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Products of Dark CO_2 Fixation in Pea Root Nodules Support Bacteroid Metabolism[1]

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ABSTRACT

Products of the nodule cytosol *in vivo* dark [^{14}C]CO_2 fixation were detected in the plant cytosol as well as in the bacteroids of pea (*Pisum sativum* L. cv "Bodil") nodules. The distribution of the metabolites of the dark CO_2 fixation products was compared in effective (fix$^+$) nodules infected by a wild-type *Rhizobium leguminosarum* (MNF 300), and ineffective (fix$^-$) nodules of the *R. leguminosarum* mutant MNF 3080. The latter has a defect in the dicarboxylic acid transport system of the bacterial membrane. The ^{14}C incorporation from [^{14}C]CO_2 was about threefold greater in the wild-type nodules than in the mutant nodules. Similarly, in wild-type nodules the *in vitro* phospho*enol*pyruvate carboxylase activity was substantially greater than that of the mutant. Almost 90% of the ^{14}C label in the cytosol was found in organic acids in both symbioses. Malate comprised about half of the total cytosol organic acid content on a molar basis, and more than 70% of the cytosol radioactivity in the organic acid fraction was detected in malate in both symbioses. Most of the remaining ^{14}C was contained in the amino acid fraction of the cytosol in both symbioses. More than 70% of the ^{14}C label found in the amino acids of the cytosol was incorporated in aspartate, which on a molar basis comprised only about 1% of the total amino acid pool in the cytosol. The extensive ^{14}C labeling of malate and aspartate from nodule dark [^{14}C]CO_2 fixation is consistent with the role of phospho*enol*pyruvate carboxlase in nodule dark CO_2 fixation. Bacteroids from the effective wild-type symbiosis accumulated sevenfold more ^{14}C than did the dicarboxylic acid transport defective bacteroids. The bacteroids of the effective MNF 300 symbiosis contained the largest proportion of the incorporated ^{14}C in the organic acids, whereas ineffective MNF 3080 bacteroids mainly contained ^{14}C in the amino acid fraction. In both symbioses a larger proportion of the bacteroid ^{14}C label was detected in malate and aspartate than their corresponding proportions of the organic acids and amino acids on a molar basis. The proportion of ^{14}C label in succinate, 2-oxogultarate, citrate, and fumarate in the bacteroids of the wild type greatly exceeded that of the dicarboxylate uptake mutant. The results indicate a central role for nodule cytosol dark CO_2 fixation in the supply of the bacteroids with dicarboxylic acids.

Symbiotic N_2 fixation in legume root nodules is fueled by carbon sources supplied by the host plant. Substantial evidence supports the hypothesis that the energy for N_2 fixation may derive primarily from plant produced dicarboxylic acids, which are taken up by the bacteroids (3, 5, 7, 13, 25). The dicarboxylic acids are most likely derived from the host cytosol, as the low O_2 tension in the infected zone of the nodule may limit mitochondrial respiration. Thus, it is unlikely that mitochondria can produce dicarboxylic acids at rates suffient to support nitrogenase activity in the bacteroids (12, 22).

The dark CO_2 fixation by PEP[2] carboxylase activity is a central anaplerotic pathway in legume root nodules as dicarboxylic acids are the immediate products of PEP carboxylase activity (10, 11, 19, 20). Nodule PEP carboxylase is located in the plant cell cytoplasm and the activity of PEP carboxylase is markedly higher in the nodule tissue than in the root tissue (10, 11, 19, 20).

Strong evidences support the theory that PEP carboxylase in legume nodules provide carbon skeletons for NH_3 assimilation and export (10, 11, 20). Furthermore, dicarboxylic acids resulting from PEP carboxylase activity have been suggested to support bacteroid metabolism in support of nitrogenase activity (11). Based on oxidation of dark CO_2 fixation products in soybean root nodules, it has been estimated, that the dicarboxylic acids produced by PEP carboxylase activity would be capable of providing 48% of the energy for nitrogenase activity (17). However, little direct evidence exists for the transport of cytosol dark CO_2 fixation products to the bacteroids.

The objectives of this work were (a) to ascertain if products of cytosol dark [^{14}C]CO_2 fixation were taken up by the bacteroids, (b) to evaluate which bacteroid metabolites were most readily labeled, and (c) to assess if the labeling patterns during nodule [^{14}C]CO_2 fixation by dct ineffective nodules were different from those of the wild-type nodules.

MATERIALS AND METHODS

Plant Materials

Peas (*Pisum sativum* L. cv "Bodil") were grown in a growth cabinet under *Rhizobium*-controlled conditions as previously described (26). The inoculation prior to sowing was with 5 mL per seed of a 3-d-old yeast mannitol broth suspension

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[2] Abbreviations: PEP, phospho*enol*pyruvate; dct, dicarboxylic acid transport; LSC, liquid scintillation counting.

culture of *Rhizobium leguminosarum* strain MNF 300 (wild type) or MNF 3080, a mutant of MNF 300, defective in dicarboxylate transport (3). Both *Rhizobium* strains were kindly provided by Dr I. A. McKay, Murdoch University, Western Australia. In plant nutrient solution (26), $CaCl_2$ was substituted by 4 mM $Ca(NO_3)_2$. Four weeks after seedling emergence, vermiculite was gently shaken from the root system and nodules picked and placed on ice. Nodule samples of 1 g (MNF 300) and 3 g (MNF 3080) were used for further experiments, which were carried out with one sample of both symbioses at a time to diminish the duration of the extraction procedure after labeling. The use of threefold more MNF 3080 dct$^-$ nodules was required in order to treat a comparable weight of bacteroids as was encountered with 1 g of effective wild-type nodules. The experiment was repeated four times.

Incubation of Nodules in [^{14}C]CO_2

Nodule samples were placed on moist filter paper at the bottom of a 10 mL reaction flask (Kontes, Vineland, NJ). The flasks were sealed with rubber stoppers. The nodules were equilibrated for about 3 min to reach room temperature. The assay was initiated by injection of 100 μL 11 N latic acid into a center well above the nodules containing 2.96×10^6 Bq of aqueous [^{14}C]Na_2CO_3 (20 μL of 2.2×10^9 Bq mmol^{-1}, The Radiochemical Centre, Amersham, UK). This resulted in an atmosphere of approximately 3.5 mL/L CO_2 within the reaction flasks. The nodules were incubated in the [^{14}C]CO_2 atmosphere for 5 min in order to obtain a brief single pulse ^{14}C labeling of the tissue.

Extraction and Compartmentation of Tissue

The incubation in [^{14}C]CO_2 was terminated by transfering the nodules to 4 mL per g tissue ice-cold extraction buffer (30 mM Hepes-KOH [pH 7.2] containing 0.3 M sucrose, 5 mM KH_2PO_4, 2 mM EDTA, 3 mM DTT, 0.8% [w/v] insoluble PVP, and 10 μL per mL of antimycin A [0.25 mg in 5 mL acetone]). The samples were homogenized in the extraction buffer using a Potter-Elvehjelm homogenizer and homogenates were filtered through four layers of Miracloth. The bacteroids were isolated by means of self-generating Percoll gradient (modified after Reibach *et al.* [24]). One mL aliquots were laid carefully on top of a 24 mL chilled prespun Percoll continuous gradient. The Percoll gradient contained 0.25 M sucrose, 8 mM KPO_4 (pH 7.2) and 0.8 mM EDTA. The Percoll had a start density of 1.09 and was prespun for 1 h at 48,000*g* in an RP70T angle rotor. The Percoll gradients including the homogenates were respun for 5 min in a GSA angle rotor at 12,000*g*, 4°C. Bacteroids formed a distinct band close to the middle of the gradient while the cytosol fraction was at the top of the gradient. The bacteroid band was collected using Pasteur pipettes and washed in five parts chilled wash buffer (50 mM KPO_4 [pH 7.6], 0.15 M NaCl). The bacteroids were pelleted in 5 min at 12,000*g*, 4°C in a GSA angle rotor. All fractionation procedures were carried out at 4°C. The cytosol fraction, the wash supernatant, and the bacteroid pellet were then individually mixed with 20 mL hot 85% (v/v) ethanol and left boiling for 15 min. The time span from termination of the [^{14}C]CO_2 incubation to mixing the individual fractions with hot ethanol never exceeded 25 min.

Fractionation of Bacteroids and Cytosol

The ethanol extracts were reduced in volume to about 2 mL by evaporation at 40°C on a rotary evaporator. The samples were reextracted in 10 mL methanol:chloroform: water:formic acid (12:5:2:1, v/v). Chloroform (2.5 mL) and water (3.5 mL) were added to split the two phases. The chloroform phase was discarded. The aqueous methanol phase was dried at 40°C on a rotary evaporator, and the extract was redissolved in 1.5 mL water. To a subsample of 20 μL was added, 100 μL 11 N lactic acid, and the acid stable radioactivity was determined by LSC.

The extracts were divided into neutral, basic (amino acids), and acid (organic acids) fractions using SP- and QAE-Sephadex ion-exchange columns (23). The neutral, basic, and acid fractions were dried on a rotary evaporator and redissolved in 1 mL water, 500 μL water:methanol (1:1, v/v), and 750 μL water, respectively. Subsamples of 10% (v/v) were withdrawn for determination of acid stable radioactivity by LSC.

Analysis of Metabolites

The amino acid content was measured as precolumn fluorescent *o*-phthaldialdehyde derivatives separated by HPLC as previously described (26). Following the spectrofluorimetric detection, the samples were collected on-line in 1 mL fractions, and the location and amount of acid stable radioactivity were determined using LSC.

The analysis of organic acids was performed by gradient elution using a Dionex 4000i (Dionex Corporation, Sunnyvale, CA) ion chromatograph. An HPIC AS5A-5μm column was used for analysis with an Anion Micro Membrane Supressor and conductivity detection. The flowrate was 0.9 mL min^{-1}. Solvent A was 0.75 mM NaOH and solvent B was 200 mM NaOH. Initial condition of 96% A and 4% B was followed by a linear gradient to 90% A and 10% B at 20 min. A steeper linear gradient followed to 57% A and 43% B at 42 min. At 42.1 min the system was eluted with 100% B for 5 min before returning to the initial condition, in which equilibrium was reached within 15 min. The regenerant in the Anion Micro Membrane Supressor was 20 mN H_2SO_4, which was used at a flowrate of 15 mL min^{-1}. The conductivity detector range was 10 μS and the sample loop, 50 μL. In this assay, 1 nmol of the individual organic acids could easily be detected. The assay provided a good separation of pyruvate, benzoate, tartrate, 2-oxoglutarate, fumarate, oxalacetate, citrate, isocitrate, *cis*-aconitate, and *trans*-aconitate. However, succinate, malate, and malonate were poorly separated. Separation of those three organic acids was markedly improved by substituting the gradient elution of the assay with an isocratic run in 3.9 mM $NaHCO_3$, 3.1 mM Na_2CO_3. Thus, individual samples were analyzed in both conditions to complete the analysis.

Post conductivity detection, the samples were collected online in 0.45 mL fractions, and acid stable radioactivity in each fraction was determined using LSC.

Protein Content and *in Vitro* PEP Carboxylase Activity

Three additional nodule samples were likewise fractionated on Percoll gradients to determine protein content of the cytosol and bacteroids (8) using Bovine serum albumine as protein standard, and *in vitro* PEP carboxylase activity of the cytosol as previously described (26).

RESULTS

Distribution of [^{14}C]CO_2 Fixation Products

The *in vivo* [^{14}C]CO_2 fixation by pea root nodules resulted in an accumulation of radioactivity in the nodule tissue. The ^{14}C incorporation was about three times as great in the wild type (MNF 300) as in the dct$^-$ mutant (MNF 3080) symbiosis (Table I). Similarly, there was a consistently higher *in vitro* PEP carboxylase activity in the cytosol of MNF 300 nodules than in the MNF 3080. On a per mg protein basis, the *in vitro* PEP carboxylase activity in the nodule cytosol was 0.35 ± 0.04 and 0.03 ± 0.003 μmol/min in the MNF 300 and MNF 3080 symbiosis, respectively. In general, the wild-type nodule tissue seemed more metabolically active than that of the mutant as the protein content was 14 mg/g fresh weight nodule of the wild type and only 4 mg/g fresh weight of the mutant nodules.

The products of [^{14}C]CO_2 fixation were detected in the cytosol as well as in the bacteroids of both symbioses (Table I). A somewhat larger proportion of the total incorporated radioactivity was detected in the bacteroids of the MNF 300 symbiosis than in bacteroids of the mutant MNF 3080.

The cytosol retained almost 90% of the radioactivity in the acid (organic acids) fraction in both symbioses (Table I). Likewise, the bacteroids of the MNF 300 symbiosis contained the largest proportion of the incorporated ^{14}C in the acid fraction. In contrast, the MNF 3080 bacteroids retained only about one-third of the radioactivity in the acid fraction, whereas the basic fraction (amino acids) constituted over 50% of the total radioactivity.

Composition of Organic Acids

The total amount of organic acids per g fresh weight pea nodule was about fourfold greater in the effective MNF 300 symbiosis as in the ineffective MNF 3080 symbiosis (Tables II and III). This difference was noted for both the plant cytosol and the bacteroid components.

Malate was the predominant organic acid in both cytosols (Table II). On a molar basis, malate constituted about half of the total cytosolic organic acid pool in both symbioses. The concentration of malate in MNF 3080 plant cytosol and bacteroids was about 30% that of effective wild-type nodules. Succinate was the second most prevalent organic acid in MNF 300 plant cytosol and the most predominant organic acid in MNF 300 bacteroids. Succinate concentrations in the ineffective MNF 3080 symbiosis appeared to be reduced to a larger extent than malate. Succinate concentrations of ineffective plant cytosol and bacteroids were reduced 92 and 85%, respectively. Malonate, the third most predominant organic acid in MNF 300 nodule plant cytosol, was reduced by 95% in MNF 3080 plant cytosol. Malonate was not consistently detected in bacteroids and when detected occurred at very low concentrations. *cis*-Aconitate was the predominant organic acid in the bacteroids of the 3080 symbiosis. This organic acid also constituted a larger proportion of the overall

Table I. *Distribution of Radioactivity Derived from* in vivo *[^{14}C]CO_2 Fixation in Pea Root Nodules*

Each value is the mean ± SE of four replicates.

Rhizobium Strain	Incorporated Radioactivity	Nodule Compartments	^{14}C in Compartments	Fraction	^{14}C in Fractions
	dpm/mg fresh wt		*% of total ^{14}C*		*% of compartment ^{14}C*
MNF 300	126 ± 44	Cytosol	90 ± 4	Neutral	0.3 ± 0.05
				Acid	89 ± 1
				Basic	11 ± 1
		Bacteroid-wash	4 ± 1	Neutral	32 ± 16
				Acid	57 ± 11
				Basic	9 ± 8
		Bacteroid	6 ± 4	Neutral	4 ± 2
				Acid	64 ± 10
				Basic	33 ± 9
MNF 3080	36 ± 15	Cytosol	80 ± 10	Neutral	0.7 ± 0.5
				Acid	86 ± 4
				Basic	13 ± 4
		Bacteroid-wash	17 ± 11	Neutral	22 ± 6
				Acid	76 ± 6
				Basic	2 ± 1
		Bacteroid	3 ± 2	Neutral	14 ± 14
				Acid	32 ± 13
				Basic	54 ± 19

Table II. *Organic Acid Composition of the Plant Cytosol of Effective and Ineffective Pea Root Nodules and the Proportion of Total ^{14}C Derived from* in vivo *Nodule [^{14}C]CO_2 Fixation Occurring in Each Compound*

Values are mean ± SE of three replicates.

Organic Acid	Effective MNF 300 Nodules		Ineffective MNF 3080 Nodules	
	Concentration	^{14}C	Concentration	^{14}C
	nmol/g fresh wt	*% total*	*nmol/g fresh wt*	*% total*
Malate	5408 ± 2254	78.8	1554 ± 476	72.2
Succinate	3080 ± 1146	0.6	247 ± 74	0.5
Malonate	945 ± 547	1.2	44 ± 3	3.6
2-Oxoglutarate	862 ± 250	2.7	223 ± 179	1.9
Fumarate	775 ± 153	4.8	81 ± 61	1.5
cis-Aconitate	479[a]	0.3	437[a]	0.0
Citrate	312 ± 77	10.2	190 ± 64	7.5
Tartrate	255[b]	0.3	155[b]	0.1
Benzoate	103 ± 78	0.2	ND[c]	
trans-Aconitate	10[a]	0.0	4[b]	0.0
Unknowns[d] (I–VII)	438	0.8	30	12.5

[a] Detected in two samples. [b] Detected in one sample. [c] Not detected. [d] Seven unidentified peaks were detected with retention time (min) corresponding to: I, 10.70; II, 13.30; III, 22.10; IV, 23.30; V, 34.60; VI, 35.90; VII, 37.70. Concentration given as the sum of arbitrary peak area units per g fresh weight.

Table III. *Organic Acid Composition of Bacteroids in Effective and Ineffective Pea Root Nodules and the Proportion of Total ^{14}C Derived from* in vivo *Nodule [^{14}C]CO_2 Fixation Occurring in Each Compound*

Values are mean ± SE of three replicates.

Organic Acid	Effective MNF 300 Nodules		Ineffective MNF 3080 Nodules	
	Concentration	^{14}C	Concentration	^{14}C
	nmol/g fresh wt	*% total*	*nmol/g fresh wt*	*% total*
Malate	90 ± 27	32.4	24 ± 11	35.3
Succinate	158 ± 39	20.3	23 ± 4	4.4
Malonate	3[b]	0.0	1[a]	2.2
2-Oxoglutarate	58 ± 19	11.7	15 ± 5	5.2
Fumarate	52 ± 1	4.1	3 ± 1	2.9
cis-Aconitate	75 ± 7	0.0	40[a]	1.8
Citrate	19 ± 6	7.1	1 ± 0.3	4.3
Tartrate	ND[c]		0.7[a]	0.8
Benzoate	15[a]	4.8	3[b]	11.6
trans-Aconitate	1[a]	0.0	ND[c]	
Unknowns[d] (I–VII)	78 ± 7	19.5	20.0 ± 2	31.6

Footnotes as in Table II.

pool of organic acids in the cytosol of MNF 3080 than in that of MNF 300.

Seven unidentified peaks were detected in the organic acid fractions (Tables II and III). Two of these (II and V) were present only in the bacteroids and not in the cytosols.

^{14}C Labeling of Organic Acids

The largest proportion of the incorporated ^{14}C in the acid fraction was detected in malate in the cytosols as well as in the bacteroids (Tables II and III). Thus, more than 70% of the cytosol radioactivity was recorded in the malate peak of both symbioses. Citrate was the second most labeled organic acid in the MNF 300 cytosol constituting 10% of the total radioactivity. In the MNF 3080, cytosol citrate contained 7.5% of the total radioactivity, whereas an unidentified organic acid (VI) retained about 11% of the cytosol radioactivity. The latter organic acid contained no radioactivity in the MNF 300 cytosol.

In both symbioses the radioactivity was more widely distributed among the organic acids in the bacteroids than in the cytosols. Malate retained the largest proportion of the bacteroid radioactivity, constituting 32 and 35% of the total radioactivity in MNF 300 and MNF 3080, respectively.

The succinate and 2-oxoglutarate peaks contained 20 and 12%, respectively, of the overall radioactivity in the bacteroids of the wild-type MNF 300. By comparison, only 4 and 5% of the total acid radioactivity in the MNF 3080 bacteroids was located in succinate and 2-oxoglutarate, respectively.

Table IV. *Amino Acid Composition of the Plant Cytosol of Effective and Ineffective Pea Root Nodules and the Proportion of Total ^{14}C Derived from* in vivo *$[^{14}C]CO_2$ Fixation Occurring in Each Compound*

Values are mean ± SE of three replicates.

Amino Acid	Effective MNF 300 Nodules		Ineffective MNF 3080 Nodules	
	Concentration	^{14}C	Concentration	^{14}C
	nmol/g fresh wt	*% total*	*nmol/g fresh wt*	*% total*
Asparagine	9497 ± 6499	3	140 ± 194	1
Glutamate	1772 ± 411	3	88 ± 48	4
Homoserine	1630 ± 1097	2	87 ± 47	3
Alanine	1101 ± 428	6	37 ± 16	1
γ-aminobutyrate	647 ± 453		103 ± 47	
Glutamine	432 ± 234	1	29 ± 23	2
Serine	304 ± 152	2	33 ± 10	3
Aspartate	203 ± 34	72	10 ± 5	72
Phenylalanine	182 ± 66		47 ± 49	
Valine	137 ± 15		29 ± 17	
Histidine	134 ± 108		31 ± 23	
Threonine / Glycine	113 ± 102	5	10 ± 4	6
Methionine	104[a]		19[a]	
Leucine	89 ± 54		26 ± 14	
Isoleucine	85 ± 48		20 ± 11	
Ornithine	83 ± 123		9[a]	
Lysine	75 ± 27		44 ± 24	
Arginine	73 ± 23	2	14 ± 10	2
Tyrosine	65 ± 34		25 ± 15	
α-aminobutyrate	32 ± 28		25 ± 21	1
β-alanine	27 ± 19		7 ± 4	1
Unknowns[b] (I–VII)	1168	2	565	1

[a] Detected in two samples. [b] Seven unidentified peaks were detected with retention times (min) corresponding to: I, 3.40; II, 11.33; III, 12.20; IV, 17.89; V, 19.83; VI, 20.35; VII, 25.73. Concentration given as the sum of arbitrary peak area units per g fresh weight.

The bacteroids of the MNF 3080 symbiosis contained about 10% of the total radioactivity in benzoate and in each of the unidentified organic acids I and III. The proportion of radioactivity in these three organic acids was considerably smaller in the bacteroids of the MNF 300 symbiosis.

Composition of Amino Acids

The amino acid content (nmol/g fresh weight) was 20 times as great in the MNF 300 cytosol as in the MNF 3080 (Table IV). In the bacteroids the amino acid content was about 10 times as high in the wild type as in the mutant MNF 3080 symbiosis (Table V). Thus, a larger proportion of the overall amino acid content of the nodule was located in the bacteroids of the MNF 3080 as compared to the MNF 300. In nodules of MNF 3080 more than 10% of the total amino acid content was located in the bacteroids, whereas the wild-type nodules contained less than 5% of the total amino acid content within the bacteroids.

Asn was the predominant amino acid in the cytosol as well as in the bacteroids of both symbioses. In the MNF 300 symbiosis Asn comprised 56 and 38% of the overall amino acid pool in the cytosol and bacteroids, respectively. The corresponding percentages in the 3080 symbiosis were 17 and 30%.

The second most abundant amino acid of the bacteroids was Glu. This amino acid comprises 15 and 18% of the amino acid pool in bacteroids of the MNF 300 and MNF 3080 symbiosis, respectively. Glu constituted about 10% of the amino acid pool in the cytosol of both symbioses.

The overall amino acid pool was more evenly distributed among the individual amino acids in the MNF 3080 symbiosis than in the MNF 300 symbiosis. This was valid in the cytosol as well as in the bacteroids.

In both nodule components of the MNF 3080 nodules γ-aminobutyrate performed a larger proportion of the amino acid pool than in the MNF 300 nodules.

Seven unidentified amino compounds were recognized (I-VII). The retention time of I corresponded to α-aminoadipate. That of IV corresponded to D,L β-aminiosobutyrate and VI had a retention time close to Trp. the unidentified II was only detected in bacteroids. Likewise, V was solely detected in bacteroids of MNF 3080.

^{14}C Labeling of Amino Acids

In both symbioses 72% of the radioactivity in the basic fraction of the cytosol was in Asp (Table IV). The peaks of Ala and Thr plus Gly retained about 5% of the basic cytosol radioactivity. The remaining ^{14}C label in the basic fraction of the cytosol was distributed fairly even at low levels among the remaining amino acids.

The label in the basic fraction of the bacteroids was somewhat more uniformly distributed among the individual amino

Table V. *Amino Acid Composition of Bacteroids in Effective and Ineffective Pea Root Nodules and the Proportion of Total ^{14}C Derived from* in vivo *[^{14}C] CO_2 Fixation Occurring in Each Compound*

Values are mean ± SE of three replicates.

Amino Acid	Effective MNF 300 Nodules		Ineffective MNF 3080 Nodules	
	Concentration	^{14}C	Concentration	^{14}C
	nmol/g fresh wt	*% total*	*nmol/g fresh wt*	*% total*
Asparagine	302 ± 314	3	27 ± 23	4
Glutamate	124 ± 98	10	16 ± 18	11
Homoserine	51 ± 43	1	5 ± 4	5
Alanine	76 ± 23	13	5 ± 4	
γ-aminobutyrate	11 ± 11		6 ± 8	1
Glutamine	10 ± 3	2	2 ± 1	3
Serine	8 ± 5	2	2 ± 1	6
Aspartate	71 ± 99	17	2 ± 2	34
Phenylalanine	67[a]	1	ND[c]	
Valine	1[a]	1	0.3[a]	2
Histidine	15[a]	1	2 ± 2	1
Threonine / Glycine	5 ± 3	9	0.8 ± 0.7	3
Methionine	3[a]	1	2[a]	
Leucine	11 ± 3	1	2 ± 2	
Isoleucine	6 ± 4	2	0.5[a]	1
Ornithine	9[a]	1	4 ± 2	1
Lysine	11 ± 2	1	3 ± 2	4
Arginine	4 ± 3	5	1.3 ± 0.2	1
Tyrosine	6 ± 2	1	5 ± 6	2
α-aminobutyrate	2 ± 1		2 ± 3	5
β-alanine	3 ± 1	19	1.3 ± 1.5	3
Unknowns[b] (I–VII)	39	9	23	13

[a, b] Footnotes as in Table IV. [c] Not detected.

acids than recorded for the cytosol (Table V). However, of the amino acids in the bacteroid component, Asp contained the major portion of label. This was particularly obvious in the MNF 3080 bacteroids, in which 34% of the total basic radioactivity was located in the Asp peak. The MNF 300 bacteroids retained 17% of the ^{14}C label in the Asp peak, proportionately similar to the radioactivity in β-Ala, Ala, and Glu.

In the bacteroids of the MNF 3080 symbiosis, Glu contained 11% of the total radioactivity. Thus, in both symbioses a larger proportion of the incorporated ^{14}C in the basic fraction of the bacteroids was located in Glu as compared to in the cytosol fractions, where only 3 and 4% of the ^{14}C label was accounted for by Glu.

DISCUSSION

The present results are the first to directly demonstrate that products of the dark CO_2 fixation, which occurs in the plant cytosol of legume root nodules, are taken up by the bacteroids (Table I). Furthermore, our detailed analyses of concentration and labeling of the various metabolites in cytosol as well as bacteroids provide new insight regarding the exchange of metabolites between the host plant and the microsymbiont in the N_2 fixing root nodules.

A brief single pulse of [^{14}C]CO_2 labeling of excised nodules was performed to elucidate which and where products from the nodule dark CO_2 fixation are initially incorporated. From this procedure the *in vivo* rate of nodule dark CO_2 fixation cannot be evaluated as (a) gas diffusion resistance increases upon excision of nodules, (b) a substantial proportion of the CO_2 available to nodule dark CO_2 fixation is likely to originate from metabolism of unlabeled nodule compounds, and (c) excised nodules respire 80% of the fixed [^{14}C]CO_2 within a 30 min cold chase (11). Thus, the overall ^{14}C incorporation from a brief single pulse underestimates the *in vivo* CO_2 fixation, but will reflect which compounds initially received the fixed CO_2. Furthermore, it is important to note that the *in vitro* PEP carboxylase activity and *in vivo* in bacteroids incorporation [^{14}] were similar in that the MNF 300 fix$^+$ symbiosis was nearly 10-fold greater than the MNF 3080 fix$^-$ symbiosis for both parameters.

Within 20 to 25 min from termination of the 5 min [^{14}C] CO_2 incubation, 63% of the total incorporated ^{14}C in the effective MNF 300 nodule was in malate of the plant cytosol. Within that same period, the majority of the ^{14}C in effective bacteroids was in malate and succinate. By comparison, in ineffective dct$^-$ MNF 3080 nodules, total incorporated ^{14}C was reduced by 71% and, of that fixed carbon, 50% was in malate of the plant cytosol. In contrast to the wild-type bacteroids, the ^{14}C in dct$^-$ bacteroids was located primarily in Asp. Such data directly implicate the uptake and use of host synthesized malate for the metabolism of effective bacteroids. Our results support the theory that energy for N_2 fixation is derived primarily from catabolism of dicarboxylic acids (3, 5, 7, 13, 25) and further verify earlier reports linking dark CO_2

fixation and nitrogenase activity in legume root nodules (11, 17, 19).

Recent results by Day and Mannix (12) suggest that the dicarboxylic acids supplied to the bacteroids during nitrogen fixation are derived from the host cytosol rather than from the mitochondria. Rawsthorne and LaRue (22) showed that nodule mitochondria are unable to produce dicarboxylic acids at rates sufficient to support nitrogenase activity in the bacteroids due to the low O_2 tension in the infected zone of the nodules. Both reports (12, 22) indicate that an anaplerotic pathway in the cytosol may be of importance to the supply of carbon substrates for bacteroid metabolism. Our data confirm this hypothesis, and put emphasis to the importance of nodule host cytosol dark CO_2 fixation in bacteroid function, as suggested in earlier reports (10, 11, 20, 26).

The bacteroids of the MNF 3080 symbiosis were considerably smaller than those of the wild type and a larger proportion of the MNF 3080 bacteria remained rodshape within the nodules as opposed to the MNF 300 symbiosis, where bacteria enlarged and became pleomorphic (data not shown). The bacteroid banding in the Percoll gradient was more diffuse in the mutant with a tendency to produce more bands in the center region of the Percoll gradient. This may reflect an incomplete transformation into bacterioids as bacteroids, transforming bacteria, and bacteria have progressively increasing densities (9).

The diffuse banding of MNF 3080 bacteroids necessitated the withdrawal of a larger volume from the center of the Percoll gradient of this symbiosis to obtain all of the bacteroid fraction. This may explain the larger proportion of ^{14}C label detected in the supernatant of the bacteroid wash from the mutant as compared to the wild type (Table I), as more cytosol contamination is likely to occur in the larger Percoll gradient volume holding the bacteroids.

Because bacteroids comprised a larger proportion of the nodule tissue in the wild type than in the mutant, threefold more nodule tissue of the mutant was required to obtain bacteroid pellets of comparable sizes upon Percoll gradient separation. The differences in the proportion of nodule tissue constituting bacteroids may have an impact on the recorded differences in the proportion of radioactivity incorporated in the bacteroids (Table I). However, the compartmentation of the incorporated ^{14}C in mainly organic acids in the MNF 300 bacteroids and mainly amino acids in the MNF 3080 bacteroids, most likely reflects the defect in the dicarboxylic acid uptake system of the MNF 3080 bacteroids.

The composition of organic acids and amino acids (Tables II, III, IV, and V) in the nodule fractions reflect the pool sizes of the individual components at the time of extraction. The turnover rate of the pools cannot be deduced from these results as a small pool of a specific compound does not necessarily imply that the compound is metabolically inactive. The present results, however, do permit a comparison of the metabolite status of the various nodule fractions between the investigated symbioses and also comparisons with the matabolite status reported for other legume nodules (2, 15, 18, 21, 28, 30).

Succinate was the predominant organic acid in the bacteroids of the MNF 300 symbiosis (Table III). Similar to soybeans (18, 28) succinate comprised a larger proportion of the organic acid pool in the bacteroids than in the cytosol.

In pea, as in soybean (18, 28) and *Phaseolus* (2), malonate was markedly more abundant in the cytosol than in the bacteroids. In fact, malonate was not always above the detection limit in the bacteroids. These data provide *in vivo* confirmation of the recent suggestion by Humbeck and Werner (15) that the peribacteroid membrane (PMB) may exclude malonate from the peribacteroid space. Although malonate, a competitive inhibitor of succinate dehydrogenase (SDH), occurs at relatively high concentrations in nodules, it is likely sequestered in the vacuole (4), and thus spatially separated from cytosolic SDH. Our data also provide *in vivo* confirmation of the exclusion of tartrate by the PBM (15).

The ineffectiveness of the MNF 3080 dct^- mutant was strikingly reflected by about 90% reduction in the total amino acid content in nodule cytosol and bacteroids as compared to the MNF 300 symbiosis (Tables IV and V). Although the decline was relatively uniform for all amino acids, it was most evident for Asn in the cytosol, which comprised 56% of the total concentration of amino acids in the cytosol of effective nodules, but only 17% of the total in the mutant cytosol. Such decreases have been observed in ineffective *Medicago* and *Lotus* (20) and have been taken to verify that a particular species falls into the category of legumes that transport symbiotically fixed N as amides. Comparable accumulations of Asn and changes during ineffective associations are not seen in ureide transporting species (2, 18, 28).

The mutant MNF 3080 contained a larger proportion of the nodule amino acid content in the bacteroids than recorded for the wild type (Tables IV and V). Also, the largest proportion of the ^{14}C label incorporated in the mutant bacteroids was in the amino acids fraction as opposed to the wild type (Table I). This suggests that amino acids may be transferred from the host cytosol to the bacteroids of the mutant. The role of amino acids as a source of reduced carbon for nitrogen fixation is a matter of controversy (6, 16, 31). The present results with pea imply a relatively greater abundance of amino acids in the bacteroids of the fix^- MNF 3080 than in the fix^+ bacteroids. However, a role of amino acids in support of nitrogenase activity cannot be excluded from the present results, since the overall amino acid concentration of the MNF 3080 nodules was markedly lower than in MNF 300.

The extensive and rapid labeling of effective pea nodule cytosolic malate and Asp confirms the reported role of nodule PEP carboxylase providing a portion of the carbon skeletons for N assimilation (10, 11, 20). The fact that these compounds contained the major proportion of the initial label in bacteroids support the hypothesis that a malate/Asp shuttle may be located in the membrane system which separates the microsymbiont from the host cytoplasm (1, 16, 27, 29). Aspartate aminotransferase (AAT), one component of such a shuttle, has been demonstrated to have nodule enhanced expression in alfalfa, another amide forming species (14).

Further evidence of a role of nodule dark CO_2 fixation by PEP carboxylase in bacteroid organic acid uptake and metabolism is the observation that the fix^- nodules induced by the dct^- mutant bacterial strain had strikingly reduced *in vivo*

and *in vitro* CO_2 fixation. This pleiotropic effect demonstrates a direct link between plant organic acid formation through anaplerotic reactions and bacteroid access to such acids.

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