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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 [U-^{14}C]succinate or [U-^{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-

Abbreviations: GAB, 4-aminobutyrate; GDH, glutamate dehydrogenase; GOGAT, glutamine:2-oxoglutarate aminotransferase; GS, glutamine synthetase; OGDH, 2-oxoglutarate dehydrogenase.

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 indicate some restriction in 2-oxoglutarate metabolism via the tricarboxylic acid cycle and possibly also a 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 N_2 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 2.6.1.1), phosphogluconate dehydrogenase (EC 1.1.1.43) and phosphoenolpyruvate carboxylase (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 (Reibach *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 l^{-1}): $CaCl_2.2H_2O$ (6·6); $MgSO_4.7H_2O$ (100); K_2HPO_4 (180); and $FeSO_4.7H_2O$ (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 l^{-1}): $CaCl_2.2H_2O$ (0·74); NaCl (1·0); $MgSO_4.7H_2O$ (1·0); and K_2SO_4 (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 polyethoxyethanol) 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 H_2O. 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 H_2O and 0·025 ml of the extract; the reaction was started by adding 0·1 ml 15 mM-oxaloacetate.

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 μM-pyridoxal phosphate, 8 mM-glutamate and about 0·25 μCi [U-^{14}C]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 × 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 μM-pyridoxal phosphate, 1 mM-dithioerythritol, 5 mM-GAB, 10 mM-pyruvate or 2-oxoglutarate and about 0·5 μCi 4-amino[U-^{14}C]butyric acid (Amersham;

220 μCi μmol^{-1}, 8·14 MBq μmol^{-1}) in a total volume of 500 μl. The controls lacked oxo acid. Following incubation for 1 h at 30 °C, reaction mixtures were passed through columns of Dowex 50 H^+ ion-exchange resin (4 × 40 mm) to stop the reaction and to separate GAB from the radioactive product succinic semialdehyde (Streeter & Thompson, 1972).

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 μl 0·5 M-aspartate (pH 7·5), 30 μl 7·1 mM-NADH, 5 units commercial malate dehydrogenase (Sigma M-2634) and enzyme sample. Control ΔA_{340} was measured, then 100 μl 0·6 M-2-oxoglutarate was added and oxalacetate formation was measured as ΔA_{340}.

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 Corex 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-H_2SO_4/0·1 M-Na_2SO_4, whereas the reduced forms are destroyed by heating for 30 min at 60 °C. Ascorbate (final concentration 30 mM) was added prior to acidification in H_2SO_4/Na_2SO_4 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 ethosulphate. 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 NAD(H) reaction mixture consisted of 2·0 ml 60 mM-Bicine [*N*,*N*-bis(2-hydroxyethyl)glycine] buffer with 1 mM-EDTA (pH 7·8), 0·3 ml 5 M-ethanol in 60 mM-Bicine, 0·2 ml 25 mM-phenazine ethosulphate, 0·2 ml 6 mM-thiazolyl blue, 0·2 ml sample, and 0·1 ml (37 units) of alcohol dehydrogenase (A-3263, Sigma).

The NADP(H) reaction mixture consisted of 2·0 ml 100 mM-Tris with 4·5 mM-$MgCl_2$ (pH 7·4), 0·3 ml 50 mM-glucose 6-phosphate, 0·2 ml 25 mM-phenazine ethosulphate, 0·2 ml 6 mM-thiazolyl blue, 0·2 ml sample, and 0·1 ml (2 units) of glucose-6-phosphate dehydrogenase (G-4134, Sigma).

Feeding and detection of labelled compounds. The feeding of ^{14}C-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 ^{14}C-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-^{14}C]malate was supplied, the label recovered in glutamate was more than 20-fold greater than that recovered in malate. Because ^{14}C would have to pass through the 2-oxoglutarate pool in 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-^{14}C]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 $^{14}CO_2$ evolved is also an underestimation, because the dissolved CO_2 is not accounted for. The $^{14}CO_2$ release is, however, proportional to the total CO_2 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% O_2 in the gas phase. After a 10 min incubation with [U-^{14}C]malate, 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% O_2 in the gas phase, 28% of the [U-^{14}C]malate 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

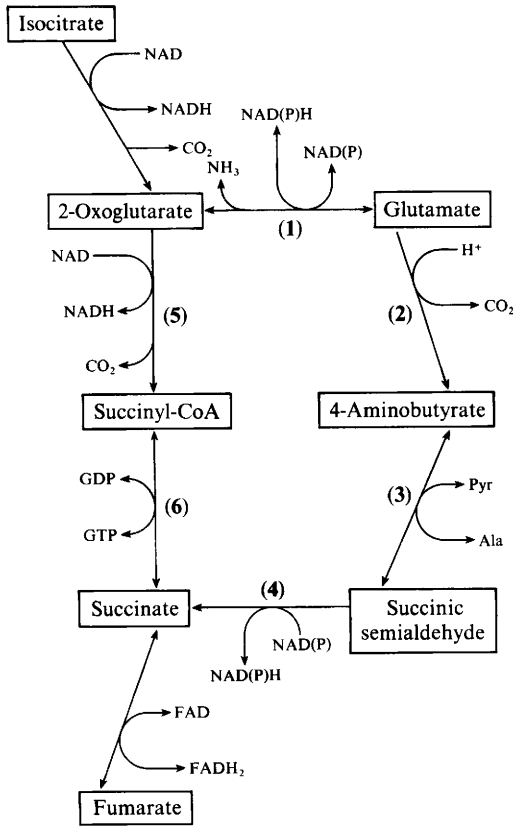

Fig. 1. 4-Aminobutyrate (GAB) shunt: (1) glutamate dehydrogenase (other enzymes, e.g. glutamine synthetase and glutamine:2-oxoglutarate aminotransferase, might also contribute to glutamate synthesis); (2) glutamate decarboxylase; (3) GAB aminotransferase; (4) succinic semialdehyde dehydrogenase; (5) 2-oxoglutarate dehydrogenase; (6) succinic thiokinase; Pyr, pyruvate; Ala, alanine.

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

The reaction mixture in a 15 ml Corex tube consisted of 1 ml bacteroids (6–8 mg protein), 0·2 ml 0·4 mM-myoglobin, 0·3 ml 0·15 M-sodium phosphate buffer pH 7·5, and 0·5 ml (1 μmol) [U-^{14}C]substrate (1 μCi μmol^{-1}). The gas phase contained 3% O_2. A KOH wick was used to capture $^{14}CO_2$. The tubes were incubated for 15 min on a rotary shaker at 200 r.p.m. at room temperature.

Distribution of label	[U-^{14}C]Substrate converted [nmol h^{-1} (mg protein)$^{-1}$]*	
	[U-^{14}C]Malate	[U-^{14}C]Glutamate
$^{14}CO_2$	14·6 ± 1·6	3·0 ± 0·8
Neutral fraction	2·3 ± 0·6	1·2 ± 0·1
Amino acid		
Fraction (total)†	34·4 ± 3·2	93·8 ± 20·3
Aspartate	0·4 ± 0·1	0·5 ± 0·0
Glutamate‡	36·2 ± 2·8	81·2 ± 21·8
Alanine	5·6 ± 0·8	0·8 ± 0·4
Organic acid		
Fraction (total)†	11·1 ± 3·0	5·2 ± 1·9
Citrate	0·3 ± 0·1	0·3 ± 0·1
2-Oxoglutarate	0·1 ± 0·1	0·2 ± 0·0
Succinate	1·7 ± 0·2	0·6 ± 0·4
Fumarate	0·8 ± 0·2	0·3 ± 0·1
Malate	1·7 ± 0·5	0·3 ± 0·2

* 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-^{14}C]malate and [U-^{14}C]glutamate feeding, respectively.

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 miocroaerobic 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. 2*a*). When the data were expressed as a function of the NADH:NAD ratio (Fig. 2*b*) 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

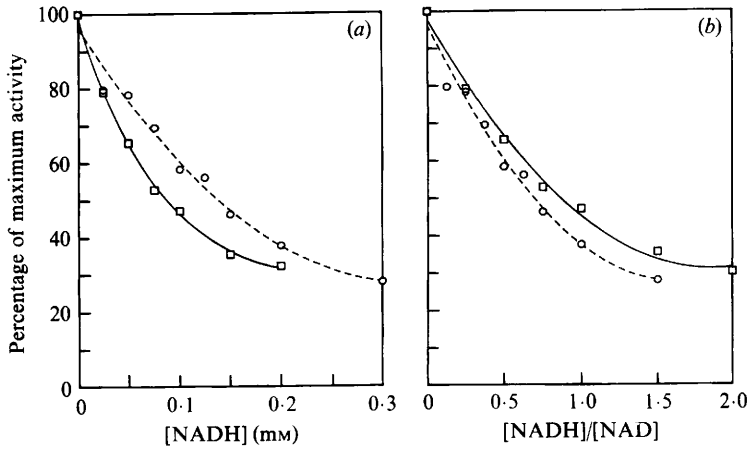

Fig. 2. (*a*) 2-Oxoglutarate dehydrogenase activity extracted from bacteroids expressed as a percentage of the maximum activity vs NADH concentration at 0·2 mM- (○) and 0·1 mM- (□) NAD. The maximum activity in nmol min^{-1} (mg protein)$^{-1}$ was 52·9 ± 1·3 at 0·2 mM- and 34·1 ± 0·0 at 0·1 mM-NAD, respectively. (*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.

Sample	Amount [nmol min^{-1} (mg protein)$^{-1}$]			
	Total NADH + NAD§	NADH	NAD	NADH/NAD ratio
Cultured bacteria*	2·79 ± 1·18	0·33 ± 0·02	3·05 ± 0·98	0·12 ± 0·03
Aerobic bacteroids†	2·35 ± 0·11	0·38 ± 0·01	2·08 ± 0·15	0·19 ± 0·01
Anaerobic bacteroids‡	2·07 ± 0·64	1·21 ± 0·29	1·51 ± 0·30	0·83 ± 0·15

* 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.

Addition	Concn (mM)	10^{-3} × Radioactivity (d.p.m.)*		
		GAB	Glutamate	Total uptake†
None		0	51 ± 7	121 ± 4
GAB	0·5	0·024 ± 0·024	87 ± 18	145 ± 4
Gabaculine	0·1	0·047 ± 0	85 ± 2	145 ± 4
2-Aminoethyl hydrogen sulphate	10	0·041 ± 0·006	117 ± 2	196 ± 19

* Mean ± SE of duplicate samples.
† Total d.p.m. in the cells + $^{14}CO_2$ released.

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 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 Δ^1-pyrroline-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

[U-^{14}C]glutamate in the reaction mixture, as well as adding inhibitors of GAB aminotransferase, gabaculine and 2-aminoethyl hydrogen sulphate (Soper & Manning, 1982), to trap [^{14}C]GAB formed. None of these treatments led to a recovery of [^{14}C]GAB beyond trace amounts, although 3% of the d.p.m. of [U-^{14}C]glutamate taken up by the bacteroids in a 15 min incubation was converted to $^{14}CO_2$. To confirm that unlabelled GAB had been taken up by the bacteroids we fed them [U-^{14}C]GAB (data not shown). In a 30 min incubation the total uptake of [U-^{14}C]GAB was 12·95 nmol h^{-1} (mg protein)$^{-1}$, of which 13·5% was found in $^{14}CO_2$. When 0·1 mM-gabaculine was included the total uptake was 21·81 nmol h^{-1} (mg protein)$^{-1}$, of which only 0·5% was found in $^{14}CO_2$. The reason for the 68% increase in the uptake of [^{14}C]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-^{14}C]glutamate (Table 3).

We further documented the lack of GAB formation by assaying glutamate decarboxylase. Its activity was too low to measure accurately in *B. japonicum* bacteroids grown with glutamate as a C and N source. Using a sensitive radioactive assay, a low limit for activity could be established; namely, specific activity was $\leqslant$0·05 nmol min^{-1} (mg protein)$^{-1}$, and the net d.p.m. in the ion-exchange column effluent (GAB) was only about 50% greater than the control. No significant glutamate decarboxylase activity could be detected in USDA strains 24, 33, 110, 136, 138 and 324 of cultured *B. japonicum*, at assay pHs of 4·0, 4·5, 5·0, 6·0 or 7·0, or with as much as 1·7 mg protein per assay using bacteroid protein from two strains (USDA 110 and 324) of *B. japonicum*. When unlabelled glutamate was omitted from reaction mixtures to maximize sensitivity, there were still only small differences between control and complete mixtures. Assays with gel-filtered (desalted) protein from nodule cytosol indicated glutamate decarboxylase activity of 4 to 5 nmol min^{-1} (mg protein)$^{-1}$ at pH 6·0, showing that a valid enzyme assay was used. Our failure to detect glutamate decarboxylase in bacteroids confirms and extends the previous observation of Stovall & Cole (1978).

Bacteroid soluble protein did contain GAB aminotransferase activity at about 4·9 nmol min^{-1} (mg protein)$^{-1}$. Activity with pyruvate was about 10-fold higher than activity with 2-oxoglutarate, and pyruvate-dependent activity was proportional to protein concentration and time. Glutamate dehydrogenase, which would lead to glutamate formation (Fig. 1), and highly active aspartate aminotransferase, which could convert glutamate to 2-oxoglutarate, were present at 9·4 $\pm$ 2·7 and 120 $\pm$ 32 nmol min^{-1} (mg protein)$^{-1}$ (mean $\pm$ SE), respectively.

Discussion

Glutamate synthesis from [U-^{14}C]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 O_2 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 V_{max} 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 [^{14}C]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 ($\sim 10^{14}$). Furthermore, N_2-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-^{14}C]glutamate to [^{14}C]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 CO_2 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 $^{15}N_2$ (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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