Uptake of pyrimidines and their derivatives into *Candida glabrata* and *Candida albicans*

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The uptake of pyrimidines and their derivatives into *Candida glabrata* and *Candida albicans* was measured using a novel technique in which the cells were rapidly separated from their suspending medium by centrifugation through a layer of an inert oil. The uptake of [14 C]cytosine was linear for 30 s for all concentrations of pyrimidine tested. In *C. glabrata* but not *C. albicans* cytosine transport was mediated by both a high affinity (K_m 0.8 ± 0.1 μ M), low capacity [V 40 ± 4 pmol (μ l cell water) $^{-1}$ s $^{-1}$] and a low affinity [K_m 240 ± 35 μ M], high capacity system [V 770 ± 170 pmol (μ l cell water) $^{-1}$ s $^{-1}$]. The cytosine permease in *C. glabrata* was specific for cytosine and 5-fluorocytosine. In *C. albicans* there was only one cytosine transport system [K_m 2.4 ± 0.3 μ M; V 50 ± 4 pmol (μ l cell water) $^{-1}$ s $^{-1}$]; this system also transported adenine, guanine and hypoxanthine. Differences in nucleoside transport were also observed for *C. glabrata* and *C. albicans*, with the uridine permease in *C. glabrata* transporting only uridine and 5-fluorouridine whereas cytidine and adenosine were also transported by the uridine permease in *C. albicans*. Studies on the effect of nucleoside analogues on uridine transport in *C. glabrata* demonstrated the importance of the sugar moiety in determining the specificity of transport, with a hydroxyl residue on C-2 being apparently essential for transport.

Introduction

The uptake of a nutrient into cells consists in most cases of two events operating in tandem; an initial transport of the nutrient across the plasma membrane followed by its intracellular metabolism. The study of nutrient transport requires a compartmentalized system and a method of uncoupling transport from further metabolism of the substrate. This uncoupling can be genetic, kinetic or by chemical manipulation. Another key element is the rapidity with which cells and suspending medium can be separated. Most studies of the transport of purines, pyrimidines and their nucleosides into yeasts have involved measuring uptake of radioactively labelled substrates after a short incubation period during which time uptake was linear, and then separating the cells from the medium by filtration. In *Saccharomyces cerevisiae* and *Candida albicans* such studies have demonstrated that uptake of purines and pyrimidines is linear for 30 s but give no indication if this is true for all concentrations used to determine the kinetic parameters

(Chevallier *et al.*, 1975; Jund *et al.*, 1977; Rao *et al.*, 1983). This technique is limited if the initial linear phase of substrate transport is very short as is the case with pyrimidine transport into mammalian cells (Wohlhueter *et al.*, 1978). Another limitation of the filtration procedure is the risk of loss of the intracellular substrate from organisms where metabolism is uncoupled from transport or a non-metabolizable substrate used.

A technique used to overcome the limitations imposed by filtration and washing involves separating the cells from the suspending medium by centrifugation through a layer of an inert oil. This ensures a rapid separation of the cells from the substrate and also obviates the requirement for washing the cells so eliminating the possibility of removing any intracellular substrate (Wohlhueter *et al.*, 1978). Centrifugation through oil was originally developed to measure substrate transport into animal cells and has been adapted in the studies reported here to investigate the transport of pyrimidines, purines and their nucleosides into *Candida* spp. Purine and pyrimidine permeases have been studied in both *S. cerevisiae* and *C. albicans* using the filtration procedure. There are a number of permeases involved which show different degrees of specificity, with cytosine, adenine, guanine and hypoxanthine being transported by a single per-

Abbreviations: 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; 5-FUrd, 5-fluorouridine; 5-FdUrd, 5-fluoro-2-deoxyuridine; YNBG, Yeast Nitrogen Base + glucose; YMA, Yeast Morphology Agar.

mease (Polak & Grenson, 1973). The transport of purines and pyrimidines into yeast is energy coupled involving an electrogenic symport; however, the stoichiometry appears to differ for cytosine and uracil (Hopkins *et al.*, 1988).

We have undertaken a study of the specificity and enzyme kinetics of nucleobase and nucleoside transport in the haploid yeast *C. glabrata* using a rapid sampling centrifugation procedure and extended these studies to re-evaluate uptake into *C. albicans*.

Methods

Organisms and preparation of cell suspensions. The organisms used in these experiments were *C. glabrata* strain 4 (Whelan *et al.*, 1984), *C. albicans* strain 72S (Whelan & Kerridge, 1984) and fluoropyrimidine-resistant strains derived from them (Fasoli & Kerridge, 1988). Details of these strains are given in Table 1. For uptake experiments cultures were grown aerobically at 37 °C in Yeast Nitrogen Base (Difco) supplemented with 1% (w/v) glucose (YNBG) to mid-exponential phase. Under these conditions *C. albicans* grew in the yeast form. After harvesting by centrifugation and washing twice in deionized water, the cells were resuspended in sodium citrate buffer (50 mM; pH 5.4) containing 2% (w/v) glucose to a final density of 5×10^7 cells ml⁻¹, as determined by haemocytometer count. The cell suspensions were shaken at 190 r.p.m. for 1 h at room temperature (21 °C), harvested by centrifugation and resuspended to the same density in fresh buffer containing glucose. This cell suspension was maintained at room temperature and used within 60 min of preparation for transport studies.

Determination of the growth inhibitory effects of nucleoside analogues on *Candida* spp. Cells of both parental and uridine-permease-deficient strains of *C. albicans* and *C. glabrata* which had been grown at 37 °C for 24 h on Yeast Morphology Agar (Difco) (YMA) were suspended in saline (0.85% NaCl) and approximately 10^6 cells spread onto 20 ml fresh YMA in a Petri dish. A paper disc containing 100 µg of the analogue was placed in the centre of the agar and after 48 h incubation at 37 °C, the agar plate was inspected for the presence of a zone of growth inhibition around the disc. For each analogue a control disc containing the solvent used to dissolve the analogue was also tested.

Determination of the effects of nucleosides on 5-fluorouridine toxicity to *Candida* spp. Overnight cultures of yeast grown in YNBG at 37 °C with

shaking were used to inoculate YNBG (5 ml) containing 5-fluorouridine (5-FUrd) at three concentrations (5, 50 or 500 µM) together with the required nucleoside (uridine, thymidine or adenosine) at 1000 µM. After 24 h at 37 °C, growth was measured as the optical density at 600 nm and compared with that of control cultures grown in the absence of 5-FUrd.

Permease assay. A variation of the method described by Harley *et al.* (1982) was employed. Cell suspensions (100 µl) were layered over 150 µl inert oil (dibutyl phthalate/dinonylphthalate, 55:45, v/v; specific gravity 1.01 g ml⁻¹) in 12 × 1.5 ml Eppendorf microfuge tubes; the tubes were placed in an Eppendorf Microfuge (model 5414) with a 12-place fixed-angle rotor. Uptake of pyrimidines or their derivatives was initiated by the rapid addition of 100 µl aliquots of radioactively labelled substrate in citrate buffer, pH 5.4, to each tube at 2 s intervals. Additions were done with a calibrated, repetitive pipette (Eppendorf Multipette 4780). Uptake was terminated by switching the centrifuge on 2 s after the addition of the reaction mixture to the last tube, and centrifuging at full speed for 30 s. After centrifugation, the supernatant fluid was aspirated with a syringe and 2 × 1 ml water added above the oil phase and then aspirated to remove any remaining radioactivity. The oil layer was removed with the last wash and the bottoms of the tubes (containing the pellets) were cut off with a razor blade and placed in plastic scintillation vials. Triton X-100 (2%, w/v) (150 µl) was added and the vials were vortexed to resuspend the pellets. Optiphase Safe scintillant (2 ml) was added to the vials, and after thorough mixing to dissolve the contents, the radioactivity was assayed in a Beckman LS3801 scintillation counter.

Formulation of the oil phase was arrived at empirically by testing several types of oil and mixtures. The criterion used was the presence of a firm cell pellet after 30 s centrifugation; this was checked by visual examination of the pellet and by microscopical examination for the absence of cells at the oil/aqueous solution interface. Non-specific binding of radioactivity to the surface of cells was measured using aliquots of cell suspensions which had been heated in a boiling bath for 2 min. The controls were run in duplicate for every concentration of substrate tested. Non-specific binding of radioactivity to cells was negligible for all compounds studied with the exception of uridine, where it represented 10–15% of the intracellular radioactivity.

Fractionation of radioactively labelled material in the low molecular mass intracellular pool. Metabolism was stopped by centrifuging the cells through an oil layer into 50 µl 0.2 M-TCA in 10% (w/v) sucrose (density 1.04 g ml⁻¹) placed beneath the oil. Following centrifugation and a water wash, the cell pellets were extracted with 1 ml ice-cold diethyl ether to remove any residual oil and most of the TCA. Fractionation into free bases, nucleosides and nucleotides was then done as previously described (Wohlhueter *et al.*, 1978).

Table 1. Characteristics of the fluoropyrimidine-resistant organisms used

<i>Candida</i> sp.	Strain	Parent strain	Phenotype*	Lesion
<i>Candida glabrata</i>	4		5-FC ^S 5-FU ^S 5-FUrd ^S	—
	cp1	4	5-FC ^R 5-FU ^S 5-FUrd ^S	Cytosine permease
	up1	4	5-FC ^S 5-FU ^R 5-FUrd ^S	Uracil permease
	udp1	4	5-FC ^S 5-FU ^S 5-FUrd ^R	Uridine permease
	udp2	4	5-FC ^S 5-FU ^S 5-FUrd ^R	Uridine permease
	cd3	4	5-FC ^R 5-FU ^S 5-FUrd ^S	Cytosine deaminase
<i>Candida albicans</i>	upy0	4	5-FC ^R 5-FU ^R 5-FUrd ^S	UMP:pyrophosphorylase
	72S		5-FC ^S 5-FU ^S 5-FUrd ^S	—
	6F1	72S	5-FC ^S 5-FU ^S 5-FUrd ^R	Uridine permease

* 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; 5-FUrd, 5-fluorouridine.

Estimation of intracellular and extracellular space in *C. albicans* and *C. glabrata*. A solution containing 5 μCi (185 kBq) [carboxyl- ^{14}C]inulin and 10 μCi (370 kBq) $^3\text{H}_2\text{O}$ per ml of citrate buffer was mixed with an equal volume of the cell suspension. Triplicate samples (200 μl) were removed after 2 min and carefully layered above 100 μl of oil in 1.5 ml Eppendorf microfuge tubes. Centrifugation and sample preparation were done as for the transport experiments. ^3H and ^{14}C were assayed using an internally standardized, dual channel Beckman LS3801 scintillation counter. The intracellular water space was calculated by difference. The data were evaluated assuming that: (i) [carboxyl- ^{14}C]inulin (M_r 5200) was distributed evenly throughout the extracellular space (outside the cell wall) (Scherrer *et al.*, 1974); (ii) $^3\text{H}_2\text{O}$ was distributed evenly throughout the total water space in the cell pellet; and (iii) the space between cell wall and plasma membrane is negligible relative to the intracellular space (Arnold, 1973).

The water space of pellets of all strains of *C. glabrata* used was $2.63 \pm 0.19 \mu\text{l}$ per 10^8 cells (mean $\pm$ SD). The comparable result for *C. albicans* strain 72S was $3.45 \pm 0.27 \mu\text{l}$ per 10^8 cells.

Sources of chemicals. 5-Fluoro[^{14}C]cytosine (5-FC) was a gift from Dr A. Polak, Hoffman-La Roche. [^{14}C]Cytosine was obtained from CEA. All other radioactively labelled compounds were obtained from Amersham. Nucleobases, nucleosides, nucleotides and their analogues were obtained from Sigma.

Results and Discussion

Characterization of pyrimidine transport into C. glabrata and C. albicans.

Studies on the uptake of nutrients into *Candida* spp. provided strong evidence that filtration is an inadequate method for the accurate measurement of the kinetic parameters of substrate transport in yeast (Fasoli, 1989).

Measurements of the rate of transport of pyrimidines in both *C. glabrata* and *C. albicans* by the oil-centrifugation technique as a function of cell density indicated that this parameter was reproducible and remained constant over a range of cell densities from 3.3×10^6 – 3.3×10^8 cells ml^{-1} . The experiments were done at 21 °C rather than the growth temperature of 37 °C; this would have had the effect of reducing the maximum rate of uptake. These results coupled with the absence of non-specific binding confirmed the suitability of this method for separating cells from the suspending medium when measuring transport of radiolabelled substrates into yeast.

The uptake of cytosine, thymine and uracil into both *C. glabrata* strain 4 and *C. albicans* strain 72S was linear for 30–60 s at all concentrations of pyrimidine examined from 1 μM –1000 μM . The curves did not extrapolate to zero radioactivity at zero time since no account was taken of the time lag between starting the centrifuge and effective sedimentation of the cells through the oil layer. This time lag was 2.5 ± 0.2 s (mean of 12 time courses in duplicate $\pm$ SD). In all experiments nominal sampling times were increased by 2.5 s for the purpose of calculating the initial rates of uptake. During the first

45 s of incubation of both *C. albicans* and *C. glabrata* with a radioactively labelled pyrimidine (cytosine, uracil and thymine) or pyrimidine nucleoside (cytidine, uridine and thymidine) less than 15% of the total radioactivity could be detected in the pyrimidine nucleotide fraction. In all determinations of the kinetic parameters of pyrimidine transport the uptake of substrate after 30 s was used as a measure of the initial rate.

Incorporation of cytosine was followed in *C. glabrata* strain 4 over a longer time period (6 min) to determine both the nature of the accumulated radioactivity and the time taken to reach steady-state levels of free intracellular pyrimidine. By 200–300 s the concentration of cytosine in the intracellular pool had achieved a steady-state value (6 mM); the increase in total radioactivity from this time onwards measured solely the combined rates of deamination of cytosine and the subsequent phosphorylation of uracil. Chromatographic analysis of the intracellular accumulated products in both *C. albicans* and *C. glabrata*, following their exposure to all the pyrimidines tested, indicated that the incorporated radioactivity could be recovered predominantly as the free base after the first 30–60 s. After this time there was a significant decrease in the percentage of radioactivity recovered as the free base and a simultaneous increase in the level of phosphorylated derivatives. This supports the concept that the initial transport of pyrimidines into the cell is functionally separate from their subsequent phosphorylation.

Initial velocities of transport of [^{14}C]cytosine into *C. glabrata* strain 4 were determined as a function of external cytosine concentration after a single 30 s incubation by the oil-centrifugation assay. The results obtained were analysed graphically by a Lineweaver–Burk plot (Fig. 1); this demonstrated the existence of two kinetic functions, corresponding to a high affinity, low capacity system, and a low affinity, high capacity system. The apparent affinities (K_m values) were 0.8 μM and 240 μM respectively (Table 2). These results contrasted with those for *C. albicans* strain 72S, where cytosine transport was a single phase system with K_m of 2.4 μM (Table 3).

Studies on the effect of substrate concentration on the accumulation of other radioactively labelled pyrimidines and their nucleoside derivatives indicated that in both *C. glabrata* strain 4 and *C. albicans* strain 72S these substances are also transported into the cell by saturable systems displaying simple Michaelis–Menten kinetics (Tables 2 and 3). The experimentally determined K_m and V values differed for all pyrimidines analysed except for cytosine and 5-FC which utilize the same permease to enter the cell. In *C. glabrata*, the cytosine permease had the highest affinity for its substrate (K_m 0.8 μM at low concentrations) and the thymine permease the lowest

Fig. 1. Cytosine uptake by *C. glabrata* strain 4. Initial velocities of uptake [v ; pmol (μ l intracellular water) $^{-1}$ s $^{-1}$] of cytosine as a function of external concentration (S ; mM) were measured in 30 s assays as described in the text; the results are plotted according to Lineweaver-Burk.

(K_m 110 μ M). The low affinity transport system for cytosine had the highest maximum velocity [770 pmol (μ l cell water) $^{-1}$ s $^{-1}$] whereas that of cytidine the lowest [8.8 pmol (μ l cell water) $^{-1}$ s $^{-1}$]. In *C. albicans*, the cytosine permease had the highest affinity for its substrate (K_m 2.4 μ M) and that for thymine the lowest (K_m 124 μ M). Cytosine transport had the highest maximum velocity [49.8 pmol (μ l cell water) $^{-1}$ s $^{-1}$] and uracil the lowest [7.2 pmol (μ l cell water) $^{-1}$ s $^{-1}$].

The transport of pyrimidines was measured in strains of *Candida* where the transport step was uncoupled from any further metabolism of the substrate once inside the cell by virtue of the loss of an enzyme of the pyrimidine salvage pathway. The K_m and V values for the transport of cytosine into strains either deficient for cytosine deaminase or UMP pyrophosphorylase, or for uracil-

Table 2. Characteristics of pyrimidine and pyrimidine nucleoside transport in *C. glabrata*

The steady-state intracellular concentration of substrate (S_i) was determined after 5 min incubation with radioactively labelled pyrimidine or pyrimidine nucleoside [50 μ M; 10 μ Ci ml $^{-1}$ (370 kBq ml $^{-1}$)]. Cells were centrifuged through an oil layer and radioactivity in the soluble pyrimidine pool and the supernatant fluid, which corresponded to the pyrimidine initially in the medium, was measured. K_m and V values were calculated from weighted, best line fits of Lineweaver-Burk plots. All values were determined with wild-type strain 4 except for cytosine, where S_i and S_i/S_0 values were determined for *C. glabrata* strain cd3 (cytosine-deaminase-deficient), as a significant amount of intracellular radioactivity was present in the form of uracil and nucleotides after 5 min incubation with [14 C]cytosine. There were no differences between K_m and V values for pyrimidine transport into strain cd3 and the parental strain 4 (Table 4). S_0 , substrate (radioactivity) concentration outside cell.

Substrate	S_i (μ M)	S_i/S_0	K_m (μ M) $\pm$ SD	V [pmol (μ l cell water) $^{-1}$ s $^{-1}$] $\pm$ SD
Cytosine	6100	120	high aff.: 0.8 $\pm$ 0.1 low aff.: 240 $\pm$ 35	39.7 $\pm$ 3.2 770 $\pm$ 170
Uracil	180	3.6	13.6 $\pm$ 1.5	23.6 $\pm$ 2.7
Thymine	200	4.4	100 $\pm$ 23	14.8 $\pm$ 2.0
Cytidine	260	5.1	87.5 $\pm$ 9.3	8.8 $\pm$ 1.9
Uridine	1100	22	17.7 $\pm$ 2.1	18.8 $\pm$ 2.6
Thymidine	290	5.8	83.1 $\pm$ 9.5	10.2 $\pm$ 1.1
5-Fluorocytosine	5800	115	high aff.: 1.1 $\pm$ 0.2 low aff.: 224 $\pm$ 31	36.6 $\pm$ 3.9 705 $\pm$ 163

Table 3. Characteristics of pyrimidine and pyrimidine nucleoside transport in *C. albicans*

The steady-state intracellular concentration of substrate (S_i) was determined after 5 min incubation with radioactively labelled pyrimidine [50 μ M; 10 μ Ci ml $^{-1}$ (370 kBq ml $^{-1}$)]. Cells were centrifuged through oil and the radioactivity in the soluble pool and the supernatant fluid, which corresponded to the pyrimidine or pyrimidine nucleoside in the medium, was measured. K_m and V values were calculated from weighted, best line fits of Lineweaver-Burk plots.

Substrate	S_i (μ M)	S_i/S_0	K_m (μ M) $\pm$ SD	V [pmol (μ l cell water) $^{-1}$ s $^{-1}$] $\pm$ SD
Cytosine	5300	106	2.4 $\pm$ 0.3	49.8 $\pm$ 4.2
Uracil	200	4.4	114 $\pm$ 15	7.2 $\pm$ 0.9
Thymine	430	8.6	124 $\pm$ 22	25.9 $\pm$ 3.1
Cytidine	340	6.8	106 $\pm$ 11	8.3 $\pm$ 0.9
Uridine	1400	28	17.7 $\pm$ 2.1	34.7 $\pm$ 3.8
Thymidine	370	7.4	76.5 $\pm$ 7.9	20.2 $\pm$ 2.0
5-Fluorocytosine	4900	98	2.7 $\pm$ 0.3	49.9 $\pm$ 5.5

Table 4. Characteristics of cytosine and uracil transport in *C. glabrata* strain 4 and mutant strains

K_m and V values were calculated from weighted, best line fits of Lineweaver–Burk plots, following transport assays as described in Methods. ND, no pyrimidine uptake detected after 30 s incubation, using a specific radioactivity of $5 \mu\text{Ci } \mu\text{mol}^{-1}$ ($185 \text{ kBq } \mu\text{mol}^{-1}$) and pyrimidine concentrations up to 1 mM.

Strain (lesion)	Substrate			
	Cytosine		Uracil	
	K_m (μM) $\pm$ SD	V [pmol (μl cell water) $^{-1}$ s^{-1}] $\pm$ SD	K_m (μM) $\pm$ SD	V [pmol (μl cell water) $^{-1}$ s^{-1}] $\pm$ SD
4 (wild-type)	high aff.: 0.8 ± 0.1 low aff.: 240 ± 35	39.7 ± 4.2 770 ± 170	114 ± 15	7.2 ± 0.9
cp1 (cytosine permease)	high aff.: ND	ND	104 ± 12	6.8 ± 0.7
up1 (uracil permease)	high aff.: 0.9 ± 0.1 low aff.: 244 ± 28	41.3 ± 4.5 764 ± 127	ND	ND
upy0 (UMP pyro- phosphorylase)	high aff.: 0.8 ± 0.1 low aff.: 259 ± 38	38.5 ± 3.9 747 ± 111	119 ± 18	7.0 ± 0.8
cd3 (cytosine deaminase)	high aff.: 0.8 ± 0.2 low aff.: 247 ± 22	35.3 ± 4.0 738 ± 101	107 ± 13	7.5 ± 1.1

permease-deficient strains, were within experimental error identical to those obtained for the parental strain 4 (Table 4). There was no detectable transport of uracil into the uracil-permease-deficient strain of *C. glabrata*. Transport of these substrates is functionally separated from their intracellular metabolism. This conclusion was true also for the other pyrimidines tested as in cells incubated with radiolabelled pyrimidines radioactivity appeared intracellularly first as the free pyrimidine and after a short lag period radiolabelled phosphorylated derivatives appeared.

Analysis of the data revealed that transport of all pyrimidines tested in *C. glabrata* strain 4 and *C. albicans* strain 72S was mediated by saturable processes, obeying Michaelis–Menten kinetics. The apparent K_m and V values differed for different pyrimidines, which suggested that these substrates may not share a common permease(s). The values for K_m and V differ from the published data but such differences could reflect the different techniques used and, in particular, differences in the incubation temperature and the pH value of the suspending medium, since it has been shown that in *S. cerevisiae* the experimentally determined values of K_m are dependent upon pH (Foret *et al.*, 1978).

Relationship between intracellular and extracellular concentrations of pyrimidines and pyrimidine nucleosides

The ratio of the intracellular and extracellular concentrations of pyrimidines at steady state gives an indication of

whether an active or passive mechanism of transport is operating to accumulate these substances within the cell. In *C. albicans* and *C. glabrata*, all pyrimidines tested reached their steady-state intracellular concentration (S_i) after 5 min incubation with the radiolabelled substrate. Since in *C. glabrata* cytosine enters the cell by two transport systems, the steady-state intracellular concentration of cytosine (S_i) was measured as a function of the cytosine concentration in the medium (S_0) (Fig. 2). These measurements were made in *C. glabrata* strain cd3 which

Fig. 2. Intracellular concentration of cytosine in *C. glabrata* strain cd3 as a function of the concentration of cytosine in the medium. Cells (5×10^7) were incubated for 5 min with different concentrations of [^{14}C]cytosine. Following centrifugation through oil into 0.2 M-TCA, cell-associated radioactivity was measured. All the radioactivity detected was in the form of cytosine, as determined chromatographically.

is totally defective in cytosine deaminase, to avoid the problem of artefacts caused by rapid intracellular metabolism of cytosine. In *C. glabrata*, the steady-state ratio S_i/S_0 for cytosine (Fig. 2) was high at low external concentrations of cytosine and decreased when S_0 exceeded 50 μM . This indicated that the high affinity transport system operated by means of a concentrative (active) mechanism whereas the low affinity system did not. More accurate data are required to determine the relative importance of passive and facilitated diffusion in cytosine transport in *C. glabrata* when cytosine is present in high concentrations in the medium.

The steady-state ratios S_i/S_0 for all pyrimidines and pyrimidine nucleosides tested in both *C. albicans* and *C. glabrata*, measured at a fixed external concentration of 50 μM are given in Tables 2 and 3. With the exception of cytosine, where the S_i/S_0 ratio was determined in a strain unable to metabolize cytosine, the S_i/S_0 values for the other compounds will not be an accurate estimation of the concentrative powers of the respective transport systems since, at the time of measurement, a significant amount of the pyrimidine had already been converted into phosphorylated derivatives. In both *C. albicans* and *C. glabrata* cytosine transport was the most efficient with S_i/S_0 values of 106 and 120 respectively, whilst uracil transport was the least efficient, with S_i/S_0 values of 4.4 and 3.6 respectively. These results are in agreement with those of other workers (Lossan *et al.*, 1987; Reichert & Foret, 1977; Hopkins *et al.*, 1988) and support the finding that pyrimidine transport is energetically coupled to a proton symport. This conclusion was further substantiated by the observation that pre-incubation of cells with glucose was necessary to obtain significant rates of transport for all pyrimidines and nucleosides tested.

Specificity of pyrimidine and pyrimidine nucleoside transport in C. glabrata and C. albicans

The specificity of transport of pyrimidine bases and nucleosides into *C. glabrata* strain 4 and *C. albicans* strain 72S was assessed by monitoring the effect of pre-incubating the cultures for 60 s with unlabelled purines, pyrimidines, their nucleosides and analogues of them (final concn 100 μM) on the subsequent incorporation of ^{14}C -labelled pyrimidines and ^{14}C -labelled pyrimidine nucleosides (final concn 50 μM). Where there was a reduction in the uptake of the ^{14}C -labelled pyrimidine or pyrimidine nucleoside it was assumed that both compounds entered the cell via the same transport system. In both *C. glabrata* and *C. albicans* the transport of individual pyrimidines occurred independently of that of the corresponding nucleosides. In addition, the pyrimidine bases did not share a common transport system, although in *C. albicans* cytosine shared a common

transport system with the purines adenine, guanine and hypoxanthine since pre-incubation with these compounds resulted in a 70–80% reduction in the uptake of cytosine. This was in agreement with results obtained by Polak & Genson (1973). In *C. glabrata*, pre-incubation with these purines had no effect on cytosine transport and presumably they were not transported into this organism by the cytosine permease. It was also clear that the fluorinated pyrimidines shared the same transport system with their unfluorinated counterparts, as the transport of the latter compounds into the cell was completely prevented in the presence of the analogues.

In *C. glabrata* transport of a pyrimidine nucleoside into the cell was not significantly affected by preincubating the cells for 60 s in the presence of other nucleosides (100 μM), suggesting that in this organism there are separate transport systems for cytidine, thymidine and uridine. In *C. albicans* there was some overlap in the transport systems for the pyrimidine nucleosides. In particular, cytidine and adenosine inhibited the transport of uridine by 69% and 73% respectively, indicating a common transporter for the three nucleosides. As thymidine had no effect on the transport of uridine, it would appear that in *C. albicans* there exists more than one nucleoside transport system, one of which is shared by adenosine, cytidine and uridine.

The specificity of uridine permeases in *C. albicans* and *C. glabrata* was further investigated. Firstly, by studying the growth inhibitory effects of other toxic nucleosides on uridine permease deficient strains; secondly, by measuring the uptake of radiolabelled nucleosides into parental and uridine-permease-deficient strains; and thirdly, by assessing the protective effects of selected nucleosides on the toxicity of 5-FUrd. Three uridine-permease-deficient strains were examined; *udp1* and *udp2* were derived from *C. glabrata* strain 4 and 6F1 from *C. albicans* strain 72S. These strains were resistant to 5-FUrd at a concentration of 1000 $\mu\text{g ml}^{-1}$ whereas growth of the parental strains was inhibited at concentrations less than 1 $\mu\text{g ml}^{-1}$. These uridine-permease-deficient strains were sensitive to the toxic effects of 5-FC and 5-FU.

Fourteen nucleoside analogues were tested for their growth inhibitory effects on both the parental and the uridine-permease-deficient strains of *Candida* by examining their ability to produce a zone of inhibition when 100 μg of the analogue was applied to the surface of an agar plate seeded with the relevant strain of yeast. 4-Aminopyrazolopyrimidine, bredinin, 5-fluorocytidine, 5-FUrd, purine riboside, sinefungin and tunicamycin all inhibited the growth of *C. albicans* strain 72S. *C. albicans* strain 6F1, which was 5-FUrd resistant, was also resistant to bredinin, 5-fluorocytidine and purine riboside, but was as sensitive to 4-aminopyrazolopyrimidine,

sinefungin and tunicamycin as the parental wild-type strain. The uridine-permease-deficient strains of *C. glabrata*, *udp1* and *udp2*, were sensitive to 5-fluorocytidine, a result consistent with the observation that cytidine is not transported into this organism by a uridine permease.

[¹⁴C]Uridine incorporation into uridine-permease-deficient strains of both *C. albicans* and *C. glabrata* was depressed to values approximately 10% of those obtained for incorporation into the parental strains. This reduction in uptake was also observed with [¹⁴C]adenosine for the uridine-permease-defective strain of *C. albicans* but not for those of *C. glabrata*. In *C. glabrata* adenosine was incorporated very poorly as was thymidine in both *C. albicans* and *C. glabrata*. As expected in both *C. glabrata* and *C. albicans* uridine gave good protection against inhibition by 5-FUrd in liquid culture. In *C. glabrata* no other nucleoside provided protection whereas in *C. albicans* adenosine counteracted the toxicity of 5-FUrd.

Little is known of the mechanisms and specificity of pyrimidine nucleoside transport systems in *Candida*, although it appears that the transport step is independent of phosphorylase activity (Rao *et al.*, 1983). The uridine permeases of *C. albicans* and *C. glabrata* have different substrate specificities. 5-FUrd, uridine, adenosine, brendin, cytidine, 5-fluorocytidine and purine riboside are substrates for this transport system in *C. albicans*, whereas in *C. glabrata* only 5-fluorouridine and uridine are transported via the uridine permease. Also, in addition to being toxic to the parental strains, 4-aminopyrazolopyrimidine, sinefungin and tunicamycin in *C. albicans*, and 4-aminopyrazolopyrimidine, 5-fluorocytidine and tunicamycin in *C. glabrata*, were toxic to strains lacking uridine permease, indicating the presence of alternative routes of nucleoside entry into these organisms. 5-Fluoro-2-deoxyuridine (5-FdUrd) was inactive against both *C. glabrata* strain 4 and *C. albicans* strain 72S, a surprising result in view of the toxicity of this drug to other eukaryotic cells (Martindale, 1982). There are two main possibilities for this lack of toxicity. Firstly, that 5-FdUrd enters the cells but is not metabolized to toxic nucleotide derivatives, and secondly, that 5-FdUrd cannot enter the cell (data not shown).

Examination of the structures of the nucleosides sharing the uridine transporter in *C. albicans* revealed wide differences in nucleobase structure with an intact sugar moiety as the common structural element between these compounds. The importance of this feature in the specificity requirements of uridine permease was confirmed by the finding that 5-FdUrd, whose only

difference from 5-FUrd is a hydrogen atom replacing a hydroxyl group at position 2 of the sugar ring, is not a substrate for the transporter. Indeed, the presence of an intact hydroxyl group at position 2 of the sugar moiety of a nucleoside may be itself an important structural requirement for substrates of this uridine transporter.

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