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Why do Chickpea (*Cicer arietinum* L. cv. Tyson) Bacteroids Contain Little Poly-β-Hydroxybutyrate?

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Poly-β-hydroxybutyrate (PHB) and enzymes related PHB metabolism have been measured in nitrogen-fixing symbiosis of chickpea and cowpea plants. Bacteroids from chickpea and cowpea contained PHB to 0.8% and 43% of their dry weight, respectively, whereas the free-living cells CC 1192 and I 16 produced 285±55 mg and 157±18 mg of PHB g (dry weight)$^{-1}$. To further understand why chickpea bacteroids contained little PHB, the enzyme activities of PHB metabolism (3-ketothiolase, acetoacetyl-CoA reductase, PHB depolymerase, and 3-hydroxybutyrate dehydrogenase), the TCA cycle (malate dehydrogenase, citrate synthase, and isocitrate dehydrogenase), and related reactions (malic enzyme, pyruvate dehydrogenase, and glutamate:2-oxoglutarate transaminase) were compared in extracts from chickpea and cowpea bacteroids and the respective free-living bacteria. Significant differences were observed between chickpea and cowpea bacteroids and between the bacteroid and free-living forms of CC 1192, with respect to the capacity for some of these reactions. It is indicated that a greater potential for oxidizing malate to oxaloacetate in chickpea bacteroids could be a factor that favors the utilization of acetyl-CoA in TCA cycle rather than for PHB synthesis.

Key words : *poly-β-hydroxybutyrate, nitrogen-fixation, chickpea, cowpea.*

Chickpea (*Cicer arietinum* L.) and cowpea (*Vigna unguiculata* L. Walp.) are two major legume crops which grow in many semi-arid and tropical regions of the world, respectively, for human livestock and consumption. These plants are able to obtain a substantial proportion of their N symbiotically, but little is known of PHB metabolism from their nodules.[1-3] To obtain information of the physiology and biochemistry of different nodules, the studies of chickpea (ureide-exporting plant) and cowpea (amide-exporting plant) are required.

The fixation of atmospheric N_2 by legume-rhizobium symbiosis requires the metabolic activities in two organisms to be closely coordinated. The host legume supplies carbon that furnishes the microsymbiont with energy and reductant for the fixation of N_2 and in return receives reduced nitrogen. This carbon is translocated predominantly as sucrose into the root nodules, where it is converted in the cytosol to organic acids, such as malate, which are the preferred substrates taken up by the bacteroid. Many of the metabolic processes concerned take place in a microaerobic environment which is maintained in the nitrogen-fixing region of nodules to protect O_2-labile nitrogen.[4,5] Regulation of the free O_2 concentration is achieved by a combination of mechanism, including the maintenance by the bacteroids of a high respiratory demand.[6] Although rhizobial bacteroids have the capacity for energy conservation at concentration of free O_2 that are too low to damage nitrogen,[7] it nevertheless seems likely that availability of O_2 to the bacteroids is a major limiting factor of metabolism processes that support nitrogen fixation.[8]

In various legume nodules, nodule bacteroids appear to take up more carbon than can be immediately utilized, and under these circumstances, they may form PHB. This polyester storage reserve of carbon and reductant is considered to be an important source of oxidizable substrates to help maintain the respiratory demand of bacteroids and support nitrogen fixation when the supply of photosynthate from the host is reduced, as may occur during extended periods of low light intensity or pod filling.[9] However, bacteroids in some nodules formed by mutants of *Rhizobium meliloty* contained little or no PHB.[10] To further understand why this reserve accumulates in some symbioses but not in other nodules, PHB capacity and enzyme activity for PHB metabolism have been determined in chickpea and cowpea nodules for comparison.

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Abbreviations: DTNB, 5,5'-dithio-bis(2-nitrobenzoic) acid; GOT, glutamate: 2-oxoglutarate transaminase; HEPES, N-2-hydroxyethylpiperazine - N'-2-ethanesulfonic acid; malate DH, malate dehydrogenase; PHB, poly- β-hydroxybutyrate; TES, N-tris (hydroxymethyl)-2-aminoethanesulfonic acid.

Materials and Methods

Materials. Bacterial cultures were obtained from the Ciba-Geigy Inoculant Research Institute in Basel, Swiss, and

peat moss cultures of inoculating bacteria were a generous gift from Dr. Mattos Braunegg, Dept. of Agriculture, Ciba-Geigy in Basel, Swiss. All enzymes used in coupled assays and other biochemicals were from Boehringer GmbH.

Growth of plants. Seeds of chickpea (*C. arietinum* L. cv. Tyson) and cowpea (*V. unguiculata* L. Walp. cv. Caloona) were surface sterilized in 0.4% (w/v) sodium hypochlorite for 10 min, rinsed thoroughly with running tap water for 15 min, inoculated with *Bradyrhizobium japonicum* I 16 (cowpea) or *Rhizobium* sp. (*Cicer*) CC 1192 (chickpea) and sown in moistened perlite in pots at a depth of approximately 2 cm and 3-4 cm apart. Plants were grown in glasshouse with average day and night temperatures of 25 and 19°C, respectively. N-free nutrient solution[11] was given to the plants every 3-4 days.

Growth of bacteria. Bacterial cultures were grown for 5 days at 30°C on agar plates made up of modified Bergersen's medium[12] and inoculated into 10 ml of modified Bergersen's medium in loosely capped McCartney bottles. After growth for 5 days at 30°C in an orbital shaker at 200 orbits per min, these cultures were added to 50 ml of the same medium in a 100-ml Erlenmeyer flask stoppered with cotton wool and grown for 3 to 5 days in an orbital shaker until late exponential phase (i.e., A_{660}, ca. 1).

Preparation of bacteroid and bacterial extracts. Bacteroids were isolated from cowpea nodules of 45 to 55 day old plants by the method of Lee and Ahn[3] as follows. Nodules (5 g) were homogenized with a mortar and pestle in 30 ml of ice-cold medium which contained 50 mM TES, 10 mM KH_2PO_4, 0.4 M mannitol, 2 mM EDTA, 30 mM ascorbic acid, 1% (wt/vol) bovine serum albumin, and 2% (wt/vol) polyvinylpyrrolidone-24. The final pH was adjusted to 7.6 with 0.1 M KOH. The homogenate was filtered through four layers of Miracloth, and the filtrate was centrifuged at 4,000 g for 5 min. The pellet was resuspended in 25 mM TES-NaOH (pH 7.3), layered over 30 ml of 70% Percoll in 25 mM TES-NaOH (pH 7.3), and centrifuged at 40,000 g for 30 min in a Sorvall SS-34 rotor. The bacteroids were isolated from a band near the bottom of the gradient, diluted fivefold with 25 mM TES-NaOH (pH 7.3), and pelleted by centrifuging at 10,000 g for 15 min. Chickpea nodule bacteroids were isolated from 37 to 43 day old plants by the same method, except that the concentration of Percoll in the gradients was 55%. Free-living cells of CC 1192 and I 16 were harvested from liquid cultures by centrifuging at 20,000 g for 15 min, and the pellets were washed by resuspension in 10 ml of 25 mM TES-NaOH (pH 7.3).

Bacteroid and bacterial pellets were resuspended in approximately 5 ml of a mixture containing 25 mM TES-NaOH (pH 7.5), 50 mM KCl, 5 mM $MgSO_4$, and 5 mM 1,4-dithiothreitol, sonicated for three 1-min periods at 75% of maximum energy (1,000 W), and centrifuged at 100,000 g for 1 hr, and the supernatant was used for enzyme assays. PHB depolymerase activity was measured in pellets from sonicated bacteroid and bacterial suspensions that were centrifuged at 40,000 g for 50 min and resuspended in 9 ml of 50 mM Tris-HCl (pH 8.5).

Analysis of PHB. To determine the PHB content of bacteroids, nodules (5 g) were crushed with mortar and pestle in 2 volumes of 50 mM Tris-HCl (pH 8.4), and the homogenate was filtered through four layers of Miracloth and centrifuged at 300 g for 10 min. The supernatant was centrifuged at 5,000 g for 20 min, and the pellet was washed twice with deionized water and dried to a constant weight at 85°C. Pellets of CC 1192 and I 16 were washed in 10 ml of deionized water before being dried to a constant weight at 85°C.

PHB analysis was performed by the method of Riis and Mae[13] as follows. Dried bacterial and bacteroid pellets (40 mg) were incubated with intermittent shaking for 2 g at 100 in tightly sealed 10-ml vials that contained a mixture of 2 ml of 1,2-dichloroethane, 2 ml of a solution of 4 parts propan-1-ol and 1 part 10 M HCl, and 200 μl of a solution of 2.0 g of benzoic acid in 50 ml of propan-1-ol, which was included as an internal standard. After cooling to room temperature, 4 ml of deionized water was added, the mixture was shaken vigorously for 30 s, and the amount of 3-hydroxybutyrate *n*-propyl ester in the lower phase was determined by gas chromatography at 150-160 with a flame ionization detector and a glass column (2 m by 4 mm) packed with Gas-Chrom Q (80/100) coated with 5% (vol/vol) DC 200 and 5% (vol/vol) QF1 (Alltech, Deerfield, III.) Analysis of a known amount of PHB (Aldrich) indicated that the recovery of the 3-hydroxybutyrate *n*-propyl ester was quantitative.

Enzyme assays. Enzyme assays were carried out at 30 °C. Unless indicated otherwise, reaction mixtures had a final volume of 1 ml, and activities were calculated from initial rates that were linear. Reaction rates were proportional to the amount of enzyme used, and control mixtures from which the substrate concerned was omitted were used to correct for nonspecific reactions.

Citrate synthase (EC 4.1.3.7) was assayed by monitoring the increase in A_{365} due to the reduction of acetylpyridine-adenine dinucleotide as described by Stitt *et al.*.[14] Reaction mixtures containing 100 mM triethanolamine-HCl (pH 8.5), 3 mM disodium L-malate, 0.22 mM acetylpyridine-adenine dinucleotide, and 20 U of malate DH were incubated until equilibrium between malate and oxaloacetate was established when enzyme extract was added. After the absorbance had reached a steady-state value, the citrate synthase reaction was initiated by the addition of 0.18 mM trilithium acetyl-CoA. Citrate synthase activity was also assayed by monitoring the increase in A_{412} due to the reaction between DTNB and mercaptide ions from CoA by the method of Parvin.[15] Reaction mixtures contained 100 mM trilithium acetyl-CoA. Both methods gave comparable results, although nonspecific activity was higher with the DTNB method.

Activity of 3-ketothiolase (EC 2.3.1.9) was assayed by monitoring the decrease in A_{303} due to the disappearance

of the enolated Mg-acetoacetyl-CoA complex by the method of Karr *et al.*[16] Reaction mixtures contained 100 mM Tris-HCl (pH 7.8), 25 mM $MgCl_2$, 50 μM acetoacetyl-CoA, and 70 μM CoA (sodium salt). Reactions were initiated with CoA, and an extinction coefficient of 14.21 $mM^{-1}cm^{-1}$ was used to calculate activity.

The remaining enzymes were assayed by monitoring the change in A_{340} due to the oxidation of NAD(P)H or reduction of $NAD(P)^+$. NADH- and NADPH-dependent acetoacetyl-CoA reductase activities (EC 1.1.1.35 and EC 1.1.1.36, respectively) were assayed by monitoring the decrease in A_{340} due to the oxidation of NAD(P)H in reaction mixtures which contained 50 mM HEPES-NaOH (pH 7.6), 0.4 mM NADH or NADPH, 20 mM Mg acetate, and 50 μM acetoacetyl-CoA. Reactions were initiated with acetoacetyl-CoA. NAD- and NADP-dependent malic enzymes (EC 1.1.1.39 and EC 1.1.1.40, respectively) were assayed as described by Copeland *et al.*,[17] except that 50 mM NH_4Cl was included in reaction mixtures. Reactions were initiated with the addition of $MnCl_2$ and L-malate for NAD- and NADP-dependent malic enzyme, respectively. Activity of chickpea bacteroid NADP-dependent malic enzyme was calculated from the steady-state rate rather than the initial rate. Pyruvate DH (EC 1.2.4.1), isocitrate DH (EC 1.1.1.42), and malate DH (EC 1.1.1.37) were assayed in the reductive direction as described by Copeland *et al.*,[17] and 3-hydroxybutyrate DH (EC 1.1.1.30) was assayed by the method of Wong and Evans.[18] Malate DH was assayed in the oxidative direction in reaction mixtures that contained 50 mM HEPES-NaOH (pH 7.0), 5 mM L-malate, 10 mM L-glutamate, and 1 mM $NAD(P)^+$. The reaction was initiated by the addition of 2 U of GOT (EC 2.6.1.1). GOT was assayed in reaction mixtures that contained 50 mM HEPES-NaOH (pH 7.0), 10 mM 2-oxoglutarate, 10 mM DL-asparate, 0.2 mM NADH, and 5 U of malate DH. Protein content was determined with Coomassie blue reagent as described in the manufacturer's instruction, with bovine serum albumin as a standard.

Results and Discussion

In an earlier microscopic study of chickpea nodules, PHB granules were not observed in bacteroids in any sections examined.[19] In the present study, the information of why PHB granules were not observed in chickpea bacteroids was studied. When PHB standard was subjected to propanolysis, two peaks were detected in addition to the solvent peak (Fig. 1A). The peak with the shorter retention time corresponded to the *n*-propyl ester of 3-hydroxybutyrate, and the other peak corresponded to the internal standard (benzoic acid) which was included to monitor the yield of the transesterification reaction. Similar chromatograms were obtained from the analysis of PHB in bacteroids from chickpea and cowpea nodules (Fig. 1B, C) and in the respective free-living bacteria (Fig. 2B, C). This result suggests that only a very small amount of

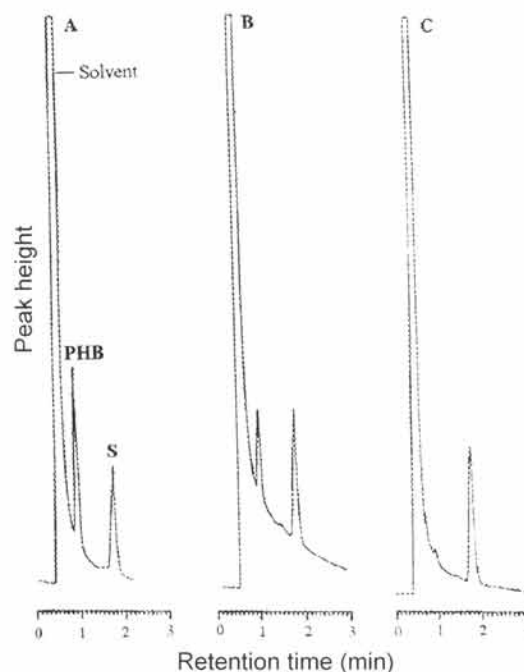

Fig. 1. Analysis of PHB in cowpea and chickpea bacteroids. The PHB standard (A) and extracts from cowpea (B) and chickpea (C) bacteroids were subjected to propanolysis and the product analyzed as described by gas chromatography using benzoic acid as an internal standard (IS). The large peak with the shortest retention time is the solvent.

PHB is present in chickpea bacteroids. However, the peak of PHB was observed in free-living cultures of the bacteria that were used to nodulate the chickpea plants (Fig. 2B). In cowpea bacteroids and free-living cultures of the nodulating bacteria, the peak of PHB was observed (Fig. 1B, 2C).

Bacteroids from nodules of 45 to 55 day old cowpea plants contained 287±41 mg of PHB g (dry weight)$^{-1}$, whereas the PHB content of bacteroids from 37 to 43 day old chickpea nodules was 10±2 mg g (dry weight)$^{-1}$. The PHB content of CC 1192 and I 16 grown in liquid culture was 285 55 mg and 157 18 mg g (dry weight)$^{-1}$, respectively (Table 1). Bacteroids isolated from Percoll gradients were intact, as indicated by activity of 3-hydroxybutyrate DH being detected only after the preparations were sonicated. The bacteroids were also essentially free of contaminating enzymes from the host cytosol; unsonicated bacteroid preparations contained less than 5% of the total nodule NADH-dependent malate DH activity, an enzymes which is highly active, mostly in the cytosol (Table 2).

Activities of enzymes in the pathway of PHB synthesis (3-ketothiolase and acetoacetyl-CoA reductase) and PHB breakdown (PHB depolymerase and 3-hydroxybutyrate DH), the tricarboxylic acid cycle (citrate synthase, isocitrate DH, and malate DH), and related metabolic reactions (NAD-malic enzyme, NADP-malic enzyme, pyruvate DH, and GOT) were measured in cell extracts from chickpea and cowpea bacteroids and from CC 1192 and I 16. When

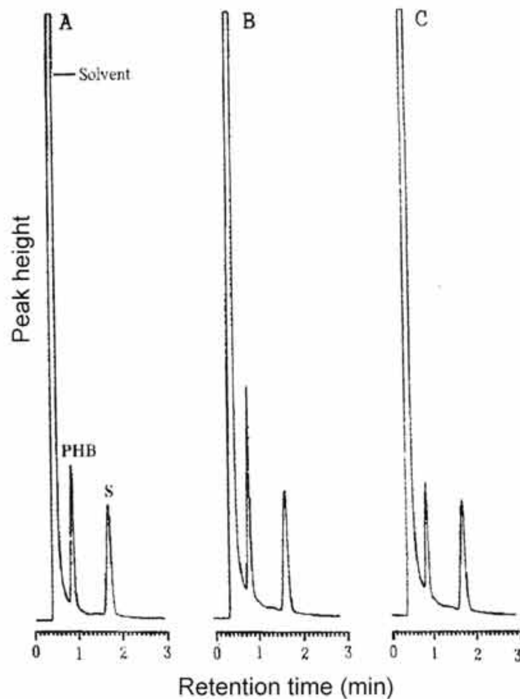

Fig. 2. Analysis of PHB in free-living CC 1192 and I 16. The PHB standard (A) and extracts from CC 1192 (B) and I 16 (C) were subjected to propanolysis and the product analyzed as described by gas chromatography using benzoic acid as an internal standard (IS). The large peak with the shortest retention time is the solvent.

Table 1. PHB content of cowpea and chickpea bacteroids, and the respective free-living bacteria. PHB content is in g mg^{-1}dry weight.[a]

Chickpea bacteroids	10±2
Cowpea bacteroids	287±41
Rhizobium sp. (*Cicer*) CC 1192	285±55
Bradyrhizobium japonicum I 16	157±18

[a]The data and SE values were determined from the number of replicate preparations.

Table 2. Enzyme activities in unsonicated bacteroids and cytosol fractions from cowpea and chickpea nodules.[a]

Enzyme	Cowpea	Chickpea
	nmol product $min^{-1}g^{-1}$ fresh wt nodule	
3-Hydroxybutyrate DH		
Unsonicated bacteroids	ND	ND
Sonicated bacteroids	1550±76	76±8
NADH-dependent malate DH		
Unisonicated bacteroids	1030±118	3678±320
Cytosol	14320±789	34970±2360

[a]The data are the means and SE values from replicate preparations.

the specific activities of these enzymes in extracts from cowpea bacteroids and I 16 were compared, statistically significant differences were noted for 3-ketothiolase, NADP-malic enzyme, GOT, pyruvate DH and NADH-dependent malate DH (Table 3). The bacteroid/free-living bacteria ratios of activities of corresponding enzymes in the two extracts ranged between 0.73 and 2.91. Much greater variability was noted in the comparison between bacteroid and free-living cells of CC 1192. NADH-dependent aceto-acetyl-CoA reductase, 3-hydroxybutyrate DH, citrate synthase, and isocitrate DH activities were significantly lower in extracts of chickpea bacteroids than in free-living CC 1192, whereas the bacteroids had higher activities of NADP-malic enzyme, NADH-dependent malate DH, and pyruvate DH (Table 3).

Specific activities of NADH-dependent acetoacetyl-CoA reductase, 3-ketothiolase, pyruvate DH, and to a lesser extent 3-hydroxybutyrate DH, and citrate synthase, were much higher in cowpea bacteroids than in chickpea bacteroids, whereas NADPH-dependent acetoacetyl-CoA reductase, NADH-dependent malate DH and NADP-dependent iso-citrate DH were more active in extracts of chickpea nodules (Table 3). The capacities to form pyruvate in the malic enzyme reactions were higher in the bacteroids than free-living forms of CC 1192, but I 16 had a greater capacity than CC 1192 to generate acetyl-CoA from pyruvate. However, even though the specific activity of pyruvate DH was 2-3 times higher in cowpea nodules than chickpea nodules, it is questioned to describe for the production of acetyl-CoA because the bacteroid/free-living bacteria ratio of specific activity of pyruvate DH was 4.5 times higher in chickpea nodules than cowpea nodules.

To identify steps important in directing carbon towards PHB synthesis, enzyme activities have been measured in extracts from chickpea and cowpea bacteroids and the respective free-living bacteria. Since such comparisons may reflect enzymatic capacity rather than *in vivo* activities, only differences that are sufficiently large (2-4 fold) may be considered to have physiological significance. These results have indicated that extracts from bacteroid and free-living cells of I 16 did not differ greatly in their capacity for the enzymes measured, whereas some differences that may have physiological significance were noted between the two forms of CC 1192.

PHB biosynthesis is initiated with the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA. The enzyme concerned, 3-ketothiolase, was present in chickpea bacteroids, but its specific activity was much lower than that in cowpea bacteroids. However, it seems unlikely that the lower activity of 3-ketothiolase in chickpea bacteroids would limit PHB synthesis, since CC 1192 had a similarly reduced capacity for this step, compared with I 16. The next reaction in the pathway of PHB synthesis is the reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA, which is the substrate of PHB synthase. NADH-dependent acetoacetyl-CoA reductase activity was significantly lower in chickpea bacteroids than CC 1192, which in turn had less activity than cowpea bacteroids and I 16.

Malate taken up by bacteroids may be decarboxylated to pyruvate by malic enzyme or oxidized by malate DH and in this way can provide both acetyl-CoA and oxalo-

Table 3. Enzyme activities in soluble extracts from cowpea bacteroids, *Bradyrhizobium japonicum* I 16, chickpea bacteroids and *Rhizobium* sp. (*Cicer*) CC 1192.

Enzyme	nmol product $min^{-1}mg^{-1}protein^{a}$					
	Cowpea bacteroids	I 16	Ratio[b]	Chickpea bacteroids	CC 1192	Ratio[c]
Acetoacetyl-CoA reductase						
NADH-dependent	773±96	821±102	0.94	42±4	132±11	0.32
NADPH-dependent	88±29	71±32	1.24	126±29	114±22	1.11
Citrate synthase	156±37	203±43	0.77	63±8	119±14	0.53
GOT	143±18	114±15	1.25	149±15	139±31	1.07
3-Hydroxybutyrate DH	81±5	80±10	1.01	51±5	143±10	0.36
Isocitrate DH	101±26	121±29	0.85	152±11	302±26	0.50
3-Ketothiolase	2634±302	905±233	2.91	584±52	575±104	1.02
Malate DH						
NADH-dependent	2287±192	1246±329	1.84	5569±387	1222±202	4.56
NAD-dependent	38±11	52±9	0.73	210±25	96±23	2.19
NADPH-dependent	1322±290	1220±329	1.10	1172±323	202±36	5.80
Malic enzyme						
NAD-dependent	53±9	104±14	0.51	49±7	46±7	1.06
NADP-dependent	47±9	32±11	1.47	42±4	9±5	4.67
Pyruvate DH	59±10	54±14	1.09	19±3	4±2	4.75

[a]Enzyme activity were from the means SE of at least 7 replicate extractions. The extraction from cowpea bacteroids and *Bradyrhizobium japonicum* I 16 contained 1.3 0.2 mg and 1.5 0.2 mg of protein, respectively, and the extraction from chickpea bacteroids and *Rhizobium* sp. (*Cicer*) CC 1192 contained 2.3 0.3 mg and 1.6 0.3 mg of protein, respectively.
[b]Ratio-cowpea bacteroid/I 16 activity.
[c]Ratio-chickpea bacteroid/CC 1192 activity.

acetate for the TCA cycle. Extracts from chickpea bacteroids contained both NAD- and NADP-dependent malic enzyme activities and in this regard were similar to cowpea bacteroids, which have two malic enzymes, one specific for $NADP^+$ and with a high affinity for malate and the other an NAD-dependent enzyme with a lower affinity for malate.[17] As has been demonstrated in other studies,[17,19] activity of malate DH was much lower in the direction of the reaction when the TCA cycle is operating. NAD-dependent malate DH activity appeared to be closer to the capacity for malate decarboxylation by the malic enzyme and to the activity of pyruvate DH and TCA cycle enzymes in Copeland *et al.*[17] and this study. The potential for oxidation of malate to oxaloacetate rather than decarboxylation to pyruvate was greater in chickpea bacteroids than in free-living CC 1192 and in free-living and bacteroid cells of I 16. This can be seen by comparing the activity of malate DH in the oxidative direction with the combined activities of NAD- and NADP-dependent malic enzyme. The activities of NADH-dependent malate DH, 3-ketothiolase and 3-hydroxybutyrate DH determined in chickpea bacteroids in this study were comparable to the maximum activities reported for these enzyme by Hong[20]; our values for citrate synthase were lower than those in the earlier study.

PHB is considered to accumulate throughout the active nitrogen-fixing period in legume nodules.[9,16,18] Earlier studies have been indicated that when cowpea bacteroids take up excess malate, an increase in malic enzyme activity leads to increase in the acetyl-CoA/CoA and $NAD(P)H/NAD(P)^+$ ratios, which in turn stimulate 3-ketothiolase and acetoacetyl-CoA reductase, decrease activity of the TCA cycle DH, and divert carbon to the PHB synthesis.[21] In chickpea bacteroids, the higher capacity for malate oxidation may produce a different balance between malate decarboxylation and oxidation, resulting in a higher steady-state concentration of oxaloacetate that favors the utilization of acetyl-CoA in the TCA cycle rather than for PHB synthesis. The present study indicates important possibility for understanding why PHB accumulates in cowpea nodules but little in chickpea nodules. Our findings will be the basis for more detailed studies. To clarify the convert of oxaloacetate to amino acid or TCA cycle and the utilization of acetyl-CoA to PHB or TCA cycle in chickpea nodules, further studies will be required.

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