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

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Summary

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

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

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

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

Introduction

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

Abbreviation: SDS, sodium dodecyl sulfate.

these proteins have to be derepressed at a certain moment during nodule development.

In this paper, we report on the appearance of leghemoglobin and nitrogenase activity, which reveals that leghemoglobin is probably essential for the functioning of nitrogenase. Further we studied the rate and the pattern of protein synthesis of bacteroids and plant tissue from root nodules during nodule development and in relation to this the quality and quantity of ribosomal RNA in bacteroids.

Materials and Methods

The growth of pea plants (*Pisum sativum*, var. Rondo), inoculation with *Rhizobium leguminosarum* (PRE), determination of nitrogenase activity of intact nodules, preparation of soluble plant proteins from nodules, [^{35}S]-sulfate labeling of pea plants, determination of protein concentration, and quantification of ^{35}S incorporated into protein, polyacrylamide gel electrophoresis (SDS: Serva Fine Biochemicals, Heidelberg, F.R.G. [4]), and staining and radioautography of the gels, were performed as described previously [5]. Estimation of the radioactivity incorporated into CI and CII of nitrogenase was performed after cutting protein bands from the polyacrylamide gel co-migrating with purified CI and CII; ^{35}S radioactivity was determined as described before [5]. Purified bacteroid preparations were obtained by collecting the bacteroids from gradients. Sucrose gradient centrifugation of bacteroids was done as described by Van den Bos et al. [4]; bacteroids were separated from contaminating plant material by centrifugation through a linear 25 ml sucrose gradient (12—30%, w/v) in 25 mM Tris-HCl, 20 mM sodium dithionite (pH 7.5) for 15 min at 1500 $\times g$. Bacteroids were pelleted by centrifugation for 5 min at 12 000 $\times g$.

Preparation of soluble bacteroid proteins. The lysis of the bacteroids was as described before [5] except for the following modifications: the bacteroids were isolated and lysed anaerobically, by flushing with argon and buffers contained 20 mM sodium dithionite, to prevent partial precipitation of nitrogenase.

RNA determination. Bacteroids were lysed by suspending a pellet of cells in 100 μl 1% SDS and heating at 100°C for 5 min. For determination of RNA in the lysate 10 μl 50% trichloroacetic acid was added; after centrifugation for 5 min at 12 000 $\times g$ the precipitate was washed with 96% ethanol, and extracted with 200 μl 5% trichloroacetic acid at 95°C. The pellet was washed once with 200 μl 5% trichloroacetic acid and the two supernatants were combined. RNA content in the extract was measured with the orcinol reaction [6]. For total protein determination 20 μl 1 N NaOH was added to 100 μl lysate and the mixture was heated for 2 h at 60°C. After precipitation of proteins with trichloroacetic acid, the protein content was measured [5].

Isolation of ribosomes. To avoid RNA degradation during the procedure all glassware was sterilized by heating for 1 h at 150°C and all manipulations were done at 0°C. Bacteroids were suspended in 10 mM Tris-HCl buffer (pH 7.4), 10 mM magnesium acetate, 1 mM dithioerythritol and, as a ribonuclease inhibitor, 0.5 mg/ml Macaloid (Langer and Co., Ritterude, F.R.G.). Cells were

disrupted with a Branson B30 cell disruptor for 3 min using a microtip. The homogenate was centrifuged for 10 min at 12 000 × *g*. The resulting supernatant was centrifuged for 1 h at 200 000 × *g*. The pellet thus obtained consists largely of ribosomes.

Isolation of ribosomal subunits. Ribosomes were suspended at a concentration of 5—10 mg/ml in a low-Mg^{2+} buffer (10 mM Tris-HCl/0.05 mM $MgCl_2$/0.5 mM NH_4Cl/0.1 mM dithioerythritol/10 mM NaCl, pH 7.4) and 1 ml of this suspension was loaded onto a linear 10—30% (w/v) sucrose gradient in the same buffer in SW27 tubes (Spinco). After centrifugation at 20 000 rev./min for 16 h at 4°C the gradient was fractionated through a Uvicord, recording the transmittance at 254 nm. Appropriate fractions containing the ribosomal subunits were pooled and the subunits were pelleted by centrifugation for 1 h at 200 000 × *g*.

Polyacrylamide gel electrophoresis of ribosomal RNA. Ribosomes or individual ribosomal subunits were suspended in 10 mM Tris-HCl (pH 7.4)/1 mM EDTA/0.5% SDS/0.1% Bromophenol Blue/10% sucrose. 10-μl samples containing 1—15 μg ribosomes were loaded on cylindrical (6 mm diameter × 80 mm length) 2% polyacrylamide gels, containing 0.5% agarose. The buffer in the gel was the same as in the two buffer compartments and contained: 36 mM Tris-H_3PO_4/30 mM $Na_2HPO_4 \cdot H_2O$/10 mM EDTA/0.2% (w/v) SDS (pH 7.7). After polymerisation of the gel approx. 1 cm was cut from the top with a razor blade. Electrophoresis was at 6 mA per gel until the Bromophenol Blue marker reached the end of the gel.

After electrophoresis absorbance at 260 nm along the gels was scanned with a Gilford linear gel transport system coupled to a Beckman DU spectrophotometer.

Immunological measurement of leghemoglobin. Leghemoglobin was purified from 80 g nodules of *P. sativum* infected with *R. leguminosarum* (PRE) according to Appleby [7]. The ferrileghemoglobins were eluted from a DEAE cellulose column (10 × 1 cm), with a linear gradient of sodium acetate buffer (0.01 M, pH 5.5, 75 ml; to 0.1 M pH 5.5, 75 ml; flow rate, 10 ml/h), generated with an Ultrograd Gradient Mixer (LKB). Further purification was performed by ACA 44 Ultrogel (LKB) chromatography (column 4 × 70 cm, 0.1 M Tris-HCl pH 7.5, flow rate, 20 ml/h).

Antiserum against leghemoglobin was prepared by injecting a rabbit intravenously with a mixture of the different, electrophoretically pure, leghemoglobins in 0.9% NaCl; 2 and 3 months later this was followed by subcutaneous injections of 1 mg leghemoglobin in 0.9% NaCl, mixed with an equal volume of Freund's complete adjuvant (Difco, Detroit, MI). The rabbit was bled through the jugular ear vein 14 days after the third injection. Under these conditions the titre allowing immunoprecipitation against 1 mg/ml leghemoglobin was 1/32.

The specificity of the leghemoglobin-antiserum was tested by immunodiffusion performed on 1.5% agar slides as described by Ouchterlony [8]. With soluble plant protein preparations, from uninfected roots of *P. sativum* an immunoprecipitate was never formed, while with a soluble plant protein preparation isolated from nodules only one precipitation line was formed, and using a non-immune serum no immunoprecipitate was formed with leghemoglobin.

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

Results

Appearance of leghemoglobin and nitrogenase activity

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

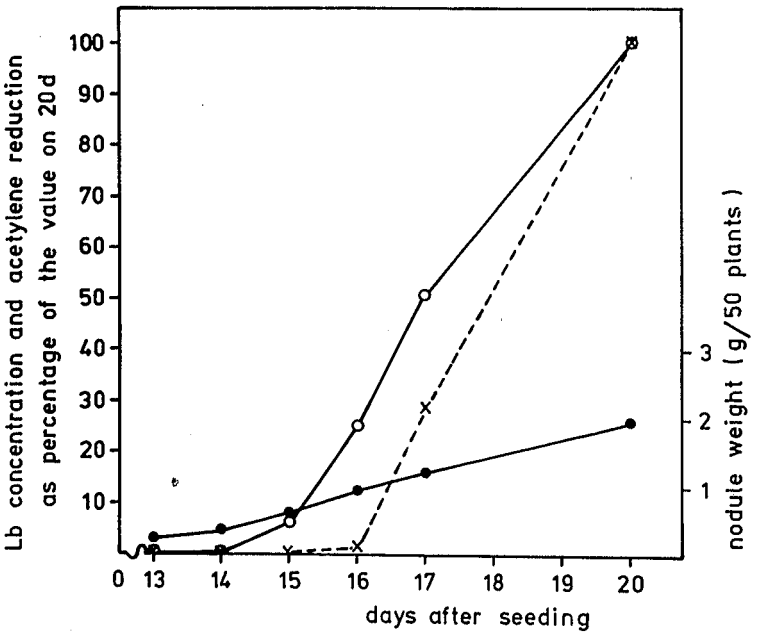

Fig. 1. Acetylene reduction, leghemoglobin content and nodule weight during development of the root system of *P. sativum*. 50 plants were used per day and nodule weight, leghemoglobin content and acetylene reduction were determined on the same plants. o———o, leghemoglobin content; X———X, acetylene reduction;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

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

Nitrogenase synthesis

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

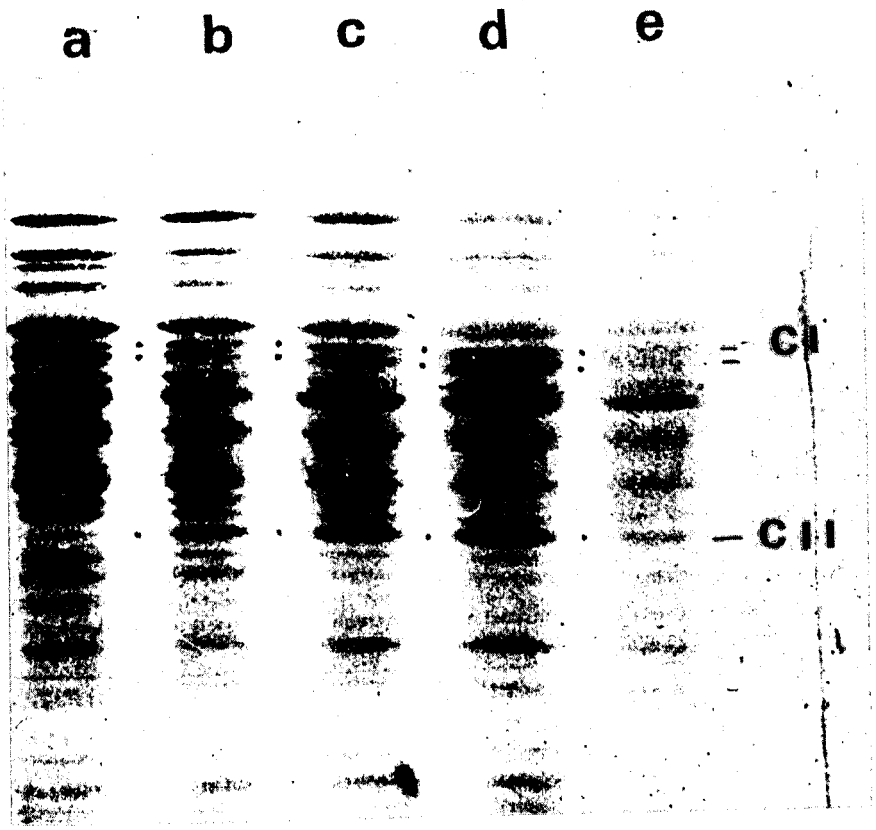

Fig. 2. Radioautograph of soluble bacteroid proteins (15 μg protein in slot a and b and 25 μg in c, d and e) analysed on 10% acrylamide gels. Bacteroids were isolated from 10 plants of different ages: a, 16 days; b, 19 days; c, 22 days; d, 23 days; e, 28 days. The direction of electrophoresis was downward.

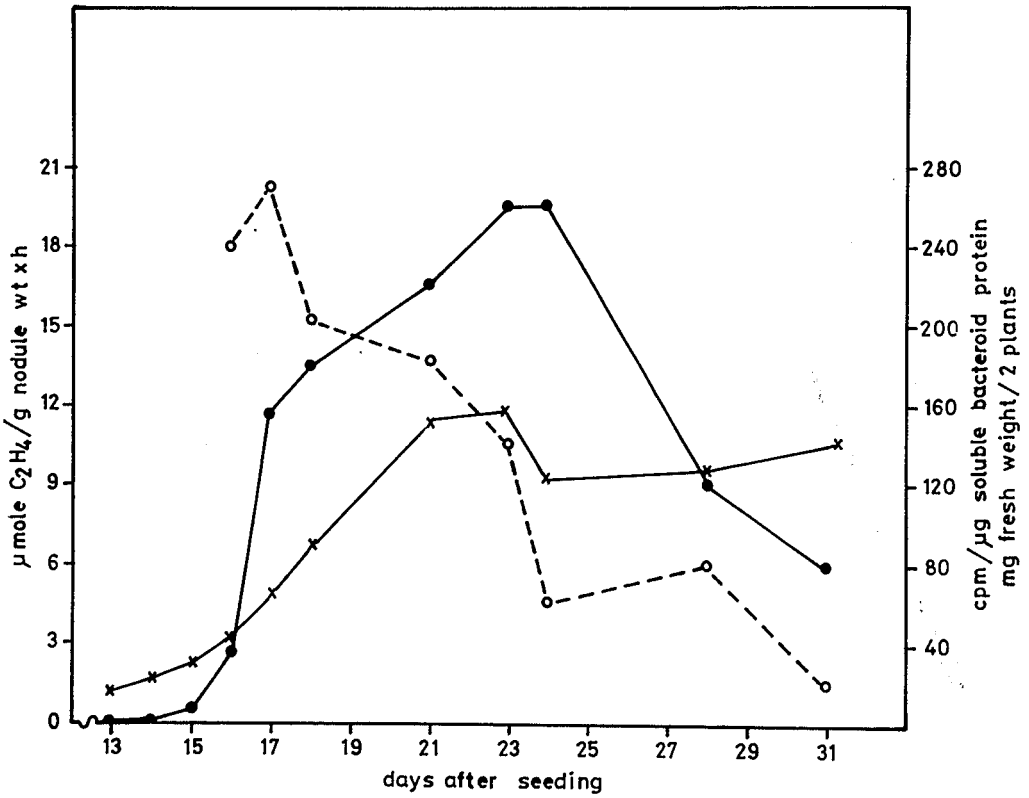

Fig. 3. Nodule weight, acetylene reduction and bacteroid protein synthesis during development of *P. sativum*. Nodule weight and acetylene reduction were determined on 35 plants and protein synthesis in 10 plants. •———•, acetylene reduction (μmol C_2H_4/g nodule fresh wt. X h); X———X, nodule weight (mg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

Protein synthesis

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

TABLE I

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

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

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

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

Amount of RNA per cell in bacteroids of different ages

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

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

Gel electrophoretic analysis of bacteroid ribosomal RNA

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

TABLE II

RNA CONTENT OF BACTEROIDS ISOLATED FROM PEA PLANTS OF DIFFERENT AGES

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

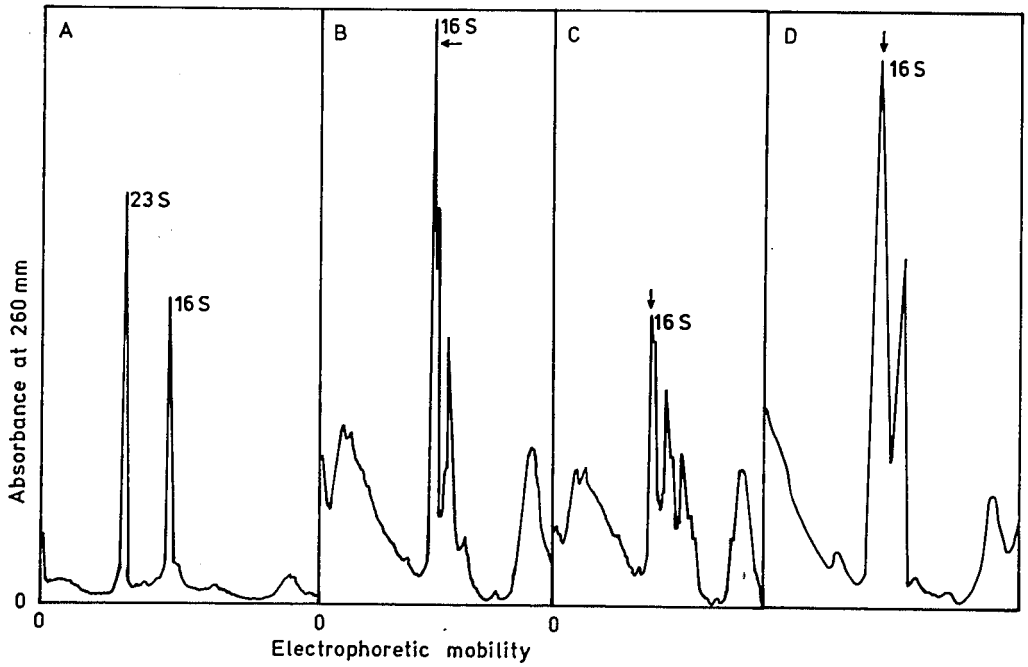

Fig. 4. SDS-polyacrylamide gel electrophoresis of rRNA from *R. leguminosarum* (PRE). Electrophoresis was from left to right. Absorbance and electrophoretic mobility are given in arbitrary units. rRNA was isolated from: logarithmic bacteria (A), bacteroids 21 days (B), bacteroids 32 days (C) and stationary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

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

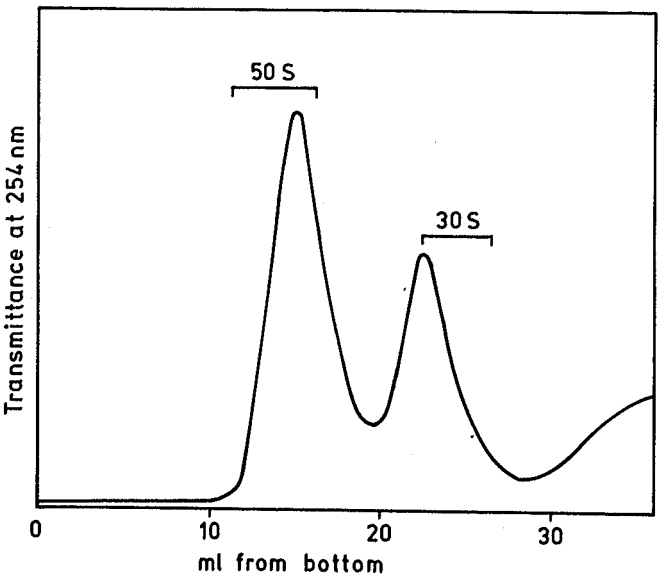

Fig. 5. Sucrose gradient centrifugation of ribosomal subunits isolated from 21 day old bacteroids.

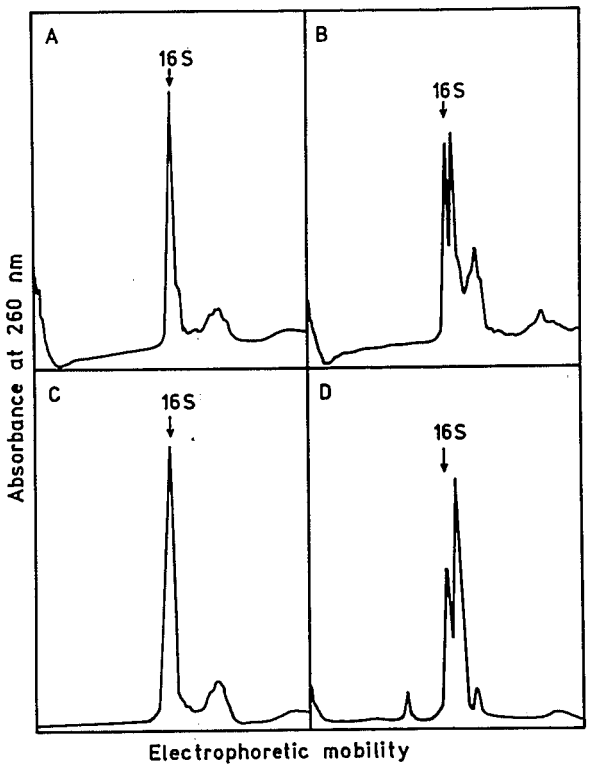

Fig. 6. SDS-polyacrylamide gel electrophoresis of ribosomal subunits. Isolation of subunits is shown in Fig. 5; 3—5 μg of subunits were electrophoresed. A_{260} was measured along the gel. A, 30-S subunits from bacteroids; B, 50-S subunits from bacteroids; C, 30-S subunits from stationary phase bacteria;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

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

Discussion

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

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

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

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

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

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

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

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

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

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