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Fructose Transport in *Neurospora crassa*

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A specific fructose uptake system (K_m = 0.4 mM) appeared in *Neurospora crassa* when glucose-grown mycelia were starved. Fructose uptake had kinetics different from those of intramycelial fructose phosphorylation, and uptake appeared to be carrier mediated. The only sugar which competitively inhibited fructose uptake was L-sorbose (K_i = 9 mM). Glucose, 2-deoxyglucose, mannose, and 3-*O*-methyl glucose were noncompetitive inhibitors of fructose uptake. Incubation of glucose-grown mycelia with glucose, 2-deoxyglucose, or mannose prevented derepression of the fructose transport system, whereas incubation with 3-*O*-methyl glucose caused the appearance of five times as much fructose uptake activity as did starvation conditions.

Two general sugar transport systems have been described so far in *Neurospora* mycelia. System I, which is present constitutively in glucose-grown mycelia, transports glucose, 3-*O*-methyl glucose, and probably L-sorbose (1, 8, 10). System II is derepressed in starving mycelia and transports glucose, L-sorbose, xylose, galactose, 3-*O*-methyl glucose, mannose, 2-deoxyglucose, and talose (7, 9, 10). We have also obtained evidence for a specific constitutive galactose uptake system in glucose-grown mycelia which is distinct from system I (7). In conidia, two glucose uptake systems which seem analogous to the mycelial systems I and II (6) have been characterized. Klingmüller's laboratory has described three distinct L-sorbose transport activities in conidia: a constitutive system in ungerminated conidia (possibly system I), one in germinated conidia which have been starved (probably system II), and a system in ungerminated conidia which have been pretreated with L-sorbose (3).

Fructose transport has not been studied in *Neurospora*, although the existence in mycelia of a derepressible fructose uptake activity which is distinct from system II was inferred by Schneider and Wiley (10, 11). Since we had shown that fructose is not transported by system II (7), it was of interest to characterize this derepressible fructose activity and compare its regulation with that of system II (7, 11).

MATERIALS AND METHODS

Reagents and chemicals. [*U*-^{14}C]fructose and 3-*O*-methyl-D-[*U*-^{14}C]glucose were purchased from New England Nuclear Corp. ATP, NADP, glucose-6-phosphate dehydrogenase, phosphohexose isomerase, and all unlabeled sugars were obtained from Sigma Chemical Co. All sugars used in this study were of the D configuration unless otherwise indicated.

Culture and growth conditions. Wild-type *Neurospora crassa*, RL3-8A, from the Rockefeller collection, was used throughout. Conidial suspensions were inoculated into 600 ml of Vogel minimal medium N (12) plus 2% glucose in a 2-liter flask and grown on a rotary shaker for 18 to 22 h at 30°C. Transfer of the mycelia to other media was accomplished by rapid vacuum filtration, washing with minimal salts solution, and resuspension in the appropriate incubation medium. This was performed in less than 1 min, and the mycelia were not permitted to dry out. Most kinetic experiments were performed on cultures which had been incubated for 3 h at 30°C in minimal salts (carbon starved).

Sugar uptake assays. The appropriate culture was harvested rapidly by vacuum filtration and washed with distilled water, and approximately 1 g (wet weight) was suspended in 49 ml of 50 mM sodium phosphate (pH 5.7) in a 125-ml flask. Assays were conducted at 30°C in a reciprocating shaker water bath. After 1 min for the mycelia to equilibrate, the assay was initiated by the addition of 1 ml of a 50-fold-concentrated solution of the radioactive sugar at a specific activity of 1 to 10 μCi/mmol. Uptake of 3-*O*-methyl glucose by system I was assayed at a final concentration of 20 mM. Fructose uptake during the derepression time courses was assayed at a 5 mM final concentration. For competition studies, the mycelia were suspended in 40 ml of the above buffer, and the radioactive fructose plus the competing sugar were added in 10 ml of buffer prewarmed to 30°C. In all cases, 5-ml samples were removed at four 30-s time points, rapidly filtered, and washed with distilled water. The mycelial pads were transferred to scintillation vials; 2 ml of water was added to disperse the samples, which were then counted in 15 ml of Aquasol (New England Nuclear Corp.) on a Nuclear-Chicago Unilux counter. A 10-ml sample taken from the assay mixture was filtered, washed, dried overnight at 90°C, and weighed. The results from each assay were plotted, and a straight line was fitted by inspection. Sugar

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uptake results are presented as nanomoles of fructose accumulated per minute per milligram (dry weight) of mycelia.

Phosphorylation assay. Mycelial extracts for the fructose phosphorylation assays were prepared from lyophilized mycelia, which were ground with an equal weight of sand and 10 volumes of 0.1 M Tris (pH 7.6) and then centrifuged at 39,000 × *g* for 20 min. The supernatant was diluted 10-fold with the same buffer and assayed for fructose phosphorylation spectrophotometrically by coupling the reaction to phosphohexose isomerase and glucose-6-phosphate dehydrogenase. The reaction mixture contained 0.1 M Tris (pH 7.6), 5 mM $MgCl_2$, 2 mM fructose, 2.5 mM ATP, 1 mM NADP, 1 U of glucose-6-phosphate dehydrogenase, 0.1 U of phosphohexose isomerase, and 10 μl of the diluted *Neurospora* extracts in a total volume of 1 ml. The progress of the reaction was monitored at 340 nm with a Zeiss PMQ II spectrophotometer equipped with a W and W recorder. The rate of the reaction was constant with time and was linearly dependent on the amount of extract added.

The protein concentration in the extracts was measured by the method of Lowry et al. (4), using bovine serum albumin as the standard, and phosphorylation rates were compared with uptake rates by assuming that 40% of the mycelial dry weight is extractable protein.

RESULTS

Fructose uptake by previously described sugar uptake systems. Sugar transport system I is a constitutive uptake system for glucose, 3-*O*-methyl glucose, and several other sugars (8, 10). It may be assayed by measuring 3-*O*-methyl glucose uptake in glucose-grown mycelia. A 20-fold excess of fructose (400 mM) did not inhibit 3-*O*-methyl glucose uptake, thus implying that fructose is not a substrate for system I. It has been shown previously that fructose is not transported by system II (7, 10).

Comparison of fructose uptake and phosphorylation. The kinetics of fructose phosphorylation were examined in crude extracts of repressed (glucose-grown) and derepressed (starved) mycelia and compared with the kinetics for fructose uptake in intact mycelia. As Fig. 1 shows, fructose phosphorylation in starved mycelia exhibited an apparent K_m from fructose 6-fold higher and a V_{max} 20-fold higher than the comparable parameters for fructose uptake. This suggested that the uptake of the sugar was a separate phenomenon from sugar metabolism. During derepression, uptake activity increased more than 100-fold, whereas the level of fructose phosphorylation remained constant (presumably due to hexokinase) (Fig. 2). Furthermore, the apparent K_m for fructose phosphorylation did not change during this time (data not shown). Therefore, the uptake of fructose appears to be carrier mediated and distinct from fructose metabolism.

Characteristics of fructose uptake. Fructose uptake in derepressed mycelia was maximal between pH 5 and 6 and was 40% less at pH 4 and 60% less at pH 7 than the maximal values. Respiratory inhibitors severely reduced fructose uptake activity: 1 mM KCN by more than 70% and 1 mM NaN_3 and 0.5 mM dinitrophenol each by more than 80%. Fructose uptake was sensitive to sodium acetate (50 mM inhibited fructose uptake by 50%) and to high ionic strength (200 mM KCl inhibited uptake by 60%). In derepressed mycelia, the K_m for fructose was 0.4 mM, and the V_{max} was 7 nmol/min per mg (dry weight) of mycelia (Fig. 1A).

Effects of other sugars. Glucose, 2-deoxyglucose, mannose, and several other sugars inhibited fructose uptake (Fig. 3), but in all but one case, the kinetics of inhibition were noncompetitive. Only L-sorbose inhibited fructose uptake competitively, with a K_i of 9 mM (Fig. 4). These and other results are shown in Table 1. In

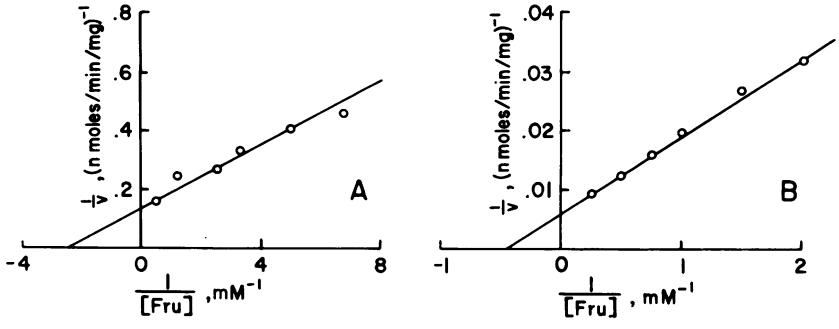

FIG. 1. *Comparison of fructose uptake in intact mycelia (A) with fructose phosphorylation in mycelial extracts (B). Glucose-grown mycelia were starved in minimal salts solution for 3 h; one portion was used for the uptake experiments, and another portion was lyophilized, ground, and assayed for fructose phosphorylation. Points represent the means of at least two determinations and are presented on a double-reciprocal plot normalized to the dry weight of mycelia.*

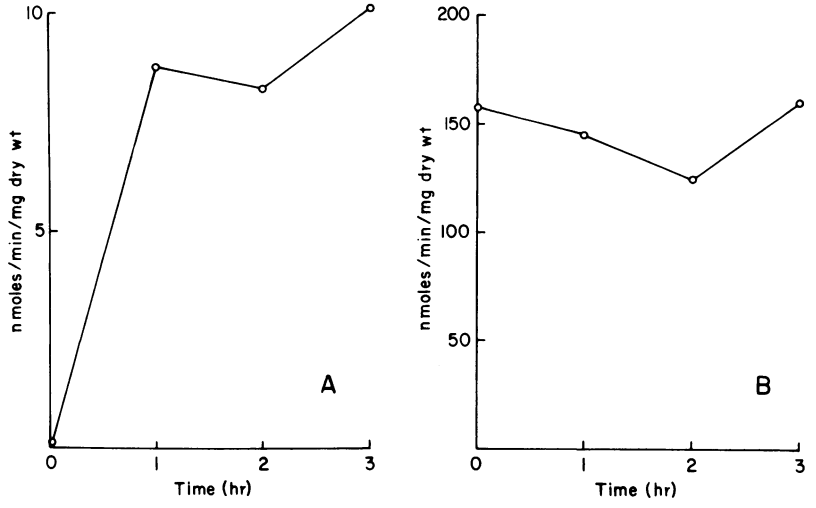

FIG. 2. *Time course of fructose uptake activity in intact mycelia (A) and fructose phosphorylation activity in mycelial extracts (B) after transfer of the mycelia from glucose growth medium to minimal salts solution. Points represent the means of at least two determinations.*

addition, dihydroxyacetone weakly inhibited fructose uptake ($K_i \sim 40$ mM), but the nature of this inhibition was not determined. DL-Glyceraldehyde, erythrose, ribose, lyxose, L-arabinose, sedoheptulose, mannitol, and sorbitol in 40-fold excess and tagatose in 13-fold excess all failed to inhibit fructose uptake.

Repression and derepression. As already shown (Fig. 2), when glucose-grown mycelia were incubated without any carbon source, the fructose uptake activity appeared within about 1 h. The appearance of this activity was completely prevented by cycloheximide (4 μg/ml), implying that the process required protein synthesis. The fructose transport level then fluctuated somewhat and gradually decreased approximately 35% by the end of 8 h. The presence of 100 mM glucose, 50 mM 2-deoxyglucose, or 100 mM mannose (Fig. 5) prevented the derepression of fructose transport activity. Fructose (100 mM) permitted partial derepression, while 100 mM xylose permitted full derepression during the time that mycelia were adapting to this new growth sugar, but produced partial repression in mycelia actively growing on the sugar (Fig. 5).

Since we had shown previously that sugar transport system II could be specifically stimulated to high levels by galactose (7), it was of interest to examine the effects of different sugars on derepression of fructose transport activity. Results of several such experiments are shown in Fig. 6. Galactose and L-sorbose were able to cause slightly elevated levels of fructose transport activity, but this effect, although reproducible, was not of great magnitude. When glucose-

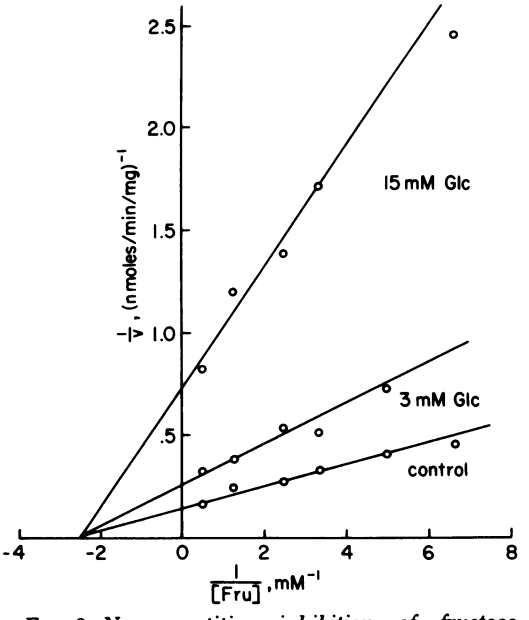

FIG. 3. *Noncompetitive inhibition of fructose transport by glucose in starved mycelia. Points represent the means of at least two determinations.*

grown mycelia were transferred to medium containing as its only carbon source the nonmetabolizable sugar 3-*O*-methyl glucose (50 mM), fructose transport activity increased at the same rate as in starving cultures, but kept rising linearly with time until the level of transport activity was almost five times that of what starvation alone could produce. This effect was abolished by cycloheximide. When already derepressed

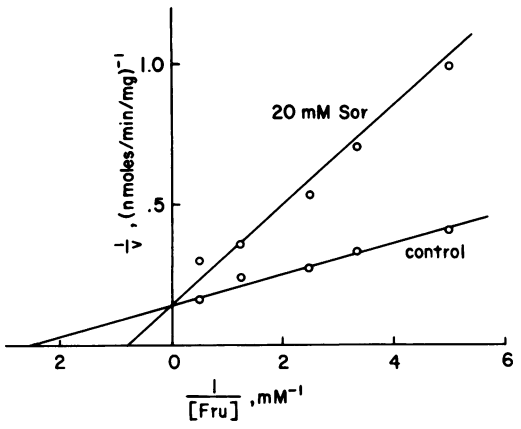

FIG. 4. *Competitive inhibition of fructose transport by* L*-sorbose in starved mycelia. Points represent the means of at least two determinations.*

TABLE 1. *Sugars which inhibit fructose uptake*

Sugar	Apparent K_i (mM)
Competitive, L-sorbose	9
Noncompetitive	
Mannose	2
3-*O*-Methyl glucose	3
Glucose	4
2-Deoxyglucose	6
Xylose	20
Arabinose	25
Galactose	~75

(starved) cultures were transferred to medium containing 50 mM 3-*O*-methyl glucose, fructose transport activity increased fourfold at this same rate (data not shown). This also was inhibitable by cycloheximide, which argued that this effect was not due to some passive interaction of 3-*O*-methyl glucose with the fructose carrier, but rather that new synthesis of the carrier or some protein which interacted with the carrier was required. Mycelia incubated as described with 3-*O*-methyl glucose had an apparent K_m for fructose uptake of 0.6 mM, quite close to the value of 0.4 mM in starved mycelia.

DISCUSSION

In studying sugar transport, it is essential to differentiate between carrier-mediated transport (or facilitated diffusion) of the sugar across the membrane and passive diffusion into the cell followed by the metabolism of the sugar in the cytoplasm. Ideally, one distinguishes between these processes by using a nonmetabolizable substrate of the uptake system and demonstrating saturation kinetics of uptake. Unfortunately, the derepressible fructose uptake activity in *Neurospora* is specific for fructose. Only one other sugar, L-sorbose, is transported by this system, as judged by its competitive inhibition of fructose uptake. Although L-sorbose is not metabolized by *Neurospora* (2), it is a substrate for at least two other sugar transport systems in *Neurospora* (1, 7, 9), both of which are active in derepressed mycelia, and so could not be used to study fructose uptake. The inability to find a suitable nonmetabolizable substrate of the fructose transport system makes it difficult to study accumulation of the free sugar inside the mycelia or the equilibrium of internal to external free sugar. Nevertheless, since (i) fructose uptake exhibited saturation kinetics, (ii) these kinetics were different from those of fructose phosphorylation, and (iii) the uptake activity only appeared after derepression, whereas phosphorylation activity was constitutive, it appears that uptake is not passive and is distinct from metabolism. A similar argument was used by Scarborough (8) to differentiate glucose uptake by system I from glucose phosphorylation in glucose-grown mycelia. Thus, there is strong evidence that fructose uptake involves a specific carrier. This is similar to fructose transport in *Aspergillus nidulans* (5), where fructose is not transported by either of two general sugar transport systems, but rather by a specific fructose system.

Although the regulation of the fructose transport system differs in several ways from that of system II (7, 11), the conditions for repression of the two systems seem comparable. These two sugar transport systems are repressed by glucose, mannose, and 2-deoxyglucose and partially repressed by fructose and xylose. In addition,

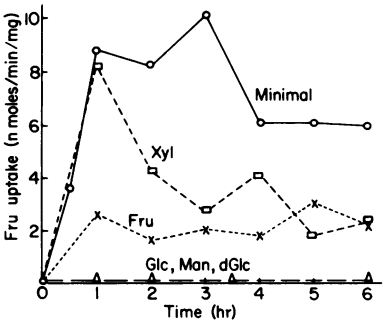

FIG. 5. *Repression of fructose transport activity. At time zero, glucose-grown mycelia were transferred to minimal salts medium* (○) *or to minimal salts containing 100 mM xylose* (□), *100 mM fructose* (×), *100 mM glucose* (△), *100 mM mannose, or 50 mM 2-deoxyglucose. Since the values for mannose and 2-deoxyglucose are identical to those for glucose, only one symbol is used for all three sugars. Points represent the means of at least two determinations.*

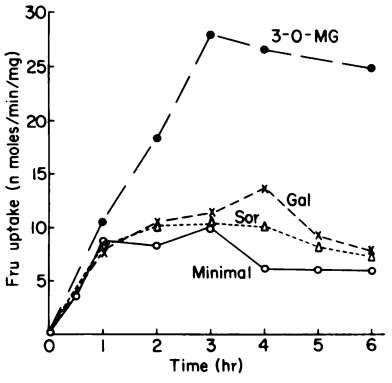

FIG. 6. *Stimulation of fructose transport activity. At time zero, glucose-grown mycelia were transferred to minimal salts medium* (○) *or to minimal salts containing 50 mM 3-O-methyl glucose* (●), *100 mM galactose* (×), *or 100 mM* L-*sorbose* (△). *Points represent the means of at least two determinations.*

both transport systems show full derepression immediately after transfer from glucose to xylose, followed by partial repression as the mycelia initiate active growth on xylose. Studies of the mechanism of catabolite repression in *Neurospora* should be aided by the availability of two distinguishable activities with the same pattern of repression.

Although L-sorbose was the only sugar to exhibit competitive inhibition of fructose transport, many sugars were found to be noncompetitive inhibitors. An examination of the structures of these noncompetitive inhibitors reveals no common basis for their action. With the exception of arabinose, they are all transported by sugar transport system II (7), but the K_i's for fructose inhibition are all 10- to 100-fold higher than their K_m's or K_i's for system II (7). Some of these sugars act as repressors of the fructose transport system, some have little effect on its regulation, and 3-*O*-methyl glucose acts in a manner similar to gratuitous inducers of bacterial transport systems.

The observation that incubation with 3-*O*-methyl glucose could cause added synthesis of fructose uptake activity was unexpected, especially since this sugar is not a substrate for the system (as judged by its noncompetitive inhibition of fructose uptake). It is still not certain whether incubation with 3-*O*-methyl glucose leads to higher levels of the same fructose transport system found in starved mycelia or whether a second fructose transport activity appears, although we favor the former alternative. In either case, protein synthesis is required for the appearance of this additional activity.

We have shown previously that galactose can stimulate the general sugar transport system II to higher levels than can starvation alone (7). A pattern thus seems to be emerging among derepressible sugar transport systems in *Neurospora*: often a sugar which is not itself a substrate for the system (or a poor substrate) can increase the activity of the system to levels higher than those achieved by starvation (derepression) alone.

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