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Ammonia (^{14}C-Methylamine) Transport across the Bacteroid and Peribacteroid Membranes of Soybean Root Nodules

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ABSTRACT

[^{14}C]Methylamine (MA; an analog of ammonia) was used to investigate ammonia transport across the bacteroid and peribacteroid membranes (PBM) from soybean (*Glycine max*) root nodules. Free-living *Bradyrhizobium japonicum* USDA110 grown under nitrogen-limited conditions showed rapid MA uptake with saturation kinetics at neutral pH, indicative of a carrier. Exchange of accumulated MA for added ammonia occurred, showing that the carrier recognized both NH_4^+ and $CH_3NH_3^+$. MA uptake by isolated bacteroids, on the other hand, was very slow at low concentrations of MA and increased linearly with increasing MA concentration up to 1 millimolar. Ammonia did not inhibit MA by isolated bacteroids and did not cause efflux of accumulated MA. PBM-enclosed bacteroids (peribacteroid units [PBUs]) were qualitatively similar to free bacteroids with respect to MA transport. The rates of uptake and efflux of MA by PBUs were linearly dependent on the imposed concentration gradient and unaffected by NH_4Cl. MA uptake by PBUs increased exponentially with increasing pH, confirming that the rate increased linearly with increasing CH_3NH_2 concentration. The results are consistent with other evidence that transfer of ammonia from the nitrogen-fixing bacteroid to the host cytosol in soybean root nodules occurs solely by simple diffusion of NH_3 across both the bacteroid and peribacteroid membranes.

Most of the ammonia[3] that is produced by nitrogen-fixing bacteroids in legume root nodules is exported to the plant cytosol where it is assimilated by plant enzymes. Enzymes for the assimilation of ammonia in bacteroids are either repressed or have low activity (3, 4, 9), precluding substantial ammonia assimilation by the endosymbiont. Thus, isolated bacteroids reducing $^{15}N_2$ liberate most of the [^{15}N] ammonia produced into the surrounding medium (1). Similarly, free-living (Brady)rhizobia induced to fix nitrogen *in vitro* also lose ammonia to the external medium (2, 17, 25).

Free-living (Brady)rhizobia and many other nitrogen-fixing bacteria possess NH_4^+ carriers which are generally only expressed under nitrogen-limited conditions (6, 7, 10, 11, 17, 19). However, recent evidence suggests that bacteroids do not express the NH_4^+ carrier but rather export ammonia via simple diffusion of NH_3 across the bacteroid membranes (10, 11, 17). On the other hand, little is known of the mechanism(s) of ammonia transport across the PBM[4] which is of plant origin and which surrounds the bacteroids in the infected cells of legume nodules.

Thus, ammonia (either NH_3 or NH_4^+) has to cross the PBM before it reaches assimilation sites in the host cytoplasm. The PBM has been shown to possess a carrier for dicarboxylate ions (5, 27) and is energized upon ATP-hydrolysis by an H^+-ATPase, in intact PBUs (bacteroids enclosed by PBM), with the interior being positive and, presumably, somewhat acidic compared with host cytosol and bacteroid cytoplasm (26). It is, therefore, reasonable to expect that at least part of the NH_3 diffusing out of the bacteroid will become protonated in the peribacteroid space and equally reasonable to expect that a transporter for NH_4^+ exists on the PBM. Such a transporter could play a key role in ammonia assimilation in soybean nodules. On the other hand, passive diffusion of NH_3 across the PBM could be adequate to support measured rates of ammonia assimilation, provided that a steep concentration gradient were maintained by the host cell glutamine synthase (24).

The ammonia analog [^{14}C]MA has proved useful in the study of ammonia transport in both bacteria (12) and plants (20, 22) where the two compounds share common carriers. In the current study, we have likewise used [^{14}C]MA to investigate the transport of ammonia across both the bacteroid and peribacteroid membranes of PBUs isolated from soybean root nodules.

MATERIALS AND METHODS

Plant Material

Soybeans (*Glycine max* L. cv Bragg) were inoculated with *Bradyrhizobium japonicum* USDA 110 and grown in pots of sand in a naturally illuminated glasshouse as described previously (5). Nodules were harvested 5 to 7 weeks after inoculation.

Chemicals

[^{14}C]MA hydrochloride (2.07 GBq/mmol), ^3H_2O (37 GBq), and [U-^{14}C]sucrose (20 GBq/mmol) were obtained from

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[3] In this paper, the words ammonia and methylamine are used to refer to each compound without defining its state of protonation. Chemical formulae are used to indicate a specific protonation state.

[4] Abbreviations: PBM, peribacteroid membrane; MA, methylamine; PBUs, peribacteroid units.

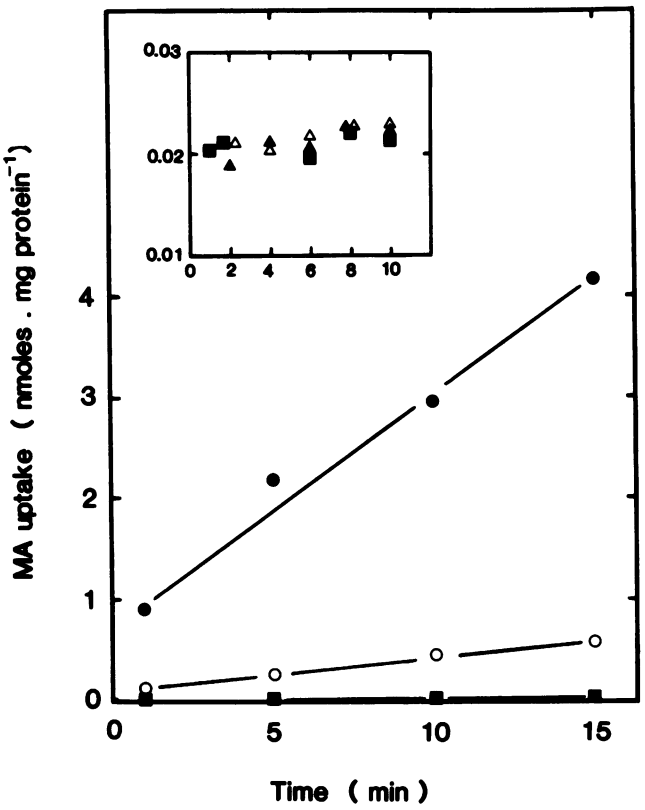

Figure 1. Time course for [^{14}C]MA uptake by free-living and bacteroid forms of *B. japonicum* strain USDA110. Free-living cells were grown to midlog phase in nitrogen-free growth medium containing either 10 mM Na-glutamate (●) or 10 mM NH_4Cl (○) as the sole source of nitrogen. Bacteroids (■) were isolated from nodules of 6-week-old soybean plants. MA was added to give an external concentration of 10 μM. The inset shows the effect of 1 mM NH_4Cl (▲) or 10 μM CCCP + 10 μM valinomycin + 1 mM KCl (△) on MA uptake by isolated bacteroids (control, ■). Data points are the means of duplicate experiments.

Amersham International. Silicon oil (AR200) was purchased from Wacker Chemie (Munich, West Germany). Other chemicals were purchased from Sigma Chemical Co.

Isolation of Peribacteroid Units (PBUs) and Bacteroids

Peribacteroid membrane-enclosed bacteroids (peribacteroid units) were isolated from soybean root nodules as previously described (5). Bacteroids were liberated from intact PBUs by vortexing for 2 min.

In Vitro Growth of Bacteria

For experiments with free-living bacteria, *B. japonicum* strain USDA110, was grown in a nitrogen-free liquid growth medium supplemented with either 10 mM glutamate or 10 mM NH_4Cl as described previously (9).

Transport Studies

Measurements of [^{14}C]MA uptake and efflux employed the technique of silicon oil filtration centrifugation (18). PBUs or bacteroids were suspended in wash buffer (see above) to which was added the required quantity of [^{14}C]MA; this was layered over 100 μL of silicon oil, which in turn was layered over 10 μL of 15% (v/v) percholic acid in a 400 μL microfuge tube. After the desired time interval, the reaction was terminated by centrifuging in a Beckman microfuge-E (14,250*g*). Bacteroids and bacteria were centrifuged for 15 s and PBUs for only 5 s, the shorter time preventing most of any contaminating free bacteroids from pelleting. AR-200 silicon oil (density 1.04 g/mL) was used undiluted. ^3H_2O and [U-^{14}C]sucrose were used to estimate the total and external water volumes of pelleted particles, respectively (5). PBU volumes were typically estimated as 4-5 μL per mg protein, while bacteroid volumes were 1-2μL per mg protein (5). This difference in volume between PBUs and bacteroids indicates that little damage to the PBM occurred during pelleting of the PBUs through the silicon oil; if rupture of the PBM and loss of peribacteroid space contents had occurred during this step, then the PBU and bacteroid volumes would have been identical. Unless stated otherwise, reactions were carried out at 22 ± 1°C and pH 7.0.

Protein Determination

The method of Lowry *et al.* (14) was used to estimate protein concentrations.

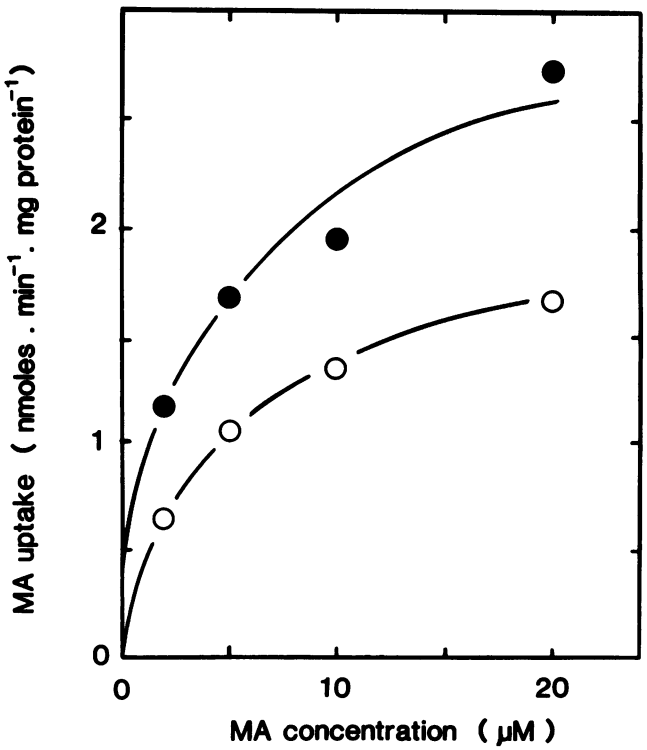

Figure 2. Concentration dependence of the rate of [^{14}C]MA uptake by strain USDA110 grown to midlog phase in nitrogen-free growth medium plus 10 mM Na-glutamate (●). Also shown is the effect of 10 μM NH_4Cl on MA uptake (○). Reactions were terminated after 10 s. Data points are the means of duplicate experiments.

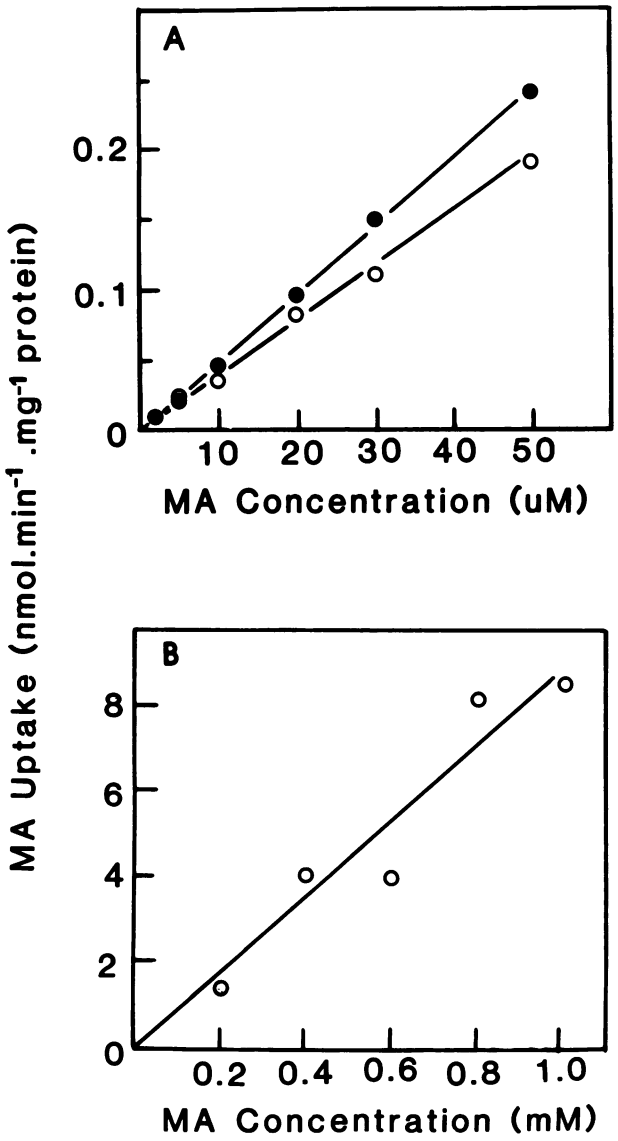

Figure 3. Concentration dependence of the rate of [^{14}C]MA uptake by USDA110 bacteroids isolated from nodules of 6-week-old soybeans. Reactions were terminated after 10 s. Data points are the means of duplicate experiments (○), Control; (●), +100 μM NH_4Cl.

RESULTS

Comparison of Methylamine Uptake by Free-Living Bacteria and Bacteroids

It was shown previously (10) that growth of slow-growing *Bradyrhizobium* (*Parasponia*) sp ANU289 on 10 mM glutamate as the sole N-source induced the expression of an ammonium transport system which can catalyze a rapid accumulation of NH_4^+ (MA) in response to the membrane electrochemical potential. Growth on high concentrations of NH_4 Cl (10 mM) represses the synthesis of the carrier.

Figure 1 shows the results of a similar set of experiments with *B. japonicum* strain USDA110. Uptake of [^{14}C]MA was rapid and linear for at least 15 min for bacteria grown under N-limited conditions (*i.e.* growth on 10 mM glutamate). In contrast, bacteria grown in the presence of 10 mM NH_4Cl showed much lower rates of MA uptake, although uptake was also linear for the first 15 min. Also shown in Figure 1 are the results obtained with bacteroids isolated from soybean root nodules. Bacteroids exhibited the lowest rates of MA uptake, with transport activity more than 100-fold lower than that of glutamate-grown cells. MA-uptake by bacteroids was insensitive to the presence of 1 mM NH_4Cl, and a mixture of uncouplers containing 10 μM CCCP, 10 μM valinomycin, and 1 mM KCl (Fig. 1, inset).

MA uptake by free-living bacteria grown on 10 mM glutamate showed Michaelis-Menten or saturation kinetics (apparent K_m = 2 μM, V_{max} = 2.3 nmol·min^{-1}·mg protein^{-1}) and was inhibited by 10 μM ammonia (Fig. 2). Since *B. japonicum* strain USDA110 cannot grow on MA as the sole N-source (results not shown), it is likely that ammonia is the natural substrate for the carrier. In contrast, MA uptake by isolated bacteroids did not show saturation kinetics but increased linearly with increasing concentrations of methylamine up to 1 mM (Fig. 3).

Ammonia transport was further investigated by counterflux experiments. Counterflux (or countertransport) is a characteristic feature of carrier-mediated transport (8). Its occurrence depends on competition between structurally similar molecules for a common carrier. For instance, addition of a large excess of compound *S* (*e.g.* ammonia) to a cell suspension

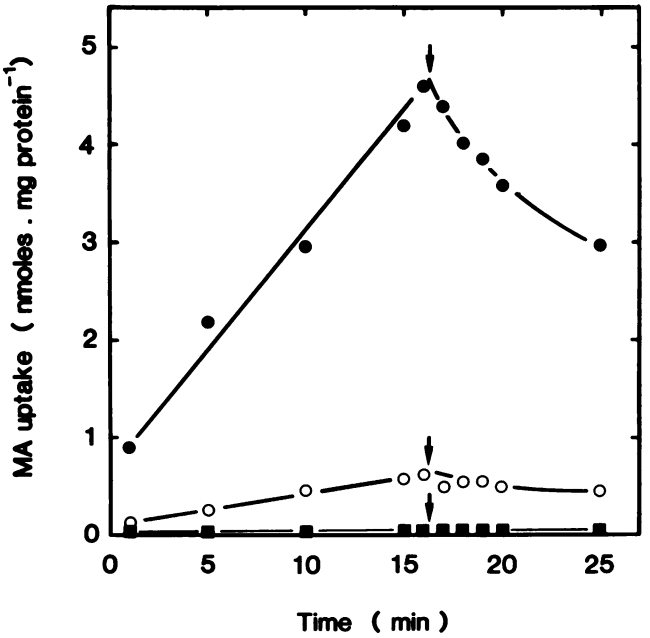

Figure 4. Counterflux experiment showing the effect of adding 1 mM NH_4Cl on the accumulation of [^{14}C]MA (10 μM external) by free-living and bacteroid forms of strain USDA110. Free-living cells were grown to midlog phase in nitrogen-free growth medium containing either 10 mM Na-glutamate (●) or 10 mM NH_4Cl (○). Bacteroids (■) were isolated from nodules of 6-week-old soybeans. Arrows indicate the time of NH_4Cl addition. Data points are the means of duplicate experiments. See text for details.

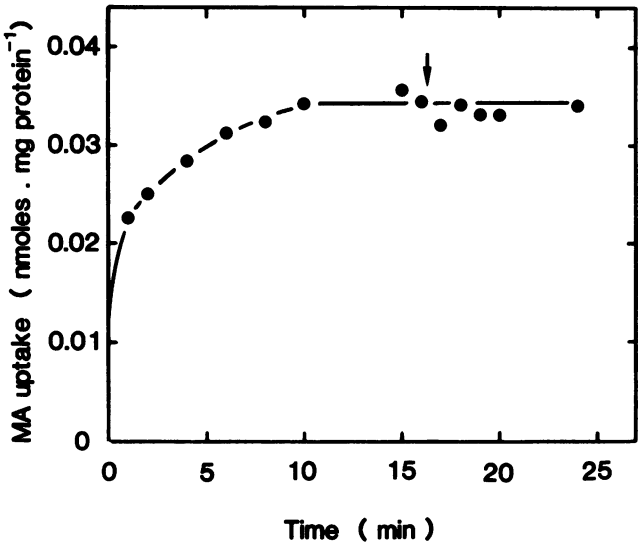

Figure 5. A counterflux experiment showing the effect of adding 1 mM NH_4Cl (indicated by the arrow) to isolated PBUs equilibrated with [^{14}C]MA (10 μM external). Data points are the means of duplicate experiments.

preincubated with radioactively labeled compound *R* (*e.g.* [^{14}C]MA) should cause a net efflux of *R* if *R* and *S* share a common carrier. Competition for the carrier by the large excess of *S* largely prevents influx of *R*, while efflux (via the carrier and/or simple diffusion) of internal *R* continues. This leads to a (transient) decrease in internal label (*R*), known as the counterflux minimum. Figure 4 shows the results of a set of counterflux experiments in which free-living bacteria or bacteroids were exposed to a low concentration (10 μM) of [^{14}C]MA for 16 min prior to the addition of a 100-fold excess (1 mM) of ammonia. Substantial efflux of [^{14}C] label from bacteria grown in the presence of glutamate occurred upon presentation of ammonia and is suggestive of carrier-mediated exchange of ammonia for [^{14}C]MA. Likewise, a slight loss of [^{14}C] label from ammonia-grown cells suggests a small amount of carrier-mediated transport of ammonia and MA. Isolated bacteroids, in contrast, did not exhibit the counterflux phenomenon (Fig. 4).

The rate of [^{14}C]MA uptake by bacteroids increased exponentially with increasing external pH and was, therefore, directly proportional to the concentration of CH_3NH_2; a plot of the log of uptake rate against external pH gave a straight line with a correlation coefficient (*r*) of 0.9 (set not shown). There was no evidence for a pH optimum for MA uptake, which might indicate the presence of an NH_4^+ carrier, such as has been shown before with free-living rhizobia (10). These results suggest that transport of MA across the bacteroid membrane occurs via simple diffusion of CH_3NH_2 alone.

Ammonia MA Import and Export by PBUs

Figure 5 shows a typical time-course for [^{14}C]MA uptake by PBUs. Two features of the time course are particularly noteworthy. First, uptake of MA occurred during the first 10 min only. Equilibrium was reached after this time. Second, the actual amount of MA taken up by PBUs was very small (less than 1%) compared with that accumulated by free-living bacteria which possess an NH_4^+ carrier (Figs. 1 and 4).

Estimates of the concentration of MA inside PBUs indicated that at equilibrium the internal concentration of MA was similar to the external concentration (Table I). The slightly higher internal concentration of ^{14}C-label may reflect either some metabolism of [^{14}C]MA by bacteroid glutamine synthetase to give γ-*N*-methylglutamine (7), a slightly more acidic interior, or nonspecific binding of $CH_3NH_3^+$ to the membranes. In an experiment analogous to the counterflux experiment shown in Figure 4, addition of a 100-fold excess of ammonia to PBUs equilibrated with [^{14}C]MA had no significant effect on the concentration of MA inside the PBUs (Fig. 5; Table I).

The rate of MA uptake by PBUs was directly proportional to MA concentration up to 1 mM (Fig. 6). The rate of [^{14}C]MA uptake by PBUs increased exponentially with increasing pH and was, therefore, directly proportional to the concentration of CH_3NH_2 (Fig. 7). Linear regression analysis of a plot of the log of uptake rate against external pH gave a correlation coefficient of 0.92 (not shown). As was the case with bacteroids, there was no evidence for a pH optimum in Figure 7, which suggests that transport of MA across the PBM occurred exclusively via simple diffusion of CH_3NH_2.

In a separate set of experiments, the rate of [^{14}C]MA efflux from PBUs preincubated with varying concentrations of MA was investigated; the rate of efflux was directly proportional to the internal MA concentration and was not affected by the addition of NH_4Cl to the external medium (results not shown).

DISCUSSION

Ammonia Transport Across the Bacteroid Membrane

The results obtained with free-living and symbiotic bacteria indicate that although *B. japonicum* is able to express an

Table I. *Concentration of [^{14}C]MA Inside PBUs Suspended in a Medium Containing 10 μM [^{14}C]MA*

NH_4Cl (1 mM) was added at t = 16.25 min.

Time	$[MA]_{I(internal)}$	$[MA]_I/[MA]_{E(external)}$
min	μM	
1	9.7	0.97
2	10.8	1.08
4	12.3	1.23
6	13.4	1.34
8	13.9	1.39
10	14.6	1.46
15	15.2	1.52
16	14.9	1.49
(1 mM NH_4Cl)		
17	13.8	1.38
18	14.6	1.46
19	14.3	1.43
20	14.2	1.42
25	14.5	1.45

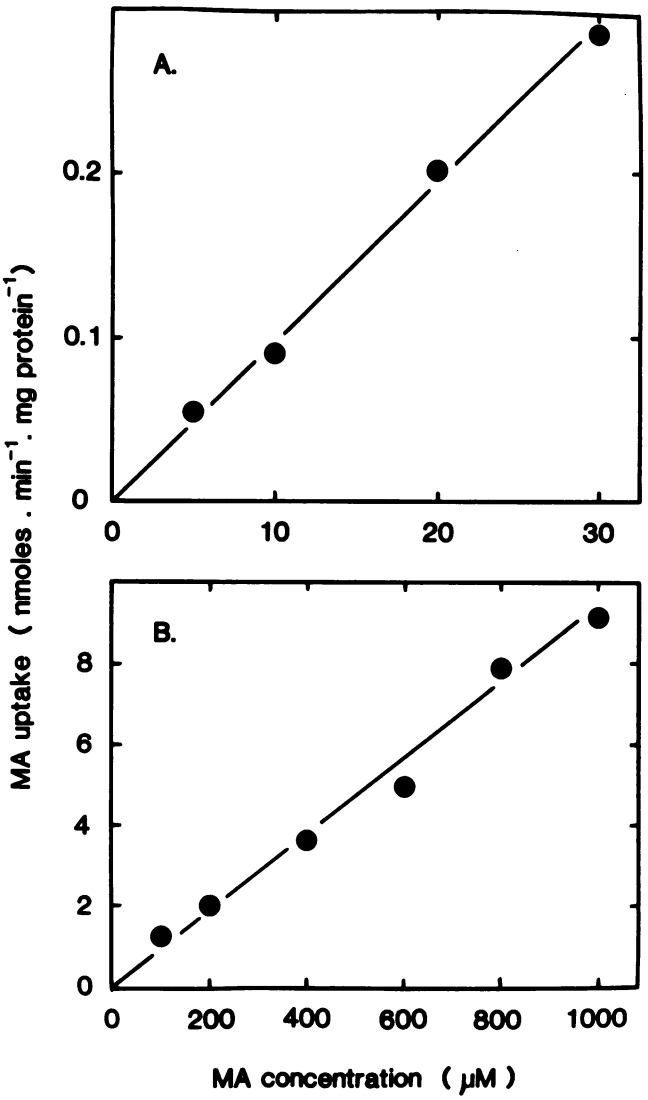

Figure 6. Concentration dependence of the rate of [^{14}C]MA uptake by PBUs isolated from nodules of 6-week-old soybeans. Reactions were terminated after 10 s. Data points are the means of duplicate experiments. Linear regression analysis gave a correlation coefficient of $r = 0.99$ for both lines.

ammonium (MA) transport system under certain growth conditions, this system is repressed in the bacteroid state. The same conclusion has been reached with different species by others (10, 11, 13, 17). We have previously shown that bacteroids isolated in the same way from soybean nodules retain functional carriers for both dicarboxylates (27) and L-glutamate (28). It is unlikely, therefore, that an ammonium carrier has been inactivated during the isolation procedure. Thus, it would appear that export of ammonia from nitrogen-fixing bacteroids in soybean nodules occurs exclusively via simple diffusion of NH_3.

Although expression of the NH_4^+ carrier in rhizobia is regulated by the nitrogen status of the cell, the mechanism(s) by which the system is repressed in the bacteroid is unknown. Reduction of N_2, at least in free-living diazotrophs, is one of the final responses to a limitation of other sources of nitrogen. Derepression of nitrogenase in these prokaryotes is generally preceded by derepression of nitrogen-scavenging systems such as the NH_4^+ carrier. Yet the NH_4^+ carrier is repressed in nitrogen-fixing bacteroids. This apparent bypass of the nitrogen-control system of rhizobia suggests that another, overiding regulatory system exists. This system may involve O_2. Indeed, Marsh *et al.* (15) found that low O_2 concentrations repressed the ammonia carrier of both the cowpea strain 32H1 and *B. japonicum*. The existence of such a novel regulatory system in rhizobia may be an essential feature of the nitrogen-fixing symbiosis.

Ammonia Transport Across the Peribacteroid Membrane

The results with isolated PBUs likewise indicate the absence of a MA carrier on the PBM. Although the absence of a carrier for MA does not absolutely preclude the presence of a carrier for ammonia, it should be noted that the plasmalemma of many plant cells has been shown to possess an NH_4^+ uniport system using MA as an ammonia analog (21–23). Such a system enables those cells to accumulate relatively high concentrations of NH_4^+ from low external concentrations of ammonia in response to membrane potential. The situation which exists in the infected nodule cell is somewhat different, since the primary source of ammonia for these cells comes from within, namely the PBM-enclosed bacteroids.

Streeter (24) has calculated that the concentration of ammonia in soybean bacteroids is approximately 12 mM, while that in the nodule cytosol is essentially zero, presumably because of the strong metabolic sink provided by the host glutamine synthetase. Under these conditions, rapid flux of ammonia through both the bacteroid membrane and the PBM could occur via simple diffusion of NH_3. Indeed, the apparent absence of an NH_4^+ carrier on both the PBM and bacteroid membrane is consistent with previous evidence that passive

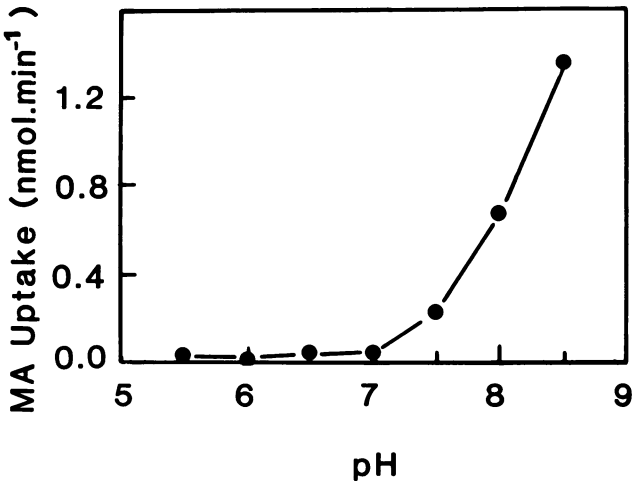

Figure 7. Effect of pH on the rate of [^{14}C]MA uptake by isolated PBUs. An external concentration of 10 μM MA was used and the reaction was terminated after 10 s.

diffusion of NH_3 is the sole mechanism for ammonia transfer from the bacteroids to the cytosol. We have previously noted that ATPase-dependent charge transfer across the PBM may be important in the transport of metabolites, such as dicarboxylates, between plant and endophyte (26). Absence of an NH_4^+ carrier on the PBM would prevent PBM depolarization due to NH_4^+ transport to the plant cytosol and avoid interference in other transport processes.

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