Fructose Metabolism in Wild-type, Fructokinase-negative and Revertant Strains of *Rhizobium leguminosarum*

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Rhizobium leguminosarum accumulates fructose by an active process sensitive to azide, 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenylhydrazone. The fructose is not phosphorylated during transport. Sorbose and glucose interfere with fructose uptake. Inside the cell fructose is metabolized via fructose 6-phosphate; there is no evidence for an alternative metabolic route via sorbitol to glucose or via sorbitol 6-phosphate to fructose 6-phosphate. Tn5-induced mutants lacking fructokinase failed to grow on fructose, mannitol or sorbitol and grew slowly on sucrose; growth was normal on all other single carbon sources tested. Growth of these mutants on a range of carbon sources was retarded by added fructose. Revertants which had regained the capacity to utilize fructose all had an unstable fructokinase which could be partially stabilized by fructose.

INTRODUCTION

Legume root nodules contain a variety of carbohydrates including sugars such as glucose, fructose and sucrose (Streeter, 1980). Fructose, both as the free sugar and as a constituent of sucrose, is potentially a carbon source for bacteroid growth and development and subsequent N₂ fixation.

The requirement for fructose catabolism for the establishment of an effective symbiosis has been investigated using mutants defective in fructose uptake or phosphorylation. In *Rhizobium trifolii* (Ronson & Primrose, 1979) and *R. leguminosarum* (Glenn *et al.*, 1984) mutants defective in fructose metabolism nodulated and fixed N₂. However, in *R. meliloti* (Duncan, 1981) a fructokinase mutant formed ineffective nodules. These data suggest that in the closely related *R. trifolii* and *R. leguminosarum* the capacity to utilize fructose is not essential for the development of an effective symbiosis. Nevertheless, they do not eliminate fructose as a substrate used simultaneously with other carbon compounds. A study of substrate selection in *R. leguminosarum* 3841 (Dilworth *et al.*, 1983) indicated that in binary mixtures of carbon sources there was simultaneous utilization even though the substrates may be utilized at markedly different rates.

Little is known about the uptake and catabolism of fructose in *Rhizobium* except for the presence of a cytoplasmic fructokinase (Martinez-de Drets & Arias, 1970; Ronson & Primrose, 1979; Glenn *et al.*, 1984). In many organisms the initial step of fructose metabolism involves a phosphoenolpyruvate-dependent phosphotransferase system (Baumann & Baumann, 1975; Sawyer *et al.*, 1977; Van Dijken & Quayle, 1977). The fructose 1-phosphate is then further phosphorylated to yield fructose 1,6-bisphosphate which can then be metabolized via the Entner–Doudoroff and/or Embden–Meyerhof pathways (Sawyer *et al.*, 1977).

During the course of a study on Tn5-induced sugar mutants of *R. leguminosarum* 3841 (Glenn *et al.*, 1984) two fructose-negative strains were isolated which grew poorly on sucrose. This paper

reports on the uptake system and metabolism of fructose in *R. leguminosarum* and provides an explanation for the poor growth of fructose non-utilizing strains on sucrose.

METHODS

Organism. *Rhizobium leguminosarum* MNF3841 is a *str^r* derivative of strain 300 (Johnston & Beringer, 1975). Mutant strains are shown in Table 1.

Media. Bacteria were grown in batch culture at 28 °C in the minimal salts medium of Brown & Dilworth (1975) with NH_4^+ (10 mM) as nitrogen source, phosphate at 0.3 mM, and carbon sources at 10 mM, except for glycerol which was at 20 mM. The medium was buffered with 40 mM-HEPES, pH 7.2.

Mutagenesis. Tn5 mutagenesis was carried out at 28 °C using the procedure developed by Beringer *et al.* (1978). Fructose-utilizing revertants were isolated by plating 10^8 mutant cells on to fructose minimal salts agar.

Nodulation and preparation of bacteroids. Pea plants (*Pisum sativum* L. cv. Greenfeast) in pots were nodulated by *R. leguminosarum* strains and bacteroids were prepared as described by Glenn *et al.* (1980); they were used immediately after isolation.

Radiochemicals. D-[U- ^{14}C]Fructose ($10.3 \text{ GBq mmol}^{-1}$), $^3\text{H}_2\text{O}$ (185 MBq g^{-1}) and [^{14}C]inulin ($185 \text{ MBq mmol}^{-1}$) were purchased from Amersham.

[^{14}C]Fructose uptake experiments. Cells were prepared and uptake experiments conducted as described by Hudman & Glenn (1980), except that the cells were centrifuged at 22 °C and washed with minimal salts (Brown & Dilworth, 1975) rather than Millipore filtered before use. The scintillant was as described by Hudman & Glenn (1980) with the addition of Triton X-100 (300 ml l^{-1}).

Determination of cell volume. The cell volume in pellets was determined using the $^3\text{H}_2\text{O}$ and [^{14}C]inulin technique described by Stock *et al.* (1977). In the suspension used for determination of intracellular fructose concentrations, the cell volume was $1.45 \text{ ml (g dry wt cells)}^{-1}$.

Chromatography. Fructose and its phosphorylated derivatives were separated by descending chromatography on Whatman 3M paper in a solvent system containing 0.1 M-EDTA, pH 5, 1 M-ammonium acetate, pH 5, and 95% ethanol in the ratio 1:30:70, by vol. (Baumann & Baumann, 1975). Chromatograms were run for 9–10 h during which the solvent front progressed 27–28 cm. Sugars were detected by using a reagent consisting of a 9:1 (v/v) mixture of phloroglucinol (0.7%, w/v, in toluene) and 40% TCA. The toluene was allowed to evaporate and the paper was heated to 120–140 °C for 5 min.

Isolation of intracellular fructose. MNF3850 was grown in succinate (10 mM) plus glycerol (20 mM) to an A_{600} of approximately 0.7; the cells were harvested by centrifugation, washed with minimal salts (Brown & Dilworth, 1975) and resuspended in minimal salts to an A_{600} of approximately 0.5. [^{14}C]Fructose (37 kBq) and cold fructose ($0.025 \text{ } \mu\text{mol}$) were added to 2.5 ml cells. After 10 min the cells were filtered on a $0.45 \text{ } \mu\text{m}$ Millipore filter, washed twice with minimal salts and resuspended in 1.0 ml salts containing 2.5 mM-unlabelled fructose. This material was heated to 100 °C for 10 min, centrifuged, and the resultant supernatant was chromatographed. Some 97% of the radioactive fructose was taken into the cells and of this about 90% was released by the hot water extraction. The chromatogram was dried, cut into 1 cm sections and the radioactivity present in each section was determined by liquid scintillation counting.

Analytical methods. Protein was determined by the Lowry method using bovine serum albumin as a standard. Fructose was estimated using the anthrone and resorcinol methods described by Herbert *et al.* (1971).

Enzyme assays. Cell-free extracts were prepared as described by Glenn *et al.* (1984). Fructokinase (EC 2.7.1.4) was measured using the glucokinase method of Lynch *et al.* (1975) with fructose as the substrate. Sorbitol dehydrogenase (EC 1.1.1.14) was measured using the technique of Wolff (1955). Sorbitol 6-phosphate dehydrogenase (EC 1.1.1.140) was measured by the method of Horwitz (1966) and aldose reductase (EC 1.1.1.21) by the method of Velle (1975).

Table 1. *Strains of R. leguminosarum*

Strain	Derivation	Growth on:	
		Fructose	Glucose
MNF3841	Str ^a mutant of wild-type strain 300*	+	+
MNF3850	Tn5 ⁺ derivative of MNF3841	—	+
MNF3855	Tn5 ⁺ derivative of MNF3841	—	+
MNF3851	Fructose-utilizing (Frk ⁺) revertant of MNF3850†	+	+
MNF3852	Fructose-utilizing (Frk ⁺) revertant of MNF 3850†	+	+

* Johnston & Beringer (1975).

† Phenotypic symbol: Frk, fructokinase.

RESULTS AND DISCUSSION

Growth properties of fructose⁻ mutants

Tn5-induced fructokinase mutants MNF3850 and MNF3855 (Glenn *et al.*, 1984) failed to grow on fructose, sorbitol or mannitol and grew poorly on sucrose. The parent, MNF3841, grew well on all these carbon sources. The growth of these mutants on other sugars such as glucose and arabinose and on organic acids like succinate and malate was indistinguishable from that for MNF3841. The poor growth of these two strains on sucrose was investigated further by comparing growth of the parent and mutants on glucose, glucose plus fructose, and sucrose. Cells were grown on 20 mM-glycerol-minimal salts to an A_{600} of approximately 0.8, centrifuged, washed and resuspended in glucose (10 mM) or glucose plus fructose (both 10 mM), or sucrose (10 mM). Samples were taken at 0.5 h intervals for determination of A_{600} . Strain MNF3841 had mean generation times of 3.5, 3.3 and 3.5 h, respectively, on glucose, glucose plus fructose, and sucrose. The mutant strains, MNF3850 and MNF3855, had generation times on glucose similar to the parent, but were markedly slower on sucrose. Fructose depressed the rate at which the mutants grew on glucose by some 50% (Table 2). This growth rate inhibition was not due solely to the structural similarity of fructose and glucose, since the same result was seen when fructose was added to glycerol or succinate (Table 2). Strain MNF3851, a fructose-utilizing revertant of MNF3850, was similar to the parent strain MNF3841. Strains MNF3841 and MNF3851 grew on mixtures of glucose plus fructose or succinate plus fructose at rates identical to growth on glucose, though growth on glycerol plus fructose, or succinate plus fructose, was faster than on the single carbon source (Table 2).

[¹⁴C]Fructose uptake

Cells of MNF3841 grown on a variety of carbon sources were able to take up and metabolize labelled fructose, whereas strains MNF3850 and MNF3855 accumulated but did not metabolize fructose. These data are consistent with the constitutive nature of fructose utilization reported previously for this strain (Glenn & Dilworth, 1981*a*). [¹⁴C]Fructose uptake by MNF3850 was an active process since it was inhibited by azide (1 mM), 2,4-dinitrophenol (1 mM) and carbonyl cyanide *m*-chlorophenylhydrazone (0.025 mM) (Table 3).

The specificity of the fructose uptake system was examined by adding various sugar and sugar phosphates 1 min before the addition of radioactive fructose. The addition of the phosphorylated sugars glucose 6-phosphate, fructose 6-phosphate, fructose 1-phosphate or fructose 1,6-bisphosphate (all at 2.5 mM) had little or no effect on [¹⁴C]fructose uptake. Of the sugars tested, sorbose (2.5 mM) had the greatest effect, consistent with its structural similarity to fructose (Table 3), while glucose (2.5 mM) decreased [¹⁴C]fructose uptake by 50%. Galactose, fucose, sucrose and mannitol had no effect (Table 3).

Isolated bacteroids of MNF3841 failed to accumulate [¹⁴C]fructose even though they were able to take up [¹⁴C]succinate. This observation is consistent with the lack of fructose oxidation observed previously in bacteroids of this strain (Glenn & Dilworth, 1981*a*). It shows that fructose, like glucose (Hudman & Glenn, 1980) and sucrose (Glenn & Dilworth, 1981*b*) cannot be transported by isolated bacteroids even though the uptake systems for all three have been shown to be constitutive in this strain (Glenn & Dilworth, 1981*a*).

Is the accumulated [¹⁴C]fructose phosphorylated?

To determine if the [¹⁴C]fructose accumulated by the fructose non-metabolizing mutant MNF3850 were phosphorylated, the cell-associated radioactive material was extracted and chromatographed. There was a single peak of radioactivity accounting for more than 95% of the label applied to the chromatogram which migrated with an R_F of 0.67 identical to that of authentic fructose. There was no evidence for phosphorylated derivatives of fructose. Identical results were obtained with strain MNF3855.

This experiment provides clear evidence that *R. leguminosarum* does not transport fructose by means of a PEP-dependent uptake system. In this respect it is different from many *Pseudomonas* species (Van Dijken & Quayle, 1977; Sawyer *et al.*, 1977). This observation together with earlier

Table 2. *Effect of fructose on the growth of various strains of R. leguminosarum*

Rhizobium leguminosarum strains were grown in glycerol-minimal salts broth to an A_{600} of approximately 0.8, centrifuged, washed and resuspended in fresh media containing different carbon sources. At 0.5 h intervals samples were taken for measurement of A_{600} .

Carbon source	Strain...	Mean generation time (h)			
		3841	3850	3851	3855
Glucose		3.3	3.8	3.65	4.0
Fructose		4.5	—	4.6	—
Sucrose		3.5	9.6	3.6	9.0
Glucose/fructose		3.3	6.25	3.65	6.5
Glycerol		3.75	3.75	4.0	3.9
Glycerol/fructose		3.0	6.45	3.45	6.7
Succinate		3.9	3.45	4.0	3.7
Succinate/fructose		ND	5.1	3.1	5.05

ND, Not determined. —, No growth.

Table 3. *The effect of metabolic inhibitors and other sugars on [14 C]fructose uptake by R. leguminosarum MNF3850*

Cells were grown on glycerol (20 mM). The data are averages from two separate experiments. The control rate of uptake was $9 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$.

Addition	Percentage of control
None	100
Carbonyl cyanide <i>m</i> -chlorophenyl hydrazone (0.025 mM)	1
2,4-Dinitrophenol (1 mM)	2
Azide (1 mM)	2
Fructose 1-phosphate (2.5 mM)	98
Fructose 6-phosphate (2.5 mM)	103
Fructose 1,6-bisphosphate (2.5 mM)	95
Glucose 6-phosphate (2.5 mM)	108
Fucose (2.5 mM)	77
Galactose (2.5 mM)	110
Glucose (2.5 mM)	50
Mannitol (2.5 mM)	98
Sorbose (2.5 mM)	22
Sucrose (2.5 mM)	107

work on glucose uptake in *R. leguminosarum* (Hudman & Glenn, 1980) shows that this organism does not have a phosphotransferase system.

Intracellular fructose concentration

The intracellular concentration of fructose in MNF3850 and MNF3855 was determined by measuring the amount of [14 C]fructose accumulated from 1 ml of a solution containing 7.4 kBq [14 C]fructose and 0.1 mM unlabelled fructose after 5, 15 and 120 min. Accumulation was essentially complete after 15 min, when the intracellular concentration of fructose was 30 mM, a 300-fold increase in concentration over that in the medium. In a comparable experiment with MNF3841, only 20% of the accumulated radioactivity was water extractable. When this soluble material was separated using the paper chromatography system described previously, about 15% was identifiable as fructose, corresponding to an intracellular concentration of 0.4 mM. Fructose 6-phosphate accounted for a further 31% of the soluble cell-associated radioactivity and 32% of the radioactivity remained at the origin, a totally different pattern to that observed in MNF3850 and MNF3855.

Fig. 1. The effect of fructose on the stability of fructokinase in parent and revertant strains of *R. leguminosarum*. Cell-free extracts were prepared from fructose-grown cells and incubated at 28 °C without further addition [MNF3841 (Δ); MNF3851 (■)], or with the addition of 2 mM-fructose [MNF3841 (▲); MNF3851 (●)]. The 100% enzyme activity was 95 and 6.8 nmol min⁻¹ (mg protein)⁻¹ for MNF3841 and MNF3851, respectively.

These experiments suggest that in MNF3850 and MNF3855 the process of accumulation of fructose, or fructose itself, or both, may cause the inhibition of growth when fructose is added to a second carbon source.

Fructose-utilizing (*Frk*⁺) revertants

Frk⁺ revertants of MNF3850 and MNF3855 were selected by plating on to fructose minimal salts plates, and arose at a frequency of about 2×10^{-6} for both mutants. All such clones examined were kanamycin resistant; thus a copy of Tn5 was retained in the genome of each. Thus, the *Frk*⁺ phenotype of pseudorevertants could arise from a suppressor mutation within Tn5, or within the associated fructokinase gene sequence or elsewhere in the genome. The pseudorevertant strains regained the capacity to grow on fructose, sorbitol or mannitol and grew well on sucrose (Table 2). The growth rate and final turbidity of MNF3851 (a *Frk*⁺ revertant of MNF3850) on fructose (2 mM) were identical to those for MNF3841. Similarly, the rate of consumption of fructose was 0.78 μmol h⁻¹ per *A*₆₀₀ unit in MNF3841 and 0.70 μmol h⁻¹ per *A*₆₀₀ unit in MNF3851, compared with 0 in MNF3850.

Since both MNF3850 and MNF3855 have been shown to lack fructokinase (Glenn *et al.*, 1984) the growth characteristics of revertants like MNF3851 and MNF3852 suggested that the fructokinase activity had been restored. The parent strain, MNF3841, had a high specific activity of fructokinase [70–80 nmol min⁻¹ (mg protein)⁻¹] when grown on glycerol. However, initial attempts to demonstrate fructokinase activity in cell-free extracts of glycerol-grown revertants were unsuccessful. Even when cell-free extracts were prepared from revertant cells grown on fructose, the fructokinase activity was relatively low [2–15 nmol min⁻¹ (mg protein)⁻¹] compared with MNF3841 [100–120 nmol min⁻¹ (mg protein)⁻¹]. This relatively low fructokinase activity in extracts of MNF3851 and MNF3852 grown on fructose was unstable with a half-life of 15 min at 28 °C. When revertant extracts were stored on ice, the fructokinase activity declined with a half-life of 20–30 min. The fructokinase activity derived from MNF3841 was completely stable at these temperatures (Fig. 1).

To test the idea that fructokinase in the revertants may be stabilized *in vivo* by its sugar substrate, fructose (2 mM) was added to extracts of MNF3851 and MNF3852. The fructose-treated and control extracts were incubated at 30 °C and samples were removed at intervals for immediate assay for fructokinase activity. The addition of fructose markedly increased fructokinase stability (half-life 2 h), but the enzyme was still less stable than that in MNF3841 (Fig. 1; data shown only for MNF3851). A further twelve independently isolated revertants of both MNF3850 and MNF3855 showed a similar instability of fructokinase. Although all the revertants grew well on fructose at 28 °C, four of the fourteen, including MNF3852, failed to grow on fructose at 33 °C. Controls showed that the parent MNF3841 grew well on fructose at 33 °C and all the revertants grew well on tryptone-yeast extract medium (Beringer, 1974), sucrose or succinate at 33 °C.

The possibility that some inhibitor of fructokinase was being produced in the revertants was investigated by a mixing experiment. An extract of glycerol-grown MNF3841 was assayed for total activity (59 nmol min⁻¹), while the activity in the revertant MNF3852 was 0. When the two extracts were mixed together, the total activity remained essentially unchanged (63 nmol min⁻¹).

Alternative pathways of fructose metabolism, via sorbitol to glucose, or via sorbitol 6-phosphate to fructose 6-phosphate were excluded because of insufficient enzyme activity. Although there is an NAD⁺-dependent sorbitol dehydrogenase induced in cells grown on fructose [80 nmol min⁻¹ (mg protein)⁻¹], NADP⁺-dependent aldose reductase activity was low [5 nmol min⁻¹ (mg protein)⁻¹] with an equilibrium markedly in favour of sorbitol formation (Velle, 1975). *Rhizobium leguminosarum* MNF3841, like *R. meliloti* (Martinez de Drets & Arias, 1970), lacks sorbitol-6-phosphate dehydrogenase. These observations support the view that in the fructose-utilizing revertants fructose is metabolized via fructokinase to fructose 6-phosphate.

MNF3850 and MNF3855 both nodulate and are effective on peas, though the onset of nodulation is delayed in comparison with the parent MNF3841.

Given the precedent of fructose inhibiting growth in the fructokinase mutants, the possibility of accumulated compounds in other mutants causing similar effects cannot be ignored. If the accumulated material were to render the bacteroids unable to fix nitrogen, that mutation might erroneously be considered significant to nitrogen fixation.

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