Movements of Ammonia in *Rhizobium leguminosarum*

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Histidine-grown cells of *Rhizobium leguminosarum* can accumulate more than 100 mM free ammonia in the medium. Growth and respiration are unaffected by these concentrations of ammonia. Measurements of internal and external ammonia concentrations for cells grown on histidine or NH_4Cl as nitrogen sources suggest rapid equilibration of ammonia across the cell membrane under conditions where actively accumulated solutes are retained. Implications of these results for movement of ammonia out of N_2 -fixing bacteroids are discussed.

INTRODUCTION

The first product of N_2 fixation in legume root nodule bacteroids is ammonia (Kennedy, 1966*a*; Bergersen, 1965). In the legume system, in liverworts (Stewart & Rodgers, 1977) and in lichens (Stewart *et al.*, 1977), ammonia appears to move to the plant symbiont for assimilation into amino acids and other compounds. Several lines of evidence suggest that ammonia assimilation into organic form occurs in the plant fraction of legume root nodules: (1) bacteroids appear to lack sufficient ammonia-assimilatory enzymes to cope with known fluxes of ammonia from N_2 fixation (Brown & Dilworth, 1975; Kurz *et al.*, 1975); (2) the plant fraction is usually high in glutamine synthetase (EC 6.3.1.2) and glutamate synthase (EC 1.4.1.13 or 1.4.1.14), and sometimes glutamate dehydrogenase (EC 1.4.1.2) as well (Brown & Dilworth, 1975; Robertson *et al.*, 1975*a, b*); (3) isolated nodule bacteroids fixing $^{15}\text{N}_2$ liberate most of the $^{15}\text{NH}_3$ into the surrounding medium (Bergersen & Turner, 1967); (4) rhizobia induced for nitrogenase under laboratory conditions also lose most of the $^{15}\text{N}_2$ they fix as $^{15}\text{NH}_3$ (O'Gara & Shanmugam, 1976; Tubb, 1976; Bergersen & Turner, 1978).

Data from bacteroids fixing N_2 *in vitro* (Bergersen & Turner, 1967) can be used to calculate that the ammonia concentrations inside the cells and in the medium were similar, although certain assumptions have to be made about the volume of bacteroids involved. Labelling of ammonia following $^{15}\text{N}_2$ fixation by serradella nodules led Kennedy (1966*a, b*) to propose two pools of ammonia in them – a small but very rapidly equilibrating pool, and a larger, more slowly equilibrating pool. If the assumption is made that the small pool represents the bacteroid ammonia closest to the site of fixation, and the larger, the pool in the plant cytoplasm, estimates can be made about the relative concentrations in the two pools, given information about bacteroid volumes in serradella nodules (Dilworth & Kidby, 1967). Such calculations, admittedly speculative, lead to a conclusion that the ammonia concentration in the plant cytoplasm could be considerably higher than that in the bacteroid. Since this ammonia distribution would imply an active excretion mechanism against a concentration gradient, studies on the exchange of ammonia between rhizobia and their environment become important.

Measurements of the efflux of radioactive methylamine from bacteroids loaded with it anaerobically indicate an oxygen-dependent export system for methylamine (Laane *et al.*, 1980). If methylamine export is a valid measure of ammonia export, an active transport system for ammonia directed outwards would be indicated.

In this paper we report on some aspects of ammonia movements into, and out of, *Rhizobium leguminosarum* WU235 cells grown under various laboratory conditions.

METHODS

Bacteriological methods. *Rhizobium leguminosarum* WU235 was from the culture collection of the Department of Soil Science and Plant Nutrition of the University of Western Australia; it effectively nodulates peas (*Pisum sativum* L., cv. Greenfeast).

The basic medium was the minimal salts medium of Brown & Dilworth (1975); for growth of cells with high concentrations of ammonia or histidine the phosphate concentration was increased fourfold. Histidine (6–64 mM) or mannitol (27 mM) were used as sole carbon sources.

Cells for methylamine uptake studies were grown on glucose (26 mM)/minimal salts with either glutamate, aspartate, glutamate plus NH_4^+ , or NH_4^+ as nitrogen source ($0.25 \text{ mg N ml}^{-1}$).

Filtration. All filtration experiments for the preparation or harvesting of cells were carried out using 47 mm diam. $0.45 \mu\text{m}$ pore size filters. Uptake experiments were carried out using 25 mm filters of similar pore size.

Methylamine uptake. Cells were harvested in late-exponential phase, filtered on to a Millipore filter and resuspended in 50 mM-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) buffer pH 7.2, containing 2 mM- MgCl_2 and 2 mM-glucose. Cells were shaken for 20 min and then used in uptake experiments. Inhibitors and analogues were added 2 min prior to addition of $^{14}\text{CH}_3\text{NH}_2$ ($1 \mu\text{Ci}$).

Samples of cells (0.1 ml) were removed at intervals, filtered and washed twice with 5 ml 50 mM-HEPES pH 7.2, containing MgCl_2 and glucose (both at 2 mM) on membranes, and the radioactivity on the membrane was determined by liquid scintillation counting as described by Hudman & Glenn (1980).

Determination of intracellular ammonia. A culture (500 ml) grown on 38 mM-histidine/minimal salts was centrifuged (6500 g, 15 min, 26°C) and the pellet resuspended in 5 ml of spent medium. A portion of this suspension (1–2 ml) was filtered through a Millipore filter and the cells were washed with 10 ml minimal salts (Brown & Dilworth, 1975). The cells were washed off the filter with minimal salts (5 ml); 4 ml of the collected cells were added to 9 ml ethanol and left for 1.5 h at 22°C . After the cell pellet had been removed by centrifugation, 0.5 or 1.0 ml samples of the supernatant were placed in 30 ml Universal bottles, and 1 ml of a 25% saturated solution of K_2CO_3 was added. Ammonia was distilled from the mixture into 2 M- H_2SO_4 held on an etched glass rod over a 4 h period during which the bottles were rotated at an angle of 28° (Dilworth *et al.*, 1965). Fully saturated K_2CO_3 solution cannot be used as it forms a two-phase system with the ethanolic sample.

Determination of cell volumes in suspensions. Cell volumes were determined either by haematocrit methods and measurement of entrained liquid, or by weighing the pellet and measuring the entrained liquid.

In the haematocrit method, samples in 50 μl haematocrit tubes were centrifuged at 11800 g for 15 min in a Clements haematocrit centrifuge (Clements Pty Ltd, Sydney, Australia).

Entrained liquid in pellets was measured by resuspending them in a known volume of 0.2% (w/v) Blue Dextran in 0.1 M-phosphate buffer (pH 7.6), centrifuging the suspension (11800 g, 15 min) and reading the A_{630} . After resuspending the cells in 0.1 M-phosphate buffer (pH 7.6), the centrifugation at 11800 g for 15 min was repeated and the A_{630} of this second supernatant also measured. Entrained liquid was then calculated from the known volumes and Blue Dextran concentrations. Values ranged from 28 to 40% in different experiments with histidine-grown cells, and between 50 and 60% for mannitol/ammonium-grown cells. This difference in entrained liquid appears to be related to the fact that histidine-grown cells assume a spherical shape and presumably pack more tightly than the rod-shaped cells observed in the mannitol medium.

All centrifugation steps were carried out at $25\text{--}28^\circ\text{C}$ to avoid release of cellular constituents from the bacteria due to temperature shock.

Oxygen uptake. Oxygen uptake rates were measured polarographically at 25°C using a Hansatech oxygen electrode (Hansatech, King's Lynn, U.K.).

Cell counting. Total cell counts were made on diluted suspensions under phase contrast at a magnification of 400 in a Neubauer-type counting chamber 0.01 mm deep.

Analytical methods. Ammonia was measured spectrophotometrically by the phenol hypochlorite method (Fawcett & Scott, 1960). Histidine interferes with the method, so media containing histidine were either diluted greater than 200-fold to eliminate the interference, or the ammonia was distilled from the samples with saturated K_2CO_3 solution.

Histidine was measured with a modified Pauly reagent (Ray, 1967), and urocanic acid after acidification of samples with perchloric acid, using a molar absorption coefficient of $18800 \text{ l mol}^{-1} \text{ cm}^{-1}$ at 268 nm (Rechler & Tabor, 1971). Protein was measured by the Lowry method using bovine serum albumin as the standard.

Chromatography. Glutamate was identified in culture supernatants by thin-layer chromatography on cellulose sheets (Eastman Kodak Co., Rochester, N.Y., U.S.A.) using the solvents propan-1-ol/concentrated NH_3 solution (7:3 by vol.) and butan-1-ol/acetic acid/water (4:1:5 by vol.).

Radiochemicals. [^{14}C]Methylamine (specific activity $40.4 \text{ mCi mmol}^{-1}$; $1.49 \text{ GBq mmol}^{-1}$) and 3-*O*-methyl-D-[1- ^3H]glucose ($7.7 \text{ mCi } \mu\text{mol}^{-1}$; $0.28 \text{ GBq } \mu\text{mol}^{-1}$) were from Amersham.

RESULTS AND DISCUSSION

Methylamine uptake

There is evidence, particularly with eukaryotic cells, that the methylamine transport system is the same as the ammonia transport system (Hackett *et al.*, 1970; Roon *et al.*, 1975; Cook & Anthony, 1978; Breiman & Barash, 1980). Radioactive methylamine has also been used as an analogue of ammonia in uptake experiments with bacteria (Stevenson & Silver, 1977; Bellion *et al.*, 1980; Laane *et al.*, 1980), but here the situation is not so clear. In *Pseudomonas fluorescens*, for example, the methylamine transport system is inducible but appears not to be the same as the ammonia system (Bellion *et al.*, 1980). Some caution may, therefore, be necessary in automatically equating methylamine uptake with ammonia uptake in bacteria.

In *R. leguminosarum* WU235, methylamine uptake was unaltered by any of the growth conditions tested; it was an active process inhibited by 8 mM-azide and 10 μM -carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), but the absolute rate of transport was very low [$0.02 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$]. Even at a 200-fold molar excess of ammonia over methylamine, uptake of radioactive methylamine was only inhibited about 22% at pH 7.2. It appears that in this strain methylamine is not satisfactory as an analogue of ammonia for use in uptake experiments.

As an alternative approach we attempted to measure intracellular and extracellular concentrations of ammonia for laboratory-grown cells of *R. leguminosarum*, to give an indication of whether ammonia transport into or out of the cells results in the establishment of a concentration gradient.

Ammonia concentrations during growth on histidine

Free-living rhizobia have been shown to utilize histidine as sole source of carbon and nitrogen, releasing up to 2.6 mol ammonia per mol histidine used (O'Gara & Shanmugam, 1976). We used this property to study the distribution of ammonia between cells and medium during this excretion process.

The production of extracellular ammonia as a function of histidine concentration is shown in Fig. 1. In this experiment, all cultures were grown until no further increase in turbidity occurred. Maximal cell yield was obtained at 38 mM-histidine, which also corresponded to a maximal yield of ammonia per unit of histidine utilized. Although final ammonia concentrations were slightly higher with 52 mM-histidine than with 38 mM, recovery of ammonia from histidine did not increase proportionately.

A time course for ammonia release during growth of *R. leguminosarum* WU235 on histidine is shown in Fig. 2. Ammonia concentrations rose slightly even after there was no further increase in cell density, and the slight decrease in ammonia concentrations in the medium late in stationary phase was demonstrated consistently (in all experiments). Small amounts of urocanic acid also appeared in the medium (Fig. 2).

The results indicate the resistance of these rhizobia to high ammonia concentrations – up to 90 mM. We confirm the observations of O'Gara & Shanmugam (1976) on ammonia release from histidine, though in our experiments using 13–38 mM histidine we have obtained virtually stoichiometric release of ammonia (3 mol per mol) from histidine. When the flask shaking speed was lowered and oxygenation consequently decreased, the pattern of metabolism altered to yield ammonia (~ 2 mol per mol histidine) and glutamate (identified qualitatively by thin-layer chromatography in two solvent systems).

Cells assayed for ammonia content after filtration and washing as described in Methods gave low apparent internal ammonia concentrations (5–10 mM). Since the cell washings contained considerable amounts of ammonia, it appeared that washing in this way merely

Fig. 1. Ammonia concentrations (●) and cell yields (○) of *R. leguminosarum* WU235 cultures grown at various concentrations of histidine.

Fig. 2. Time course of changes in culture density (○) and concentrations of histidine (△), urocanic acid (▲) and ammonia (●) during growth of *R. leguminosarum* WU235 on 38 mM-histidine.

eluted ammonia from the cells, although organic solutes such as 3-*O*-methylglucose, succinate, disaccharides and amino acids are not so eluted (Glenn *et al.*, 1980; Hudman & Glenn, 1980; Glenn & Dilworth, 1981). We therefore sought to measure internal and external ammonia concentrations without washing.

Dilution experiments

Cells were grown either on a 38 mM-histidine/minimal salts medium or on a 27 mM-mannitol/minimal salts medium containing 3.8 mM- NH_4Cl .

In the initial experiment (Fig. 3) 1000 ml of a culture grown on 38 mM-histidine were centrifuged and resuspended in 16 ml minimal salts medium (suspension A) containing 55 mM-glucose and the same concentration of ammonia as that found in the supernatant (final concentration, 84.6 mM-ammonia). This suspension was shaken vigorously for 40 min, with samples taken every 10 min for determination of ammonia concentration in the supernatant; ammonia concentration in the supernatant was shown to be stable. A 2 ml sample of

Fig. 3. Flow chart for dilution experiments with *R. leguminosarum* WU235.

suspension A was removed to measure cell volume and entrained liquid by weighing the pellet and using the Blue Dextran method. An 8 ml sample of suspension A was centrifuged (11 800 g, 15 min) and the pellet resuspended in minimal salts medium containing 55 mM-glucose without ammonia (suspension B). This suspension contained, from the entrained liquid/cell volume measurements, 1.092 ml cells and 0.736 ml entrained liquid containing 62.3 μ mol ammonia. Suspension B was again centrifuged (11 800 g, 15 min) and the ammonia concentration in the supernatant determined.

We can consider two extreme cases for ammonia distribution. In the first, the ammonia concentration in the cells is maintained at a relatively constant and low level; for the calculations in Table 1 we have arbitrarily used 5 mM. In the second case, the ammonia concentration within the cells equilibrates freely with the outside medium, i.e., in this experiment it starts at 84.6 mM and finishes at 16.2 mM (Fig. 3). For these two assumptions, we calculate the predicted ammonia concentration in the supernatant of suspension B (Table 1). It is clear that the first assumption cannot adequately explain the amount of ammonia found in the supernatant of suspension B; the second explanation is consistent with the observed final ammonia concentration, and leads to a calculated intracellular ammonia concentration of 88.8 mM.

Table 1. *Ammonia concentrations predicted for final supernatant in dilution experiments*

Case 1	
Ammonia contributed by entrained liquid	$0.736 \times 84.6 = 62.3 \text{ } \mu\text{mol}$
Ammonia present in cells	$1.092 \times 5.0 = 5.5 \text{ } \mu\text{mol}$
Total ammonia in suspension B	67.8 μmol
Volume of liquid containing ammonia	8.736 ml
Predicted ammonia concentration in supernatant	7.8 mM
Case 2	
Ammonia contributed by entrained liquid	$0.736 \times 84.6 = 62.3 \text{ } \mu\text{mol}$
Ammonia present in cells	$1.092 \times 84.6 = 92.4 \text{ } \mu\text{mol}$
Total ammonia in suspension B	154.7 μmol
Volume of liquid containing ammonia	9.83 ml
Predicted ammonia concentration in supernatant	15.7 mM
Ammonia concentration found in supernatant	16.2 mM

Table 2. *Predicted and observed ammonia concentrations for suspensions of R. leguminosarum WU235 after dilution experiments with cells grown on 38 mM histidine/minimal salts*

	Ammonia concentration (mM)			
Concentration in supernatant of suspension A	30.7	38.2	47.8	84.6
Concentration predicted for supernatant of suspension B assuming:				
(a) free equilibration	7.7	9.6	11.7	15.7
(b) active exclusion	3.5	4.1	5.3	7.1
Concentration observed in supernatant of suspension B	8.0	8.0	14.4	16.2

Table 3. *Predicted and observed ammonia concentrations for cells of R. leguminosarum WU235 grown on 27 mM mannitol/minimal salts plus 3.8 mM NH₄Cl*

	Ammonia concentration (mM)		
Concentration in supernatant of suspension A	1.77	4.22	17.4
Concentration predicted for supernatant of suspension B assuming:			
(a) free equilibration	0.15	0.39	1.52
(b) active exclusion	0.09	0.16	0.94
Concentration observed in supernatant of suspension B	0.21	0.38	1.73

No account has been taken in these calculations of the volume of periplasmic space inaccessible to Blue Dextran. Stock *et al.* (1977) reported that for *Escherichia coli* and *Salmonella typhimurium* 20–40% of the cellular water was inaccessible to inulin, and presumably therefore to Blue Dextran (since its molecular weight is far higher). Since the lower estimate was for conditions most comparable to ours, we calculated what difference a 20% periplasmic space would have made. In the example summarized in Table 1, the predicted ammonia concentration in the supernatant would have been 9.5 mM for case 1 and 15.7 mM for case 2. As the agreement with the observed value is still far better for case 2 than for case 1, no further corrections for the volume of the periplasm have been made in later tables, since the effects would have been of comparable order.

Further experiments with histidine-grown cells were conducted where the minimal salts/glucose medium used to prepare suspension A contained varied concentrations of added ammonia. In each case, the ammonia concentration observed in the final supernatant corresponded to the value calculated from the cell volume, entrained liquid and ammonia concentration of the supernatant of suspension A, and was consistent with free equilibration of ammonia (Table 2).

Since histidine-grown cells had been exposed to high ammonia concentrations during growth, we also studied cells grown at low ammonia concentrations (3.8 mM) and then

incubated with shaking in the presence of varying concentrations of external ammonia (2–20 mM). Subsequent dilution experiments (Table 3) showed that the observed ammonia concentrations agreed well with those expected from the free equilibration model, but were inconsistent with a low internal ammonia concentration.

Equilibration of ammonia appears to be a rapid process, since stable values for ammonia concentrations in the supernatants in the dilution experiments were found after 2 min, the minimum time interval studied.

One possible explanation of the apparently free equilibration for ammonia was that in the heavy cell suspensions used in these experiments there was inadequate oxygen available to support an energy-driven ammonia export system. This was tested by pre-loading cells grown on 27 mM-mannitol/minimal salts plus 3.8 mM NH_4Cl with 3-*O*-methyl-D-[1- ^3H]glucose for 60 min and then following the centrifugation, resuspension and incubation conditions used in the ammonia experiments (Fig. 3). The resuspension medium in this case was 27 mM-mannitol/minimal salts instead of glucose/minimal salts, because the glucose might have caused exchange efflux of 3-*O*-methyl-D-glucose from the cells. The cell densities used were similar to those used in the dilution experiments with ammonia. Samples were taken from the initial culture, from the resuspended culture (suspension A stage) after 30 min incubation, and after treatment of the suspension with toluene [0.017 ml (ml culture) $^{-1}$]. Samples were filtered on to Millipore membranes and washed twice with 5 ml minimal salts, and the radioactivity was determined by scintillation counting. The cells accumulated label: cells sampled from the initial culture contained 6160 c.p.m. (mg protein) $^{-1}$; those sampled after 30 min incubation contained 6525 c.p.m. (mg protein) $^{-1}$. After toluene treatment only 150 c.p.m. (mg protein) $^{-1}$ remained associated with the cells. The experiment indicated that under these conditions the cells retain non-metabolizable 3-*O*-methyl-D-glucose accumulated from the medium; since the transport system for this analogue is an active one and rapid unloading occurs when the cell membrane is de-energized (Hudman & Glenn, 1980), sufficient energy must be available to retain this solute. Destruction of the membrane with toluene released 98% of that radioactivity, indicating that it is not bound. Accordingly, under conditions where the cell membrane is sufficiently energized to retain 3-*O*-methyl-D-glucose, ammonia is apparently not excluded or retained, but equilibrates across the membrane.

Ammonia effects on growth and substrate oxidation

The mean generation time (3.5 h) of *R. leguminosarum* WU235 on mannitol/minimal salts was unaffected by NH_4Cl up to 90 mM and only reached 4.2 h at a concentration of 140 mM. The effect of NH_4Cl on mannitol-dependent O_2 uptake was examined in cells grown on low (2 mM) and high (100 mM) concentrations of NH_4Cl in the mannitol/minimal salts medium. In cells grown on both media a 20% increase in O_2 consumption occurred with 20 mM- NH_4Cl , followed by a progressive decrease with increasing NH_4Cl concentration, reaching 90% of the control at 100 mM. By contrast, the addition of the uncoupling agent CCCP (10 μM) to cultures grown on mannitol/minimal salts resulted in a doubling of the O_2 uptake.

Our results indicate that in this strain of *R. leguminosarum* ammonia tends to equilibrate rapidly under conditions where an actively accumulated solute (3-*O*-methyl-D-glucose) remains within the cells. Filtration and washing techniques appear inappropriate for measuring internal ammonia concentrations; rapid equilibration leads to a continuous leaching of ammonia from the cells – this explains why the histidine-grown cells treated in this way appeared to maintain low internal ammonia concentrations.

The implication for nodule metabolism would be that ammonia efflux from N_2 -fixing bacteroids should result from rapid equilibration between intra- and extracellular environments. A diffusive loss of ammonia from bacteroids may explain ammonia movement from bacteroids to plant cytoplasm in legume root nodules. Rapid transport outwards would depend on maintenance of a low ammonia concentration in the plant cytoplasm through rapid assimilation by a system with a low Michaelis constant for ammonia. Nodule glutamine

synthetase appears to meet these conditions; it has a low apparent K_m for ammonia (McParland *et al.*, 1976; Boland *et al.*, 1980) and the activities are high in nodules (Brown & Dilworth, 1975; McParland *et al.*, 1976; Boland *et al.*, 1980). Attempts to define ammonia concentrations in cytoplasm and bacteroids after nodule disruptions are open to serious doubts because of leakage from bacteroids. Indeed, if equilibration does occur, as our results with free-living rhizobia suggest, the concentrations in diluted cytoplasm and bacteroids will be identical. Measurements on extracted nodule sap may provide a better answer.

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