Reassessment of major products of N₂ fixation by bacteroids from soybean root nodules

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NH₃/NH₄⁺ was the principal product from soybean bacteroids, prepared by various procedures, when assayed in solution in a flow chamber under N₂ fixation conditions. In addition, small quantities of alanine were produced (reaching 20% of NH₃/NH₄⁺ under some conditions). Some ¹⁵N was assimilated by bacteroids purified from soybean root nodules on Percoll density gradients and shaken with ¹⁵N₂ and 0.008 atm O₂. Under these conditions, ¹⁵NH₄⁺ accounted for 93% of the ¹⁵N fixed into the soluble fraction. This fraction contained no measurable [¹⁵N]alanine. Neither these bacteroids nor those prepared by the previously used differential centrifugation method, when incubated with exogenous alanine under non-N₂-fixing conditions, gave rise to NH₃ from alanine. Therefore, contamination of bacteroid preparations with enzymes of plant cytosolic origin and capable of producing NH₃ from alanine cannot explain the failure to detect [¹⁵N]alanine [as reported elsewhere: Waters, J. K., Hughes, B. L., II, Purcell, L. C., Gerhardt, K. O., Mawhinney, T. P. & Emerich, D. W. (1998). *Proc Natl Acad Sci USA* 95, 12038–12042]. Cell-free extracts of the bacteroids as used in the ¹⁵N experiments contained alanine dehydrogenase and were able to produce alanine from pyruvate and NH₄⁺. Other experiments with alanine dehydrogenase in extracts of cultured rhizobia and bacteroids are reported and discussed in relation to the ¹⁵N experiments. Possible reasons for the differences between laboratories regarding the role of alanine are discussed. It is concluded that NH₃ is the principal soluble product of N₂ fixation by suspensions of soybean bacteroids *ex planta* and that NH₄⁺ should continue to be considered the principal product of N₂ fixation which is assimilated *in vivo* in soybean nodules.

Keywords: alanine, alanine dehydrogenase, ammonia, nitrogen fixation, ¹⁵N balance

INTRODUCTION

For many years it has been accepted that the fixation of atmospheric N₂ by the root nodules of legumes involved the production of NH₃ by the symbiotically diazotrophic bacteria (bacteroids) within the cells of the central tissue of the nodules. It was recognized that, due to a pK of 9.26 for the NH₃–NH₄⁺ equilibrium, at physiological pH only a very small proportion of this nitrogen existed as NH₃. The NH₄⁺ ion would not diffuse across biological membranes, giving rise to concerns about the mechanism by which fixed N₂ left bacteroids (e.g. Kahn *et al.*, 1985). Subsequently, an NH₄⁺ channel was identified on the peribacteroid membrane (PBM) (Tyerman *et al.*, 1995) and the accepted interpretation persisted. That is, the acidic nature of the symbiosome space acts as an acid trap to form NH₄⁺ ions, thereby creating a gradient for

diffusion of NH₃ out of the bacteroids, with NH₄⁺ subsequently transported to the plant via the PBM channel.

The basis for this interpretation was substantially due to the following experimental observations. (1) NH₃ was the earliest ¹⁵N-labelled product of N₂ fixation when detached root nodules of soybean (*Glycine max* Merr.; Bergersen, 1965) or serradella (*Ornithopus sativa* L.; Kennedy, 1966a, b) were incubated for short periods of time in atmospheres containing ¹⁵N₂. (2) With anaerobically prepared soybean nodule bacteroids (the symbiotic form of *Bradyrhizobium japonicum*) in micro-aerobic, shaken assays with ¹⁵N₂ in the gas phase, the principal product was ¹⁵NH₃ (Bergersen & Turner, 1967). (3) Later, ¹⁵NH₃ was the principal product of ¹⁵N₂ fixation by soybean bacteroids in an open, flow-reaction

system in which a well-stirred suspension of soybean bacteroids was perfused with solutions containing dissolved O_2 and $^{15}N_2$; no ^{15}N -labelled amino acids were detected. The long-accepted view was challenged by Waters *et al.* (1998) who found that alanine, not NH_3 , was the principal ^{15}N -labelled product when N_2 -fixing soybean bacteroids, purified on anaerobic sucrose density gradients, were supplied with malate and shaken in a gas mixture containing $^{15}N_2$ and 0.008 atm O_2 . These authors concluded also that the earlier results arose from the use of bacteroid preparations that were contaminated with cytosolic enzymes from the host tissue, which released NH_3 from the primary product, alanine. Allaway *et al.* (2000) subsequently showed that both [^{15}N]alanine and $^{15}NH_3$ were produced by bacteroids prepared from nodules of *Pisum* sp. on anaerobic Percoll density gradients, but that the proportion of these products was altered by the conditions applied. When conditions were optimized for N_2 fixation, NH_3 was the first and major product formed, but alanine was formed at high bacteroid densities when NH_3 accumulated (Allaway *et al.*, 2000). These authors also used a mutant strain of *Rhizobium leguminosarum*, defective in alanine catabolism, to produce N_2 -fixing nodules on peas. Such plants fix N_2 , but grow more slowly than plants nodulated by the wild-type, although the basis for this was not determined. Bacteroids from these nodules produced only NH_3 in shaken assays. It was concluded that alanine was a secondary product of N_2 -fixing bacteroids; alanine amino-nitrogen arose from NH_3 , derived from N_2 fixation, which accumulated in the experimental system used. More recently, Atkins & Thumfort (2001) have reported that assimilation of $^{15}N_2$ by nodulated roots of cowpea produced ^{15}N -labelled products which showed no evidence that alanine was a precursor of the amide groups of glutamine or of the purine ring of ureides (the major N_2 -fixation product translocated from the nodules).

In the present paper we report experiments with density-gradient-purified soybean bacteroids, which were undertaken to verify the results of Waters *et al.* (1998). Verification was not achieved. Instead, we found that NH_3 was the sole significant ^{15}N -labelled product of $^{15}N_2$ fixation accumulated during 30 min in shaken assays with 0.008–0.01 atm O_2 . Alanine, although sometimes found in low concentrations in flow chamber reactions, was not labelled with ^{15}N in shaken, closed-system experiments. We conclude also that these and earlier results (Bergersen & Turner, 1967, 1990) were not due to contamination with host cytosolic enzymes as suggested by Waters *et al.* (1998) and that NH_3 is the principal product of N_2 fixation in soybean.

METHODS

Bacteria. *Bradyrhizobium japonicum* strain USDA110c is the strain used in this laboratory for many years. Strain USDA110de was kindly supplied by D. W. Emerich, University of Missouri, USA, and strain SU1014/1 (CB1809) by the SUNFix culture collection, University of Sydney, Australia. *Rhizobium leguminosarum* bv. *viciae* strains 3841 and RU1327

(*aldA*⁻) were gifts from P. S. Poole, University of Reading. Cultures were maintained on yeast extract/mannitol agar (Dalton 1980) and on Brown & Dilworth's defined liquid medium (Dalton 1980), but with 10 mM NH_4^+ and succinate, respectively, as nitrogen and carbon sources (Allaway *et al.*, 2000) when rhizobia were grown for preparation of cell-free extracts for determination of alanine dehydrogenase activity.

Nodules and bacteroid suspensions. Nodules aged about 35 days were picked from roots of soybean (*Glycine max* Merr. cv. Stevens, and sometimes for comparison, cv. Williams) inoculated at planting with *B. japonicum* strain USDA110c and grown in pots of sand, supplied twice weekly with McKnight's nutrient solution free of combined nitrogen (Gibson, 1980). The nodules were washed in tap water, drained and blotted dry with paper towels and used immediately for preparation of bacteroid suspensions: (i) by the standard method of anaerobic homogenization in phosphate buffer (0.1 M, pH 7.4) containing sucrose (0.2 M), Mg^{2+} (2 mM), granular PVP (Polyclar) and filtration through Miracloth in a closed system under flowing argon [bacteroids were obtained by differential centrifugation and washed as described by Bergersen & Turner (1990)]; or (ii) by homogenization in the same solution under argon in an anaerobic glove box, followed by separation of bacteroids by anaerobic centrifugation on Percoll (Pharmacia) density gradients and washing, essentially as described by Udvardi *et al.* (1988; cf. Allaway *et al.*, 2000). Finally, the bacteroids were dispersed in reaction solution and an aliquot was saved for determination of dry weight after centrifugation and washing with distilled water.

Experimental systems. Experiments in closed systems were based on those described by Waters *et al.* (1998) and Allaway *et al.* (2000), but some of the buffer constituents and concentrations were different. N_2 fixation experiments were run in conical flasks (100 ml) closed with Suba Seals. Reaction solutions (9.0 ml per flask) contained 50 mM MOPS/KOH buffer, pH 7.4, 2 mM DL-malate and 0.2 M sucrose. After inserting the Suba Seals, the flask contents were degassed under vacuum for 10 min with periodic agitation on a manifold fitted with hypodermic needle connections and an Hg manometer (Turner & Gibson, 1980). The flasks were flushed twice with Ar and then filled with gas mixtures containing 0.008–0.02 atm O_2 and N_2 to 1 atm, from a screw-piston reservoir (Turner & Gibson, 1980). They were brought to the reaction temperature (26 °C) before injecting 1.0 ml bacteroid suspension containing 3–6 mg (dry wt) bacteroids and shaking at 100 or 150 r.p.m. in a rotary shaker. Reactions were terminated by removing the seals, admitting air to inactivate nitrogenase, immediately chilling on ice and then centrifuging at 0 °C and 10000 g to separate bacteroids from the reaction solution. Products of N_2 fixation were measured (below) in the supernatant reaction solution.

Experiments in the flow chamber system (Bergersen & Turner, 1990) were performed as described by Li *et al.* (2001), but samples of effluent were analysed for NH_3 , alanine and other amino acids as described below.

Detection of alanine-degrading enzymes adhering to bacteroids. The presence of enzymes able to deaminate alanine and which adhered to bacteroids was sought in bacteroids prepared by differential centrifugation or by density gradient purification. Dehydrogenases were detected by following the reduction of NAD^+ spectrophotometrically at 340 nm in assays containing 100 mM K-CAPS buffer (pH 10.0), alanine (4 mM), NAD^+ (3 mM) and 3 mg (dry wt) bacteroids. Also, any alanine-dependent production of NH_4^+ was sought in

similar aerobic (non-N₂-fixing) assays devoid of exogenous NAD⁺.

Endogenous alanine dehydrogenase. Alanine dehydrogenase activities were determined in cell-free extracts prepared by disruption of suspensions in a French press. Liquid cultures of various strains of rhizobia were harvested in mid-exponential phase by centrifugation and resuspended in breakage buffer. Bacteroids were prepared from soybean nodules as described above and resuspended in breakage medium (TES/NaOH buffer, pH 7.5, containing 50 mM KCl, 5 mM MgSO₄ and 5 mM DTT). The homogenates were clarified by centrifugation at 35 000 *g* at 5 °C for 1 h to yield crude soluble extracts. Alanine dehydrogenase activity was estimated at 25 °C according to Allaway *et al.* (2000) and Smith & Emerich (1993). The apparent *K_m* values for pyruvate and NH₄⁺ were determined.

¹⁵N experiments. ¹⁵N₂ gas was prepared from (¹⁵NH₄)₂SO₄ (57.8 atoms % ¹⁵N; Isotec, Miamisburg, OH, USA) by oxidation with alkaline hypobromite (Bergersen, 1980) and purified by storage over alkaline potassium permanganate solution (to remove oxides of nitrogen) and then over dilute H₂SO₄ (to remove any residual NH₃) (Burris, 1976). Contamination of this gas with O₂ was measured electrometrically as described below and its concentration adjusted to the desired level by addition of Ar and air after transfer at measured pressure to the screw-piston reservoir (see above). Typically, reaction gas mixtures used contained 0.008 atm O₂, 0.5 atm Ar and 0.49 atm N₂ (54.6 atoms % ¹⁵N) [At the elevation of Canberra, 1 atm is 705–715 mm Hg (94–95.3 kPa).] For experiments in shaken flasks (see above), a blank, unsealed control flask, containing all reaction components except ¹⁵N₂, was placed on ice at zero time and thereafter treated in the same manner as the reaction flasks. Reactions were terminated as above after shaking for 30 min. The flask contents were analysed for N₂ fixation products (see below).

Chemicals and analytical methods. Except where indicated, all chemicals were of analytical grade purchased from Sigma. NH₃ in reaction solutions and in fractions after Kjeldahl digestion and recovery by steam distillation (Bergersen, 1980) or by diffusion at pH 11 onto filter paper strips (Whatman No. 1; 15 × 4 mm) soaked in 0.5 M H₂SO₄, was determined colorimetrically by the Chaney–Marbach method (Bergersen, 1980). Alanine and other amino acids were determined by HPLC analysis after derivitization at the Nucleic Acid and Applied Protein Chemistry Unit, Department of Plant Science, Waite Campus, University of Adelaide, Australia, or at the Ecosystems Research Group, Department of Botany, University of Western Australia.

The concentration of O₂ contaminating the ¹⁵N₂ used in N₂ fixation experiments was determined in a sample (20 ml) of the gas collected in a gas-tight syringe and flushed through the previously Ar-flushed gas space (volume 5 ml) above 1 ml of stirred distilled water in the chamber of a pre-calibrated O₂ electrode (Rank Bros.). The chamber was sealed before electrode currents were recorded and the zero checked after measurements by addition of a few crystals of sodium dithionite to the water layer.

In the ¹⁵N experiments, samples (1.0 ml) of the reaction flask contents were taken for analysis of total nitrogen by Kjeldahl digestion and distillation (Bergersen, 1980). The rest of the reaction mixture was separated into the supernatant (soluble) and bacteroid fractions by centrifugation at 10 000 *g* for 10 min. The small quantities of free NH₃–NH₄⁺ in the soluble fraction, being insufficient for ¹⁵N analysis, were recovered by

diffusion onto paper strips (below) and after drying were supplemented with carrier nitrogen (50 µg) as (NH₄)₂SO₄ of accurately known natural nitrogen abundance. For these analyses, the atoms % ¹⁵N before addition of carrier nitrogen (*x*) was calculated as:

$$x = [c(a + b) - bd] / a \quad (1)$$

in which *a* = µg NH₃ nitrogen in the sample before adding carrier, *b* = µg carrier nitrogen added, *c* = atoms % ¹⁵N measured in the sample plus carrier and *d* = atoms % ¹⁵N of the carrier nitrogen.

Bacteroid total nitrogen, soluble total nitrogen and the total nitrogen of the residue of the soluble fraction after removing NH₃ nitrogen, were recovered by Kjeldahl digestion and distillation. Thus the distribution of all ¹⁵N incorporated from ¹⁵N₂ could be determined. NH₃ in distillates and in samples of supernatants was recovered by diffusion for > 24 h onto strips of filter paper soaked in 0.5 M H₂SO₄ (see above; Bergersen, 1980). The filter paper strips were dried in tin capsules and submitted to total nitrogen and ¹⁵N analysis using an ANCA SL stable isotope analytical system (Europa Scientific) in the CSIRO Division of Plant Industry, Canberra, Australia. In addition to these analyses, the presence of [¹⁵N]alanine in the soluble fraction was sought by purifying any amino acids present by using chromatography on Sephadex (Pharmacia) SP-25 (Redgwell, 1980), lyophilizing eluates and submitting them to GC-MS analysis after derivitization with *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (Allaway *et al.*, 2000). The amount of ¹⁵N incorporated into alanine was assessed from the ratios of the mass peaks at *m/e* 260 and 261 (the principal fragments of the derivative of alanine), as follows:

$$\text{atoms \% excess} = 100[R_s / (1 + R_s) - R_c / (1 + R_c)] \quad (2)$$

in which *R_s* and *R_c* are, respectively, the ratios mass 261/mass 260 for the mass spectral peaks of the sample (*s*) and the control (*c*). The calculation assumes that, apart from nitrogen, the isotopic ratios for all elements present in the fragment are the same in sample and control.

RESULTS AND DISCUSSION

¹⁵N experiments

There were two similar ¹⁵N₂ experiments with Percoll-gradient-purified bacteroids shaken in stoppered flasks using conditions designed to be close to those reported to produce [¹⁵N]alanine (Allaway *et al.*, 2000; Waters *et al.*, 1998). These experiments were conducted 6 months apart; the results of the first (bacteroids from summer-grown nodules) are presented in Table 1. The data for analysis of the total nitrogen of the experimental system indicated that digestion of the morpholino-moiety of the MOPS buffer may have been incomplete. However, this was a constant error and did not affect calculation of the nitrogen-weighted ¹⁵N balance in which a matrix of determinations of ¹⁵N-labelled fractions and no ¹⁵N₂ controls was used. Also, as noted elsewhere (Bergersen & Turner, 1967; Waters *et al.*, 1998) there was a substantial background of endogenous NH₃ in these reaction mixtures (perhaps including NH₃ adsorbed on the untreated filter paper strips onto which the ¹⁵NH₃ diffused). These factors contributed to the relatively low ¹⁵N enrichment of soluble NH₃ (1.2 atoms % excess; Table 1) compared with the enrichment of the

Table 1. Incorporation of ^{15}N from $^{15}\text{N}_2$ (54.6 atoms % ^{15}N) by USDA110c bacteroids in 30 min at 26 °C in flasks (100 ml) containing 0.008 atm O_2 , shaken at 100 r.p.m.Values are means $\pm$ SEM.

Fraction analysed	Nitrogen per assay (μg)	^{15}N excess (atoms %)	^{15}N excess	
			ng per assay	Percentage recovered
Total nitrogen	7344 $\pm$ 382	0.0130 $\pm$ 0.0010	958 $\pm$ 71	100
Bacteroid total nitrogen	4141 $\pm$ 813	0.0059 $\pm$ 0.0016	243 $\pm$ 122	25.4
Soluble total nitrogen*	1676 $\pm$ 153	0.0287 $\pm$ 0.0016	481 $\pm$ 41	50.2
Soluble NH_3 nitrogen†	36 $\pm$ 0.8	1.2336 $\pm$ 0.0347	448 $\pm$ 23	46.8
Soluble residue total nitrogen‡	1061 $\pm$ 84	0.0053 $\pm$ 0.0007	57 $\pm$ 13	5.9

* After centrifuging bacteroids.

† Recovered from soluble fraction by diffusion.

‡ After removal of NH_3 .**Table 2.** Data for GC-MS analysis of the *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide derivatives of alanine from standard alanine (Sigma), control (purified from supernatants of bacteroid reactions in assay flasks in air, stopped in ice at zero time), and purified from soluble fractions of 30 min reactions with $^{15}\text{N}_2$ Data are from the same experiment presented in Table 1. *R* is the ratio (ion-current counts at *m/e* 261)/(ion-current counts at *m/e* 260).

Sample analysed	<i>R</i> ($\pm$ SEM)	<i>n</i>	Excess ^{15}N ($\pm$ SEM) (atoms %)
Standard alanine	0.22956 $\pm$ 0.00028	6	–
Control	0.24293 $\pm$ 0.00252	16	0
Reactions with $^{15}\text{N}_2$	0.24326 $\pm$ 0.00044	4	0.00076 $\pm$ 0.00053

$^{15}\text{N}_2$ supplied (54.6 atoms % ^{15}N). The analysis accounted for 79.2 and 75.4 %, respectively, of total nitrogen and ^{15}N that was fixed. About 25 % of the ^{15}N that was fixed was incorporated in the bacteroids (similar to previous reports; Bergersen & Turner, 1967, 1990). The soluble fraction after removal of the bacteroids contained > 50 % of the fixed ^{15}N , 93 % of which was $^{15}\text{NH}_3$ with enrichment of 1.2 atoms % excess. The residue of the soluble fraction, after removal of NH_3 , where any amino acids would have been located, contained only 6 % of the total ^{15}N excess.

The second experiment, using winter-grown nodules, produced an almost identical distribution and enrichment of fixed ^{15}N , but the total amount of ^{15}N fixed was 25 % lower, perhaps reflecting lower rates of fixation during winter.

In both experiments alanine was present in the soluble fraction, but the GC-MS analysis revealed no significant excess of ^{15}N in flasks containing bacteroids exposed to $^{15}\text{N}_2$ for 30 min, compared with control flasks (Table 2). Values of *R* for the standard (Sigma) alanine were lower than those for the control (no $^{15}\text{N}_2$) alanine. This may have been due to different isotopic composition of any of the elements in the Sigma alanine, but the *m/e* signals

were free of signals from other chemicals which, at very low concentration, may alter the *m/e* signals of control or enriched experimental alanine. Additionally, the data for pure alanine were obtained at slightly higher concentration than for control and experimental alanine. In all cases, alanine was the most significant compound derivitized. It should be noted that had the standard alanine (Sigma; as used by Waters *et al.*, 1998) been used as the control at natural ^{15}N abundance, the alanine from the reactions would appear to have been enriched with ^{15}N (0.88 atoms % excess).

Allaway *et al.* (2000) used bacteroids from pea root nodules to show that alanine was synthesized from the soluble pool of $\text{NH}_3/\text{NH}_4^+$ in shaken assays. In our experiments, although $\text{NH}_3/\text{NH}_4^+$ had by far the greatest atoms % excess ^{15}N of any fraction (Table 1), it was only 1.2 atoms % excess. This may have contributed to our failure to measure significant [^{15}N]alanine by the relatively insensitive GC-MS method (Table 2). Nevertheless, Table 1 shows that there was little room in the ^{15}N balance of the soluble fraction for [^{15}N]alanine. In this experiment, an increment of $2.0 \pm 0.8 \mu\text{g}$ NH_3 nitrogen was measured between zero time and 30 min samples. Therefore it was possible to calculate that

Table 3. Examples of bacteroid activities during steady states in flow chamber experiments

Bacteroids were prepared anaerobically by the standard differential centrifugation method or by centrifugation through Percoll density gradients. Variations in respiration rates (O_2 consumption) were achieved with different rates of flow through the reaction chamber containing media containing different concentrations of dissolved O_2 . In the flow chamber (vol. 12 ml) there were 72 and 96 mg dry wt of bacteroids, respectively, prepared by the two methods.

Bacteroid preparation	$[O_2 \text{ free}]$ (nM)	Bacteroid activities [$\text{nmol min}^{-1} (\text{mg dry wt})^{-1}$]		
		O_2 consumption	NH_3 production	Alanine production
Differential centrifugation	7	0.94	0.87	0.11
	8	1.62	1.18	0.06
	4	2.52	2.09	0.06
Percoll density gradients	5	1.48	0.69	0.09
	9	2.33	0.28	0.04
	8	8.05	1.01	0.19

newly fixed NH_3/NH_4^+ had 22.8 (95 % confidence limits 1.8–43) atoms % excess ^{15}N . This rather imprecise estimate reflects the analytical difficulty of determining small increments of NH_3 nitrogen.

Flow chamber experiments

Waters *et al.* (1998) stressed the need to use gradient-purified bacteroids which earlier work had shown to be free of host cytosolic enzyme activity and that the critical pO_2 for alanine production by density-gradient-purified bacteroids in their shaken, closed system was 0.008 atm. Therefore, we sought alanine production in gradient-purified bacteroids and in bacteroids prepared by differential centrifugation, in flow chamber reactions at low steady-state concentrations of dissolved O_2 ($[O_2 \text{ free}]$). Examples from these experiments are presented in Table 3. It has been shown that N_2 fixation into NH_3 by bacteroids in the flow chamber system depends on respiration rates (Bergersen, 1997). Sucrose-density-gradient-purified bacteroids, as used by Waters *et al.* (1998), generally had lower rates of respiration and NH_3 production, but yielded no more alanine than bacteroids prepared by other methods (data not presented). Percoll-gradient-purified bacteroids were more active metabolically than sucrose-gradient-purified bacteroids and produced alanine at up to 19% of rates of NH_3 production (Table 3), whilst bacteroids prepared by the differential centrifugation method, although producing more NH_3 per unit of respiration (Table 3), produced little alanine at rates unrelated to respiration. N_2 fixation into NH_3 by these bacteroids in shaken assays continued for 30–40 min, declining gradually thereafter (Fig. 1), but in the flow chamber, steady rates were sustained easily for several successive periods of > 20 min. Allaway *et al.* (2000) suggested that failure to detect significant alanine in flow chamber effluents (Bergersen & Turner, 1990) could have been due to continuous removal of NH_3 from the flow chamber, thus preventing NH_3 reaching a concentration needed for significant alanine formation.

Fig. 1. Production of NH_3 and alanine by USDA110c bacteroids in microaerobic shaken assays. (a) Bacteroids prepared by differential centrifugation, with 0.02 atm O_2 in the gas phase. (b) Bacteroids prepared on a Percoll density gradient, with 0.008 atm O_2 in the gas phase. ●, NH_3 ; ▲, alanine.

Alanine in closed shaken bacteroid systems

As noted above, some alanine is present in the soluble fractions from such experiments. However, it is unlikely that alanine production was directly due to N_2 fixation because there was little difference between the concentrations of alanine in 1 h assays with Percoll-gradient-

Table 4. Activities of alanine dehydrogenase in the amination direction in cell-free extracts of strains of *B. japonicum* grown in succinate broth and in bacteroids of strain USDA110c prepared from fresh nodules on cv. Stevens or cv. Williams

Values are means $\pm$ range of two separate experiments. ND, Not determined.

Source	Activity [nmol NADH min ⁻¹ (mg protein) ⁻¹]	K_m (pyruvate) (mM)	K_m (NH ₄ ⁺) (mM)
Cultures			
<i>B. japonicum</i>			
USDA110c	25.4 $\pm$ 0.2	0.29 $\pm$ 0.06	4.7 $\pm$ 0.3
USDA110de	30.6 $\pm$ 2.5	0.39 $\pm$ 0.01	2.9 $\pm$ 0.05
SU1014(CB1809)	132.7 $\pm$ 3.5	1.2 $\pm$ 0.07	4.0 $\pm$ 0.2
<i>R. leguminosarum</i> bv. <i>viciae</i>			
TY3841 (<i>aldA</i> ⁺)	18.8 $\pm$ 0.2	0.84 $\pm$ 0.04	2.6 $\pm$ 0.1
RU1327 (<i>aldA</i> ⁻)	0.3 $\pm$ 2.6	ND	ND
Bacteroids			
USDA110c on cv. Forrest	633 $\pm$ 108	0.9 $\pm$ 0.2	6.8 $\pm$ 0.9
USDA110c on cv. Williams	154 $\pm$ 6	1.3 $\pm$ 0.1	5.2 $\pm$ 0.3

purified bacteroids in air (in which nitrogenase activity would have been destroyed) and at a pO_2 of 0.008 atm at which production of alanine by soybean bacteroids has been reported to be optimal (Waters *et al.*, 1998). In this experiment, alanine comprised, respectively, 72 and 90% of the total of 10 amino acids detected (data not presented). Alanine was always produced at much lower rates than NH₃ (Fig. 1). When up to 5 mM NH₄⁺ was supplied in such experiments, the rates of alanine production doubled, but remained much lower than NH₃ production with no added NH₄⁺ (data not shown). We often found that bacteroid suspensions contained significant amounts of alanine (and NH₃; Fig. 1) upon isolation. We suggest that this arises during the isolation procedure. Experiments with extracts from bacteroids prepared by a Percoll density gradient and other methods (see below) indicated that alanine dehydrogenase was present internally in our bacteroid preparations, but produced only limited amounts of alanine in the medium from exogenous malate and NH₄⁺. This suggests that *B. japonicum* USDA110c, maintained in our laboratory, has little potential for alanine formation under N₂-fixing conditions.

Alanine dehydrogenase activity

An unpurified, soluble extract of the bacteroid preparation, as used in the ¹⁵N experiment (Table 1), contained endogenous alanine dehydrogenase activity [> 500 nmol NADH min⁻¹ (mg protein)⁻¹ in the aminating direction], with an apparent K_m of 0.9 and 7 mM for pyruvate and NH₄⁺, respectively (Table 4). This activity was greater than the activity of extracts of the same strain grown in succinate medium. These results indicated clearly that the enzyme activity had not been lost in the symbiotic state or during the isolation of bacteroids, and the failure to detect substantial alanine

excretion from N₂-fixing bacteroids did not result from such loss.

Alanine dehydrogenase activities in extracts of cultured *B. japonicum* strains

This work was undertaken to test whether the failure to detect [¹⁵N]alanine as a product of ¹⁵N₂ fixation (Table 1; Bergersen & Turner, 1990) may have been due to the absence of alanine dehydrogenase activity from the bacteria used, either constitutively or due to differences in symbiotic expression. Therefore, a comparison was made of cultures from a variety of sources, some of which had known alanine dehydrogenase phenotypes, grown in succinate medium (Allaway *et al.*, 2000), which is known to promote expression of *aldA* in *R. leguminosarum* (P. S. Poole, personal communication). Also, a comparison was made between bacteroids from nodules on cv. Williams and cv. Stevens, in case there may have been a difference between *aldA* expression in the two varieties when nodulated by the same strain of *B. japonicum* (cf. Stripf & Werner, 1978). The results are presented in Table 4. It is clear that in all strains in which alanine dehydrogenase is known to be present and in the strains used in this work (USDA110c) or previously [USDA110de (Waters *et al.*, 1998); CB1809 (Bergersen & Turner, 1990)] alanine dehydrogenase was present. The *aldA* mutant strain (RU1327; Allaway *et al.*, 2000) had negligible activity. The USDA110c bacteroids from nodules of cv. Stevens had high AldA activity when freshly prepared (although those from winter-grown plants had lower activity, causing differences between experiments; Table 4). After storage at -70 °C for several months, bacteroids prepared as for the ¹⁵N experiment (Table 1) yielded cell-free extracts with alanine dehydrogenase activity and kinetic values (data not shown) similar to those in Table 4. Bacteroids prepared from USDA110c nodules on cv. Stevens and

cv. Williams both had active alanine dehydrogenase (Table 4). These results show that it is most unlikely that the data in Table 1 or of Bergersen & Turner (1990) were due to defects in expression of *aldA* or to lack of alanine dehydrogenase activity in the bacteroids.

The need for density-gradient bacteroid preparation

Waters *et al.* (1998) reported that bacteroids prepared by a differential centrifugation method (as used by Bergersen & Turner, 1967, 1990) were contaminated with enzymes originating from the host cells of the nodules. These contaminants may have degraded alanine to produce NH_3 , thus preventing identification of alanine as a major product of N_2 fixation. It seemed to us that Waters *et al.* (1998) may not have used exactly the same procedure as the other workers and so we tested bacteroid preparations, as used in the present paper, for the presence of such contamination. In neither Percoll-gradient-purified bacteroids nor bacteroids prepared by our fractional centrifugation method was alanine-dependent reduction of NAD^+ detected (data not shown). There was a slow production of NH_3 ($<5 \text{ nmol NH}_3 \text{ mg}^{-1} \text{ h}^{-1}$) from bacteroids prepared by fractional centrifugation, but this was not stimulated in the presence of alanine concentrations up to 10 mM. Therefore, contamination of bacteroids with enzymes degrading alanine was not the cause of the failure to detect alanine as a major product of N_2 fixation in the present work (Tables 1, 2) or in previous work (Bergersen & Turner, 1967, 1990). The finding suggests that the density gradient methods, which take longer and result in bacteroids with impaired metabolic properties (Table 3), may not be necessary.

Conclusion

We conclude that NH_3 is the principal product of N_2 fixation by bacteroids from soybean nodules under a range of different conditions *ex planta*, including those used by Waters *et al.* (1998). The reason for this difference between the two laboratories could not be determined from our experiments, but the possibility remains that sucrose-density-gradient-purified bacteroids (Waters *et al.*, 1998), in closed shaken assays, differ from those purified on Percoll density gradients.

ACKNOWLEDGEMENTS

The authors thank Richard Phillips and members of the analytical services group of the CSIRO Division of Plant Industry, Canberra, Australia, for the ^{15}N analyses, and J. Lahnstein (Nucleic Acid and Applied Protein Chemistry Unit, Department of Plant Science, Waite Campus, University of Adelaide, Australia) and C. Warren (Department of Botany, University of Western Australia) for amino acid analyses. Gifts of strains of *B. japonicum* were received from D. W. Emerich (University of Missouri, Columbia, MO, USA) and I. R. Kennedy (University of Sydney, NSW, Australia) and *R. leguminosarum* from P. S. Poole (University of Reading). Seeds of the soybean cv. Williams were a gift from Dr P. Lawrence (Australian Tropical Crops Genetic Resource Centre, Biloela, Qld, Australia). The research was supported

by a grant from the Australian Research Council to D.A.D. and F.J.B. Youzhong Li acknowledges the award of an International Postgraduate Research Scholarship and an ANU PhD scholarship. F.J.B. thanks the Australian National University for support as a Visiting Fellow.

REFERENCES

- Allaway, D., Lodwig, E. M., Crompton, L. A., Wood, M., Parsons, R., Wheeler, T. R. & Poole, P. S. (2000). Identification of alanine dehydrogenase and its role in mixed secretion of ammonium and alanine by pea bacteroids. *Mol Microbiol* **36**, 509–515.
- Atkins, C. A. & Thumfort, P. P. (2001). Assimilation of fixed N in a ureide-forming symbiosis. In *Proceedings of the 13th International Conference on Nitrogen Fixation*, Hamilton, Ontario, July 2001. Dordrecht: Kluwer.
- Bergersen, F. J. (1965). Ammonia – an early stable product of nitrogen fixation by soybean root nodules. *Aust J Biol Sci* **18**, 1–9.
- Bergersen, F. J. (1980). Measurements of nitrogen fixation by direct means. In *Methods for Evaluating Biological Nitrogen Fixation*, pp. 65–110. Edited by F. J. Bergersen. Chichester: Wiley.
- Bergersen, F. J. (1997). Physiological and biochemical aspects of nitrogen fixation by bacteroids in soybean nodule cells. *Soil Biol Biochem* **29**, 875–880.
- Bergersen, F. J. & Turner, G. L. (1967). Nitrogen fixation by the bacteroid fraction of breis of soybean root nodules. *Biochim Biophys Acta* **141**, 507–515.
- Bergersen, F. J. & Turner, G. L. (1990). Bacteroids from soybean root nodules: respiration and N_2 -fixation in flow-chamber reactions with oxyleghaemoglobin. *Proc R Soc Lond B* **238**, 295–320.
- Burris, R. H. (1976). Nitrogen fixation by blue-green algae of the Lizard Island area of the Great Barrier Reef. *Aust J Plant Physiol* **3**, 41–51.
- Dalton, H. (1980). The cultivation of diazotrophic microorganisms. In *Methods for Evaluating Biological Nitrogen Fixation*, pp. 13–64. Edited by F. J. Bergersen. Chichester: Wiley.
- Gibson, A. H. (1980). Methods for legume in glasshouse and controlled environment cabinets. In *Methods for Evaluating Biological Nitrogen Fixation*, pp. 140–184. Edited by F. J. Bergersen. Chichester: Wiley.
- Kahn, M. L., Kraus, J. & Somerville, J. E. (1985). A model of nutrient exchange in the *Rhizobium* – legume symbiosis. In *Nitrogen Fixation Research Progress*, pp. 193–199. Edited by H. J. Evans, P. J. Bottomely & W. E. Newton. Dordrecht: Nijhoff.
- Kennedy, I. R. (1966a). Primary products of symbiotic nitrogen fixation I. Short-term exposures of serradella nodules to $^{15}\text{N}_2$. *Biochim Biophys Acta* **130**, 285–294.
- Kennedy, I. R. (1966b). Primary products of symbiotic nitrogen fixation II. Pulse-labelling of serradella nodules with $^{15}\text{N}_2$. *Biochim Biophys Acta* **130**, 295–303.
- Li, Y., Green, L. S., Holtzappffel, R., Day, D. A. & Bergersen, F. J. (2001). Supply of O_2 regulates demand for O_2 and uptake of malate by N_2 -fixing bacteroids from soybean nodules. *Microbiology* **147**, 663–670.
- Redgwell, R. J. (1980). Fractionation of plant extracts using ion-exchange Sephadex. *Anal Biochem* **107**, 44–50.
- Smith, M. T. & Emerich, D. W. (1993). Alanine dehydrogenase from soybean nodule bacteroids, kinetics, mechanism and pH studies. *J Biol Chem* **268**, 10746–10753.

Stripf, R. & Werner, D. (1978). Differentiation of *Rhizobium japonicum*, II. Enzymatic activities in bacteroids and plant cytoplasm during the development of nodules of *Glycine max*. *Z Naturforsch* **33C**, 373–381.

Turner, G. L. & Gibson, A. H. (1980). Measurement of nitrogen fixation by indirect means. In *Methods for Evaluating Biological Nitrogen Fixation*, pp. 111–138. Edited by F. J. Bergersen. Chichester: Wiley.

Tyerman, S. D., Whitehead, L. F. & Day, D. A. (1995). A channel-like transporter for NH_4^+ on the symbiotic interface of N_2 -fixing plants. *Nature* **378**, 629–632.

Udvardi, M. K., Salom, C. L. & Day, D. A. (1988). Transport of L-glutamate across the bacteroid membrane but not the peribacteroid membrane from soybean root nodules. *Mol Plant–Microbe Interact* **1**, 250–254.

Waters, J. K., Hughes, B. L., II, Purcell, L. C., Gerhardt, K. O., Mawhinney, T. P. & Emerich, D. W. (1998). Alanine, not ammonia, is excreted from N_2 -fixing soybean nodule bacteroids. *Proc Natl Acad Sci USA* **95**, 12038–12042.

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Received 31 December 2001; revised 10 February 2002; accepted
18 February 2002.