Factors contributing to the accumulation of glutamate in *Bradyrhizobium japonicum* bacteroids under microaerobic conditions

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Previous studies with labelled N and C have indicated synthesis and accumulation of glutamate in *Bradyrhizobium japonicum* bacteroids under microaerobic conditions similar to those found in soybean nodules. Low 2-oxoglutarate dehydrogenase (OGDH) activity might have accounted for this observation, but similar levels of enzyme activity were found in bacteroids isolated anaerobically or aerobically and in cultured bacteria. However, OGDH from *B. japonicum* bacteroids was strongly inhibited by NADH, and the degree of inhibition depended on the NADH:NAD ratio. Determination of endogenous levels of NAD and NADH gave NADH:NAD ratios of 0.19 and 0.83 in bacteroids isolated under aerobic and anaerobic conditions, respectively. A ratio of 0.83 resulted in more than 50% inhibition of OGDH *in vitro*, and this would be consistent with channelling of 2-oxoglutarate to glutamate. 

$[^{14}C]$Glutamate supplied to bacteroids was metabolized to CO$_2$ slowly relative to the respiration of malate, and essentially no labelling of products of glutamate metabolism such as arginine, proline, glutamine and 4-aminobutyrate (GAB) was found. Attempts to trap $^{14}$C in GAB by supplying unlabelled GAB or transaminase inhibitors with $[^{14}C]$glutamate were unsuccessful. The finding that glutamate decarboxylase was essentially absent in six different strains of *B. japonicum* was consistent with the labelling results and indicated that conversion of glutamate to succinate via GAB is slow or nil. The inhibition of OGDH by a high NADH:NAD ratio and the absence of the GAB shunt are complementary mechanisms which probably account for the accumulation of glutamate.

Introduction

Various lines of evidence indicate that dicarboxylic acids are important sources of reduced carbon for N$_2$-fixing bacteroids in legume nodules (Arwas *et al.*, 1985; Ronson *et al.*, 1981; Saroso *et al.*, 1984; Tuzimura & Meguro, 1960; Watson *et al.*, 1988). However, we found that, when *Bradyrhizobium japonicum* bacteroids were isolated anaerobically and supplied with $[^{1-14}C]$succinate or $[^{14}C]$malate under microaerobic conditions, 20 to 40% of the label taken up was converted to glutamate (Salminen & Streeter, 1987). When only the $^{14}$C remaining in bacteroids is considered ($^{14}$CO$_2$ subtracted), glutamate accounted for 75% of the radioactivity recovered. This unusual result is supported by evidence that glutamate is the most highly labelled compound in bacteroids following incubations of intact soybean [*Glycine max* (L.) Merr.] nodules with $^{15}$N$_2$ (Ohyama & Kumazawa, 1980). Synthesis of glutamate has been proposed to be a requirement for bacteroids capable of fixing N$_2$ (Donald & Ludwig, 1984).

*B. japonicum* bacteroids can absorb and respire glutamate (Bergersen & Turner, 1988; Salminen & Streeter, 1987), and some workers have even suggested that glutamate may be an important carbon source for bacteroids (Kahn *et al.*, 1985). However, glutamate transport through the peribacteroid membrane may be restricted (Udvardi *et al.*, 1988), so it would appear that glutamate accumulation in bacteroids may be unrelated to glutamate imported from the host cytoplasm.

The accumulation of label in glutamate in bacteroids (Ohyama & Kumazawa, 1980; Salminen & Streeter, 1987) probably reflects a relatively large pool size, and quantitative data indicate that glutamate is a major metabolite in *B. japonicum* (Kouchi & Yoneyama, 1986; Streeter, 1987). Glutamate can readily be synthesized from 2-oxoglutarate either by glutamate dehydrogenase (GDH) or by the combined action of glutamine synthetase (GS) and glutamine:2-oxoglutarate amino-
transferase (GOGAT). Other aminotransferases could also contribute to glutamate formation. The catabolism of glutamate could occur via the tricarboxylic acid cycle following conversion of glutamate to 2-oxoglutarate by GDH, by various transaminases or by deamination. Another potential route is the 4-aminobutyrate (GAB) shunt (Fig. 1), which leads to succinate formation. Glutamate metabolism via glutamic semialdehyde to arginine or proline is also a possibility. The rapid labelling of glutamate may also indicate some restriction in the breakdown of glutamate. We report here that B. japonicum bacteroids do, in fact, have NADH:NAD ratios which appear sufficient to inhibit 2-oxoglutarate dehydrogenase (OGDH), and also have essentially no capability to decarboxylate glutamate.

**Methods**

**Bacterial cultures.** Bradyrhizobium japonicum strains USDA 24, 33, 110, 136, 138 and 324 were obtained from the Nitrogen Fixation and Soybean Genetics Laboratory, US Department of Agriculture, Beltsville, MD, USA.

**Growth of plants.** Soybean plants [Glycine max (L.) Merr. cv. Beeson 80] were inoculated with Bradyrhizobium japonicum USDA 110 and grown in a greenhouse in pots of silica sand. The pots were irrigated three times per day with N-free nutrient solution (Streeter, 1989).

**Isolation of bacteroids and cultured bacteria.** Root nodules were macerated anaerobically (in a glove box under N2 flow) or aerobically (normal air) in a mortar with 0.15 M-sodium phosphate buffer, pH 7.5 (2 ml per g nodule fresh wt). The brei was filtered through two Miracloth discs in a 50 ml syringe into a cappable 40 ml centrifuge tube. The closed tube was centrifuged at 4400 g at 0°C for 10 min. The centrifuge tube was opened in the glove box (for anaerobic bacteroids) and the supernatant poured off. The pellet was rinsed twice by pipetting 1 ml of the phosphate buffer over the pellet. The pellet was resuspended in the phosphate buffer with a camel-hair brush using a final volume of 2 ml of buffer per g nodules. Three volumes of the phosphate buffer containing bovine serum albumin (2 mg ml⁻¹) were added to facilitate removal of host cytosolic enzymes adsorbed to the surface of the bacteroids. The beneficial role of bovine serum albumin in the purification of bacteroids was established by following the activity of four enzymes, malate dehydrogenase (EC 1.1.1.37), aspartate aminotransferase (EC 2.6.1.19), phosphoglucononate dehydrogenase (EC 1.1.1.43) and phosphoenolpyruvate carboxylyase (EC 4.1.1.31), in the bacteroid soluble protein fraction and in the wash buffer after each centrifugation. The tube was capped and centrifuged as above. The pellet was rinsed and resuspended in the phosphate buffer (without bovine serum albumin) using 2 ml per g nodules, and centrifugation was repeated as above. The final pellet was resuspended in phosphate buffer using 2 ml per g nodules for use in labelling studies, or 1 ml per g nodules for enzyme studies following sonication. For some studies bacteroids were purified using Percoll gradients (Reichab et al., 1981).

In studies of glutamate decarboxylase (EC 4.1.1.15), aspartate 2-oxoglutarate aminotransferase (EC 2.6.1.21) and 4-aminobutyrate:pyruvate aminotransferase (GAB aminotransferase; EC 2.6.1.19), a different protocol was used. The bacteroids were isolated aerobically at 2°C using a grinding medium consisting of 0.15 M-Tris buffer pH 7.5 containing 0.15 M-mannitol, 1 mM-EDTA, 2 mM-dithioerythritol and 0.5% bovine serum albumin. The crude homogenate was filtered and centrifuged at 4400 g. The supernatant solution was discarded and the bacteroids resuspended in the grinding buffer (about 25 ml per g fresh wt of nodules) and centrifuged again at 4400 g for 10 min. Washed bacteroids were suspended in 2 ml 10 mM-sodium phosphate buffer, pH 6.8, containing 1 mM-dithioerythritol and subjected to sonication. The defined medium used for growth of B. japonicum contained 25 mM-glutamate as the sole source of C and N, and Bishop's salts (mg 1⁻¹): CaCl₂, 2H₂O (6-0); MgSO₄·7H₂O (100); K₂HPO₄ (180); and FeSO₄·7H₂O (0-12). Vitamins and micronutrients were as described by Manhart & Wong (1979).

For assays of glutamate decarboxylase and GAB aminotransferase, B. japonicum strains USDA 24, 33, 110, 136, 138 and 324 were grown in the defined medium. Bacteria were collected in the exponential phase by centrifugation at 15000 g for 15 min. Cells were resuspended in a 20-fold volume of wash medium containing 40 mM-succinate, and salts (g 1⁻¹): CaCl₂, 2H₂O (0-74); NaCl (1-0); MgSO₄·7H₂O (1-0); and K₂HPO₄ (1-50). Cells were collected by centrifugation at 12000 g for 10 min and resuspended in a small volume (2-5 ml) of wash medium. Dithioerythritol and Triton X-100 (octylphenoxy polyethoxylate) were added to give final concentrations of 2 mM and 0.05%, respectively. The cell suspension was then sonicated.

**Sonication and gel filtration.** The sonication of bacteroids and cultured bacteria was carried out at 0°C using a Branson Sonic Power Co. sonifier for 5 min in a pulse mode. The sonicated samples were centrifuged at 27000 g and the supernatant solutions to be used for enzyme assays were then gel filtered. One or two drops of blue dextran solution were added to the samples, which were then loaded onto a Sephadex G-25 column and eluted with 0.01 M-sodium phosphate buffer, pH 7.5. The eluate was then used in enzyme assays.

**Determination of enzyme activities.** The 2-oxoglutarate dehydrogenase (EC 1.2.4.2) assay was based on the method of Sanadi et al. (1959). The reaction mixture (final volume 3 ml) consisted of 1.8 ml 25 mM-potassium phosphate pH 7.5, 0.2 ml 5 mM-coenzyme A, 0.2 ml 30 mM-dithiothreitol, 0.2 ml of 3 mM-NAD, 0.1 ml gel-filtered extract (linearity of the assay with the extract was established), 0.06 ml 1% Triton X-100 and 0.24 ml of other additions plus H₂O. The reaction was started with 0.2 ml 50 mM-2-oxoglutarate. The reduction of NAD was followed at 340 nm using a Hewlett-Packard HP8452A diode array spectrophotometer.

The reaction mixture for the malate dehydrogenase assay consisted of 2.5 ml 100 mM-potassium phosphate buffer (pH 7.4), 0.1 ml 6 mM-NADH, 0.275 ml H₂O and 0.025 ml of the extract; the reaction was started by adding 0.1 ml 15 mM-oxyacetate.

Assays of glutamate decarboxylase and GAB aminotransferase were essentially as described by Streeter & Thompson (1972) and involved the use of radioactive substrates. The reaction mixture for glutamate decarboxylase contained 50 mM-citrate/sodium phosphate buffer (variable pH), 30 mM-pyridoxal phosphate, 8 mM-glutamate and about 0.25 µCi [U-¹⁴C]glutamate (New England Nuclear; 290 µCi µmol⁻¹, 10-7 MBq µmol⁻¹) in a total volume of 500 µl. The control was boiled bacteroid soluble protein. Following incubation for 1 h at 30°C, reaction mixtures were placed for 5 min in a boiling water bath, cooled, and passed through columns of Dowex 1-formate ion-exchange resin (4 x 40 mm) which separated glutamate and GAB (Streeter & Thompson, 1972). Radioactive GAB formed was determined by liquid scintillation counting. The reaction mixture for GAB aminotransferase contained 50 mM-Tris (HCl) pH 8.5, 20 mM-pyridoxal phosphate, 1 mM-dithioerythritol, 5 mM-GAB, 10 mM-pyruvate or 2-oxoglutarate and about 0.5 µCi 4-aminobutyric acid (Amersham;
Glutamate dehydrogenase (EC 1.4.1.2) activity was measured in the direction of glutamate formation as described by Kanamori et al. (1988). Aspartate aminotransferase (EC 2.6.1.21) was assayed in a reaction mixture containing 2 ml 0.1 M-sodium phosphate buffer pH 7.5, 360 μM 5-oxo-aspartate (pH 7.5), 30 μM 7-1 mm-NADH, 5 units commercial malate dehydrogenase (Sigma M-2634) and enzyme samples. Control AAΔAD was measured, then 100 μM 2-oxo-glutarate was added and oxalacetate formation was measured as ΔAAΔAD.

Determination of NAD(P)(H) levels. Nodule brei and a pellet of cultured bacteria were obtained as described above, ‘isolation of bacteroids and cultured bacteria’. The brei was filtered as above and centrifuged for 1 min at 0°C and 48400 g, with a maximum brake setting, using a JA-20 rotor in a Beckman J2-21 centrifuge. The pellet was rinsed twice with the extraction buffer and resuspended in 0.04 M-NaOH containing 0.5 mm-cysteine (cysteine was added just prior to use) using a glass rod. The volume was adjusted to 1 ml per g nodules. The mixture was transferred to a 15 ml Corol centrifuge tube and sonicated for 3 min at 0°C. A sample was removed for protein determination and the rest centrifuged at 27000 g for 15 min. The supernatant was used for assaying NAD(P)(H) levels. The assay was based on the methods of Burch et al. (1967) and Bernofsky & Swan (1973). The oxidized forms are stable in 0.01 M-H2SO4/0.1 M-Na2SO4, whereas the reduced forms are destroyed by heating for 30 min at 60°C. Ascobate (final concentration 30 mm) was added prior to acidification in H2SO4/Na2SO4 to prevent oxidation of the reduced forms by leghaemoglobin.

NADH and NADPH are very stable in 0.04 M-NaOH, whereas their oxidized forms are destroyed by heating for 10 min at 60°C. Cysteine (0.5 mm) was added to prevent oxidation of the reduced forms during heating. The oxidized forms are stable in 0.04 M-NaOH for several hours at 0°C. Thus it is possible to make an independent determination of the total amount of NAD + NADH, and of NADP + NADPH, in the sample. The pH of these samples as well as that of the cooled samples from heating treatments was quickly adjusted to 7.0–7.1. This appeared critical even though the assays were run at alkaline pHs.

The specificity of the assay for either NAD(H) or NADP(H) is established by the enzyme and substrate used: NAD(H) levels were determined with alcohol dehydrogenase and ethanol; for NADP(H) determination glucose-6-phosphate dehydrogenase and glucose 6-phosphate were employed.

The recycling assay does not distinguish between the oxidized and reduced forms. It is based on repeated oxidation and reduction of NAD(P)(H) in the presence of enzymes and substrates given below with thiazolyl blue acting as the terminal electron acceptor. The reduction of thiazolyl blue is mediated by phenazine ethosulfate. The cycling provides sensitivity and linearity to picomole levels, whereas the enzymes provide specificity for either NAD(H) or NADP(H). The assays were carried out in minimal light and the reduction of thiazolyl blue was followed at 570 nm. Control analyses were carried out with mixtures of NAD(H) and NADP(H) reagents.

The NADH reaction mixture consisted of 2.0 ml 60 mm-Bicine [N,N,N-tris(2-hydroxyethyl)glycine] buffer with 1 mm-EDTA (pH 7.8), 0.3 ml 5% ethanol in 60 mm-Bicine, 0.2 ml 25 mm-phenazine ethosulfate, 0.2 ml 6 mm-thiazolyl blue, 0.2 ml sample, and 0.1 ml (2 units) of glucose-6-phosphate dehydrogenase (G4134, Sigma).

Feeding and detection of labelled compounds. The feeding of 14C-labelled substrates and the methods used for the analysis of labelling of individual metabolites has been described previously (Salminen & Streeter, 1987).

Results

Typical results for the addition of 14C-labelled metabolites to anaerobically isolated bacteroids under microaerobic conditions are shown in Table 1. Although label in the organic acid fraction was distributed in small amounts among numerous compounds, label in the amino acid fraction was concentrated in glutamate. There was some labelling of aspartate and alanine, but other amino acids (not shown) were not significantly labelled. Even when [U-14C]glutamate was supplied, the label recovered in glutamate was more than 20-fold greater than that recovered in malate. Because 14C would have to pass through the 2-oxoglutarate pool to transit to glutamate from malate, we interpret these results to indicate that the 2-oxoglutarate pool is very small relative to the glutamate pool. There was virtually no label in 2-oxoglutarate even when [U-14C]glutamate was the metabolite supplied. The uptake rates reported here are low. The concentration of the substrates in the reaction mixture, 0.5 mm, would not give maximal rates of uptake. The 14CO2 evolved is also an underestimation, because the dissolved CO2 is not accounted for. The 14CO2 release is, however, proportional to the total CO2 respired and shows a linear increase after a lag period (Salminen & Streeter, 1987).

In order to determine whether the rapid and substantial labelling of glutamate is related to the microaerobic conditions in nodules, we isolated bacteroids in air and incubated them as described in Table 1 but with 21% O2 in the gas phase. After a 10 min incubation with [U-14C]glutamate, 13% of the label taken up was recovered in glutamate. For a second sample of bacteroids isolated anaerobically at the same time and incubated with 3% O2 in the gas phase, 28% of the [U-14C]glutamate taken up was recovered in glutamate following a 10 min incubation (data not shown; based on two replicate samples).

Glutamate is synthesized by amination or transamination of 2-oxoglutarate. Thus it seemed logical to consider the possibility that in the bacteroids 2-oxoglutarate was diverted from the tricarboxylic acid cycle to glutamate (Fig. 1). This could be the consequence of the bacteroids having low levels of OGDH activity. The OGDH complex in B. japonicum had a pH optimum around pH 7.5, and a broad temperature optimum reached at about 30°C (data not shown). Triton X-100 stimulated the activity in gel-filtered extracts, reaching a
maximum threefold enhancement at 0.02% (w/v). The increase in OGDH activity was observed immediately after adding the Triton X-100, and this would suggest a conformational 'opening' of the OGDH complex making the active site more available.

A comparison of OGDH activity extracted from cultured bacteria, aerobically or anaerobically isolated bacteroids, and anaerobically isolated bacteroids purified in a Percoll gradient, gave rates of 48.7 ± 0.4, 29.7 ± 2.0, 36.2 ± 3.0 and 56.1 ± 2.2 nmol min⁻¹ (mg protein⁻¹) (mean ± SE), respectively. The corresponding values for malate dehydrogenase, measured to guard against the possibility of significant differences in enzyme extractability, were 3575 ± 216, 2966 ± 158, 3917 ± 86 and 3114 ± 82 nmol min⁻¹ (mg protein⁻¹) (mean ± SE). The results for OGDH did not support the idea that the diversion of 2-oxoglutarate to glutamate in bacteroids under microaerobic conditions is due to insufficient activity of the key TCA cycle enzyme.

A low level of OGDH activity could also result from in vivo inhibition of OGDH in bacteroids under the microaerobic conditions occurring in the nodules. The enzyme was inhibited by NADH (Fig. 2), inhibition being stronger when the NAD concentration was decreased from 0.2 to 0.1 mM (Fig. 2a). When the data were expressed as a function of the NADH : NAD ratio (Fig. 2b) the results obtained with the two NAD concentrations were similar, suggesting that the NADH : NAD ratio controls the OGDH activity. Inhibition of OGDH activity by NADPH was not observed.

Endogenous levels of NAD and NADH were determined to see whether they would be relevant to the in vitro inhibition shown in Fig. 2. Bacteroids isolated under anaerobic conditions had an NADH : NAD ratio > 0.83, whereas the ratio in cultured bacteria and aerobically isolated bacteroids was 0.12 and 0.19, respectively (Table 2). In order to minimize changes in

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**Table 1. Distribution of 14C from [U-14C]malate or [U-14C]glutamate in B. japonicum USDA 110 bacteroids**

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>14CO₂</td>
<td>14.6 ± 1.6</td>
<td>3.0 ± 0.8</td>
</tr>
<tr>
<td>Neutral fraction</td>
<td>2.3 ± 0.6</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Amino acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction (total)</td>
<td>34.4 ± 3.2</td>
<td>93.8 ± 20.3</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>Glutamate</td>
<td>36.2 ± 2.8</td>
<td>81.2 ± 21.8</td>
</tr>
<tr>
<td>Alanine</td>
<td>5.6 ± 0.6</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>Organic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction (total)</td>
<td>11.1 ± 3.0</td>
<td>5.2 ± 1.9</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.7 ± 0.2</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>Fumarate</td>
<td>0.8 ± 0.2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Malate</td>
<td>1.7 ± 0.5</td>
<td>0.3 ± 0.2</td>
</tr>
</tbody>
</table>

* Mean ± SE of two experiments. The calculation of the amounts in nanomoles was based on the assumption of a constant specific activity of 1 μCi μmol⁻¹.

† The total label in the fraction was determined before applying samples to TLC plates. The difference in the total label and the sum of individual compounds is not significant at 2 SE.

‡ The amounts of substrate converted to glutamine were 0.1 and 0.2 nmol h⁻¹ (mg protein⁻¹) with [U-14C]malate and [U-14C]glutamate feeding, respectively.
Glutamate accumulation in bacteroids

Fig. 2. (a) 2-Oxoglutarate dehydrogenase activity extracted from bacteroids expressed as a percentage of the maximum activity vs NADH concentration at 0.2 mM (○) and 0.1 mM (□) NAD. The maximum activity in nmol min\(^{-1}\) (mg protein\(^{-1}\)) was 52.9 ± 1.3 at 0.2 mM and 34.1 ± 0.0 at 0.1 mM-NAD, respectively. (b) The relative activity expressed as a function of the NADH/NAD ratio at the same NAD concentrations as in (a).

Table 2. Endogenous NAD(H) levels in cultured B. japonicum USDA 110, and in bacteroids isolated under aerobic and anaerobic conditions

The results are means ± SE.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Amount [nmol min(^{-1}) (mg protein(^{-1}))</th>
<th>NADH</th>
<th>NAD</th>
<th>NADH/NAD ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured bacteria*</td>
<td>2.79 ± 1.18</td>
<td>0.33 ± 0.02</td>
<td>3.05 ± 0.98</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>Aerobic bacteroids†</td>
<td>2.35 ± 0.11</td>
<td>0.38 ± 0.01</td>
<td>2.08 ± 0.15</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>Anaerobic bacteroids‡</td>
<td>2.07 ± 0.04</td>
<td>1.21 ± 0.29</td>
<td>1.51 ± 0.30</td>
<td>0.83 ± 0.15</td>
</tr>
</tbody>
</table>

* Two experiments.
† Three experiments.
‡ Four experiments.
§ The assay for the total NADH + NAD was independent of the individual NADH or NAD determinations.

Table 3. Accumulation of \([^{14}C]GAB\) from \([U-^{14}C]\)glutamate in B. japonicum USDA 110 bacteroids in a 30 min incubation

Other experimental conditions were the same as given in Table 1.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Concentration (mM)</th>
<th>GAB Radioactivity (d.p.m.)*</th>
<th>Glutamate Radioactivity (d.p.m.)</th>
<th>Total uptake†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>0</td>
<td>51 ± 7</td>
<td>121 ± 4</td>
</tr>
<tr>
<td>GAB</td>
<td>0.5</td>
<td>0.024 ± 0.024</td>
<td>87 ± 18</td>
<td>145 ± 4</td>
</tr>
<tr>
<td>Gabaculine</td>
<td>0.1</td>
<td>0.047 ± 0.0</td>
<td>85 ± 2</td>
<td>145 ± 4</td>
</tr>
<tr>
<td>2-Aminoethyl sulphate</td>
<td>10</td>
<td>0.041 ± 0.006</td>
<td>117 ± 2</td>
<td>196 ± 19</td>
</tr>
</tbody>
</table>

* Mean ± SD of duplicate samples.
† Total d.p.m. in the cells + \(^{14}C\)CO\(_2\) released.

NAD(H) in mitochondria could have a significant influence on the results in Table 2. This is also supported by similar ratios for cultured bacteria and bacteroids isolated under aerobic conditions.

For glutamate to accumulate, its catabolism must be restricted. If OGDH is inhibited, this would slow down glutamate metabolism through the tricarboxylic acid cycle following conversion to 2-oxoglutarate. Glutamate could be metabolized via glutamic semialdehyde and ornithine to arginine or via Δ⁵-pyroline-5-carboxylic acid to proline, but no labelling of arginine or proline was found (data not shown). A pathway capable of supporting the tricarboxylic acid cycle activity by bypassing a restriction at the OGDH step would be the GAB shunt (Fig. 1). We investigated metabolism via the GAB shunt by measuring \([^{14}C]GAB\) formation from \([U-^{14}C]\)glutamate (Table 3). No label was recovered in GAB in an initial experiment. The possibility that this was a consequence of a small GAB pool with a rapid turnover was further examined using unlabelled GAB with concentrations of NAD(P)(H) during extraction no effort was made to exclude mitochondria from the bacteroid preparations. The numbers of bacteroids far exceed those of mitochondria, so it is unlikely that the relative results in Table 2 are due to an influence of NAD(H) in mitochondria.
[U-14C]glutamate in the reaction mixture, as well as adding inhibitors of GAB aminotransferase, gabaculine and 2-aminoethyl hydrogen sulphate (Soper & Manning, 1982), to trap [14C]GAB formed. None of these treatments led to a recovery of [14C]GAB beyond trace amounts, although 3% of the d.p.m. of [U-14C]glutamate taken up by the bacteroids in a 15 min incubation was converted to 14CO2. To confirm that unlabelled GAB had been taken up by the bacteroids we fed them [U-14C]GAB (data not shown). In a 30 min incubation the total uptake of [U-14C]GAB was 12.95 nmol h⁻¹.treatments led to a recovery of [14C]GAB beyond trace [U-14C]GAB (data not shown). In a 30 min incubation had been taken up by the bacteroids we fed them converted to 14C02. To confirm that unlabelled GAB taken up by the bacteroids in a 15 min incubation was amounts, although 3% of the d.p.m. of [U-14C]glutamate was found in 14C02. The reason for the 68% increase in the uptake of [14C]GAB in the presence of gabaculine is not clear. A smaller (20%) enhancement of glutamate uptake by gabaculine was also seen after the addition of [U-14C]glutamate (Table 3).

Discussion

Glutamate synthesis from [U-14C]malate is considered here to be the consequence of diversion of 2-oxoglutarate away from the tricarboxylic acid cycle. However, the OGDH activity in cultured bacteria was similar to that observed in bacteroids isolated under either aerobic or anaerobic conditions. OGDH has been shown to be inhibited by NADH in Acetobacter xylinum (Kornfeld et al., 1977), Acinetobacter (Hall & Weitzman, 1977) and Dictyostelium discoideum (Heckert et al., 1989). Our results also indicate control of OGDH in B. japonicum by NADH and implicate the NADH : NAD ratio in the cell as a factor governing enzyme activity.

Endogenous levels of NADH and NAD (Table 2) are consistent with OGDH activity being inhibited in the bacteroids under microaerobic conditions. This pattern of the oxidation-reduction state of NAD agreed with that measured by Jackson & Dawes (1976) in Azotobacter in response to O2 limitation. A high NADH : NAD ratio in B. japonicum bacteroids relative to that in soybean whole nodule or root tissue has also been reported by Tajima & Kouzai (1989). The NADH : NAD ratio seen in anaerobic bacteroids, 0·83 (Table 2), would result in more than 50% inhibition of in vitro OGDH activity. Assuming the inhibition is similar in vivo this would partially restrict metabolism of 2-oxoglutarate via the tricarboxylic acid cycle. A comparison between the rates of substrate utilization (Table 1) of 0·6 to 1·5 nmol min⁻¹ (mg protein)⁻¹ and OGDH activities of 36 to 56 nmol min⁻¹ (mg protein)⁻¹ would seem to suggest that even at 50% inhibition there would be a huge excess of OGDH activity. However, the in vitro OGDH rates are Vₘₐₓ rates obtained at substrate concentrations unlikely to be present in vivo (see below).

The inhibition of OGDH in bacteroids would be consistent with the formation of [14C]glutamate from labelled substrates (Salminen & Streeter, 1987). No accumulation of radioactivity in 2-oxoglutarate was seen (Table 1). If the conversion to glutamate was carried out by GDH then this could reflect the equilibrium constant of the reaction (~ 10⁻¹⁴). Furthermore, N₂-fixing bacteroids have high ammonium concentrations (Klucas, 1974; Streeter, 1989) which, together with the high NADH : NAD ratios (Table 2) would favour glutamate synthesis by GDH. The high glutamate and NADH levels would also support glutamate formation by the GS/GOGAT pathway.

Generation of reductant and ATP for nitrogenase is thought to depend on the operation of the TCA cycle (Duncan & Fraenkel, 1979; Ronson et al., 1981; Salminen & Streeter, 1987). Thus, a mechanism which would permit conversion of 2-oxoglutarate to succinate, bypassing OGDH and permitting continued operation of
the TCA cycle, would be highly important. The GAB shunt (Fig. 1) for bypassing OGDH has been reported as a major pathway for 2-oxoglutarate metabolism in *Escherichia coli* under anaerobic conditions (Rosenqvist et al., 1973). There was no convincing evidence for the conversion of any [U-14C]glutamate to [14C]GAB by bacteroids. Assays of glutamate decarboxylase indicated that the enzyme may be absent from *B. japonicum*, although we cannot rule out the possibility that a trace of activity was present. Thus, our data are consistent with those of Ta et al. (1988) for *Rhizobium meliloti* bacteroids, in which glutamate is largely converted via 2-oxoglutarate to CO₂ through the tricarboxylic acid cycle.

An important unanswered question is whether the regulation of the tricarboxylic acid cycle at the OGDH step is operating in intact nodules. Perhaps the most direct evidence on this point is the demonstration of substantial labelling of glutamate in *B. japonicum* bacteroids after supplying intact nodules with 15N₂ (Ohyama & Kumazawa, 1980). Also, the glutamate pool in *B. japonicum* bacteroids is large relative to other amino and organic acids (Kouchi & Yoneyama, 1986; Streeter, 1987). Thus, our results here for the inhibition of OGDH by high NADH concentration in bacteroids are consistent with data from intact systems (Kouchi & Yoneyama, 1986; Ohyama & Kumazawa, 1980; Streeter, 1987), and we suggest that slow operation of the TCA cycle may provide a constraint for the generation of ATP in bacteroids.

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References

**References**


