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Sucrose transport and hydrolysis in *Rhizobium tropici*

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Abstract

The *Rhizobium tropici* strain CFN 299 was maintained on PY medium and was grown in minimal medium (MM) with sucrose, glucose, fructose and glutamate (or their combination) as carbon sources. Bacteria were able to simultaneously use different carbon sources and, with a combination sucrose and glutamate, the growth rate was faster than with either carbon source alone. Sucrose transport was induced by sucrose and partially repressed by glucose and glutamate if they were included in MM as additional carbon sources. The transport of sucrose was active because both an uncoupler (dinitrophenol, DNP) and inhibitors of terminal oxidation (KCN, NaN_3) severely reduced sucrose uptake. Sucrose transport was also sensitive to a functional sulfhydryl reagent but was much less sensitive to EDTA and arsenate. We obtained nonlinear Lineweaver-Burk plots for the uptake of sucrose (by sucrose-grown bacteria), and this implied the existence of at least two uptake mechanisms. Invertase (EC 3.2.1.26) is the main enzyme for sucrose hydrolysis in this organism. This enzyme was induced by sucrose and had high activity in mid-log phase cells when sucrose was the sole carbon source (0.2%). Invertase activity was not detected in growth medium. In general, the results obtained support the idea, that *R. tropici* is adapted to sucrose utilization and to multicarbon nutrition during its interaction with plants.

Introduction

In legume-*Rhizobium* interactions, the bacteria use carbon compounds supplied by host plants. Sucrose is a major component of the carbon material translocated in the phloem to the roots and nodules, and is a major carbon compound in nodules (Streeter, 1991). Sucrose synthase, the major enzyme for sucrose breakdown in nodules has been identified as nodulin 100 in soybean (Thummer and Verma, 1987). Whether sucrose is a potential carbon substrate for bacteria inside the host cell would be important to investigate.

A general dogma of symbiotic nitrogen fixation in legumes is that bacteroids use only C_4-dicarboxylates as carbon and energy sources, primarily malate and succinate (Day and Copeland, 1991; Streeter, 1991; Vance and Hiechel, 1991). However, some reports do not prove the concept of a exclusive role of C_4-dicarboxylates in the carbon nutrition of bacteroids (Duncan, 1981; Herrada et al., 1989; Romanov et al.,1985). *Rhizobium* may use sucrose as carbon source at least during the infection process and the early stages of nodule development. This process could be important in the formation of effective nodules, but information concerning sucrose metabolism in Rhizobia is very limited. This is why we decided to begin this project.

Rhizobium tropici CFN 299 is a broad host range *Rhizobium*, that nodulates *Phaseolus vulgaris* bean and *Leucaena* spp. (Martínez et al.,

1991). In the present paper we suggest that this organism possesses at least two active transport systems for sucrose uptake. These bacteria lack catabolite repression by glucose and are able to use glucose simultaneously with sucrose or glutamate.

Materials and methods

Chemicals

($U^{14}C$)-sucrose (475 mCi/mmol) and ($U^{14}C$)-glucose (320 mCi/mmol) were from DuPont & Co. Inc.; L-($U^{14}C$)-glutamate (262 mCi/mmol) and (6,6(n)^{3}H)-sucrose (14.4 Ci/mmol) were from Amersham. All other chemicals were reagent grade and were purchased from standard sources.

Cell Growth

One day old cultures of *R. tropici* CFN 299 grown on PY medium (Noel et al., 1984) agar plates were used to inoculate liquid MM. A basal MM of the following composition was used: K_2HPO_4 - 0.25 g; KH_2PO_4 - 1.0 g; $MgSO_4$ $7H_2O$ - 0.2 g; KNO_3 - 0.7 g; $CaCl_2$ - 0.2 g; $FeCl_3$ - 0.01 g; Biotin - 1 mg; H_3BO_3 - 3.17 mg; Na_2MoO_4 - 1.0 mg; $MnSO_4 4H_2O$ - 1.52 mg; $ZnSO_4 7H_2O$ - 0.25 mg; $CuSO_4$ $5H_2O$ - 0.087 mg; 1.0 liter demineralized water; the pH was adjusted to 6.8 with 1*N* NaOH. The basal MM was usually supplemented with 2 g of carbon source per liter. When two carbon sources were used, an equal mass of each carbon compound was added. In the MM containing glutamate, KNO_3, was omitted. Cultures were grown at 30°C (220 rpm). Growth rate was determined at 600 nm and/or by protein measurement.

Transport assays

A modified sugar uptake procedure (Wong, 1990) was used to analyze the transport kinetics of sucrose, glucose and glutamate. Cells were harvested at mid-log phase and washed two times with 0.05 *M* K-phosphate buffer, pH 6.6. Washed cells were resuspended in fresh buffer to 0.65–1.0 mg protein per mL. Suspensions were incubated 1 h with gentle stirring to deplete endogenous substrates. Twenty-four well tissue culture plates (Costar, Cambridge, Mass.) were used. Each duplicate well contained 0.8 mL carbon-free MM with radiolabeled sucrose, glucose, glutamate or its combinations; the concentrations of sucrose and glucose were 0.1 m*M* and glutamate was 0.05 m*M*. Specific radioactivity of each substrate was always kept at 0.1 mCi/mmol when either one or two substrates were used. The plates were taped on a standard shaker at 23–25°C, at 200 rpm. Transport experiments were started by adding 0.2 mL of cell suspension to each well. When the effect of inhibitors was studied, transport was started by adding (U^{14})-sucrose, inhibitors were added 1 min before the addition of radioactivity and uptake was measured for 5 min. Without inhibitors, after 1, 2, 3 or 5 min 0.15–0.3 mL cell samples from each well were transferred to 1 mL ice-cold 2% sucrose or glucose, or glutamate or its combination in MM. Cells were washed twice by centrifugation in a Microfuge at 4° C with the same MM, transferred to POPOP-PPO-toluene scintillator solution and counted in a Beckman LS 7000 scintillation counter at least two times. Data presented in the figures are the means calculated from four time points of duplicates if not specially mentioned. Boiled cells were used to determine the background counts for non specific binding after incubation with radiolabeled compounds during 5 min. This control usually shows very low radioactivity, less than 1% of the experiments.

Other assays

Washed cell suspensions in 0.05*M* K-phosphate buffer pH 6.6 were sonicated on ice, centrifuged for 10 min in a Microfuge in cold and the supernatant was used to determine invertase activity (Hoelzle and Streeter, 1990). Sucrose phosphorylase was assayed using the method of Mieyal (1972). The concentration of the sucrose, glucose, fructose and glutamate during the bacteria growth was measured in culture supernatants, using Boehringer kits. Protein was determined by the method of Bio-Rad laboratories.

Table 1. Invertase activity in *R. tropici* grown in the presence of different carbon sources[a]

Carbon sources	Invertase
Glucose	22.1±1.4
Fructose	24.6±1.6
Glutamate	32.0±4.7
Glucose + Fructose	19.6±0.9
Glucose + Sucrose	63.1±2.1
Sucrose	81.9±1.9

[a]Units are glucose liberated per minute per mg protein and are average of four replicate assays.

Results and discussion

There is a report in the literature that some of the fast-growing Rhizobia have a constitutive sucrose uptake system, while others have an inducible system (Glenn and Dilworth, 1981). In *R. tropici*, sucrose uptake is inducible (Fig. 1a) and an unexpected finding was that in glutamate-grown cells, sucrose transport was markedly higher than that of fructose- or glucose-grown cells. To obtain more information, cells were grown in two carbon sources and it was confirmed that glucose repressed sucrose transport more strongly than glutamate (Fig. 1b).

Invertase activity in *R. tropici* was found in cells grown on any of the carbon sources tested, but this enzyme was also strictly induced by sucrose (Table 1). Our attempts to show sucrose phosphorylase activity failed and this indicates that invertase is the main enzyme for sucrose hydrolysis in this organism. There was no invertase activity found in the culture supernatant. From the data presented in Figure 1 and Table 1, it may be speculated that in *R. tropici*, sucrose transport and invertase synthesis may be coordinately regulated.

The results from Figure 1 and Table 1 lead us to suggest that *R. tropici* is probably able to utilize different carbon substrates simultaneously, as it is known for *R. trifolii* (De Hollander and Stouthamer, 1979) and *R. leguminosarum* bv. *viciae* (McKay et al., 1989). First, it was shown that during the growth of bacteria in the presence of two carbon sources, the concentrations of sucrose and glucose (Fig. 2b) or of sucrose and glutamate (Fig. 2c) in the culture supernatant decreased simultaneously. The same result was obtained for glucose + glutamate or glucose + fructose as carbon sources (data not shown). Other evidence for this phenomenon was obtained from the uptake experiments.

Sucrose + glucose -grown bacteria show uptake activity for both these carbon substrates measured either independently (Fig. 3a) or simultaneously (Fig. 3b). From the results of the co-transport experiment, it is clear that when present in equimolar concentrations (0. 1 m*M*), glucose does not influence sucrose transport, but sucrose sharply reduces glucose uptake. This was confirmed in experiments using ($U^{14}C$)-sucrose + glucose or with ($U^{14}C$)-glucose + sucrose. Glucose at a concentration 10 fold higher than sucrose reduced sucrose uptake by only 20% (data not shown).

Sucrose + glutamate grown bacteria show uptake activity of both substrates (Fig. 4a) and co-transport measurements demonstrate only weakly diminished glutamate uptake (Fig. 4b). It appears that these systems are probably working independently. It is important to note that with a combination of sucrose and glutamate, the bacteria growth rate was faster than with either of them independently (Fig. 2a and 2c). This could be important in the course of interaction of the bacteria with host legume.

A nonlinear (biphasic) Lineweaver-Burk plot was obtained for sucrose-grown cells when sucrose uptake was measured at different sucrose concentrations (Fig. 5). This suggested that a high-affinity (K_m = 0.04 m*M*) and a low-affinity (K_m = 1.0 m*M*) transport systems are present in *R. tropici*. The apparent K_m values for transport did not vary significantly in different experiments and high V_{max} values for both systems (56.8±4.9 and 19.0±1.6 nmol·min^{-1} mg protein^{-1}) were consistently obtained with sucrose-grown cells. This data indicates that *R. tropici* has a very effective system for sucrose uptake.

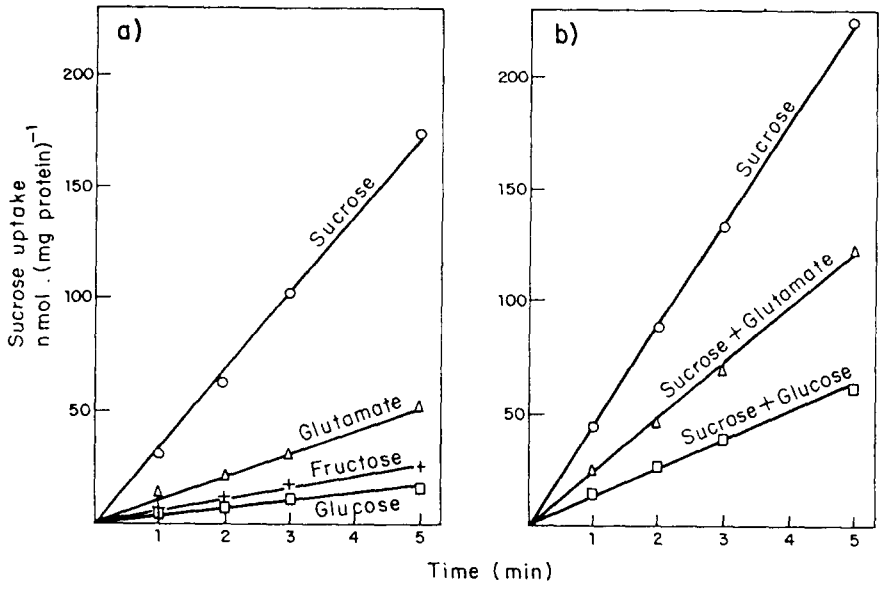

Fig. 1. Kinetics of (^{14}C)-sucrose uptake by *R. tropici*, grown on minimal medium plus **a** sucrose (O), glutamate (△), fructose (+), glucose (□), and **b** sucrose+glutamate (△) and sucrose+glucose (□). **a** - experiment 1; **b** - experiment 2.

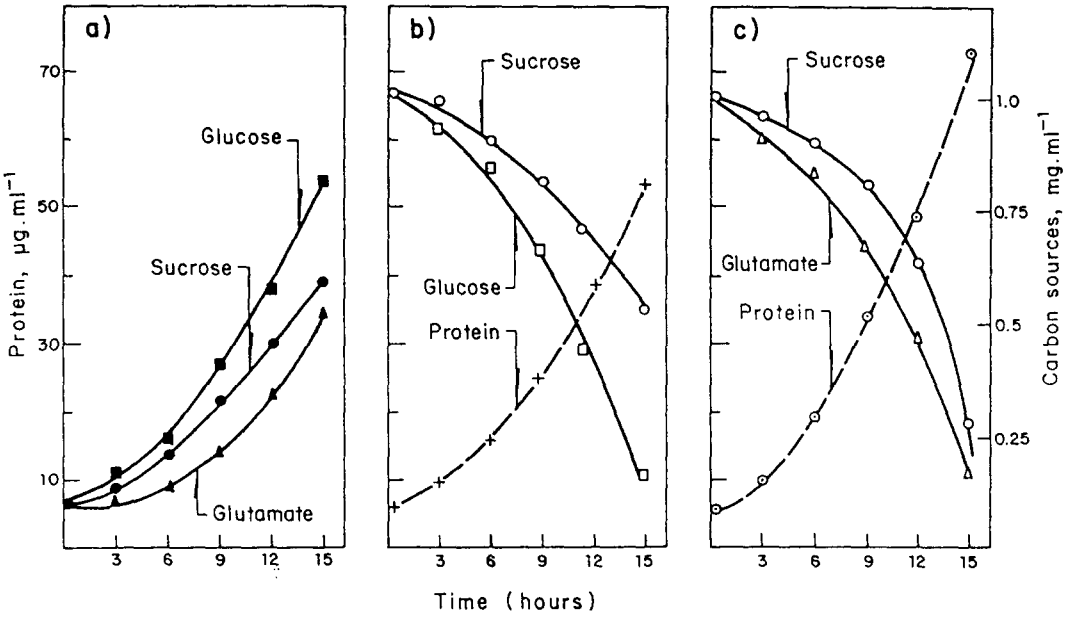

Fig. 2. Growth of *R. tropici* and carbon source concentration in minimal medium, containing single (**a**) or double (**b** and **c**) carbon sources. Growth was determined by protein measurements of cultures and plotted as function of time. a - growth on MM plus glucose (■), sucrose (●) or glutamate (▲). b - growth on MM plus glucose+sucrose (+); (○) sucrose and (□) glucose concentration in culture. c - growth on MM plus sucrose+glutamate (⊙); (○) - sucrose and (△) - glutamate concentration in culture. This figure represents the data from one of the two experiments which shows very similar results.

Both an uncoupler and inhibitors of terminal oxidation severely reduced the uptake of 0.1 m*M* sucrose in sucrose-grown cells (Table 2). The sulfhydryl reagent N-ethylmaleimide inhibited sucrose uptake by more than 96%. EDTA and arsenate however, had little effect. These data

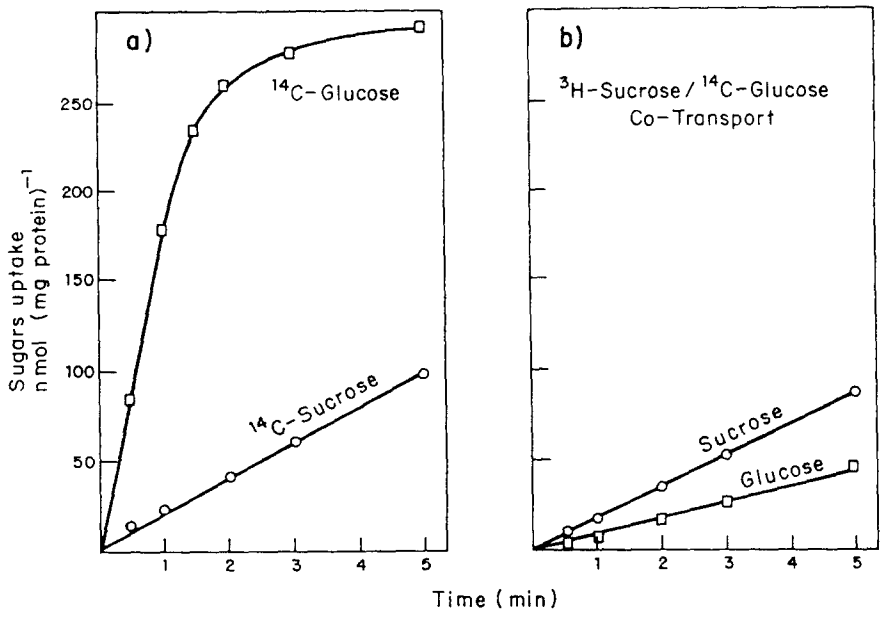

Fig. 3. Uptake of sucrose and glucose by *R. tropici* grown on minimal medium containing sucrose+glucose.**a**- independent measurements of (^{14}C)-sucrose and (^{14}C)- glucose uptake. **b** - simultaneous uptake of (^{3}H)-sucrose and (^{14}C)-glucose.

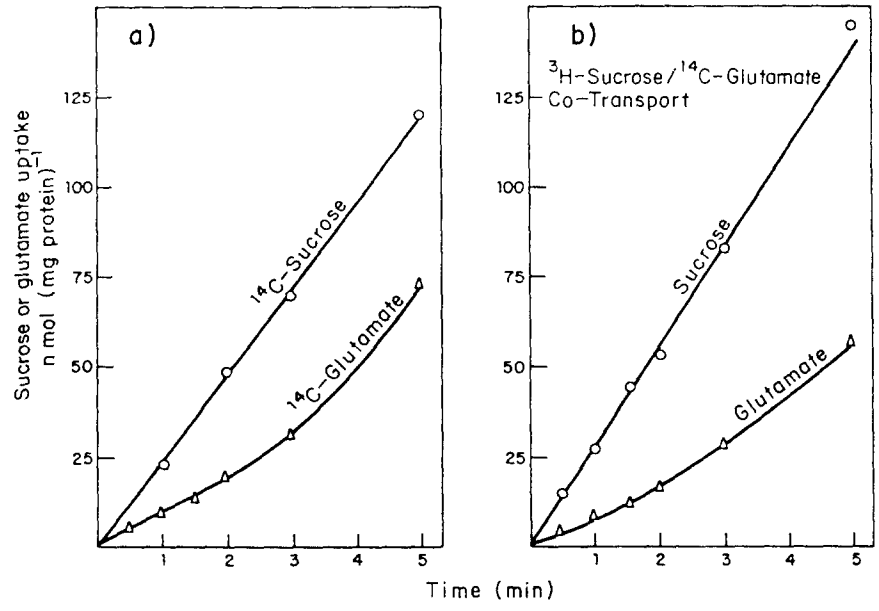

Fig. 4. Uptake of sucrose and glutamate by *R. tropici* grown on minimal medium containing sucrose+glutamate. **a**- independent measurements of (^{14}C)-sucrose and (^{14}C)-glutamate uptake. **b** - simultaneous uptake of (^{3}H)-sucrose and (^{14}C)-glutamate.

suggested that the uptake of sucrose in *R. tropici* is via an active process.

As for organic acids, in particular C_4-dicarboxylates, it was shown that sucrose+malate-grown bacteria were able to take up both substrates (data not shown), but that the presence of malate in MM caused the bacteria to aggregate. To address this question, other growth condition for *R. tropici* would be needed.

In summary, we stress two important and interesting points: (a) *R. tropici* strain CFN 299 is well adapted for the uptake of sucrose in a broad

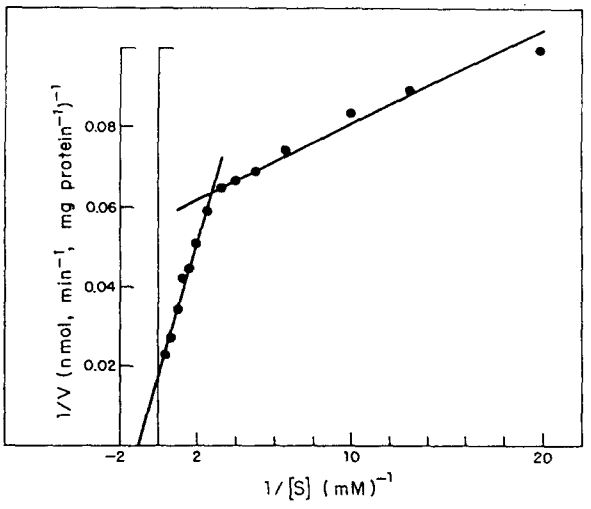

Fig. 5. Lineweaver-Burk plot of the rate of sucrose transport by sucrose-grown *R. tropici*. Specific radioactivity 0.1 mCi/mmol.; concentration range 0.05 to 3.0 m*M*. Uptake was determined at 2 min. This figure represents the data from one of the five experiments which shows similar results.

Table 2. Effect of metabolic inhibitors on sucrose transport by sucrose grown *Rhizobium tropici*[a]

Inhibitor	Inhibition %
Dinitrophenol (1 m*M*)	98.7±0.4
Potassium cyanide (1 m*M*)	95.8±0.2
Azide (2 m*M*)	88.8±0.2
N-Ethylmaleimide (1 m*M*)	96.7±1.5
EDTA (10 m*M*)	16.0±4.7
Arsenate (5 m*M*)	2.9±1.3

[a]Inhibitors were added 1 min before the addition of radioactivity and uptake was measured for 5 min. Results are average of three experiments and control rate was 42.6±5.1 nmol/min per mg protein.

range of sucrose concentration; (b) this organism lack catabolite repression control by glucose and, probably, could utilize different sugars and aminoacids (at least glutamate) simultaneously.

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