C₄-Dicarboxylate Metabolism in Free-living and Bacteroid Forms of *Rhizobium leguminosarum* MNF3841

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The transport and catabolism of C₄-dicarboxylic acids have been further studied in *Rhizobium leguminosarum* MNF3841. Uptake of [¹⁴C]succinate was induced in free-living cells of strain MNF3841 within 12 min of exposure to succinate and reached a maximum rate within 60 min. Free-living cells of strain MNF3841 oxidizing fumarate accumulated pyruvate when treated with arsenite, an inhibitor of pyruvate dehydrogenase. Generation of pyruvate from C₄-dicarboxylates was accomplished by malic enzyme. Although malic enzyme was present in free-living cells grown on sucrose, higher activities were observed when fumarate or L-arabinose was the growth substrate. In crude extracts, malic enzyme activity required either NAD⁺ or NADP⁺ as cofactor, together with Mn²⁺, and was stimulated by K⁺.

Bacteroids from pea nodules transported [¹⁴C]succinate immediately after isolation, and contained twofold higher activities of both malic enzyme and pyruvate dehydrogenase than those found in fumarate-grown free-living cells. These data indicate that C₄-dicarboxylates are available to nodule bacteroids and that they are catabolized through the tricarboxylic acid cycle using malic enzyme and pyruvate dehydrogenase to generate acetyl-CoA.

INTRODUCTION

C₄-dicarboxylic acids are essential for N₂ fixation by *Rhizobium* bacteroids in legume nodules. Mutants unable to transport C₄-dicarboxylates are ineffective in N₂ fixation (Glenn & Brewin, 1981; Ronson *et al.*, 1981; Finan *et al.*, 1983; Arwas *et al.*, 1985). On the other hand, mutants blocked in carbohydrate metabolism, with one exception (Duncan, 1981), form fully effective nodules on their hosts (Ronson & Primrose, 1979; Glenn *et al.*, 1984*a, b*; Arwas *et al.*, 1986; Dilworth *et al.*, 1986), indicating that the ability to metabolize sugars is not an essential requirement for symbiotic N₂ fixation.

In free-living cells of *R. leguminosarum*, the utilization of C₄-dicarboxylates for growth involves not only systems for dicarboxylate transport and oxidation, but also a gluconeogenic system with phosphoenolpyruvate carboxykinase (PEPCK) and fructose-bisphosphate aldolase as essential enzymes. This gluconeogenic system is completely repressed when concentrations of sucrose as low as 0.4 mM are introduced into chemostat cultures growing on C₄-dicarboxylates. Pea bacteroids do, however, show measurable activities of PEPCK, implying that only limited amounts of sugars reach the bacteroid (McKay *et al.*, 1985).

Despite the importance of C₄-dicarboxylates for N₂ fixation, the biochemical route by which they are metabolized has not been elucidated for *Rhizobium* or *Bradyrhizobium* spp. This paper describes the requirements for C₄-dicarboxylate catabolism by *R. leguminosarum* MNF3841 in both the free-living cell and the bacteroid.

Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; TCA, tricarboxylic acid.

METHODS

Organisms. *Rhizobium leguminosarum* MNF3841 is a Str^R derivative of strain 300 described by Johnston & Beringer (1975). MNF3070 and MNF3085 are, respectively, pyruvate carboxylase and PEPCK deficient mutants derived from strain 300 by transposon Tn5 mutagenesis (Arwas *et al.*, 1985, 1986). *Bradyrhizobium japonicum* CB1809 and cowpea *Bradyrhizobium* CB756 were from CSIRO, Brisbane, Australia. *Azotobacter vinelandii* WU102 was obtained from the University of Western Australia.

Growth conditions. Media were based on the minimal salts medium of Brown & Dilworth (1975) but with 10 mM-NH₄⁺ as the nitrogen source, and 10 mM-arabinose, 10 mM-sucrose or 15 mM-fumarate as the carbon source. The pH was maintained at 7.2 with either 10 mM-phosphate (MSP medium) or 40 mM-HEPES/NaOH (MSH medium) with 0.5 mM-K₂HPO₄ as the phosphate source.

Batch cultures were grown aerobically at 28 °C in MSH or MSP with carbon sources at 3–15 mM as indicated. Cell growth was measured as optical density at 600 nm.

Continuous cultures used the same MSH medium except for a limiting phosphate concentration (0.08 mM). The chemostat had a working volume of 400 ml and was operated at a dilution rate of 0.1 h⁻¹ at 28 °C.

Harvesting of cells. Cells grown to an OD₆₀₀ of 0.7–1.0 were centrifuged (6000 *g* for 10 min) and washed twice in the minimal salts medium of Brown & Dilworth (1975) from which carbon and nitrogen sources had been omitted and the phosphate concentration reduced to 0.5 mM. They were resuspended to the cell density (OD₆₀₀) indicated in the text.

Nodule production. Pre-germinated peas (*Pisum sativum* L. cv. Greenfeast) were planted into pots of sterilized yellow sand and inoculated with MNF3841. The plants were regularly watered with nitrogen-free nutrient solution (Broughton & Dilworth, 1971) until harvesting 28–35 d after inoculation.

Bacteroid isolation. Bacteroids used for the preparation of cell-free extracts were isolated on a Percoll gradient as described by McKay *et al.* (1985). Bacteroids used for C₄-dicarboxylate uptake and metabolism experiments were isolated by grinding nodules in a mortar and pestle with an equal amount of 0.1 M-sodium phosphate buffer, pH 7.6, containing 0.25 M-mannitol. The slurry was filtered through four layers of medical gauze, centrifuged at 6000 *g* for 10 min in a Sorvall SS34 rotor at 22 °C, and the bacteroids washed twice in the above medium.

Uptake experiments. Cells were harvested, washed twice in MSP and, following starvation for 30 min at 28 °C, incubated with shaking in the presence or absence of 3 mM-succinate. Samples (2.8 ml) were taken at various times up to 60 min, centrifuged (8000 *g* for 1 min) in a benchtop Eppendorf centrifuge and washed twice in MSP. The harvesting and washing process was completed within 8 min and the washed cells were immediately tested for their ability to transport [¹⁴C]succinate as described by Glenn *et al.* (1984a).

Arsenite inhibition. Cells of strain MNF3841 were grown in batch culture in MSH with 15 mM-fumarate, harvested, and resuspended to an OD₆₀₀ of 0.6. Cells were added to a mixture of 3 mM-fumarate and 1.25 mM-arsenite; this concentration of arsenite gave the greatest oxoacid accumulation over the range 0–4 mM (results not shown). After incubation on an orbital shaker at 28 °C, for various times up to 60 min, samples (2.8 ml) were centrifuged (8000 *g* for 2 min), and the supernatants used to monitor fumarate disappearance and oxoacid production. The accumulating oxoacid was identified as pyruvate with lactate dehydrogenase, which gave a close correspondence with the chemical method using dinitrophenylhydrazine. Paper chromatography (Dilworth *et al.*, 1986) revealed that pyruvate was the only oxoacid present. Similar experiments with MNF3085 (a PEPCK-deficient mutant of MNF300) used cells grown on sucrose and incubated in fumarate for 5 h to allow induction of dicarboxylate metabolism before addition of arsenite.

Labelling of pyruvate from [¹⁴C]succinate. Strain MNF3841 was grown in batch culture in MSH with 10 mM-succinate as the sole carbon source, harvested and resuspended to an OD₆₀₀ of 1; 2 ml of this suspension was added to Warburg vessels containing 1.25 mM-arsenite, 1 mM-succinate and 1.25 µCi (46.25 kBq) [1,4-¹⁴C]succinate in a final volume of 3 ml. The centre well contained a concertina-shaped wick (Whatman no. 42, 12 × 30 mm) plus 0.1 ml 20% (w/v) KOH to trap released CO₂. Controls lacking either cells or arsenite were run simultaneously. The flasks were incubated at 28 °C in a reciprocating water bath for 60 min and the reaction was stopped by the addition of 0.1 ml 0.1 M-H₂SO₄. Following a further 30 min incubation to allow complete trapping of the CO₂, the mixture was neutralized by the addition of KOH and centrifuged (8000 *g* for 2 min). Pyruvate in the supernatants was assayed colorimetrically and total radioactivity was determined using liquid scintillation counting as described by Glenn *et al.* (1984a). Pyruvate was chromatographically separated from succinate (see below) and the radioactivity in each fraction was determined.

Separation of succinate and pyruvate. Succinate and pyruvate were chromatographed on columns (45 mm × 6 mm diam.) of Dowex-1 × 8 (100–200 mesh chloride form) prepared as described by Von Korf (1967). Neutralized cell supernatants (1.5 ml) containing up to 1.5 µmol organic acids were applied to the columns, which were washed with 1.0 ml water. Succinate was eluted with 12 × 1.0 ml volumes of 10 mM-HCl, and pyruvate with a subsequent 8 × 1.0 ml of 50 mM-HCl.

Analytical methods. Pyruvate was determined either as the 2,4-dinitrophenylhydrazone as described by Arwas *et al.* (1985) or, when 2-oxoglutarate was present, by using lactate dehydrogenase. In mixtures, 2-oxoglutarate was measured as the 2,4-dinitrophenylhydrazone after subtraction of pyruvate.

L-Malate was measured using a technique derived from Bernt & Bergmeyer (1974) for L-glutamate estimation. In a final volume of 1.5 ml were 0.75 mmol glycine, 0.60 mmol hydrazine monohydrate, 4 µmol NAD⁺ and 3 units of malate dehydrogenase. Samples were incubated for 90 min at 30 °C before reading absorbance at 340 nm. Minus-enzyme controls were run as blanks. Fumarate was measured at 253 nm using sodium fumarate in 40 mM-HEPES/NaOH pH 7.2 as the standard. Protein was measured by the method of Lowry using bovine serum albumin as the standard.

Preparation of cell-free extracts. Cell-free extracts were prepared as described by McKay *et al.* (1985); for malic enzyme assays they were dialysed overnight against 40 mM-Tris/HCl (pH 8) containing 1 mM-dithiothreitol.

Enzyme assays. Oxaloacetate decarboxylase (EC 4.1.1.3) was measured by monitoring the production of pyruvate in an assay mixture of 0.5 ml containing 20 µmol HEPES (pH 7.8), 10 µmol oxaloacetate (pH 7.8) and cell extracts. The tubes were incubated for 5 min at 30 °C and the reaction was stopped by the addition of 0.1 ml 1.4 M-NaOH. The pH was lowered using 0.4 ml of 1.0 M-HEPES pH 7.5 and the amount of pyruvate produced was measured at 340 nm using NADH and lactate dehydrogenase.

Malic enzyme [malate dehydrogenase (oxaloacetate decarboxylating)] was assayed in two ways. The first monitored the increase in absorption at 340 nm due to the formation of NADPH or NADH. The assay mixture contained (in a final volume of 1 ml) 100 µmol glycylglycine buffer (pH 8.5), 1 µmol MnCl₂, 50 µmol KCl, 1 µmol NADP⁺ or NAD⁺ and cell extract. The reaction was initiated with the addition of 20 µmol L-malate pH 7.1. The other method measured the amount of pyruvate produced from malate following a 20 min incubation at 30 °C. The assay mixture contained (in a final volume of 0.5 ml) 25 µmol Tris/HCl (pH 8.5), 0.5 µmol MnCl₂, 25 µmol KCl, 0.5 µmol NADP⁺ or NAD⁺ and 10 µmol L-malate pH 7.1. The reaction was started by the addition of 0.05 ml cell extract and terminated with 0.5 ml of 0.1% 2,4-dinitrophenylhydrazine in 2 M-HCl. After a 10 min incubation, 2 ml 2 M-NaOH was added, the solution centrifuged (6000 g for 5 min) and the absorbance of the supernatant recorded at 580 nm.

Pyruvate kinase (EC 2.7.1.40) was assayed using a modification of the method of Bucher & Pfeleiderer (1955), in a final volume of 1 ml containing 50 µmol phosphate buffer (pH 7.5), 2 µmol ADP, 0.2 µmol NADH, 5 µmol MgCl₂, 5 units of lactic dehydrogenase (commercial), 5 µmol phosphoenolpyruvate and cell-free extract. PEPCK (phosphoenolpyruvate carboxykinase, EC 4.1.1.49) was assayed using the method of Krebs & Bridger (1980), pyruvate dehydrogenase (EC 1.2.4.1) by the method of Visser *et al.* (1982), and pyruvate carboxylase [pyruvate:CO₂ ligase (ADP-forming), EC 6.4.1.1] as described by Arwas *et al.* (1986).

RESULTS AND DISCUSSION

Fumarate metabolism in the presence of arsenite

Oxidation of dicarboxylates via the TCA cycle in MNF3841 is likely to require the formation of pyruvate from the dicarboxylate, as well as the formation of oxaloacetate. Arsenite, a potent inhibitor of dithiol enzymes like the lipoamide dehydrogenase components of the pyruvate and 2-oxoglutarate dehydrogenase complexes (Sanadi *et al.*, 1959), should then cause the accumulation of pyruvate when added to fumarate-utilizing cells.

After exposure to arsenite for 1 h, fumarate-grown cells of strain MNF3841 had consumed 3.5 µmol fumarate (mg protein)⁻¹ and had accumulated 2.4 µmol pyruvate (mg protein)⁻¹ and 0.7 µmol malate (mg protein)⁻¹ (Fig. 1a), indicating that part of fumarate metabolism in MNF3841 is via malate to pyruvate. In the absence of arsenite, cells of MNF3841 grown on fumarate did not accumulate pyruvate.

Fumarate conversion to pyruvate in the presence of arsenite was also studied in cells grown on sucrose and therefore not induced for C₄-dicarboxylate metabolism. After 1 h only 0.24 µmol fumarate (mg protein)⁻¹ had been utilized and 0.06 µmol pyruvate (mg protein)⁻¹ had accumulated. These data indicate that only cells already induced for C₄-dicarboxylate metabolism accumulate significant quantities of pyruvate in the presence of arsenite.

The assumption implicit in these experiments was that the pyruvate that accumulated was derived entirely from the decarboxylation of malate. The specific activity of pyruvate accumulated in the presence of arsenite when [1,4-¹⁴C]succinate was the substrate was therefore determined. In the presence of arsenite, succinate-grown cells of MNF3841 converted [1,4-¹⁴C]succinate to [¹⁴C]pyruvate with a mean specific activity (0.52 × 10⁶ d.p.m. µmol⁻¹) half that of the initial [1,4-¹⁴C]succinate (1.08 × 10⁶ d.p.m. µmol⁻¹). The overall recovery of radioactivity was 99%. These data indicate that the pyruvate that is accumulated by MNF3841 in the presence of arsenite is derived entirely from the decarboxylation of a C₄-dicarboxylic acid.

Similar experiments with fumarate-grown cultures of *Bradyrhizobium japonicum* CB1809 and

Fig. 1. Fumarate consumption (●) and the accumulation of malate (○), pyruvate (■) and 2-oxoglutarate (□) in the presence of 1.25 mM-arsenite by fumarate-grown cells of *R. leguminosarum* MNF3841 (a) and fumarate-incubated cells of PEPCK-deficient *R. leguminosarum* MNF3085 (b).

Fig. 2. Potential routes of pyruvate formation from C_4 -dicarboxylates. 1, Malate dehydrogenase; 2, oxaloacetate decarboxylase; 3, pyruvate carboxylase; 4, PEPCK; 5, pyruvate kinase; 6, malic enzyme.

cowpea *Bradyrhizobium* CB756 also showed pyruvate accumulation from fumarate in the presence of arsenite.

Route of fumarate conversion to pyruvate

The most obvious routes for pyruvate formation from C_4 -dicarboxylates in rhizobia (Fig. 2) include (i) the decarboxylation of oxaloacetate by oxaloacetate decarboxylase; (ii) the decarboxylation of oxaloacetate by PEPCK followed by the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase; (iii) production of pyruvate from oxaloacetate via a reversible pyruvate carboxylase; and (iv) the decarboxylation of malate to pyruvate by malic enzyme.

Cells of both *A. vinelandii* (which produces high activities of oxaloacetate decarboxylase; Plaut & Lardy, 1949) and MNF3841 were grown in batch culture on MSH with fumarate and cell extracts were prepared. While large activities of oxaloacetate decarboxylase [$500 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] were detected in extracts of *A. vinelandii* WU102, none was evident in extracts of MNF3841, implying that this is not the route to pyruvate used by MNF3841.

MNF3841 contained significant activities of both PEPCK [$120 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] and pyruvate kinase [$290 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] when grown on a C_4 -dicarboxylate. The significance of this potential route was examined using MNF3085 (a PEPCK-deficient mutant of MNF3841 unable to grow on succinate; Arwas *et al.*, 1985). Like the wild-type, strain MNF3085 accumulated pyruvate and malate in the presence of arsenite (Fig. 1b); in addition, 2-oxoglutarate was identified as a product by paper chromatography (Dilworth *et al.*, 1986). Of the fumarate consumed, 86% was accounted for in these three products. The simplest explanation of the production of 2-oxoglutarate in MNF3085 but not in the wild-type lies in the ability of

Table 1. *NADP*⁺- and *NAD*⁺-dependent activities of malic enzyme and pyruvate dehydrogenase from pea bacteroids isolated on a Percoll gradient and of free-living cells of *R. leguminosarum* MNF3841 grown on sucrose, arabinose or fumarate (10 mM)

The results are means $\pm$ standard deviation ($n = 3$). ND, Not determined.

Source of enzyme	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]		
	NADP ⁺ -dependent malic enzyme	NAD ⁺ -dependent malic enzyme	Pyruvate dehydrogenase
Sucrose-grown free-living cells	38 $\pm$ 7	61 $\pm$ 11	68 $\pm$ 5
Arabinose-grown free-living cells	92 $\pm$ 8	108 $\pm$ 9	ND
Fumarate-grown free-living cells	103 $\pm$ 11	127 $\pm$ 12	40 $\pm$ 4
Bacteroids	209 $\pm$ 9	243 $\pm$ 13	99 $\pm$ 7

MNF3085 to metabolize fumarate but not to grow on it. Thus, in the 5 h induction period, cells of MNF3085 consume fumarate without growth, and are therefore likely to accumulate intracellular carbon reserves. If such reserves can be used to generate acetyl-CoA without prior formation of pyruvate, arsenite would then cause the accumulation of 2-oxoglutarate by complete inhibition of 2-oxoglutarate dehydrogenase (Dilworth *et al.*, 1986). By contrast, MNF3841 cells grow exponentially on fumarate and do not accumulate reserves capable of giving rise to acetyl-CoA independent of pyruvate formation. Irrespective of the reason for 2-oxoglutarate formation in MNF3085, the fact remains that it generates pyruvate from fumarate despite a total absence of PEPCK activity.

Pyruvate carboxylase activity has been found in cells grown on sucrose [106 nmol min⁻¹ (mg protein)⁻¹; Arwas *et al.*, 1986] or succinate [60 nmol min⁻¹ (mg protein)⁻¹]. Since a pyruvate-carboxylase-negative mutant (MNF3070) grows as well as the wild-type on succinate (Arwas *et al.*, 1986), and dialysed cell-free extracts of succinate-grown MNF3841 produce pyruvate from malate without added adenine nucleotides (results not shown), this enzyme cannot be necessary for C₄-dicarboxylate metabolism.

Malic enzyme activity was measured in dialysed cell-free extracts of MNF3841 grown in batch culture on MSH with fumarate, arabinose or sucrose as carbon source, using both NAD⁺/NADP⁺ reduction and pyruvate production. In crude extracts, however, NADP⁺ and NAD⁺ reduction underestimated the activity of malic enzyme; NADH oxidase activity can adequately explain the discrepancy for the NAD-linked activity, but the underestimation by NADPH formation is not understood. It is impractical to use cyanide to inhibit NADH oxidase activity as the malic enzyme itself is cyanide-sensitive (results not shown). Consequently pyruvate production was used as the principal method of assaying malic enzyme in crude extracts. Malic enzyme was constitutive in MNF3841, although its activity was higher when fumarate or L-arabinose was the growth substrate (Table 1). Malic enzyme activity (pyruvate formation) was optimal at pH 8.0–8.5 in Tris/HCl buffer, and totally dependent on the presence of NAD⁺ or NADP⁺. Enzyme activity with either NAD⁺ or NADP⁺ was dependent on the concentration of Mn²⁺, with an apparent K_m of 54–71 μ M over the range 10–100 μ M. K⁺ (50 mM) stimulated either NAD⁺- or NADP⁺-dependent malic enzyme activity approximately threefold, while Na⁺ inhibited malic enzyme at concentrations above 5 mM.

Induction of C₄-dicarboxylate catabolism

The kinetics of C₄-dicarboxylate consumption were examined by following fumarate utilization by cells of MNF3841 grown on sucrose in batch culture. Cells were harvested and resuspended (OD₆₀₀ approximately 0.3) in MSH containing fumarate as the sole carbon source. Exponential growth (mean generation time 3.3 h) was evident within an hour of resuspension into fumarate. A control experiment where cells were inoculated into MSH lacking any carbon source showed that endogenous reserves were insufficient to support any significant growth.

Fig. 3. Fumarate consumption by cells of *R. leguminosarum* MNF3841 following transfer from sucrose.

Fig. 4

Fig. 4. Induction of [¹⁴C]succinate uptake by *R. leguminosarum* MNF3841 grown on MSH arabinose and incubated in the presence (●) or absence (○) of 3 mM-succinate.

Fig. 5

Fig. 5. Fumarate consumption (●) and the accumulation of malate (○) and pyruvate (■) by isolated pea bacteroids of *R. leguminosarum* MNF3841 incubated in the presence of 1.25 mM-arsenite.

The capacity to utilize fumarate was rapidly induced and was observed 30–60 min after resuspension. Fumarate utilization was linear when corrected for growth by plotting $\int \text{dry weight}. dt$ versus μmol fumarate consumed (Fig. 3). NADP⁺- and NAD⁺-dependent malic enzyme activity increased from 38 and 61 nmol min⁻¹ (mg protein)⁻¹ to 66 and 104 nmol min⁻¹ (mg protein)⁻¹ over the 8 h period; activity was not maximized until 12 h (Table 1).

The induction of C₄-dicarboxylate transport in *R. leguminosarum* was examined using cells grown in phosphate-limited chemostat culture with 10 mM-arabinose as the carbon source to an OD₆₀₀ of 0.7. Arabinose was chosen as the carbon source because *R. leguminosarum* is known to metabolize L-arabinose via 2-oxoglutarate semialdehyde, 2-oxoglutarate and the TCA cycle (Dilworth *et al.*, 1986). Consequently cells of MNF3841 grown on L-arabinose contain the enzymes of both C₄-dicarboxylate metabolism and gluconeogenesis, but will not be induced for dicarboxylate transport. Dicarboxylate transport activity was present at very low rates in cells grown on arabinose but showed an eightfold induction within 60 min in the presence of 3 mM-succinate (Fig. 4). This induction is much more rapid than the 6–8 h required to induce either histidase or the enzymes for *p*-hydroxybenzoate metabolism in this strain (Dilworth *et al.*, 1983). The enzymes for gluconeogenesis in *R. leguminosarum* are also derepressed by growth on C₄-dicarboxylates, PEPCK reaching full activity within 2 h and fructose-bisphosphate aldolase after 8 h (McKay *et al.*, 1985).

C₄-dicarboxylate metabolism by bacteroids

Bacteroids were able to transport succinate immediately after isolation, although the rate of uptake [$15 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] was lower than that observed in fully induced free-living cells. When bacteroids were incubated at 28°C in the presence of 3 mM -fumarate and 1.25 mM -arsenite, a higher proportion of the fumarate utilized remained as malate. Nevertheless, the accumulation of both pyruvate and malate compared with the free-living form (Fig. 5) indicates that bacteroids also convert C₄-dicarboxylates to pyruvate. Of the fumarate utilized, 85% could be accounted for as malate and pyruvate, although the rate of pyruvate accumulation was slower than that for derepressed free-living cells.

Cell extracts derived from bacteroids isolated on a Percoll gradient had activities of 209 and $243 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ for NADP⁺- and NAD⁺-dependent malic enzyme and $99 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ for pyruvate dehydrogenase, levels which are some twofold higher than those found in extracts from free-living cells grown on fumarate (Table 1).

Since pyruvate formation from malate in cell-free extracts has the same cofactor requirements (NAD⁺/NADP⁺, Mn²⁺) as malic enzyme, this activity appears to be critical for dicarboxylate metabolism. Oxidation of dicarboxylates through the TCA cycle would then involve the conversion of one mole of dicarboxylate to pyruvate and acetyl-CoA via malic enzyme and pyruvate dehydrogenase, and another to oxaloacetate. While malic enzyme appears to be the critical enzyme in pyruvate formation, we cannot eliminate the possibility that in wild-type cells the PEPCK-pyruvate kinase route may function in parallel with it. In bacteroids of MNF3841, where PEPCK activity is very low [$10 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$; McKay *et al.*, 1985] relative to malic enzyme [$210\text{--}240 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$; this paper], the PEPCK route does not appear to be quantitatively significant.

It is unclear whether the NAD⁺ and NADP⁺-dependent malic enzyme activities in crude extracts of MNF3841 are properties of two different proteins, as the genetic evidence suggests in *Escherichia coli* (Hansen & Juni, 1975). In MNF3841, we have failed to isolate transposon Tn5-induced mutants in malic enzyme, despite screening of 20000 transconjugants. No mutant with the expected phenotype (able to grow on pyruvate, but not on succinate, L-arabinose or L-glutamate) has been found, suggesting that in MNF3841 there may also be two different enzymes. Attempts to clarify this question in cell-free extracts using electrophoretic analysis or the additivity of NAD⁺- and NADP⁺-linked activities have proven unsatisfactory; purification of the enzyme(s), currently in progress, should resolve the situation.

Since bacteroids are believed to require dicarboxylates for active N₂ fixation, the route of dicarboxylate breakdown is important. Arsenite inhibition resulted in the accumulation of pyruvate from fumarate by isolated pea bacteroids. Malic enzyme was also present in pea bacteroids, at activities twice those found in free-living cells grown on fumarate, suggesting that it is responsible for pyruvate formation. The importance of the elevated activities of malic enzyme and pyruvate dehydrogenase is not clear, since dicarboxylate transport rates in bacteroids are lower than those in free-living cells (Glenn *et al.*, 1980; Finan *et al.*, 1981, 1983; Saroso *et al.*, 1984). Isolated pea bacteroids thus have the transport system for dicarboxylates and malic enzyme seems to be one of the catabolic enzymes necessary for their metabolism. Mutants defective in malic enzyme should therefore, like mutants in the *dct* system, be unable to fix N₂.

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