Reassessment of major products of $N_2$ fixation by bacteroids from soybean root nodules

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INTRODUCTION

For many years it has been accepted that the fixation of atmospheric $N_2$ by the root nodules of legumes involved the production of $NH_3$ 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 $NH_3$–$NH_4^+$ equilibrium, at physiological pH only a very small proportion of this nitrogen existed as $NH_3$. The $NH_4^+$ ion would not diffuse across biological membranes, giving rise to concerns about the mechanism by which fixed $N_2$ left bacteroids (e.g. Kahn et al., 1985). Subsequently, an $NH_3^+$ 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 $NH_4^+$ ions, thereby creating a gradient for diffusion of $NH_3$ out of the bacteroids, with $NH_4^+$ subsequently transported to the plant via the PBM channel.

The basis for this interpretation was substantially due to the following experimental observations. (1) $NH_3$ was the earliest $^{15}$N-labelled product of $N_2$ 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 $^{15}N_2$. (2) With anaerobically prepared soybean nodule bacteroids (the symbiotic form of *Bradyrhizobium japonicum*) in microaerobic, shaken assays with $^{15}N_2$ in the gas phase, the principal product was $^{15}NH_3$ (Bergersen & Turner, 1967). (3) Later, $^{15}NH_3$ was the principal product of $^{15}N_2$ fixation by soybean bacteroids in an open, flow-reaction.

$NH_3/NH_4^+$ was the principal product from soybean bacteroids, prepared by various procedures, when assayed in solution in a flow chamber under $N_2$ fixation conditions. In addition, small quantities of alanine were produced (reaching 20% of $NH_3/NH_4^+$ under some conditions). Some $^{15}N$ was assimilated by bacteroids purified from soybean root nodules on Percoll density gradients and shaken with $^{15}N_2$ and 0.008 atm $O_2$. Under these conditions, $^{15}NH_4^+$ accounted for 93% of the $^{15}N$ fixed into the soluble fraction. This fraction contained no measurable $[^{15}N]$alanine. Neither these bacteroids nor those prepared by the previously used differential centrifugation method, when incubated with exogenous alanine under non-$N_2$-fixing conditions, gave rise to $NH_3$ from alanine. Therefore, contamination of bacteroid preparations with enzymes of plant cytosolic origin and capable of producing $NH_3$ from alanine cannot explain the failure to detect $[^{15}N]$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 $^{15}$N experiments contained alanine dehydrogenase and were able to produce alanine from pyruvate and $NH_4^+$. Other experiments with alanine dehydrogenase in extracts of cultured rhizobia and bacteroids are reported and discussed in relation to the $^{15}$N experiments. Possible reasons for the differences between laboratories regarding the role of alanine are discussed. It is concluded that $NH_3$ is the principal soluble product of $N_2$ fixation by suspensions of soybean bacteroids *ex planta* and that $NH_4^+$ should continue to be considered the principal product of $N_2$ fixation which is assimilated *in vivo* in soybean nodules.

Keywords: alanine, alanine dehydrogenase, ammonia, nitrogen fixation, $^{15}$N balance
system in which a well-stirred suspension of soybean bacteroids was perfused with solutions containing dissolved O₂ and ¹⁵N₂; no ¹⁴N-labelled amino acids were detected. The long-accepted view was challenged by Waters et al. (1998) who found that alanine, not NH₃, was the principal ¹⁴N-labelled product when N₂-fixing soybean bacteroids, purified on anaerobic sucrose density gradients, were supplied with malate and shaken in a gas mixture containing ¹⁵N₂ and 0.008 atm O₂. 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 NH₄ from the primary product, alanine. Allaway et al. (2000) subsequently showed that both ¹⁴N-alanine and ¹⁴NH₄ 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 N₂ fixation, NH₄ was the first and major product formed, but alanine was formed at high bacteroid densities when NH₄ accumulated (Allaway et al., 2000). These authors also used a mutant strain of Rhizobium leguminosarum, defective in alanine catabolism, to produce N₂-fixing nodules on peas. Such plants fix N₂, 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 NH₄ in shaken assays. It was concluded that alanine was a secondary product of N₂-fixing bacteroids; alanine amino-nitrogen arose from NH₄, derived from N₂ fixation, which accumulated in the experimental system used. More recently, Atkins & Thumfort (2001) have reported that assimilation of ¹⁵N₂ by nodulated roots of cowpea produced ¹⁵N-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 N₂-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 NH₄ was the sole significant ¹⁴N-labelled product of ¹⁵N₂ fixation accumulated during 30 min in shaken assays with 0.008–0.01 atm O₂. Alanine, although sometimes found in low concentrations in flow chamber reactions, was not labelled with ¹⁵N 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 NH₄ is the principal product of N₂ 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 NH₄ 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), MgCl₂ (2 M), 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 dispensed 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. N₂ 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, 2·0 M 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 O₂ and N₂ 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 N₂ 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 NH₄, 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 NH₄ 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 ℃ for 1 h to yield crude soluble extracts. Alanine dehydrogenase activity was estimated at 25 ℃ according to Allaway et al. (2000) and Smith & Emerich (1993). The apparent Kᵦ 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 electrochemically 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 (94–953 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 derivatization 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 ¹⁴N 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 ¹⁴N 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 % ¹⁴N before addition of carrier nitrogen (x) was calculated as:

\[ x = \frac{[c(a + b) - bd]}{a} \]  

in which \( a = \mu g \) NH₃ nitrogen in the sample before adding carrier, \( b = \mu g \) carrier nitrogen added, \( c = \text{atoms} % ^{15}\text{N} \) measured in the sample plus carrier and \( d = \text{atoms} % ^{15}\text{N} \) 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 ¹⁴N 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 ¹⁴N 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 derivatization with N-(tert-butylmethylsilyl)-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:

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

in which \( R_c \) and \( R_s \) 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 determinations of ¹⁵N-labelled fractions and no ¹⁵N 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 $^{15}$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.

<table>
<thead>
<tr>
<th>Fraction analysed</th>
<th>Nitrogen per assay (µg)</th>
<th>$^{15}$N excess (atoms %)</th>
<th>$^{15}$N excess (ng per assay)</th>
<th>Percentage recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total nitrogen</td>
<td>7344 ± 382</td>
<td>0.0130 ± 0.0010</td>
<td>958 ± 71</td>
<td>100</td>
</tr>
<tr>
<td>Bacteroid total nitrogen</td>
<td>4141 ± 813</td>
<td>0.0059 ± 0.0016</td>
<td>243 ± 122</td>
<td>25.4</td>
</tr>
<tr>
<td>Soluble total nitrogen</td>
<td>1676 ± 153</td>
<td>0.0287 ± 0.0016</td>
<td>481 ± 41</td>
<td>50.2</td>
</tr>
<tr>
<td>Soluble NH$_4$ nitrogen‡</td>
<td>36 ± 0.8</td>
<td>1.2336 ± 0.0347</td>
<td>448 ± 23</td>
<td>46.8</td>
</tr>
<tr>
<td>Soluble residue total nitrogen‡</td>
<td>1061 ± 84</td>
<td>0.0053 ± 0.0007</td>
<td>57 ± 13</td>
<td>5.9</td>
</tr>
</tbody>
</table>

*After centrifuging bacteroids.
‡Recovered from soluble fraction by diffusion.
‡After removal of NH$_4$.

Values are means ± SEM.

**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 $^{15}$N$_2$

<table>
<thead>
<tr>
<th>Sample analysed</th>
<th>$R$ (± SEM)</th>
<th>n</th>
<th>Excess $^{15}$N (± SEM) (atoms %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard alanine</td>
<td>0.22956 ± 0.00028</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>Control</td>
<td>0.24293 ± 0.00252</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Reactions with $^{15}$N$_2$</td>
<td>0.24326 ± 0.00044</td>
<td>4</td>
<td>0.00076 ± 0.00053</td>
</tr>
</tbody>
</table>

$^{15}$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 $^{15}$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 $^{12}$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 $^{15}$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 NH$_4$/NH$_3$ in shaken assays. In our experiments, although NH$_4$/NH$_3$ 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 $[^{15}N]alanine. In this experiment, an increment of 2.0 ± 0.8 µg NH$_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.

<table>
<thead>
<tr>
<th>Bacteroid preparation</th>
<th>[O$<em>2$]$</em>{free}$ (nM)</th>
<th>Bacteroid activities [nmol min$^{-1}$ (mg dry wt)$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>O$_2$ consumption</td>
</tr>
<tr>
<td>Differential centrifugation</td>
<td>7</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.52</td>
</tr>
<tr>
<td>Percoll density gradients</td>
<td>5</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>8.05</td>
</tr>
</tbody>
</table>

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$_2$]$_{free}$). 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.

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

<table>
<thead>
<tr>
<th>Source</th>
<th>Activity [nmol NADH min⁻¹ (mg protein)⁻¹]</th>
<th>$K_m$ (pyruvate) (mM)</th>
<th>$K_m$ (NH₃) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cultures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. japonicum</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USDA110c</td>
<td>25.4 ± 0.2</td>
<td>0.29 ± 0.06</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>USDA110de</td>
<td>30.6 ± 2.5</td>
<td>0.39 ± 0.01</td>
<td>2.9 ± 0.05</td>
</tr>
<tr>
<td>SU1014(CB1809)</td>
<td>132.7 ± 3.5</td>
<td>1.2 ± 0.07</td>
<td>4.0 ± 0.2</td>
</tr>
<tr>
<td><em>R. leguminosarum</em> bv. <em>viciae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TY3841 (addA⁻)</td>
<td>18.8 ± 0.2</td>
<td>0.84 ± 0.04</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>RU1327 (addA⁺)</td>
<td>0.3 ± 2.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Bacteroids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USDA110c on cv. Forrest</td>
<td>633 ± 108</td>
<td>0.9 ± 0.2</td>
<td>6.8 ± 0.9</td>
</tr>
<tr>
<td>USDA110c on cv. Williams</td>
<td>154 ± 6</td>
<td>1.3 ± 0.1</td>
<td>5.2 ± 0.3</td>
</tr>
</tbody>
</table>

Values are means ± range of two separate experiments. ND, Not determined.

purified bacteroids in air (in which nitrogenase activity would have been destroyed) and at a pH 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 NH₃ 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 ¹⁴N 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 [¹⁴N]alanine as a product of ¹⁴N₂ 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 addA 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 addA 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 addA 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 ¹⁴N 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 nmol NH$_3$ mg$^{-1}$ 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.

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