EFFECT OF COMBINED NITROGEN ON SYMBIOTIC NITROGEN FIXATION IN PEA PLANTS

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des namiddags te vier uur in de aula
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STELLINGEN

1. Het teruglopen van de symbiontische stikstofbinding in leguminosen na opname van stikstof uit de grond berust niet op een direkte invloed van de opgenomen stikstofverbindingen op de microsymbiont.

   Dit proefschrift.

2. De veelvuldig ter ondersteuning van de "fotosynthaat-onttrekkings-theorie" aangehaalde experimenten van Small en Leonard zijn in feite middellange termijnproeven en tonen niet aan dat het primaire effekt van gebonden stikstof een vermindering van het koolhydraat-transport naar de wortelknollen is.

   Small, J.G.C. en Leonard, O.A. (1969)
   Amer. J. Bot. 56: 187-194.

3. Het door Ryle en medewerkers veronderstelde verband tussen de kooldioxydeproduktie door het wortelstelsel en de efficiëntie van de stikstofbinding is onjuist, aangezien andere energieverbruikende processen, verspillende ademhaling en carboxylatiereakties verwaarloosd worden.

   Ryle, G.J.A., Powell, C.E. en Gordon, A.J. (1979)
   J. Exp. Bot. 30: 135-153.

4. Het feit dat de aanwezigheid van een opname-hydrogenase in een stikstofbindende symbiose gecorreleerd is met een hogere opbrengst van de plant bewijst nog niet dat waterstofopname van belang is voor de stikstofvoorziening van de plant.

   Schubert, K.R., Jennings, N.T. en Evans, H.J. (1978)
   Plant Physiol. 61: 398-401.

5. Het wordt onvoldoende onderkend dat ingrepen, die bedoeld zijn om de produktie of het verbruik van fotosyntheseprodukten te veranderen, de symbiontische stikstofbinding mede kunnen beïnvloeden via de hormonale toestand van de plant.

   Bethlenfalvay, G.J., Abu-Shakra, S.S., Fishbeck, K. en Phillips, D.A. (1978) Physiol. Plant. 43: 31-34.

6. De nadruk die gelegd wordt op de maatschappelijke relevantie van wetenschappelijk onderzoek leidt vaak tot het wekken van onjuiste verwachtingen ten aanzien van de toepassingsmogelijkheden van fundamenteel onderzoek.

7. De hoop dat windenergie kan bijdragen tot grootschalige energielevering is vervlogen; alleen bij kleinschalige energievoorziening kan de wind nog een partijtje meeblazen.

8. Zowel aan de meestal geringe als aan de soms overdreven zorg die aan fietspaden wordt besteed is te zien dat het fietsverkeer hoofdzakelijk vanuit de auto wordt bestuurd.

F. Houwaard
Effect of combined nitrogen on symbiotic
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Wageningen, 12 oktober 1979.

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1. GENERAL INTRODUCTION

Nitrogen fixation and nitrogenase

Biological nitrogen fixation, which is realized by procaryotic microorganisms - free-living or in symbiosis with higher plants - contributes a good deal to the maintenance of life. It plays a significant role in the nitrogen cycle and thus in the nitrogen balance - recent estimates amount to a global total of 175×10^9 kg of nitrogen fixed per year (13). This function, that is, to ensure a continuous input of combined nitrogen into the biosphere, accounts for its great importance to the food supply. More than 50% of the biological nitrogen fixation occurs in agricultural land: 45×10^9 kg of N in grasslands, 35×10^9 kg of N in grain legumes and 10×10^9 kg of N in other arable land, e.g. rice fields (13). Therefore, it is not surprising that mankind has been greatly interested in the process and has tried to divulge its secrets even since its discovery, which can be traced back to 1838 when Boussingault demonstrated the capture of nitrogen from the air by leguminous plants. Understanding of the mechanism and the regulation of the nitrogen-fixing process (introducing the possibility of intervention) could help to improve the nitrogen nutrition of plants, animals and consequently of man.

In view of the above remarks, nitrogen fixation by symbiotic associations between microorganisms and higher plants is by far the most important, being responsible for the majority of biological nitrogen fixation. On the other hand, most research on regulatory mechanisms of the nitrogen-fixing system as a whole and the responsible enzyme complex - nitrogenase - itself has been performed with free-living nitrogen fixers. Involvement of a symbiotic association induces more complications: two different organisms participate in the process and affect each other, making the process more intricate. Tracing the similarities and differences between the two nitrogen-fixing systems could lead to a better comprehension of the process anyways.

Physical and (bio)chemical investigations on the properties of the (more or less purified) nitrogenase complex have revealed important similarities

between the enzymes of free-living and symbiotic microorganisms. It is generally assumed that the structures of both enzyme complexes and their catalytic mechanisms are basically identical (18, 49, 81). As in free-living microorganisms the nitrogenase complex of bacteroids consists of two subunits, an Fe and an Fe-Mo protein (34, 88); nitrogenase activity in cell-free extracts of bacteroids requires anaerobic conditions and the presence of Mg-ions, ATP and reductants (4, 39); kinetics of the bacteroid enzyme are comparable to those of the enzyme of free-living nitrogen fixers (5, 36). Therefore, differences in apparent characteristics have to be sought in ancillary metabolic processes or in the regulation of enzyme synthesis via repression or derepression of its genes. Influences of the host plant in symbiotic systems can be expected to become manifest particularly in these processes: the plant has the potency to regulate the environmental conditions of the microsymbiont, thus greatly determining the actual nitrogenase activity. These crucial conditions, determining the establishment and the functioning of the nitrogen-fixing system in both free-living and symbiotic microorganisms, include the oxygen concentration (nitrogenase is sensitive to free oxygen), the supply of carbohydrates (to deliver ATP, reductants and carbon skeletons) and the presence of combined nitrogen (the product of nitrogenase and thus a potential regulatory factor). As a matter of fact these environmental conditions are interrelated via various mechanisms (transport processes, respiration, nitrogen assimilation).

Comparison of symbiotic and free-living nitrogen fixers

When the characteristics of nitrogenase of a free-living bacterium - viz. an *Azotobacter* sp. - and that of a bacteroid - viz. a *Rhizobium* sp. - are compared, striking differences can be observed (50). These differences can be assumed to arise from one main distinction: bacteroids are non-growing organisms which nevertheless possess the ability to fix nitrogen. As a consequence, nitrogen fixation can be more efficient in terms of substrate used per unit nitrogen fixed - no energy is needed for growth processes of the microorganisms. However, there is also the expenditure of energy by the symbiosis as a whole; when this factor is included the efficiency might well be similar to that of free-living nitrogen fixers (60). Again, this emphasizes the dependency of the bacteriods on the substrate supply by the plant. One of the intentions of the experiments described in the following sections was the investigation of the efficiency of the nitrogen fixation,

expressed in terms of the utilization of carbohydrates by the bacteroids and the nitrogen-fixing root nodules.

The plant supplies the nitrogen-fixing microsymbiont with carbohydrates (derived from photosynthesis), and at the same time it has to transport the fixed nitrogen to other parts. The latter circumstance constitutes another difference with the free-living nitrogen-fixing bacteria, which fix nitrogen for their own use only; when they stop growing, nitrogenase activity is reduced as the ammonium ions formed are not consumed (16, 18, 76). This inhibition by ammonium ions has been investigated extensively during the last decades, and at least two different mechanisms have been suggested. One mechanism is based on the observation that ammonium ions bring about an immediate drop in nitrogenase activity. This effect has been found in aerobic microorganisms, viz. *Azotobacter* spp. (11, 52), and does not occur in anaerobic organisms (15, 52). It could be explained by assuming that ammonium ions block the synthesis of ATP and/or reducing equivalents through the uncoupling of oxidative phosphorylation; a similar effect of ammonium ions on photophosphorylation has been demonstrated in isolated chloroplasts (27, 40).

The second mechanism, the repression of nitrogenase synthesis by ammonium ions, produces a gradual decrease in nitrogenase activity (15, 19, 56). The repression is suggested to be interrelated with the assimilation of ammonia, especially with the activity of glutamine synthetase; this has been concluded from experiments with nitrogenase-derepressed mutants (82, 86) and from the effect of methionine sulfoximine - an inhibitor of glutamine synthetase activity - on the repressive action of ammonium ions (28, 79). Besides glutamine synthetase certain amino acids have been suggested to be involved in the regulation of nitrogenase synthesis (75). In symbiotic systems regulation of nitrogenase activity by means of the presence or absence of combined nitrogen will be exercised by the macrosymbiont which is assumed to be responsible for the assimilation of fixed nitrogen. However, it remains questionable whether mechanisms comparable with those in free-living nitrogen fixers are effective in symbiotic systems as well. Keeping in mind the basic similarities between the nitrogenase complexes from various organisms this might be true; on the other hand completely different mechanisms exerted via the plant could also be assumed. A major question addressed in the study presented here, it to what extent the effect of combined nitrogen on nitrogen fixation by a symbiotic system is comparable to that occurring in free-living nitrogen fixers.

Effect of combined nitrogen on symbiotic nitrogen fixation

The adverse effect of combined nitrogen on the establishment and on the functioning of the nitrogen-fixing symbiosis between bacteria and leguminous plants has been recognized ever since the discovery of the symbiosis. Even before the function of root nodules was known, it was described that nodule development was inhibited by ammonium salts or nitrates (67). After the identification of the symbiotic association and its significance by Hellriegel (33), many communications appeared on the effect of nitrogenous salts on the infection of plant roots by bacteria, the number and growth of root nodules and nitrogenase activity - see for instance the references given by Fred, Baldwin and McCoy (22) and by Wilson (91). Since that time, investigators in various fields (agricultural, physiological, biochemical) have tackled with the subject and have constructed different hypotheses to explain the phenomenon. The hypotheses which have been proposed, and which have not been essentially changed since the beginning of the research on nitrogen fixation, can be divided into several types. Those exclusively dealing with the prologue of the symbiosis (growth of bacteria near the roots, attachment of bacteria, infection process) will not be mentioned in detail, as they are not essential in this study. On the other hand a survey of the hypotheses concerning the influence of combined nitrogen on the active symbiosis seems to be relevant, as this effect constitutes the major object of the present study.

One of these suggestions states that combined nitrogen (in most studies a restriction to nitrate has been made) would be injurious to the bacteria as such, thus influencing either their infection capacity or their functioning in the nodule. However, a detrimental effect of nitrate on rhizobia has not been found when concentrations were applied which could reasonably be expected in the soil (43, 65). Elevation of the nitrate concentration does have an effect on nodulation anyways. The nitrate effect was suggested to be a local external effect; this assumption is based on experiments with the split root technique (89), with local applications of nitrate (32) and with nitrate addition to excised root systems (66). However, if the nitrate effect would be exerted after nitrate had been taken up by the plant, internal influences would be involved which might operate on the functioning of the nitrogenase system as well (51, 83).

The adverse effect of added nitrate might otherwise be exercised by nitrite, formed by dissimilatory nitrate reduction. Nitrite might influence nodulation by destruction of indole-acetic acid, which can be produced by the bacteria from tryptophane excreted by the plant (84) and which might be essential for the process of nodule development (35). The nitrogenase activity of an already established symbiotic system could also be inhibited by nitrite, if this compound would form a complex with leghemoglobin (87), would oxidize leghemoglobin (69) or would inactivate nitrogenase (70). However, the importance of nitrite as factor to explain the nitrate effect is doubtful, since it has been demonstrated that nitrate is also effective when *Rhizobium* mutants deficient in nitrate reductase are involved in the symbiosis (25). Moreover, the hypothesis does not explain the effect of ammonium ions, which is comparable to that of nitrate in many aspects.

A hypothesis related to the foregoing states that accumulation of fixation products in the nodules causes inhibition of nitrogenase activity; accumulation would result when the added nitrate is consumed instead of the fixation products (26), brought about, for instance, by a shortage of carbon skeletons (92). However, a feedback inhibition on nodule nitrogenase activity has not been demonstrated, for instance, when plants were artificially maintained in the vegetative state by removing flower primordia (58, 73). Moreover, no inhibition of *in vitro* nitrogenase activity by ammonium ions or amino acids has been demonstrated (36).

The importance of the carbohydrate supply to sustain nitrogenase activity - and thus the interrelationship between photosynthesis and nitrogen fixation - has been recognized many years ago (e.g. 21). Wilson suggested that the carbohydrate-nitrogen ratio would determine the nodulation capacity and the nitrogenase activity (90, 91). This theory explains the influence of combined nitrogen as an effect on the internal or external C to N ratio, and seemed to be confirmed by experiments where in combination with addition of nitrogenous compounds the carbohydrate concentration was raised. These experiments included supplemental addition of glucose or sucrose (74, 84), CO_2 enrichment of the atmosphere (23, 47) or increase in light intensity (7, 68). However, the theory does not pay attention to the actual mechanism of the combined nitrogen effect and thus explains too little. When applied to local variations in either carbohydrate or nitrogen concentration - taking into consideration the heterogeneous character of the plant - an indication as to the regulation of nitrogen-fixing activity might be found.

Photosynthate deprivation

Recent theories, also based on ideas from some fifty years ago, accentuate the role of carbohydrate supply to the bacteroids as a natural regulator of nitrogenase activity - see, for instance, the reviews of Pate (60) and Hardy *et al.* (30). The transport of newly formed photosynthates to the root nodule and their use in amino acid synthesis have been demonstrated with $^{14}CO_2$ (2, 45, 62). A disproportional quantity of photosynthates is transported to the nodules in view of their weight (48). The significance of carbohydrate supply has further been substantiated in experiments with so-called source-sink manipulations, for instance defoliation or depodding of the plant (8, 44) and grafting tests (80). Results of experiments where light intensity was varied or the plant was shaded (1, 78), or where the CO_2 concentration was increased (29) point into the same direction.

In view of the above-mentioned theories addition of combined nitrogen to a nitrogen-fixing symbiosis would decrease nitrogenase activity by depriving the nodules of photosynthate (24, 59). An analogous photosynthate deprivation might account for diurnal and seasonal variations in nitrogenase activity (3, 31). A decreased translocation of carbohydrates to the root nodule is supposed to occur while carbohydrates are consumed in roots or shoots during the assimilation of newly absorbed nitrogenous compounds. Alteration of the translocation pattern of photosynthate upon feeding leguminous plants with combined nitrogen was demonstrated indeed in experiments using $^{14}CO_2$: less newly formed carbohydrates were found in the nodules (42, 77). Other data supporting the idea of regulation via the translocation system can be derived from nitrogenase measurements of intact plants when fed with combined nitrogen. The time scale of the effect of nitrates on nitrogenase activitiy and the reversibility of the inhibition upon termination of the external supply of nitrogenous compounds both suggest such a regulation (24, 54). Further evidence for the photosynthate deprivation theory can be derived from results of investigations in which C- and N-balance studies (61) or ^{15}N-dilution techniques (55) were used.

In addition to the altered translocation of carbohydrates, various other phenomena have been demonstrated which result from the presence of combined nitrogen in nodulated legumes. The most striking effect is the change of colour of the nodules from pink to green, which is often called senescence of the nodule (14, 38). The same discolourization of nodules can be seen when plants are in or beyond the fruit ripening stage. This effect might be

understood as being derived from photosynthate deprivation: nitrogenase activity of the nodule is switched off due to starvation, and the nodule dies. Destruction of the nodule as expressed in its fine structure can be considered as a similar starvation process (19). The decrease in the amount of leghemoglobin in the nodule might also be initiated by the changed carbohydrate translocation pattern caused by the addition of nitrogenous compounds (10, 57).

Assimilation of nitrogen in the root nodule

Finally, one might think of an influence of nitrogen metabolism in the nodule as regulating factor on nitrogenase synthesis, comparable to regulating effects displayed by free-living nitrogen-fixing microorganisms (82, 86). Involvement of glutamine synthetase as a regulator has been considered doubtfull, since no correlation between the adenylylation state of this enzyme and nitrogenase activity was found in soybean bacteroids (9). An adenylylation control mechanism of glutamine synthetase in pea seeds could be demonstrated neither (37). On the other hand, the specific glutamine synthetase activity in pea bacteroids has been shown to bear an inverse relationship with the development of nitrogenase activity (63). Results of experiments with glutamine-auxotrophic mutants of *Rhizobium* spp. (46) and with nitrogen-fixing *Rhizobium* spp. in continuous culture (6) also suggest a role for glutamine synthetase in the gene expression of nitrogenase activity. Nevertheless, in the case of symbiotic nitrogen fixation in leguminous plants, the regulation would be expected to be effectuated via the plant cells, where ammonia-assimilating enzymes are found to be mainly localized (12, 41, 64). On the other hand, very low concentrations of such enzymes still present in the bacteroids might function in regulating nitrogenase synthesis or activity. A connection between the activities of the enzymes of ammonia assimilation in the root nodule and nitrogenase has been suggested, since the level of these enzymes (glutamine synthetase, glutamate synthase) changes during the development of the symbiosis (71, 72). Moreover, activities of the ammonia-assimilating enzymes alter when combined nitrogen is added to the plant (20). A function of amino acids - like glutamate - in the regulation of the export of fixed nitrogen from the bacteroids to the plant cells has been suggested: amino acids would switch off the ammonia assimilation within the bacteroids (53). However, data about regulatory functions of enzymes are scarce. As yet, a primary function of the ammonia assimilating metabolism on the regulation of nitrogenase synthesis and activity seems to be unlikely.

Outline of the investigations

The following experiments were performed to investigate the effect of ammonium and nitrate ions on nitrogenase activity of the pea-*Rhizobium* symbiosis. As regulation of nitrogenase activity is exerted by the symbiotic system as a whole, via mutual interactions between the microsymbiont and the host plant, it was important to minimize disturbances of the system. On the other hand, a detailed study concerning characterization and localization of the effect of combined nitrogen has never been carried out and seemed preferable. These starting points implied work with intact nodulated plants, detached nodules and isolated bacteroids, separating the microsymbiont to a gradually higher degree from its host. Regulation of nitrogen fixation via carbohydrate supply was used as working hypothesis.

First of all, isolated bacteroid suspensions were studied, to investigate possibly direct effects of ammonium chloride on the microorganism or on nitrogenase (Chapter 2). Secondly, the photosynthate deprivation theory was tested in various experiments where ammonium chloride and potassium nitrate were added to intact plants (Chapters 3 and 4).In an extension of these experiments, the effect of ammonium chloride on detached root nodules was examined (Chapter 5). Finally, the relation between carbohydrate utilization and nitrogenase activity - in combination with the effect of ammonia addition - was studied in detached nodules and in isolated bacteroids, in view of the efficiency of nitrogen fixation (Chapter 6).

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2. ACETYLENE REDUCTION AND RESPIRATION OF ISOLATED PEA BACTEROIDS AS AFFECTED BY AMMONIUM CHLORIDE AND OTHER FACTORS

Bacteroids were isolated from root nodules of pea plants. To study the effect of addition of carbon substrates and ammonium chloride, optimum conditions for acetylene reduction were used. Several dicarboxylic intermediates of the tricarboxylic acid cycle could be used as substrate for bacteroids; glucose, pyruvate and citrate were not active as such. Efficient substrates were present in the nodule cytosol. Ammonium chloride had no specific inhibitory effect on the acetylene reducing activity. Neither the enzyme nitrogenase as such, nor the physiologically active bacteroids showed a decrease in acetylene reduction when ammonium chloride was added. Respiration of bacteroid suspensions was not always positively correlated with acetylene reduction. Ammonium chloride had no effect on bacteroid respiration, or caused a slight enhancement.

INTRODUCTION

Nitrogenase activity (acetylene reduction) of free-living nitrogen-fixing microorganisms is adversely affected by combined nitrogen. When ammonium ions or other easily assimilable nitrogenous compounds are present in the growth medium, nitrogenase is not synthesized owing to enzyme repression. This has been demonstrated for different organisms, like *Azotobacter* spp. (9), *Klebsiella* spp. (25), *Clostridium* spp. (8) and *Anabaena* spp. (21). On the other hand, when ammonium ions are added to an actively nitrogen-fixing organism, nitrogenase activity is reduced or entirely eliminated. At least two different effects causing this decrease can be distinghuished. First of all, an immediate drop in acetylene-reducing activity was shown in whole organisms, but not in cell-free extracts; this effect is thought to be due to a decrease in the supply of ATP or reducing equivalents (7). Secondly, a long-term decrease in nitrogenase activity is caused by repression of enzyme synthesis and dilution of existing activity (9). The assimilation of ammonium (via glutamine synthetase) may be interrelated with the repression and derepression of nitrogenase synthesis (23).

Influence of combined nitrogen on dinitrogen fixation can also be demonstrated in symbiotic systems of legumes with *Rhizobium* spp. The

presence of nitrogenous compounds inhibits the development of an effective symbiosis or decreases the activity of an existing nitrogen-fixing system (12). However, it is difficult to decide whether ammonium acts directly on the bacterial part of the symbiosis, or indirectly via the plant. If a direct effect would be involved the mechanism of nitrogenase inhibition might be comparable to that in free-living organisms. If ammonium would act in an indirect way, entirely different processes might be involved. In a previous study (14) it has been shown that addition of ammonium chloride to pea plants diminishes the nitrogen fixation of the intact system, whereas the potential nitrogenase activity of the bacteroids does not decrease. Other processes reported to be influenced by the addition of ammonium salts to legumes include the distribution of photosynthates (18), the synthesis of leghemoglobin (6) and the assimilation of ammonia (10). These findings suggest that effects of nitrogenous compounds on nitrogen fixation are brought about by influences on the physiology of the plant.

However, it can not be concluded that the microsymbiont is fully insensitive to influences of ammonium. For example it is unknown how many ammonium ions, when added to the plant, reach the bacteroids. One way to approach this problem is to study the effect of ammonium on nitrogenase activity of free-living *Rhizobium* spp. Results of these studies are not unequivocal and the following observations upon NH_4Cl addition are reported: no repression of nitrogenase at all (16), a stimulation at low NH_4Cl concentrations and a long-term repression at higher concentrations (11, 13), or repression and a fast decrease in activity at moderate NH_4Cl concentrations (5, 24). Apart from these differences it is questionable whether the results are applicable to the symbiotic system, regarding the differences between free-living rhizobia and bacteroids (for instance in environmental conditions and in actual nitrogenase activity).

Another way to address this question is to study detached nodules or isolated bacteroids, in order to minimize influences of the plant. In a previous study it was demonstrated that addition of ammonium chloride to detached pea root nodules results in a decrease in acetylene-reducing activity within a few hours (15). In the present study ammonium chloride was added to isolated bacteroids, to see whether ammonium ions might influence nitrogenase activity. Both the effect on nitrogenase as such and the effect on physiologically active bacteroids was studied.

MATERIALS AND METHODS

Plants

Pea plants (*Pisum sativum* L. cv. Rondo), inoculated with *Rhizobium leguminosarum* PRE, were grown in gravel with a nitrogen-free nutrient solution (19). They were cultured in a growth chamber at 18-20°C and a light intensity of 12,000 lux, with a 16 h light - 8 h dark period. Plants were harvested 26-31 days after sowing, when nodules had fully developed and nitrogenase activity was maximal.

Bacteroid suspensions

Freshly picked nodules were pressed in a Bergersen press (2) under anaerobic (argon) conditions. The buffer used (pH 7.2) contained tris-(hydroxy methyl)-aminomethane (50 mM), magnesium chloride (2.5 mM) and polyvinyl-pyrrolidone (4% w/v); in the aerobic assay in addition sucrose (0.3 M) was given. Per ml of buffer 40 mg (aerobic assay) or 80 mg (anaerobic assay) of nodules, fresh weight, was used. Nodule brei was centrifuged (10 min at 5,000 x g), the pellet washed with buffer (same buffer, without PVP) and the suspension centrifuged again (10 min at 5,000 x g). Finally bacteroids were suspended in buffer solution (without PVP), kept at 0°C under argon and used as soon as possible. In some experiments the latter buffer contained 1% (w/v) bovine serum albumin (essentially fatty-acid-free, Sigma Chemical Company, St. Louis, Missouri, USA).

Nitrogenase activity

Nitrogenase activity was determined with the acetylene reduction assay; ethylene produced was measured gaschromatographically. Acetylene reducing activity of isolated bacteroids is calculated per g of corresponding fresh weight nodules (µmoles C_2H_4 per g of fresh weight nodule). Bacteroid suspensions were incubated in 16.5-ml Hungate tubes in a shaker bath at 24°C, shaking rate 200 strokes/min. In the anaerobic assay the reaction mixture (total volume 1 ml) contained 0.5 ml of EDTA-toluene-treated bacteroid suspension (22), 50 µmoles of tris-HCl (pH 7.2), 15 µmoles of $MgCl_2$, 18.4 µmoles of creatine-phosphate, 5.6 µmoles of ATP, 0.03 mg of creatine phosphokinase (Boehringer Mannheim, W. Germany) and 20 µmoles of $Na_2S_2O_4$. The gas phase was 10% acetylene in argon. In the aerobic assay the reaction mixture (total volume 1 ml) contained 0.5 ml

of bacteroid suspension, 50 µmoles of tris-HCl (pH 7.2), 2.5 µmoles of $MgCl_2$, 300 µmoles of sucrose and 10 µmoles of sodium succinate. The gas phase was 10% acetylene and 1% O_2 in argon. Myoglobin (from whole skeletal muscle, type II; Sigma Chemical Company, St. Louis, Missouri, USA) was reduced (ferric to ferrous) with sodium ascorbate (20 mM), and dialysed three times against a 200 x volume of buffer solution.

Respiratory activity

Respiration (oxygen consumption) of nodule brei and of isolated bacteroids was determined with an oxygen electrode (Model 53, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio, USA). The reaction mixture (total volume 3 ml) contained 150 µmoles of tris-HCl (pH 7.2), 7.5 µmoles of $MgCl_2$, 900 µmoles of sucrose and 40-60 mg of fresh weight nodules. Reaction temperature was 24°C. The mixture was saturated with air, and oxygen consumption monitored. Sodium succinate and ammonium chloride were added during the assay by injection of 30 µl of a 1 M solution of each.

RESULTS

Effect of ammonium ions in the anaerobic assay

The acetylene-reducing activity of isolated bacteroids in the anaerobic assay, when treated with EDTA and toluene as described by van Straten and Roelofsen (22), can be considered to be the potential nitrogenase activity of the bacteroids *in vivo*. With this assay method a 100%, or more, recovery of the activity of the intact plant can be found (14, 22); when the EDTA-toluene treatment is omitted, the recovery is only 10%. Therefore, this "EDTA-toluene-assay" seemed to be a suitable method for checking whether ammonium ions had any effect on nitrogenase itself. Fig. 1 shows that addition of NH_4Cl (10 mM) had no inhibiting effect on the acetylene-reducing activity. During an incubation of 2 h the activity was not influenced by NH_4Cl or by KCl.

Optimum conditions for the aerobic assay

In the aerobic assay, energy and reducing equivalents, necessary for nitrogenase activity, are produced through the oxidation of succinate by the isolated bacteroids. This situation was thought to be more or less comparable with that in the intact nodule, and therefore the aerobic assay was used

to study physiologically active bacteroids. Figure 2 shows that the optimum oxygen concentration for isolated bacteroids was 1% O_2 in the gas phase, with a bacteroid concentration corresponding with 20 mg of fresh weight nodules per

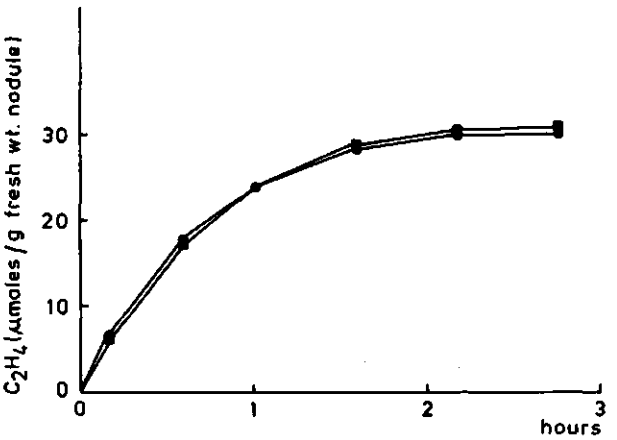

Fig. 1. Acetylene reduction by isolated bacteroids of pea root nodules. Bacteroid suspensions were treated with EDTA-toluene and incubated in the anaerobic assay. Assays were performed with KCl, 10 mM (●) and NH_4Cl, 10 mM (■).

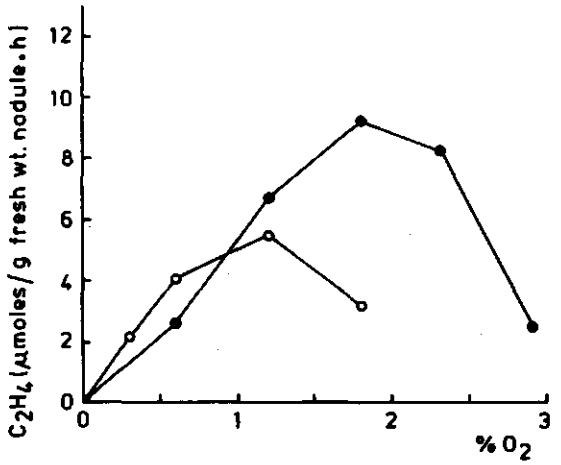

Fig. 2. Acetylene reduction by isolated bacteroids of pea root nodules vs. oxygen concentration. Bacteroid suspensions were tested in the aerobic assay under different O_2 concentrations in the gas phase; incubation time was 15 min. Nodule brei (●) and isolated, washed bacteroids (o).

ml. For nodule brei the optimum oxygen concentration was somewhat higher. In the standard assay 1% O_2 in the gas phase was used, both for bacteroids and for nodule brei. With this oxygen concentration a shaking rate of 200 strokes per min was necessary to achieve highest activities. Results in Table 1 show the effect of succinate and sucrose. When succinate was omitted, acetylene reduction of isolated bacteroids was negligible. The presence of sucrose during isolation of bacteroids was essential. However, when nodule brei was assayed, a relatively high activity was detected without addition of sucrose and/or succinate. When nitrogenase was determined in nodule brei, succinate had a negative effect. Fatty-acid-free bovine serum albumin (BSA) enhances the acetylene-reducing activity of isolated bacteroids (17). BSA is thought

Table 1. Acetylene reduction and respiration of root nodule fractions[a]

Preparation	Activity (μmoles/g of nodule fresh wt. per h)			
	C_2H_4 production		O_2 consumption	
	-sucrose	+sucrose	-sucrose	+sucrose
Nodule Brei (N.B.)	17.9	12.7	27.6	62.1
N.B. + succin. (10 mM)	9.9	9.2	30.5	92.8
N.B. + succin. (100 mM)	0.7	7.8	-	-
Nodule Cytosol (N.C.)	0.0	0.0	3.6	5.9
N.C. + succin. (10 mM)	0.0	0.0	3.6	5.9
Bacteroids (B)	0.3	0.7	10.2	19.4
B. + succin. (10 mM)	0.4	3.9	31.9	74.2
B. + succin. (10 mM) + BSA (1%)	0.2	9.6	33.4	88.8

[a]Nodule brei, nodule cytosol and isolated bacteroids were prepared with or without sucrose (10 % w/v) in the buffer solution. Succinate and BSA were added to the assay mixture. Incubation time was 10-15 min. Values are averages of three determinations; standard deviations 10 - 20%.

to act by binding free fatty-acids, which are uncouplers of oxidative phosphorylation. Table 1 shows that addition of 1% (w/v) BSA to the bacteroid suspension resulted in a threefold increase in nitrogenase activity (the optimum oxygen concentration was not changed by BSA). These effects of BSA were also found by Laane *et al.* (17).

Addition of supernatant and various substrates

Addition of nodule cytosol (supernatant obtained from the first centrifugation in the isolation procedure) resulted in an important increase in activity of isolated bacteroids (Table 2); the effect of cytosol was additive to the influence of BSA (cf. Table 1). This result suggests an important function of the plant material in supporting nitrogenase activity. The function might be the supply of substrates, or the protection against damage of membranes or enzymes. The effect can not be explained by the presence of leghemoglobin in the supernatant, since at the used oxygen concentrations (1%) leghemoglobin has no effect (see the effect of myoglobin in Fig. 3). When cytosol was heated (15 min at 100°C followed by centrifuging, 10 min at 20,000 x g), the stimulating effect on nitrogenase activity was diminished. However, dialysis of cytosol (overnight, against a 200 x volume of buffer solution) eliminated the stimulation of acetylene reduction almost completely, suggesting a low molecular weight compound (possibly a substrate), which can support nitrogenase activity.

Table 2. Acetylene reduction and respiration by isolated bacteroids with various substrates and cytosol[a]

Addition[b]	Activity (μmoles/g of nodule fresh wt per h)[c]	
	C_2H_4 production	O_2 consumption
None	0.1	11.0
Glucose	0.1	11.0
Pyruvate	0.3	17.0
Citrate	0.3	11.8
2 Oxoglutarate	2.3	11.5
Succinate	8.7	83.5
Fumarate	9.8	43.3
Malate	10.8	48.6
Oxaloacetate	5.6	31.7
Cytosol	18.2	71.2
Cytosol + Succinate	14.8	81.6
Cytosol (10 x diluted)	6.5	53.4
Cytosol (heated)	10.7	58.2
Cytosol (dialysed)	0.9	59.2

[a]Assays as described in methods; BSA (1% w/v) was added; sucrose (10% w/v) was present during isolation and in the assay.
[b]Substrates (sodium salts) were added in a 10 mM concentration, cytosol was added to give the concentration of the original nodule brei.
[c]Values are averages of three determinations, standard deviations 10 - 20%.

Various substrates, added to the assay mixture, were able to stimulate the nitrogenase activity of isolated bacteroids (Table 2). The tricarboxylic acid cycle intermediates 2-oxoglutarate, succinate, fumarate, malate and oxaloacetate, all dicarboxylic acids, were efficient. Glucose, pyruvate and the tricarboxylic acid citrate had no effect. Addition of ATP to the assay mixture did not markedly increase nitrogenase activity; on the contrary, in the presence of succinate the nitrogenase activity actually is inhibited by the addition of ATP (10 mM), while low concentrations of ATP (1 mM) did not affect nitrogenase activity at all (results not shown).

Effect of myoglobin

In the root nodule, bacteroids are surrounded by leghemoglobin; this protein which can bind oxygen reversibly, probably has a function in the regulation of the oxygen concentration around the bacteroids, creating optimum conditions for nitrogenase activity (3). Leghemoglobin and other oxygen-binding proteins like myoglobin increase the efficiency of acetylene reduction by isolated bacteroids by facilitating the oxygen flux to the bacteroid at a low free oxygen concentration (26). In this study, the addition of reduced (i.e. Fe^{2+}-containing) myoglobin at a concentration of 250 µM, increased the acetylene-reducing activity at oxygen concentrations below 0.5% O_2 in the gas phase (Fig. 3). In the assay

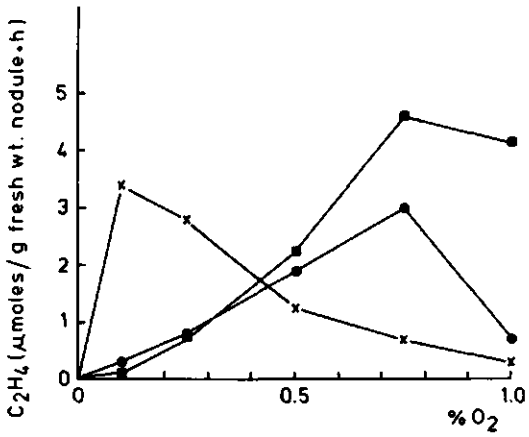

Fig. 3. Acetylene reduction by isolated bacteroids of pea root nodules in the presence of myoglobin. Nitrogenase activity was measured in the aerobic assay, at different oxygen concentrations in the gas phase; incubation time was 10 min. No addition (●); 250 µmoles of reduced (Fe^{2+}) myoglobin added (✕); 250 µmoles of oxidized (Fe^{3+}) myoglobin added (■).

with myoglobin, addition of BSA had a positive effect (cf. the activity in Fig. 4D as compared to that in Fig. 3); the same results are reported by Laane *et al.* (17). At an oxygen concentration of 1% O_2 in the gas phase, reduced myoglobin had no stimulating effect. However, oxidized (i.e. Fe^{3+}-containing) myoglobin did enhance the activity at the latter O_2 concentration (Fig. 3). This suggests a different function of myoglobin, because the oxidized form is much less active in binding oxygen. Perhaps the effect is comparable with the effect of BSA (250 μM myoglobin corresponds to 0.4% of protein).

Effect of ammonium ions in the aerobic assay

Preliminary experiments showed that neither ammonium chloride nor ammonium acetate (at a 1 mM concentration) had any effect on the acetylene-reducing activity of isolated bacteroids. Fig. 4 shows the results of experiments where higher concentrations of NH_4Cl were tested. No significant inhibitory effect

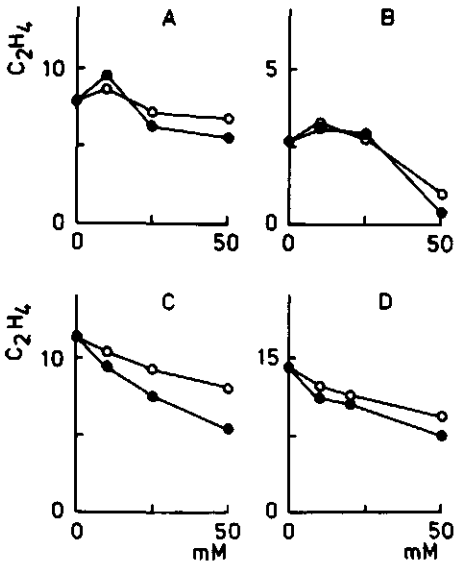

Fig. 4. Influence of NH_4Cl on the rate of acetylene reduction by nodule brei (A), isolated bacteroids (B), isolated bacteroids with BSA (C) and isolated bacteroids with BSA and myoglobin (D). Nitrogenase activity was measured in the aerobic assay (1% O_2 in assays without myoglobin, 0.1% O_2 in the assay with myoglobin); incubation time was 15 min. Concentration of salts as stated, KCl (o) or NH_4Cl (•). Activities are given in micromoles of C_2H_4 formed per g of fresh weight nodule per h.

of ammonium ions could be detected with any of the following preparations: nodule brei, isolated bacteroids, isolated bacteroids with BSA added, and isolated bacteroids with BSA and myoglobin added. High concentrations of NH_4Cl did reduce nitrogenase activity, but this was a non-specific salt effect, as KCl had the same effect. In isolated bacteroids a concentration of 50 mM (NH_4Cl or KCl) is almost completely inhibitory; in nodule brei or when BSA was present, the salt effect was much less pronounced.

Respiration of root nodule fractions

Respiratory activity (measured as oxygen consumption) was determined in nodule brei, in isolated bacteroids and in nodule cytosol (Table 1). Oxygen consumption of the nodule cytosol was only a small fraction (about 10%) of the total (nodule brei) activity, and was not increased by the addition of succinate. Respiration of nodule brei and of isolated bacteroids was highest when the isolation procedure was performed in the presence of sucrose. Oxygen consumption of these fractions was enhanced by the addition of 10 mM sodium succinate, especially when sucrose was present. Other substrates could be oxidized by bacteroids, and addition of nodule cytosol also enhanced respiration (Table 2). When the cytosol was heated or dialysed the stimulation of respiration was decreased, but not eliminated completely. In other experiments

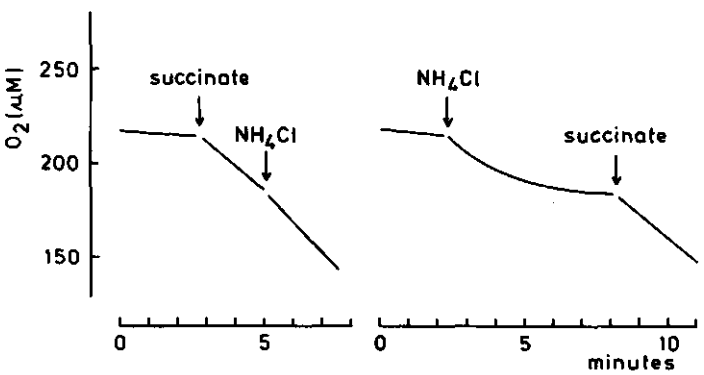

Fig. 5. Oxygen consumption by isolated bacteroids, measured with an O_2-electrode. Recorder traces are shown. Buffer solution (3 ml), containing bacteroids corresponding with 40 mg of fresh weight nodules, was saturated with air, and the assay started at time 0. During the assay 30 µl of a 1 M solution of succinate or NH_4Cl was added, at the time indicated.

it was shown that respiration was not oxygen-limited until the O_2 concentration fell below 6 μM. This suggests that respiration at high O_2 concentrations can be compared with that at low concentrations, as used in the assay for acetylene reduction. Addition of NH_4Cl (at a 10 mM concentration) during the respiration measurements had no effect, or gave a slight enhancement of the oxygen consumption (Fig. 5) when bacteroids were isolated without sucrose. When no succinate was present, the increase was only temporary, when succinate was present the higher oxygen consumption was continued.

DISCUSSION

As part of a study on the influence of ammonium ions on the nitrogenase activity of symbiotic nitrogen-fixing systems, isolated bacteroids from pea root nodules were examined. Acetylene-reducing activity and the effect of ammonium on this activity were studied under different conditions.

The anaerobic assay method with EDTA-toluene-treated bacteroids (22) can be considered to be an enzymic determination. Results (Fig. 1) show that nitrogenase itself, supplied with ATP and reducing equivalents, was not inhibited by ammonium ions. This is in accordance with the characteristics of nitrogenase isolated from free-living organisms, which shows no specific inhibition by NH_4Cl measured in cell-free extracts (20).

In the aerobic assay the bacteroids can be considered to occur under more or less natural conditions, where nitrogenase activity is coupled to respiration. The method used is based on descriptions by Bergersen and Turner of nitrogenase activity measurements of isolated bacteroids of soybean nodules (4). Results described in this paper show that pea bacteroids behave in a similar way like soybean bacteroids. However, bacteroids from pea seem to be more sensitive to the absence of sucrose; omittance of this compound resulted in a total loss of acetylene-reducing activity, which could not be restored by the addition of succinate (Table 1). Probably, the presence of sucrose is necessary to protect the cell against an osmotic shock. Oxygen consumption was also largely decreased when sucrose was omitted (Table 1). Addition of sucrose in the assay with EDTA-toluene-treated bacteroids reduces acetylene reducing activity (1), so sucrose is not necessary for the enzyme activity as such.

Myoglobin was used as a substitute for leghemoglobin, which surrounds the bacteroids in the nodule (3). When myoglobin was added, maximum

acetylene-reducing activity was reached at a lower oxygen concentration (Fig. 3, see also (17) and (26)). This is in accordance with the function of myoglobin as an oxygen carrier, regulating optimum oxygen concentration at the bacteroids. A lower oxygen concentration may have the advantage that oxygen damage of nitrogenase is less likely to occur. Bovine serum albumin increases nitrogenase activity of isolated bacteroids, as described by Laane *et al.* (17); results reported here are in accordance with their findings. With the addition of myoglobin and serum albumin a nitrogenase activity of isolated bacteroids could be detected which represented about 50% of the activity of the intact system (cf. Fig. 4D in this paper and Fig. 2 in (14)).

Experiments with NH_4Cl added to isolated bacteroids in the aerobic assay showed no specific ammonium effects on acetylene reduction (Fig. 4). Assuming that conditions in this assay were comparable to those in the nodule, it can be concluded that NH_4Cl has no influence on the functioning of bacteroids. At least this applies to external NH_4Cl, as nothing can be said about the internal concentration of ammonium ions in the bacteroids. Nevertheless, these results demonstrate that the decrease of acetylene reduction after addition of NH_4Cl to intact plants (14) or to nodules (15), cannot be attributed to an effect of ammonium ions on the bacteroids. Therefore, this decrease must be due to other factors, for instance, the decreased supply of carbon sources.

Nitrogenase is provided with ATP and reductants via the respiration of substrates. Consequently, a low respiratory activity resulted in a low acetylene-reducing activity (Tables 1, 2). However, in some cases no positive correlation between respiration and acetylene reduction was observed. This can be seen, for instance, when the effect of different substrates (fumarate, malate, succinate) on C_2H_2 reduction and O_2 consumption are compared. Another example can be found in the effects of nodule cytosol, after different treatments. It is concluded that respiration as such does not support nitrogenase activity, but respiration of special substrates does. These substrates might be citric acid cycle intermediates (like succinate, fumarate or malate) which probably are present in the nodule cytosol. Moreover, factors other than respiration might be important in determining nitrogenase activity.

As could be expected, ammonium chloride had no negative effect on respiratory activity of bacteroid suspensions. The slight temporary enhancement of respiration when bacteroids isolated without sucrose were used (Fig. 5) is

difficult to explain and its importance, if any, to nitrogen fixation is unknown. Possibly, it points to an uncoupling of oxidative phosphorylation from the respiratory chain, which can only occur when the bacteroids occur under less favourable conditions (isolated without sucrose).

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3. INFLUENCE OF AMMONIUM CHLORIDE ON THE NITROGENASE ACTIVITY OF NODULATED PEA PLANTS (*PISUM SATIVUM*).

A study was made on the short-term effect of ammonium ions on the nitrogenase activity of pea root nodules. Nodulated pea plants (*Pisum sativum*), having reached maximum acetylene-reducing activity, were supplied with NH_4Cl (20 mM). Nitrogenase activity of intact plants, detached nodules, and isolated bacteroids was measured at different time intervals. A significant drop (20 to 40%) in the acetylene-reducing activity of treated intact plants and their detached nodules was observed after 1 day. No drop in the nitrogenase activity of bacteroids (assayed aerobically, or anaerobically after treatment with ethylenediamine-tetraacetic acid and toluene) occurred for 2 to 4 days after the addition of NH_4^+ to the plants, depending on cultural conditions. From these results it is concluded that the adverse effect of NH_4^+ on acetylene reduction by intact plants and detached nodules during the first 2 days is not due to a decrease in the amount of nitrogenase in the bacteroids. It is suggested that the effect has to be attributed to a reduced supply to the bacteroids of energy-delivering photosynthates.

INTRODUCTION

Biological nitrogen fixation, by free-living organisms as well as by symbiotic systems, is affected by combined nitrogen. Generally the nitrogen-fixing activity is reduced on addition of N-compounds such as NH_4^+, NO_3^-, or amino acids. In free-living nitrogen-fixing bacteria two effects of the addition of NH_4^+ are known: an immediate decrease of nitrogenase activity (3) and a long-term decrease by repression of nitrogenase synthesis (7, 17).

In symbiotic nitrogen-fixing systems, the regulation of nitrogenase activity by nitrogen compounds is more complex. Addition of combined nitrogen may cause physiological changes in the plant which in turn may influence nitrogen fixation (10). It has been shown that the addition of NH_4^+ or NO_3^- to soybeans results in a changed distribution pattern of photosynthates, less C-compounds reaching the root nodules (12). The same phenomenon has been observed with pea plants upon addition of NO_3^- (15). Furthermore, morphological changes in the nodule may occur; these have been found when clover plants were treated with ammonium nitrate (6).

Except for a decrease in nitrogenase activity upon addition of nitrogen compounds, it is not known what happens to the enzyme shortly after the addition.

The present study was carried out to determine whether the decrease in nitrogenase activity of the intact plant following addition of NH_4^+ is accompanied by a concomitant decrease in the amount of nitrogenase present in the root nodule.

MATERIALS AND METHODS

Plants

Pea plants (*Pisum sativum* L. cultivar Rondo), inoculated with *Rhizobium leguminosarum* PRE, were grown in gravel with nitrogen-free nutrient solution (13) in a growth chamber at 18 to 20°C with an 8 h dark-16 h light period. The plants were used for the experiments during the period from 25 to 30 days after sowing, when nitrogenase activity had reached maximum values and remained at this level. At that time the nodule fresh weight per plant was about 100 mg. In some experiments, 14-day-old plants were transferred from the gravel into 30-ml tubes containing 15 ml of nutrient solution (single plants) or into 300-ml Erlenmeyer flasks containing 200 ml of nutrient solution (sets of five or six plants). Plants were supported by a plug of cotton, keeping the root nodules above the solution to prevent oxygen limitation.

NH_4^+ treatment

Plants growing in gravel, in pots with perforated bottoms, were treated by wetting the gravel with nutrient solution containing 20 mM NH_4Cl or 20 mM KCl (control). This treatment was repeated once a day. Plants growing in nutrient solution were treated by replacing the medium by a similar medium containing 20 mM NH_4Cl or 20 mM KCl (control). This solution was refreshed daily. The effect of L-methionine-sulfoximine (MSX) was tested by adding MSX (Sigma Chemical Co.) to the nutrient solution at a concentration of 10 μM.

Bacteroid suspensions

Bacteroid suspensions for the anaerobic assay were prepared as described by van Straten and Roelofsen (16). For the aerobic assay the same preparation method was used, with the following modifications: the 0.05 M tris(hydroxymethyl)-aminomethane-HCl buffer (pH 7.4) contained 2.5 mM $MgCl_2$, 0.3 M sucrose and 4% polyvinyl-pyrrolidone ("Kollidon 25", Fluka AG, Buchs, Switzerland),

and the nodule concentration was 40 mg of nodule tissue, fresh weight, per ml of buffer. The final bacteroid suspension contained in addition 1% bovine serum albumin (essentially fatty acid-free, Sigma Chemical Co., St. Louis, Missouri), which increases acetylene-reducing activity of bacteroid suspensions (C. Laane, H. Haaker and C. Veeger, Eur. J. Biochem., in press).

Nitrogenase activity

Nitrogenase activity was determined with the acetylene reduction assay. Intact plants were incubated in 200-ml Erlenmeyer flasks (single plants) or 1,000-ml Erlenmeyer flasks (sets of five or six plants). The gas phase was 10% acetylene in air. Ethylene produced was measured chromatographically (11). Detached nodules were incubated on wet filter paper in Erlenmeyer flasks with a gas phase of 10% acetylene in air. With intact plants and with detached nodules, ethylene production was shown to be linear for at least 30 min, after a lag phase of 3 min. Production in the first 15 min was used to calculate the activity on an hourly basis. Activity of bacteroid suspensions was determined in 16.5-ml Hungate tubes (Bellco Glass, Inc.). The reaction was carried out in a GFL shaker bath (shaking rate, 200 strokes per min) at 24°C. Ethylene production was measured after 10 min of incubation. In the aerobic assay, the reaction mixture contained 0.5 ml of bacteroid suspension, 50 μmol of tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4), 2.5 μmol of $MgCl_2$, 300 μmol of sucrose, 10 μmol of sodium succinate, and 5 mg of bovine serum albumin in a total volume of 1 ml. The gas phase was 10% acetylene and 1% oxygen in argon. In preliminary experiments these conditions were found to be optimal. In the anaerobic assay, the reaction mixture contained 0.5 ml of ethylenediaminetetraacetic acid (EDTA)-toluene-treated bacteroid suspension, 50 μmol of tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4), 15 μmol of $MgCl_2$, 18.4 μmol of creatine phosphate, 5.6 μmol of adenosine 5'-triphosphate, 0.03 mg of creatine phosphokinase (Boehringer, Mannheim, W. Germany), and 20 μmol of $Na_2S_2O_4$ in a total volume of 1 ml. The gas phase was 10% acetylene in argon.

Uptake of NH_4^+

The uptake of NH_4^+ by the plants was determined using $^{15}NH_4Cl$ (VEB-Berlin-Chemie). Sets of five plants were placed in 300-ml Erlenmeyer flasks with

100 ml of nutrient solution containing 20 mM NH_4Cl with 24 atom % ^{15}N. After 24 h the ^{15}N enrichment was determined in roots, shoots and root nodules separately, using emission spectrometic analysis as described by Akkermans (Ph. D. Thesis, University of Leiden, Leiden, The Netherlands). Calculations were performed according to Ferraris and Proksch (9).

RESULTS

Nitrogenase activity of intact plants and detached nodules

The results with plants growing in culture solution (Fig. 1) show that the addition of NH_4Cl (20 mM) caused a significant decrease in nitrogenase activity of intact plants during the subsequent 5 days. With 10 mM NH_4Cl (not shown in the figure), the decrease was about half the inhibition obtained with 20 mM NH_4Cl. Four or 5 days after the start of the NH_4^+ treatment, the root nodules turned green (as a result of decomposition of leghemoglobin).

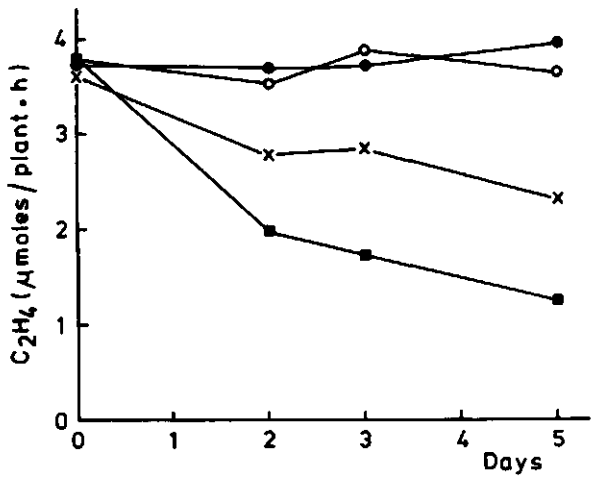

Fig. 1. Influence of NH_4Cl and MSX on the acetylene-reducing activity of intact pea plants growing in nutrient solution. At day 0 plants were placed in tubes with nutrient medium containing: (●) 20 mM KCl; (■) 20 mM NH_4Cl; (○) 20 mM KCl + 10 µM MSX; (x) 20 mM NH_4Cl + 10 µM MSX. Per treatment, 10 single plants were used, the same plants throughout the experiment. Activities are expressed in micromoles of C_2H_4 formed per plant per hour; standard deviations vary from 0.4 to 0.8.

The depressing effect of NH_4^+ on nitrogenase activity was also found with detached nodules from treated plants, in spite of the lower acetylene reduction values of these nodules (Table 1).

To know whether the inhibitory effect of NH_4^+ was due to the ammonium ion per se or was related to its assimilation, the effect of MSX, a strong inhibitor of glutamine synthetase (2), was studied. Addition of MSX at a concentration of 10 µM together with NH_4Cl diminished the effect of NH_4^+ (Fig. 1). At days 3 and 5, the differences in the activities of the treatments (KCl, NH_4Cl plus MSX, NH_4Cl) were significant at the 1% level. Linear regression coefficients of these treatments differed significantly at the 5% level. There was no effect on nitrogenase activity of the plants upon addition of MSX (in combination with KCl) at a concentration of 10 µM, and the plants looked healthy. Higher concentrations of MSX could not be used because they damaged the plants seriously.

Table 1. Influence of NH_4Cl and KCl on acetylene-reducing activity of intact plants, detached nodules, and bacteroid suspensions[a].

Expt no.	Assay	Activity				
		Day 0	Day 2		Day 4	
			KCl	NH_4Cl	KCl	NH_4Cl
I	Intact plants	46	55	21	46	16
	Detached nodules	11	14	6	15	5
II	Intact plants	39	38	19	31	12
	Bacteroids (aerobic)	10	10	9	8	5
III	Intact plants	40	42	26	45	15
	Bacteroids (anaerobic)	21	16	17	20	10

[a] NH_4Cl (20 mM) and KCl (20 mM; control plants) were added to pea plants growing in nutrient solution. Activities of detached nodules, aerobic bacteroids, and anaerobic bacteroids were determined in different experiments (I, II, and III); in each experiment, measurements of intact plants were included (sets of six plants). Activities are expressed in micromoles of C_2H_4 formed per gram of nodule fresh weight per hour.

To determine how much NH_4Cl was taken up by the plants, and where this N could be found in the plant, experiments with $^{15}NH_4Cl$ were performed (Table 2). After 24 h of NH_4Cl treatment, an average uptake of 0.74 mg of N per plant was found. Nearly all of this N was present in the roots and the green parts of the

plants, and only a small part in the root nodules. When the plants were provided with MSX (10 μM) plus NH_4Cl, the amount of ^{15}N in the plants appeared to be diminished by about 25%. This decrease was apparent in both the shoots and the roots.

Nitrogenase activity of bacteroid suspensions

Bacteroid suspensions, isolated from plants treated with NH_4Cl for 2 days and assayed under aerobic conditions, showed only a slight decrease in nitrogenase activity, which was less pronounced than that of intact plants. The nitrogenase activity of EDTA-toluene-treated bacteroid suspensions in the anaerobic assay behaved in the same way (Table 1). Although the activity of bacteroids from both KCl- and NH_4Cl-treated plants was lower after 2 days, no specific inhibitory effect of NH_4^+ was observed. After 4 days of NH_4Cl treatment, the activity of both aerobically and anaerobically assayed bacteroid suspensions had decreased, but still less than did the activity of intact plants.

Because of the low recovery of nitrogenase activity of EDTA-toluene-treated bacteroid suspensions from plants in Erlenmeyer flasks (about 40%), the experiment was repeated with plants grown in gravel for the entire duration of the experiment. Under these conditions the nitrogenase activity of isolated bacteroids was about 100% of that of intact plants when calculated per gram of nodule fresh weight. Bacteroids isolated from KCl-treated plants and those from NH_4Cl-treated plants did not differ significantly in nitrogenase activity, not

Table 2. Uptake of NH_4Cl by pea plants[a]

Plant part	20 mM NH_4Cl		20 mM NH_4Cl + 10 μM MSX	
	Atom % ^{15}N excess	N absorbed (mg per plant)	Atom % ^{15}N excess	N absorbed (mg per plant)
Roots	2.80 ± 0.21	0.42 ± 0.04	2.20 ± 0.13	0.31 ± 0.02
Shoot	0.76 ± 0.08	0.31 ± 0.02	0.63 ± 0.04	0.23 ± 0.01
Root nodules	0.18 ± 0.03	0.009 ± 0.002	0.18 ± 0.03	0.009 ± 0.001

[a] Plants were treated with a 20 mM NH_4Cl solution with a ^{15}N-enrichment of 24%. After 24 h, the ^{15}N content was determined in roots, shoots and root nodules. Given values (± standard deviations) are averages of six determinations, performed in two experiments with triplicate measurements.

even after 4 days (Fig. 2). At days 2 and 3 the activity of NH_4Cl-treated plants differed significantly at the 1% level from the activity of bacteroids isolated from these plants. The activity of KCl-treated plants did not differ from that of their isolated bacteroids. One day after the treatment with NH_4Cl or KCl was started, the EDTA-toluene-treated bacteroids had a rather low nitrogenase activity. This may have been an effect of wetting the nodules by flushing the pots with nutrient solution. Before starting the treatment, the gravel was kept relatively dry to advance nodule formation and development.

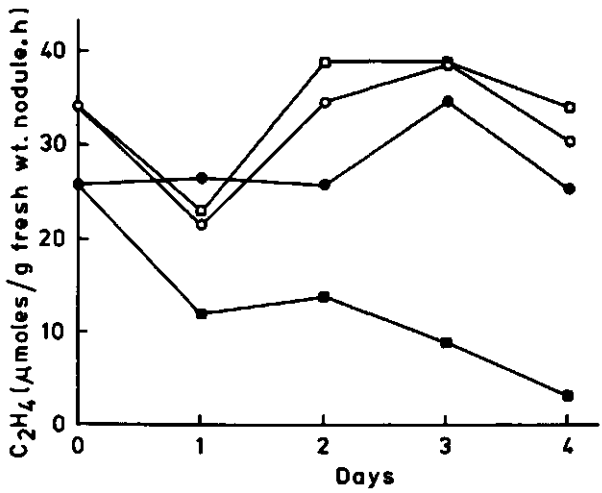

Fig.2. Influence of NH_4Cl on the *in vivo* and the *in vitro* acetylene-reducing activity of bacteroids from pea root nodules. For each determination sets of eight plants were used, treated with 20 mM KCl (●) or 20 mM NH_4Cl (■). Treatment started at day 0. The *in vitro* activity was measured with bacteroids isolated from these eight plants and after treatment with EDTA-toluene: bacteroid suspensions from KCl-treated plants (○) and from NH_4Cl-treated plants (□). Activities of intact plants and isolated bacteroids have been calculated per gram of nodule fresh weight per hour. Values are mean activities of duplicated determinations; standard deviations vary from 2 to 5.

DISCUSSION

The well-known fact that nitrogenase activity of nodulated legumes decreases upon addition of nitrogenous compounds has been the subject of many studies. Most of these studies are concerned with long-term effects of nitrogen fertilizers on the yield of plant material. The mechanism of the regulation of nitrogenase activity is still unclear, although some datailed studies have

been performed in recent years. These studies suggest a possible role in this regulation for the supply of photosynthate (12) or the leghemoglobin concentration (2a, 4); a regulation mediated through the NH_4^+-assimilating enzymes is doubtful (1, 8). The time after which the effect of added NH_4^+ or NO_3^- is expressed through nitrogenase activity differs in these studies, depending on concentration of the N-compound, plant species, bacterial strain, and probably other factors. In the experiments described in this paper there was an apparent effect within a short period of time: 1 day after the addition of NH_4Cl, nitrogenase activity of intact plants was inhibited by 20 to 40% (Fig. 2). Therefore, it was possible to study short-term effects of NH_4^+. The acetylene-reducing activity of detached nodules responded in a way similar to that of intact plants (Table 1).

The activity of EDTA-toluene-treated bacteroid suspensions represents a recovery of 100% or more of the activity of whole pea plants when using plants grown in gravel (16). Consequently, this activity can be used as a measure of the amount of potentially active enzyme present in the bacteroids. By comparing the activity of intact plants with that of isolated, EDTA-toluene-treated bacteroids, it was concluded that during the first days after the addition of NH_4Cl the decrease in activity of the plants was not a result of a decrease in the amount of nitrogenase (Fig. 2). It is unknown whether this would be due to continued turnover of nitrogenase in the presence of NH_4^+ or to the great stability of nitrogenase, which would cause the constancy of the enzyme level even when the synthesis would be repressed.

For unknown reasons the recovery of nitrogenase activity was constantly much less than 100% when bacteroid suspensions were derived from plants grown in nutrient solution. Nevertheless, the results with pea plants grown in culture solution (when cultural conditions actually are better defined) confirm those of gravel-grown plants (Table 1). The activity of EDTA-toluene-treated bacteroids was affected less by the addition of NH_4^+ than was the activity of intact plants. Moreover, the same tendency was shown for isolated bacteroids in the aerobic assay. Therefore, it can be concluded that not only the nitrogenase itself is unaffected by NH_4^+, but also the process in the bacteroids providing energy for nitrogenase activity. After 4 days, the activity of both anaerobically and aerobically assayed bacteroid suspensions had decreased (as opposed to the results of the experiments with gravel-grown plants). At this time root nodules turned somewhat green. This was due to a decrease in leghemoglobin concentration, which was shown to occur upon addition of NH_4^+ or

NO_3^- to nodulated pea plants (2a, 4). These are rather long-term effects which are beyond the scope of this study.

The question arises of which regulatory process is primarily responsible for the inhibition of the nitrogenase activity of whole plants after treatment with NH_4Cl. From this study it can be concluded that this regulation is not at the bacteroid level. Moreover, almost no $^{15}NH_4^+$ was taken up by the root nodules. Therefore it is likely that the effect of added NH_4^+ is not brought about in the nodules. Probably changes in the physiological condition of the plant as a whole are of more importance in the regulation of nitrogenase activity. According to Small and Leonard (15), in pea plants the amount of photosynthate transported to the nodules diminishes in the presence of NO_3^-. This is also shown to occur in soybeans with both NO_3^- and NH_4^+ (12). If the same processes are taking place in pea plants with the addition of ammonium chloride, a shortage of C-compounds may be created in the nodules, thus causing a decrease in energy production. Since energy charge in the nodules is thought to be a regulatory factor in nitrogenase activity (5), this could explain the demonstrated effect of ammonium ions. Assuming that the assimilation of NH_4^+ induces this translocation of photosynthates, the effect of MSX can be explained (Fig. 1). Recent studies on the assimilation of NH_4^+ by plants strongly suggest that glutamine synthetase plays a dominant role in this process (14). Inhibition of this enzyme by the addition of MSX will cease the NH_4^+ assimilation and thus the translocation of C-compounds. However, the uptake of NH_4^+ was influenced by MSX (Table 2). This might be a result of inhibition of NH_4^+ assimilation, but other influences of MSX, not coupled to this assimilation, cannot be excluded. On the other hand, a shortage of C-compounds can also be brought about by a decrease in photosynthetic activity of the plant. Such a decrease might be a result of NH_4^+, if these ions reach the chloroplasts.

Addition of MSX to the control plants does not affect acetylene reduction. In these plants, NH_4^+, from N_2 fixation, is assimilated in the root nodule. Inhibition of the assimilation (assuming that MSX reaches the nodule) will not influence the translocation of photosynthates, as is suggested to occur when the assimilation of added NH_4^+ (probably in the roots) is inhibited.

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4. INFLUENCE OF AMMONIUM AND NITRATE NITROGEN ON NITROGENASE ACTIVITY OF PEA PLANTS AS AFFECTED BY LIGHT INTENSITY AND SUGAR ADDITION.

Addition of ammonium chloride or potassium nitrate to nodulated pea plants resulted in a decrease of the acetylene-reducing activity. Both nodule growth and specific activity of the nodule were diminished. Acetylene-reducing activity of isolated bacteroids, treated with EDTA-toluene and supplied with ATP and dithionite, had not decreased after 3 days of NH_4Cl or KNO_3 treatment of the plants. The effect of combined nitrogen could be counteracted by raising the light intensity or by the addition of sucrose to the growth medium. The latter treatment reduced the nitrogen uptake by the plants. It is concluded that combined nitrogen affects symbiotic nitrogen fixation via the carbohydrate supply to the bacteroids.

INTRODUCTION

Dinitrogen fixation by nodulated legumes is counteracted by the presence of combined nitrogen in the growth medium, like soil or a synthetic nutrient solution. As demonstrated more than a century ago, nodulation is inhibited by ammonium salts and by nitrates (20). The effect of nitrogenous compounds on the establishment of an effective symbiosis between leguminous plants and root nodule bacteria has been studied frequently (19, 21, 26). Many stages in the process of infection and nodule development are adversely affected by combined nitrogen, as is shown particularly with nitrate (5, 21, 26).

On the other hand, the nitrogen-fixing activity of an already established symbiosis is also affected by added nitrogenous compounds. Apart from the effect on further nodule development, a more general and non-specific effect can be assumed to arise from so-called photosynthate deprivation (18). This deprivation - a diminished translocation of carbohydrates to the root nodule when combined nitrogen is absorbed by the plant - would result from nitrogen assimilation and concomitant carbohydrate consumption in the roots and the shoots (18). Experimental evidence for this theory has come from investigations with $^{14}CO_2$ on the translocation of photosynthates and their distribution among the different parts of the plant. With both peas (23) and soybeans (12) it was shown that in plants with added nitrogenous compounds less carbohydrates were transported to the nodules than in plants without this

addition. The hypothesis of photosynthate deprivation is founded upon the generally accepted idea that carbohydrate supply is the natural regulator of nitrogenase activity in symbiotic systems (8, 18). This idea is supported, for instance, by the diurnal fluctuations in nitrogenase activity (3, 9), by the effect of removal of flowers or pods (13, 14) and by grafting experiments with two shoots on a single root (25).

Furthermore, the value of the photosynthate deprivation theory can be demonstrated with more or less indirect tests. For instance, the reversibility of the nitrogenase inhibition by nitrate and the period of time during which it is effectuated are in accordance with the assumption and show that no irreversible damage is done (7). The fact that nitrogenase *in vitro* is not inhibited by ammonium ions or by amino acids (11) suggests that such an inhibition does not occur in the *in vivo* system. Finally, it has been demonstrated that the decrease in the nitrogen-fixing activity of pea plants upon the addition of ammonium chloride was not accompanied by a decrease in the *in vitro* activity of isolated bacteroids supplied with ATP and reductants (10).

In this communication the arguments mentioned above are substantiated by experiments concerning the interaction of the effect of ammonium chloride with other physiological factors which may influence the carbohydrate supply of the nodule.

MATERIALS AND METHODS

Plants

Pea plants (*Pisum sativum* cv. Rondo) inoculated with *Rhizobium leguminosarum* PRE were grown in gravel with a nitrogen-free nutrient solution (15). They were cultured in a growth chamber at 18-20°C with a 16 h light-8 h dark period, light intensity on the average being 12,000 lux. Two weeks after sowing, plants were transferred from gravel to 300-ml Erlenmeyer flasks with 200-ml nutrient solution (5 plants per Erlenmeyer, sustained with a plug of cotton wool). Treatments with combined nitrogen and/or sucrose were performed by replacing the nutrient solution by a medium containing the desired compound. This medium was refreshed daily during the experiment to prevent drop of pH, usually occurring with supply of ammonium salts.

Bacteroids

Root nodules were pressed in a Bergersen-press (2) under argon. The buffer contained 50 mM Tris-HCl (pH 7.2), 2.5 mM $MgCl_2$, 4% polyvinyl-pyrrolidone (PVP) and 20 mM sodium dithionite. Bacteroids were spinned down (10 min at 5,000 x g), washed with buffer (same buffer without PVP), spinned down again (10 min at 5,000 x g) and resuspended in buffer without PVP. A quantity of bacteroids corresponding to 80 mg of fresh weight nodules was used per ml of buffer. Immediately prior to the start of the assay bacteroids were treated with EDTA and toluene (24). Protein was determined with the Lowry method, with bovine serum albumin as a standard.

Acetylene reduction

Intact plants were incubated in closed Erlenmeyer flasks (5 plants in 1000-ml flasks) with 10% acetylene in air. After 15-20 min, ethylene produced was measured gas-chromatographically. Bacteroids were incubated in 16.5-ml Hungate tubes in a shaker bath (25°C, 200 strokes/min). The assay mixture contained 0.5 ml of EDTA-toluene-treated bacteroid suspension, 50 µmoles of Tris-HCl, 15 µmoles of $MgCl_2$, 18.4 µmoles of creatine phosphate, 5.6 µmoles of ATP, 0.03 mg of creatine phosphokinase and 20 µmoles of $Na_2S_2O_4$, in a total volume of 1 ml. The gas phase was 10% acetylene in argon and the incubation time was 10 min.

Uptake of NH_4Cl

The uptake of ammonium chloride by the plant was determined using ammonium chloride (20 mM) enriched with 25 atom % ^{15}N in the nutrient solution. After the ammonium treatment, parts of the plant (roots, shoots and nodules) were harvested separately and dried. Total nitrogen was determined after destruction of the plant material using the Kjeldahl method. Atom % ^{15}N excess was assayed using emission spectrometric analysis (1) and calculated according to Ferraris and Proksch (6). From this figure the amount of N taken up by the plant was computed.

RESULTS

Effect of NH_4Cl and KNO_3 on acetylene reduction

Ammonium chloride or potassium nitrate was added to 4-weeks-old pea plants growing in culture solution. After 1 and 3 days the acetylene-reducing activity of plants with different treatments (10 mM salt, 20 mM salt or no salt addition) were compared (Table 1). At a salt concentration of 10 mM, potassium nitrate had a more pronounced effect than ammonium chloride, as appears from the values measured after 3 days. A concentration of 20 mM of either of the salts gave identical results: 50-60% inhibition. In further experiments a concentration of 20 mM was applied to obtain significant and pronounced short-term effects.

Table 1. Influence of NH_4Cl and KNO_3 on the acetylene reducing activity of intact pea plants[a]

Addition	Day 1	Day 3
None	4.0 *a*	4.4 *a*
NH_4Cl (10 mM)	3.1 *b*	3.1 *b*
NH_4Cl (20 mM)	1.5 *d*	1.9 *d*
KNO_3 (10 mM)	2.5 *bc*	1.9 *d*
KNO_3 (20 mM)	2.1 *cd*	1.7 *d*

[a]Activities in micromoles C_2H_4 per plant per h.
Values are averages of 5 determinations; standard deviations 0.2-0.9.
Values not significantly different at the 5% level according to Tukey's test are indicated with the same letters.

The influence of nitrogenous salts on bacteroid activity itself (when the salts were added to the intact plant) was determined with the acetylene reduction test with EDTA-toluene-treated bacteroids (Table 2). When plants were treated with NH_4Cl or KNO_3, nitrogenase activity of isolated bacteroids supplied with ATP and dithionite did not decrease, whereas the specific activity of the intact plant was inhibited by about 50% after 3 days. This result suggests that the decrease of the nitrogenase activity does not originate from a drop of the amount of potentially active enzyme.

Table 2. Influence of NH_4Cl and KNO_3 on the *in vivo* and the *in vitro* nitrogenase activity of pea bacteroids

Treatment	Activity/plant[a]	Specific activity[b]	*In vitro* activity[c]
Control	4.1	47.6	15.6
NH_4Cl (20 mM, 3 days)	2.4	25.2	18.1
KNO_3 (20 mM, 3 days)	2.4	24.9	16.0

[a] µmoles C_2H_4/plant per h; standard deviations 0.3–0.9
[b] µmoles C_2H_4/g fresh weight nodule per h; standard deviations 3.2–13.6
[c] µmoles C_2H_4/mg protein per min; standard deviations 0.4–1.3
Values are averages of three determinations.

Effect of NH_4Cl on plant growth

In a 6-day experiment the influence of ammonium chloride (at a 20 mM concentration) on the growth of roots, shoots and nodules of pea plants was studied, and compared with the acetylene-reducing activity of the intact plants (Fig. 1). A linear regression of dry weight against time (regression coefficient b, correlation coefficient r) was assumed and straight lines were traced to obtain a surveyable view. Growth of root nodules was retarded when ammonium chloride was added, whereas the growth of roots and shoots was not affected. In the same experiment the acetylene reduction was measured and plotted in the same way. A decrease in the activity per plant was found, arising in part from the diminished increase of the nodule mass and in part from the reduced specific nitrogenase activity (see the following section).

Uptake of nitrogen and nitrogenase inhibition

Plants which had developed an active nitrogenase system were fed with ammonium chloride enriched with ^{15}N. The distribution of the absorbed nitrogen was determined after 1, 2 and 3 days of treatment, in an attempt to find any correlation between the uptake of ammonia and the decrease in nitrogenase activity. Fig. 2 gives the enrichment in ^{15}N of different parts of the plant and the specific acetylene-reducing activity of the nodules. In the roots the concentration of newly absorbed nitrogen seemed to be built up to a maximum, whereas the concentration in the shoot and in the nodules increased almost linearly, but at a low rate, during the experiment. In the nodules, the concentration of absorbed nitrogen was low compared to that in the root and in the shoot. The decrease of the specific acetylene-reducing activity was most

pronounced during the first day of the treatment and less steeply afterwards, suggesting an inverse proportionality with the nitrogen uptake by the roots.

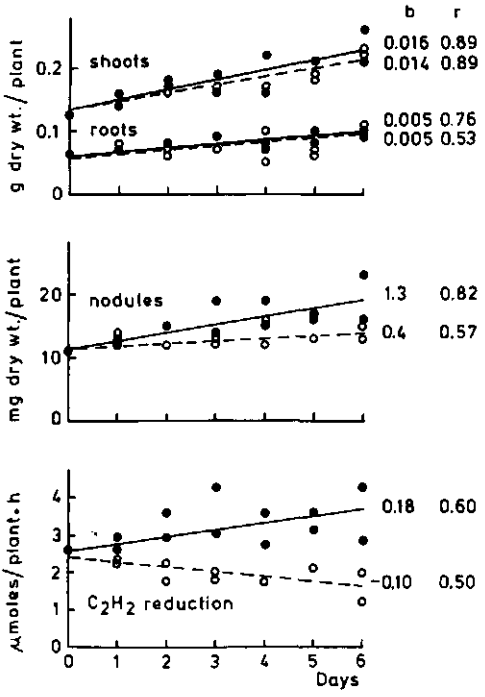

Fig. 1. Influence of NH_4Cl on the growth of roots, shoots and root nodules of pea plants, and on the nitrogenase activity of the intact plants. Each mark represents the average of 3 plants, treated with 20 mM KCl (•——•) or 20 mM NH_4Cl (o---o). Linear regression coefficients (b) and correlation coefficients (r) are given; upper number applies to KCl-, lower number to NH_4Cl-treated plants.

Effect of light intensity

The interaction between the effect of combined nitrogen and that of light intensity was investigated with two groups of four-week-old plants of which one group was placed under a twice as high light intensity as the other. After 3 days the effect of added ammonium chloride (20 mM) was traced in both groups. Table 3 shows that doubling the light intensity increased the nitrogenase activity by about 45% and weakened the inhibitory effect of ammonium chloride. After 2 days the nitrogenase activity of ammonium-treated plants at 13,000 lux was significantly lower than that of the control plants. At 26,000 lux this

claim was not true. When the decrease in the acetylene-reducing activity after NH_4Cl application was assumed to be a linear regression with time, the regression coefficients at the two distinct light intensities were significantly different at the 5% level. Therefore, it is concluded that the inhibition of nitrogenase activity by ammonium chloride can be counteracted by raising the light intensity.

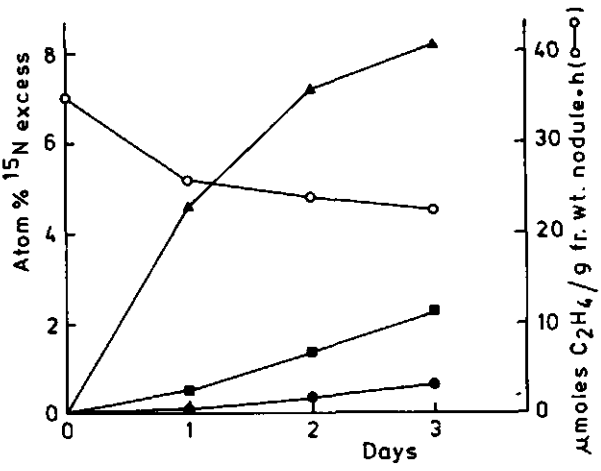

Fig. 2. Uptake of ^{15}N-enriched ammonium nitrogen and distribution of the absorbed nitrogen among roots (▲), shoots (■) and nodules (●) of pea plants, and the influence of NH_4Cl on the specific acetylene-reducing activity of the nodules (○). Each mark represents the average of duplicate determinations.

Table 3. Inhibition of nitrogenase activity by NH_4Cl as affected by light intensity[a]

Light intensity (lux)	Salt added (20 mM)	Day			
		0	1	2	3
13,000	KCl	2.3 *ab*	2.1 *ab*	2.2 *a*	2.5 *a*
13,000	NH_4Cl	2.5 *a*	1.7 *abc*	1.3 *bc*	1.0 *c*
26,000	KCl	3.4 *de*	3.5 *de*	3.6 *d*	3.8 *d*
26,000	NH_4Cl	3.5 *de*	3.1 *de*	2.9 *de*	2.4 *e*

[a] Activities in µmoles C_2H_4 produced per plant per h.
Values are averages of 8 determinations; standard deviations 0.6-1.0
Values not significantly different at the 5% level according to Tukey's test (within any of the light intensities) are indicated with the same letters.

Effect of sucrose

In another attempt to relieve a possible shortage in photosynthates, sucrose was added to the nutrient solution of the plants. To oppose bacterial growth, 12 mg/l of chloramphenicol was applied in these experiments; with this precaution acidification of the solution owing to bacterial activity could be prevented (Table 5). Sucrose was added 2 days before the start of the NH_4Cl treatment; this addition resulted in an increase of the nitrogenase activity by about 30% (Fig. 3). It is shown that the inhibitory effect of NH_4Cl on the nitrogenase activity was less pronounced when sucrose

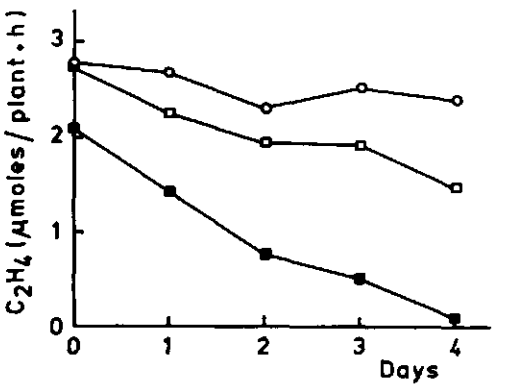

Fig. 3. Influence of NH_4Cl on the acetylene-reducing activity of intact pea plants, as affected by sucrose: no (■), 0.5% (□) and 2% sucrose (o). Each mark represents the average of 6 determinations; standard deviations 0.2-0.6.

was present. Linear regression coefficients of the acetylene-reducing activity against time were 0.54, 0.27 and 0.11 with 0, 0.5 and 2% of sucrose, respectively; these coefficients were significantly lower in the presence of sucrose than without sucrose addition.

However, the presence of sucrose also affected the uptake of nitrogen by the plant, as was shown with ^{15}N-enriched NH_4Cl (Table 4). After 1 day of ammonium chloride treatment less newly absorbed nitrogen was found in the shoot when 0.5% sucrose was added, whereas the concentration of absorbed nitrogen in the root was unaffected. This effect was even more pronounced when 2% sucrose was added (not shown). These results suggest a decreased transport stream of N-compounds from the root to the shoot as a consequence of the presence of sucrose

in the medium. The same conclusion can be drawn from results of transpiration measurements (Table 5): when sucrose was present in the nutrient solution less medium was taken up by the plant as compared to the control.

Table 4. Uptake of ^{15}N-enriched NH_4Cl by pea plants as affected by sucrose[a]

Plant part	Atom % ^{15}N excess		N taken up/plant (mg)	
	no sucrose	0.5% sucrose	no sucrose	0.5% sucrose
Roots	5.07	5.48	0.64	0.75
Shoots	0.81	0.42	0.26	0.17
Root nodules	0.27	0.25	0.02	0.02

[a] Plants were treated with a NH_4Cl solution (20 mM) with a ^{15}N-enrichment of 24% for 24 h.

Table 5. Influence of sucrose on the uptake and on the pH of nutrient solution[a]

Sucrose addition	Solution used/plant (ml)	pH of solution
None	5.9 ± 0.3	6.5 ± 0.1
0.5 %	5.4 ± 0.4	6.4 ± 0.1
1.0 %	4.9 ± 0.6	6.4 ± 0.1
2.0 %	3.6 ± 0.4	6.3 ± 0.1

[a] Plants were treated with a 20 mM NH_4Cl solution, with sucrose. During 4 days the daily uptake of solution (determined by means of the decrease in weight of the nutrient medium) and the pH of the solution were measured. Average values over these 4 days, ± standard deviations, are given.

DISCUSSION

Inhibition of nitrogen-fixing activities of legume-*Rhizobium* symbiotic systems by ammonium- and nitrate-ions - as well as other, less frequently studied nitrogenous compounds - has drawn the attention of several investigators since many years. In many cases the effect of ammonium salts is less pronounced than that of nitrates (16, 21). This difference was also found in the present study with peas, when the salt was added at a concentration of 10 mM (Table 1). It could be accounted for by a better uptake

mechanism for nitrates compared to that for ammonium salts. A higher concentration of ammonium ions would overcome this apparent disparity (see Tables 1 and 2, results of a 20 mM treatment), thus allowing an investigation on the fundamental effects of nitrogenous salts independent of concentration effects.

The decrease of the nitrogenase activity of nodulated pea plants caused by added ammonium chloride (Fig. 1) partly originated from a decreased nodule growth. Apart from this effect on nodule development, the specific activity of the nodules (C_2H_2 reduced per unit nodule weight) also diminished. This drop was not brought about by a decreased quantity of potentially active nitrogenase: the activity of isolated bacteroids, treated with EDTA and toluene and provided with ATP and reductants, had not declined (Table 2). Therefore, it is concluded that the decrease in specific nitrogenase activity of the root nodules was a result of plant influences. When the time course of nitrogenase inhibition after addition of NH_4Cl is followed and compared with the uptake of nitrogen, a correlation with the concentration of newly adsorbed nitrogen in the roots was observed (Fig. 2).

As mentioned in the introduction, a theory to explain the effects of nitrogenous compounds on nitrogen fixation of symbiotic systems emphasizes the carbohydrate supply as a major regulatory factor. According to this theory an increase of photosynthate supply might counteract the inhibitory effect of nitrogen salts. Table 3 shows that variation of photosynthetic activities by changing the light intensity, affected the drop of nitrogenase activity of intact plants caused by NH_4Cl according to this prediction. Similar relationships were found by other investigators, using somewhat different techniques (4). Variation of the carbohydrate supply affects both specific nitrogenase activity and growth of the nodules.

Another way to provide the plant with additional carbohydrates is the addition of sugars to the rooting medium. This treatment enhances the nodulation of legumes (22); an increase of the C_2H_2-reducing activity by added sucrose can be observed in Fig. 3. Sugars have been utilized in investigations on the nodulation process, in an attempts to alter the C to N ratio; addition of sugars to intact plants or to isolated root systems was found to favour nodulation in the presence of combined nitrogen (19, 26). Fig. 3 shows that the decrease of the acetylene-reducing activity caused by added ammonium chloride is counteracted by sucrose. As sucrose apparently also affects the uptake and transport of nitrogen (Tables 4 and 5), influences

of sugars can not merely be ascribed to an increase of the internal carbon concentration (additional supply of carbohydrates).

Summarizing the results reported here and in a former publication (10), it can be concluded that ammonium chloride (and probably potassium nitrate as well) when added to nodulated leguminous plants has no direct effect on nitrogenase activity. Its effect, when added to whole plants, may be ascribed to creating a shortage of carbohydrates in the nodules.

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5. EFFECT OF AMMONIUM CHLORIDE AND METHIONINE SULFOXIMINE ON THE ACETYLENE REDUCTION OF DETACHED ROOT NODULES OF PEAS (*PISUM SATIVUM*).

Acetylene-reducing activity of detached pea nodules was determined by submerging the nodules in buffer solution (tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.4) containing 100 mM sodium succinate and incubating under a gas phase of 90% O_2 and 10% C_2H_2. The nitrogenase activity was 4 to 8 µmoles of C_2H_4 formed per g of nodule fresh weight per h and remained constant for at least 4 h. Addition of NH_4Cl to the buffer solution (at a concentration of 10 mM or more) resulted in a significant decrease of nitrogenase activity, which was more pronounced at higher concentrations of ammonium chloride. The inhibition of nitrogenase activity by NH_4Cl was reversible; when the NH_4Cl-containing buffer solution was replaced by buffer without NH_4Cl, the original activity was partly restored. Treatment of the nodules with NH_4Cl had almost no effect on the amount of nitrogenase, as measured by the acetylene-reducing activity of ethylenediamine-tetraacetate-toluene-treated bacteroid suspensions. The effect of NH_4Cl was largely eliminated by simultaneous addition of 10 mM methionine sulfoximine to the assay solution. This suggests that the assimilation of ammonium ions by glutamine synthetase controls the functioning of nitrogenase activity in the nodules. However, no effect of glutamine, glutamate or aspartate on the acetylene reduction by detached nodules could be detected.

INTRODUCTION

Addition of ammonium or nitrate ions to a legume-*Rhizobium* symbiosis influences the capacity of the system to fix atmospheric nitrogen (11). If combined nitrogen is added when the symbiosis is still developing, further development is disturbed. If it is added when the symbiosis is fully developed, nitrogenase activity decreases gradually. The plant seems to prefer the uptake of combined nitrogen over the fixation of dinitrogen, possibly because it is more efficient in terms of energy consumption.

The physiological mechanism of this regulation in symbiotic systems is not well understood. More information is available about this process in free-living, nitrogen-fixing microorganisms like *Azotobacter* (8), *Klebsiella* (26), and *Clostridium* spp. (7). In these, nitrogenase synthesis is blocked in the presence of NH_4^+. This repression is thought to be mediated by the NH_4^+-assimilating system, either by the enzyme glutamine synthetase itself or by

the products of NH_4^+ assimilation, amino acids (20, 23). An immediate effect of added NH_4^+ on nitrogenase activity, which was not due to repression, was observed by S. Brotonegoro (Ph.D. Thesis, Agricultural University, Wageningen, The Netherlands, 1974). This effect is thought to be due to interference with the energy supply. Comparison of the nitrogen-fixing system of free-living bacteria with that of symbiotic systems is complicated by the regulation by the host plant of nitrogenase activity in the latter. Examples of such regulation are the supply of photosynthates to,and the transport of fixed nitrogen from, the nodules (18).

Several effects resulting from the addition of combined nitrogen to a legume-*Rhizobium* symbiosis have been demonstrated. The distribution pattern of photosynthates in the plant is changed (14), the synthesis of leghemoglobin in the nodule decreases (4, 6), and the activity of certain ammonia-assimilating enzymes in roots and nodules is altered (9). However, these studies do not show which is the primary effect.

In a previous study (13) it was demonstrated that the amount of nitrogenase in pea nodules is not diminished when nitrogenase activity of intact plants is reduced by the addition of ammonium chloride. It was also suggested that most of the nitrogen taken up by the plant does not reach the nodules and is probably assimilated in roots or shoots. To avoid the problem of interfering processes in the plant, it seems desirable to study isolated parts of the symbiotic system, in which the influence of the plant is at least partially eliminated. However, it should be clear that results obtained with detached nodules cannot be related directly to results obtained with whole plants. In the present study nitrogen compounds were added directly to detached nodules, and the effect on nitrogenase activity in these nodules was determined.

MATERIALS AND METHODS

Plants

Pea plants (*Pisum sativum* L. cultivar 'Rondo') inoculated with *Rhizobium leguminosarum* PRE were grown in gravel with a nitrogen-free nutrient solution (15) in a growth chamber at 18 to 20°C and with a light intensity of 12,000 lx and an 8-h dark period. They were harvested at 27 to 31 days after sowing.

Detached nodules

Root nodules were carefully detached from the roots and kept on wet filter paper until use. The acetylene-reducing activity of detached nodules was measured as follows. Nodules (20 to 50 mg, fresh weight) were submerged in 1 ml of a 50 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer solution (pH 7.4) containing 2.5 μmol of $MgCl_2$ and 100 μmol of sodium succinate in a 16.5-ml Hungate tube. The tubes were flushed with pure O_2 for 10 min, and the reaction was started by the addition of acetylene, giving a gass phase of 90% O_2 and 10% C_2H_2. During the assay, the tubes were shaken in a shaker bath at 24°C at a rate of 200 strokes per min. At regular time intervals ethylene was measured chromatographically. The effect of compounds to be tested was studied by adding these compounds to the assay solution before starting the acetylene reduction.

NH_4Cl uptake

The uptake of ammonium chloride by detached nodules when submerged in an NH_4Cl solution was determined by using NH_4Cl enriched with 50.9% ^{15}N. After the $^{15}N_4Cl$ treatment nodules were rinsed with water and dried, and their ^{15}N content was determined as described by A.D.L. Akkermans (Ph.D. Thesis, State University of Leiden, Leiden, The Netherlands, 1971); the amount of NH_4Cl taken up was calculated by the method of Ferraris and Proksch (10).

Bacteroid suspensions

The potential nitrogenase activity of the nodules was measured with ethylenediaminetetraacetate (EDTA)- and toluene-treated bacteroids, as described originally by van Straten and Roelofsen (22). The exact methods for preparing bacteroid suspensions and for the determination of acetylene-reducing activity after EDTA-toluene treatment were described previously (13).

Nitrogen determination

For the determination of the total N content of the nodules, digestion was performed by the Kjeldahl method, followed by distillation of the NH_4^+ formed and determination of ammonia with Nessler reagent. The amount of free ammonium

nitrogen was assayed after vacuum distillation (at 50°C and pH 9.4) of an 80% methanol extract of nodule brei.

RESULTS

Acetylene reduction by detached and submerged nodules

To test the effect of added N compounds on acetylene reduction by detached nodules, it was necessary to develop a method which would give reproducible results and a constant activity for at least several hours. In addition, these compounds had to enter the nodule. The acetylene reduction method with submerged soybean nodules as described by Sprent (21) seemed to be suitable for this purpose. Some preliminary experiments were carried out to find optimum conditions for the assay. An important factor was the oxygen concentration in the gas phase; highest activities were found with 90% O_2 (Fig. 1). When sodium succinate

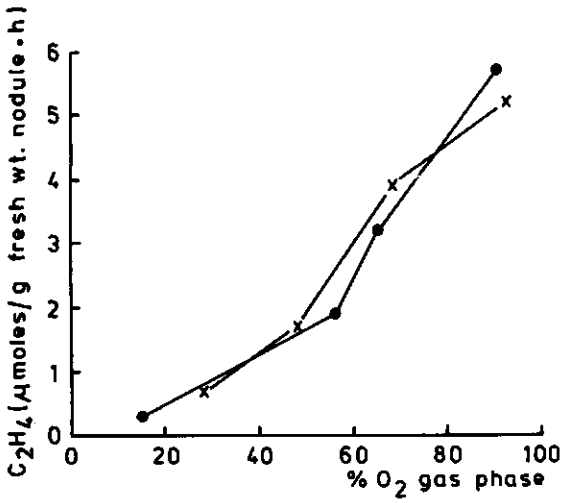

Fig. 1. Acetylene reduction of detached pea root nodules submerged in buffer solution in relation to the oxygen concentration in the gas phase. Two separate experiments were performed (× and ●). The activity was measured between 2 and 4 h after starting the assay. A total of 20 mg of nodules was used per assay. Values are averages of triplicate determinations; standard deviations varied from 0.2 to 0.5 μmole per g of nodule fresh weight per h.

(final concentration, 100 mM) was added to the assay solution, the acetylene-reducing activity remained constant for 4 h. Lower concentrations of sodium succinate had less effect; sucrose was fully ineffective in stimulating the activity (Fig. 2). With 100 mM succinate an activity of 5 to 10 µmol of C_2H_4 per g of nodule fresh weight per h was measured. This is about 15 to 30% of the activity of the intact system (plant with nodulated roots).

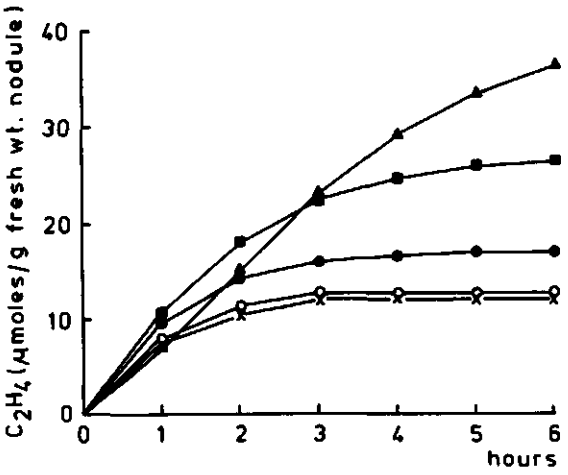

Fig. 2. Acetylene reduction of detached pea root nodules with different concentrations of carbon sources added to the assay solution. Symbols: O, no carbon source added; ●, 10 mM sodium succinate; ■, 50 mM sodium succinate; ▲, 100 mM sodium succinate; x, 100 mM sucrose. A total of 20 mg of nodules was used per assay. Values are averages of triplicate determinations; standard deviations varied from 0.5 to 3.8 µmol/g of nodule fresh weight.

Effect of ammonium chloride

Results of an experiment with different concentrations of NH_4Cl added to the assay solution are shown in Fig. 3. There was a significant decrease in activity with NH_4Cl at a concentration of 10 mM or more. Moreover, in the presence of NH_4Cl the rate of the reaction decreased more than the rate without NH_4Cl (Fig. 4). The inhibitory effect of NH_4Cl at a concentration of 10 mM was not significant until after 3 h. To obtain a more pronounced and faster effect, a concentration of 25 mM was used in most experiments. Control experiments

were performed with KCl at the same concentrations. The rate of acetylene reduction with KCl was only slightly decreased as compared with the activity of nodules without salt addition (Fig. 3 and 4). Ammonium acetate tested at a

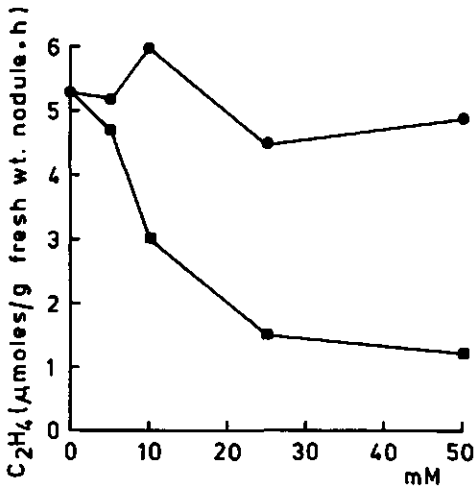

Fig. 3. Influence of KCl and NH_4Cl on the acetylene reduction of detached pea root nodules. The activity was measured between 2 and 3 h after starting the assay. A total of 40 mg of nodules was used per assay. Values are averages of duplicate determinations. Symbols: ●, KCl; ■, NH_4Cl.

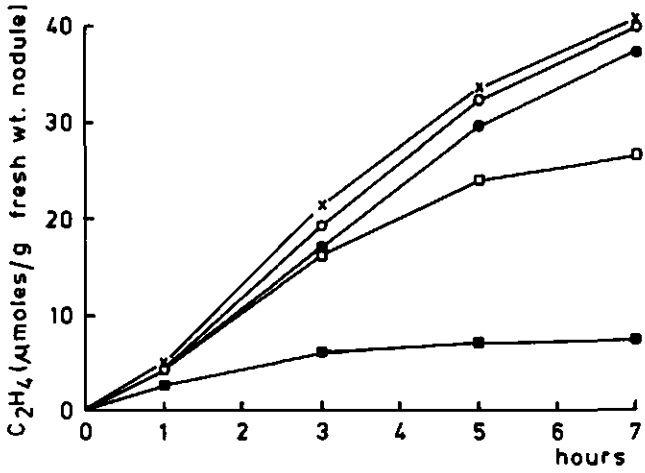

Fig. 4. Time course of the acetylene reduction of detached pea root nodules and the influence of different concentrations of KCl and NH_4Cl. A total of 30 mg of nodules was used per assay. Values are averages of duplicate determinations. Symbols: ✖, no salt; ○, 10 mM KCl; ●, 50 mM KCl; □, 10 mM NH_4Cl; ■, 50 mM NH_4Cl.

10 mM concentration gave the same effect as NH_4Cl at 10 mM (data not shown). Even with the highest concentration of NH_4Cl, there was no change in pH of the assay solution after 6 h; with both KCl and NH_4Cl the pH was 7.3.

Reversibility of the ammonium effect

It was of interest to determine whether the inhibition of nitrogenase activity by NH_4Cl was reversible or irreversible. To decide between the two possibilities, nodules were incubated with 20 mM NH_4Cl, and after several hours the solution was replaced by a solution without NH_4Cl. The acetylene-reducing activity was slowly restored after the removal of NH_4Cl (Fig. 5). Activity increased to about 65% of the activity of the control when NH_4Cl was removed after 2 h, and to about 45% when removal took place after 4 h. When NH_4Cl was not removed, the activity decreased to about 5%.

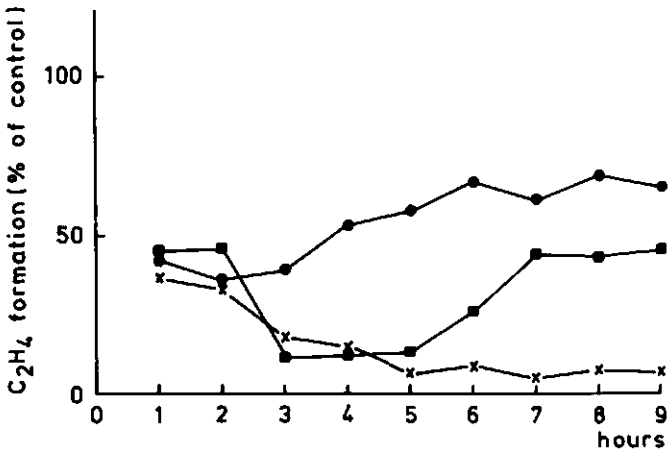

Fig. 5. Reversibility of the ammonium inhibition of the acetylene reduction of detached pea root nodules. The activity in the presence of 20 mM NH_4Cl is expressed as the percentage of the activity of untreated nodules. Percentages were calculated from average values of five determinations. A total of 15 mg of nodules was used per assay.
Symbols: ✕, NH_4Cl present during 9 h; ■, NH_4Cl removed after 4 h; ●, NH_4Cl removed after 4 h.

Potential nitrogenase activity of the nodules

Another way to obtain information on the nature of the ammonium inhibition of nitrogenase activity is to measure the potentially active nitrogenase present in the nodules. This nitrogenase activity was determined with isolated bacteroids after treating them with EDTA and toluene (22). Detached nodules were incubated in buffer containing 25 mM NH_4Cl or 25 mM KCl (control), and acetylene reduction was measured. After 2 and 3 h, nodules were washed, and bacteroids were isolated. As shown in Table 1, the decrease in potential nitrogenase activity was much less pronounced than the decrease in nitrogenase activity of intact nodules. When the activity of the nodules was inhibited by NH_4Cl to 40 and 80% (after 2 and 3 h, respectively), the activity of EDTA-toluene-treated bacteroids from these nodules was only 5 and 20%, respectively, less than that of the control. Thus, the decreasing activity, measured in the presence of NH_4Cl, was not due to a decreasing amount of nitrogenase in the nodules.

Table 1. Acetylene reduction of detached pea root nodules and isolated EDTA-toluene-treated bacteroids[a]

Expt	Incubation time (h)	Amt of C_2H_4 formed (μmoles/g fresh weight nodule per h)			
		Control (KCl)		NH_4Cl treated	
		Nodules	Bacteroids	Nodules	Bacteroids
1	2	4.5	27.6	2.6	26.5
2	2	4.9	33.2	2.8	28.7
3	3	3.7	24.6	0.8	18.2
4	3	5.0	17.9	2.5	16.6

[a] Activity of detached nodules (60 mg of nodule fresh weight per assay) was measured in the presence of 25 mM KCl or 25 mM NH_4Cl. After 2 and 3 h nodules were washed, bacteroids were isolated (300 mg of nodule fresh weight per assay) and activity was measured after EDTA-toluene treatment.

Effect of MSX

To determine whether the assimilation of ammonium ions was interrelated with the effect of NH_4Cl on nitrogenase activity, methionine sulfoximine (MSX) was used. This glutamine analog is known to be an inhibitor of glutamine synthetase in bacteria (5, 12) and higher organisms (16); the enzyme is present in root nodules of leguminous plants and probably has a key role in the assimilation of fixed nitrogen in higher plants (16). Figure 6

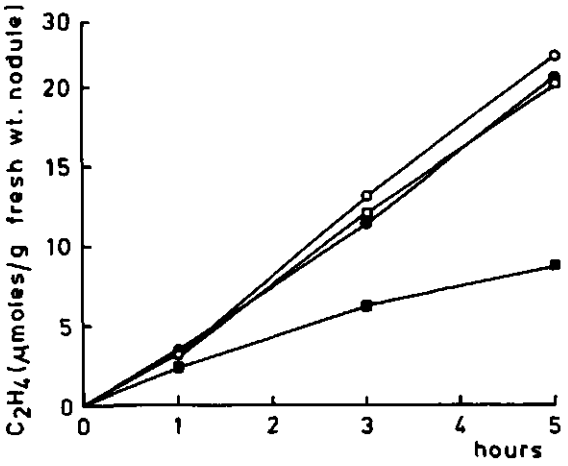

Fig. 6. Influence of MSX on the acetylene reduction of detached pea nodules in the presence of KCl or NH_4Cl. Symbols: ●, 25 mM KCl; ■, 25 mM NH_4Cl; ⊖, 25 mM KCl + 10 mM MSX; □, 25 mM NH_4Cl + 10 mM MSX. A total of 40 mg of nodules was used per assay. Values are averages of duplicate determinations.

shows that the inhibitory effect of NH_4Cl at a concentration of 25 mM on the acetylene-reducing activity of detached nodules could be prevented by simultaneous addition of MSX at a 10 mM concentration. Addition of MSX to the control assay with KCl had no effect on the nitrogenase activity. A lower concentration (1 mM) had no significant effect, whereas a higher concentration (100 mM) decreased acetylene-reducing activity both in the assay with NH_4Cl and in the control with KCl. Methionine sulfone, another inhibitor of glutamine synthetase, which is less effective (5), did not show the same effect as MSX in combination with ammonium chloride (Table 2).

Table 2. Acetylene reduction of detached pea nodules and the influence of KCl, NH_4Cl, MSX and methionine sulfone[a]

Addition to assay solution	Amount of C_2H_4 formed (μmoles/g of nodule fresh wt per h)
KCl (25 mM)	4.1 ± 0.5
KCl (25 mM) + MSX (10 mM)	4.5 ± 0.4
KCl (25 mM) + MSX (100 mM)	2.1 ± 0.2
NH_4Cl (25 mM)	1.6 ± 0.7
NH_4Cl (25 mM) + MSX (1 mM)	1.4 ± 0.4
NH_4Cl (25 mM) + MSX (10 mM)	4.3 ± 0.3
NH_4Cl (25 mM) + MSX (100 mM)	1.9 ± 0.5
NH_4Cl (25 mM) + MSF (10 mM)	1.2 ± 0.2
NH_4Cl (25 mM) + MSF (100 mM)	2.2 ± 0.5

[a] The values (mean of two determinations, ± standard deviations) were measured as the activity between 1 and 5 h after starting the reaction by the addition of acetylene. A total of 30 mg of nodules was used per assay.
MSF, methionine sulfone.

Influence of amino acids on acetylene reduction

To determine whether the inhibition of acetylene reduction by detached nodules in the presence of NH_4Cl was brought about by the ammonium ion itself or by compounds formed during its assimilation, the effects of some amino acids were tested. Glutamine, glutamate, and aspartate (key amino acids in the nitrogen metabolism of the plant) at concentrations of 10 mM, together with KCl or NH_4Cl (also 10 mM), had no marked effect on the acetylene-reducing activity (Table 3). In other experiments (data not shown) it was found that higher concentrations of amino acids (up to 20 mM) were also ineffective in influencing acetylene reduction. The effect of MSX (prevention of the inhibition by NH_4Cl) was also found in the presence of glutamine together with NH_4Cl.

Uptake of ammonium ions by detached nodules

The uptake of NH_4^+ by detached nodules when submerged in an NH_4Cl solution was determined in experiments with $^{15}NH_4Cl$. Detached nodules were treated with NH_4Cl (or NH_4Cl plus MSX) as described above. At 1, 2, or 3 h after starting the treatment, nodules were analyzed for their ^{15}N content. Table 4 gives the amount of NH_4Cl absorbed by the nodules, calculated from these analyses.

Table 3. Acetylene reduced by detached pea nodules in the standard assay after 8 h of incubation in the presence of amino acids[a]

Addition	Amount of C_2H_4 formed (μmoles/g of nodule fresh wt)
KCl (10 mM)	53.2 ± 8.4
KCl (10 mM) + glutamine (10 mM)	50.1 ± 2.1
KCl (10 mM) + glutamate (10 mM)	54.1 ± 6.9
KCl (10 mM) + aspartate (10 mM)	54 0 ± 16.7
NH_4Cl (10 mM)	31.6 ± 5.1
NH_4Cl (10 mM) + glutamine (10 mM)	28.9 ± 4.5
NH_4Cl (10 mM) + glutamate (10 mM)	36.2 ± 7.7
NH_4Cl (10 mM) + aspartate (10 mM)	28.7 ± 5.7

[a] Additions of KCl, NH_4Cl and amino acids to the buffer solution were as shown. Values are averages of three determinations, ± standard deviations. A total of 15 mg of nodules was used per assay.

Table 4. Uptake of NH_4Cl by detached pea nodules when submerged in 20 mM NH_4Cl or 20 mM NH_4Cl plus 10 mM MSX[a].

Incubation time (h)	Amt of uptake (mg of N taken up/g of nodule dry wt)	
	Incubation without MSX	Incubation with MSX
0	0.38 ± 0.13	0.40 ± 0.13
1	1.30 ± 0.06	1.31 ± 0.12
2	1.72 ± 0.54	1.59 ± 0.28
3	2.60 ± 0.04	1.72 ± 0.18

[a] NH_4Cl was enriched with 50.9% ^{15}N; the amount of NH_4 taken up by the nodules was calculated from the ^{15}N analyses. The values are means of two determinations in separate experiments ± standard deviations of the mean. A total of 80 mg of nodules (fresh weight) was used per determination.

For comparison, the total N content and the free NH_3-N content in untreated nodules were determined. Nodules contained 89.1 ± 1.5 mg of N per g of nodule dry weight and 0.86 ± 0.03 mg of NH_3-N per g of nodule dry weight (averages of six determinations ± standard deviations). The uptake of NH_4^+ by nodules submerged in 20 mM NH_4Cl was continued for 3 h. At that time about 2.5% of the amount of nitrogen originally present in the nodule was adsorbed. In the presence of MSX the adsorption of NH_4^+ was reduced after 3 h of incubation.

DISCUSSION

The effect of nitrogen compounds on acetylene reduction by detached root nodules was studied by a method in which the nodules were submerged in buffer solution under increased oxygen tension, as described first by Sprent for soybeans (21). This assay method has some advantages as compared with the method of incubating nodules on wet filter paper (1): acetylene reduction is prolonged for a longer period of time, results are more reproducible, and the addition of compounds is easier. However, the nodules are kept under rather unnatural conditions, and this must be considered when interpreting the results. Nevertheless, it seemed to be the best method to determine the nitrogenase activity of detached nodules, and, keeping in mind the restrictions, results can be compared with the effects of N compounds on nitrogenase activity of intact plants.

The oxygen concentration was an important factor for the acetylene-reducing activity of submerged nodules. Even at 90% O_2 in the gas phase, oxygen supply to the nodules might be the limiting factor (Fig. 1). However, this limitation also determines the activity of nodules on filter paper (1). The energy production necessary for the activity of nitrogenase can be achieved by the respiration of endogeneous substrate still present in the nodule. This substrate seemed to be exhausted within 3 h (Fig. 2). Prolonged acetylene reduction could be achieved by adding succinate. The effect of succinate was not osmotic, because sucrose was not effective. Other workers have found a prolonged activity of detached nodules (*Lupinus* and *Lotus*) when kept on agar with sucrose (19, 24). Under those circumstances sucrose perhaps exerts an osmotic effect. The influence of succinate might be due to a prolonged energy production for nitrogenase activity; another explanation might be that oxygen damage of nitrogenase is reduced by an increased oxygen consumption in the nodule (but outside the bacteroids).

When ammonium chloride was added to the assay solution, the acetylene-reducing activity was lower than that in control assays with potassium chloride. This inhibition was more pronounced with higher concentrations of NH_4Cl and with prolonged incubation periods (Fig. 3 and 4). Therefore, ammonium ions had an effect on the nitrogenase activity of detached nodules comparable with the effect on the nitrogenase activity of intact plants (3, 4, 6, 13). With detached nodules, the effect was detectable within a few hours, but with intact plants it took at least 24 h. In the plant, NH_4^+ has

to be taken up via the roots and is transported to other parts of the plant. In the meantime, there may be other effects of NH_4^+ on processes more or less related to nitrogenase activity, and, moreover, NH_4^+ will be assimilated to amino acids. With detached nodules the effect of NH_4^+ is expected to be more direct, and secondary effects due to the plant are thought to be excluded. Ammonium chloride can be directly taken up by the nodules, possibly via the wounds caused by detaching the nodules. When NH_4Cl is added to the roots of intact plants, only transport via the shoot to the nodules is possible. It is not sure, in fact it is even rather improbable, that free NH_4^+ reaches the nodules. It is, nevertheless, interesting to know whether the action of NH_4Cl responsible for the nitrogenase inhibition in intact plants is the same as it is in detached nodules; comparison with NH_4^+ effects on nitrogenase activity of free-living nitrogen fixers - *Azotobacter* (8), *Klebsiella* (26), *Clostridium* (7), *Rhizobium* (17, 25) - might be interesting.

The observed effect of NH_4Cl on nitrogenase activity of detached nodules could be due to repression of nitrogenase synthesis, which is one of the effects of added NH_4Cl on free-living nitrogen fixers. The short period of time after which added NH_4Cl exerts its inhibition on nitrogenase activity of detached nodules makes this suggestion unlikely. Also, results of the experiments with EDTA-toluene-treated bacteroids (Table 1) and the observed reversibility of the inhibition (Fig. 5) point into the same direction.

In a previous study (13) it was shown that primary effects of combined nitrogen on the nitrogenase activity of whole plants are not due to repression. Here, other processes were thought to be responsible for the inhibition, like the repressed synthesis of leghemoglobin (4, 6) or the reduced supply to the nodules of carbon sources (14). There may also be effects on NH_4^+-assimilating enzymes in the nodules (9), which in turn may influence nitrogen fixation.

Inhibition of nitrogenase by NH_4^+ does not seem to explain the observed effect of NH_4Cl on nitrogenase activity of detached nodules either. A direct inhibition of nitrogenase activity in cell-free extracts of nitrogen-fixing bacteria has never been demonstrated. In addition, the activity of isolated bacteroids is also unaffected by NH_4Cl (2; Houwaard, unpublished data). A reasonable explanation for the ammonium effect could be an effect of NH_4Cl on processes connected with nitrogenase, e.g., the provision of energy or reducing power for nitrogenase activity.

In view of the above statements, the observed effect of MSX (Fig. 6) is rather unexpected. This compound is thought to act by changing the conformation

and/or activity of glutamine synthetase,which is connected with the synthesis of nitrogenase in free-living nitrogen fixers (12). It might be that products of the ammonium assimilation are in fact the cause of the nitrogenase inhibition and that prevention of the assimilation would prevent this inhibition. On the other hand, it was observed that these products, amino acids such as glutamine and glutamic acid, are not inhibitory as such when added to detached nodules (Table 3). An explanation of these contradictory results might be that the decrease in nitrogenase activity in the presence of NH_4Cl is due to a lack of energy. This might result from the consumption of C compounds at the ammonia assimilation level (as carbon skeletons or for energy production). Inhibition of NH_4^+ assimilation would restore energy supply without NH_4^+. Another explanation of the observed effects might be that amino acids are still the inhibitory compounds, but are unable to penetrate the nodule or to reach the regulating site when added. An effect of MSX appeared to be the reduction of the uptake of NH_4^+ by the nodules. However, this reduction cannot explain the effect of MSX on NH_4^+ inhibition of acetylene reduction. The added MSX hardly had affected the NH_4^+ uptake after 2 h, when the effect of NH_4^+ on nitrogenase activity was already very pronounced. In experiments extending for a longer period of time, this effect of MSX cannot be ignored. The inhibition of NH_4^+ uptake by MSX might be due to a buildup of NH_4^+ inside the nodule when the assimilation is blocked by inhibition of glutamine synthetase.

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In free-living rhizobia (when not fixing dinitrogen) the functioning of different pathways of glucose breakdown (19) and of the tricarboxylic acid cycle enzymes (13, 14) has been demonstrated. Less is known about the energy-yielding processes in bacteroids which seem to be limited to the use of special substrates (3, 29). This limitation may be due either to a restricted transport system for the uptake of substrates, or to the lack of some enzymes. The tricarboxylic acid cycle is functioning in bacteroids, at least in part, and perhaps in combination with other metabolic pathways (26). A relation between nitrogenase activity and the presence of isocitrate dehydrogenase in the bacteroids has been found, suggesting that the latter enzyme provides reductants for nitrogen fixation (15). Bacteroids contain an electron transport chain, although some of the carriers differ from those of free-living rhizobia (2). This might represent an adaptation to changed environments, like a low free oxygen concentration and the presence of leghemoglobin. The modified electron transport chain, accompanied by a change in oxygen affinity, might be a decisive factor in the determination of the energy efficiency.

This communication describes experiments concerning the use of various carbohydrates by detached nodules; results of these experiments are compared with data on substrate use by isolated bacteroids. The efficiency of substrate use (as expressed in the relation between respiration, acetylene reduction and hydrogen evolution) and the influence of ammonium chloride on nitrogen fixation are also investigated; these factors are involved in the regulation of nitrogenase activity, as was suggested by earlier studies (11, 12).

MATERIALS AND METHODS

Plants and nodules

Pea plants (*Pisum sativum* L. cv. Rondo) inoculated with *Rhizobium leguminosarum* PRE were grown in gravel in a growth chamber (18-20°C, 12,000 lux, 8 h dark - 16 h light) as described before (11).

Root nodules were detached carefully and stored on wet filter paper until use. In the acetylene reduction assay (12) nodules were submerged in buffer solution (Tris-HCl, pH 7.4), 20-40 mg of fresh weight nodules per ml; the gas phase contained 10% C_2H_2 or 10% argon in O_2. Ethylene production, hydrogen production and carbon-dioxide production were measured gas-chromatographically (flame ionization detection for C_2H_4, katharometer detection for H_2 and CO_2).

Bacteroids

Bacteroid suspensions were prepared anaerobically with a Bergersen-press. For the aerobic assay the buffer solution contained 50 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$, 4% polyvinylpyrrolidone (PVP) and 10% sucrose; isolated bacteroids were stored anaerobically in a buffer which contained in addition 1% bovine serum albumin. The acetylene reduction assay was performed with 10 mM succinate present and a gas phase of 1% O_2 in argon (11). Oxygen consumption of bacteroids was measured with an oxygen electrode. The reaction mixture, containing the same buffer, was saturated with air and oxygen uptake was monitored at 24°C. For the anaerobic assay the buffer solution contained 50 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$, 4% PVP and 20 mM Na-dithionite. Isolated bacteroids were treated with 0.5 mM EDTA and 1% toluene for 1 min (27). In the acetylene reduction assay an ATP-generating system (creatine phosphate/creatine phosphokinase) and additional $MgCl_2$ were present (11); the gas phase was 10% C_2H_2 in argon.

RESULTS

Substrate use by detached nodules

In a previous study it was shown that after incubation of detached nodules, submerged in buffer solution, under a gas phase of 90% O_2 and 10% C_2H_2 high and reproducible levels of nitrogenase activity were obtained; when succinate was added to this assay, the acetylene-reducing activity was maintained for a prolonged period of time (12). The same assay method was used to measure the production of CO_2 as an indication of respiratory activity. As shown in Table 1, about 40 µmoles of CO_2 were produced per g of nodule fresh weight per h. This corresponds with an efficiency of about 5 moles of CO_2 produced per mole of C_2H_2 reduced, or a consumption of 16 g of carbohydrate per g of N fixed (using an equivalency between the reduction of 3 moles of C_2H_2 and the fixation of 1 mole of N_2, and a conversion factor of 6 moles of CO_2 out of 180 g of carbohydrates). The same value could be calculated from the acetylene reduction and O_2 consumption rates of isolated bacteroids, when succinate was added (Table 1). However, in the intact root system the carbohydrate use by the nodules per g of N fixed is about 5 g. The respiration and nitrogenase activities of this system were determined by subtraction of the activities of denodulated roots from the activities of nodulated roots.

Table 1. Efficiency of substrate utilization in nitrogen fixation by the pea-*Rhizobium* symbiosis

System	Succinate (mM)	Acetylene reduction	Respiration	mole $CO_2(O_2)$ per mole C_2H_4	mg N_2 per g (CHO)
Nodules on roots	-	45.7	68.4	1.5	207
Detached nodules	-	8.0	43.2	5.4	58
Detached nodules	100	8.1	40.0	4.9	63
Isolated bacteroids	-	0.5	37.6	75.2	4
Isolated bacteroids	10	13.8	69.8	5.1	61

Acetylene reduction in µmoles C_2H_4 per g fresh weight nodule per h.
Respiration in µmoles CO_2 (nodules) or O_2 (bacteroids) per g fresh weight nodule per h.

The effect of different carbon compounds on nitrogenase and respiratory activities of detached nodules, as measured with the assay method described is shown in Table 2. Nodules were incubated with the indicated substrates for 5 h; during this period nitrogenase activity of nodules without added substrate declined to about 10% of the activity at the start of the assay. Hereupon an assay period of an hour followed, after which the data recorded in Table 2 were measured. Most of the carbon compounds tested slightly stimulated respiration as contrasted with nitrogenase activity which was stimulated much more

Table 2. Nitrogenase activity and respiration by detached pea root nodules

Substrate added (100 mM)	C_2H_4	H_2	CO_2
None	0.8	1.6	22.9
Glucose	3.0	4.6	29.2
Pyruvate	1.5	3.8	34.4
Citrate	3.0	-	-
2-Oxoglutarate	2.8	-	-
Succinate	6.2	6.8	26.4
Fumarate	5.6	5.3	25.6
Malate	2.3	4.7	29.3

Acetylene reduction: gas phase 90% O_2 and 10% C_2H_2.
Hydrogen and carbondioxide production: gas phase 90% O_2 and 10% argon.
Activities in µmoles per g fresh weight nodules per h.
Values are averages of 3 determinations; standard deviations 0.5-4.0.

clearly; acetylene reduction and hydrogen production were influenced to about the same extent. Succinate was most effective (100%), followed by fumarate (70-90%), malate (30-60%) and 2-oxoglutarate (40%). Glucose (40-60%), pyruvate (10-40%) and citrate (40%) were also effective, in contrast with the influence of these substrates on isolated bacteroids (Houwaard, results to be published).

Long term experiments with detached nodules

When the assay with detached nodules was continued for 10 h an apparent divergency of nitrogenase activity and respiration was observed (Fig. 1).

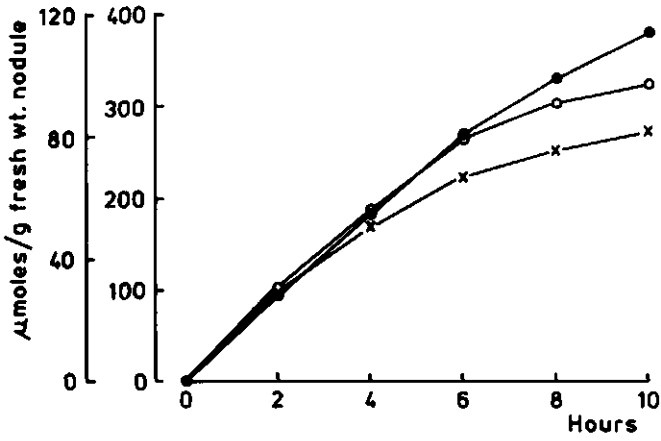

Fig. 1. Acetylene reduction (×), hydrogen production (○) and CO_2 production (●) by detached root nodules (left axis applies to C_2H_4 and H_2, right axis to CO_2).

After 10 h of incubation the nitrogenase activity (both C_2H_2 reduction and H_2 production) was about 20% of that at the start of the assay, whereas respiratory activity was still about 50% (Fig. 1). In this experiment sodium succinate was added to the assay solution, and , to prevent bacterial growth, the assay tubes received 20 µg/ml chloramphenicol. It was investigated to what extent the decrease of the apparent nitrogenase activity was coupled with a similar decrease of the potential nitrogenase activity. The latter activity was estimated by measuring the acetylene reduction by isolated bacteroids after treatment with Tris-EDTA and toluene (27). Results are shown in Fig. 2; detached nodules were incubated in assay tubes, with sodium succinate added to

the buffer solution (100 mM). After 1, 3, 5 and 7 h parts of these nodules were washed and bacteroids were isolated. As can be seen from Figure 2, the

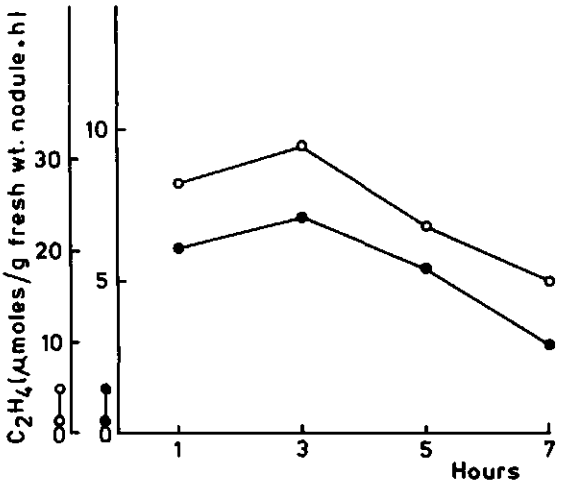

Fig. 2. Acetylene-reducing activity of detached root nodules (●) and of the bacteroids isolated from these nodules (○); bacteroids were treated with EDTA-toluene before the assay.

potential nitrogenase activity also declined during the incubation of the nodules. Therefore, the drop in acetylene-reducing activity in detached nodules might be due to a reduction of the amount of potentially active nitrogenase present in the nodule.

Effect of NH_4Cl on detached nodules

Addition of ammonium chloride (25 mM) to detached nodules reduces the nitrogenase activity within a few hours, as reported before (12). Figure 3 shows that this effect was found with either an efficient substrate (fumarate) or a less efficient one (glucose), both at a 100 mM concentration. With other substrates present a similar inhibition by NH_4Cl could be demonstrated. Table 3 shows the results of another experiment where acetylene reduction, hydrogen production and carbon-dioxide production were measured over an incubation period of 5 h with succinate as substrate. Both acetylene reduction and hydrogen production (under an argon-oxgyen atmosphere) were decreased by NH_4Cl to the same extent. Thus the ammonium effect can be considered to be a general inhibition of nitrogenase activity.

On the other hand, respiration (CO_2 production) of detached nodules was not influenced by NH_4Cl at all (Table 3). Again no close connection between respiration and nitrogenase activity was found, as was demonstrated when nodules were incubated for 10 h.

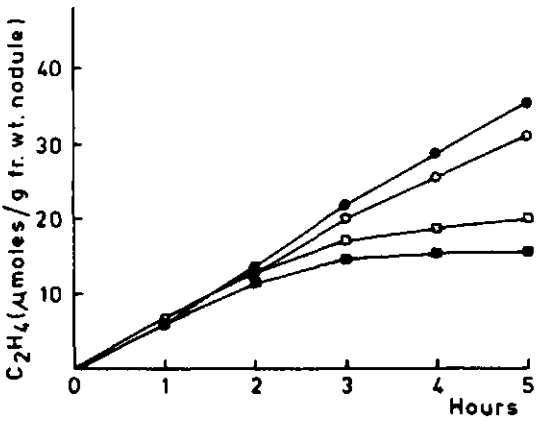

Fig. 3. Influence of NH_4Cl on the acetylene reduction by detached nodules. Addition of fumarate (●,■) or glucose (○,□) and NH_4Cl (■,□) or KCl (●,○).

Table 3. Effect of NH_4Cl on nitrogenase and respiratory activities of detached pea root nodules

Atmosphere	Gas produced	μmoles C_2H_4/g fresh wt.nodule per 5 h	
		+KCl (25 mM)	+NH_4Cl (25 mM)
90% O_2 - 10% C_2H_2	C_2H_4	31.4	15.4
	H_2	0.0	0.0
	CO_2	132.4	116.6
90% O_2 - 10% A	H_2	32.7	19.6
	CO_2	115.2	118.4

Assays as described in methods, 100 mM Na-succinate present; addition of KCl or NH_4Cl as stated.
Values are averages of 3 determinations; standard deviations 10-20%.

The inhibition of acetylene reduction by NH_4Cl seemed to be dependent on the oxygen concentration. Results given in Table 4 show that reduction of the

oxygen concentration in the gas phase from 90% to 50% raised the inhibition by NH_4Cl from 8% to 41% (after 2 h of incubation) and from 43% to 74% (after 4 h of incubation). Apart from this, the acetylene-reducing activity of the control was about 75% lower at decreased oxygen concentration.

Table 4. Influence of NH_4Cl and the O_2 concentration on the acetylene-reducing activity of detached pea root nodules

O_2 concentration	Addition (25 mM)	0 - 2 h	2 - 4 h
90%	KCl	12.4	12.3
90%	NH_4Cl	11.4	7.0
50%	KCl	3.4	3.5
50%	NH_4Cl	2.0	0.9

Activities in µmoles C_2H_4 per g of fresh weight nodule, produced during the first 2 h and during the subsequent 2 h of incubation, respectively. Values are averages of 5 determinations; standard deviations 10-20%.

Effect of NH_4Cl on isolated bacteroids

As ammonium chloride decreases the acetylene-reducing and hydrogen-producing activities of detached nodules, the question arose whether this is an effect on the bacteroids as such or rather an indirect effect. When isolated bacteroids were tested (in an aerobic assay, provided with succinate) no short-term effect of added NH_4Cl was detected (Houwaard, results submitted for publication). Figure 4 shows results of such an experiment where the acetylene reduction by isolated bacteroids was followed for two hours. The nitrogenase activity was the same during this period with KCl (10 mM), NH_4Cl (10 mM) or without salt added. Thus NH_4Cl has no effect on the nitrogenase activity of isolated bacteroids.

Relative efficiency of nitrogen fixation

Nodulated pea plants incubated in air fix dinitrogen, and at the same time produce hydrogen; this is a general characteristic of both free-living and symbiotic nitrogenase-containing organisms. The production of hydrogen by nitrogenase can be considered to dissipate energy. Symbiotic systems of pea and *Rhizobium leguminosarum* strains contain hydrogenase which

oxidizes the H_2 formed, thus diminishing the loss of energy; the presence or absence of hydrogenase depends on the microsymbiont (21). No uptake of H_2 could be detected in the symbiosis used in this study, indicating that *Rhizobium leguminosarum* PRE does not contain hydrogenase.

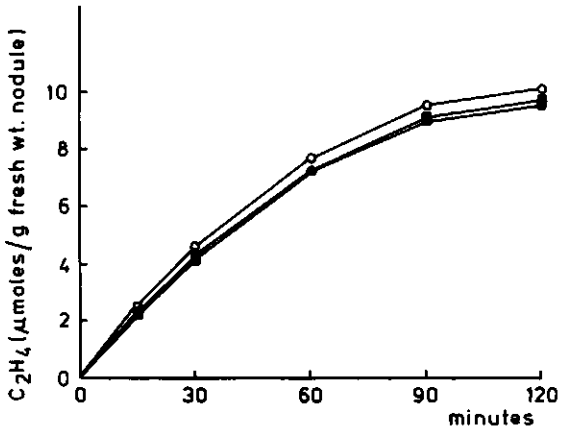

Fig. 4. Influence of ammonium chloride on the acetylene reduction by isolated bacteroids. No salt (○), 10 mM KCl (●) or 10 mM NH_4Cl (■) added.

By comparing the H_2 production in an N_2/O_2 atmosphere with the H_2 production in an A/O_2 atmosphere or with the C_2H_2 reduction (when nitrogenase produces only H_2 or C_2H_4, respectively) a "relative efficiency" can be calculated as proposed by Schubert *et al.* (22). These experiments have been performed with the intact system, with detached nodules and with isolated

Table 5. Relative efficiency of intact pea plants, detached nodules and isolated bacteroids

System	H_2(argon)	C_2H_4	H_2(nitrogen)	R.E.	
				(a)	(b)
Intact plants	22.6	19.7	8.2	0.64	0.58
Detached nodules	11.8	12.2	7.9	0.33	0.35
Isolated bacteroids	12.2	11.1	4.3	0.65	0.61

Activities are expressed in μmoles per g of fresh weight nodule per h; values are averages of 3 determinations.
Hydrogen production in argon/O_2 and nitrogen/O_2 atmosphere, respectively.
Relative efficiency (R.E.): $1 - \frac{H_2(\text{nitrogen})}{H_2(\text{argon})}$ (a) or $1 - \frac{H_2(\text{nitrogen})}{C_2H_4}$ (b).

bacteroids. Results are given in Table 5; the relative efficiency of intact nodulated plants and of isolated bacteroids was about 60-65%. This means that 35-40% of the nitrogenase capacity was lost by the production of H_2. Detached nodules, under the assay conditions used, were less efficient and had a relative efficiency of only 30-35%. It can be concluded that the relative efficiency can be affected by the conditions under which root nodules occur and that less favourable circumstances increase energy dissipation.

DISCUSSION

In this study some aspects of the nitrogen-fixing symbiosis between pea plants and *Rhizobium leguminosarum* were investigated. Emphasis was laid on the interrelationship between nitrogen fixation (acetylene reduction) and substrate as affected by environmental factors (oxygen concentration, substrate supply and combined nitrogen), and intrinsic factors like hydrogen production.

In the intact system carbon compounds used by the bacteroids, are provided by the host plant and originate from photosynthesis. Sucrose, which is transported from the shoot to the root system, is one of the main carbon compounds in root nodules and its presence seems to be correlated with nitrogenase activity (1, 28) although the bacteroids are unable to oxidize it. They can use citric acid cycle intermediates, as has been described in a previous communication (Houwaard, submitted for publication). An explanation of the need for special substrates would be that bacteroids lack enzymes to take up or to degrade substrates like glucose. This in contrast with free-living rhizobia, which can grow with many different substrates (13, 14). However, when free-living rhizobia are fixing dinitrogen (whether or not in association with plant cells), a function of succinate as energy supplier to nitrogenase has been suggested (5, 20).

It is reasonable to assume that bacteroids are provided with utilizable substrates by the plant cells. These substrates might be formed in the nodule from other carbon compounds. An indication of this assumption is the observed stimulation of the acetylene reduction of detached nodules (Table 2) by substrates which are not oxidized by isolated bacteroids. On the other hand, this table shows that the respiration of detached nodules is not substantially enhanced by the added substrates. This could be explained by assuming that the oxidation in the nodule measured and reported in Table 2 as

CO_2 production, largely concerns endogenous substrate and is not coupled to nitrogenase activity. The effect of added carbon compounds would be the supply of substrates which stimulate nitrogenase, whether or not after conversion in plant cells of the nodule. Such a substrate might be succinate, fumarate or malate which stimulate acetylene reduction by isolated bacteroids (Houwaard, submitted for publication) and which are found in root nodules (1, 16). When detached nodules are incubated for some hours, this substrate will be exhausted and acetylene-reducing activity decreases unless substrates are supplemented by external sources, as can be seen in Table 2 and in a previous paper (12).

When substrate (succinate) was present, nitrogenase activity of detached nodules decreased with time anyways (Fig. 1). This decrease resulted from a decline of the amount of potentially active nitrogenase (Fig. 2). The long-term inactivation or decay of nitrogenase might be initiated by the "waterlogging" conditions under which the nodules were brought and which might induce changes in their metabolism (25). The decrease of respiration during this long-term incubation was less pronounced than the decrease of nitrogenase activity.

Other conditions under which nodule respiration and acetylene reduction behaved in a different way were found in the experiments with added NH_4Cl (Table 3). Nitrogenase activity of detached nodules was inhibited by the addition of NH_4Cl, without an accompanying inhibition of the respiration. In contrast to the actual nitrogenase activity no decrease of the amount of potentially active nitrogenase was found upon the addition of NH_4Cl (12). Moreover, no direct inhibitory effect of NH_4Cl on nitrogenase activity of isolated bacteroids was detected (Fig. 4). It was suggested before that the inhibition of nitrogen fixation of detached nodules by NH_4Cl was caused via interaction with the supply of carbon compounds (12). This interaction might also be deduced from the results of experiments with different O_2 concentration: at a lower oxygen concentration NH_4Cl had a more pronounced inhibiting effects (Table 4). A similar connection between O_2 concentration and effect of NH_4Cl has been reported for a free-living N_2-fixing *Rhizobium* sp. (23). However, in *Azotobacter chroococcum* the adverse effect of ammonium acetate on acetylene reduction was most pronounced at higher partial pressure of oxygen (8).

Two ways of expressing the efficiency of nitrogen fixation have been used. One of these is the calculation of the amount of carbon substrate consumed per unit of nitrogen fixed by nitrogen fixing organisms. In the present study

acetylene reduction was compared with respiration (Table 1). The efficiency was found to be influenced by the detachment of the nodules and by the isolation of the bacteroids and by such factors like NH_4-supply and pO_2. It may be concluded that *in vivo* efficiency is also affected by environmental conditions, comparable to those encountered under *in vitro* conditions. The efficiency measured with the intact system (about 200 mg N fixed per g carbohydrate consumed) approaches the theoretical value. Slightly higher (18) or lower (6, 17) values have been reported in the literature, which emphasizes the variation in efficiency of nitrogen fixation according to experimental conditions and theoretical approach.

The "relative efficiency" which includes the energy waste occurring upon hydrogen production was also influenced by manipulation of the symbiotic system (Table 5). More energy seemed to be wasted by detached nodules (i.e. at decreased O_2 supply) than by intact plants or isolated bacteroids. The influence of environmental factors on the relative efficiency has also been shown for the intact system, upon varying the light intensity and nitrogen nutrition of pea plants (6, 7).

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7. GENERAL DISCUSSION

Inhibitory effect of ammonium chloride

In the experiments described in the previous chapters, the effect of combined nitrogen (with emphasis on ammonium chloride) on nitrogenase activity of pea bacteroids was investigated. This effect was shown to depend on experimental conditions (oxygen concentration, presence of substrates or addition of an inhibitor of ammonium assimilating enzymes) and on the degree of purification of the bacteroids. It is interesting to compare the applied conditions with the *in vivo* situation in order to explain the observed effects in physiological terms (supply of carbohydrates, respiratory activity, transport of fixed nitrogen, assimilation of nitrogen).

No inhibitory effect of ammonium chloride on the nitrogenase activity of isolated bacteroids could be demonstrated (Chapters 2 and 6). Thus, it can be concluded that *Rhizobium* bacteroids, unlike *Azotobacter* cells (5), are insensitive to ammonium ions: no immediate drop in acetylene-reducing activity is brought about by ammonium chloride (Chapter 2, Fig. 4). At a concentration of 10 mM NH_4Cl no effect could be demonstrated even after 2 h (Chapter 6, Fig. 4). This concentration of NH_4^+ seems to be rather high, but according to the measured C_2H_2 reduction rates it might be built up in the nodule by reduction of N_2 within 1 h, assuming that no assimilation would occur. The effect of ammonium ions in *Azotobacter* spp. probably arises from a decrease in the flow of reducing equivalents to nitrogenase, by interference of NH_4^+ with membrane energization (14). The absence of this effect in bacteroids might mean that bacteroids do not take up ammonium ions, thereby prohibiting ammonium ions to exert their damaging effect (14).

In contrast with the foregoing, ammonium chloride does have an inhibitory effect on nitrogenase activity of whole, detached nodules (Capter 5, Fig. 3). Since no immediate inhibition of acetylene-reducing activity occurs in this case, it might be explained as repression of nitrogenase biosynthesis. However, repression of enzyme synthesis in detached nodules is doubtful, because of the

relatively rapid decline in acetylene reduction rate compared with the turnover rate of nitrogenase in bacteroids - which has a half-live of about 2 days (4). Moreover, the reversibility of the inhibitory effect (Chapter 5, Fig. 5) and the negligible decrease in potential nitrogenase activity (Chapter 5, Table 1) point into the same direction. On the other hand, repression of nitrogenase synthesis might be accompanied by inactivation of the enzyme present; such an inactivation has been found to occur in free-living nitrogen-fixing aerobic miroorganisms - *Azotobacter chroococcum* (8), *Anabaena cylindrica* (28) - where the decrease in nitrogenase activity after addition of ammonium ions exceeds the decline of activity that would be expected if nitrogenase synthesis would stop. Inactivation of the enzyme might be brought about by oxygen in these organisms. It is expected, that further study of detached nodules, concerning the mechanisms of nitrogenase inhibition and including the enzymes of nitrogen assimilation and of carbohydrate degradation, may give valuable information about nitrogenase regulation, which would apply to the *in vivo* situation under stress conditions - e.g. waterlogging in wet soils.

Photosynthate deprivation

The work with intact pea plants has demonstrated that the decrease in nitrogenase activity, when ammonium chloride or potassium nitrate are added to the plants, does not result from a decrease in nitrogenase content of the nodule (Chapter 3, Fig. 2; Chapter 4, Table 2). this result strongly supports the photosynthate deprivation theory as the explanation of the influence of combined nitrogen on the nitrogenase activity of leguminous plants. As a consequence, a decrease in nitrogenase activity arises from a shortage of carbohydrate in the nodule. This explanation is in agreement with the observation that nodules receive less carbohydrates when potassium nitrate is added to pea plants (25). The reason why less photosynthate is transported to the the nodule has to be discussed in some more detail.

A plausible explanation of the altered carbohydrate distribution over the plant is the consumption of the assimilates before they reach the nodule. Nitrate reduction can be brought about all over the plant. At low nitrate concentrations in the rooting medium the main portion of the nitrate reductase is induced in the roots, where most of the nitrate is assimilated. At higher nitrate concentrations, part of it is transported to the shoot and reduced in the upper parts of the plant (30). Ammonium ions, though studied less intensively, are also likely to be assimilated in the roots (20). In both instances

carbohydrates are consumed - to deliver reductants for nitrate reduction and carbon skeletons for the synthesis of amino acids - and carbohydrate transport to the nodules is decreased.

The plant obviously prefers the assimilation of nitrogenous compounds taken up via the roots to the reduction of dinitrogen. The reason for this preference might be an energetic advantage. Ammonium ions are profitable in this respect, as no energy for reduction is needed. Nitrate ions do not have this advantage: reduction of nitrate theoretically consumes as much energy as does reduction of dinitrogen (10). The advantage of nitrate consumption over nitrogen fixation might be the rapid induction and the more widespread occurrence of nitrate reductase (18). Moreover, nitrate reductase which is present in the plant cell cytoplasm may be associated with the plastids (9), thus enabling a more direct transfer of reducing equivalents. Nitrite reductase is even enclosed within root plastids and chloroplasts (9, 31). Nitrate reduction, as well as nitrite reduction and ammonia assimilation are closely associated with photosynthesis; in green leaves, these processes have been shown to be light-dependent (15, 17) and coupled to oxygen evolution (1), demonstrating the close association.

When high concentrations of ammonium chloride are applied, transport of ammonium ions to the shoot might occur. This situation could induce specific effects in view of photosynthate production. When free ammonium ions would reach the photosynthetic apparatus, uncoupling of photosynthetic phosphorylation might reduce the formation of ATP (13). However, this is unlikely to have occurred in the experiments described in the present study, since no adverse ammonium effects on plant growth and appearance have been found (Ch. 4, Fig. 1). On the other hand, ammonium ions may affect the carbon metabolism in the shoot. It has been demonstrated that ammonium chloride decreases the synthesis of sucrose in chloroplasts, whereas more amino acids are produced (22). The regulation is probably exerted via a decrease in pyruvate kinase activity (21). This regulatory mechanism might play a role in the reduction of carbohydrate (sucrose) transport to the nodules, when relatively high ammonium chloride concentrations are applied. To which degree photosynthesis and carbon metabolism in the shoot are affected by ammonium ions added to the root system of plants remains questionable and has to be further investigated.

Methionine sulfoximine

Special reference should be given to the effect of methionine sulfoximine (MSX) which counteracts the inhibitory effect of ammonium chloride on nitrogenase activity both in detached nodules and in intact plants (Chapters 3 and 5). MSX, an inhibitor of the enzyme glutamine synthetase (19), has been discovered in mammalian brain research on account of its harmfull effect when taken up with the food (3). Inhibition occurs when methionine sulfoximine, a structural analog of glutamate, is phosphorylated and bound irreversibly to the enzyme (23, 24). Thus, the enzymic activity of glutamine synthetase is prevented and at the same time adenylylation and deadenylylation is prohibited. The latter process might play an important role in the regulation of the synthesis of enzymes involved in nitrogen metabolism (16), including nitrogenase. This role has been suggested, among other indications, by MSX studies with *Azotobacter*, *Klebsiella* and *Anabaena* spp. (11, 27).

In the present experiments, methionine sulfoximine was applied to intact pea plants (Chapter 3, Fig. 1) and to detached nodules (Chapter 5, Fig. 6). Little is known as to possible effects of this application on the *in vivo* situation of higher plants. The detrimental effect found when high concentrations were used dictates a careful interpretation of the results; in this context the apparent influence on the uptake of exogenous ammonium chloride by intact plants and detached nodules should also be mentioned (Chapter 3, Table 2; Chapter 5, Table 4). During the experimentations it was found that MSX at a concentration of 2 mM did not affect acetylene reduction or respiration of isolated bacteroids (unpublished results). Inhibition by MSX of higher plant glutamine synthetase has been mentioned in literature for the pea chloroplast enzyme and for whole plants of duckweed (1, 26), but the universality of the effect can be assumed to extend to other plants as well.

Methionine sulfoximine affects the acetylene-reducing activity of pea (Chapter 3, Fig. 1). Assuming that the effect is due to an inhibition of glutamine synthetase - and perhaps of glutamate synthase (6) - the explanation of this phenomenon remains obscure. As repression of enzyme synthesis is rejected as an explanation of the effect of ammonium chloride, the influence of MSX on nodulated pea plants supplied with ammonium ions can not be connected with this process. Another possibility would be that products of ammonia assimilation are responsible for the inhibition of nitrogenase: blocking the assimilation would relieve the inhibition. This theory is also less plausible - as has been discussed in more

detail in the introduction. Especially the absence of any effect of amino acids on nitrogenase activity of detached nodules, and the slow entrance of exogenous nitrogenous compounds into the nodules when intact plants are used, do not favour this hypothesis. As stated before, the supply of carbohydrates might be a major factor regulating nitrogenase activity; this theory seems to be valuable in the explanation of the MSX effect as well. Carbon compounds are consumed during the assimilation of ammonia, added to the plant or to the nodules; this consumption is injurious to the nitrogenase activity which becomes short of carbohydrates, causing reduced supply of ATP, reductants and carbon skeletons. In this view, inhibition of ammonia assimilation relieves this shortage and restores nitrogenase activity.

Disintegration of the symbiosis

Obviously, the ultimate result of the presence of combined nitrogen is the disintegration of the nitrogen-fixing symbiosis (29). This process leads to separation of the partners, which survive as 'free-living' organisms. In the root nodule of leguminous plants the disintegration starts with destruction of the cytoplasm of bacteroid-containing plant cells, followed by a gradual disorganization of the symbiotic complex (7). During this process the number of (rod-shaped) bacteria increases (7). Similar phenomena are displayed during senescence of bacteroids under *in vivo* conditions (12) and during premature deterioration of an ineffective symbiosis (2).

The studies described in the present communication were short-term experiments, extending over a few days. During this period a gradual disintegration might have occurred. However, it is assumed that the results have not been affected significantly, since this disintegration is only in an initial stage during the experiments. This assumption is based on data concerning clover and medic (7), where disintegration phenomena were observed after 2 days. It might be expected to take at least the same period of time in pea plants, although data on these processes are not available in the literature. Morphological studies on the influence of combined nitrogen on the bacteroids and the surrounding plant cells in pea root nodules will supply a valuable addition to the present research.

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SUMMARY

The nitrogen-fixing activity of the symbiotic system of *Pisum sativum* with *Rhizobium leguminosarum* is adversely affected by combined nitrogen. Both ammonium chloride and potassium nitrate, when added to the roots, lower the nitrogenase activity (acetylene-reduction) of intact pea plants. During a 3-day treatment of the plants with combined nitrogen, when the *in vivo* nitrogenase activity falls to less than 50%, the nitrogenase activity of isolated bacteroids, treated with toluene and supplied with ATP and reductants, does not decrease. Thus, the potential nitrogenase activity of the root nodules is unaffected by short-term combined-nitrogen treatment of the plants. The adverse effect of ammonium chloride on the nitrogenase activity of pea plants is counteracted by the addition of sucrose or of methionine sulfoximine (an inhibitor of ammonia assimilation) to the rooting medium. A higher light intensity also diminishes the effect of ammonium chloride on nitrogenase activity.

Ammonium chloride has no specific inhibitory effect on the nitrogenase activity of isolated pea bacteroids, neither in the anaerobic, nor in the aerobic assay. On the other hand, ammonium chloride does inhibit the nitrogenase activity of detached root nodules within a few hours. At a lower oxygen concentration in the assay this inhibition is more pronounced. The effect of ammonium chloride on detached nodules is relieved by simultaneous addition of methionine sulfoximine.

Various carbon compounds (glucose and tricarboxylic acid cycle intermediates) stimulate the nitrogenase activity of detached nodules; only dicarboxylic acids of the tricarboxylic acid cycle support the nitrogenase activity of isolated bacteroids. Efficiencies of nitrogen fixation as to consumption of carbon compound are similar in both systems, although lower than in the intact system. Ammonium chloride does not affect respiratory activities of detached nodules or of isolated bacteroids.

It is concluded from the above-mentioned results that combined nitrogen, added to intact plants or detached nodules, does not affect nitrogenase activity directly. The results support the photosynthate theory, i.e. the photosynthate supply of the nodules is reduced and consequently the nitrogenase activity decreased, owing to the consumption of carbon compounds for the assimilation of the added combined nitrogen.

SAMENVATTING

De aanwezigheid van anorganische stikstofverbindingen heeft een ongunstige invloed op de stikstofbindende aktiviteit van het symbiontische systeem van *Pisum sativum* met *Rhizobium leguminosarum*. Zowel ammoniumchloride als kaliumnitraat, toegevoegd aan de wortels, verlagen de nitrogenase-aktiviteit(acetyleenreductie) van intakte erwteplanten. Wanneer de planten gedurende 3 dagen met gebonden stikstof behandeld worden en de *in vivo* nitrogenase-aktiviteit tot minder dan 50% is gedaald, is de nitrogenase-aktiviteit van de uit deze planten geïsoleerde bacteroiden, gemeten na behandeling met tolueen en onder toevoeging van ATP en reductie equivalenten, niet afgenomen. De potentiële nitrogenase-aktiviteit van de wortelknollen wordt dus niet beinvloed door een kortdurende behandeling van de planten met gebonden stikstof. De nadelige werking van ammoniumchloride op de nitrogenase-aktiviteit van erwteplanten is minder groot wanneer saccharose of methionine-sulfoximine (een remstof van de ammoniumassimilatie) aan het groeimedium worden toegevoegd. Een hogere lichtintensiteit verzwakt het remmende effekt van ammoniumchloride op de nitrogenase-aktiviteit eveneens.

Ammoniumchloride oefent geen specifiek remmende werking uit op de nitrogenase-aktiviteit van geïsoleerde bacteroiden; dit geldt zowel voor de anaërobe als de aërobe bepaling. De nitrogenase-aktiviteit van afgeplukte wortelknollen wordt daarentegen wel geremd door ammoniumchloride (binnen enkele uren). Deze remmende werking is sterker bij een lagere zuurstofspanning tijdens de test. Toevoeging van methionine-sulfoximine aan de afgeplukte knollen, heft de schadelijke werking van ammoniumchloride op. Verschillende koolstofverbindingen (glucose en intermediairen van de citroenzuurcyclus) stimuleren de nitrogenase-aktiviteit van afgeplukte wortelknollen; slechts de dicarbonzuren van de citroenzuurcyclus ondersteunen de nitrogenase-aktiviteit van geïsoleerde bacteroiden. De rendementen van het substraatverbruik bij de stikstofbinding zijn in beide gevallen gelijk, maar lager dan die bij de stikstofbinding van het intakte systeem. Ammoniumchloride heeft geen invloed op de ademhalingsaktiviteit van afgeplukte knollen of geïsoleerde bacteroiden.

Op grond van bovenvermelde resultaten wordt de conclusie getrokken dat anorganische stiksyofverbindingen, toegediend aan intakte erwteplanten of afgeplukte wortelknollen, geen direkte invloed uitoefenen op de nitrogenase-aktiviteit. De resultaten stemmen overeen met de theorie dat de knollen van carbohydraten beroofd worden: het verbruik van fotosynthaat voor de assimilatie van de opgenomen stikstofverbindingen veroorzaakt een vermindering van de toevoer van koolstofverbindingen naar de wortelknollen, met als gevolg een daling van de nitrogenase-werking. Regulatie van de stikstofbinding in erwteplanten door de aanwezigheid van gebonden stikstof vindt derhalve plaats via processen in de waardplant.

CURRICULUM VITAE

De auteur van dit proefschrift werd op 7 april 1950 te Amsterdam geboren. In 1968 behaalde hij het diploma gymnasium β aan het Cartesius Lyceum, eveneens te Amsterdam. In hetzelfde jaar begon hij zijn studie scheikunde aan de Universiteit van Amsterdam; in juni1975 studeerde hij af, met als hoofdvak microbiologie en als bijvak biochemie. Van 1976 tot 1979 was hij als gastmedewerker verbonden aan het Laboratorium voor Microbiologie van de Landbouwhogeschool te Wageningen.