MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 1, No. 6, pp. 250-254, 1988

Transport of L-Glutamate across the Bacteroid Membrane but Not the Peribacteroid Membrane from Soybean Root Nodules

Michael K. Udvardi, Caroline L. Salom, and David A. Day

Botany Department, Australian National University, GPO Box 4, Canberra A.C.T. 2601.
Received 11 April 1988. Revised 12 July 1988. Accepted 9 September 1988.

Uptake of L-[U-^{14}C] glutamate by both peribacteroid membrane-enclosed bacteroids (peribacteroid units) and free bacteroids from soybean root nodules was studied. Free *Bradyrhizobium japonicum* USDA110 bacteroids were able to accumulate L-glutamate rapidly via a high affinity transport system (K_m = 0.8 μM, V_{max} = 23 nmoles.min^{-1}.mg protein^{-1}). However, the peribacteroid membrane prohibited supply of L-glutamate to enclosed bacteroids. The bacteroid transport system appeared to be relatively specific for carboxy-amino acids, as judged by inhibitor sensitivity, and exhibited a broad pH optimum around pH 6.5. The transport system was not stimulated by ATP or Na^+ ions. Bacteroid L-glutamate uptake was, however, inhibited by uncoupler and respiratory poisons. We conclude that, although bacteroids have the potential to accumulate L-glutamate rapidly, the peribacteroid membrane is essentially impermeable to L-glutamate. The results suggest that L-glutamate does not play a role in carbon supply to the bacteroid during nitrogen fixation.

Additional keywords: Metabolite exchange, *Glycine max* (L.).

Symbiotic nitrogen fixation requires carbon compounds for the generation of ATP and electrons needed by nitrogenase. In legume-(*Brady*)*rhizobium* symbioses, carbon compounds also provide the carbon skeletons for incorporation and transport of fixed nitrogen to the plant shoot. Much research has been done in recent years to establish the major carbon compounds supplied by the plant to bacteroids for nitrogen fixation, and a variety of potential sources of reduced carbon have been identified in legume root nodules. These include sugars such as sucrose, glucose, and fructose; organic acids, including malate and succinate; amino acids; and the fermentation products ethanol and acetaldehyde (Streeter 1981; Stumpf and Burris 1979; Tajima and La Rue 1982).

Bacteroids within infected cells of legume root nodules are enclosed by the peribacteroid membrane (PBM), a membrane of plant origin. The PBM, which effectively excludes the bacteroids from the host cell cytoplasm, is an essential feature of the symbiosis and has the potential to control carbon supply to the bacteroid. Several approaches have suggested that dicarboxylic acids from the plant play an essential role in bacteroid nitrogen fixation (Dilworth and Glenn 1985: Quispel *et al.* 1985; Stowers 1985). Others have suggested that amino acids such as glutamate may be the primary source of reduced carbon for nitrogen fixation (Kahn *et al.* 1985). Free-living rhizobia are capable of rapid rates of glutamate (and other amino acids) uptake (Poole *et al.* 1985), but as yet, no direct study of glutamate transport across bacteroid and peribacteroid membranes has been reported. In addition to being a potential source of carbon (Kahn *et al.* 1985), glutamate could also be involved in provision of fixed nitrogen to the bacteroid, at least before commencement of nitrogen fixation.

In the present study we have measured uptake of L-glutamate by bacteroids and peribacteroid units (PBUs) isolated from soybean nodules. The results are discussed in the context of symbiotic nitrogen fixation.

Address correspondence to Michael K. Udvardi.

MATERIALS AND METHODS

Materials. Soybean (*Glycine max* (L.) Merr.) cultivar Bragg seeds were inoculated with *Bradyrhizobium japonicum* USDA110 and grown in pots in a greenhouse as previously described (Price *et al.* 1987). Plants were harvested when 6–8 wk old. L-[U-^{14}C] glutamic acid, [U-^{14}C] sucrose, and ^3H_2O were obtained from Amersham International (Amersham, England). [carboxyl-^{14}C]-dextran was obtained from New England Nuclear (Boston, MA). Silicon oil (AR-200) was purchased from Wacker Chemie (Munich, West Germany). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of PBUs and bacteroids. PBUs were prepared from soybean root nodules as previously described (Price *et al.* 1987) with the following alterations. Glycerol was omitted, and mannitol increased to 350 mM, in all solutions. PBUs were collected as a band at the 60%/80% interface on a Percoll step gradient after centrifugation for 15 min at 5,000 rpm (4,000 × *g*) in a Sorvall HB-4 swing-out rotor. PBU enrichment was achieved by dispersing the PBUs gently into 20 ml of wash buffer and pelleting onto an 80% Percoll cushion by centrifugation for 15 sec at 5,000 rpm (4,000 × *g*) in the same rotor. This band was resuspended in wash buffer (350 mM mannitol, 25 mM MES, pH 7.0, 3 mM $MgSO_4$) before uptake studies.

The integrity of PBU preparations was routinely checked by light microscopy. Bacteroids were prepared isotonically from intact PBUs by vortexing for 2–3 min.

Transport studies. Measurements of L-[U-^{14}C] glutamate uptake employed the silicon oil filtration technique (Palmieri and Klingenberg 1979). PBUs or bacteroids were suspended in wash buffer (see above) to which was added the required quantity of ^{14}C-glutamate (10 mCi per mmol). After the desired time interval, the reaction was terminated by centrifuging in a Beckman microfuge -E (14,250 × *g*).

Bacteroids were centrifuged for 10 sec and PBUs for only 5 sec (the shorter time preventing most of any contaminating free bacteroids from pelleting). The lag time of 5 sec, required for bacteroids to move through the oil layer before being acid killed, was taken into account when calculating uptake rates. AR-200 silicon oil was used undiluted. Total and external water volumes of the pelleted particles were estimated by using $^{3}H_2O$ and either [U-^{14}C] sucrose or [U-^{14}C] dextran, respectively (Palmieri and Klingenberg 1979). Unless otherwise stated, reactions were carried out at a temperature of 24 ± 1° C and pH 7.0. Where pH was varied, this was achieved by resuspending 0.1 ml PBUs or bacteroids in 1 ml of wash buffer containing either 100 mM MES (pH 5.5, 6.0, 6.5) or 100 mM MOPS (pH 7.0, 7.5).

Protein estimation. Protein concentration was estimated by the method of Lowry *et al.* (1951).

RESULTS

Initial experiments established that in the presence of saturating concentrations of L-glutamate, uptake of L-glutamate was linear for at least the first 30 sec in preparations of both free bacteroids and PBUs (Fig. 1). However, at sub-saturating concentrations of L-glutamate, the period of time during which bacteroid L-glutamate uptake was linear was reduced (results not shown). This effect was more marked with higher bacteroid concentrations and more dilute substrate concentrations. For this reason, bacteroid uptake reactions at sub-saturating concentrations of L-glutamate were terminated after 5 sec.

Figure 1 shows that the rate of L-glutamate uptake by preparations of PBUs was far less than that by bacteroids. This indicates that under the conditions employed, the PBM restricted the supply of L-glutamate to the bacteroids. It should be noted that the protein content of the PBM is insignificant compared with that of the enclosed bacteroids.

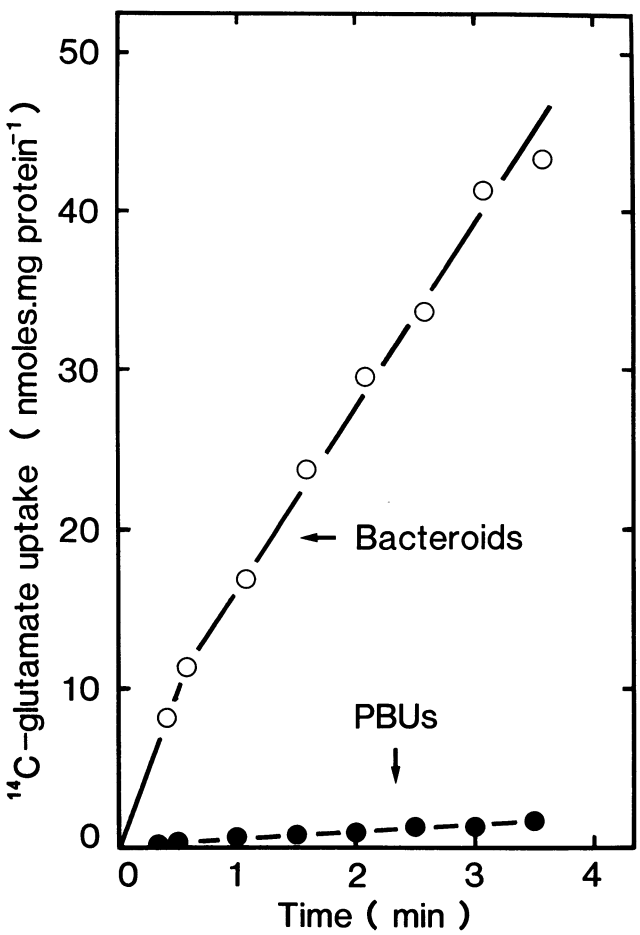

Fig. 1. Time course of ^{14}C-glutamate uptake. Uptake was measured as described in the text, with 100 μM added glutamate. o, free bacteroids ●, peribacteroid units (PBUs).

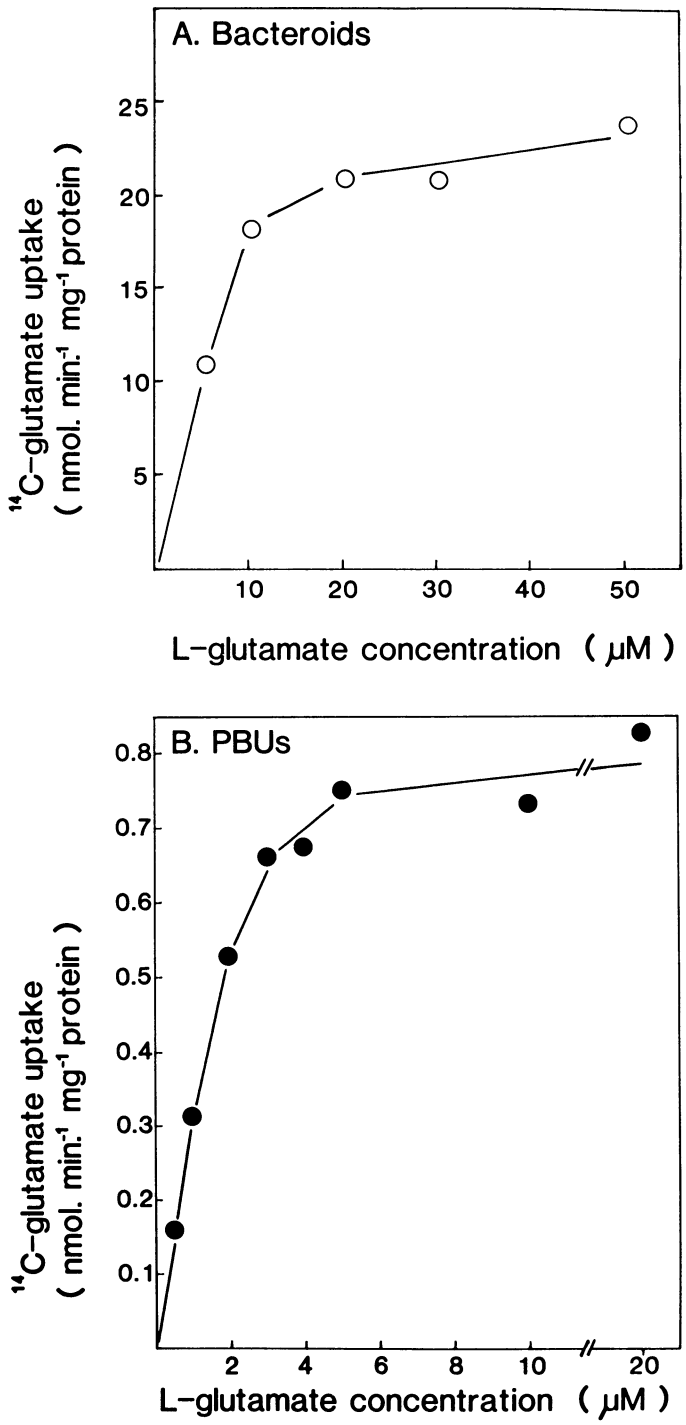

Fig. 2. The effect of L-glutamate concentration on the rate of uptake of ^{14}C-glutamate. Uptake was measured over an interval of 5 sec. Note the difference in rate of uptake between **A** and **B**.

Thus, direct comparisons can be made between the rates of PBU and bacteroid melabolite uptake expressed per unit of protein.

Figure 2 illustrates the relative kinetics of L-glutamate uptake by preparations of PBUs and bacteroids in a typical experiment. The saturation kinetics displayed by free bacteroids implicates a carrier-mediated mechanism for L-glutamate uptake. From several such experiments, the K_m was found to be 0.8 ± 0.3 μM ($\pm$ the 95% confidence interval) and the V_{max} 23 ± 7 nmoles.min^{-1}.mg protein^{-1}. PBU preparations showed an identical response to glutamate concentration (K_m = 0.8 μM), but the V_{max} was much less (Fig. 2B). Microscopic examination of our PBU preparations has shown that some free bacteroids are also present (Price *et al.* 1987). This contamination was usually 5–10%. When uptake experiments were carried out with a dilute suspension of free bacteroids (corresponding to the quantity likely to be present in the PBU preparations), identical kinetics to those shown in Fig. 2B were obtained (results not shown). There was little evidence for simple diffusion of L-glutamate across either the PBM or bacteroid membrane (BM), even at concentrations up to 1 mM.

The effect of a variety of inhibitors and other compounds on L-glutamate uptake by preparations of free bacteroids and PBUs is shown in Table 1. PBU preparations responded in the same way as free bacteroids to every compound tested. ATP and NaCl, added alone or together, had no effect on L-glutamate uptake, but the uncoupler, CCCP, and the respiratory poison, cyanide, strongly inhibited. The latter result suggests that membrane energization is required for L-glutamate uptake by bacteroids. From estimates of bacteroid volume per unit of protein (1.7 μl.mg protein^{-1}), it was calculated that bacteroids were able to accumulate L-glutamate to concentrations up to 600-fold greater than those in the surrounding medium. This accumulation was presumably driven by the electrochemical membrane potential and metabolism of the glutamate taken up. The inhibition of bacteroid L-glutamate uptake by the analogue dimethyl-glutamate ester (Table 1) lends support to the idea of carrier-mediated uptake.

Table 2 shows the effect of a range of different amino acids on L-glutamate uptake. Again,the pattern of inhibition displayed by the PBU preparation was identical to that of the free bacteroids. Uptake of L-glutamate by bacteroids was inhibited substantially by L-aspartate, slightly by L-glutamine and L-asparagine, and not at all by the other amino acids tested. This pattern suggests that the BM carrier is relatively specific in its binding for acidic amino acids and to a lesser extent their amide analogues.

L-glutamate uptake by bacteroids and preparations of PBUs showed similar pH profiles with relatively broad pH optima around 6.5–7.0 (Fig. 3).

Attempts were made to measure possible exchange of L-glutamate for other metabolites within the PBUs and bacteroids. A concentrated preparation of PBUs or bacteroids was preloaded with potential exchange metabolites by incubating for 1 min with 100 μM of either L-malate, oxoglutarate, or L-aspartate. This was then diluted 10-fold with reaction medium containing 1 mM ^{14}C-glutamate to minimize possible competition between glutamate and the exchange metabolite remaining in the

Table 1. Effect of inhibitors and other compounds on L-glutamate uptake by PBUs and bacteroids[a]

Compound	Percentage of control glutamate uptake	
	Peribactoid units	Bacteroids
3 μM CCCP	23	20
1 mM KCN	25	15
5 μM valinomycin + 1 mM KCl	84	93
3 μM CCCP + mM KCN	5	16
1 mM ATP	100	100
1 mM NaCl	100	100
1 mM ATP + 1 mM NaCl	100	100
1 mM methyl-glutamate ester	58	53

[a]The PBUs and bacteroids were preincubated with the various compounds for 1 min before addition of L-glutamate (at 100 μM). Results are expressed as percentage of uptake with L-glutamate alone (control).

Table 2. Effect of other amino acids on uptake of L-glutamate[a]

Amino acid	Percent inhibition	
	Peribacteroid units	Bacteroids
L-aspartate	54	47
L-asparagine	23	18
L-glutamine	21	19
L-leucine	0	0
L-alanine	0	0
Glycine	0	0
L-serine	0	0

[a]Assay conditions were as described in Table 1. All amino acids were added at 1 mM final concentration.

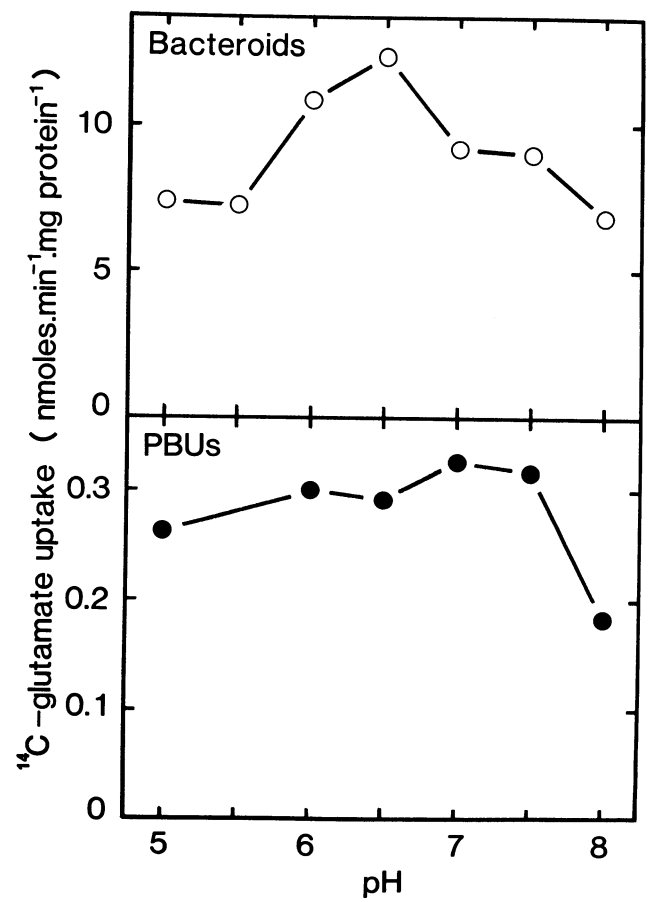

Fig. 3. The effect of external pH on ^{14}C-glutamate uptake. The concentration of added glutamate was 100 μM, and the duration of the reaction was 30 sec.

incubation medium. Such treatments had no appreciable effect on L-glutamate uptake (data not shown).

DISCUSSION

Our results indicate that *B. japonicum* (strain USDA110) bacteroids from mature soybean nodules possess a high affinity transport system that catalyses a rapid accumulation of L-glutamate. In contrast, L-glutamate uptake by PBUs was very slow. The similarity of the response of L-glutamate uptake by PBUs and free bacteroids to substrate concentration, external pH, inhibition by other amino acids, and a substrate analogue lead us to conclude that the PBM is impermeable to L-glutamate and that the small amount of uptake seen with PBU preparations was due to contamination by a few free bacteroids in those preparations. Thus, *in vivo*, the PBM would prevent L-glutamate uptake into the bacteroids.

It is always difficult with any membrane preparation to eliminate the possibility of damage to transport systems during isolation. It is also possible that our experimental conditions may not have allowed proper activation of a PBM carrier. Nonetheless, it should be noted that uptake of the organic acids malate and succinate by the same PBU preparations under similar conditions was very rapid (Udvardi *et al.* 1988). The transport studies with L-glutamate also support previous results obtained with that substrate in oxygen electrode experiments (Price *et al.* 1987). Our results to date, plus the relatively high concentrations of malate and succinate found in the soybean nodule (Stumpf and Burris 1979), make the dicarboxylic acids much more likely candidates for the primary carbon source of the bacteroid during nitrogen fixation.

The importance of glutamate catabolism for bacteroid function has been emphasized by studies with *Rhizobium* and *Bradyrhizobium* mutants (Kahn *et al.* 1985), but recent work by Salminen and Streeter (1987) has shown that L-glutamate can be formed in large quantities within the bacteroid from imported malate and succinate. Our transport studies fit well with the latter findings and suggest that schemes such as the malate-aspartate cycle (Kahn *et al.* 1985), which require rapid glutamate uptake across the PBM (balancing equally rapid malate uptake), are unlikely to operate.

The nitrogen reduced in the bacteroid is mostly lost, as ammonia, to the plant cell cytoplasm. This ammonia is initially incorporated into glutamine and glutamate by the enzymes glutamine synthetase and glutamate synthase (Miflin and Cullimore 1984). In *Glycine max* the amino side chain of glutamine may enter the ureide biosynthetic pathway that produces nitrogen-containing compounds for export to the rest of the plant. The absence of a transporter for L-glutamate on the PBM would ensure that the bacteroids are unable to scavenge the reduced nitrogen that they originally lost as ammonia.

Although elegant, the model of nutrient exchange proposed by Kahn *et al.* (1985) requires quite a subtle mechanism for the overall control of the invading (*Brady*)*rhizobia*. The model suggests that by providing its microsymbiont with a form of reduced carbon obligatorily bound to reduced nitrogen (such as amino acids), the plant is able to demand the loss of fixed nitrogen from the bacteroid. We suggest that such a subtle control system is not required. Instead, the microaerobic environment of the nodule probably provides the key that locks the endosymbiont into a nitrogen-exporting program. Indeed, a good deal of evidence in support of oxygen control of *nif* gene expression is now emerging (Fischer and Hennecke 1987; Pawlowski *et al.* 1987; Ditta *et al.* 1987; Adams and Chelm 1988). Derepression of nitrogenase synthesis within the nodule is accompanied by loss of an ammonium transport system (O'Hara *et al.* 1985; Howitt *et al.* 1986) and suppression of ammonia assimilating enzymes (Miflin and Cullimore 1984) in the bacteroid. These events, together with exclusion of L-glutamate at the PBM, ensure that the bacteroid has no option but to export most of its fixed nitrogen. The low activity of glutamine synthetase that remains in bacteroids would probably be sufficient to meet the needs of bacteroid maintenance. Because transport of dicarboxylates across the PBM is probably mediated by facilitated diffusion in response to a metabolic sink within the bacteroid (Udvardi *et al.* 1988), energy-demanding nitrogen fixation by the endosymbiont would ensure it a supply of carbon. Thus, the bacteroid and host plant manipulate each other to their mutual advantage.

Although the mature, nitrogen-fixing bacteroid may not require an external source of fixed nitrogen (see above), during replication and differentiation of the invading rhizobia in the developing nodule, some form of nitrogen must be able to cross the PBM. This suggests that the permeability of the PBM to nitrogenous compounds changes during nodule development. Of course, even in the mature nodule, forms of fixed nitrogen other than glutamate may be able to penetrate the PBM.

The results presented here suggest that the PBM of soybean root nodules prohibits the supply of L-glutamate to the enclosed bacteroids that otherwise can rapidly accumulate L-glutamate via a high-affinity transport system. These results and others (Udvardi *et al.* 1988) emphasize the key role of the PBM in the exchange of metabolites between bacteroid and plant, and show that the PBM can control the rate of supply of nutrients to the microsymbiont.

ACKNOWLEDGMENTS

We thank Joanne Perks for typing the manuscript and Catherine Elderidge for technical assistance.

LITERATURE CITED

Adams, T. H., and Chelm, B. K. 1988. Effects of oxygen levels on the transcription of *nif* and *gln* genes in *Bradyrhizobium japonicum*. J. Gen. Microbiol. 134:611-618.

Dilworth, M. J., and Glenn, A. R. 1985. Transport in *Rhizobium* and its significance to the legume. Pages 13-61 in: Nitrogen Fixation and CO_2 Metabolism. W. Ludden and J. E. Burris, eds. Elsevier, New York.

Ditta, G., Virts, E., Palomares, A., and Kim C.-H. 1987. The *nifA* gene of *Rhizobium meliloti* is oxygen regulated. J. Bacteriol. 169:3217-3223.

Fischer, H. M., and Hennecke, H. 1987. Direct response of *Bradyrhizobium japonicum nifA*-mediated *nif* gene regulation to cellular oxygen status. Mol. Gen. Genet. 209:621-626.

Howitt, S. M., Udvardi M. K., Day, D. A., and Gresshoff, P. M. 1986. Ammonia transport in free-living and symbiotic *Rhizobium* sp. ANU289. J. Gen. Microbiol. 132:257-261.

Kahn, M. L., Kraus, J., and Somerville, J. E. 1985. A model of nutrient exchange in the *Rhizobium*-legume symbiosis. Pages 193-199 in: Nitrogen Fixation Research Progress. H. J. Evans, P. J. Bottomley, and W. E. Newton, eds. Martinus Nijhoff Publ., Dordrecht.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randal, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem.

193:265-275.
Miflin, B. J., and Cullimore, J. V. 1984. Nitrogen assimilation in the legume-*Rhizobium* symbiosis: A joint endeavor. Pages 129-178 in: Genes Involved in Microbe-Plant Interactions. D. P. S. Verma and T. Hohn, eds. Springer-Verlag, Vienna.
O'Hara, G. W., Riley, I. T., Glenn, A. R., and Dilworth, M. J. 1985. The ammonium permease of *Rhizobium leguminosarum* MNF3841. J. Gen. Microbiol. 131:757-764.
Osburne, M. S., and Signer, E. R. 1980. Ammonium assimilation in *Rhizobium meliloti*. J. Bacteriol. 143:1234-1240.
Palmieri, F., and Klingenberg, M. 1979. Direct methods for measuring metabolite transport and distribution in mitochondria. Pages 279-301 in: Methods in Enzymology. Vol LVI. S. Fleischer and L. Packer, eds. Academic Press, New York.
Pawlowski, K., Ratet, P., Schell, J., and de Bruijn, F. J. 1987. Cloning and characterisation of *nifA* and *ntrC* genes of the stem nodulating bacterium ORS571, the nitrogen fixing symbiont of *Sesbania rostrata*: Regulation of nitrogen fixation (*nif*) genes in the free-living versus symbiotic state. Mol. Gen. Genet. 206:207-219.
Poole, P. S., Franklin, M., Glenn, A. R., and Dilworth, M. J. 1985. The transport of L-glutamate by *Rhizobium leguminosarum* involves a common amino acid carrier. J. Gen. Microbiol. 131:1441-1448.
Price, G. D., Day, D. A., and Gresshoff, P. M. 1987. Rapid isolation of intact peribacteroid envelopes from soybean nodules and demonstration of selective permeability to metabolites. J. Plant Physiol. 130:157-164.
Quispel, A., van Brussel, A. A. N., Hooymans, J. J. M., Priem, W. J. E., and Staal, H. J. M. 1985. Inter-symbiotic transport processes in nitrogen-fixing root nodules. Pages 193-202 in: Nitrogen Fixation and CO_2 Metabolism. W. Ludden and J. E. Burris, eds. Elsevier, New York.
Salminen, S. O., and Streeter, J. G. 1987. Involvement of glutamate in the respiratory metabolism of *Bradyrhizobium* bacteroids. J. Bacteriol. 169:495-499.
Stowers, M. D. 1985. Carbon metabolism in *Rhizobium* species. Annu. Rev. Microbiol. 39:89-108.
Streeter, J. G. 1981. Seasonal distribution of carbohydrates in nodules and stem exudate from field-grown soybean plants. Ann. Bot. 48:441-450.
Stumpf, D. K., and Burris, R. H. 1979. A micromethod for the purification and quantification of organic acids of the tricarboxylic acid cycle in plant tissues. Anal. Biochem. 95:311-315.
Tajima, S., and LaRue, T. A. 1982. Enzymes for acetaldehyde and ethanol formation in legume nodules. Plant Physiol. 70:388-392.
Udvardi, M. K., Price, G. D., Gresshoff, P. M., and Day, D. A. 1988. A dicarboxylate transporter on the peribacteroid membrane of soybean nodules. FEBS Lett. 231 (1):36-40.