

Location of Nitrogenase and Ammonia-assimilatory Enzymes in Bacteroids of *Rhizobium leguminosarum* and *Rhizobium lupini*

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Cytoplasmic and membrane fractions were isolated from bacteroids of *Rhizobium leguminosarum* and *R. lupini* disrupted by lysozyme-induced lysis and sonication. The release of nitrogenase, glutamine synthetase (EC 6.3.1.2), glutamate synthase (EC 2.6.1.53) and glutamate dehydrogenase (EC 1.4.1.4) activities was compared with NADH oxidase and 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) activities as markers for membrane and cytoplasmic enzyme activities respectively. Nitrogenase was localized in the cytoplasm. Most of the glutamate dehydrogenase activity was released on lysozyme treatment of intact bacteroids although some was released from membrane fractions on sonication, indicating a very superficial localization. Glutamate synthase was present at very low levels in *R. leguminosarum* bacteroids; the level in *R. lupini* bacteroids was higher and the enzyme was probably weakly membrane-bound. About 95% of the glutamine synthetase activity was in the nodule cytosol, and the supernatant of washed bacteroids had a high specific activity; the remaining activity was unevenly distributed between soluble and membrane fractions.

INTRODUCTION

Bacteroids are the site of nitrogen fixation in nodules of leguminous plants (Kennedy, Parker & Kidby, 1966), and after short, pulsed exposures of intact nodules to $^{15}\text{N}_2$ (Kennedy, 1966*a, b*) glutamate, glutamine and aspartate showed the highest enrichment with ^{15}N . Subsequently glutamate synthase (Nagatani, Shimizu & Valentine, 1971; Robertson, Warburton & Farnden, 1975*b*), glutamate dehydrogenase (Grimes & Fottrell, 1966) and glutamine synthetase (Kennedy, 1973; Dunn & Klucas, 1973) were found both inside and outside the bacteroids.

Bacteroids of *Rhizobium leguminosarum* are sensitive to lysis by lysozyme, but the free-living bacteria require the addition of EDTA before they become sensitive to lysozyme, indicating that bacteroids have a modified outer membrane (van Brussel, 1973; van Brussel, Planqué & Quispel, 1977). In free-living nitrogen-fixing *Rhizobium japonicum* and 'cowpea' *Rhizobium* most of the newly-fixed NH_4^+ is exported out of the organism (O'Gara & Shanmugam, 1976). If this occurs in bacteroids, the excreted ammonia could be converted to asparagine by a co-operative system comprising glutamine synthetase, glutamate synthase and asparagine synthetase (Scott, Farnden & Robertson, 1976).

The levels of glutamine synthetase, glutamate synthase and glutamate dehydrogenase are controlled by the NH_4^+ concentration in the culture medium in *Klebsiella aerogenes* (Tempest, Meers & Brown, 1973) and several other bacteria including the free-living form of *Rhizobium* (Brown & Dilworth, 1975). The levels of these enzymes were different in the

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bacteroid forms of five *Rhizobium* species investigated. This could indicate different ammonia levels inside the respective bacteroids or, more importantly, a different response in bacteroids to varying ammonia concentrations. The latter point could have important implications since glutamine synthetase has both an assimilatory and a regulatory role in nitrogen metabolism (Streicher *et al.*, 1974). Glutamine synthetase is involved in the regulation of nitrogenase synthesis in *Klebsiella pneumoniae* (Tubb, 1974; Shanmugam & Valentine, 1975). If a comparable control mechanism operates in *Rhizobium*, the differences in glutamine synthetase activities in different bacteroid species reported by Brown & Dilworth (1975) could be explained if the ammonia-assimilating enzymes had a specific location inside the bacteroids which resulted in an ammonia excess in one part of the cell and an ammonia limitation in another.

To evaluate this possibility, we have attempted to determine the location of nitrogenase, glutamate dehydrogenase, glutamate synthase and glutamine synthetase by fractionating bacteroids into membranes and cytoplasm and comparing the release of these enzymes with NADH oxidase and 3-hydroxybutyrate dehydrogenase as markers for membrane-bound and cytoplasmic enzyme locations respectively (Osborn *et al.*, 1972; Brown & Dilworth, 1975).

METHODS

Plants and production of nodules. Yellow lupins (*Lupinus luteus*), field-grown at the State Forestry Nurseries at Baarn, The Netherlands, were inoculated with *Rhizobium lupini*; nodules were harvested 2 to 6 weeks before flowering. Green peas (*Pisum sativum* cv. Rondo; Cebeco, Rotterdam, The Netherlands) were inoculated with *Rhizobium leguminosarum* strain A171, and were cultivated as described by Planqué & van Brussel (1976); nodules were harvested at 21 days.

Preparation of nodule fractions and bacteroids

Root systems of lupin and pea plants were thoroughly washed with water. Bacteroids were released from the root nodules by homogenizing the root system in a Waring blender for 2 min in a medium containing 0.05 M-potassium phosphate buffer pH 7.4, 0.3 M-sucrose and 2% (w/v) soluble polyvinyl pyrrolidone K90 (Fluka, Buchs, Switzerland). When nitrogenase activity was to be assayed, 0.1 mg sodium dithionite ml⁻¹ and 0.1 mg dithiothreitol ml⁻¹ were added, and the medium was sparged with N₂ before adding the nodules. All subsequent operations were under N₂.

Nodule cytosol. Large plant fragments and other debris were removed by filtration through cheese cloth, and centrifuging the filtrate at 500 g for 2 min. Bacteroids, together with membranous plant material, were collected by centrifuging at 5000 g for 10 min. The supernatant, designated nodule cytosol, was stored at 0 °C or frozen under N₂. It contained leghaemoglobin and other constituents of infected and uninfected cells of root tissue. All centrifugations were at 4 °C.

Bacteroid preparation. The pellet obtained by centrifuging at 5000 g was resuspended and washed twice in sucrose/phosphate buffer using a buffer to pellet ratio of 10:1 (v/v) and then centrifuged for 10 min at 4000 g. Phase contrast microscopy and electron microscopy of the supernatants, designated first and second wash supernatants, showed the absence of bacteroids although a considerable quantity of membranous material was present (Fig. 1). The washed pellet was suspended in sucrose/phosphate buffer pH 7.4, and designated bacteroid suspension. All fractions were stored under N₂; the wash supernatants were kept at 0 °C or frozen.

Preparation of bacteroid fractions

Sonication. Suspensions of lupin bacteroids were sonicated for 2 to 3 min under a stream of N₂ in an ice bath, using a Branson model L sonifier. The sonicate was separated into soluble and insoluble fractions by centrifuging at 105000 g for 30 min at 4 °C. Suspensions of pea bacteroids were sonicated for six periods of 15 s with intermittent cooling. Separation into soluble and insoluble fractions was achieved by centrifuging the supernatant at 50000 g for 20 min. The supernatant and pellet fractions were designated bacteroid sonicate supernatant and bacteroid sonicate pellet.

Lysozyme lysis. Osmotic shock caused extensive lysis of bacteroids after the bacteroid suspension had been incubated with lysozyme. The techniques used were based on those employed by van Brussel (1973). Freshly-prepared bacteroid suspension (20 ml) in 0.05 M-potassium phosphate buffer pH 7.0, containing 0.3 M-sucrose was incubated at 30 °C for 30 min with 1.0 mg lysozyme ml⁻¹ (Sigma, grade I). At the end of the

incubation the bacteroids were removed by centrifuging at 5000 g for 10 min to give a bacteroid-free supernatant (designated lysozyme incubation supernatant) which was removed and stored under N_2 . The pellet was then 'shocked' by rapid resuspension in 20 ml de-aerated distilled water. The resulting preparation of completely lysed bacteroids was separated into a membrane and a soluble fraction (designated lysozyme-shock pellet and lysozyme-shock supernatant) by centrifuging at 105 000 g. Under phase contrast microscopy, transparent bacteroid forms were seen in the lysozyme-shock pellet; these were comparable to ghost preparations of *Salmonella* (Obsorn *et al.*, 1972). The ghosts can be separated into cytoplasmic membrane and outer membrane preparations (K. Planqué, A. v. d. Werff, T. v. Slogteren, unpublished results). Sonication of the lysozyme-shock pellet for 45 s followed by centrifuging at 50000 g for 20 min resulted in a lysozyme-shock sonication pellet and a lysozyme-shock sonication supernatant.

Enzyme assays

Nitrogenase. The nitrogenase activity of bacteroids and extracts was measured by the acetylene reduction technique as described by Akkermans (1971). Assays on intact bacteroids routinely used 12 ml rubber-sealed vials containing (in 1.5 ml) 75 μmol potassium phosphate buffer pH 7.4, 50 μmol sodium succinate, and a suitable quantity of bacteroids in phosphate buffer, under 0.1 atm O_2 and 0.2 atm acetylene in N_2 . Extracts, and sometimes whole bacteroids, were assayed in a similar manner but anaerobically using an ATP-generating system and sodium dithionite assay as described by Yates & Planqué (1975).

Glutamine synthetase (EC 6.3.1.2). Both γ -glutamyl transferase activity and synthetase activity were measured. Transferase activity was assayed as described by Shapiro & Stadtman (1970), except that 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid (HEPES) buffer pH 7.3, was used instead of imidazole/HCl. Synthetase activity was assayed as described by Rowe *et al.* (1970) except that ATP was replaced by an ATP-generating system comprising (per 0.4 ml assay mixture) 1.8 μmol Na_2ATP , 250 μmol creatine phosphate and 90 μg creatine kinase. The production of γ -glutamyl hydroxamate was determined spectrophotometrically by calibration against authentic material. Under the conditions used 2.5 mol product had an E_{540} of 0.001.

NADH oxidase, glutamate dehydrogenase (EC 1.4.1.4), **glutamate synthase** (EC 2.6.1.53) and **3-hydroxybutyrate dehydrogenase** (EC 1.1.1.30). These enzymes were assayed spectrophotometrically at 340 nm by measuring NADH oxidation. All assays contained (in 2 ml) 50 μmol HEPES buffer pH 7.5, 0.2 μmol NADH, and enzyme preparation. The NADH oxidase activity was measured, and then various compounds were added to the assay mixture to allow measurement of other enzyme activities: 20 μmol NH_4Cl and 20 μmol 2-oxoglutarate pH 7.0 were added to assay glutamate dehydrogenase activity; 20 μmol 2-oxoglutarate and 2 μmol glutamine to assay glutamate synthase activity; and 2 μmol acetoacetate to assay 3-hydroxybutyrate dehydrogenase activity.

All assays were corrected for non-specific NADH oxidation measured in the absence of only the NH_3 donor. No difference in this activity was observed between enzyme preparations which were fresh, stored, or passed over Sephadex G-25 (2.5 \times 30 cm column) to remove inhibitors and endogenous substrates. The ammonia-assimilating enzymes were assayed within 3 h of the start of nodule isolation.

Protein content. This was measured according to Lowry *et al.* (1951), using bovine serum albumin as a standard.

Electron microscopy. Electron micrographs of membranes were prepared on formvar-coated grids by negative staining with phosphotungstic acid (Brenner & Horne, 1959). Thin sections of bacteroid suspensions were prepared after mixing the suspension with 2% (w/v) agar and fixation as described by Kijne (1975).

Other methods. Membranes were centrifuged on a sucrose gradient according to Osborn *et al.* (1972) and bacterial outer membrane was identified by heptose determinations according to Dische (1962).

RESULTS

Preparation of bacteroid fractions

After the homogenization of root nodules, many bacteroids were still retained in clumps of cytoplasm. This was especially true for very young (9 day) pea nodules. Only after washing were bacteroids completely released, and a supernatant containing most of the plant cell membranes was separated from the bacteroid pellet by centrifuging. The membranes released on washing the bacteroid pellet (Fig. 1) are probably not outer membranes of the bacteroid cell wall since heptose determinations were negative and no material was found in sucrose gradients at the characteristic density of bacteroid membranes.

The bacteroid suspension was practically free from starch granules and plant debris

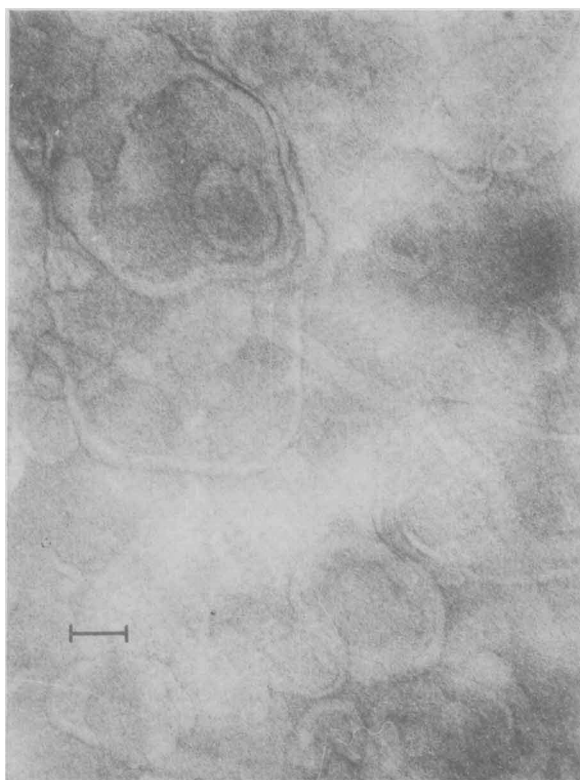


Fig. 1

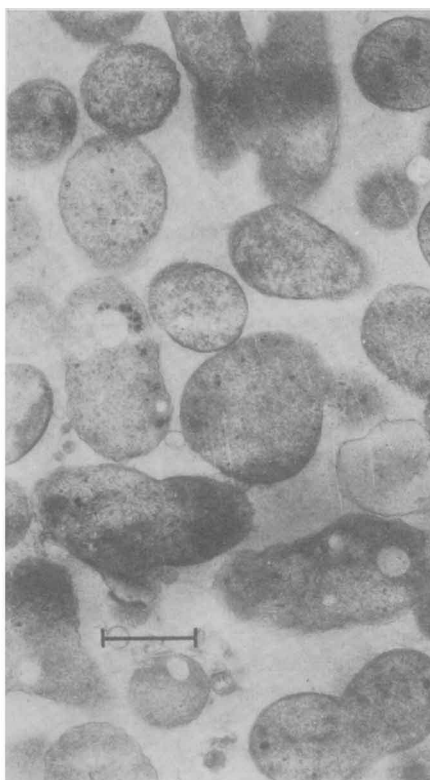


Fig. 2

Fig. 1. Electron micrograph of negatively-stained material released in the first wash supernatant on washing pea bacteroids obtained by homogenization. Bar marker represents $0.1 \mu\text{m}$.

Fig. 2. Electron micrograph of the preparation of pea bacteroids used in the experiments. Bar marker represents $1 \mu\text{m}$.

when examined by phase contrast microscopy. No starch granules or plastids, and only small amounts of membranous material, were observed by electron microscopy (Fig. 2). Lysozyme incubation followed by osmotic shock caused a rapid decrease in the absorbance of lupin and pea bacteroid suspensions (Fig. 3). Pea and lupin bacteroids were also easily ruptured by sonication whereas free-living *R. leguminosarum* was not disrupted under the conditions used. Protein recovery from all treatments was similar, indicating that no cell material was lost.

Location of enzyme activities

The measured activities of nitrogenase in fractions of lupin and pea bacteroids varied slightly but the overall distribution pattern (Table 1) was similar in different experiments. The activity of each fraction was determined at several protein concentrations since each had an optimum protein concentration (Fig. 4). Intact pea and lupin bacteroids had low nitrogenase activities unless oxygen and succinate were used as substrates in place of ATP and dithionite.

Lupin bacteroids became permeable to ATP and dithionite on treatment with lysozyme, without an osmotic shock treatment (Table 1). About the same total activity was found as when nitrogenase was released by sonication or by osmotic shock following lysozyme treatment. The distribution of nitrogenase throughout the fractions examined supports the view

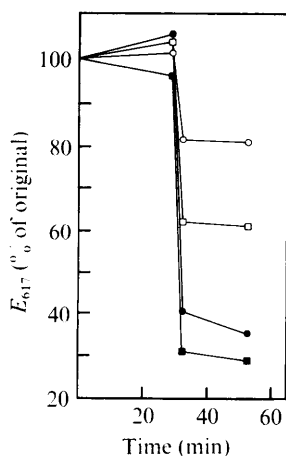


Fig. 3

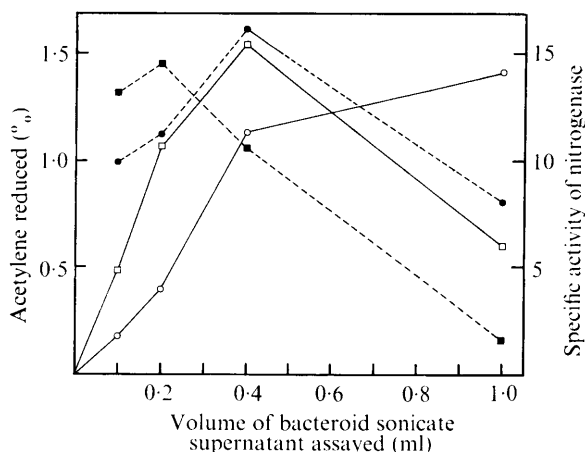


Fig. 4

Fig. 3. Effect of osmotic shock on the absorbance (E_{617}) of bacteroid suspension after 30 min incubation in the presence (●, ■) or absence (○, □) of lysozyme: ●, ○ *R. lupini* bacteroids; ■, □ *R. leguminosarum* bacteroids. Note that the ordinate scale starts at 20%.

Fig. 4. Effect of enzyme concentration and incubation time on nitrogenase assay. A bacteroid sonicate supernatant containing $16.3 \text{ mg protein ml}^{-1}$ was used. Acetylene reduced: ○, in the first 9 min; □, in the next 20 min of reaction. Specific activity of nitrogenase [$\text{nmol C}_2\text{H}_2$ reduced $\text{min}^{-1} (\text{mg protein})^{-1}$]: ●, in the first 9 min; ■, in the next 20 min of reaction.

Table 1. Distribution of nitrogenase in fractions of lupin and pea bacteroids

Specific activities are expressed as $\text{nmol C}_2\text{H}_2$ reduced $\text{min}^{-1} (\text{mg protein})^{-1}$; total activities are expressed as $\text{nmol C}_2\text{H}_2$ reduced min^{-1} .

Fraction	Lupin		Pea	
	Specific activity	Total activity	Specific activity	Total activity
Bacteroid suspension	0.2	237	*	*
Bacteroid sonicate supernatant	20.9	7768	21.0	1733
Bacteroid sonicate pellet	0	0	0	0
Lysozyme incubation suspension	8.0	6458	ND	ND
Lysozyme incubation supernatant	5.5	1479	12.5	31
Lysozyme-shock supernatant	13.9	3156	72.1	2631
Lysozyme-shock pellet	0	0	ND	ND
Lysozyme-shock sonication supernatant	0	0	1.5	42
Lysozyme-shock sonication pellet	0	0	0	0

ND, Not determined. * Very low irreproducible values were recorded.

that nitrogenase is in the soluble cytoplasm of bacteroids, although in lupin bacteroids it is quite accessible to substrates after lysozyme treatment.

Table 2 shows the specific and total activities of glutamine synthetase in nodule and bacteroid fractions of lupins and green peas. As crude extracts of bacteroids possessed a high ATPase activity which interfered with the assay of Shapiro & Stadtman (1970) for glutamine synthetase (I. Kennedy & S. Stone, unpublished results), we followed the formation of γ -glutamyl hydroxamate from glutamate in the presence of Mg^{2+} and ATP. The synthetase activity of glutamine synthetase in pea bacteroids was too low to measure in the bacteroid fractions; that of the nodule cytosol and first wash supernatant was 20 to 30 % of

Table 2. *Distribution of glutamine synthetase activity in plant and bacteroid fractions of pea and lupin nodules*

Specific activities are expressed as nmol γ -glutamyl hydroxamate formed min^{-1} (mg protein) $^{-1}$;
total activities are expressed as nmol γ -glutamyl hydroxamate formed min^{-1} .

Fraction	Lupin				Pea	
	Transferrase assay		Synthetase assay		Transferrase assay	
	Specific activity	Total activity	Specific activity	Total activity	Specific activity	Total activity
Nodule cytosol	41.8	327800	4.4	34025	305	68750
First wash supernatant	737.5	82500	21.6	2425	171	3854
Second wash supernatant	1.1	900	0	0	0	0
Bacteroid suspension	43.5	55500	0.4	465	ND	ND
Bacteroid sonicate supernatant	134.3	50000	1.3	480	5.7	265
Bacteroid sonicate pellet	45.8	13000	1.3	615	0	0
Lysozyme incubation suspension	76	20800	1.8	1500	ND	ND
Lysozyme incubation supernatant	31.7	8500	1.5	390	26.9	94
Lysozyme-shock supernatant	118.6	24900	3.9	825	2.8	77
Lysozyme-shock pellet	26.8	4500	1.7	285	ND	ND
Lysozyme-shock sonication supernatant	18.3	1200	0.8	52	2.8	38
Lysozyme-shock sonication pellet	4.3	1200	0.9	250	1.7	29

ND, Not determined.

Table 3. *Distribution of NADH oxidase, glutamate dehydrogenase, glutamate synthase and 3-hydroxybutyrate dehydrogenase in plant and bacteroid fractions of pea and lupin nodules*

The activities shown are total activities and are expressed as nmol NADH oxidized min^{-1} .

Fraction	NADH oxidase		Glutamate dehydrogenase		Glutamate synthase		3-Hydroxybutyrate dehydrogenase	
	Lupin	Pea	Lupin	Pea	Lupin	Pea	Lupin	Pea
	Nodule cytosol	14 190	8 360	7740	0	0	0	ND
Bacteroid sonicate supernatant	1 810	2 500	2 060	428	271	0	ND	31 912
Bacteroid sonicate pellet	*	928	774	192	0	30	ND	264
Lysozyme incubation supernatant	1 376	72	1 583	216	0	12	ND	732
Lysozyme-shock supernatant	426	1 648	503	56	0	0	ND	3 632
Lysozyme-shock sonication supernatant	387	2 048	0	240	774	0	ND	1 292
Lysozyme-shock sonication pellet	*	1 136	0	0	0	0	ND	84

ND, Not determined. * Not measurable due to interference of membranes.

the transferase activity. In lupin nodules approximately 75 % of the transferase and 95 % of synthetase activity was localized in the nodule cytosol, in agreement with the results of Brown & Dilworth (1975). A high specific activity was found in the first wash supernatant from both pea and lupin nodules. In lupin the ratio of transferase to synthetase activity in this fraction differed from that found in the nodule cytosol, suggesting that glutamine synthetase located very close to the bacteroids and released by the first wash is in a different state to that found in the nodule cytosol. In peas, about 99 % of the transferase activity was located within the nodule cytosol.

Most of the glutamine synthetase was localized in the cytoplasm of lupin bacteroids, as indicated by the activity released into the lysozyme-shock supernatant. In contrast, pea bacteroids released about one-third of their total glutamine synthetase activity on incubation with lysozyme, a further 40 % was released on osmotic shock treatment and the rest remained membrane-bound. The release of enzyme into the lysozyme incubation supernatant of pea bacteroids was not due to lysis by this treatment, since only 13 % of the total 3-hydroxybutyrate dehydrogenase activity, a soluble enzyme in pea bacteroids, was released compared with 39 % of the total bacteroid glutamine synthetase.

High levels of glutamate dehydrogenase were present in lupin nodule cytosol, but this enzyme was absent from the cytosol of pea nodules (Table 3). Up to 60 % of the total activity leaked out of lupin bacteroids on treatment with lysozyme and approximately 40 % leaked out of pea bacteroids. A little activity remained membrane-bound in pea bacteroids but in lupin all residual activity was released after osmotic shock.

Glutamate synthase was NADH-linked in lupin bacteroids, as was found by Brown & Dilworth (1975). The enzyme was present only in very small amounts in pea bacteroids (Table 3), in agreement with the results of Brown & Dilworth (1975) and Kurz, Rokosh & LaRue (1975). To determine optimal assay conditions for these enzymes both NADH and NADPH were tested. When extracts were passed through a Sephadex G-25 column to remove possible inhibitors no increase in activity was observed. In lupin bacteroids, glutamate synthase was released in the bacteroid sonicate supernatant and in the lysozyme-shock sonication supernatant, indicative of a loosely membrane-bound localization.

NADH oxidase, chosen as a marker enzyme for membranes, was present in all fractions of lupin and pea nodules (Table 3), suggesting that a soluble form of the enzyme occurred. 3-Hydroxybutyrate dehydrogenase was present only as a soluble enzyme, since no significant activity was found in the bacteroid sonicate pellet (Table 3).

DISCUSSION

The total activity of nitrogenase in extracts of pea bacteroids was similar to the activity reported for the intact root system ($2.5 \mu\text{mol h}^{-1}$ per plant) by Planqué & van Brussel (1976). Sonication or lysozyme treatment gave a good recovery of nitrogenase from bacteroids. Detached root systems of 21-day-old plants reduced acetylene at a constant rate of 30 to 45 nmol min^{-1} per plant for up to 2 h, compared with a nitrogenase activity *in vitro* of 20 to 30 nmol min^{-1} per plant. The specific activities *in vitro* were comparable to those for cell-free soybean bacteroids (Dunn & Klucas, 1973) but were much lower than those reported by Kurz *et al.* (1975) for intact peas and vetches. The nitrogenase was non-particulate, in agreement with reports for nitrogenase of *Klebsiella pneumoniae* and *Clostridium pasteurianum* (Ljones, 1974). Particulate nitrogenases have been reported from '*Mycobacterium flavum*' (Biggins & Postgate, 1969) and from *Azotobacter vinelandii* (Bulen, Burns & LeComte, 1964) although the latter has been questioned by Oppenheim *et al.* (1970) who used osmotic lysis instead of a French pressure cell to disrupt the organism. The soluble nature of Rhizobium nitrogenase is also supported by the rapid release of nitrogenase on incubation of bacteroids with 1 % Triton X-100; this also released 95 % of the total 3-hydroxybutyrate dehydrogenase activity (de Vries, unpublished results).

The total transferase activity of glutamine synthetase in pea bacteroids was negligible compared with that of the nodule cytosol (less than 0.2 %). However, in lupin bacteroids the total transferase and synthetase activities of glutamine synthetase were much higher. In fractions prepared by sonication or lysozyme incubation, levels were 17 % and 10 % respectively for transferase activity and 3 to 4 % for synthetase activity (as percentages of nodule cytosol activity). This relative amount of total activity is not affected by the presence of non-nodulated root material, since root tissue of lupin does not contain a highly active glutamine synthetase while the nodule tissue does (Robertson *et al.*, 1975*a*). These authors found synthetase activities similar to those reported in this paper.

The transferase activity in lupin bacteroid sonicate supernatant was 100 times greater than the synthetase activity, indicating that most of the glutamine synthetase is in the bio-synthetically inactive form. The absence of synthetase activity in pea bacteroids supports the hypothesis of Brown & Dilworth (1975) that glutamine synthetase is not involved in NH_4^+ assimilation inside the bacteroids. They suggested that the low activity of bacteroids is due to absorption of enzyme from the nodule cytosol. The different ratios of transferase to synthetase activity in lupin nodule cytosol and bacteroid fractions reported here are inconsistent with this view. We are aware of the danger of comparing the ratios of synthetase and transferase activities between plant and bacteroid glutamine synthetases as considerable differences have been reported for the optimal assay conditions of the two enzymes (MacParland *et al.*, 1976). Care must also be taken in interpreting the γ -glutamyl transferase activity determined in this assay since it can be separated into several proteins by isoelectric focusing (Darrow, Knotts & Jarrel, 1976).

Glutamine synthetase in bacteroid fractions showed a predominantly cytoplasmic localization, as most of the total activity was released either on sonication or on lysozyme lysis and very little activity remained membrane-bound. The leakage of some enzyme from the bacteroids on incubation might indicate a superficial localization of part of the enzyme. The high specific activity of the synthetase found in the first wash supernatant is consistent with the view that most of the newly-fixed nitrogen is assimilated between the bacteroid and the host membrane envelope.

Glutamate synthase activities were lower than glutamine synthetase activities in lupin bacteroids and were virtually absent in pea bacteroids. Kurz *et al.* (1975) reported very low glutamate synthase levels in bacteroids of *Rhizobium leguminosarum* and doubted the presence of the enzyme. This suggests that the glutamine synthetase–glutamate synthase pathway does not operate within the bacteroids.

Although assimilation of newly-fixed NH_4^+ will take place mainly outside the bacteroids (Lea & Mifflin, 1976; Scott *et al.*, 1976) some assimilation inside the bacteroids cannot be ruled out completely. The amount of N_2 reduced, calculated as described by Bergersen (1970) from the acetylene reduction data (Table 1), can be compared with the glutamate dehydrogenase activities. For lupin bacteroids we calculated that 14.4 nmol NH_3 were formed and 27 nmol NADH were oxidized per min^{-1} per plant. For pea bacteroids the values were 7.7 and 1.3 respectively. From these values one could conclude that some ammonia assimilation occurred inside the bacteroids. However, it may be argued that the assay conditions are not physiological, in particular for glutamate dehydrogenase for which apparent K_m values for NH_4^+ of 13.9 mM (Brown & Dilworth, 1975) and 73 mM (Dunn & Klucas, 1973) have been reported. The rate of efflux of ammonia from the bacteroids will determine whether bacteroid glutamate dehydrogenase can play an important role in the assimilation of the newly-fixed N_2 . In pea bacteroids no significantly active assimilatory pathway could be detected.

In pea and lupin bacteroids only 30 to 50 % of the total glutamine synthetase activity and 10 to 25 % of the total glutamate dehydrogenase activity was clearly cytoplasmic. The remaining activity was either membrane-bound or leaked out on incubation with lysozyme, indicative of a superficial or periplasmic localization. The localization of nitrogenase in the cytoplasm might lead to a relative high ammonia concentration inside the bacteroids.

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