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Kinetic Characteristics of the Two Glucose Transport Systems in *Neurospora crassa*

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Glucose is transported across the cell membrane of *Neurospora crassa* by two physiologically and kinetically distinct transport systems. System II is repressed by growth of the cells in 0.1 M glucose. System I is synthesized constitutively. The apparent K_m for glucose uptake by system I and system II are 25 and 0.04 mM, respectively. Both uptake systems are temperature dependent, and are inhibited by NaN_3 and 2,4-dinitrophenol. Glucose uptake by system II was not inhibited by fructose, galactose, or lactose. However, glucose was shown to be a noncompetitive inhibitor of fructose and galactose uptake. The transport rate of [^{14}C]3-0-methyl-D-glucose (3-0-MG) was higher in cells preloaded with unlabeled 3-0-MG than in control cells. The rate of entry of labeled 3-0-MG was only slightly inhibited by the presence of NaN_3 in the medium. Further, NaN_3 caused a rapid efflux of accumulated [^{14}C]3-0-MG. These data imply that the energetic step in the transport process prevents efflux.

Present evidence suggests that carbohydrates, as well as other nutrilites, are transported across the cell membrane of *Neurospora crassa* by specific transport systems which are dependent on metabolic energy (2, 3, 8, 10, 20). Recent work by Scarborough has shown that glucose uptake in *Neurospora* is mediated by two kinetically distinct systems (14, 15). One of the systems was reported to be a facilitated diffusion system with a K_m for glucose of about 8 mM (14); the other was reported to be an active transport system with a K_m of about 10 μM (15). The low K_m system was apparently absent in cells grown on 50 mM glucose, implying that high concentrations of glucose repressed synthesis.

Simultaneous studies in our laboratory yielded results which are similar to those of Scarborough (Abstr. Bacteriol. Proc., p. 145, 1970). The high K_m system and the low K_m system were designated system I and system II, respectively. The rate of uptake of fructose, galactose, and lactose in *Neurospora* also increased during starvation for sugar (17). This report describes the results of a detailed study of the characteristics of uptake by the low K_m transport system in *Neurospora*. Our data suggest that active transport of glucose occurs via a "pull" mechanism.

MATERIALS AND METHODS

Organisms. The organism used in these experiments was *N. crassa* strain Em a.

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Culture conditions. Methods for the maintenance of stock cultures and the harvest of conidia were described previously (20). Germinated conidia for the uptake experiments were prepared by inoculating 1 liter of Vogel's mineral salts medium (19) containing 2% glucose with conidia (approximately 5×10^7 conidia/ml). The conidia were germinated and grown at 30 C for 16 to 17 hr on a reciprocal shaker. Germinated conidia were harvested and washed by filtration. The washed cells were suspended in five times the original volume of Vogel's medium without glucose. The cells were either stored at 0 C (4 hr or less; repressed cells) or derepressed by incubation at 30 C for 1.5 to 2 hr. Cells were washed three times in distilled water before initiating the uptake experiments.

Uptake experiments. Uptake experiments were conducted at 30 C. The experiments were initiated by rapidly injecting the radioactively labeled sugar into 25 ml of Vogel's medium containing 0.4 to 1 mg (dry weight) of cells/ml. Mixing and aeration of the culture were insured by the use of an immersible magnetic stirrer. The time course of uptake was measured by removing samples (2 ml) of the cell suspension at appropriate intervals (usually 30 sec). The samples were expelled into 15 ml of water (0 C), and the mixture was rapidly filtered through glass fiber filters (Whatman GF/A, 2.4 cm). The filters were washed five times in 3 to 5 ml of cold distilled water (4 C), dried, and added directly to scintillation vials for radioactivity measurements. All radioactivity measurements were made in a Nuclear-Chicago liquid scintillation counter (60% efficiency). The scintillation fluid contained 0.1 g of 1,4-bis-2-(5-phenyl oxazolyl)-benzene and 4 g of 2,5-diphenol oxazole in 1 liter of toluene; each vial contained 10 ml of scintillation fluid. Samples were counted for 1 min or for a time adequate to give at least 1000 counts per sample.

Reagents and chemicals. The following radioactive sugars were purchased from New England Nuclear (Boston): glucose-*U*-^{14}C, fructose-*U*-^{14}C, galactose-*1*-^{14}C, lactose-*1*-^{14}C. The 3-0-methyl-D-glucose-*methyl*-^{14}C was purchased from I.C.N., Irvine, Calif., and cycloheximide (Acti-dione) was purchased from Upjohn Co., Kalamazoo, Mich.

RESULTS

Concentration dependence. A comparison of the kinetics of glucose uptake in glucose-grown (repressed cells) and starved cells (derepressed cells) is shown in Fig. 1. The curve describing the concentration dependence of uptake by starved cells is not a classical hyperbola and, except for the higher rate at concentrations less than about 2 mM, is similar to the curve for glucose-grown cells. When one looks at the results shown in Fig. 2, it becomes clear that the curve for derepressed cells in Fig. 1 is probably biphasic. Figure 2 shows the kinetics of uptake in repressed and derepressed cells for glucose concentrations between 0 to 1 mM. A classical hyperbolic curve can be resolved in this lower range of concentrations in derepressed cells but not in repressed cells (Fig. 2). The increase in the initial

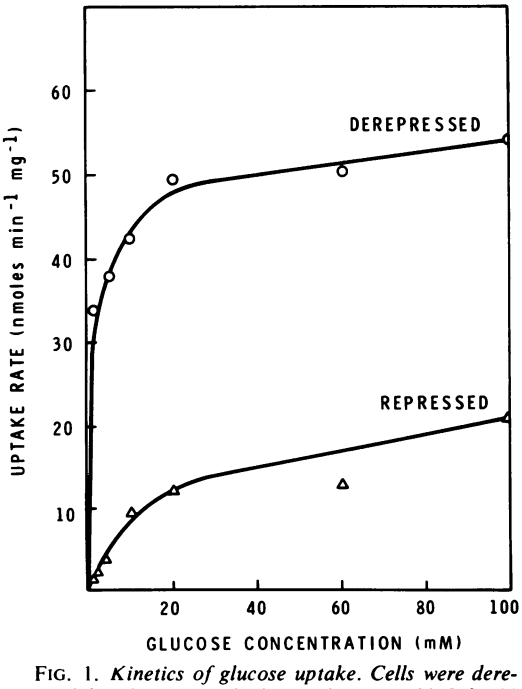

FIG. 1. *Kinetics of glucose uptake. Cells were derepressed for glucose uptake by incubation at 30 C for 90 min in the absence of a carbon source. Repressed cells were grown on 0.1 M glucose, washed and assayed. The rate was calculated from six 2-ml samples taken at 30-sec intervals using 1 × 10^{-4} M [^{14}C]glucose (specific activity, 0.01 μCi/μmole).*

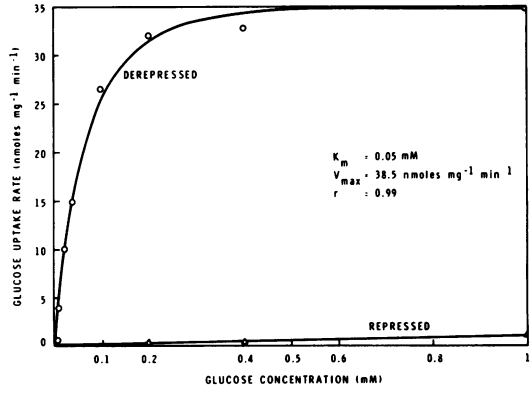

FIG. 2. *Kinetics of glucose uptake by system I and system II at low substrate concentration. The cells used in this experiment were identical to those described by the experiment in Fig. 1. The uptake rates were measured with [^{14}C]glucose (specific activity, 0.05 μCi/μmole).*

rate of uptake after derepression is, then, the result of the appearance of another uptake system with 600-fold-lower K_m than that observed in repressed cells. Hereafter, we will refer to the low K_m system as system II and the constitutive (high K_m system) as system I. The accompanying paper (17) provides a description of the regulation of system I in greater detail. At concentrations less than 1 mM, the transport rate of system I is negligible, meaning that system II can be studied by using concentrations of substrate less than 1 mM and system I can be studied by working with repressed cells.

In transport studies of the type described in this report, it is advantageous to use nonmetabolizable analogues to distinguish between accumulation and metabolism. The data shown in Fig. 3 suggest that 3-0-methyl-D-glucose (3-0-MG) is a competitive inhibitor of glucose uptake and is consequently transported by system II. Figure 3 is a double-reciprocal plot of glucose uptake rate versus glucose concentration in the absence and presence of 0.2 mM 3-0-MG, a nonmetabolizable substrate. The apparent K_i for 3-0-MG is 0.03 mM, reasonably close to the measured K_m of 3-0-MG (Table 1). If, indeed, glucose and 3-0-MG are transported by the same system, we should observe a proportionality between the increase in glucose and 3-0-MG transport during derepression. The results of such an experiment are shown in Fig. 4. In this experiment, cells were incubated in the absence of a carbon and energy source, samples were removed from the culture at various intervals and assayed for the uptake of 10^{-4} M glucose and 3-0-MG. The plot of the rate of 3-0-MG transport versus glucose transport rate (Fig. 4) shows a linear relation-

ship, indicating that the ratios of the specific transport activities were consistent over a wide range of activities. These data provide additional support for the contention that 3-0-MG enters the cell via the glucose transport system (system II). The data also suggest that the use of glucose for the measurement of initial rates is valid, although glucose can be metabolized by the cell. Chromatographic evidence that 3-0-MG is not metabolized is described below.

Table 1 summarizes the kinetic properties of system I and system II. The apparent K_m for glucose uptake by system II is approximately 40 μM, for 3-0-MG, it is about 80 μM. The activity of system I was derived by subtracting V_{max} of system II from the total rate of each concentration above 1 mM (*see* Fig. 2). The apparent K_m and V_{max} of system I for 3-0-MG were much higher in derepressed cells than in repressed cells; both K_m and V_{max} were intermediate in cells growing on glycerol before and during the assay of transport activity. The reason for this is not clear; it is possible that it is related to the metabolic state of the cells. Our results are consistent with those of Scarborough (14, 15) except for minor differences in the kinetic constants.

Time course of uptake of 3-0-MG. The time course of uptake by derepressed cells from solutions containing 10^{-5} M and 10^{-4} M 3-0-MG is illustrated in Fig. 5. Accumulation of the sugar from 10^{-5} M 3-0-MG was linear for about 3 min, after which an apparent steady state of 6 to 7 nmoles per mg (dry weight) was maintained. Accumulation from 10^{-4} M substrate was linear for about 5 to 6 min; the pool was saturated at about 70 nmoles per mg (dry weight). By using a published (18) value of 2.54 for the ratio of cell water to dry weight of *Neurospora*, we estimate intracellular concentrations at steady state to be 2.8 and 28 nmoles per kg of cell water, respectively. The cells removed 20% of the sugar from the medium, leaving 8 and 80 nmoles per ml, respectively. The ratio of intracellular concentration to extracellular concentration at steady state was about 350 for both concentrations of 3-0-MG. These data suggest that intracellular 3-0-MG does not regulate its intracellular concentration by feedback inhibition of the uptake system. Since unaltered sugar was accumulated against a concentration gradient, we conclude that the transport of glucose is an active process. That 3-0-MG was not metabolized was demonstrated by incubating cells in 10^{-4} M [^{14}C]3-0-MG for 3, 10, and 30 min. The cells were lyophilized and extracted with boiling water for 3 min. Paper chromatography and subsequent autoradiography of each of the extracts revealed a single radioactive spot with the same R_F as that of standard 3-0-MG. Also, 98 to 100% of the radioactivity applied to the paper was recovered by elution of the chromatographic spots. Apparently 3-0-MG was not phosphorylated during transport. The solvent systems used for chromatography were 1 M sodium acetate (*p*H 3.8)-ethanol (2:5) and 200:1 phenol-NH_3 (4).

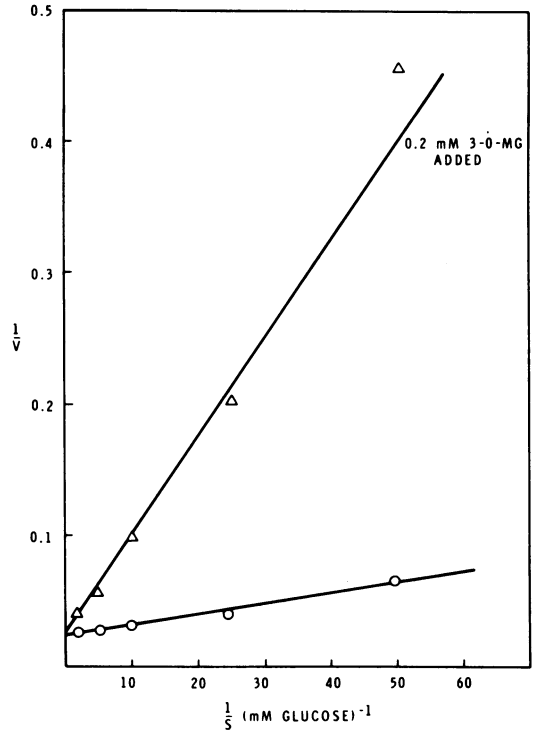

FIG. 3. *Competitive inhibition of glucose uptake by 3-0-MG. The cells were starved for 2 hr before the assay. The reciprocal of initial uptake rate is plotted against the reciprocal of external glucose concentration. Assay was conducted as described for Fig. 2.*

Uptake of glucose and 3-0-MG from 10^{-4} M solutions was inhibited completely by 10 mM NaN_3 and 2,4-dinitrophenol. Transport of 10 mM 3-0-MG by repressed cells (system I) was inhibited 80% by NaN_3. These data suggest that glucose uptake by both systems is dependent on metabolic energy.

The time course of uptake of 20 mM 3-0-MG by repressed cells is shown in Fig. 6. The intracellular concentration of the sugar in the unpoisoned cells was about twice the external concentration at the end of 1 hr. The concentration in the cells inhibited by NaN_3, however, approached the external concentration; further, the initial rate of uptake by the poisoned cells was inhibited by more than 85%. Since the sugar was accumulated by the cells and since both accumulation and rate of uptake were inhibited by

TABLE 1. *Kinetic properties of system I and system II*

State of cells	Substrate	System	Concn range (mM)	K_m	V_{max} (nmoles per mg per min)	r^b
Repressed[a]	Glucose	I	4-100	23	24	0.97
	3-0-MG	I	1-200	64	65	0.95
		I	2-100	48	24	0.99
Derepressed[c]	Glucose	II	0.004-1	0.05	39	0.99
		II	0.02-0.4	0.04	46	0.99
		II	0.04-4	0.03	70	0.89
		II	0.04-4	0.04	73	0.89
		I[d]	4-100	29	30	0.99
	3-0-MG	II	0.01-2	0.08	16	0.99
		II	0.01-4	0.09	18	0.99
		I[d]	18-165	265	460	0.98
		I[d]	4-100	156	363	0.99
		I[d]	1-100	161	417	0.99
Glycerol[e]		I[d]	2-100	122	153	0.98

[a] Cells were grown on glucose until assayed.
[b] Correlation coefficient.
[c] Cells were starved at 30 C for 90 min before assay.
[d] V_{max} of system II subtracted from velocity at each concentration of substrate above 1 mM.
[e] Cells were growing on glycerol during the assay.

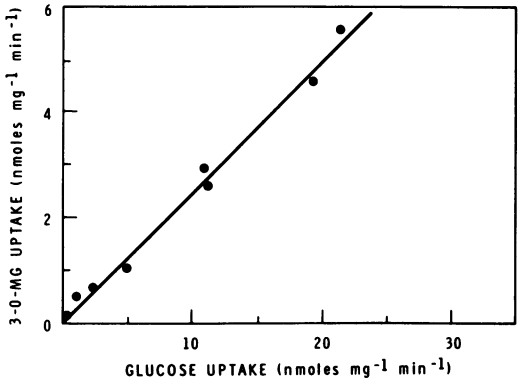

FIG. 4. *Proportionality between 3-0-MG uptake and glucose uptake during derepression. Cells undergoing derepression in the manner described for Fig. 1 were removed from the culture at 10 min intervals, and the rates of uptake of 3-0-MG and glucose measured for each interval. The specific activity of the labeled sugars was 0.05 μCi/μmole.*

NaN_3, we conclude that system I is also an active uptake system.

Specificity. We demonstrated that the rate of glucose, fructose, galactose, and lactose uptake by *Neurospora* cultures increases after glucose deprivation and that this increase is the result of derepression of synthesis of the uptake systems (17). If a single uptake system were responsible for this increase, competitive inhibition between the sugars would be expected. The data shown in Table 2 suggest that four distinct systems are operative. This experiment was performed on cells which were derepressed for 90 min, by incubation in the absence of a carbon and energy source. As indicated, fructose, galactose, and lactose failed to inhibit the uptake of each other. Glucose and 3-0-MG, however, inhibited the uptake of fructose and galactose. Lester et al. (10) reported that galactose inhibited lactose uptake; however, the long incubation period (3 hr) used made it difficult to ascertain whether the effect was on transport or metabolism of lactose. Our results are consistent with those of Marzluf and Metzenberg (11), who showed that glucose inhibited the uptake of fructose and that fructose had no effect on the initial rate of glucose uptake.

Figure 7 illustrates the rate of fructose uptake as a function of 3-0-MG concentration. The inhibition is not competitive. The K_i (one-half maximal inhibition) for 3-0-MG was the same as the apparent K_m for 3-0-MG transport. This is consistent with the contention that transport of 3-0-MG rather than some nonspecific inhibition resulted in the inhibition of fructose transport. Koch (9) reported inhibition of galactoside transport by glucose in *Escherichia coli* and suggested that glucose and galactosides share a common step or site, possibly the carrier site. In view of

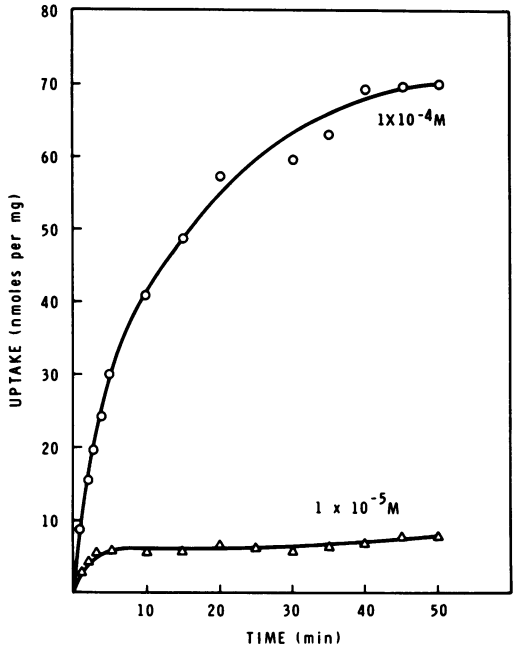

FIG. 5. *Effect of initial 3-0-MG concentration in the external medium on intracellular pool size at steady state. The methods for measuring the time course of uptake were described in the text. The time course measurements were made for initial concentration of 10^{-5} and 10^{-4} M 3-0-MG (specific activity, 0.05 μCi/μmole). The cells, 0.174 mg (dry weight)/ml, were derepressed 90 min before the uptake experiments were initiated.*

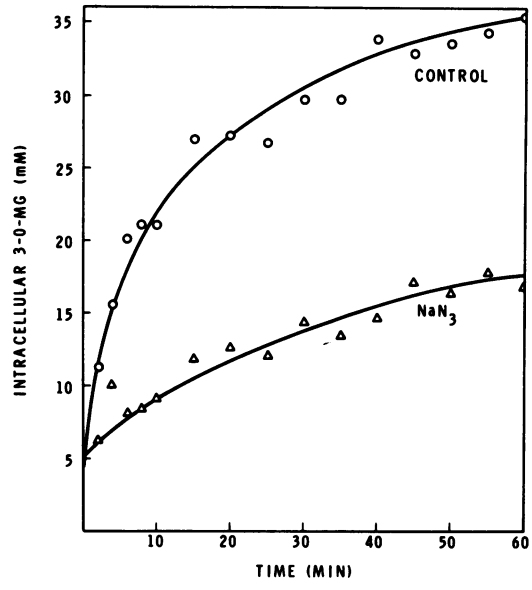

FIG. 6. *Time course of uptake of 20 mM 3-0-MG by repressed cells. The cells were retained in medium containing 0.1 M glucose until the experiment was initiated. The incubation medium contained 4 μg of cycloheximide per ml to prevent synthesis of system II during the course of the experiment. The concentration in the cells was calculated from the radioactivity inside the cells (specific activity, 0.01 μCi/μmole) and from the dry weight, assuming a ratio of intracellular water to dry weight of 2.5.*

the similarities in the K_i and K_m for 3-0-MG transport, it is possible that the explanation proposed by Koch may apply to glucose and fructose transport in *Neurospora*.

Efflux of accumulated 3-0-MG. The study of exit and counterflow reactions of transport systems is frequently useful in suggesting possible sites of energy input to active transport. Figure 8 shows the time course of uptake from 10^{-4} M 3-0-MG by derepressed cells and the subsequent efflux from the cells after the addition of unlabeled 10 mM 3-0-MG and 10 mM NaN_3 to the cell suspension. The data suggest that the maintenance of the 3-0-MG is dependent on metabolic energy. The efflux of labeled 3-0-MG when unlabeled 3-0-MG was added to the external medium indicates an exchange between accumulated pool and the sugar in the external environment.

The efflux of 3-0-MG in the presence of NaN_3 is temperature dependent (Fig. 9). The data in Fig. 9 were obtained by loading derepressed cells with labeled 3-0-MG (1 mM) for 30 min, washing, and measuring efflux of intracellular radioactivity at various intervals during incubation in sugar-free medium containing 10 mM NaN_3.

TABLE 2. *Specificity of sugar transport systems*

Addition[a]	Rate of sugar uptake (per cent of control)[b]			
	Glucose	Fructose	Galactose	Lactose
Glucose		26	4.6	65
3-0-MG	49	46	20	120
Fructose	95		94	79
Galactose	88	80		78
Lactose	99	87	112	

[a] Labeled test sugars (0.1 mM) and unlabeled competitors (1 mM) were added to the uptake medium simultaneously.

[b] Control initial rate was measured in the absence of another sugar.

The strong temperature dependence indicates that the efflux is mediated and not simply diffusion from the cells. The linearity of the semilogarithmic plot of efflux suggests that efflux is a first-order rate process and the 3-0-MG is contained in a single intracellular compartment.

The data shown in Table 3 summarize the efflux experiments. In these experiments, cells were loaded with 10^{-4} M 3-0-MG for 30 min, washed,

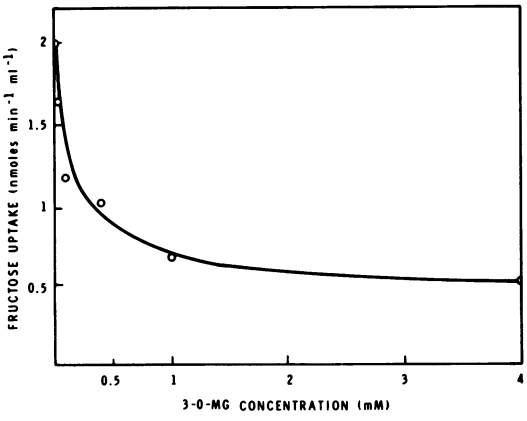

FIG. 7. *Effect of 3-0-MG on the transport of fructose by derepressed cells. Various concentrations of unlabeled 3-0-MG and 10^{-4} M [^{14}C]fructose (specific activity, 0.5 μCi/μmole) were added simultaneously to the uptake medium. The rates of uptake were determined from time course studies of the type described for Fig. 5.*

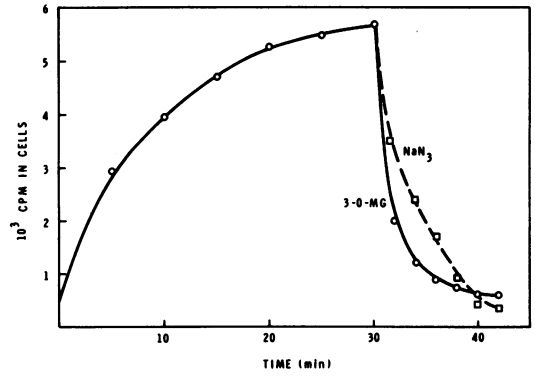

FIG. 8. *Effect of 3-0-MG and NaN_3 in the external medium on the efflux of intracellular 3-0-MG. Derepressed cells were incubated in 10^{-4} M [^{14}C]3-0-MG (specific activity, 0.05 μCi/μmole); at 30 min, the culture was divided into two portions. Unlabeled 10 mM 3-0-MG was added to one, and 10 mM NaN_3 was added to the other.*

and placed in media containing the appropriate additives (Table 2). Efflux in media without NaN_3 or sugar was about 20 times slower than that into medium containing unlabeled 3-0-MG or glucose. Efflux was not stimulated by the addition of 10 mM fructose or lactose to the medium. These results provide additional support for the contention that glucose and 3-0-MG enter the cell via the same transport sites and that those are not the same as sites for fructose and lactose uptake.

Preloading the cells with unlabeled 3-0-MG resulted in an increase in the rate of uptake of

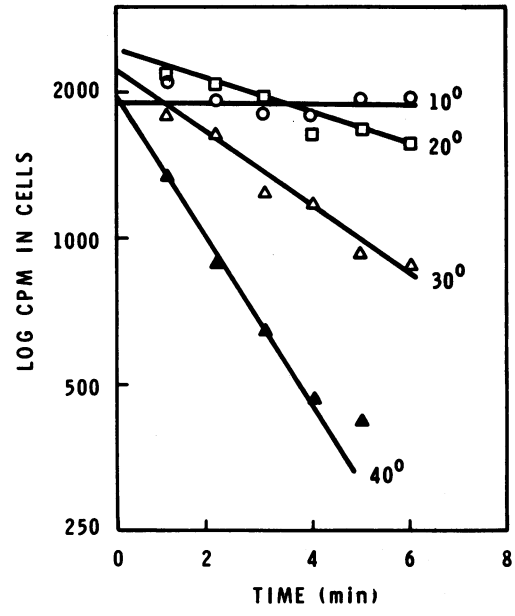

FIG. 9. *Effect of temperature on the retention of intracellular pools of 3-0-MG in the presence of NaN_3. The decrease in intracellular 3-0-MG was assayed by removing 2.0-ml samples at appropriate intervals for the measurment of radioactivity. Derepressed cells were loaded by incubating in 10^{-4} M [^{14}C]3-0-MG (specific activity, 0.05 μCi/μmole) for 30 min, washed, and resuspended in medium containing 10 mM NaN_3 at the various temperatures. Samples were removed for radioactivity measurements as described for Fig. 8.*

radioactive 10^{-4} M 3-0-MG. In addition, the preloaded cells were able to drive the uphill transport of radioactive 3-0-MG in the presence of NaN_3. These data are shown in Fig. 10. In unpoisoned cells, the increased uptake may be attributed to the same facilitated diffusion component which results in the extrusion of accumulated label from preloaded cells. Since the efflux in the presence of NaN_3 is independent of unlabeled 3-0-MG in the medium, the efflux and influx must be uncoupled under these conditions. We infer from these data that accumulation of labeled 3-0-MG by preloaded NaN_3-treated cells resulted from exchange of unlabeled intracellular 3-0-MG with labeled 3-0-MG outside the cell (counterflow; 21). If this inference is correct, such a process would result in a net active movement of labeled sugar into the cell. This explanation is also consistent with the fact that influx is only moderately inhibited by NaN_3. It seems reasonable to speculate that mediated exchange between external and internal 3-0-MG in the presence of NaN_3 (influx and efflux) represents the operation of a passive component which was uncoupled from some undefined energy step in

TABLE 3. *Exit of accumulated 3-0-MG from intracellular pool*[a]

Additions to medium	Addition concn (mM)	$T_{1/2}$ of exit (min)[b]
None		40
NaN_3	10	3
NaN_3 + 3-0-MG	10	3
3-0-MG	1	3.3
3-0-MG	10	2.4
Glucose	10	2.2
Fructose	10	36
Lactose	10	49

[a] Cells were derepressed for 2 hr then incubated in 10^{-4} M [^{14}C] 3-0-MG (specific activity, 0.05 μCi/μ mole) for 30 min, washed, and suspended in Vogel's medium (30 C) containing the appropriate additions.

[b] $T_{1/2}$, half-time.

the process. All attempts to demonstrate the presence of a carrier with the kinetic properties of system II in repressed cells failed. For example, when repressed cells were incubated with 10 mM ^{14}C-3-0-MG for 1 hr in the presence of cycloheximide to prevent the synthesis of system II, efflux was independent of either NaN_3 or 10 mM 3-0-MG. Repressed cells, preloaded with unlabeled 3-0-MG in cycloheximide and then incubated in labeled 10^{-4} M 3-0-MG with or without NaN_3, did not take up labeled 3-0-MG.

DISCUSSION

The work described in this report confirms the observations made by Scarborough (14, 15) that *Neurospora* has a high K_m and a low K_m uptake system and that the low K_m system is repressed by glucose. Our data, however, suggest that the high K_m system in germinated conidia is energy dependent. Uptake by this system is inhibited by NaN_3, and the intracellular concentration of 3-0-MG exceeds the external concentration at steady state.

We also found that phosphorylation of 3-0-MG during transport does not occur in *Neurospora*. These results are in agreement with the results reported by Scarborough for *Neurospora* (14) and by Brown and Romano for *Aspergillus nidulans* (1).

In addition to the lack of transport related phosphorylation (7), system II has other properties in common with the β-galactoside permease of *E. coli*. Intracellular pools of accumulated galactosides are released from the cells in the presence of NaN_3, whereas influx is not inhibited by the inhibitor (9). Accumulated pools of α-methyl glucose in *E. coli* (6) and yeast (12), on the other hand, are retained in the presence of

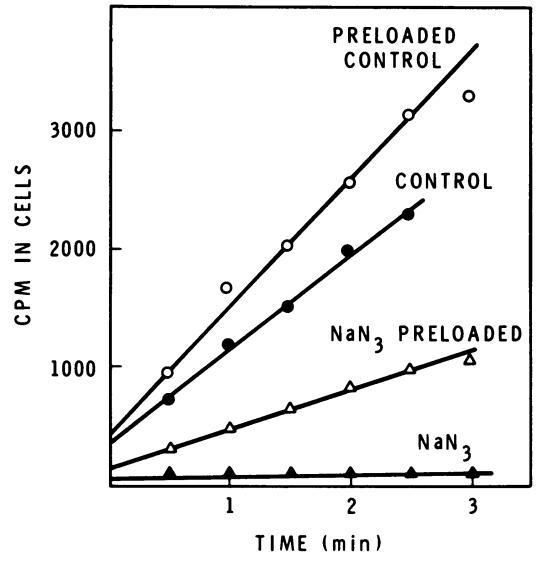

FIG. 10. *Effect of preloading with unlabeled 3-0-MG on uptake of labeled 3-0-MG. The cells used in this experiment were derepressed, washed, and incubated in medium containing 10^{-2} M 3-0-MG for 30 min. After the 30-min incubation, the cells were washed and suspended in fresh medium, and the time course of [^{14}C]3-0-MG (10^{-4} M; specific activity, 0.05 μCi/μmole) uptake was measured in the presence and absence of 10 mM NaN_3.*

NaN_3. Efflux of α-methyl glucose is energy dependent in both organisms.

Our data on system II are consistent with the models proposed by Fox and Kennedy (5), Koch (9), and Schacter and Mindlin (16) for the β-galactoside permease of *E. coli*. An important feature of the model is the pull mechanism, i.e., the site of energy input is on the inside of the membrane preventing efflux. The model of Schacter and Mindlin differs from the others in that it does not propose a decrease in affinity of the carrier for the substrate on the inside face of the membrane. Our data do not permit us to choose between the two alternatives.

We were not able to measure facilitated diffusion in unloaded, poisoned cells because of the complication arising from the use of high substrate concentration. As indicated previously, high concentrations of substrate would result in interference from system I. Thus, we have used counterflow in the presence of NaN_3 as a measure of entry in metabolically poisoned cells (21). The rate of influx was less than that of unpoisoned cells but efflux of unlabeled sugar leaking from the poisoned cells could have competed with uptake of label due to isotopic mixing in the periphytic space of the cell (13). Since counterflow could be demonstrated in 10^{-4} M 3-

0-MG in derepressed but not repressed cells, a system II carrier component was apparently absent in repressed cells. Koch (9) suggested that β-galactoside carrier activity in uninduced *E. coli* cells was composed of a relatively nonspecific carrier, and induction resulted in the synthesis of a permease which conferred specificity to the system. Although much of our data may be consistent with this view, it is not necessary to invoke such a mechanism to explain our data.

ACKNOWLEDGMENTS

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