Biochimica et Biophysica Acta, 740 (1983) 313–322
Elsevier

BBA 91238

CONSTITUTIVE NITROGENASE SYNTHESIS FROM DE NOVO TRANSCRIBED mRNA IN ISOLATED *RHIZOBIUM LEGUMINOSARUM* BACTEROIDS

ROMMERT C. VAN DEN BOS *, ARJEN SCHOTS, JAN HONTELEZ and ALBERT VAN KAMMEN

Laboratory of Molecular Biology, Agricultural University, Wageningen (The Netherlands)

(Received January 17th, 1983)

Key words: Nitrogenase synthesis; Gene expression; mRNA half-life; Translational regulation; Bacteroid; (R. leguminosarum)

Nitrogenase synthesis was studied in *Rhizobium leguminosarum* by incubation of bacteroids isolated from *Pisum sativum* nodules in a medium containing succinate, myoglobin and [^{35}S]methionine. Protein synthesis analysed by SDS-polyacrylamide gel electrophoresis and autoradiography revealed a strong nitrogenase synthesis in bacteroids isolated from root nodules 16 days after infection. The labeled proteins were identified as nitrogenase by immunoprecipitation with specific antisera. Nitrogenase synthesis appeared not to be repressed by oxygen concentrations up to 100 μM; neither was repression found using 25 mM NH_4^+ or by NO_2^- or NO_3^- at 10 mM. The ionophores valinomycin, and triphenylphosphonium bromide do have an immediate effect on acetylene reduction by bacteroids but were found to have no repressive influence on nitrogenase structural gene expression. In bacteroids isolated 22 days after infection acetylene reduction under a low pO_2 was much stronger than at 15 days, but overall protein synthesis and label incorporated into nitrogenase proteins were reduced. Evidence is presented that synthesis of nitrogenase proteins in isolated bacteroids takes place by translation of de novo synthesized mRNA. By inhibiting RNA-transcription with rifampicin and assuming that the rate of nitrogenase synthesis at a given moment is proportional to the amount of *nif* mRNA present, the decay of *nif* mRNA was found to proceed with a biological half-life of approx. 1.6 min.

Introduction

Rhizobium bacteria are capable of fixing atmospheric nitrogen in symbiotic association with legume roots. Since the genes coding for nitrogenase, the enzyme that catalyses the nitrogen fixation reaction, are only expressed in the *Rhizobium* bacteroids which are located within the root nodules, the regulation of the synthesis and the activity of nitrogenase is difficult to study and poorly understood.

For free-living nitrogen-fixing bacteria, particularly the facultative anaerobically nitrogen fixing *Klebsiella pneumoniae* the regulation of the synthesis and the activity of nitrogenase has been extensively studied. In this organism the nitrogen fixation (*nif*) genes are under strict control by oxygen and nitrogenous compounds. Both oxygen and nitrogenous compounds repress the synthesis of components I and II of the nitrogenase complex (for reviews see Refs. 1 and 2). In the aerobic nitrogen-fixing *Azotobacter vinelandii* the *nif* genes are expressed when nitrogenous compounds are absent from the culture medium; oxygen has no repressive effect on nitrogenase synthesis, but can affect the activity of the enzyme.

In the case of slow-growing rhizobia it is possible to induce nitrogen-fixing activity in free-living cultures. Such induction requires a low oxygen concentration and specific carbon compounds in the medium [3–6]; in contrast to other free-living nitrogen fixing organisms the presence of fixed

nitrogen appears to have no inhibitory effect in this case [7].

The regulation of nitrogenase synthesis in root nodules has been studied by analyzing bacteroid protein synthesis in plants grown in [^{35}S]sulfate; in these experiments [8] ammonium reduced nitrogen fixation without repressing nitrogenase synthesis. The immediate effect on the bacteroids of ammonium addition to the whole plant remained unclear, however. In the present paper we describe experiments to study nitrogenase synthesis in isolated bacteroids under various conditions. Our results indicate that nitrogenase synthesis in bacteroids isolated from nodules in an early stage of development is not repressed by oxygen concentrations up to 100 μM, which completely abolish enzyme activity [9]. Also ammonium did not repress nitrogenase synthesis.

We present evidence that in the experimental system we use nitrogenase is translated from de novo synthesized mRNA; the $T_{1/2}$ of this mRNA is slightly lower than the average $T_{1/2}$ of mRNAs in bacteroids under these conditions.

Materials and Methods

Cultivation of nodulated P. sativum plants and isolation of bacteroids. Growth of *P. sativum* in gravel and inoculation with *R. leguminosarum* PRE were as described by Bisseling et al. [8]. Bacteroids were isolated under strictly anaerobic conditions at 0°C by a modification of the method described by Laane et al. [9]; all solutions were flushed with nitrogen gas before use. Pieces of main root carrying nodules were ground in a beaker with the flat backside of a mortar pestle in the presence of isolation buffer (50 mM 2(*N*-morpholino)ethane sulfonic acid (Mes)·KOH/20 mM sodium dithionite/4% (w/v) poly(vinyl pyrolidone)/0.1 mM dithiothreitol/0.3 M sucrose, pH 7.3; (20 ml/g nodules)). The slurry was filtered through two layers of miracloth and the filtrate was centrifuged at 2000 × *g* for 5 min at 4°C. The pellet containing bacteroids was washed in buffer 1 (25 mM Mes·KOH/2.5 mM $MgCl_2$/0.3 M sucrose, pH 7.3) with 2% (w/v) fatty acids free bovine serum albumin. The bacteroids were suspended in buffer 1 at a density corresponding to A_{660} = 10–20, stored on ice and used the same day.

Incubation and labeling of bacteroids under defined oxygen concentrations. Bacteroids were incubated in an oxygraph cell as described by Laane et al. [9]. The standard incubation medium consisted of 0.9 ml buffer 1, containing 50 mM sodium succinate; 50 μl reduced 2 mM myoglobin were added [10]. The cell was flushed with N_2 gas to reduce [O_2] to 1–2 μM and 100 μl bacteroid suspension were added. During a preincubation of 5 min the pO_2 was brought to the desired level by injection of air or O_2 in the gas phase and/or by changing the stirring speed. The temperature of the cell was kept at 26°C. Acetylene reduction was determined as described by Laane et al. [9]. Bacteroids were incubated in the presence of [^{35}S]methionine, to measure protein synthesis, or [^{3}H]uridine for RNA synthesis for different periods as described under Results. Incorporation of label was stopped by addition of a 1000-fold excess of the corresponding unlabeled compound followed by centrifugation of the bacteroids in an Eppendorf centrifuge at 4°C for 3 min. Pellets were kept frozen at −80°C.

Immunoprecipitations. Bacteroids were lysed in 1% (w/v) sodium dodecyl sulphate (SDS); then a 10-fold excess of buffer 2 without SDS was added (buffer 2 is: 50 mM sodium phosphate, pH 7.5/0.15 M NaCl/0.2% (w/v) bovine serum albumin/1% (w/v) Triton X-100/0.1% (w/v) sodium desoxycholate/0.1% (w/v) SDS/10 mM NaN_3). Antiserum specific against nitrogenase component I or II induced in rabbits was added [11]; incubation was at room temperature overnight. The amount of antiserum used depended on the titer of the antiserum. Antigen-antibody complexes were precipitated with *Staphylococcus aureus* cells. The *S. aureus* cells were washed once in buffer 2 before use and suspended in buffer 2 as a 10% (w/v) cell suspension. A 5-fold excess of this suspension was added to the mixture and incubation was continued in a rotary mixer for 2 h at 4°C. The suspension was then carefully layered on a sucrose cushion in an Eppendorf tube consisting of layers of, respectively, 0.5 ml 1 M sucrose and 0.2 ml 0.5 M sucrose, both in buffer 2 and spun for 15 min. The pellet was washed twice in 0.25 ml buffer 2, lysed in sample buffer (10 mM Tris HCl/1 mM EDTA/10% (w/v) glycerol/2% (w/v) SDS/5% (v/v) mercaptoethanol/0.001% bromophenol blue,

pH 7.5) and electrophoresed in a polyacrylamide gel.

Polyacrylamide gel electrophoresis. Bacteroids were lysed in sample buffer and kept at 100°C for 5 min; after centrifugation for 3 min at 10000 × *g* the supernatant was loaded on a 12.5% polyacrylamide gel [12]. Gels were stained with Coomassie brilliant blue and dried; radioactivity incorporated in the nitrogenase proteins was estimated as described by Bisseling et al. [8].

Quantitation of total incorporation. 15 μl portions of cell lysate (in sample buffer) were spotted on Whatman 3 MM filters and kept in 10% (w/v) trichloroacetic acid for 2 h at 4°C under stirring. The filters were washed twice in ethanol, once in diethyl ether and dried. Radioactivity on the filters was determined in a Hewlett Packard scintillation counter with 7 ml Instafluor (Packard).

Protein determination. Protein concentrations were determined with the BioRad protein assay (Bio-Rad laboratories, Richmond, CA). Proteins were precipitated in 5% (w/v) trichloroacetic acid, pelleted by centrifugation, washed twice with acetone (at −20°C) and dissolved in 0.8 ml H_2O; 0.2 ml dye reagent concentrate was added and after a 30 min incubation A_{595} was determined. As a standard bovine serum albumin solutions of known concentration were used.

Materials. All chemicals used were reagent grade; rifampicin, bovine serum albumin and myoglobin (whale skeletal muscle) were from Sigma. The following radioisotopes were used: [^{35}S]methionine (1000 Ci/mmol), and [^{3}H]uridine (26 Ci/mmol) from the Radiochemical Centre, Amersham. *S. aureus* cells were obtained from the Enzyme Center (33 Harrison Ave, Boston, MA 02111).

Results

Acetylene reduction by isolated bacteroids

Rhizobium bacteroids isolated under anaerobic conditions and washed with fatty acid-free bovine serum albumin are able to reduce acetylene when incubated under a low [O_2] and with succinate as a carbon source [9,13]. Bacteroids were isolated from pea root nodules at different times after infection and incubated in an oxygraph cell under standard conditions. Acetylene reduction was measured at

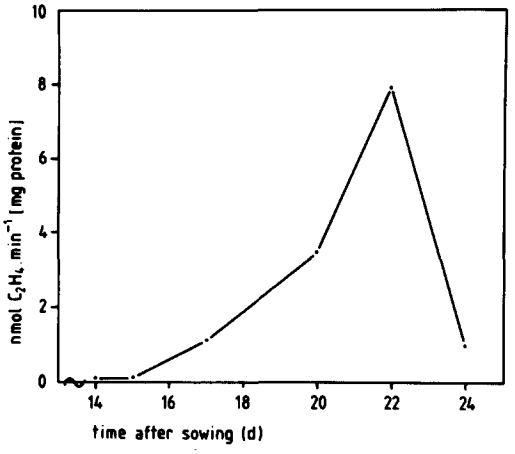

Fig. 1. Acetylene reduction by isolated *R. leguminosarum* bacteroids as a function of nodule age. Bacteroids were isolated and incubated in the standard incubation medium as given in Materials and Methods. Acetylene reduction was measured at [O_2] = 2 μM as described by Laane et al. [9].

[O_2] = 2 μM and plotted against the age of the plants; at this O_2 concentration nitrogenase activity of isolated bacteroids is optimal [9]. The rate of acetylene reduction was maximal in bacteroids isolated from 22-day-old plants (Fig. 1). This agrees with our earlier observations that inoculated pea plants show a maximal rate of acetylene reduction around 22 days after infection [8]. The decrease of activity at 24 days found in these experiments is stronger than in the in vivo situation and occurs at an earlier stage than in root nodules [8,14].

Protein synthesis by isolated bacteroids

Protein synthesis in isolated *R. leguminosarum* bacteroids was studied in the oxygraph system by labeling the bacteroids with [^{35}S]methionine. Bacteroids were isolated anaerobically and preincubated in the oxygraph at [O_2] = 2 μM in buffer 1 for 5 min. Then 15 μCi [^{35}S]methionine were added and incubation was continued for 10 min. Further incorporation of label was stopped by addition of a 1000-fold excess of unlabeled methionine and immediate centrifugation at 4°C. Cell pellets were lysed in sample buffer and bacteroid proteins were separated by polyacrylamide gel electrophoresis; the synthesized proteins were made visible by autoradiography of the dried gel. The resulting autoradiograph of an experiment

using bacteroids from roots at 16 days after infection is shown in Fig. 2. The bands migrating at the same velocity as purified nitrogenase components

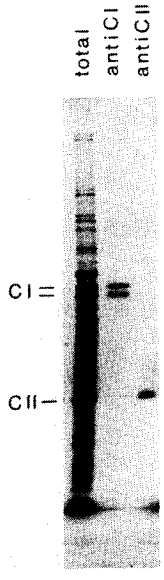

Fig. 2. Immunoprecipitation of nitrogenase components. Bacteroids from pea root nodules 16 days after inoculation were labeled as given in Materials and Methods. The labeled proteins were immunoprecipitated with antiserum specific against the nitrogenase components; after electrophoresis the polyacrylamide gel was autoradiographed. Top: total labeled proteins. Middle: proteins precipitated with antiserum specific against component CI. Bottom: proteins precipitated with antiserum specific against component CII.

CI (FeMo-protein) and CII (Fe-protein) comprise an appreciable fraction of the total incorporated label. Total incorporation as quantitated by protein precipitation with trichloroacetic acid at different time intervals was found to be linear up to 90 min (data not shown).

Immunoprecipitations

The labeled protein bands comigrating with components CI and CII were identified as nitrogenase proteins by incubation of the bacteroid proteins after [^{35}S]methionine labeling with antiserum specific against component CI or CII [11]. The antigen-antibody complexes were precipitated by incubation with *S. aureus* cells and the precipitates were subjected to SDS-polyacrylamide gel electrophoresis. The autoradiographs of the gels are shown in Fig. 2. Incubation with antiserum specific against component CII resulted in a simple band corresponding to the nitrogenase component in question. Incubation of the labeled bacteroid proteins with antiserum specific against component I resulted in the precipitation of the two component I subunits. It is visible that the immunoprecipitate of component II contains also a minor amount of component I and vice versa; this is probably due to an interaction between the nitrogenase components during the precipitation. We conclude that at low [O_2] isolated bacteroids continue to synthesize nitrogenase proteins.

Influence of oxygen on nitrogenase synthesis

The influence of [O_2] on the nitrogenase activity in isolated bacteroids is very pronounced [9,13]. Acetylene reduction is maximal at approx. 2 μM [O_2] and decreases rapidly with increasing [O_2]; at oxygen concentrations of 10 μM and higher acetylene reduction is completely inhibited. The synthesis of nitrogenase proteins under different dissolved oxygen concentrations was measured by labeling bacteroids from 16-day-old plants with [^{35}S]methionine. The rate of protein synthesis as measured by incorporation of ^{35}S into trichloroacetic acid-insoluble material (Table I) increased with [O_2], probably as a consequence of an increased rate of respiration, and hence energy production in the bacteroid. No appreciable specific effect of [O_2] variation was apparent on the percentage of incorporation into the nitrogenase components (Table I, Expts. 1–3); this suggests that nitrogenase synthesis in isolated bacteroids is insensitive to oxygen concentrations (10–100 μM) which completely inhibit nitrogenase activity. The insensitivity of nitrogenase synthesis to free dissolved oxygen might be explained by assuming that preexisting *nif* mRNAs are being translated, whilst oxygen might only be effective in repressing *nif* mRNA synthesis. In that case a preincubation of the bacteroids for 30 min at high [O_2] and subsequent labeling would be expected to result in a reduced incorporation of label into nitrogenase proteins because of degradation of mRNAs during this preincubation period. As seen in Table I (Expt. 4) after a 30 min preincubation at low [O_2] the pattern of protein synthesis is unchanged com-

TABLE I

INFLUENCE OF OXYGEN CONCENTRATION AND VARIOUS ADDITIONS ON THE RATE OF SYNTHESIS OF TOTAL PROTEIN AND NITROGENASE IN *R. LEGUMINOSARUM* BACTEROIDS ISOLATED FROM PEA ROOT NODULES 16 DAYS AFTER INOCULATION

For incorporation of ^{35}S in total protein bacteroids were incubated in 1.05 ml standard incubation medium for 10 min with 15 μCi [^{35}S]methionine. After centrifugation the cell pellet was dissolved in 250 μl sample buffer; trichloroacetic acid-precipitable radioactivity was determined in 15 μl aliquots. The control value (100%) corresponds to 30500 cpm/15 μl. The percentage of total incorporated ^{35}S-label in components I and II was estimated as follows: fraction of total label incorporated in the nitrogenase proteins was estimated by cutting the corresponding bands from polyacrylamide gels. The amount in component I comprises incorporation in both subunits. Values given are averages from three independent labeling experiments.

Experiment	Addition	$[O_2]$ (μM)	Incorporation of ^{35}S in total protein (% of control)	Percentage of total incorporated ^{35}S-label in:	
				CI	CII
1	—	2	100	6.5	10.3
2	—	10	103	7.8	13.1
3	—	100	167	5.9	13.9
4 [a]	—	2	102	8.0	10.1
5	—	2 [b]	101	5.9	9.8
6 [a]	—	100	139	5.5	9.5
7	10 mM NH_4Cl	2	227	5.0	9.5
8	25 mM NH_4Cl	2	217	6.3	12.9
9	10 mM KNO_3	2	110	6.4	10.2
10	10 mM KNO_2	2	108	7.3	11.2
11	0.05 μM valinomycin	2	38	6.5	10.4
12	0.2 mM tetraphenylphosphoniumbromide	2	81	6.4	11.8
13 [a]	0.2 mM cGMP	2	47	6.8	8.8
14	rifampicin (400 μg/ml)	2	35	6.3	10.3
15 [a]	rifampicin (400 μg/ml)	2	4	n.d.	n.d.

[a] Labeling after a preincubation of the bacteroids for 30 min.

[b] In this experiment bacteroids were preincubated for 30 min at 100 μM $[O_2]$. Immediately before the addition of label the oxygen concentration was readjusted to 2 μM by flushing with N_2.

pared to that of the control, suggesting that *nif* mRNA is continually synthesized or very stable under these conditions. After a preincubation at 100 μM $[O_2]$ and subsequent labeling at 2 or 100 μM $[O_2]$ (Expts. 5 and 6), also no relative decrease of ^{35}S incorporation into nitrogenase is apparent. Thus, high $[O_2]$ does not seem to have a specific influence, neither on the synthesis or stability of *nif* mRNA nor on its translation into nitrogenase proteins.

Influence of nitrogenous compounds on nitrogenase synthesis

Ammonium has a repressive effect on the function and synthesis of nitrogenase in *Azotobacter vinelandii* and *Klebsiella pneumoniae* [1,15]. With isolated *R. leguminosarum* bacteroids the addition of 10 mM ammonium does not influence acetylene reduction [9], although in whole nodulated pea plants nitrogenase activity is reduced by added ammonium nitrate [8]. To study the effect of ammonium on the synthesis of nitrogenase proteins, bacteroids were labeled at $[O_2] = 2$ μM and $[NH_4^+]$ = 10 or 25 mM with [^{35}S]methionine. The overall rate of [^{35}S]methionine incorporation (Table I, Expts. 7 and 8) is stimulated by NH_4^+ but no specific effect on the incorporation of label into nitrogenase proteins is evident. The result of this experiment was the same whether label was added at $t = 0$ or after a 30 min preincubation in NH_4^+ (data not shown). It appears that NH_4^+ has an overall positive effect on protein synthesis but does not specifically influence nitrogenase synthesis. Incubation of bacteroids in 10 mM nitrate of

nitrite (Expts. 9 and 10) also did not show an influence on nitrogenase synthesis.

Influence of ionophores on nitrogenase synthesis

Ionophores change the pH difference (ΔpH) or the charge difference ($\Delta\Psi$) across the bacteroid membrane and thereby influence nitrogen fixation in isolated bacteroids [16]. Valinomycin and tetraphenylphosphoniumbromide both decrease $\Delta\Psi$ and inhibit nitrogen fixation. Valinomycin at a concentration of 0.05 μM (Table I, Expt. 11), which has only a slightly inhibitory effect on the rate of acetylene reduction in bacteroids [16] did not selectively inhibit the synthesis of nitrogenase proteins, but it strongly inhibited overall protein synthesis. When 0.2 mM tetraphenylphosphoniumbromide was added acetylene reduction is strongly inhibited (about 95%) but under these conditions protein synthesis is relatively unaffected (Expt. 12) and again no selective influence on nitrogenase synthesis was apparent.

Influence of cyclic GMP on nitrogenase synthesis

A specific effect of cGMP was reported by Lim et al. [17] on the synthesis of nitrogenase components in free-living *R. japonicum* without a general effect on the rate of protein synthesis. In isolated *R. leguminosarum* bacteroids we found a marked decrease in the rate of protein synthesis upon addition of 0.2 mM cGMP but no specific effect on component I or II synthesis was detectable upon labeling after a 30 min preincubation with this compound (Expt. 13).

Structural nif-genes are transcribed in isolated bacteroids

The observed synthesis of structural nitrogenase proteins will be due partly to translation of *nif* mRNA already present at the moment of bacteroid isolation and also to the translation of de novo synthesized mRNA. To assess the relative importance of these two contributions and thus to substantiate the conclusion (from Table I, Expts. 4–6) that in bacteroids mRNA is continually synthesized bacteroid proteins were pulse-labeled in the presence of rifampicin, which inhibits initiation of RNA synthesis in prokaryotes.

The concentration of rifampicin necessary to inhibit incorporation of [^{3}H]uridine into trichloroacetic acid-precipitable material by isolated bacteroids for more than 95% was found to be 400 μg/ml. This value was determined after a preincubation of bacteroids in the presence of rifampicin for 5 min and a pulse-labeling with [^{3}H]uridine for 10 min (data not shown). Rifampicin at 400 μg/ml strongly reduced protein synthesis in isolated bacteroids as measured by labeling with [^{35}S]methionine (Table I, Expt. 14), while incorporation into nitrogenase decreased to the same extent as total incorporation into protein. A 30 min preincubation with rifampicin results in a 96% inhibition of incorporation. (Table I, Expt. 15), while a preincubation without rifampicin (Table I, Expt. 4) resulted in an unimpaired protein and nitrogenase synthesis. These results are best explained by assuming that for a continued protein synthesis transcription of mRNA is needed. Rifampicin then effectively blocks initiation of mRNA synthesis in isolated bacteroids; when [^{35}S]methionine is added together with rifampicin at time zero an appreciable incorporation into protein is still found. This is probably the result of two effects. During the 10 min labeling period preexisting mRNAs are being transcribed and besides that synthesis of mRNA which had been initiated before the addition of rifampicin continues.

Stability of nif-mRNA in isolated bacteroids

In nitrogen-fixing *K. pneumoniae* the *nif* mRNA appears to be unusually stable with a half-life of 10–18 min [18]. In the following experiment we tried to estimate the biological half-life of *nif* mRNA in isolated bacteroids. It is based on the assumptions that the rate of nitrogenase synthesis at any given moment is proportional to the amount of *nif* mRNA present; and that, as shown before, mRNA synthesis can be effectively blocked by rifampicin. Under these conditions a gradual (probably exponential) decrease of the mRNA concentration may be expected after an initial lag in the decline due to termination of mRNA molecules initiated before the addition of rifampicin. The oxygen electrode cell was filled with 3.6 ml standard incubation medium; 200 μl 2 mM myoglobin were added and the oxygen concentration was reduced to 100 μM; 0.4 ml bacteroid suspension was added (from 16-day-old plants)

and rifampicin to a final concentration of 400 μg/ml. At 2 min intervals 250 μl portions were transferred to 6 ml incubation vials containing 5 μCi [^{35}S]methionine. After a 2 min labeling period further incorporation was stopped; bacteroids were lysed in 250 μl sample buffer, and the mixture was heated at 100°C for 5 min and centrifuged. 15 μl of the supernatant were taken for measuring incorporation of label into acid-precipitable radioactivity. Another sample of 15 μl was analysed by SDS-polyacrylamide gel electrophoresis and incorporation into components I and II was determined. The result of this experiment is shown in Fig. 3. Total incorporation of label decreases approximately exponential; supposing a linear relationship between the incorporation and the amount of mRNA present an estimated half-life of 2.4 min can be calculated for the average mRNA in bacteroids. Incorporation of [^{35}S]methionine into the nitrogenase proteins similarly decreases by an approximately exponential mode but slightly faster than the total incorporation. The average half-life of *nif* mRNAs in bacteroids estimated from this curve is 1.6 min. As a control the same experiment was repeated in the absence of rifampicin. The incorporation of labeled methionine into total protein increased slightly after longer preincubation times (cf. Table I, Expt. 6) while the percentage of total label in the nitrogenase components was essentially constant (data not shown). It has to be noted that the $T_{1/2}$ determined in the reported experiment was obtained under high oxygen conditions. A similar experiment under low [O_2] is technically difficult to perform, because then a series of pulse-labelings would have to be done under precisely the same oxygen concentrations. In a separate experiment (data not shown) we have incubated bacteroids with [^{35}S]methionine and rifampicin in an oxygraph under low [O_2] and taken samples every minute. The increase in label incorporated into nitrogenase proteins between successive samples was measured and taken as the rate of protein synthesis at that moment. The $T_{1/2}$ value graphically determined in this way was approx. 2 min and thus did not differ significantly from the value under a high oxygen concentration.

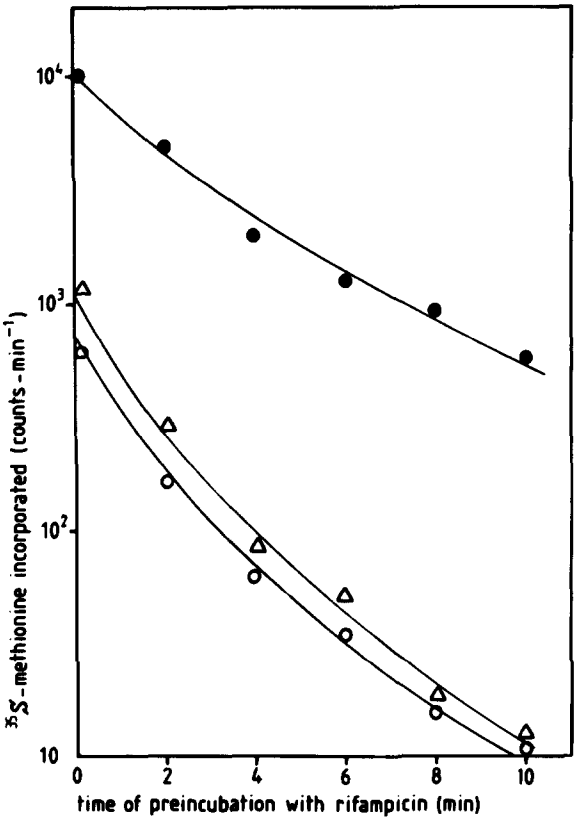

Fig. 3. Rate of total protein and nitrogenase synthesis in isolated bacteroids in the presence of rifampicin. For details see text. ●, incorporation of label in total bacteroid protein. △, incorporation of label in component II of nitrogenase. ○, incorporation of label in component I of nitrogenase.

Discussion

The most striking result of our present study is that nitrogenase synthesis in *R. leguminosarum* bacteroids isolated from pea root nodules 16 days after inoculation is not repressed by oxygen concentrations which completely abolish the function of the nitrogenase complex. At a free oxygen concentration of 100 μM overall protein synthesis was stimulated, probably as a result of a higher energy production, and nitrogenase synthesis was unimpaired which is remarkable because in other nitrogen fixing organisms nitrogenase synthesis is repressed by free oxygen. In *K. pneumoniae* and *A. chroococcum* [1,15,18] and also in free-living *R. japonicum* [19] nitrogenase is only expressed when the free oxygen concentration is very low or zero. Recently Shaw (J. Gen. Microbiol., in the press)

reported that in bacteroids of *Rhizobium* strain NZP2257 isolated from *Lupinus angustifolius* nodules nitrogenase synthesis is repressed by a high oxygen concentration. This result differs from the data presented here and we have no explanation for this discrepancy apart from the fact that two widely different *Rhizobium* strains are involved.

We conclude from the experiments in Table I that translation of *nif* mRNA is not inhibited by oxygen concentrations up to 100 μM but also that *nif* mRNA transcription is not repressed by oxygen, because even after a 30 min preincubation of bacteroids in 100 μM [O_2] nitrogenase synthesis continues unaffected (Expt. 6). In this case nitrogenase synthesis cannot be due to the translation of preexisting *nif* mRNA as a possible pool of *nif* mRNA would be depleted after 30 min, because of the $T_{1/2}$ of about 1.6 min found for this mRNA. We also exclude the possibility that ^{35}S-incorporation into nitrogenase polypeptides only results from peptide chain elongation as nitrogenase synthesis depends upon de novo mRNA synthesis (Table I, Expts. 14 and 15) and does not decrease with time for up to 30 min (Table I, Expts. 1 and 5).

In the experiments shown in Table I myoglobin was added as oxygen carrier because this stimulates acetylene reduction [9]; omission of myoglobin from the medium in labeling experiments at either 2 or 100 μM [O_2] did not change the incorporation in nitrogenase proteins (data not shown), however; this suggests once more that nitrogenase synthesis in isolated bacteroids does not depend upon low oxygen.

As for the effect of nitrogenous compounds (NH_4^+, NO_3^-, NO_2^-) on nitrogenase synthesis we also conclude that a distinct difference exists between bacteroids on the one hand and free-living nitrogen fixers on the other. In *K. pneumoniae* ammonia represses nitrogenase synthesis [15] and decreases *nif* mRNA half-life [18]. In free-living rhizobia ammonia appears to inhibit the appearance of nitrogenase activity only under low oxygen conditions [1,3,20]. In *Rhizobium* sp. 32H1 nitrogenase activity was present in a medium containing ammonia; probably fixed nitrogen is largely excreted as ammonia [7]. Laane et al. [21] reached the same conclusion for isolated bacteroids; no accumulation of ammonia by bacteroids was found. Our finding that ammonia stimulates overall protein synthesis might indicate that it is taken up to a certain extent by bacteroids.

We also could not detect a specific influence of cyclic GMP on nitrogenase in *R. leguminosarum* bacteroids as was reported for free-living *R. japonicum* by Lim et al. [17]. It may be important in this respect that the effect of cGMP was only observed in the initial stages of *nif* expression; bacteroids have obviously passed this stage of development.

The ionophores valinomycin and tetraphenylphosphoniumbromide are known to decrease the membrane potential and inhibit acetylene reduction [16]. Our results indicate that both compounds decrease protein synthesis in bacteroids but have no selective effect on nitrogenase synthesis. The mechanism by which valinomycin probably inhibits protein synthesis is possibly by allowing dissipation of potassium ions from the cell; certainly the amount of ATP will not be the limiting factor, because valinomycin slightly increases the ATP/ADP ratio [16].

We conclude from the present data that in *R. leguminosarum* bacteroids isolated from pea root nodules 16 days after inoculation regulation of the *nif* genes probably no longer takes place and that nitrogenase protein synthesis is 'constitutive' under these circumstances. This might be explained by considering the position of the endosymbiotic *Rhizobium*. Large fluctuations in oxygen concentration or fixed-nitrogen compounds will probably not occur, nor appreciable changes in *trans*-membrane ΔpH or $\Delta\Psi$, while fixed N is exported to the plant as NH_4^+ [22]. An important question then is why a set of *nif*-genes would be constitutively expressed in bacteroids and not in bacteria. Maybe a parallel could be drawn between the process of bacteroid development and differentiation of *Bacillus subtilis* during sporulation [23]. In the latter case a modified RNA polymerase seems to be responsible for the selective transcription of a set of sporulation genes. It would be very interesting to find out whether an altered bacteroid RNA polymerase is responsible for the transcription of *nif* genes in *R. leguminosarum* bacteroids; though this RNA polymerase would have the same sensitivity to rifampicin as that in *Rhizobium* bacteria.

Labeling of isolated bacteroids with [^{35}S]methionine results in the production of many radioactive protein bands in SDS-polyacrylamide gels, of which the most prominent ones comigrate with the nitrogenase component I and II proteins. Shaw and Sutton [24] reported the synthesis of ^{35}S-labeled proteins by *R. lupini* bacteroids but they were unable to further identify the protein bands as nitrogenase proteins. The present results show that in *R. leguminosarum* bacteroids the putative nitrogenase proteins are precipitable with antisera specific against the nitrogenase components [14], which proves that nitrogenase is synthesized in this system. The use of antisera to quantify the percentages of total incorporated label found in the individual nitrogenase components was used in a number of experiments (7–10 and 13 in Table I) and gave the same values as the method we have used earlier, where we cut out the bands from a gel and assayed the radioactivity in a scintillation counter [8].

The ratio of incorporation into component I and II was slightly variable (cf. Table I), but in all cases more label was found in CII than in CI. If the methionine content and the molecular weights of the nitrogenase components of *R. leguminosarum* are similar to those in *R. lupini* [25] then the ratio of ^{35}S-label in component CII/CI would be 0.255, based on a 1 : 1 molar ratio of the components. The different results in Table I show an average ratio of 1.76, which suggests that component II is synthesized in about 7-fold molar excess over CI. These results are in accordance with our earlier observations [14,26] where component II was shown to be synthesized and present in a more than equimolar ratio respective to CI, although the difference was less drastic than in the present study. This phenomenon may be explained by assuming that regulation takes place at the translational level or that a second promoter is located upstream of genes D and K. Analogous experiments on *K. pneumoniae* [15] and *A. chroococcum* [27] show that in these organisms an excess of component II is also synthesized; in *A. vinelandii* [28] for component II a molar excess of 6–8 is reported. Recently Shaw (J. Gen. Microbiol., in the press) also reported in similar experiments on *R. lupini* bacteroids that in this case considerably more [^{35}S]methionine label is incorporated in component II than CI.

Bacteroids from plants about 22 days after inoculation show a maximal acetylene reduction. When bacteroids of this age were ^{35}S-labeled the amount of incorporation was significantly lower than at 16 days and the relative amount of nitrogenase was variable, but always lower than at 22 days. Therefore all further labeling experiments were done with bacteroids from nodules isolated at 16 days after infection. This result agrees with our earlier observations on protein synthesis in bacteroids 'in planta' [14,26], where bacteroids in an early stage of development were found to be most active. After 22 days the capacity to reduce acetylene and to synthesize proteins declines more steeply in isolated bacteroids than is the case inside the intact plant. An explanation for this phenomenon could be that older bacteroids become more sensitive to environmental changes and thus are more easily damaged during isolation. Similar changes in properties are reported by Trinchant et al. [13], where older bacteroids appear to have a lower [O_2]-optimum for acetylene reduction. *Rhizobium* bacteroids also lose their colony-forming capacity after a certain stage of development [29].

Because in the present study the biological half-life of *nif* mRNA plays an important role we tried to estimate this value from the rate of nitrogenase synthesis during inhibition of RNA synthesis with rifampicin. Experiments designed to pulse label *nif* mRNA in bacteroids with [^{32}P]phosphate or [^{3}H]uridine failed to yield sufficient radioactive RNA for a quantitation by hybridization. The value of 1.6 min for the *nif* mRNA half-life in bacteroids is in the same range as that found in bacteria in general but greatly differs from values (11–18 min) reported for *nif* mRNA in *K. pneumoniae* measured after repression of nitrogenase synthesis [15] or by quantitative hybridization of *nif* mRNA [18].

The present study shows that repression and derepression of *nif* genes in mature *Rhizobium* bacteroids is not an important regulating mechanism in the cellular economy; it will be interesting to study which factors are involved in derepression of the *nif* genes. Possibly nodule-specific plant proteins (nodulins) are involved in this process.

Acknowledgements

The authors thank Mrs. Scott Israel and Marcel Vos for skillful technical assistance during the initial stages of this investigation. We are grateful to Mr. H.J. Wassink for help in the purification of bacteroids. Dr. T. Bisseling and Dr. N.C.M. Laane are thanked for helpful discussions. We thank Dr. B.D. Shaw for making available his results on *R. lupini* bacteroids before publication. Mr. P. Madern is thanked for making the illustrations and Mrs. M.J. Van Neerven for typing the manuscript.

References

1 Eady, R. (1981) in Proc. 4th Int. Symp. Nitrogen Fixation (Gibson, A.H. and Newton, W.E., eds.), pp. 172–182, Australian Academy of Sciences, Canberra
2 Kennedy, C., Cannon, F., Cannon, M., Dixon, R., Hill, S., Jensen, J., Kumar, S., McLean, P., Merrick, M., Robson, R. and Postgate, J. (1981) in Proc. 4th Int. Symp. Nitrogen Fixation (Gibson, A.H. and Newton, W.E., eds.), pp. 146–156, Australian Academy of Sciences, Canberra
3 Bergersen, F., Turner, G.L., Gibson, A.H. and Dudman, W.F. (1976) Biochim. Biophys. Acta 444, 164–174
4 Child, J.J. (1980) in Recent advances in biological nitrogen fixation (Subba Rao, N.S., ed.), pp. 325–343, London, Arnold
5 Evans, W.R. and Keister, D.L. (1976) Can. J. Microbiol. 22, 949–952
6 Kaneshiro, T., Crowell, C.O. and Hanrahan, R.F. (1978) Int. J. System. Bacteriol. 28, 27–31
7 Tubb, R.S. (1976) Appl. Environ. Microbiol. 32, 483–488
8 Bisseling, T., Van den Bos, R.C. and Van Kammen, A. (1978) Biochim. Biophys. Acta 539, 1–11
9 Laane, C., Haaker, H. and Veeger, C. (1978) Eur. J. Biochem. 87, 147–153
10 Wittenberg, J.B., Bergersen, F.J., Appleby, C.A. and Turner, G.L. (1974) J. Biol. Chem. 249, 4057–4066
11 Bisseling, T., Moen, A.A., Van den Bos, R.C. and Van Kammen, A. (1980) J. Gen. Microbiol. 118, 377–381
12 Zabel, P., Moerman, M., Van Straaten, F., Goldbach, R. and Van Kammen, A. (1982) J. Virol. 41, 1083–1088
13 Trinchant, J.C., Birot, A.M. and Rigaud, J. (1981) J. Gen. Microbiol. 125, 159–165
14 Bisseling, T., Van den Bos, R.C., Weststrate, M.W., Hakkaart, M.J.J. and Van Kammen, A. (1979) Biochim. Biophys. Acta 562, 515–526
15 Eady, R.R., Rafick, J., Kennedy, C., Postgate, J.R. and Ratcliffe, H.D. (1978) J. Gen. Microbiol. 104, 277–285
16 Laane, C., Krone, W., Konings, W.N., Haaker, H. and Veeger, C. (1979) FEBS Lett. 103, 328–332
17 Lim, S.T., Hennecke, H. and Scott, D.B. (1979) J. Bacteriol. 139, 256–263
18 Kaluza, K.B. and Hennecke, H. (1981) Arch. Microbiol. 130, 38–43
19 Scott, D.B., Hennecke, H. and Lim, S.T. (1979) Biochim. Biophys. Acta 565, 365–378
20 Scowcroft, W.R., Gibson, A.H. and Pagan, J.D. (1976) Biochem. Biophys. Res. Commun. 73, 516–523
21 Laane, C., Krone, W., Konings, W., Haaker, H. and Veeger, C. (1980) Eur. J. Biochem. 103, 39–46
22 O'Gara, F. and Shanmugam, K.T. (1976) Biochim. Biophys. Acta 437, 313–321
23 Haldenwang, W.G. and Losick, R. (1979) Nature 282, 256–260
24 Shaw, B.D. and Sutton, W.D. (1979) Biochim. Biophys. Acta 563, 216–226
25 Whiting, M.J. and Dilworth, M.J. (1974) Biochim. Biophys. Acta 371, 337–351
26 Van den Bos, R.C., Bisseling, T. and Van Kammen, A. (1978) J. Gen. Microbiol. 109, 131–139
27 Robson, R.L. (1979) FEMS Microbiol. Lett. 5, 259–262
28 Tianqin, G. and Fazhu, W. (1981) Acta Microbiol. Sinica 21, 452–456
29 Van den Bos, R.C. and Broughton, W.J. (1981) Arch. Microbiol. 129, 349–352