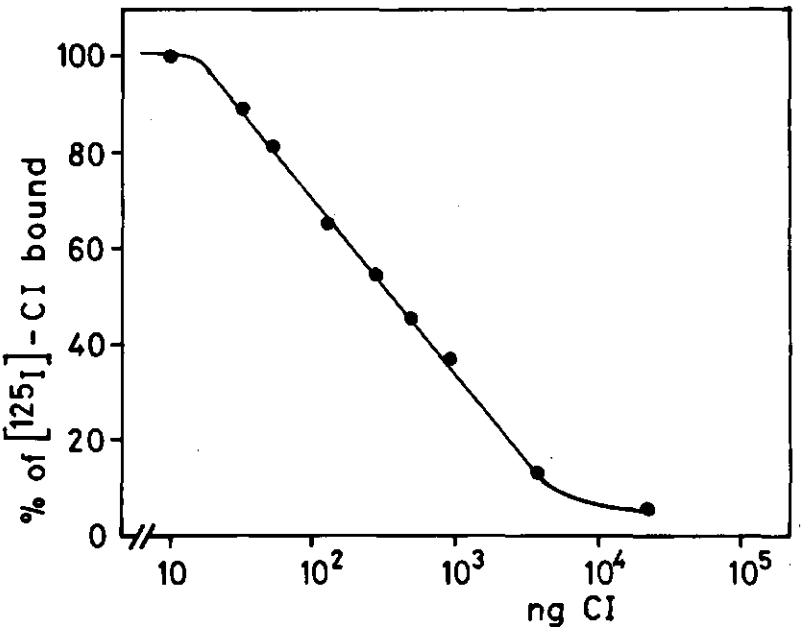

Fig. 1. Inhibition of binding of ^{125}I- CI to anti-CI-serum as a function of the amount of non-radioactive CI.

For both the leghaemoglobin RIAs, root proteins from non-inoculated plants were used as a blank. A maximum of 5% of the binding could be inhibited by root proteins, and the amounts of leghaemoglobin quantified with the RIAs are corrected for this factor.

Appearance of leghaemoglobin and nitrogenase

On pea plants inoculated with *R. leguminosarum*, root nodules are large enough to be picked with forceps about 10 d after sowing. Pea plants are cultured in such a way that root nodules are preferentially formed on the main root. To extend quantitative measurements of the amounts of CI, CII and leghaemoglobin to plants younger than 10 d, bacteroids and soluble plant proteins were isolated from pieces of the main root, where root

nodules normally appear.

Fig. 2 shows the amounts of CI, CII and leghaemoglobin, together with the nitrogenase activity at different times after sowing. At day 10 leghaemoglobin was first detected, while acetylene reduction appeared 2 d later. The appearance of the separate nitrogenase components immediately

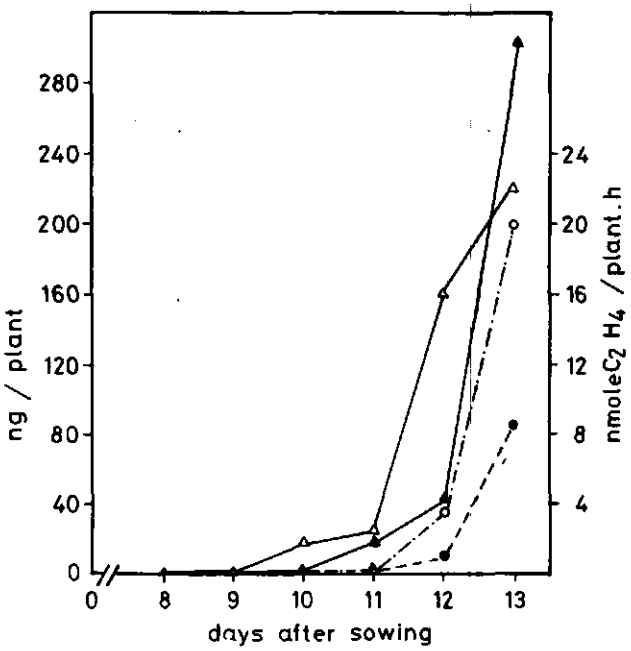

Fig. 2. Acetylene reduction, leghaemoglobin, component I and component II content during development of the root nodule system of *P. sativum*. 75 plants were used for each estimation. Δ——Δ, leghaemoglobin content; ▲——▲, component I content; ●---●, component II content; 0··0, acetylene reduction.

after the onset of nodule formation, indicated that CI was first detectable at day 10, the same day as leghaemoglobin, while CII was first detectable at day 11. Acetylene reduction became measurable at day 12 (Fig. 2).

Table 1. Component I and component II content during development of the root nodule system of *P. sativum*.

days after sowing	CI ng/plant	CII ng/plant	ratio of CI/CII
8	n.d.	n.d.	-
9	n.d.	n.d.	-
10	1.4	n.d.	-
11	20	1.2	17
12	41	10	4.1
13	302	83	3.6

n.d. not detectable.

Molecular weights of CI and CII are about 200,000 D and 65,000 D (Whiting, 1974). This implies that at ratio 3.1, CI and CII are present in equimolar ratios.

At day 11 the ratio of the amounts of CI and CII was 17, while by days 12 and 13 (Fig. 2) this ratio had rapidly decreased to 4.1 and 3.6 respectively (Table 1). At day 10, the amount of CII (per 75 plants) was below the limit of detection (20 ng); taking into account the amount of CI found on this day this implies that the CI/CII ratio is certainly larger than 4. Table 1 suggests that during root nodule formation the ratio of the amounts of CI and CII is not constant. Initially CI can easily be detected but CII

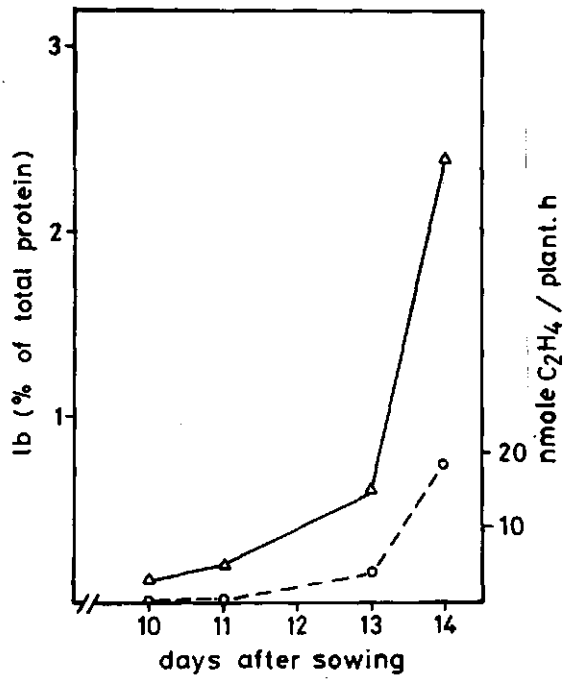

Fig. 3. Acetylene reduction and leghaemoglobin content during development of the root nodule system of *Vigna unguiculata*, 25 plants were used for each estimation. Δ——Δ, leghaemoglobin content; 0---0, acetylene reduction.

not, which implies that CI is synthesized before CII.

This experiment was performed 3 times; in all cases CI appeared at the same time as leghaemoglobin on day 10, while CII always appeared later. In two experiments CII was detectable at day 12 together with the first acetylene reduction, while in the experiment of Table 1 CII was detected at day 11. In the two experiments when CII could not be detected at day 11, the bacteroids from 75 plants contained 140 and 200 ng of CI. This means that if the amount of CII present in these bacteroids was below the detection limit of 20 ng, the ratios of the amounts of CI and CII are at least 7 and 10.

The appearance of nitrogenase activity and leghaemoglobin was also studied in cowpea plants inoculated with *R.* sp. 32H1. Ten days after sowing nodule formation was observed; the root nodules are preferentially formed on the main root. At day 10 leghaemoglobin was detectable by a RIA in these small root nodules. This was followed 1 d later by the first acetylene reduction. Thus in this system nitrogenase activity is also preceeded by leghaemoglobin synthesis (Fig. 3).

DISCUSSION

We have suggested earlier, that in pea root nodules leghaemoglobin synthesis starts before nitrogenase activity is measurable (Bisseling *et al.*, 1979). This conclusion was based on an extrapolation of data, in which leghaemoglobin was first perceptible at the same day as nitrogenase activity. This extrapolation was necessary because of the relative insensitivity of the Mancini immunodiffusion assay as compared to the RIA described here, which is a hundred times more sensitive. The results in this paper are strong evidence that leghaemoglobin is synthesized in root nodules of pea plants before an active nitrogenase complex is discernible. In cowpea root nodules, leghaemoglobin synthesis also preceeded acetylene reduction. These results do not prove, but strongly suggest, that leghaemoglobin is essential for nitrogen fixation in legumes in symbiosis with slow-growing as well as fast-growing rhizobia.

The fact that leghaemoglobin synthesis preceedes nitrogenase activity is in accordance with the results of Godfrey *et al.* (1975) (*R. lupini*, leghaemoglobin was determined immunologically), Robertson *et al.* (1975) (*R. lupini*), Bergersen & Goodchild (1973) (*R. japonicum*, pyridine hemochrome assay) and Verma (1979) (*R. japonicum*, leghaemoglobin-complementary

DNA probe), who all used slow-growing rhizobia. However, Broughton *et al.* (1978) (*R.* sp., Mancini immunodiffusion assay) reported that leghaemoglobin appeared a few days later than nitrogenase activity. The discrepancy between this result and ours may be explained by the relative insensitivity of the Mancini immunodiffusion assay used by Broughton *et al.* (1978).

We could not detect CI or CII of nitrogenase in proteins prepared from broth-cultured *R. leguminosarum* bacteria. This result is contradictory to experiments described by Bishop *et al.* (1975), who showed that a protein cross-reacting with anti-CI-serum is synthesized by broth-cultured bacteria, which do not reduce acetylene. In our opinion these authors did not regourously exclude the possibility that not CI but another protein is precipitated, since more than one precipitation band is observed in their immunodiffusion experiments and also a precipitation band could be obtained with protein preparations of NH_4^+-grown *Azotobacter vinelandii*.

In pea plants inoculated with *R. leguminosarum*, we found that at the beginning of root nodule formation, CI is synthesized preferentially. CII could not be detected in young non nitrogen-fixing root nodules (days 10 and 11), while CI was present and the ratio of the amounts of CI and CII was much higher than in nitrogen-fixing bacteroids (days 12 and 13). This result indicates that CI is synthesized before CII and might have some regulatory function possibly for the complete derepression of the nif-genes. It has also been reported that in *Clostridium pasteurianum* CI is synthesized before CII after derepression of the nif genes (Seto & Mortenson, 1974).

The fact that CI appeared before CII confirms our earlier experiments (Bisseling *et al.*, 1979; Van den Bos *et al.*, 1978) in which protein synthesis of bacteroids was studied during module development.

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by subtracting TCA precipitable ^{35}S from total ^{35}S.

Protein concentrations were determined according to Lowry (7) after precipitation with 5% (w/v) TCA (3).

Polyacrylamide gel electrophoresis was performed according to Laemmli (8) and quantification of ^{35}S incorporated into component I (CI) and component II (CII) of nitrogenase and leghemoglobin was carried out as described previously (3).

Specific antisera against CI and CII of *Rhizobium leguminosarum* (PRE) were prepared by injecting a rabbit intravenously with 0.5 mg electrophoretically pure CI or CII in 0.9% NaCl; 1 month later this was followed by subcutaneous injection of 2 mg CI or CII respectively in 0.9% NaCl, mixed with an equal volume of Freund's complete adjuvant (Difco, Detroit, MI). The rabbits were bled through the jugular ear vein 7 days after the second injection. Under these conditions the titers allowing immunoprecipitation with 1 mg/ml CI or CII are 1/64 and 1/32 respectively.

Radioimmunoassays against CI and CII were performed as described by Hunter (9). Electrophoretically pure CI and CII were iodinated with Na ^{125}I (Radiochemical Centre Amersham, U.K.). 5 µg protein in 20 µl 0.25 M phosphate buffer pH 7.5 was labeled with 1 mCi Na ^{125}I (10 µl) by the addition of 30 µg chloramine T in 10 µl 0.05 M phosphate buffer pH 7.5. The reaction was stopped with 400 µg Na_2SO_3 in 10 µl 0.05 M phosphate buffer pH 7.5 and 1 ml of 0.05 M phosphate pH 7.5 containing 2 mg bovine serum albumin (BSA) was added. Iodinated protein was separated from unreacted iodine by dialysis for 16 h against 5 l of 0.05 M phosphate buffer pH 7.5, 0.85% (w/v) NaCl. The specific activity of the protein was about 10^7 cpm/µg protein. Per assay 20 ng of iodinated protein was used. With the amounts of antisera used in the assays 70% of the maximal binding was achieved. A mixture of iodinated CI or CII antiserum and bacteroid proteins in which CI or CII concentrations had to be determined were incubated at 4°C in 0.05 M phosphate buffer pH 7.5, 0.85% (w/v)

NaCl, 0.2% (w/v) BSA, 0.01% (w/v) thimerosal, 1% (w/v) triton X 100, 0.1% (w/v) SDS and 0.1% (w/v) sodium deoxycholate (RIA buffer). After 16 h the free and bound CI or CII molecules were separated with protein A-sepharose CL-4B (Pharmacia) by centrifugation (1 min, 10,000 g) and the precipitate was washed 3 times with RIA buffer. For quantification of the amounts of CI and CII purified nitrogenase components were used as a standard. CI and CII concentrations were determined after the bacteroids were incubated with lysozyme and lysed with an osmotic shock (4).

RESULTS

Kinetic analysis of ^{35}S incorporation into nodule protein

In experiments in which the kinetics of $^{35}SO_4^=$ incorporation into plant and bacteroid protein were studied, one hundred pea plants (21 days old) were labeled with 6 mCi $^{35}SO_4^=$. Batches of 20 plants were removed ½, 1, 2, 3 and 6 days after addition of $^{35}SO_4^=$ and the incorporation of ^{35}S into soluble plant and bacteroid proteins was examined. Incorporation of ^{35}S into plant and bacteroid proteins was linear for 1 day, but declined thereafter (Fig. 1). It reached a constant value after 3 days. Plant and bacteroid protein showed similar kinetics of incorporation of ^{35}S.

The reason for the decline of the rate of incorporation of ^{35}S into protein during the labeling period could be: and exhaustion of the ^{35}S precursor pool for protein synthesis during the incubation period, a decrease in protein synthesizing capacity, or turnover of proteins. In order to investigate the possible exhaustion of the ^{35}S precursor pool, we determined also the amounts of soluble ^{35}S in plant and bacteroid preparations in the same experiment in which the kinetics of ^{35}S incorporation was studied. Fig. 1 shows that the amount of soluble ^{35}S in bacteroids increased during the first day, after which it remained constant. The amount of soluble ^{35}S in the plant fraction

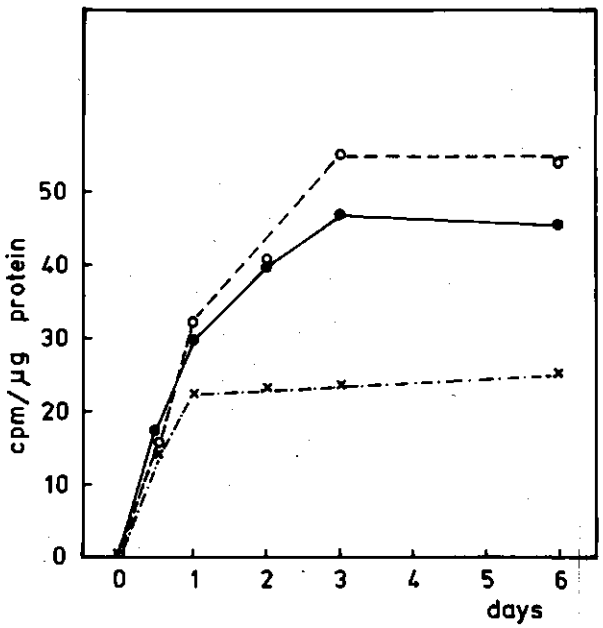

Fig. 1. Incorporation of ^{35}S into plant and bacteroid proteins from pea root nodules, during different incubation periods. o---o ^{35}S cpm/µg plant nodule protein. ●——● ^{35}S cpm/µg bacteroid protein. x-·-x soluble ^{35}S cpm/µg bacteroid protein (soluble means: non trichloroacetic acid precipitable).

(not shown) had a similar course. This result indicates that there is no exhaustion of soluble ^{35}S during the incubation period. This means that exhaustion cannot be the reason for the decline of the rate of ^{35}S incorporation into protein. The distribution of label in ^{35}S-containing compounds was analysed by chromatography on thin layer plates (DC plastikrolle Cellulose, 0.1 mm, Merck); a mixture of butanone, pyridine, water and acetic acid (70: 15:15:2) was used as an eluens. More than 95% of the radioactivity co-migrated with sulfate.

Protein synthesizing capacity, measured as ^{35}S incorporated per µg protein during a 24 h labeling of pea plants of different ages with $^{35}SO_4^=$, decreased

Table 1.

plant age (days)	^{35}S cpm/µg	
	bacteroid protein	plant protein
21	34 (100%)	65 (100%)
22	31 (96%)	59 (90%)
23	26 (76%)	54 (83%)
27	18 (52%)	29 (45%)

Table 1. Relation between plant age and the rate of soluble bacteroid and plant nodule protein synthesis. Protein synthesis was determined with $^{35}SO_4^=$ labeling of groups of 50 pea plants for each day.

Table 2.

days	% ^{35}S in					
	CI		CII		leghemoglobin	
	A	B	A	B	A	B
21-22	9.6%	9.4%	8.9%	9.3%	11.5%	10.7%
21-23	10.0	9.7	8.6	9.2	10.4	10.7
21-24	11.7	11.9	10.9	10.4	11.9	9.5
21-27	12.9	9.6	11.2	10.3	13.8	11.1

Table 2. Amounts of ^{35}S incorporated into CI, CII and leghemoglobin, expressed as percentages of total ^{35}S incorporated into bacteroid and plant nodule proteins respectively. Bacteroid and plant proteins were isolated from nodules of 6 pea plants. The percentages are average values of 2 experiments. A: $^{35}SO_4^=$ labeling during different periods. B: different chase periods. The pea plants were labeled 24 h and the chase was started at day 22.

by about 50% from 21 to 27 d (Table 1). However, this decrease in protein synthesis cannot explain why the rate of ^{35}S incorporation becomes zero between days 3 and 6 (Fig. 1). Therefore this decline must be due, at least in part, to turnover of nodule proteins. The definition of turnover we have used is: the flux of amino acids through proteins (2).

An estimation of the turnover rates of bacteroid and plant proteins can be made in the following way. The amount of ^{35}S incorporated into protein (P) depends on the rate of protein synthesis (V_s) and degradation (V_d), so $\frac{dP}{dt} = V_s - V_d$. From day 3 to day 6 (Fig. 1) the amount of ^{35}S incorporated per µg protein is constant; $\frac{dp}{dt} = 0$, therefore $V_s = V_d$. Since the ^{35}S precursor pool is constant during this period V_s is theoretically zero order ($V_s = K_s$) and will depend only on the age of the pea plants (Table 1). V_d is theoretically of first order ($V_d = K_d P$) (2). Protein synthesis declines when pea plants grow older, and decreases by about 50% from day 21 to 27 (Table 1). From day 3 to 6, the pea plants are 24 to 27 days old. From day 3 to 6 protein synthesis will be lower than during the first day of the labeling. Assuming that protein synthesis decreases linearly in time from 21 to 27 days, the results of Table 1 indicate that protein synthesis during day 3-6 will be about 70-50% of the synthesis at the first day of the labeling. Assuming that during the first day (21 d) protein degradation can be neglected, V_s in the bacteroids during the first day of labeling will be 30 cpm/µg protein·day. For the calculation of $t_{\frac{1}{2}}$ of bacteroid proteins we take the average value of the protein synthesis from day 3 to 6. This will be 60% of the synthesis at the first day. Therefore from day 3 to 6 the average $V_s = 6/10 \times 30 = 18$ cpm/µg protein·day. From day 3 to 6, P = 45 cpm/µg. Since $V_s = V_d$, $V_s = K_d P$, $K_d = 18/45$ $K_d = 2/5$. Since in first order reactions $t_{\frac{1}{2}}$ of the reaction is given by $t_{\frac{1}{2}} = 0.693/K_d$, $t_{\frac{1}{2}} = 1.7$ days. A similar calculation for plant proteins results in $t_{\frac{1}{2}} = 1.9$ days. In other experiments $t_{\frac{1}{2}}$ of plant and bacteroid proteins varied between 1.5 and 2.5 days.

Pulse-chase kinetics of plant and bacteroid proteins

In the pulse-chase experiments plant and bacteroid proteins were labeled with $^{35}SO_4^{2-}$, after which the decrease in specific radioactivity of the proteins was followed during a chase with an excess of unlabeled sulfate.

One hundred plants, 21 days old, were labeled with 6 mCi $^{35}SO_4^{=}$. Plants of that age were chosen, because from 21 days after sowing and inoculation onward, nodule weight remains constant (4), which probably means that the amount of nodule protein per plant will be constant during the pulse-chase experiment. Loss of radioactivity during the chase should follow first-order kinetics. After 24 hours the incubation medium with $^{35}SO_4^{=}$ was removed and replaced by a medium with 2 mM unlabeled sulfate. This is 10^6 times the concentration of sulfate present in the labeling medium. Nodules from 20 plants were harvested 0, 2, 3, 4 and 6 days after the beginning of the chase.

Fig. 2 shows the specific radioactivity of plant and bacteroid protein and the amount of soluble ^{35}S in bacteroids after different chase period. The amount of ^{35}S incorporated per µg protein continued to increase for two days after the removal of $^{35}SO_4^{=}$; from then on the specific radioactivity of bacteroid and plant proteins decreased continually at the same rate. The amount of soluble ^{35}S in the plant protein fraction followed a similar pattern as the soluble ^{35}S in the bacteroid tissue, but it was about 6 times higher (not shown).

If a $t_{\frac{1}{2}}$ value could be determined from these results it would only be possible 2 days after the beginning of the chase. From day 2 to 6 the radioactivity incorporated into plant and bacteroid proteins decreases from 77 to 45 cpm/µg and from 73 to 43 cpm/µg respectively. So about 40% of the radioactivity incorporated into plant and bacteroid protein is lost from day 2 to 6. If the decrease of radioactivity would have continued in a similar way, at day 7 about 50% of the radioactivity would have been lost. This indicates that the $t_{\frac{1}{2}}$ value of soluble plant and bacteroid proteins would be about 5

In order to estimate the turnover rates of CI, CII and leghemoglobin, both the amounts of ^{35}S incorporated into these proteins and the absolute amounts of these proteins have to be determined during the period of the pulse-chase and the "kinetics of ^{35}S incorporation" experiment. These absolute amounts were determined by radioimmunoassays developed for CI, CII and leghemoglobin. The absolute amounts of all three proteins increased from day 22 to 28 by about 25% (Table 3). Both the amount of radioactivity incorporated into leghemoglobin, CI and CII as well as the absolute amount of leghemoglobin increase by about 25% during the "kinetics of ^{35}S incorporation" experiment. Therefore, in this experiment the amount of ^{35}S/µg CI, CII and leghemoglobin is constant during the experiment, so $t_{\frac{1}{2}}$ of CI, CII and leghemoglobin are virtually identical to $t_{\frac{1}{2}}$ of bacteroid and plant proteins. If the $t_{\frac{1}{2}}$ of these proteins would be lower or higher, the amount of ^{35}S per µg CI, CII, or leghemoglobin would have increased or decreased respectively. As these are similar, this means that $t_{\frac{1}{2}}$ of CI, CII and leghemoglobin are about 2 days.

The results of the pulse-chase experiment will be discussed later.

Chloramphenicol treatment

In this last approach to determine the turnover rate of nitrogenase, we tried to inhibit bacteroid protein synthesis with 0.3 mM chloramphenicol.

Protein synthesis in bacteroids was measured by $^{35}SO_4^=$ labeling of 6 groups of 12 pea plants, 3 of which were supplied with chloramphenicol, and the amount of ^{35}S incorporated into soluble plant and bacteroid protein was determined. Bacteroid protein synthesis was inhibited by 50, 44 and 58% when 0.3 mM chloramphenicol was applied to the pea plants for 1, 2 and 3 days respectively, while plant protein synthesis in root nodules was unaffected. The inhibition of synthesis of CI and CII was determined after polyacrylamide gel

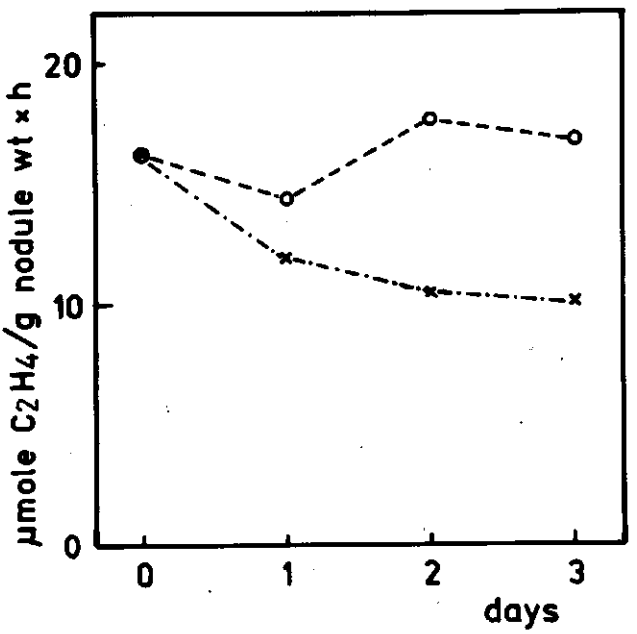

Fig. 3. Nitrogenase activity of toluene/EDTA treated bacteroid isolated from control pea plants cultured for different periods on 0.3 mM chloramphenicol. o——o control. x——x 0.3 mM chloramphenicol.

electrophoresis of bacteroid proteins. The protein bands comigrating with purified CI or CII were cut from the gel and the amount of ^{35}S incorporated into CI and CII was determined. CI and CII synthesis were inhibited by 55% and 50% respectively.

On day 23 chloramphenicol was supplied to the pea plants. On day 24, 25 and 26 nodules were harvested from 12 plants, bacteroids were isolated and nitrogenase activity of bacteroids was measured. Therefore bacteroids were treated with toluene/EDTA, since with this method the amount of active nitrogenase is determined. Possible side effects of the chloramphenicol treatment on *in vivo* nitrogenase activity, e.g. by a reduced photosynthesis, will not influence nitrogenase activity of toluene/EDTA treated bacteroids. Fig. 3 shows that the nitrogenase activity of bacteroids isolated

period to determine protein synthesis also some turnover of protein will take place. This implies that V_s is somewhat greater than the value used in the determination of turnover rates, so the value of $t_{\frac{1}{2}}$ for nitrogenase and leghemoglobin is probably somewhat smaller than 2 days.

When the incorporation of ^{35}S into the nitrogenase components and leghemoglobin was studied, it was shown that the percentages of radioactivity incorporated into the nitrogenase components increased by 25% from day 22 to 28 (Table 2). Since also the amounts of CI, CII and leghemoglobin protein per µg soluble bacteroid and plant protein increased by 25%, we concluded that per µg CI, CII and leghemoglobin the amount of ^{35}S incorporated is constant during the "kinetics of ^{35}S incorporation" experiment. In a good pulse-chase experiment an increase of 25% of the amount of CI, CII and leghemoglobin protein should have resulted in a decrease of 25% of the percentage of radioactivity in each protein during the chase. However, in our pulse-chase experiment we observed that the amounts of ^{35}S incorporated into component I and II and leghemoglobin remained constant during the pulse-chase. We think that this result can be explained by the fact that our pulse-chase experiments are a combination of a pulse-chase and a "kinetic of ^{35}S incorporation" experiment, and it confirms our statement that pulse-chase experiments are not suitable for determination of turnover rates in pea root nodules.

In the experiments in which bacterial protein synthesis was inhibited by chloramphenicol, we have tried to use a rather low concentration of chloramphenicol to minimize unwanted side effects. We have used 0.3 mM chloramphenicol which gave 50% inhibition of bacteroid protein synthesis. This degree of inhibition is similar to the results of Coventry and Dilworth (11), who used 1 mM chloramphenicol for inhibition of protein synthesis of bacteroids of *Rhizobium lupini*.

After chloramphenicol addition acetylene reduction of intact root nodules would not be a good measure for the amount of nitrogenase, because of possible side effects of chloramphenicol; for example protein synthesis in chloroplasts

will also be inhibited by chloramphenicol and photosynthesis might decrease. This implies that the energy supply to the bacteroids will be less, and this will influence nitrogenase activity, while the amount of nitrogenase can be unaffected. Also if any other protein necessary for nitrogenase activity would have a high turnover rate, this turnover rate will determine the turnover of the nitrogenase activity of whole nodules. To avoid these problems, we have measured acetylene reduction on EDTA/toluene treated bacteroids, in the presence of dithionite and ATP.

Root nodules of pea plants contain bacteroids of different ages. E.M. observations on pea root nodules have shown that young bacteroids are localized in the apical part of the nodule while the older ones are present in the basal part (12,13). In this pea rhizobium system acetylene reduction can first be detected 9 days after seeding, while the beginning of degradation of bacteroids and plant nodule cells can be observed 23 days after seeding (12). This indicates that nitrogen fixing bacteroids might have a life-time of about 11 days.

So the average life-time of a bacteroid in a pea root nodule is probably about 11 days and the $t_{\frac{1}{2}}$ of bacteroid proteins is about 2 days. This implies that a bacteroid has to resynthesize its proteins several times during its life.

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CHAPTER VII

PROTEIN SYNTHESIS IN DETACHED PEA ROOT NODULES AND ISOLATED BACTEROIDS

INTRODUCTION

In this thesis the regulation of nitrogenase and leghemoglobin (Lb) synthesis in pea root nodules were investigated. Some difficulties in using the intact plant were encountered. Radioactive amino acids could not be used to follow protein synthesis in root nodules, since the specific radioactivity of the proteins nver reached sufficiently high levels. Only $^{35}SO_4^=$ resulted in sufficient incorporation of radioactivity into proteins. But $^{35}SO_4^=$ was incorporated into proteins after a 6 h lag period (Chapter II) and only after 24 hours sufficient label was incorporated to allow analysis of newly synthesized proteins with polyacrylamide gel electrophoresis and autoradiography. The exposure time of the autoradiographs was about three weeks (Chapter II). Another problem with the use of intact pea plants was the interpretation of the effects of environmental conditions on protein synthesis. For instance waterlogging of the root system repressed synthesis of component II. It is suggested that a decrease of the amount of O_2 was responsible for this repression (6). Since it is difficult to test this idea with intact pea plants, less complex systems derived from the root nodules of pea plants were sought.

The simplest system to study nitrogenase synthesis would have been nitrogen fixing *Rhizobium* bacteria. Since nitrogen fixation *ex planta* has really only been reported for slow-growing rhizobia, however, this was not a feasible method with *Rhizobium leguminosarum*.

Instead we compared the proteins that were synthesized in pea root nodules using: 1. intact pea plants, 2. nodules attached to pieces of the main root, 3. detached root nodules and 4. isolated nitrogen fixing bacteroids.

MATERIALS AND METHODS

Pea plants were grown and inoculated as described before (Chapter II). In all experiments 21 days old pea plants were used, that were cultured from day 18 on on a sulfur deficient medium.

Pea plants. Pea plants were labeled in two ways: for 24 h in plastic trays as described before. Four pea plants were incubated with 1 mCi $^{35}SO_4^=$ in 200 ml of sulfur deficient medium (Ch. II); plants were also labeled with $^{35}SO_4^=$ (1 mCi/4 plants) for 24 h in a glass tube (1½ x 10 cm) after removal of the lateral roots and the lower part of the main root, in 5 ml of a sulfur deficient medium (Ch. II) supplied with 0.1 M sucrose and 50 mM succinate. The nodules were kept above the medium.

Pieces of main root. Pieces of main root carrying nodules were labeled in a similar way as for the intact pea plants in a glass tube. Three ml of sulfur deficient medium was used per four pieces.

Detached root nodules. Detached root nodules were incubated as described by Houwaard (1). Nodules from four plants were submerged in 1 ml of 50 mM phosphate pH 7.0, 2.5 mM $MgCl_2$, 0.1 M sucrose, 50 mM succinate and were labeled with 100 µCi ^{35}S-methionine (1035 Ci/mmole) for 6 h.

Bacteroids. Bacteroids were isolated according to Laane *et al.* (2), washed anaerobically in 25 mM TES/KOH (pH 7.4), 2 mM $MgSO_4$, 0.3 M sucrose (TMS) containing 2.5% (w/v) fatty-acid-free BSA, suspended in TMS and kept on ice. For labeling under low pO_2, the apparatus described by Laane *et al.* (2) was used. The temperature was kept at 30°C and pO_2 at 0.25-1.0 µM; the same buffer and amount of bacteroid protein was taken as in Laane *et al.* (2). Myoglobin (130

μM) was used as an oxygen buffer. Bacteroids were labeled with 20 μCi ^{35}S-methionine for 1 h.

After labeling, bacteroids and plant proteins were isolated as described previously (Ch. IV). Bacteroids were purified by sucrose gradient centrifugation (3) and lysed with 1% SDS.

Acetylene reduction of pieces of main root was determined as described previously. Acetylene reduction of detached root nodules and bacteroids was measured as described by Houwaard (1) and Laane (2) respectively.

Polyacrylamide gel electrophoresis and autoradiography were performed as described previously (Ch. II).

RESULTS

Autoradiographs of bacteroid proteins from pea plants with the complete root system and after removal of the lateral roots are shown in Fig. 1a and b respectively. The exposure times of the autoradiographs for Fig. 1a and b were 4 weeks and 3 days respectively. The nitrogenase bands were identified with purified nitrogenase components. In both autoradiographs, the bands of CI and CII were the most intense. This indicates that removal of the lateral roots introduced no great changes in the relative rates of synthesis of CI and CII. The two bacteroid protein preparations of Fig. 1a and b were separated in different runs. Therefore it is not possible to compare the synthesis of other bacteroid proteins under the two conditions at which pea plants were labeled. The plant proteins isolated from root nodules of the labeled pea plants were also analysed. Fig. 2a and b show plant proteins from intact pea plants (3 weeks exposure) and after removal of the lateral roots (3 days exposure) respectively. The Lb bands were identified with purified Lb. Fig. 2a shows that the two Lb bands belong to the most intensely labeled proteins, and this is still the case after removal of the lateral roots (Fig. 2b).

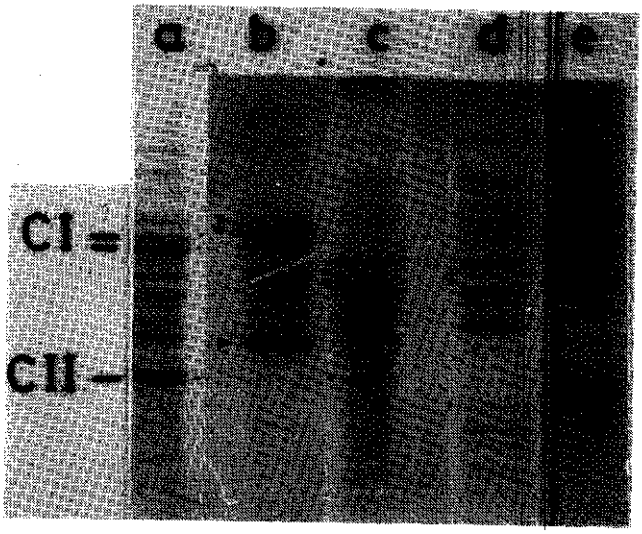

Fig. 1. Autoradiograph of soluble bacteroid proteins analysed on 10% polyacrylamide gels. Bacteroids were isolated from: a. intact pea plants ($^{35}SO_4^{=}$), b. pea plants without lateral roots ($^{35}SO_4^{=}$), c. pieces of main root with root nodules ($^{35}SO_4^{=}$), d. detached nodules (^{35}S-methionine), e. isolated bacteroids (^{35}S-methionine).

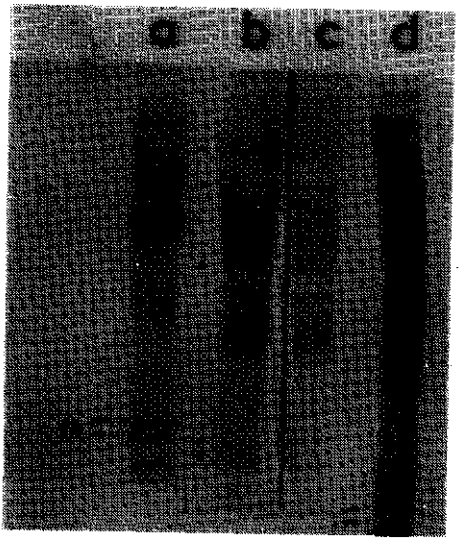

Fig. 2. Autoradiograph of plant proteins analysed on 13% polyacrylamide gels. Plant nodule proteins were prepared from: a. intact pea plants ($^{35}SO_4^{=}$), b. pea plants without lateral roots ($^{35}SO_4^{=}$), c. pieces of main root with nodules ($^{35}SO_4^{=}$), d. detached root nodules (^{35}S-methionine).

Pieces of main root carrying nodules were labeled with $^{35}SO_4^=$ for 24 hours. At the start of this incubation acetylene reduction was equal to the activity of the intact plants, but decreased by 50% during the 24 h of the incubation. Comparison of bacteroid as well as plant nodule proteins isolated from pieces of main root and pea plants, separated by polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue, demonstrated that no significant changes in protein composition had taken place during the incubation (result not shown). Also after incubation of detached root nodules or isolated bacteroids no changes in the stained protein patterns were observed.

Fig. 2c shows that in pieces of main root much less $^{35}SO_4^=$ is incorporated into the Lb bands than in pea plants (Fig. 2b). Equal amounts of radioactivity were loaded on both gels (Fig. 2b and c). This result indicates that the relative Lb synthesis is strongly decreased in nodules attached to pieces of main root. The plant proteins from pea plants without lateral roots (Fig. 2b) and from pieces of main root were separated on the same slabgel, which allowed a good comparison of the proteins synthesized. Besides Lb, the relative synthesis of a number of other plant nodule proteins, marked with dots in Fig. 2b, was decreased in pieces of main root. Bacteroid protein synthesis in pieces of main root and pea plants can be compared in Fig. 1c and b (both 3 days exposure) respectively. The autoradiographs (Fig. 2b and c) demonstrate that the relative synthesis of component I (CI) and component II (CII) are decreased in pieces of main root. Equal amounts of radioactivity were loaded on the gels, but the intensities of the CII and the two CI bands were decreased.

Protein synthesis in detached root nodules was followed by labeling with ^{35}S-methionine, since $^{35}SO_4^=$ was poorly incorporated into protein. Nitrogenase activity (2 µmoles C_2H_4/g fresh nodule wt. h) of detached root nodules was linear during the first 6 hours, and in this period the nodules were labeled. The proteins synthesized in the bacteroid and plant fraction in

detached nodules are shown in Figs. 1d and 2d respectively. A comparison with protein synthesis in nodules on pea plants (Figs. 1b and 2b) and pieces of main root (Figs. 1c and 2c) shows, that the relative synthesis of the nitrogenase components and Lb is decreased in detached root nodules just as is the case in pieces of main root.

Isolated bacteroids were active in nitrogen fixation (20 nmoles/min. mg bacteroid protein) for 1 h. During this period the bacteroids were labeled with ^{35}S-methionine. Protein synthesis in isolated bacteroids is shown in Fig. 1e (exposure 3 days). From the isolated bacteroids an equal amount of radioactivity was loaded on the gel as in the case of bacteroid proteins labeled in pea plants (Fig. 1b). Since the intensities of the CI and CII bands are much lower in the "isolated bacteroids" (Fig. 1e), than in "pea plants" (Fig. 1b), it can be concluded that the relative synthesis of CI and CII is decreased in isolated bacteroids.

DISCUSSION

In order to enable the study of the regulation of nitrogenase and Lb synthesis at the cellular level, systems more simple than the intact pea plants were examined, if it would be possible to increase the efficiency of protein labeling with radioactive precursors and to enable a more direct interference with the nitrogenase and/or Lb synthesis. Systems with a gradually decreasing complexity were used: from pea plants without lateral roots to isolated bacteroids. Only in pea plants without lateral roots the relative nitrogenase and Lb synthesis were similar to the synthesis in whole pea plants. In pieces of main root the relative synthesis of nitrogenase and Lb was already considerably decreased. This result implies that the regulation of nitrogenase and Lb synthesis can only be studied as yet in whole pea plants or in pea plants without lateral roots. Plants without lateral roots have the advantage

that the specific radioactivity of bacteroid and plant proteins can be 5-10 times higher than with whole pea plants.

Pea plants and pieces of main root were labeled with $^{35}SO_4^=$, which is incorporated into sulfur containing amino acids. Detached root nodules and bacteroids were labeled with ^{35}S-methionine. The use of these two different precursors could have introduced differences in the labeling patterns. The nitrogenase components of *Rhizobium* (4) contain "normal" amounts of methionine and cysteine and FeS clusters of nitrogenase will probably have been lost by polyacrylamide gel electrophoresis in the presence of SDS. Therefore it is unlikely that the relative amounts of ^{35}S incorporated into the nitrogenase proteins will be influenced by the use of ^{35}S-methionine instead of $^{35}SO_4^=$. In nodules on pieces of main root, labeled with $^{35}SO_4^=$, a strong decrease of the relative Lb synthesis was observed. Therefore it appears unlikely that a low amount or absence of methionine in Lb proteins (5) is responsible for the decrease of the relative synthesis of Lb in detached root nodules. This low amount of methionine will probably only corroborate the relative decrease of incorporation of ^{35}S into Lb in detached root nodules.

Nitrogenase activity in isolated bacteroids equals that of whole pea plants. The fact that no N-ase synthesis is observed suggests that the conditions necessary for nitrogenase activity (low pO_2, and energy in the form of ATP and reduction equivalents) are not sufficient for nitrogenase synthesis.

In all the systems where nitrogenase and/or Lb synthesis was decreased, acetylene reduction declined during (pieces of main root) or directly after the incubation (detached root nodules, isolated bacteroids). This suggests that nitrogenase and Lb synthesis are more sensitive to the factors that diminish acetylene reduction, than the nitrogenase activity itself.

The fate of bacteroid RNA during nodule development

During nodule development bacteroid protein synthesis decreases with time. This was shown by $^{35}SO_4^=$ labelling of pea plants of different ages (Ch. IV). Van den Bos, Bisseling and Van Kammen (7) showed that bacteroids isolated from pea plants of 21 days old could be separated according to age by sucrose gradient centrifugation. Their results demonstrated that older bacteroids have a lower protein synthesizing capacity than the younger bacteroids, and maximal protein synthesis of the bacteroids precedes maximal nitrogen fixation.

Since protein synthesis decreased during nodule development the fate of bacteroid RNA was analysed. The RNA content of bacteroids decreased during nodule development. The rRNA (especially the 23S RNA) was partially degraded in young as well as in old bacteroids, although to a greater extent in the latter. Degraded 23S could be isolated from the large ribosomal subunit.

The regulation of nitrogenase and Lb synthesis

The regulation of nitrogenase and Lb synthesis in pea root nodules was studied by $^{35}SO_4^=$ labelling of whole pea plants. In other more simple systems relative nitrogenase and Lb synthesis was strongly decreased (Ch. VII), making them unusable for such a study.

a. The effect of NH_4^+ on nitrogenase and Lb synthesis

NH_4^+ had no effect on either the relative synthesis of nitrogenase or the amount of nitrogenase. However, the amount of apo-Lb and heme decreased with the addition of NH_4^+. In spite of the fact that the amount of apo-Lb decreases with the addition of NH_4^+ the relative synthesis of apo-Lb is not affected. Therefore the measured decline in the amount of apo-Lb has to be caused by either a modification of apo-Lb resulting in a decreased recognition by anti-Lb-serum or an induced degradation of apo-Lb (Ch. II).

The fact that nitrogenase synthesis is not affected by NH_4^+ seems contrary to the finding that regulation of nitrogenase synthesis in free-living nitrogen fixing organisms like *Klebsiella* and *Azotobacter* is repressed by low amounts of NH_4^+.

In vitro there is no uptake of NH_4^+ by the bacteroids (7). If this is also the case *in vivo* it explains why NH_4^+ does not inhibit nitrogenase synthesis in *Rhizobium* bacteroids *in vivo*.

The mechanism of the decrease of nitrogenase activity of root nodules with the addition of NH_4^+ is not yet clear. The decline in the amount of Lb is probably not the only reason for the decrease of nitrogenase activity, but will certainly contribute to its decrease in the presence of NH_4^+.

b. The effect of waterlogging on nitrogenase and Lb synthesis

Waterlogging causes the synthesis of CII of nitrogenase to be repressed, while the synthesis of CI and Lb are not affected (Ch. III). The fact that the synthesis of CII is repressed while the synthesis of CI seems unaffected indicates that the synthesis of CI and CII can be regulated rather independently in *Rhizobium*.

c. The sequence of appearance of the nitrogenase components and Lb during nodule formation

The sequence of appearance of the nitrogenase components and Lb during nodule formation was followed with radioimmunoassays for these proteins (Ch. V). It was shown that Lb was present before nitrogenase activity, suggesting that Lb is necessary for the expression of nitrogenase activity in root nodules of legumes. CI of nitrogenase could be detected on the same day as Lb while CII was first detected 1 or 2 days later. These results indicate that the synthesis of Lb does not depend on the presence of nitrogenase and the synthesis of CI and CII can be regulated rather independently as is the case

menten bleek dat tijdens de knolvorming eerst leghemoglobine en component I gemaakt werden en dat 1-2 dagen later component II voor het eerst aantoonbaar was. De stikstofbinding was tegelijk met de aanwezigheid van component II aantoonbaar. Het feit dat leghemoglobine aantoonbaar is voordat de stikstofbinding begint, is een aanwijzing dat de werking van het enzym nitrogenase afhankelijk is van de aanwezigheid van leghemoglobine. Verder blijkt uit deze proeven dat net zoals bij de 'waterlogging' experimenten de synthese van component I en II weer niet strikt gekorreleerd is.

Wanneer men de regulatie van de synthese van bepaalde eiwitten wil bestuderen, dan wil men uiteindelijk weten hoe de regulatie op het DNA niveau in zijn werk gaat. M.a.w. welke moleculen en welke mechanismen spelen een rol bij het tot expressie komen van de betreffende genen. De experimenten die in dit proefschrift beschreven staan hebben niet tot doel gehad om antwoorden van dergelijke aard op te leveren. De doelstelling van de proeven die in het proefschrift beschreven staan was aanwijzingen te verkrijgen over welke factoren mogelijk een rol spelen bij de regulatie van de nitrogenase en leghemoglobine synthese. Zo blijkt bv. uit de 'waterlogging' experimenten dat de zuurstofconcentratie mogelijk een rol speelt bij de expressie van het component II gen. Hoe de regulatie van de synthese op moleculair niveau in zijn werk gaat en welke rol de zuurstofconcentratie hierin speelt is niet te concluderen uit deze experimenten.

De aanwijzingen over de regulatiemechanismen van de nitrogenase en leghemoglobine synthese die uit de experimenten die in dit proefschrift beschreven staan naar voren komen, kunnen wel een basis vormen voor proeven die gedaan zullen moeten worden om het moleculaire mechanisme van de nitrogenase en leghemoglobine synthese op te helderen.

CURRICULUM VITAE

Ton Bisseling werd op 20 april 1952 te Nijmegen geboren. In 1969 heb ik het eindexamen HBS-B behaald en in hetzelfde jaar ben ik in Nijmegen biologie gaan studeren. In 1972 haalde ik het kandidaatsexamen B_4 (cum laude) en in 1975 het doctoraalexamen (cum laude) met als hoofdvak genetica en als bijvakken exobiologie en biochemie.

Na het behalen van het doctoraalexamen ben ik 4 maanden werkzaam geweest als wetenschappelijk medewerker bij de afdeling genetica van de Katholieke Universiteit Nijmegen. Vanaf oktober 1975 ben ik verbonden geweest aan de vakgroep moleculaire biologie van de Landbouwhogeschool te Wageningen, in dienst van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO), onder auspiciën van de Stichting Biologisch Onderzoek in Nederland (BION). In 1980 zal op het Max-Planck-Institut für Züchtungsforschung, Keulen, het Institut für Mikrobiologie und Biochemie der Universität Erlangen-Nürnberg en de vakgroep moleculaire biologie, Landbouwhogeschool Wageningen, onderzoek worden verricht. Hiertoe ben ik in staat gesteld door een stipendium van de Niels Stensen Stichting.