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Transport of C_4-Dicarboxylates and Amino Acids by *Rhizobium meliloti* Bacteroids

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The rates of transport of C_4-dicarboxylic acids, aspartate, glutamate, alanine, and other compounds into mature bacteroids isolated from alfalfa nodules were determined. Of the compounds tested, only malate, succinate, pyruvate, and aspartate were accumulated at significant rates. Apparent K_m values for these compounds indicated that the concentrations of the dicarboxylates which half-saturated the transport carrier (60–70 μM) were at least 200-fold less than that required for aspartate. Malate, succinate, and aspartate were found to be present in the cytoplasm of nodule cells in the range of 150–810 nmol per gram fresh weight (0.1–0.6 mM). At these concentrations malate and succinate completely inhibited aspartate uptake by mature bacteroids. It is unlikely that any active uptake of aspartate occurs *in vivo* in view of the relative K_m values and physiologic concentrations of these metabolites. Major differences in the uptake of amino acids were noted in direct comparisons of bacteroids isolated from soybean and alfalfa nodules. Only dicarboxylates supported nitrogenase activity of the *R. meliloti* bacteroids, while succinate, aspartate, and glutamate all supported activity with *B. japonicum* bacteroids. The significance of these experimental findings is discussed in terms of energy supply, the fate of carbon sources, and nutrient exchange between host and microsymbiont.

The supply of photosynthate-derived carbon and energy-bearing substrates to bacteroids residing in root nodules of Leguminosae species has been the subject of extensive investigation from the standpoint of the cost of symbiotic nitrogen fixation to the host plant. The importance of defining the support requirements for and practical limitations on symbiotic nitrogen fixation has led to the application of both genetic and physiologic approaches to the study of the carbon and nitrogen metabolism of the microsymbiont in both the free-living, nonfixing state and in the bacteroid, fixing state.

All symbiotic species of *Rhizobium*, *Azorhizobium*, and *Bradyrhizobium* undergo a postinfection process of differentiation that is only partially defined before the active nitrogenase enzyme system is expressed in mature bacteroids. Following release from the infection thread, the transforming nodule bacteria remain enclosed in a membrane derived from the host plasmalemma (Paau *et al.* 1978). Changes to the bacteroid cell wall and cytoplasmic membrane (Miller and Tremblay 1983) accompany the expression of the nitrogenase proteins and associated support systems. Succinate and metabolites derived from succinate may play a key role in the initiation of differentiation of transforming cells in nodules in alfalfa and red clover symbioses (Urban and Dazzo 1982; Gardiol *et al.* 1987). It is necessary, therefore, to conduct studies of nutrient transport and respiratory activities that specifically support the nitrogenase system directly in mature, isolated bacteroids.

Comparisons of studies of the *B. japonicum* (Buchanan) Jordan-*Glycine max* (L.) Merr. and the *R. meliloti* Dangeard-*Medicago sativa* L. symbioses suggest that differences exist in both the process of differentiation and the functional characteristics of bacteroids formed from these slow- and fast-growing microsymbionts, respectively. Bacteroids of soybean nodules are held within a host-derived peribacteroid membrane (PBM), several cells within each sac (Verma *et al.* 1978); the enlarged bacteroids of alfalfa nodules appear to be separately enclosed in the PBM and can be isolated in this form (Miller *et al.* 1988). *Bradyrhizobium* bacteroids retain the ability to divide and grow (Sutton and Paterson 1983); isolated, mature bacteroids derived from alfalfa nodule bacteria are not viable in this manner (McRae *et al.* 1989). Although such comparisons are useful, the characteristics of one host-microsymbiont pair cannot be extrapolated or attributed to other systems, and each symbiosis must be characterized according to carbon and nitrogen fluxes across the peribacteroid and bacteroid cytoplasmic membranes. Only with such information can a rational process of improvement of host and microsymbiont compatibility be developed to include selection and genetic modification of both partners of the symbiotic system.

Genetic analysis of *R. meliloti* transposon mutants has shown that C_4-dicarboxylates are transported in bacteroids and free-living cells by a common carrier protein and that mutations in the carrier gene (DctA) are Fix^- in symbiotic association with the alfalfa host (Engelke *et al.* 1987, 1988). This transport system, the genes for which are located on the *exo* megaplasmid of *R. meliloti*, is also responsible for aspartate import in free-living bacterial cells (Watson *et al.* 1988). Bacteroids isolated from a selected field strain of *R. meliloti* (strain Balsac) had high levels of respiration-supported nitrogenase activity, but utilized only C_4-dicarboxylates as an energy source because neither sugars nor amino acids elicited significant acetylene-reducing activity over a wide range of oxygen concentrations (Miller *et al.* 1988). Although the available evidence suggests that C_4-dicarboxylates are the major source of photosynthate-derived carbon for nitrogen-fixing bacteroids *in vivo*, a

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recent report provided genetic evidence that a mutant deficient in glutamate decarboxylase had reduced nitrogenase activity in symbiotic association. This mutant had reduced ability to support the growth of host plants through export of fixed nitrogen (Fitzmaurice and O'Gara 1988). Hence, glutamate and other amino acids may also play an important but as yet undefined role in the alfalfa-*R. meliloti* symbiosis.

The supply of fixed nitrogen to the host is thought to occur primarily through the export of ammonia (O'Gara and Shanmugam 1976). However, the assimilation of ammonia into amino acids within functioning bacteroids before export remains a possibility. This would not necessarily require net mass transfer of amino acid carbon across the bacteroid cytoplasmic membrane, provided that small pools of keto acids were shuttled back across this membrane from the peribacteroid space. Kahn *et al.* (1985) proposed that such a cyclical exchange of nutrients between bacteroids and host might be required via such a shuttle mechanism.

In this study, *R. meliloti* bacteroids that were isolated and purified to remove other forms of nodule bacteria have been used to define the properties of the dicarboxylate transport system as it is expressed following differentiation of nodule bacteria. In all experiments reported here, the criterion for full bacteroid functionality has been the support of nitrogenase activity through respiration of carbon substrates supplied to the isolated system as previously described (Miller *et al.* 1988).

MATERIALS AND METHODS

Growth of alfalfa plants and bacteria, inoculation with *R. meliloti*, strain Balsac, harvesting of nodules, and isolation of mature bacteroids having nitrogenase activity coupled to respiration were conducted as previously described (McRae *et al.* 1989; Miller *et al.* 1988). The anaerobic isolation medium contained 50 mM potassium, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonate (TES) buffer, pH 7.0, 180 mM KCl, and 0.10% (w/v) bovine serum albumin, but no succinate. The mature bacteroid fraction was obtained from a two-step Percoll (Sigma, St. Louis, MO) gradient centrifugation procedure, and enzyme assays were performed as previously reported (Miller *et al.* 1988; McRae *et al.* 1989) except that the acetylene-reducing activity of bacteroid nitrogenase was determined under nitrogen instead of argon.

Soybean plants (*G. max* cv. Harasoy C_3) were grown in 25-cm plastic pots in a vermiculite medium and watered with nitrogen-free Hoagland's solution. The germinated seedlings were inoculated with a commercial soybean inoculant (Nitragin, Madison, WI) and grown in a greenhouse under supplemental illumination (18 hr photoperiod) with sodium lamps at 300 $\mu E \cdot cm^{-2} \cdot sec^{-1}$. Soybean nodules were harvested at 6 wk and processed in the same manner as alfalfa nodules (Miller *et al.* 1988). The bacteroids were isolated in an anaerobic chamber and tested for nitrogenase activity and transport capabilities. Free-living *R. meliloti* cells were grown to late exponential growth phase (log phase, 36 hr) at 30° C on yeast extract-mannitol medium as previously described (Miller *et al.* 1988).

Reaction mixtures containing the specified concentration of ^{14}C-labeled substrate, specific activity 0.15 $\mu Ci/\mu mol$, dissolved in TES buffer containing 180 mM KCl, pH 7.0, were sealed in 10-ml Wheaton serum vials. The vials were flushed with nitrogen, and oxygen was added with a syringe to compose 4% of the headspace at atmospheric pressure. The reaction mixtures were equilibrated in a reciprocal shaking water bath at 20° C. Uptake experiments were initiated by adding mature bacteroids containing 1–2 mg of protein suspended in the same buffer to give a final reaction volume of 1.0 ml. The vials were shaken at 120 strokes per minute, and 0.1-ml samples were withdrawn with nitrogen-flushed plastic 1.0-ml syringes at 15-min intervals up to 1 hr. The samples were deposited immediately on cellulose acetate filters (3 μm; Millipore, Bedford, MA, Catalog No. SMWP 02500), and the bacteroids were washed twice with 5-ml aliquots of buffer.

For uptake times of up to 1 min, the entire reaction mixture was transferred immediately, after equilibration and mixing, to a modified repeating dispenser. An Eppendorf pipette equipped with an Eppendorf Combitip was adapted to fit a 3-cm, 20-gauge disposable syringe needle, and this assembly was brought to 20° C in a covered beaker in the water bath. After combining the reactants, each mixture was taken into the modified Combitip pipette along with 0.1 ml of the gas phase of the vial. Samples (0.1 ml) were then transferred to the filters at 10-sec intervals up to 1 min. The samples were washed as before to remove externally labeled substrate. The filter discs were dried at 65° C and placed in plastic counting vials with 10 ml of Econofluor (NEN Research Products, Boston, MA) scintillation fluid. Radioactivity was determined with a Beckman LS 8000 liquid scintillation counter.

Fractionation of the plant cell cytosolic supernatant fluid, resulting from the bacteroid isolation procedure, into amino acids, organic acids, and neutral compounds was performed by sequential ion exchange chromatography on Dowex-50 (hydrogen form) and Dowex-1 (acetate form) resins. Analysis of the amino acid fraction obtained by elution of the Dowex-50 columns was conducted by high performance liquid chromatography on a Waters μBondapak C-18 column following precolumn derivatization with *o*-phthalaldehyde. The identification of amino acids was confirmed by gas chromatography (GC)-Mass spectral analysis. Dicarboxylic acids were eluted from the Dowex-1 column and determined by high pressure liquid chromatography with an ion exclusion column (Aminex HPX-87H, Bio-Rad, Richmond, CA) and ultraviolet absorbance detector (Guerrant *et al.* 1982). The identity of the major organic acid components was confirmed by comparison with authentic standards and ultraviolet absorbance properties in this standard analytical procedure.

RESULTS

Isolated, mature bacteroids had respiration-supported nitrogenase activity, morphology, and osmotic properties equivalent to those previously reported (Miller *et al.* 1988). The majority of the bacteroids appeared to be enclosed in the PBM as determined from transmission electron micrographs. Two percent or less of the vegetative bacteria were present as determined by phase contrast microscopy and by viability tests as previously reported (McRae *et al.* 1989). Metabolites that were found to be actively accumulated by the isolated bacteroids fell into two general

categories. These categories were based on apparent K_m values for uptake as determined from plots of reciprocal substrate concentration vs reciprocal uptake velocity. Rates of uptake are expressed as nanomoles of substrate accumulated per minute per milligram of bacteroid protein. Those substrates taken up with apparent K_m values between 60 and 100 μM were designated as having a high carrier protein affinity, while those taken up with K_m values in the millimolar range were considered to have significantly lower affinities. Table 1 gives apparent K_m values for three ^{14}C-labeled substrates and illustrates the range of K_m and maximum uptake velocity values observed.

The time period of active uptake of C_4-dicarboxylic acids, pyruvic acid, and amino acids under a nitrogen-4% oxygen atmosphere is shown in Figure 1. No significant difference in the results was observed when uptake rates were determined under an argon-4% oxygen atmosphere. Succinate and malate gave results typical of high-affinity substrates when taken up from a medium containing 0.5 mM dicarboxylate. Equivalent rates of uptake of low-affinity substrates such as pyruvate and aspartate required concentrations of 5 mM in the reaction medium. L-glutamate, L-alanine, γ-aminobutyric acid, L-glycine, L-leucine, L-serine, L-asparagine, and L-glutamine were not taken up at a significant rate from reaction mixtures containing 5 mM substrates.

Succinate uptake was shown to be energy dependent, because it was severely inhibited by dinitrophenol or azide (1.0 mM) or by omitting oxygen from the reaction medium headspace as shown in Figure 2. KCN (1 mM) had no significant effect on the rate of succinate uptake by bacteroids, however, implying that the electron transport chain responsible for energization of the bacteroid membrane in support of succinate uptake was not dependent on a cyanide-sensitive terminal oxidase. Heat treatment of the bacteroids for 5 min at 60° C eliminated

Table 1. Active transport of succinate, L-malate, and L-aspartate by isolated bacteroids

Metabolite	Cytoplasmic concentration	K_m	V_{max}	Regression equation	Correlation coefficient
	nmol[a] / g fresh wt	μM[b]	nmol[b] / min·mg		R
Succinate	137	68.6	22.1[c]	$y = 2.21X + 0.0322$	0.99
Malate	810	61.7	18.7[c]	$y = 3.31X + 0.0535$	0.98
Fumarate	93	N.D.[d]	N.D.	N.D.	N.D.
Aspartate	200	10,400	75.8[e]	$y = 0.138X + .0132$	0.99

[a]Mean value of three determinations, standard deviation = 40 nmol per gram fresh weight.
[b]Average standard error in values as determined from double reciprocal plots was 10%.
[c]Linear rate of uptake measured over a 1-min period.
[d]The transport of ^{14}C fumarate was not determined (N.D.).
[e]Rate of uptake measured over a 1-hr period.

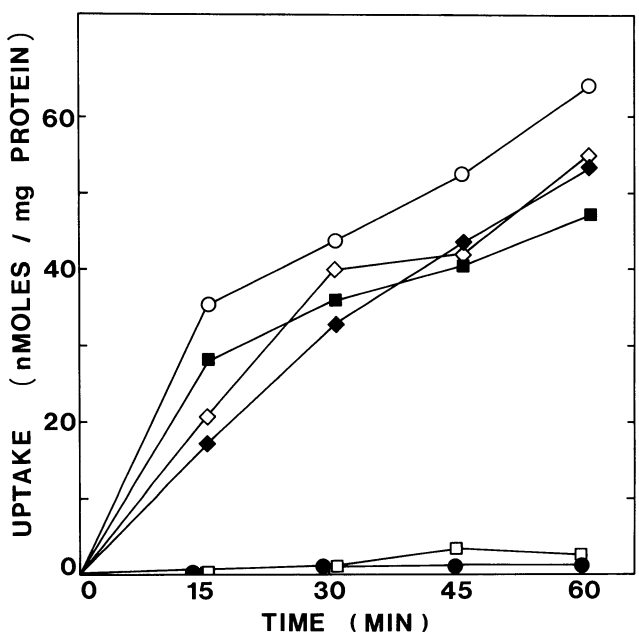

Fig. 1. Transport of metabolites by mature bacteroids. Bacteroids were isolated and separated from undifferentiated nodule bacteria, and the uptake of ^{14}C-labeled metabolites was determined as a function of time at 20° C under 4% O_2 in a buffered isotonic medium as described in the text. Indicated data and curves are for: succinate, 0.5 mM (○—○); pyruvate, 5.0 mM (◇—◇); L-aspartate, 5.0 mM (◆—◆); L-malate, 0.5 mM (■—■); L-alanine, 5.0 mM (□—□); and L-glutamate, γ-aminobutyrate, L-serine, and L-leucine, all 5.0 mM (●—●).

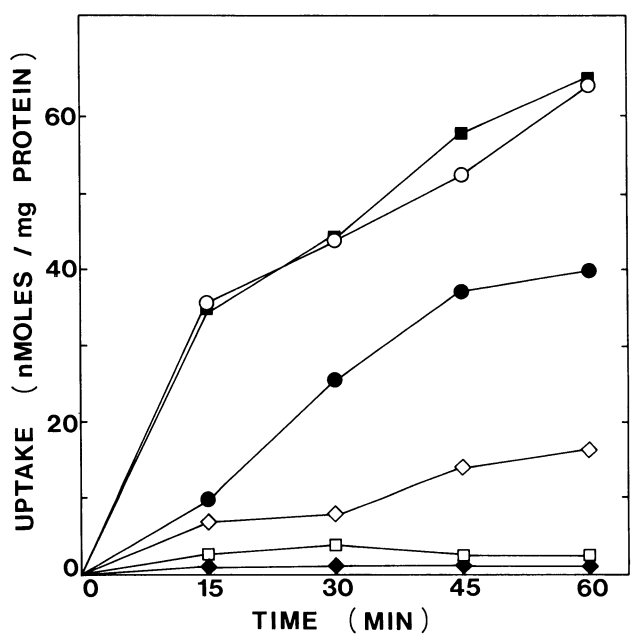

Fig. 2. Effect of inhibitors, heat, and low oxygen tension on the uptake of succinate by mature bacteroids. Experimental conditions are the same as described for Figure 1. The indicated inhibitor (1.0 mM) was added to the bacteroid suspension 1 min before the addition of 0.5 mM succinate. Inhibitors tested were: none, control (○—○); KCN (■—■); sodium azide (●—●); and 2,4-dinitrophenol (□—□). Oxygen was omitted (◇—◇) and the reaction mixture was heated to 60° C (◆—◆) in two vessels to which no inhibitor had been added.

all uptake activities. Figure 3 shows the inhibitory effect of the lack of oxygen on L-aspartate uptake. Succinate, L-malate, and fumarate (0.5 mM) inhibited uptake of L-aspartate completely despite a 10-fold excess of aspartate in relation to the dicarboxylates. Conversely, 5 mM aspartate added to reaction mixtures in 10-fold excess in relation to succinate had no discernible effect on the energy-dependent uptake of succinate (data not shown).

Free-living *R. meliloti* cells were washed with the isotonic bacteroid reaction mixture buffer (TES-KCl), and the rates of uptake of alanine, aspartate, and glutamate were determined. Figure 4 shows that all three amino acids were accumulated by the free-living cells in contrast to results obtained with the isolated, mature bacteroids. When the Percoll band containing both the transforming and undifferentiated nodule bacteria (McRae *et al.* 1989) was used to determine transport activities, uptake curves for alanine, aspartate, and glutamate similar to those found for the free-living bacteria were obtained (data not shown). The results of these experiments amply demonstrate the necessity for conducting transport determinations with bacteroid preparations that are essentially free of free-living or undifferentiated nodule bacterial cells.

For comparison with results obtained with *R. meliloti* bacteroids, *B. japonicum* bacteroids were isolated from soybean nodules and tested for succinate, glutamate, and aspartate uptake. Figure 5 illustrates the uptake activities of these bacteroids after isolation using our procedure. In contrast to alfalfa bacteroids, both L-aspartate and L-glutamate were actively taken up although not as rapidly as succinate when all subtrates were tested at 5 mM. The pronounced differences between *R. meliloti* bacteroids and the soybean bacteroids in terms of specificity for Krebs cycle dicarboxylates in supporting nitrogenase activity linked to respiration can be seen from the data in Figure 6. Nitrogenase activity was supported in the alfalfa

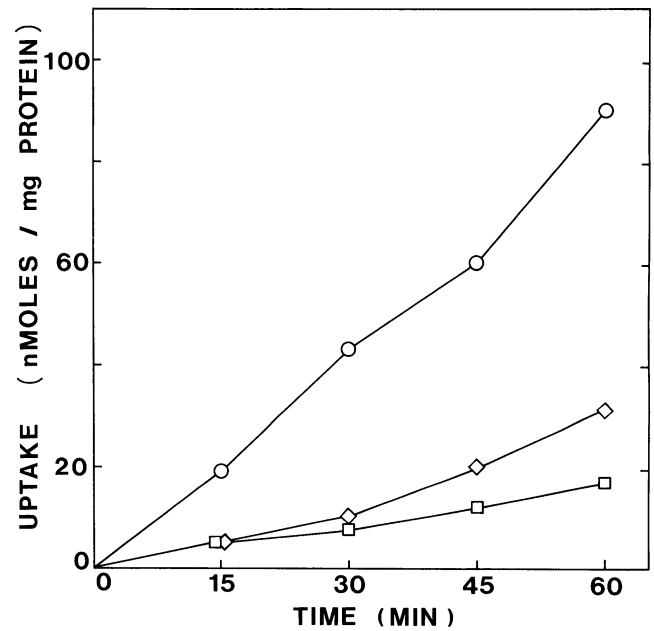

Fig. 4. Uptake of L-alanine, L-glutamate, and L-aspartate by free-living *Rhizobium meliloti* cells. The uptake of amino acids (5 mM) was determined at the indicated times at 20° C with washed, free-living cells under 4% O_2 as described in the text. The top curve was obtained with alanine (○—○), the intermediate curve with glutamate (◇ — ◇), and the lower curve with aspartate (□—□). Substrate concentration was 5 mM in all cases.

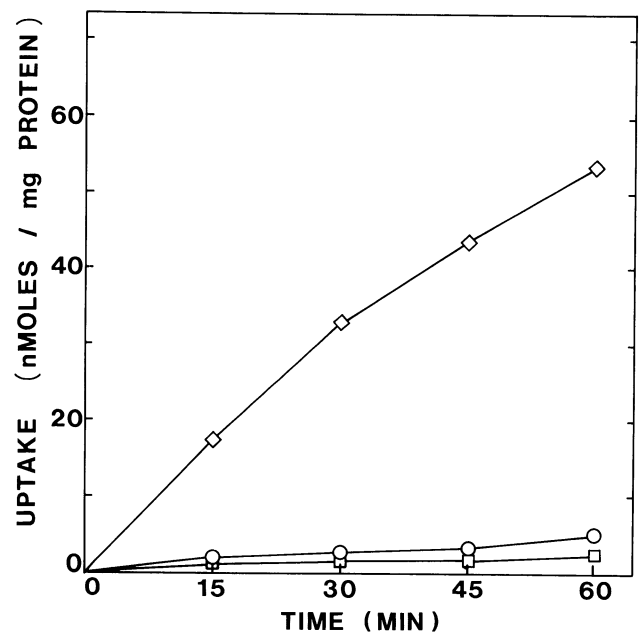

Fig. 3. Effect of oxygen and succinate, L-malate, and fumarate on the uptake of aspartate by mature bacteroids. The uptake of L-aspartate from a reaction mixture that contained 5 mM substrate was determined as a function of time as described for Figure 1. The upper curve (◇ — ◇) represents the control rate of aspartate uptake. Aspartate uptake was inhibited completely by 0.5 mM succinate, malate, or fumarate within experimental error as shown by the lowest curve (□—□). Omission of oxygen from the reaction mixture headspace resulted in an intermediate curve (○—○).

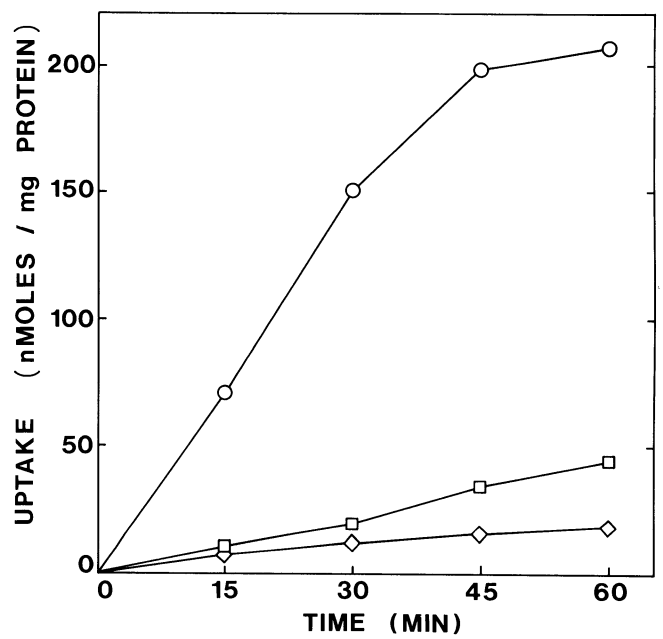

Fig. 5. Uptake of ^{14}C-labeled succinate, L-aspartate, and L-glutamate by *Rhizobium japonicum* bacteroids. Amino acid and dicarboxylate uptake was determined under conditions similar to those used for *R. meliloti* bacteroids following isolation and resuspension of bacteroids by the same procedure as that described in Figure 1. All substrates (5.0 mM) were incubated with the bacteroids at 20° C. Upper curve, succinate (○—○); intermediate curve, glutamate (□—□); and lower curve, aspartate (◇ — ◇).

bacteroids only by Krebs cycle dicarboxylates, whereas with soybean bacteroids, succinate, glutamate, and aspartate supported nitrogenase activity above control levels, which were obtained with no added substrate.

DISCUSSION

In summarizing previous reports, it is desirable to clearly delineate the known characteristics of isolated *B. japonicum* bacteroids from the properties of free-living *B. japonicum* cells. These well-characterized bacteroids actively import Krebs cycle C_4-dicarboxylates (malate, succinate, and α-ketoglutarate) as well as L-glutamate and L-aspartate (Reibach and Streeter 1984; Salminen and Streeter 1987). Accumulation of succinate by free-living cells of *B. japonicum* apparently involves two distinct transport systems having high (K_m = 2.4 μM) and low (K_m = 172 μM) carrier affinities, both of which are inhibited by KCN (Humbeck and Werner 1987). Evidence for two succinate uptake systems having saturable (K_m = 40 μM) and unsaturable, low-affinity components in *B. japonicum* bacteroids has been reported (Reibach and Streeter 1984). The K_m values for succinate and malate of the higher affinity system of *B. japonicum* bacteroids are comparable to the values obtained with *R. meliloti* bacteroids.

All transport processes of the isolated alfalfa bacteroids were shown to be dependent on an energized bacteroid membrane, as shown by inhibition with dinitrophenol and azide ions, in accordance with similar results reported for *R. leguminosarum* Frank (Glenn *et al.* 1980) and *B. japonicum* (Reibach and Streeter 1984) bacteroids. Succinate transport by free-living *R. leguminosarum* was inhibited 98% by 1.0 mM KCN. The lack of sensitivity of dicarboxylate transport activities to inhibition by KCN demonstrated by *R. meliloti* bacteroids further indicates the replacement of cytochrome a as a terminal oxidase in the transformation of vegetative cells to bacteroids (Miller and Tremblay 1983).

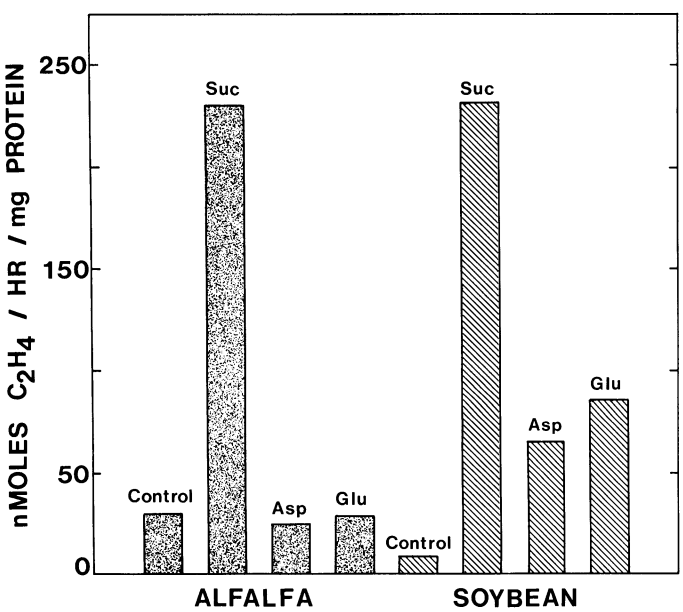

Fig. 6. Respiration-supported nitrogenase activity of bacteroids isolated from alfalfa and soybean nodules. Bacteroids were isolated in the absence of any added dicarboxylate, and nitrogenase activity supported by the indicated added metabolites was determined as previously reported (Miller *et al.* 1988). All substrate concentrations were 5 mM, and reaction mixtures were incubated at 20° C under 4% O_2. Controls contained no added respiratory substrate, and activity levels observed represent nitrogenase activity supported by endogenous substrates.

Glutamate, aspartate, and C_4-dicarboxylates stimulated respiration and supported nitrogenase activity of *B. japonicum* bacteroids at controlled substrate and oxygen concentrations (Salminen and Streeter 1987; Kouchi and Fukai 1988; Bergersen and Turner 1988). In soybean nodules, the amino acids L-glutamate and L-aspartate as well as C_4-dicarboxylates of the Krebs cycle therefore might serve as energy sources for nitrogen fixation in *B. japonicum* bacteroids. The results reported here and previously (Miller *et al.* 1988) indicate that the dicarboxylates are major energy sources for the support of nitrogenase activity in the alfalfa-*R. meliloti* symbiosis, while amino acids, sugars, and pyruvate are not. Moreover, analytical results obtained with the soluble supernatant fluid resulting from the breakage of nodule cells established that malate and succinate are the predominant dicarboxylates in the host cell cytoplasm (Table 1). Because fumarate concentrations were lower (100 nmol or less per gram fresh weight), malate and succinate may be of greater importance. As shown in Table 1, free L-aspartate was found to be present in nodule cell cytoplasm in amounts similar to succinate but less than malate. Thus, the uptake of aspartate is likely to be precluded *in vivo* through inhibition by malate and succinate.

The majority of bacteroids isolated from alfalfa nodules using the method previously described (Miller *et al.* 1988) appeared to retain the PBM in transmission electron micrographs, and therefore, the accumulation of carbon compounds by these cells may have required passage through this membrane as well as transport across the bacteroid cytoplasmic membrane. It was recently reported that while soybean bacteroids lacking the PBM actively accumulated glutamate and aspartate, the PBM was impermeable to glutamate, and hence only the dicarboxylates are likely to be major carbon sources for bacteroids *in situ* (Udvardi *et al.* 1988a, 1988b). This finding implies that some of the previously mentioned preparations of *B. japonicum* bacteroids may have contained a significant number of cells lacking the PBM.

In this study, isolated *R. meliloti* bacteroids took up aspartate only at relatively high concentrations (greater than 2 mM) and were unable to use this compound to support nitrogenase activity in direct contrast to *B. japonicum* bacteroid preparations. The latter, however, were not shown to retain the PBM. Nevertheless, identical isolation and assay procedures have been applied to both bacteroid systems precluding differential loss of coupling between nitrogenase and bacteroid respiration during isolation. There appear to be clear and important differences in metabolism between the soybean and alfalfa nitrogen-fixing symbioses.

The fate of the carbon skeleton of dicarboxylates subsequent to the primary oxidative steps catalyzed by the specialized bacteroid respiratory chain remains a question of which metabolic pathways predominate in mature bacteroids as opposed to those that are constitutive in free-living *R. meliloti* cells. Understanding the necessity for an operational citric acid cycle and specifically succinate and succinate dehydrogenase for establishing an effective

symbiosis in alfalfa requires a more comprehensive knowledge of the pathways involved in dicarboxylate metabolism in *R. meliloti* bacteroids. In *B. japonicum* bacteroids, radiolabeled dicarboxylates were rapidly converted to amino acids, with the labeled carbon atoms accumulating most rapidly in the bacteroidal pools of glutamate and to a minor extent in aspartate and alanine (Salminen and Streeter 1987). Whether these same metabolic pathways apply to *R. meliloti* bacteroids remains to be determined.

The possibility of export of amino acids or other nitrogenous metabolites following assimilation of ammonia has been considered only in the case of soybean bacteroids (Streeter and Salminen 1988). A previous report (McRae *et al.* 1989) showed a 10-fold enhancement in alanine dehydrogenase in isolated, mature *R. meliloti* bacteroids as compared to free-living cells. Current work is designed to determine the importance of this enzyme and other bacteroid assimilatory enzymes in incorporating ammonia into compounds that could be exported to the host cell cytoplasm along with ammonia. The necessary fluxes of carbon and nitrogen compounds that occur during alfalfa-*R. meliloti* symbiotic nitrogen fixation must be fully characterized to accurately calculate the energy cost of biological nitrogen fixation in this system. Advantageous modification of such systems can be approached only after the importance of these pathways has been established.

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