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Review:

Recent Developments in Carbon Transport and Metabolism in Symbiotic Systems

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Abstract

Significant recent developments relating to the integration of bacterial and plant metabolism in nitrogen fixing systems are reviewed. For legume nodules, topics include (1) progress in understanding the regulation of the *dct* genes and the localization and operation of the gene products, (2) the failure of engineered strains with amplified DctA to improve (in general) N input or plant growth, (3) demonstration of an anion channel protein and an H^+-pumping ATPase in the symbiosome membrane – thus providing a possible mechanism for the transport of dicarboxylic acids through this major barrier, and (4) additional evidence (malic enzyme mutants, very short term labeling studies, etc) supporting the concept that C4 dicarboxylic acids are the principal source of reducing equivalents for bacteroids. In non-legume systems, recent developments in the *Azospirillum*/wheat and the *Acetobacter*/sugarcane associations are reviewed.

Keywords: Carbon metabolism, dicarboxylate transport, *Rhizobium, Azospirillum, Acetobacter diazotrophicus, dct* genes

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1. Introduction

In this review an attempt is made to discuss much of the literature on carbon transport and metabolism that has been published in the past four or five years. For legume nodules, evidence continues to build for the importance of C_4 dicarboxylic acids in legume/rhizobia systems, and this will become evident in the sections below. Since most of the details of asparagine and ureide transport and metabolism have been elucidated, there is much less recent activity in this subject area and the relatively few recent papers on nitrogen metabolism are not considered here. Some aspects of this subject area have recently been reviewed (Vance and Gantt, 1992; Vance et al., 1994).

2. Recent studies – carbon metabolism in legume nodules

Carbohydrate metabolism

Incoming sucrose is the primary source of reductant in legume nodules, and starch may provide a significant temporary storage form of carbohydrate in nodules (Streeter, 1991). Comparison of plant genotypes having very high or very low nodule mass show an inverse relationship between starch accumulation and nodule mass (Müller et al., 1995). An analysis of starch synthase, α-amylase, and starch phosphorylase activities over time in Fix^+ and Fix^- nodules of soybean (*Glycine max*) showed that N_2 fixation was correlated with the ability of nodules to rapidly break down starch (Forrest et al., 1991). A recent detailed study of three α-glucosidase activities in soybean nodules indicates only weak hydrolysis of starch; these enzymes are probably involved in hydrolysis of glucosyl oligosaccharides of low molecular weight (Kinnback and Werner, 1992).

Based on a comparison of enzyme levels in infected versus cortical tissues of soybean nodules, it appears that most of the sucrose breakdown occurs in the infected zone (Gordon, 1991). That sucrose hydrolysis is restricted to the host cytoplasm (see Streeter, 1991) has recently been confirmed by the analysis of enzymes of carbohydrate metabolism in Chickpea (*Cicer arietinum*) nodules (Copeland et al., 1995). Glycolytic activity within the infected zone is probably high in both infected and uninfected cells, based on a study of the localization of glyceraldehyde-3-phosphate dehydrogenase (Zammit et al., 1992). The importance of the pentose phosphate pathway in the breakdown of hexose appears to be greater in ureide-exporting nodules based on the activity patterns of the relevant enzymes in *Pisum sativum, Lupinus luteus, Vicia faba, Vigna unguiculata, Cajanus cajan*, and *Cicer arietinum* nodules (Hong and Copeland, 1990). The Copeland group continues to document biochemical properties of the

glycolytic enzymes in host cytosol, with recent reports on the purification and properties of NAD-dependent glyceraldehyde-3-P dehydro-genase (Copeland and Zammit, 1994), phosphofructokinase (Vella and Copeland, 1993), and glucose-6-P dehydrogenase (Hong and Copeland, 1991) from soybean nodules.

Previous reports indicate that rhizobial mutants in carbohydrate metabolism generally give Fix^+ nodules whereas mutants defective in some step of the TCA cycle give Fix^- nodules (Day and Copeland, 1991; Streeter, 1991). Recent reports of carbohydrate transport mutants of *R. meliloti* (Fix^+, Hornez et al., 1994) support this view. Other mutants of *R. meliloti* lacking gluconeogenic enzymes showed complex phenotypes as bacteroids; the overall conclusion from this work is that gluconeogenesis is probably not essential in the symbiotic state, but some metabolism of hexose maybe required for full symbiotic competence (Finan et al., 1991). Molecular analysis of the gene for the key enzyme of gluconeogenesis, phosphoenolpyruvate carboxykinase, has recently been reported (Østerås et al., 1995).

C_4 dicarboxylic acids

Among studies on enzymes of dicarboxylate metabolism, work on malic enzyme in bacteroids has been most revealing. Note that, if 4-carbon acids are the main carbon source for bacteroids, this enzyme would play a key role in supplying the pyruvate needed to operate the TCA cycle (Fig. 1). Both NAD- and NADP-dependent enzymes have been described and partially purified; these two enzymes have very different Km values for malate, the NAD enzyme having a Km of about 2 mM and the NADP enzyme having a Km of about 0.1 mM (Kimura and Tajima, 1989; Copeland et al., 1989; Trinchant and Rigaud, 1990). A mutant of *R. meliloti* lacking the NAD-dependent malic enzyme but retaining the NADP-dependent activity has recently been reported (Driscoll and Finan, 1993). This mutant is Fix^-, thus providing strong support for the notion that 4-carbon dicarboxylic acids are the principal substrates for these bacteroids. However, whereas there is low NADP-dependent activity in *R. meliloti* bacteroids (Driscoll and Finan, 1993), the activity of the NAD- and NADP-dependent malic enzymes are similar in *B. japonicum* bacteroids (Copeland et al., 1989); thus, it will be useful to determine the phenotype of other rhizobia lacking NAD-malic enzyme.

Genetic analysis of TCA cycle enzymes has begun with the cloning of a fumarase gene from *B. japonicum*; because mutants retain some fumarase activity, a second enzyme is present in this organism (Acuña et al., 1991). In general, glycolytic enzyme activities are low and TCA cycle enzyme activities are high in bacteroids (Day and Copeland, 1991; Streeter, 1991; Suganuma and

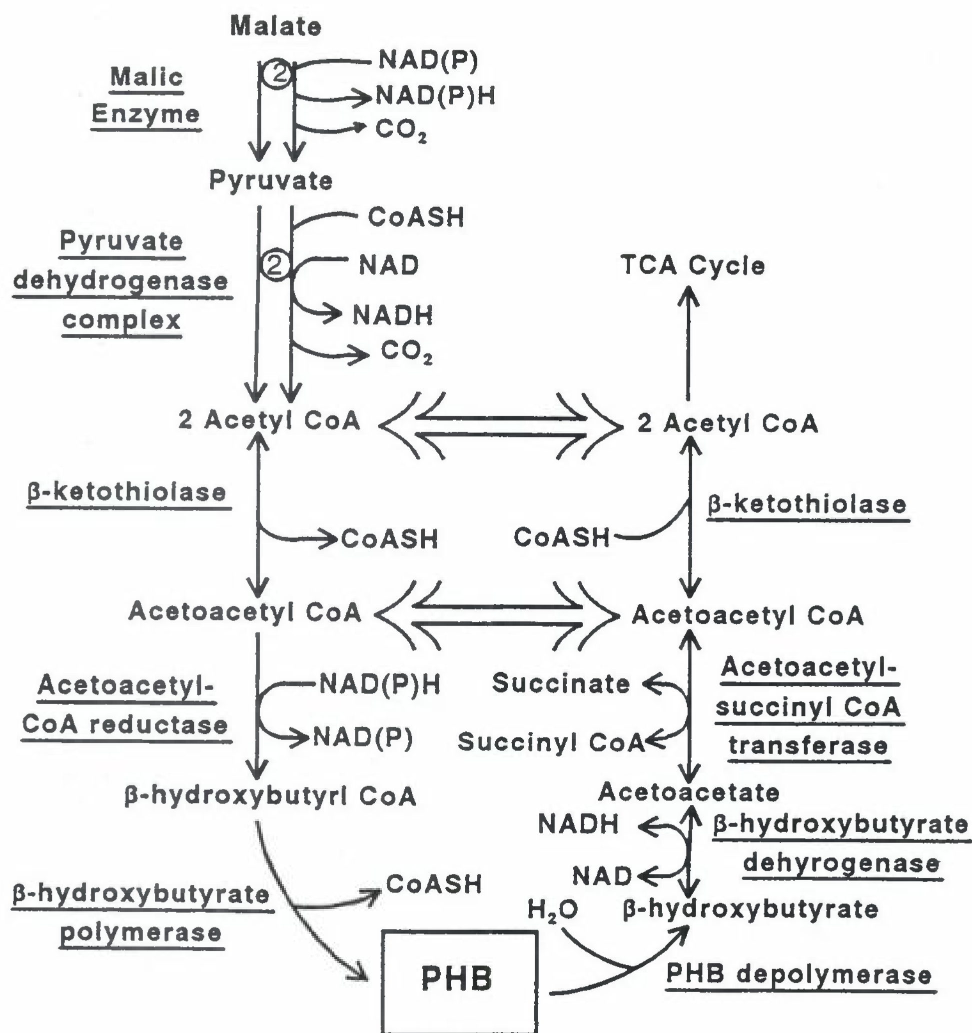

Figure 1. The pathway for synthesis and breakdown of poly-β-hydroxybutyrate (PHB) in rhizobia and the relationship of PHB to the TCA cycle (CoA = coenzyme A).

LaRue, 1993), and this is consistent with evidence for the repression of glycolytic enzymes in several strains of *Rhizobium* and *Bradyrhizobium* that were supplied with succinate (Mandal and Chakrabartty, 1993). Recent bacterial mutants that emphasize the importance of dicarboxylates include a succinate uptake mutant of *B. japonicum* (Fix^-, El-Din, 1992) and an isocitrate dehydrogenase mutant of *R. meliloti* (Fix^-, McDermott and Kahn, 1992). However, in the case of *R. tropici*, activities of glycolytic and TCA cycle

enzymes in bacteria grown with glucose, glutamate, or malate when compared with activities in bacteroids suggest that this organism could use either dicarboxylates or sugars in the nodule (Romanov et al., 1994).

Labeling studies involving intact nodules are very useful in elucidating overall nodule function because results integrate the activity of multiple enzymes, metabolic "compartments", and transport activities within the nodule. Carbohydrate metabolism in *C. arietinum* nodules has been explored by injection of labeled glucose, fructose or sucrose and analysis of labeling patterns in the same sugars; the conclusion was that sucrose synthesis may occur in nodules, but the significance of this is not clear (Singh et al., 1994). Two groups have recently taken advantage of the high activity of PEP carboxylase in nodules to rapidly label intact *P. sativum* and *G. max* nodules with $^{14}CO_2$; following very short labeling periods, bacteroids were rapidly isolated and examined for patterns of labeling in amino acid and organic acid fractions (Rosendahl et al., 1990; Salminen and Streeter, 1992). The labeling of metabolite pools in bacteroids showed a very high initial level of radioactivity in malate and declining proportional radioactivity over a 6-minute period (Table 1). This suggests that malate was the compound entering bacteroids and that other metabolites were labeled as a result of malate metabolism. (The relatively high labeling of glutamate is due to inhibition of 2-oxoglutarate dehydrogenase by high NADH concentration in bacteroids (Salminen and Streeter, 1990)).

Table 1. Changes in labeling of metabolite pools in *Bradyrhizobium japonicum* bacteroids from 1 to 6 minutes after feeding $^{14}CO_2$ to intact soybean nodules. Units are radioactivity in each compound as per cent of total radioactivity in bacteroids[a].

Compound	Initial level (1 min)	Slope (Δ%/min)[c]
Malate	55.8	–3.79
Glutamate	29.8	NS[b]
Aspartate	7.6	+ 1.25
Succinate	2.1	+ 1.67
Alanine	1.0	+ 1.92
Fumarate	0.5	+ 0.35
Citrate	0.3	NS[b]

[a]Adapted from Salminen and Streeter, 1992. The compounds shown account for about 97% of the total label in bacteroids. [b]The change in radioactivity with time was not statistically significant. [c]Increase or decrease in the proportion of radioactivity in each compound over the period from 1 to 6 minutes.

Other carbon substrates

Although it is not a substrate supplied by the host, the possibility that poly-β-hydroxybutyrate (PHB) is an important metabolite in *B. japonicum* bacteroids is indicated by recent studies of Bergersen and Turner (1992; 1993). Their results show that, depending on O_2 concentration and the concentration of carbon substrate (e.g. malate) supplied to bacteroids, PHB may be accumulated or rapidly broken down. They propose that PHB may act as a "buffer" for the TCA cycle (see McDermott et al., 1989), alternately serving as a sink for or supply of pyruvate (Bergersen et al., 1991). A linkage between PHB accumulation and hydrogenase activity in *B. japonicum* bacteroids has recently been suggested (Bergersen et al., 1995), but it should be noted that isogenic strains were not employed in this study.

The close relationship of PHB synthesis and breakdown to the TCA cycle is illustrated in Fig. 1. PHB synthesis is probably regulated at the β-ketothiolase step because of the regulation of the enzyme activity by CoASH and NADH (Suzuki et al., 1987). PHB breakdown is probably regulated at β-hydroxy-butyrate dehydrogenase, an enzyme that is sensitive to physiological concentrations NADH, pyruvate, and 2-oxoglutarate (Senior and Dawes, 1973). Overall regulation of the diversion of carbon into PHB may be linked to the NAD(P)H/NAD(P) ratio in bacteroids, a high value favoring PHB synthesis (Day and Copeland, 1991). The very high level of β-hydroxybutyrate dehydrogenase reported in *R. tropici* bacteroids (Romanov et al., 1994) is consistent with the importance of PHB turnover in nodules. However, PHB appears to be unimportant in *R. meliloti* because mutants unable to synthesize PHB have normal nodulation and N_2 fixation activity (Povolo et al., 1994). Because PHB does not normally accumulate in *R. meliloti* bacteroids (see McDermott et al., 1989), the analysis of PHB^- mutants needs to be extended to other rhizobia.

Proline is still considered by some to be an important carbon source for *B. japonicum* bacteroids based on the stimulation of acetylene reduction activity following supply of proline to nodulated roots (Zhu et al., 1992). The gene coding for the key enzyme of proline synthesis has been cloned from soybean nodules and the enzyme is mainly localized in the cytoplasm of infected cells (Delauney and Verma, 1990; Szoke et al., 1992). That proline is not an important carbon source is indicated by the failure of proline to be transported into symbiosomes from soybean nodules (Udvardi et al., 1990), to be transported into bacteroids from cowpea nodules (Glenn et al., 1991), and to be labeled in soybean nodules under conditions of rapid and substantial labeling of the dicarboxylate and amino acid pools in host cytoplasm and bacteroids (Salminen and Streeter, 1992). Thus, the situation with respect to proline remains

confusing and unresolved. It has been suggested that proline may be an important carbon source for bacteroids in environmentally stressed nodules (Kohl et al., 1994) and this is consistent with elevated levels of mRNA coding for pyrroline-5-carboxylate reductase in osmotically stressed soybean roots (Delauney and Verma, 1990).

Sucrose has been suggested as an important carbon source for *R. tropici* bacteroids on the basis of active uptake of sucrose by cultured *R. tropici* and the presence of invertase in this organism (Romanov and Martinez-Romero, 1994). This work needs to be extended to bacteroids. Earlier reports of the labeling of sucrose in *B. japonicum* bacteroids have recently been explained as an artifact based on the adsorption of ^{14}C-sucrose to the surface of bacteroids during their isolation from nodules (Streeter and Salminen, 1993).

Malonate has been suggested as a component of a transport system for amino acids and ammonium based on compartmentation of the acid and on other data for *L. luteus* nodules (Schramm, 1992). An ammonium shuttle system involving malonamate was previously suggested by Kim and Chae (1990), and a novel malonyl-CoA synthetase has been identified in *B. japonicum* and *R. trifolii* (Kim and Chae, 1991). However, malonate is not labeled in nodules on soybean plants supplied with $^{14}CO_2$, suggesting that malonate does not play an important role in carbon metabolism in nodules (see Streeter, 1991).

Oxalate has been shown to be a major acid in *V. faba* nodules and to support nitrogenase activity in bacteroids isolated from these nodules (Trinchant et al., 1994). This is curious because oxalate is such a highly oxidized form of carbon that it is not a logical source of reducing equivalents in bacteroids. Finally, glucose has been suggested as an important carbon source for bacteroids from *L. luteus* nodules based on labeling patterns following feeding with specifically labeled ^{14}C-glucose (Fedulova et al., 1990). For additional carbon sources proposed to be important for bacteroid function, see the review by Streeter (1991).

Two conclusions seem appropriate. First, we should remain open to the probability that bacteroids are exposed to a variety of carbon substrates and to the possibility that the main sources of reduced carbon for bacteroids are not the same in all legume nodules. This possibility becomes increasingly plausible as we are able to more clearly discern the large genetic differences among species of rhizobia. Second, data supporting the importance of dicarboxylic acids are very extensive and convincing; other "candidates" for important carbon sources need to be supported by data from a wide variety of experimental approaches. Questions that need to be answered before a particular carbon source can be considered as a likely source of reductant for bacteroids include: 1) Is the compound rapidly labeled to high specific activity in nodules following exposure of plants to $^{14}CO_2$, (2) Are enzymes required for synthesis of the

compound present with high activity in the host cytoplasm of the nodule?, (3) Does the compound readily pass through the symbiosome membrane?, (4) Is the compound absorbed under microaerobic conditions *in vitro* by purified bacteroids via an active mechanism?, (5) Are the enzymes required for catabolism of the compound present with high activity in bacteroids? (6) Is the labeled compound, when supplied to pure bacteroids, rapidly converted to $^{14}CO_2$ (see Salminen and Streeter, 1991)? (7) Does the compound, when supplied to bacteroids under appropriate conditions *in vitro*, support respiration and nitrogenase activity?, (8) Are bacterial mutants unable to transport or metabolize the compound Fix$^-$? Also, experimental tests of these questions should compare the compound of interest to malate or succinate in the appropriate situations.

3. Recent studies – carbon transport in legume nodules

Bacteroid cytoplasmic membrane

Previous analyses of the *dct* genes in rhizobia also provide strong support for the notion that dicarboxylic acids are the principal form of reduced carbon supplied to bacteroids (Day and Copeland, 1991; Streeter, 1991). Briefly, the DctA protein is the dicarboxylate transport protein ("permease") intrinsic to the bacteroid cytoplasmic membrane (Ronson et al., 1987); the DctB protein is anchored in the cytoplasmic membrane and senses the presence of substrate, and the DctD protein, when phosphorylated and in concert with other components, activates the expression of *dctA* gene (see Jording et al., 1994). Regulatory circuits for the three *dct* genes are complex and the reader is referred to recent papers by Jording et al. (1992; 1994) for details. Recent studies on the *dctA* gene confirm its essentiality for nitrogen fixation (van Slooten et al., 1992) and predict the actual orientation of the DctA protein in the bacteroid cytoplasmic membrane (Jording and Pühler, 1993). Whereas the expression of *dctA* is amplified by the availability of substrate (Jording et al., 1992), the expression of *dctB* is constitutive and the gene product has strong auto-phosphorylation activity (Giblin et al., 1995). The DctB protein controls the activity of DctD, and molecular mechanisms are being pursued by deletion analysis of *dctD* (Huala et al., 1992; Lee et al., 1994). A regulatory link between *dctB* and the expression of *nifHDK* in *R. meliloti* has also been suggested (Birkenhead et al., 1990). Previous confusing results relating to the growth of Dct$^-$ mutants on aspartate have recently been clarified with the demonstration of a second mechanism (independent of the dct mechanism) for aspartate (and glutamate) transport in *R. meliloti* (Watson et al., 1993).

Since the first demonstration of the importance of the Dct system to nitrogen fixation, it has been of obvious interest to construct and test rhizobial strains having increased capacity of the uptake of dicarboxylic acids. Two reports of such tests have recently appeared. In the first, a *R. meliloti* strain having 5-fold greater expression of *dctA* was used to form nodules on *Medicago sativa* plants. Bacteria having amplified DctA and grown with glucose as the carbon source had 10-fold greater succinate transport and nodules containing the construct had 30 to 60% greater acetylene reduction activity per plant (Table 2). However, there was no increase in plant growth (Rastogi et al., 1992). Note (Table 2) that, when bacteria were grown with succinate, there was no difference in succinate transport rate, suggesting that normally induced *dct* gene expression is sufficient to maintain high rates of succinate transport.

In the second report, a *R. meliloti* strain having additional copies of both *nifA* and *dctABD* was used to form nodules on *M. sativa* plants in a field study. Nodule formation by the engineered strain was good where levels of indigenous bacteria were low (Table 3). At one site where soil organic matter was low (0.9%), there was a positive effect of the engineered strain on plant biomass but, at the other two sites, there was no significant effect (Table 3). Thus, where organic matter and N levels in the soil were "typical" of U.S. soils and apparently sufficient to provide a portion of the N required, there was no advantage where the dicarboxylate transport system was amplified.

There are two possible interpretations of these two studies. First, perhaps dicarboxylate transport at the symbiosome membrane and not at the bacteroid cytoplasmic membrane limits carbon uptake by bacteroids. Second, perhaps nitrogen fixation may limit the transport of dicarboxylates to bacteroids and not vice versa (Jording et al., 1994).

Symbiosome membrane (SBM)

This major control point for metabolite transport continues to receive attention. Recent reports have emphasized the selectivity of the SBM in *G. max* (OuYang et al., 1990) and in *L. luteus* (Tomaszewska et al., 1991), in that symbiosomes take up malate or succinate at rates far in excess of the rates for the transport of sugars or malonate or amino acids. In studies of symbiosomes from pea nodules, transport of ^{14}C-malate was stimulated 3-fold by the addition of unlabeled glutamate, suggesting the possibility of a malate/aspartate shuttle mechanism (Rosendahl et al., 1992). Appels and Haaker (1991) have also suggested a shuttle mechanism based on their studies with isolated bacteroids. However, extensive labeling studies with *B. japonicum* bacteroids gave results inconsistent with the presence of a shuttle mechanism (Streeter and Salminen, 1990). Furthermore, the localization of aspartate

Table 2. Properties of a *Rhizobium meliloti* strain having amplified expression of the dicarboxylate transport gene dctA[a]

Parameter	Strain *trpPO-dctA*	Wild type	% change
^{14}C-succinate uptake (nmole × min^{-1} × mg protein^{-1})[b]	72	7	+1,000
Acetylene reduction (μmol C_2H_2 × h^{-1} × plant^{-1})[c]	3.93	2.84	+38
Shoot dry weight (mg × plant^{-1})[c]	434	446	−3

[a]Adapted from Rastogi et al., 1992. *dctA* was fused to the *Salmonella typhimurium* trp promoter. [b]Cultures grown with glucose (i.e., uninduced). When grown with succinate as the carbon source, both strains had approximately the same succinate transport rate. Both strains had similar growth rates on succinate. [c]Mean of 50 replicates in three independent experiments. The difference in acetylene reduction activity was significant at the 10% level in two experiments and at the 5% level in one experiment. Plants were *Medicago sativa* cv. Saranac.

Table 3. Effect of amplified *dctABD* plus *nifA* in *Rhizobium meliloti* on alfalfa plant yield at three field sites in Wisconsin (USA)[a]

Parameter	Site 1	Site 2	Site 3
Soil organic matter (% dry wt.)	0.9	1.9	3.2
Total extractable nitrogen (μg of NH_4^+ plus NO_3^-/g dry soil)	17.6	35.3	30.8
Forage yield (kg dry wt./ha)			
wild type	8.9	6.9	6.8
dctABD/Ω/nifA	10.1	7.1	6.7

[a]Adapted from Bosworth et al., 1994. At each of the three sites, nodule occupancy was ≥95% because indigenous *R. meliloti* were ≤60 cells/g dry wt. of soil; at a fourth site, nodule occupancy by the test strains was very low because of high indigenous populations (data not shown). For constructions involving amplification of *dctA* alone, *dctABD* alone or *nifA* alone, there was no effect of bacterial genotype on plant growth.

amino transferase in plastids, not cytoplasm, in infected cells (Appels and Haaker, 1991; Robinson et al., 1994) does not support the concept of a malate/aspartate shuttle as an important carbon transport mechanism.

The mechanism by which dicarboxylates are transported across the SBM is coming into focus. Analysis of host gene expression in nodules revealed the presence of a major 26-kD protein in the SBM several years ago and, more recently, this protein has been identified as a membrane-spanning channel protein (Miao et al., 1992; Miao and Verma, 1993). Phosphorylation of this protein is correlated with malate transport (OuYang et al., 1991) and recent studies with the protein incorporated into artificial lipid bilayers indicates that the protein is, in fact, an anion channel (Weaver et al., 1994). It is possible that the nodulin 26 protein provides the mechanism by which dicarboxylates are transported through the SBM, even though there is still no direct evidence for this.

Studies by Australian workers using intact symbiosomes from soybean nodules have demonstrated ATP-dependent formation of an electrical potential gradient over periods of a few minutes and the formation of a ΔpH over a period of hours (Udvardi et al., 1991; OuYang and Day, 1992). These results suggested the presence of an ATP-dependent proton pump in the SBM. Recently, Dubrovo et al. (1992) have succeeded in preparing well-sealed SBM vesicles from *L. luteus* nodules and, using a proton-specific fluorescent probe, very short time periods and appropriate controls, have demonstrated clearly the presence of ATP-dependent proton pumps in the SBM. The orientation of the pumps would result in H^+ transfer from host cytoplasm to the symbiosome space. Although activity had a pH optimum of about 5.6, about 50% of maximum activity was sustained in the physiologically important range of pH 6.5 to 7.5. A proton-pumping ATPase has also recently been reported in symbiosome membrane vesicles isolated from *Pisum sativum* nodules (Szafran and Haaker, 1995).

Most of the above concepts are summarized in Fig. 2. Proton pumps in the bacteroid cytoplasmic membrane and the host symbiosome membrane would create a proton gradient (high [H^+] in the symbiosome space) and this gradient probably supplies the driving force for dicarboxylate transport across the SBM. Transport of dicarboxylates via an anion channel in the symbiosome membrane, driven by the charge gradient, is suggested; this type of mechanism is considered very likely in the tonoplast where malate transport is not directly coupled to ATP hydrolysis but is clearly dependent on a proton gradient (Martinoia, 1992; Sze et al., 1992). [Note that the symbiosome membrane is thought to be a "mosaic" membrane with properties of both plant plasma membrane and tonoplast; see Streeter, 1991).] Rhizobia absorb dicarboxylic acids via a H^+/dicarboxylate co-transport system (i.e. DctA) in the cytoplasmic membrane (Tremblay and Miller, 1984; Bhandari and Nicholas, 1985).

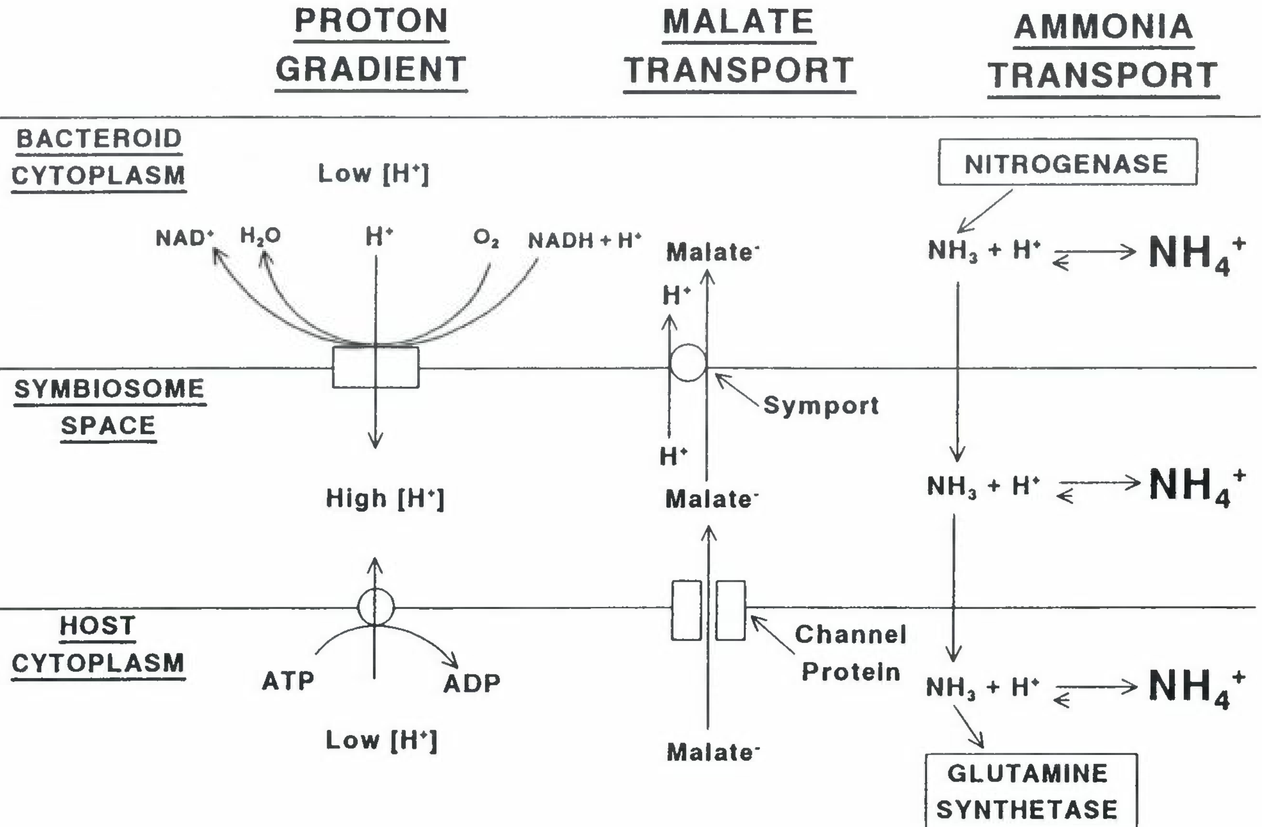

Figure 2. Proton gradients and the transport of dicarboxylates and ammonia across the symbiosome membrane and the bacteroid cytoplasmic membrane. Although most of the proton pumping by bacteroids is probably coupled to respiratory electron transport, bacteroid cytoplasmic membrane would also contain an ATPase capable of proton transport to the symbiosome space (see Nelson, 1992).

With both bacteroid and host contributing to the proton gradient across the bacteroid cytoplasmic membrane, one can imagine complex systems for the control of dicarboxylate transport. Treatments of nodules affecting ATP generation in mitochondria or bacteroids (e.g. oxygen supply) may perturb these H^+ gradients and have rapid and dramatic effects on nitrogenase activity (Bergersen, 1994; Streeter, 1995).

Also illustrated in Fig. 2 is the current concept of ammonia transport. Attempts to demonstrate an ammonium permease in the bacteroid membrane have provided uniformly negative results (see Streeter, 1991). Recent studies on ammonium transport through the symbiosome membrane from soybean nodules also showed the lack of a mechanism for transport of ammonium (Udvardi and Day, 1990). Thus, N transport to the host is probably via passive diffusion of ammonia through the two membranes and this is consistent with a very high level of glutamine synthetase in host cytoplasm and with a steep gradient of ammonium from bacteroid cytoplasm to host cytoplasm (Streeter, 1989). Because the dissociation constant for ammonium is $10^{-9.25}$ (Kleiner, 1981), relatively large pools of ammonium in bacteroid cytoplasm and in the symbiosome space may be required to accommodate the transport of N as ammonia (Fig. 2).

4. Carbon transport and metabolism – other associations

Azospirillum/wheat

Stimulation of the growth of grasses by inoculation with *Azospirillum* has been known for over a decade and attempts to explain the basis for this response continue. Some workers report major increases in yield in field studies although the basis for these increases does not appear to be increased N input (e.g. Caballero-Mellado et al., 1992). A recent suggestion is that nitrite formed in nitrate respiration by *Azospirillum* in combination with auxin provides the growth stimulus (Bothe et al., 1992). In some studies, increases in N content of the plants (Stancheva and Dinev, 1992) or incorporation of ^{15}N from $^{15}N_2$ (Christiansen-Weniger and Van Veen, 1991) indicate that N_2 fixation in the bacterium does contribute to plant N nutrition. Recently, a *nifH-gusA* fusion in *A. brasilense* has been used to study the patterns of colonization on wheat roots (Van de Broek et al., 1993).

Recent results indicate that N_2 fixation may be enhanced by release of organic acids by roots or by NH_4^+-excreting strains of *Azospirillum* (Christiansen-Weniger et al., 1992). The effect of organic acids in root exudate is illustrated in Table 4; if other aluminum tolerant genotypes also show

Table 4. Effect of exudation of organic acids by wheat roots on N input from *Azospirillum brasilense* and on plant growth[a]

Cultivar	Acid produced (µg/g dry wt. root) Malic	Succinic	Total N fixed (mg/plant)[b]	Total plant growth (g dry wt./plant)
Al tolerant	566	218	2.49	8.0
Al sensitive	117	42	1.42	4.8

[a]Adapted from Christiansen-Weniger, C. et al., 1992. The Al tolerant cultivar was 'Carasinho' and the Al sensitive cultivar was 'Buck Bolivar'. All differences between the sensitive and tolerant cultivar are statistically significant. [b]These values represent about 4 to 6% of total N in the plants.

improved associative N_2 fixation, it may be possible to exploit this trait in the development of new wheat cultivars. It is also encouraging that a genotype of *A. brasilense* with superior N_2 fixation has recently been reported (Katupitiya et al., 1995). However, a recent study of 23 soil types led to the conclusion that this organism survives poorly in soils in the absence of growing plant roots (Bashan et al., 1995).

It has also been known for some time that exposure of wheat roots to very low concentrations of the synthetic auxin 2,4-D inhibits lateral root growth and results in the formation of nodule-like structures ("*para*-nodules"). Recent studies on 2,4-D application coupled with *Azospirillum* inoculation have demonstrated that these structures may provide a low $[O_2]$ environment for the bacteria (Zeman et al., 1992; Christiansen-Weniger, 1992). Although acetylene reduction rates are typically very low in these studies (Zeman et al., 1992), use of ^{15}N has demonstrated the transfer of fixed nitrogen to the plant (Christiansen-Weniger, 1992; Yu et al., 1993). Because natural auxins like indoleacetic acid substitute only poorly for 2,4-D (Sriskandarajah et al., 1993), it is difficult to see how these systems can be exploited directly.

Acetobacter/sugar cane

Although N_2 fixation activity associated with the roots of sugarcane plants has been known for many years, only recently has the microsymbiont been isolated and identified. The bacterium, *Acetobacter diazotrophicus*, was first identified by Gillis et al. (1989) and the identification has recently been confirmed by more detailed analyses (Dong et al., 1995). Remarkably, this

organism shows optimum growth in culture with 10% sucrose and a pH of 5.5 and will actually grow at sucrose concentrations as high as 30% (Gillis et al., 1989). Two recent studies using electron microscopy suggest localization of the bacterium either in the vascular apoplast (Dong et al., 1994) or within the xylem elements of the shoots (James et al., 1994). Dong et al. (1994) have analyzed the apoplastic fluid from sugar cane stems and found a sucrose concentration of 12% and a pH of 5.5 – remarkably similar to optimum conditions for growth in culture. Because this interesting organism also produces indoleacetic acid, stimulation of sugarcane growth may be due to both hormone and nitrogen effects (Fuentes-Ramirez et al., 1993).

5. Summary

Although advances in knowledge continue in several directions, two elements of progress stand out in my view. The first is the steady accumulation of evidence supporting the key role of C_4 dicarboxylates in carbon nutrition of bacteroids in legume nodules. The main recent evidence consists of new Fix^- mutants (TCA cycle and malic enzyme), labeling patterns using intact nodules, and the emergence of plausible mechanisms for dicarboxylate transport from host to bacteroids. As stated earlier, we still need to be open to some utilization of substrates other than dicarboxylates and the (remote?) possibility that there is some legume system where dicarboxylates are not employed at all in bacteroid carbon nutrition. As an example, the recent data of Romanov et al. (1994) strongly suggest that *R. tropici* bacteroids are fully capable of carbohydrate metabolism and this constitutes a major departure from data for many other systems.

The second element that stands out is the beautiful integration of nitrogen-fixing systems. This is reflected in *Acetobacter diazotrophicus*, an unusual N_2-fixing organism that has evolved to occupy a highly specialized niche in the sugarcane apoplast, in the integration of plant and microbe activities to channel directional movement of carbon and nitrogen across two barriers in an efficient manner (e.g. Fig. 2), and in the failure of amplified *dct* genes to increase N_2 fixed (i.e., gene expression is probably already optimum). As we learn more and more about this integration at the molecular level, it seems increasingly improbable that we can expect, by manipulation of the symbionts, to make major increases in N input to the host plant – beyond merely increasing nodule weight per plant. If the nodule is considered as a machine with thousands of component parts, it seems that all of the parts fit very well together and operate in concert with nearly maximum efficiency.

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