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Characterization of Cytosine Permeation in *Saccharomyces cerevisiae*

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Cytosine permeation in *Saccharomyces cerevisiae* has been studied. Cytosine uptake is mediated by a permease which is also responsible for purines transport. The K_m for the transport of various substrates of this permease have been determined. By means of appropriate selective techniques, mutants with altered K_m and mutants lacking the permease have been selected. Cytosine transport is active and is inhibited by 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, and by *N*-ethylmaleimide, a reagent of —SH group. Internal labeled cytosine is chased by addition of unlabeled cytosine in the medium. These results support the hypothesis of a carrier-mediated transport, with reduced internal affinity, allowing the release and accumulation of cytosine in the inner compartment. The efflux of cytosine from cytosine permease-less cells has also been studied and shows first order kinetics. A diffusion coefficient of 5.7 per 10^{-8} cm per s^{-1} has been evaluated for this efflux.

The uptake of pyrimidines in the yeast *Saccharomyces cerevisiae* has been described to occur by at least two permeases: one permease is specific for cytosine and the other for uracil (4, 6). In view of the increasing interest in the functioning of membranes and of the great suitability of yeast for genetical and biochemical experimentation, it seemed valuable to study in more detail the pyrimidine permeases in this organism. The availability of all the radioactive substrates, as well as the possibility of avoiding cytosine or uracil metabolism by appropriate mutations, were especially convenient for such studies.

This paper presents results which have been obtained in the study of the cytosine permease. A comparison between the uracil and cytosine permeation in *S. cerevisiae* will be presented elsewhere (R. Jund, M. R. Chevallier, and F. Lacroute, manuscript in preparation).

MATERIALS AND METHODS

Strains and media. Most strains used in this work were derived from *Saccharomyces cerevisiae*, strain FL 100 (haploïd, *a* mating type), or from an isogenic strain FL 200 (haploïd, α mating type) (8). The strains carrying the *fcy 1-1* mutation derived from a wild-type strain obtained from F. Sherman. The strains used in the present study and their characteristics are listed in Table 1.

Yeast nitrogen base (Difco), without amino acids, supplemented with 2% glucose was used as minimal medium.

Complete medium (YPG) consisted of (per liter): yeast extract (Difco), 10 g; peptone (Difco), 10 g; and glucose, 20 g per liter. For solid media, 20 g of agar (Difco) was added per liter.

Mutagenesis and mutant selection. About 10^6 cells were plated on YNB agar adequately supplemented (see below). The plates were irradiated for 10 to 30 s with a Philips Germicidal lamp (TUV, 15 W) at 40-cm distance. The plates were then incubated at 28 C for at least 3 days. Clones resistant to the selective agent were reisolated once on complete agar and subclones were retested.

Mutagenesis was also performed with ICR 170F as mutagenic agent. ICR 170F is an acridine mustard synthesized at the Institute for Cancer Research in Philadelphia. It was the generous gift of H. J. Creech. The cells in late exponential phase (optical density = 200 Klett, blue filter) were collected by centrifugation, washed once with distilled water, and resuspended in 0.1 M sodium phosphate buffer, pH 7. One milliliter of ICR 170F solution at 1 mg/ml was added to a 10-ml cell suspension which was incubated for 30 min at 28 C with shaking and then plated on selective media. Care was taken to avoid exposure to visible light during incubation and plating.

The cytosine permease mutants were usually selected from FL 521 (*a*) or (α), a strain carrying a deletion at the *ura-2* gene and requiring uracil or cytosine for growth. In *S. cerevisiae*, the utilization of cytosine occurs only through its deamination into uracil, which is then converted in uridine 5'-monophosphate. Therefore starting from a *ura*$^-$ strain, all the mutations which give resistance to the fluoroanalogue by changing the regulation of the novo biosynthesis of the pyrimidines are not selected.

TABLE 1. *Strains used*[a]

Strains	Genotype	Phenotype
FL 100 (*a*)	Wild type	
FL 200 (*α*)	Wild type	
FL 442-2D (*a*)	*fcy 1-1*	5-FC resistance, upper limit 3×10^{-5} M
FL 442-4B (*α*)		
FL 521-sp1 (*a*)	*ura 2-60*	Uracil auxotroph with a deletion at the *ura 2* locus; growth on cytosine inhibited by MC
FL 521-sp2 (*α*)		
FL 509-sp1 (*a*)	*ade 1-1*	Adenine auxotroph; growth on adenine inhibited by cytosine
FL 509-sp2 (*α*)		
FL 480-1B (*a*)	*fcy 2-3*	5-FC resistance
FL 480-1D (*α*)		
FL 502-1A (*a*)	*ura 2-60 fcy 2-10*	Uracil auxotroph; resistant to inhibition by 5-MC when grown on cytosine; slightly resistant to 5-FC
FL 502-1B (*α*)		
FL 503-2B (*a*)	*ura 2-60 fcy 2-13*	Uracil auxotroph; resistant to inhibition by 5-MC when grown on cytosine; slightly resistant to 5-FC
FL 503-1D (*α*)		
NC 1-6D (*a*)	*fcy 2-10*	Slightly resistant to 5-FC; confers resistance to inhibition by 5-MC to *ura*$^-$ cells grown on cytosine
NC 1-2A (*α*)		
NC 12-1E (*a*)	*fcy 2-31*	Resistance to 5-FC
NC 12-1A (*α*)		
NC 31-sp2 (*α*)	*fcy 2-20*	Slightly resistant to 5-FC; confers resistance to inhibition by cytosine to *ade*$^-$ cells grown on adenine
NC 32-3A (*a*)	*fcy 2-21*	Slightly resistant to 5-FC; confers resistance to inhibition by cytosine to *ade*$^-$ cells grown on adenine
RJ 16-sp2 (*α*)	*fcy 1-1, fcy 2-3*	Resistance to 5-FC
NC 5-3C (*a*)	*fcy 1-3*	Resistance to 5-FC
NC 20-2A (*α*)	*fcy 1-3, fcy 2-5*	Resistance to 5-FC

[a] The products of genes *fcy 1*, *fcy 2*, *ura 2*, and *ade 1* are, respectively, cytosine deaminase, cytosine permease, aspartate carbamoyltransferase, and phosphoribosyl-aminoimidazole succinocarboxamide synthetase.

(i) Mutants resistant to 5-FC. Using a strain *ura 2-60*, it is possible to select mutants resistant to the cytosine analogue, 5-fluorocytosine (5-FC), if uracil is added to support the growth. As the phosphorylation step is essential for an *ura*$^-$ strain, the 5-FC-resistant mutants obtained are generally not impaired at this step and are not resistant to 5-fluorouracil. They belong essentially to two classes: either they are impaired at the entry step or they have deficient cytosine deaminase.

The 5-FC-resistant mutants were selected on minimal medium supplemented with 3×10^{-5} M uracil and concentrations of 5-FC ranging from 3×10^{-6} to 10^{-4} M.

(ii) Mutants expected to have cytosine permease with modified affinity. If cytosine is the source of uracil for an *ura*$^-$ strain, its uptake by the cell may be inhibited by competition with other substrates of the cytosine permease. If these substrates are not toxic to the cell, mutants carrying cytosine permease with specifically modified affinities might well be resistant to this inhibition. Two substrates for the cytosine permease, adenine (10) and 5-methylcytosine (5-MC), were used for this kind of selection. Cytosine concentration in YNB was 3×10^{-5} M, 5-MC was 4×10^{-3} M, and adenine was at concentrations ranging from 2×10^{-4} to 10^{-3} M. In the same way adenine$^-$ cells resistant to inhibition produced by excess cytosine relative to the adenine concentration were selected. In this case, adenine concentration in minimal medium was 2.5×10^{-5} M and cytosine was about 10^{-3} M.

Genetic techniques. The methods described by Mortimer and Hawthorne (9) were used throughout.

Uptake measurements. All experiments were carried out at 30 C with cells growing in exponential phase (i.e., below 100 Klett units, blue filter) in minimal medium. For the measurement of the initial velocity of uptake, 1 ml of cell culture was pipetted into a test tube containing the radioactive solution, shaken manually for 30 s, filtered through a membrane filter (Millipore Corp., pore size 0.8 μm), and quickly washed twice on the filter with 10 ml of cold water. Blanks with portions of the radioactive solution were also filtered to determine the radioactivity retained by the filters alone. Kinetic experiments have shown that during the first 30 s, accumulation is proportional to the time of incubation, which is no longer true at 60 s. The filters were dried and counted in toluene plus 2,5-diphenyloxazole (5 g/liter) in a Beckman liquid scintillation spectrometer LS 50. To calculate the internal concentration of cytosine an intracellular volume of 8×10^{-4} ml/100 Klett units (blue filter) was taken. This value has been estimated independently by R. Jund using a method based on determination of intracellular water in pellets of yeast cells, and by F. Lacroute using direct determination of mean cellular volume. [2-^{14}C]cytosine (45 mCi/mmol) was from Commissariat à l'Energie Atomique,

Saclay, France. It was usually diluted into nonlabeled cytosine to obtain a specific radioactivity of 10 mCi/mmol.

RESULTS

Cytosine uptake in wild-type, cytosine deaminase-less, and cytosine permease-less strains. (i) Wild-type mutants. Measurements of initial velocities of uptake of [^{14}C]cytosine were carried out on the wild type. The apparent K_m for cytosine uptake was found to be $\simeq 1.7 \times 10^{-6}$ M (Table 2). Inhibition of cytosine uptake by several cytosine derivatives has also been measured on the same strain (Table 2) and the inhibitions found were competitive.

(ii) Cytosine deaminase-less mutants. By means of selection on 5-FC, a 5-FC-resistant mutant (*fcy 1-1*) has been isolated. The biochemical block of this mutant has been previously investigated and shown to be a lack of cytosine deaminase activity; it does not incorporate significantly exogenous [^{14}C]cytosine into macromolecular components, but a high cytosine pool is formed in the presence of added external cytosine. No cytosine deaminating activity was detected in cell free extracts, in conditions where extracts of the wild-type strain showed a high cytosine deaminase activity (6). Moreover it was shown that no other label than [^{14}C]cytosine is found in the soluble pool if external [^{14}C]cytosine is added in the incubation medium (Jund, Thesis Universite Louis Pasteur, Strasbourg, France, 1973). Uptake of [^{14}C]cytosine in this mutant occurs therefore without further metabolism, a prerequisite for study solely of cytosine uptake. Initial velocities of uptake of cytosine in this mutant are similar to those observed for wild type (see Table 2). Even with high external concentrations of cytosine (up to 8 μmol/ml), only one straight line was observed in Lineweaver-Burk plots (Fig. 1). This contrasts with preliminary results where no corrections were made for absorption of radioactive cytosine by the filters, in which two

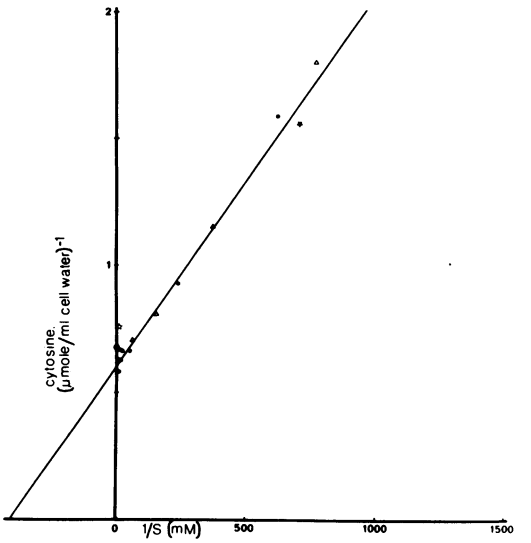

FIG. 1. *Initial velocities of uptake of cytosine as function of external concentration in strain NC 5-3C. Results are plotted according to Lineweaver-Burk. S, Cytosine concentration in the medium (in mM). V is expressed in micromoles per milliliter of cell water per 30 s of incubation. Different symbols refer to independent experiments. The extrapolated K_m is 2.4 × 10^{-6} M.*

TABLE 2. *K_m and K_i values for several substrates of the cytosine permease measured in various strains*

Strains	Genotype[a]	No. of expt	K_m cytosine[b] (10^{-6} M)	V_{max}[c]	K_m adenine[b] (10^{-6} M)	V_{max}[c]	K_i 5 methyl cytosine (10^{-6} M)	K_i deoxycytidine (10^{-6} M)	K_i isocytosine (10^{-6} M)
FL 100 (*a*)	Wild type	4	1.67 ± 0.23	1.20 ± 0.28	1.4	1.66	5	250	330
FL 442-4B	*fcy 1-1*	3	2.50 ± 0.80	1.72 ± 0.42					
NC 5-3C	*fcy 1-3*	4	2.38 ± 0.30	1.57 ± 0.52	2.2×10^{-6}				
FL 521-sp1	*ura 2-60*	3	1.87 ± 0.11	0.85 ± 0.39					
NC 1-6D	*fcy 2-10*	1	15	0.61	12	0.77	900		5,000
NC 31-sp2	*fcy 2-20*	1	50	0.65	5.9	0.77			
NC 32-3A	*fcy 2-21*	1	12	1.90	1.7	1.5			
NC 20-2A	*fcy 2-5*	3	7.13 ± 2.49	0.165 ± 0.05					
FL 480-1B	*fcy 2-3*	3	L[d]						
NC 12-1E	*fcy 2-31*	1	L[d]						

[a] See Table 1 for explanations on the strain genotype.
[b] The K_m values and V_{max} values are followed by standard deviation whenever possible.
[c] V_{max} are in micromoles per milliliter of intracellular fluid per 30 s.
[d] L, Too low to be measured.

different slopes were found. The apparent K_m as well as V_{max} are similar to those observed for the wild type. Internal cytosine concentrations, even after only 1 min of uptake, are much higher than the external concentrations, showing that cytosine transport is active (see the upper curve of Fig. 2).

(iii) Cytosine permease-less mutants. Measurements of initial velocities of uptake of cytosine were carried out on several strains resistant to 5-FC which belong to the same complementation group and are presumably blocked at cytosine entry (see Table 2). Each mutation segregated as a single gene and they were designated *fcy 2-3, fcy 2-31.* Initial velocities of uptake were not measurable in strains FL 480-1B (*fcy 2-3*) and NC 12-1E (*fcy 2-31*) in the standard conditions, where uptake is highly significant in wild-type and in cytosine deaminase-less strains. This confirms that cytosine uptake is impaired in the *fcy 2* mutants. Uracil entry in the mutant strain FL 480-1B was unaffected. Measurements of [^{14}C]cytosine retention by strain FL 480-1B at a high cell density and for 60 s showed a slight uptake. This uptake increased linearly with the external concentration without any tendency to saturation even at 1.3×10^{-5} M cytosine (see Fig. 2).

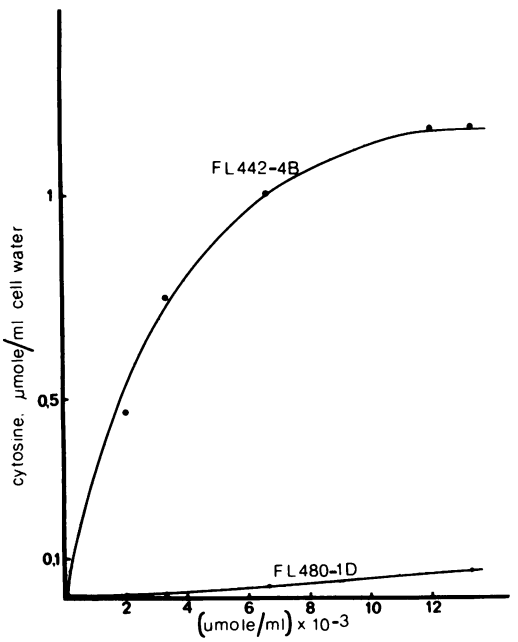

FIG. 2. *Comparison of velocities of uptake in strains FL 442-4B and FL 480-1D. The standard technique was used except that in these experiments the cells were sampled after 60 s of incubation in* [^{14}C]*cytosine and that the cellular density was about twice the usual one (≃130 Klett units).*

Specificity of the cytosine uptake system. The kinetics of inhibition of the entry of cytosine by 5-MC, deoxy-cytidine, and cytidine proved to be of the competitive type, both in the wild-type and in the cytosine deaminase-less mutants. In addition, it has been shown by Polak and Grenson (10) that in *S. cerevisiae* adenine and hypoxanthine are transported by the same system. In fact, uptake of cytosine in the wild type is competitively inhibited by adenine and vice versa. The entry of several purines and their inhibitory effect on cytosine entry has been investigated in another deaminase-less strain, NC 5-3C (see Table 3 for K_m and K_i values). In accordance with these results, adenine, guanine, or hypoxanthine can relieve the inhibition of growth produced by 5-FC. Moreover, in a cytosine permease-less strain (FL 480-1B), the uptake of adenine, hypoxanthine, and guanine was also barely detectable. This confirms that these three compounds are taken up by the same system as cytosine. The possible existence of other low-affinity systems for these compounds has not yet been investigated.

Mutants with modified cytosine-adenine uptake, altered K_m, and nearly normal V_{max}.

(i) Mutants with reduced affinity for 5-MC. Uptake of cytosine is competitively inhibited by 5-MC, and therefore *ura*$^-$ strains are unable to grow on minimal medium supplemented with 3×10^{-5} M cytosine plus 4×10^{-3} M 5-MC. We have selected mutants of *ura*$^-$ strains which can grow on this medium (see above). Genetical and physiological analyses were performed on two such mutants (FL 502-1A and FL 503-2B). Crosses with the wild-type strain showed 2:2 segregation, and crosses with the cytosine permease-less strain (*fcy 2-3*) yielded spores which were either 5-FC resistant, or 5-MC resistant and slightly 5-FC resistant. Complementation

TABLE 3. *Entry of purines and inhibition of cytosine entry by purines (strain NC5-3C)*

Substrate	K_m for entry	K_i for cytosine
Adenine	2.2×10^{-6} M	4.2×10^{-6} M
Guanine	3.1×10^{-6} M	6.8×10^{-6} M
Hypoxanthine	3.6×10^{-6} M	14×10^{-6} M
2-APP[a]		53×10^{-6} M
Cytosine	2.4×10^{-6} M	
Deoxyadenosine		910×10^{-6} M

[a] 2-APP, 2-Amino-pyrazolopyrimidine, analogue of adenine.

tests, using 5-FC resistance as criterion, showed no complementation. Hence these mutants are most likely alleles of the *fcy 2* gene.

Initial velocities of uptake of cytosine and inhibition of uptake of cytosine by 5-MC were determined on strain *fcy 2-10*. As expected a modified K_i for 5-MC was found as well as slightly modified K_m for cytosine (see Table 2). V_{max} was only slightly reduced, and in view of the variability observed in the determination of V_{max}, this reduction seems not significant.

(ii) Mutants resistant to adenine inhibition of cytosine utilization. Following the principle outlined above, we selected mutants of *ura*$^-$ strains which can grow on minimal medium supplemented with 3×10^{-5} M cytosine plus 8×10^{-4} M adenine. However, in this instance, none of the mutants found displayed even a slight resistance to 5-FC. Moreover, the mutants belong to at least two complementation groups different from the cytosine permease locus. Two of the mutants belonging to each complementation group were crossed with *fcy 2-3:* in each case *ura*$^-$ spores possessing neither the cytosine permease-less mutation nor the mutation giving the resistance to adenine were isolated. This shows that these mutants are not allelic to the *fcy 2-3* mutation. One of the mutants, *ura*$^-$ *adR 1-1*, was tested for cytosine and adenine uptake and normal K_m and V_{max} values were found. If these mutants are therefore not relevant to the present study, they are interesting for the study of the linkage between purine and pyrimidine pathways and will be examined in the future.

(iii) Mutants with reduced affinity for cytosine. By an analogous procedure, an adenineless strain (FL 509 sp1) was used to select mutants resistant to the inhibition of growth produced by cytosine. Two mutants (NC 31-sp2 and NC 32-3A) were analyzed. They proved to be slightly resistant to 5-FC, did not complement with the *fcy 2* mutations, and yielded no 5-FC fully sensitive clones in crosses with *fcy 2* mutants. They are therefore allelic of the cytosine permease locus. As expected, the K_m and K_i for several substrates of the cytosine transport system were modified in these strains (Table 2). The V_{max} were only slightly impaired. It is interesting to note that mutants with altered K_m are readily isolated using the appropriate selections.

Mutants with modified cytosine-adenine uptake, with V_{max} more altered than K_m. Using ICR 170 as mutagen and a low concentration of 5-FC for the selection, several mutants were isolated which were shown to be alleles of the cytosine permease gene by complementation tests. Among these mutants, most of which showed no detectable concentrating activity for cytosine, one NC 20-2A had a slightly modified K_m for cytosine or a significantly reduced V_{max} (see Table 2 and Fig. 3). This mutant might have either a reduced efficiency in energy coupling, or a reduced number of carrier molecules. Further studies to elucidate this point are in progress.

Involvement of active transport in cytosine uptake. Measurements of initial velocities of cytosine uptake in a cytosine deaminase-less strain clearly show that the transport of cytosine occurs against its concentration gradient. Additional experiments were carried out in which cytosine uptake was measured on cells suspended in: complete minimal medium, minimal medium without glucose, distilled water plus 2% glucose, or distilled water. The results (Fig. 4) show that the level of accumulation depends on a metabolic energy source. The levels attained without glucose are 10 times lower than with glucose. It should be noted that, as no prior starvation was made, the residual accumulation of cytosine in absence of glucose is probably due to residual energy available at the beginning of the experiment. To see whether

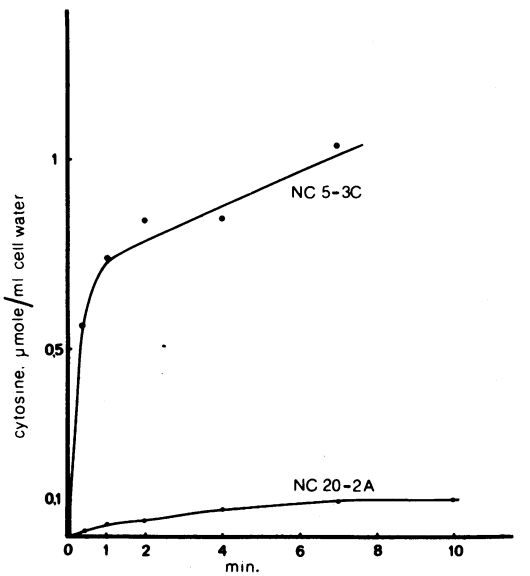

FIG. 3. *Uptake of cytosine as a function of time in strain NC 5-3C and in strain NC 20-2A. The strains were grown in YNB medium.* [^{14}C]*cytosine* (2.1×10^{-3} *µmol/ml*) *was added in each culture; 1 ml of cell culture were filtered through membrane filters (Millipore Corp.), and the filters were washed, dried, and counted in the standard way.*

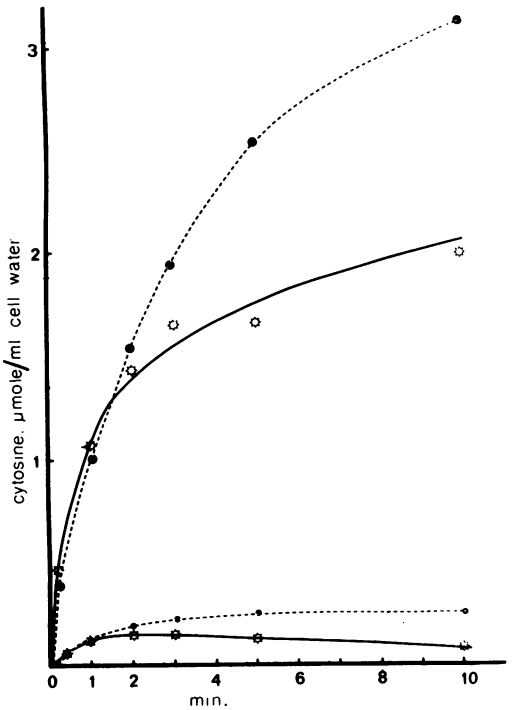

FIG. 4. *Cytosine uptake by strain FL 442-4B in several conditions. Cells grown in YNB were filtered through membrane filters and resuspended in distilled water (•, lower curve); distilled water plus glucose 0.5% (●, upper curve), YNB without glucose (☆, lower curve); YNB plus 0.5% glucose (☆, upper curve). Incubation was then carried out with cytosine at 2.7 × 10⁻³ µmol/ml and 1-ml culture samples were taken, filtered, and washed in standard way, at the indicated time intervals.*

the equilibrium level reached after prolonged incubation in cytosine is a dynamic one, cytosine deaminase-less cells were incubated in unlabeled cytosine (0.046 µmol/ml) until equilibrium was reached, then trace amounts of [^{14}C]cytosine (0.002 µmol/ml) were added to the culture and the uptake was measured. The initial uptake rate was measured in a parallel culture. The results (Fig. 5) clearly show that cytosine is constantly being recycled and do not suggest the existence of transinhibition.

Effect on cytosine uptake of inhibitors acting on energy production. The effects on cytosine uptake of 2,4-dinitrophenol (DNP) and iodoacetic acid have been compared. DNP uncouples oxidative phosphorylation, stimulates respiration, and is a proton conductor (for a review see 12). Iodoacetic acid reacts with unmasked sulfhydryl groups and inhibits glycolysis mainly by its action on 3-phosphoglyceraldehyde dehydrogenase (14). We found that 10^{-3} M DNP, added together with the cells into the incubation medium, strongly inhibited the uptake of cytosine; in fact, the uptake of cytosine is no longer accurately measurable in the standard conditions. This effect of DNP was similar whether the cells were ρ^+ or ρ^-, suggesting that the effect is not linked to respiration (see Table 4). On the contrary, 10^{-3} M iodoacetic acid added in the same conditions was without effect on the uptake. It should be noted that in our cultural conditions (YNB containing 2% glucose; optical density $\leq$ 100 Klett units, blue filter), the yeast cells were essentially fermenting.

Action of n-ethylmaleimide (NEM) on cytosine uptake. NEM is a reagent with high specificity for sulfhydryl groups and binds covalently to the proteins. Fox and Kennedy (3) have shown that the lac permease of *Escherichia coli* is inactivated by NEM and that the addition of β-D-galactosyl-1-thio-β-D-galactoside, a substrate of the permease, prevented the inactivation. We studied the effects of NEM on cytosine uptake and efflux in cytosine permease$^+$ and permease$^-$ cells. In cytosine

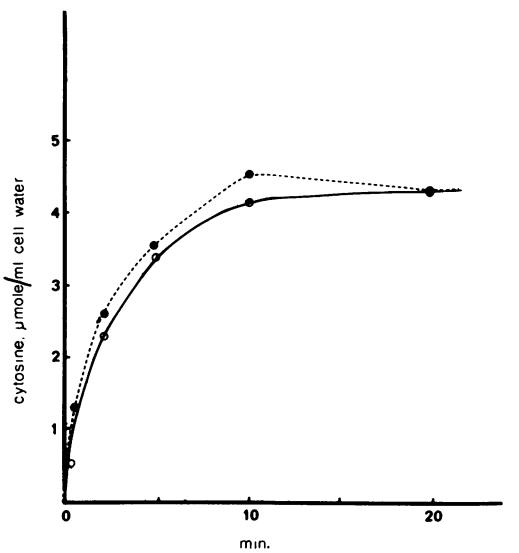

FIG. 5. *Comparison of cytosine uptake in strain FL 442-4B, loaded and not loaded with cytosine. Upper curve: strain FL 442-4B in YNB. At zero time, [^{14}C]cytosine (0.046 µmol/ml, 3.31 × 10^6 counts/min per µmol) is added, with sampling at the indicated time intervals. Lower curve: an aliquot of the culture has been incubated with cold cytosine (0.046 µmol/ml) for 40 min. At this time a trace amount of [^{14}C]-cytosine has been added (final specific radioactivity 2.8 × 10^6 counts/min per µmol) with sampling at the indicated time intervals.*

TABLE 4. *Action of inhibitors on cytosine uptake; action of DNP and IA on initial velocity of uptake of cytosine*[a]

Strain	[^{14}C]cytosine (nmol/ml)	Uptake in % control	
		DNP (10^{-3} M)	IA[b] (5×10^{-4} M)
FL 442-4B ρ^+	2.0	4.1	100
	5.0	5.9	94
FL 100 ρ^+	2.0	4.7	
	5.0	4.6	
FL 442-4B ρ^-	1.9	1.3	
	3.8	2.0	
	9.6	3.7	

[a] DNP (10^{-3} M) or IA were added in the tubes containing the radioactive cytosine at the indicated concentration. Samples (1 ml) of the culture were added and, after 30 s of incubation (with shaking), the samples were filtered and washed. The radioactivity was then determined. Control samples were treated identically except for the inhibitors.

[b] IA, Iodoacetic acid.

permease$^+$, it was found that incubation in minimal medium plus NEM 10^{-4} M decreases the initial velocity of uptake of cytosine. The addition of cytosine or adenine, before or together with the NEM did not prevent this decrease of the initial velocity of uptake (see Table 5). However, these experiments showed that incubation with cytosine reduced the initial velocity to about 70% of the control value, and this effect was already observed after 1 min of incubation. The incubation with adenine had a more pronounced effect which increased as a function of the length of preincubation (Fig. 6). If NEM is added to cells equilibrated with [^{14}C]cytosine, and the radioactivity of the cells is measured, one observes that after a lag the radioactivity of cells drops with a rate constant nearly the same as the diffusion constant found for cytosine permease-less cells (compare Fig. 7 and Fig. 11). This suggests that NEM blocks the carrier protein without strongly influencing diffusion. The action of NEM on cytosine efflux in permease-less cells previously equilibrated with [^{14}C]cytosine was measured to verify that it does not affect the passive diffusion process: the efflux was not significantly affected in the presence of NEM 10^{-4} M (see below).

The size of cytosine pool at steady-state. Measurements of the cytosine pool at steady-state have been made in strain FL 442-4B (*fcy 1-1*). The values in Lineweaver-Burk representation are given in Fig. 8. The results, which are similar to those observed for β-galactoside uptake in *E. coli* (7), can be formally described according to Stein (13). At steady-state, where influx is equal to efflux, if the efflux is proportional to internal concentration S_i, and since influx obeys the Michaelis-Menten kinetics, we may write:

$$kS_i = (V_{max} \cdot Q\, S_e)/(K_m + S_e) \qquad (1)$$

where S_i and S_e are, respectively, internal and external concentrations of cytosine, K_m is the Michaelis constant as calculated using initial velocities measurements, V_{max} is the maximum velocity of uptake, and Q represents the efficiency of the energy-coupling mechanism (the V_{max} measured experimentally are $V_{max} \cdot Q$). This equation rearranged gives:

$$\frac{1}{S_i} = \frac{k}{V_{max}\, Q} + \frac{k\, K_m}{V_{max}\, Q} \cdot \frac{1}{S_e} \qquad (2)$$

The constant k is the ratio of the slopes of the two curves $1/S_i$ and $1/S_e$. In our case, $k = 0.37$ which is about 10 times higher than the rate constant for exit of cytosine by diffusion which has been measured on permease-less strains (see below).

TABLE 5. *Action of inhibitors on cytosine uptake; initial velocity of uptake of cytosine of cells incubated in NEM 10^{-4} M for various lengths*[a]

Time (min)	10^{-4} M NEM (flask 1)	Cytosine (0.1 mol/ml) with 10^{-4} M NEM added (flask 2)	Adenine (0.1 mol/ml) with 10^{-4} M NEM added (flask 3)
0	100		
15	36	100[b]	100[c]
25	29	52	58
35	24	39	38
45	21	22	33
55	17	14	20

[a] Strain FL 442-4B was used. The culture in exponential growth phase was divided into four parts. At zero time, 10^{-4} M NEM was added to flask 1, 0.1 μmol of cytosine per ml to flask 2, 0.1 μmol of adenine per ml to flask 3. Flask 4 was the control without addition. After 15 min of incubation, 10^{-4} M NEM was also added in flasks 2 and 3. For the cytosine uptake measurements, 2-ml samples were withdrawn, filtered, washed with 2 × 2 ml of prewarmed YNB, and resuspended quickly in 2 ml of YNB; 1 ml was then taken immediately to measure cytosine uptake in the standard conditions.

[b] After the incubation in cytosine (0.1 μmol/ml for 15 min), the initial velocity of uptake of cytosine was reduced to 64% of the control which remained constant during the length of the experiments.

[c] After the incubation with adenine (0.1 μmol/ml for 15 min), the initial velocity of uptake of cytosine was reduced to 45% of the control.

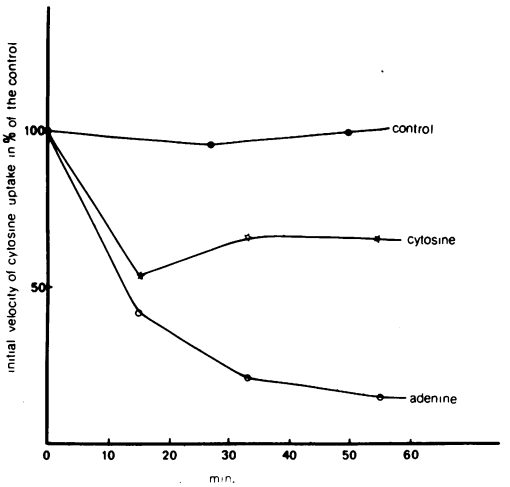

FIG. 6. *Effect of incubation in adenine and cytosine on the initial velocities of cytosine uptake. Strain NC 5-3C was grown in YNB. To 20-ml portions of the culture was added 0.1 μmol of cytosine per ml, 0.1 μmol of adenine per ml, or no addition (control). At intervals, 2 ml of the culture was filtered, washed twice with 5 ml of prewarmed YNB, resuspended in 2 ml of YNB, and well mixed. 1 ml was then used immediately to measure the initial velocity of uptake of cytosine at a concentration of 1.2 × 10^{-3} μmol/ml. The initial velocity is expressed as percentage of that in the control tube which did not change significantly during the incubation period.*

Efflux of cytosine. The efflux of cytosine has been studied in permease$^+$ and permease-less cells. Both strains carry an impaired cytosine deaminase to avoid any cytosine metabolism.

(i) Exit of cytosine from the permease$^+$ strain. If the permease$^+$ strain is first equilibrated with [^{14}C]cytosine, washed free of cytosine, and resuspended in minimal medium, cytosine is lost slowly and a new equilibrium is reached (Fig. 9 and 10).

The apparent rate constant at which cytosine leaves the cell before the new equilibration is smaller than the passive diffusion rate. However, if DNP is added after equilibrium is reached, cytosine efflux is induced at a rate which is significantly higher than the passive diffusion rate (see Fig. 9) and may reach the initial uptake rate (Fig. 7). Again, this shows that the equilibrium is a dynamic one and that the slow exit rate measured after the cytosine wash is a net rate (exit rate minus reentry rate). As DNP induces an efflux which is higher than the diffusion rate, this efflux is expected to be carrier mediated. This indicates that the inhibition of the uptake by DNP is not due to an inactivation of the carriers, but more probably to an effect on the energy-coupling mechanism.

A chase experiment of [^{14}C]cytosine by non-radioactive cytosine has been carried out (see Fig. 10). In this experiment, cells were first equilibrated with labeled cytosine and were then transferred to medium without cytosine. After a new equilibrium was reached, non-radioactive cytosine was added to a portion of the culture and the radioactivity of the cells was measured as a function of time. The addition of non-radioactive cytosine provokes an efflux of the internal [^{14}C]cytosine, which is best explained by a competition between the recycled molecules for reentry

Using the data of Fig. 8 and knowing the concentration of cytosine added, it is possible to calculate the new internal concentration expected at equilibrium and the final dilution of the internal labeled cytosine. This has been done for the experiment described in Fig. 10 (see Table 6). These calculations again indicate

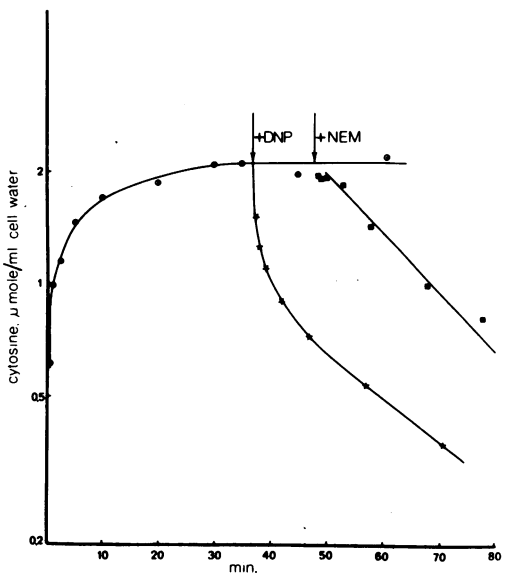

FIG. 7. *Compared action of DNP and NEM on intracellular concentration of cytosine. To a 50-ml culture of strain NC 5-3C in YNB medium, [^{14}C]cytosine at a final concentration of 1.4 × 10^{-3} μmol was added. 1-ml samples were then taken, filtered, and washed at the times indicated. At the time indicated by the first arrow, 10 ml of the culture were taken and added to a flask containing DNP (final concentration 10^{-3} M) and sampling was continued. When indicated by the second arrow, 10 ml of the control flask were added to a flask containing NEM (final concentration 1.3 × 10^{-4} M). Sampling was then continued as indicated. During the first 30 s after the addition of DNP, the cells lost 0.55 μmol of cytosine per ml of cell water, whereas the initial uptake rate was 0.62 μmol/ml of cell water per 30 s. In the presence of NEM the slope for the cytosine efflux was 0.037/min.*

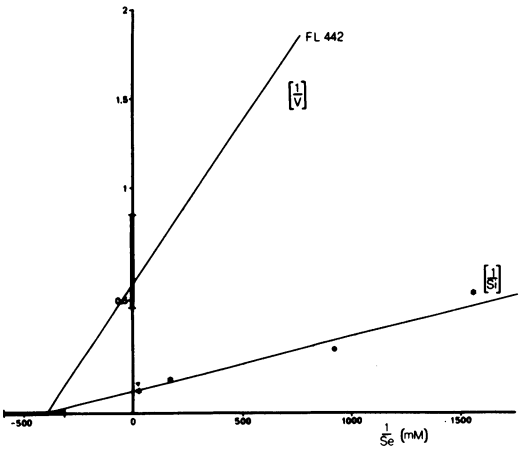

FIG. 8. *Measurements of internal concentration of cytosine at equilibrium in strain FL 442-4B. The lower curve represents the reciprocal of internal cytosine concentration (in micromoles per milliliter of cell water) at the plateau, versus reciprocal of external cytosine concentration (mM) at the plateau. Different symbols refer to different experiments. The upper curve represents the reciprocal of initial velocities of uptake versus reciprocal of external cytosine concentration using mean K_m and V_{max} given in Table 2 for this strain. The horizontal and vertical bars indicate the standard deviation of 1/S and $1/V_{max}$, respectively.*

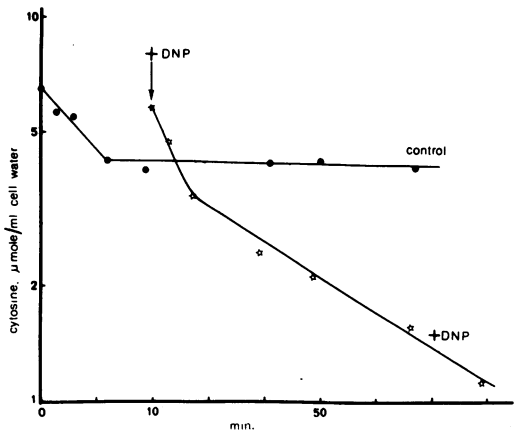

FIG. 9. *Effect of DNP on the internal cytosine level in strain FL 442-4B. Procedure similar to that reported in Fig. 10. Cellular [^{14}C]cytosine concentration at the plateau: 6.5 µmol/ml. Efflux of cytosine: k, 0.031 in the control. Initial efflux after addition of DNP 10^{-3} M: k, 0.071 in the first part of the efflux.*

that cytosine is constantly recycled contrary to what occurs, for instance, with the histidine permease in *S. cerevisiae* (1).

(ii) Exit of cytosine from the permease-less cells. The strain RJ 16 sp 1, first equilibrated

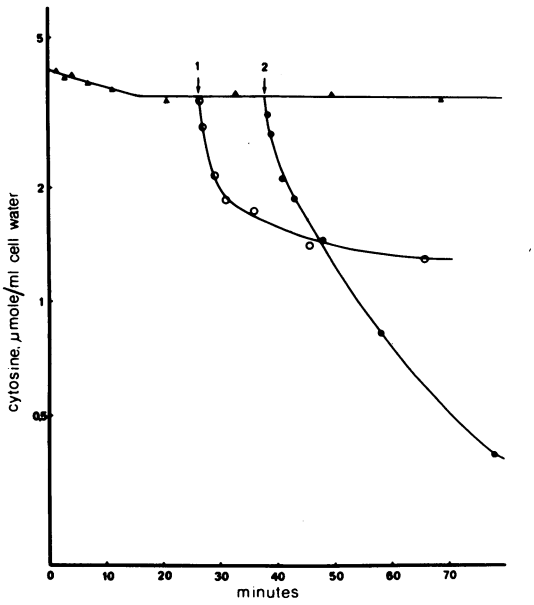

FIG. 10. *Chase experiment. 50 ml of strain FL 442-4B was incubated in minimal medium with [^{14}C]cytosine (2×10^{-3} µmol/ml) for 60 min. After filtration the cells were quickly resuspended in minimal medium, 1-ml samples were withdrawn at time intervals, and their radioactivity determined as indicated. Where indicated by the first and second arrow, 2×10^{-2} and 2×10^{-1} µmol of non-radioactive cytosine per ml was added to 15 ml of the culture and the cells were sampled as indicated, for the determination of radioactivity.*

with [^{14}C]cytosine at high external cytosine concentration, was washed free of cytosine and resuspended in minimal medium; the loss of radioactivity from the cells was then followed. First order kinetics was observed with a rate constant of 0.040/min (Fig. 11). This rate constant was reduced by 30% if the exit was measured in the presence of either DNP (10^{-3} M), NaN_3 (10^{-3} M), or *N*-ethylmaleimide (10^{-4} M). These results are opposite to those obtained with permease-mediated transport (in which DNP enhances cytosine efflux) and support the idea that only passive diffusion of cytosine was occurring in the permease-less strain. A diffusion coefficient of 5.7×10^{-8} cm per s has been calculated using our experimental data.

Possible regulation of the cytosine permease level. To determine if the cytosine permease level in the cell may be regulated, the initial velocities of uptake of cytosine were compared in cells cultivated in different media: wild-type cells were grown in minimal medium without ammonium and with cytosine as sole nitrogen source, in minimal medium with pro-

TABLE 6. *Chase of [^{14}C]cytosine by non-radioactive cytosine*

Concn of cytosine added for chase (μmol/ml)	[^{14}C]cytosine/cold cytosine	Internal concn of cytosine expected after equilibration with cold cytosine	Internal concn of [^{14}C]cytosine expected at equilibrium	Internal concn of [^{14}C]cytosine found after a 40-min chase
0.02	0.15	≃8–9 μmol/ml of cell water	1.2 μmol/ml of cell water	1.3 μmol/ml of cell water
0.2	0.0175	≃10 μmol/ml of cell water	0.175 μmol/ml of cell water	0.39 μmol/ml of water[a]

[a] The value of 0.39 μmol/ml of cell water was found after a 40-min chase, and presumably the expected value would have been obtained after a longer incubation (see fig. 10).

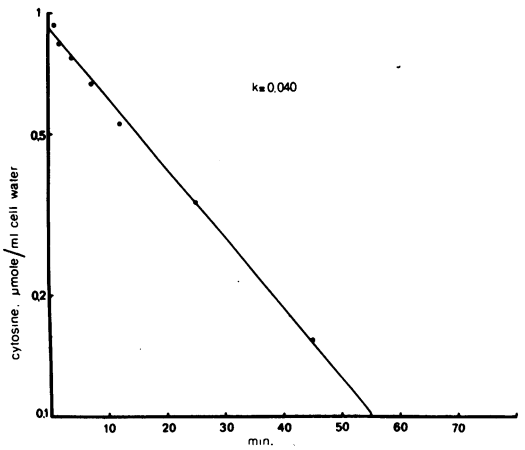

FIG. 11. *Cytosine efflux from strain RJ 16-sp2. The cells were loaded with cytosine by incubation in YNB medium plus [^{14}C]cytosine (2 μmol/ml) for 2 h. 20 ml were then filtered through a membrane filter and quickly resuspended in the same volume of YNB at the same temperature. At the indicated time intervals, 2 ml of cell suspension were filtered and washed in the standard way. Just before filtering the 20 ml, 1 ml was sampled to determine the internal level before filtration. First order kinetics of cytosine efflux with a rate constant k = 0.040/min was found.*

line as sole nitrogen source, and in minimal medium with ammonium sulfate as nitrogen source (standard conditions). Cells in exponential growth phase were used for the uptake measurements. The culture with cytosine was filtered on membrane filters (Millipore Corp.), washed with minimal medium without ammonium, and resuspended in the medium without ammonium. The uptake measurements were carried out during the 5 min after the wash. The results given in Table 7 show that in medium with proline as nitrogen source there is less than a twofold reduction of the velocities, whereas in the minimal medium or minimal medium with cytosine, no significant differences were found.

It seemed therefore that the permease level could be only slightly modified. But in the

TABLE 7. *Uptake of cytosine by S. cerevisiae FL 100 cultivated in minimal medium with different nitrogen sources*

Cytosine concn (μmol/ml)	Counts/min[a]		
	MM	MM[b] plus cytosine (1 mg/ml) as nitrogen source	MM[b] plus proline (1 mg/ml) as nitrogen source
1.44×10^{-2}	770	697	581
2.88×10^{-2}	970	862	591
5.76×10^{-2}	1,090	951	640
14.4×10^{-2}	1,111	1,078	660

[a] Incorporated per 30 s per 1 ml of culture at 100 Klett units. The growth rate was 290 min in medium with either cytosine or proline as nitrogen source and 160 min in minimal medium (MM).

[b] Without $SO_4(NH_4)_2$.

experiments with strain FL 442-4B, where a protective effect toward NEM of adenine and cytosine was looked for, it was reproducibly found that incubation with adenine (0.1 μmol/ml) reduced the velocity of uptake about fivefold, the maximal effect being reached after about 1 h. Cytosine at the same concentration reduced the activity by only 30 to 50% (see Fig. 11). Maximal inhibition with adenine is obtained after 60 min i.e., half the doubling time. This indicates that the cytosine permease is inhibited either by adenine itself or more probably by a metabolic derivative of it. Another possibility could be a repression by adenine, if one assumes that the permease has a much faster turnover than average yeast proteins.

DISCUSSION

Previous genetic and physiological studies have shown that cytosine and uracil entry in *S. cerevisiae* are mediated by two distinct transport systems (4, 6). We have now studied in more detail the cytosine transport system which is also responsible for purine transport (10). It is

striking that this system, which transports such different compounds as adenine, hypoxanthine, and cytosine, has about the same high affinities for all these compounds, and that these affinities are about 10 times higher than the affinity of the uracil transport system which seems much more specific (4, 6). Interestingly, mutants with modified affinities for cytosine and adenine are readily obtained; for instance, a mutant in which the K_m for adenine is nearly normal whereas the K_m for cytosine is increased by a factor of seven has been isolated, but mutants with decreased affinity for adenine only have not yet been found.

The affinity of the corresponding deoxynucleosides for the transport system are considerably lower, as shown by their low competitive inhibition (see Table 2 for deoxycytidine, and Table 3 for deoxyadenosine). Moreover, the cytidine and adenosine either display no competitive inhibition towards cytosine or their effects are so small that they could not be visualized in the concentration range studied (cytosine at concentration $<K_m$ and with concentration ratios of nucleoside/cytosine ranging from 160 to 1,000). These results are in accordance with the growth response of the cells on minimal medium supplemented with 5-FC 10^{-6} M and nucleosides, 200 μg/ml: after 24 h of incubation at 28 C, adenosine, inosine, and cytidine do not relieve the inhibition due to the 5-FC, whereas deoxycytidine and deoxyadenosine do, and with deoxycytidine growth is nearly as good as the control.

Models currently proposed to account for transport in bacteria are of three kinds (7). One type of model is the "mobile carrier" model, where the carrier is supposed to undergo a cycle of transformation such that the affinity of the carrier is much less on the internal side of the membrane than on the external side. Such models have been proposed by Fox and Kennedy (3) and by Winkler and Wilson (16) for the lactose permease in *E. coli*. This model has been thoroughly described (2, 5, 13). A second type of model, "the vectorial" model, postulates that accumulation is linked to chemical transformation of the substrate itself, as is found in the accumulation of phosphorylated sugars by the phosphoenolpyruvate-linked phospho-transferase mechanism (7). The last type of model involves periplasmic proteins carrying affinity sites for the transported substance (binding proteins), but in addition, a second protein molecule located in the membrane itself is thought to interact with the binding protein for releasing the substrate in the cytoplasm (7). We have tried to classify the cytosine permease according to one of these models.

Up to now, all the mutants deficient in cytosine transport which have been looked for belong to the same chromosomal locus. This suggests that cytosine permease does not function according to the third model. Moreover, since cytosine itself is accumulated in the internal pool, the vectorial model is also not tenable. Therefore we think that the functioning of this permease may be best described by the mobile carrier model. In this model, the transport reaction follows a Michaelis-Menten kinetics and the initial rate of entry v_{influx} is dependent of the external substrate concentration S_e, so that

$$v_{\text{influx}} = [S_e\, V_{max(e)}\, Q]/[S_e + K_{m(e)}] \quad (3)$$

where $K_{m(e)}$ is the affinity of the carrier on the external side of the membrane, $V_{max(e)}$ is proportional to the quantity of carrier proteins per unit area of the membrane, and Q represents the efficiency of the energy-coupling mechanism (13). The efflux of the substrate is also considered to be carrier mediated, with the effect of the energy-coupling mechanism being to decrease the affinity of the carrier at the internal side of the membrane. Therefore, it is expected that

$$v_{\text{efflux}} = \frac{S_i\, V_{max(i)}}{S_i + K_{m(i)}} + K S_i \quad (4)$$

where S_i stands for the internal concentration, $K_{m(i)}$ is the affinity of the carrier modified by the energy-coupling mechanism, $V_{max(i)}$ is the maximal velocity of exit when all the carriers are modified by the coupling mechanism (11) and K is the rate constant of noncarrier-mediated exit. It has been found in several transport systems that preincubation with a substrate of a given permease dramatically reduces its activity (1, 2, 3). This phenomenon has been termed transinhibition. According to Hunter and Segel (5) it is expected for any carrier-mediated transport system in which (i) the carrier exists in at least two conformations or locations, one accessible to external substrate and one accessible to internal substrate, and (ii) the internal conformation has a reasonable affinity for the substrate. In the case studied here, the initial velocity of uptake was similar for cells not preloaded with cytosine and for cells incubated during 40 min with non-radioactive cytosine (0.046 μmol/ml to which 0.002 μmol of radioactive cytosine were then added (Fig. 5). In this condition, transinhibition

should have been detected since the internal concentration is approaching its maximum level. This suggests that transinhibition is not very important for this permease, but more detailed experiments are needed to determine quantitatively the importance of this phenomenon in this particular transport system. If, provisionally, transinhibition is neglected, at least for low substrate concentrations, we can write that at equilibrium:

$$\frac{V_{max(e)}\, Q\, S_e}{K_{m(e)} + S_e} = \frac{V_{max(i)}\, S_i}{K_{m(i)} + S_i} + K S_i \qquad (5)$$

Under the assumption that $V_{max(i)} \simeq V_{max(e)}$, it is possible to evaluate $K_{m(i)}$, a constant of interest. Table 8 gives the calculated values $K_{m(i)}$ with this formulation. On the other hand, for the mobile carrier model, it has been shown that the equilibrium constant for the active transport process $K_{eq} = (V_{max(e)}/V_{max(i)}) \cdot (K_{m(i)}/K_{m(e)})$ (2). Using the data of Table 8, K_{eq} has been calculated (see Table 8, last column). It is noticeable that, at low external concentration, $(S_i)/(S_e)$ is nearly constant, but for high concentration of substrate the ratio $(S_i)/(S_e)$ decreases markedly. This may be due to the simultaneously increasing importance of passive diffusion and possibly of transinhibition. Using the K_{eq} obtained with the lowest concentration of cytosine and again with the assumption that $V_{max(i)} \simeq V_{max(e)}$, $K_{m(i)}$ calculated from K_{eq} is 8.5 μmol/ml, a value similar to the one found using equation (5), and rather high compared to the $K_{m(e)}$ of 0.0025 μmol/ml. We have found that

TABLE 8. *Estimation of* $K_{m(i)}$

	External concn (μmol/ml)	Internal concn (μmol/ml)	$K_{m(i)}$[a]	$K_{eq} = (S_i)/(S_e)$[b]
1	0.064×10^{-2}	1.9	8.36	2,967
2	0.070×10^{-2}	3.7	15.83	5,285
3	0.126×10^{-2}	4.0	9.85	3,171
4	0.152×10^{-2}	4.35	8.93	2,861
5	0.157×10^{-2}	4.3	8.50	2,738
6	0.370×10^{-2}	7.0	6.58	1,891
7	0.600×10^{-2}	6.7	3.97	1,166
8	4.30×10^{-2}	7.7	1.30	179
9	4.5×10^{-2}	10.2	2.10	226

[a] $K_{m(i)}$ was calculated using equation (5) with: $K_{m(e)}$, 2.5×10^{-3} μmol/ml; $V_{max(e)}$ Q, 3.44 μmol/min per ml of cell water; $V_{max(i)}$, 6.9 μmol/min per ml of cell water; K, 0.04/min.

[b] $K_{eq} = (S_i)/(S_e)$, where (S_i) stands for internal concentration expressed in micromoles per milliliter of cell water, and (S_e) for external concentration in micromoles per milliliters of medium.

DNP affects entry of cytosine, such that entry can no longer be measured under standard conditions in the presence of DNP 10^{-3} M. However, with a high cellular concentration and high external cytosine concentration, we found that uptake occurs with kinetics which show rapid equilibration of internal versus external concentration. In experiments with permease-less cells in identical conditions, the equilibration time could not be measured due to the slow increase in radioactivity of the cells. This indicates that, in DNP-treated cells, the entry of cytosine is still carrier mediated. That DNP does not inactivate the carrier is confirmed by the experiments where DNP is added to preloaded cells: in this case DNP induces an efflux of cytosine which is more rapid than the efflux occurring through passive diffusion in permease-less cells. Again these experiments are in accordance with the mobile carrier model where the effect of uncoupling is thought to prevent the change of K_m at the internal face of the membrane. That DNP acts immediately, whether the cells are ρ^+ or ρ^-, suggests that no functional mitochondria are needed for the active transport of cytosine and that the effects of DNP must be looked for in the plasma membrane itself.

The inhibitory effect of NEM differs markedly from DNP action. It is by no means instantaneous, 100% inhibition could not be reached even after prolonged incubation. Moreover, whereas DNP added to cells equilibrated with [^{14}C]cytosine induces an immediate and rapid efflux, NEM causes a slow efflux. This is best explained if one supposes that inactivation of the carriers allows passive diffusion in the direction imposed by the concentration gradient of cytosine. As no protective effect of the substrate of the permease towards NEM was found, we tentatively conclude that no —SH groups are in the binding-site area.

The existence of an inhibitory effect of some metabolic derivative of adenine raises interesting possibilities for future research. A search for a similar effect with cytosine derivatives will be made using a strain carrying the impaired cytosine deaminase and aspartate transcarbamylase no longer sensitive to feedback inhibition by uridine triphosphate. In such a strain the acid-soluble pool contains a high level of the various pyrimidine compounds.

A comparison of the properties of uracil and cytosine transport, especially of the efflux, studied in permease$^+$ and permease-less cells will be reported elsewhere.

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