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^{19}F Nuclear Magnetic Resonance Study of Fluoropyrimidine Metabolism in Strains of *Candida glabrata* with Specific Defects in Pyrimidine Metabolism

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Flucytosine (5-FC)-resistant strains were isolated from the haploid opportunistic pathogen *Candida glabrata* by UV-induced mutation and fluoropyrimidine selection. These strains were characterized biochemically, and the metabolism of fluorinated pyrimidines was studied by ^{19}F nuclear magnetic resonance spectroscopy. No evidence was obtained from these studies for degradative metabolism of the fluorinated derivatives. In the parental susceptible strain of *C. glabrata*, 5-fluorouracil but not 5-FC was detected within the cells. 5-Fluorouracil was also present in the culture supernatant after incubation of the cells with 5-FC. The distribution of fluorinated derivatives within the 5-FC-resistant strains was consistent with their genotype. Two strains of *C. glabrata* which had only a partial loss of cytosine deaminase and UMP pyrophosphorylase activity had high levels of resistance to 5-FC. Both *C. glabrata* and *Candida albicans* were susceptible to 5-fluorouridine. This compound but not the anticancer drug 5-fluoro-2-deoxyuridine was shown to be transported into susceptible cells by a specific uridine permease.

Nuclear magnetic resonance (NMR) spectroscopy is a powerful method for identifying drug metabolites (33) and investigating drug-receptor interactions in cell-free solutions (13). It can also be used noninvasively to study metabolism in vivo in microorganisms, isolated tissues, intact animals, or humans (18). The practical application of NMR spectroscopy in these living systems, however, is limited by sensitivity considerations.

The ^{19}F nucleus has the second-highest NMR sensitivity of the stable nuclei (ca. 85% of that of ^{1}H at constant field). It is the naturally occurring isotope of fluorine (100% natural abundance) and is not present in living systems to any significant degree. The ^{19}F chemical shift range is large so that the resonance of any compound labeled with ^{19}F can often be unambiguously assigned. ^{19}F NMR is therefore particularly suitable for in vivo investigations of exogenously administered fluorinated chemicals. For example, it provides a most sensitive method for the accurate in vivo measurement of many cations of biological importance (1, 12) and has allowed direct observation of the fluorinated anticancer drug 5-fluorouracil (5-FU) and its metabolites in the liver and in implanted lung carcinomas in mice (23), in cultured tumor cells (11), in *Escherichia coli* (6) and, more recently, in the liver (22, 31), plasma, and urine (8) of patients undergoing chemotherapy. In addition, it has been used to demonstrate tumor trapping of 5-FU in tumor-bearing humans and rabbits (32). NMR spectroscopy has also been successful in demonstrating the existence of two distinct pathways of 5-FU degradation in *E. coli* (14).

^{19}F NMR spectroscopy has also been employed in a preliminary study of the uptake and metabolism of the clinical antifungal drug 5-flucytosine (5-FC) in the medically important yeast *Candida albicans* (10). A similar but more quantitative approach was adopted to study 5-FC metabolism in three *C. albicans* strains and one *Candida tropicalis* strain (25). Results from these studies complemented each other and were consistent with the assigned genotypes of the strains used; in addition, they established the basic conditions under which ^{19}F NMR spectroscopy may be used to obtain significant information on 5-FC metabolism in viable cells of *Candida* spp. Similar studies have been reported with *Aspergillus* spp. in which it has been shown that the main pathway of 5-FC metabolism is via the pyrimidine salvage pathway to 5-fluorouridine triphosphate (5-FUTP). In *Aspergillus fumigatus*, the catabolite and α-fluoro-β-alanine were also detected (3). Although 5-FC and its derivatives can be readily detected in the soluble pool of intact cells, it is not possible to detect these compounds once they are incorporated into cellular macromolecules without prior degradation (20, 26).

From biochemical studies, it is known that in yeasts 5-FC is deaminated to 5-FU and that 5-FU is subsequently phosphorylated to 5-FUMP and 5-FUDP. This latter compound can be converted to either 5-FUTP or to 5-fluoro-2′-deoxyuridine-5′-monophosphate (5-FdUMP), which, together or independently, determine the antifungal activity of 5-FC via production of aberrant RNA or inhibition of DNA synthesis, respectively (21, 25) (Fig. 1). The antifungal activity of 5-fluorouridine (5-FUrd) can be explained on a similar basis, following its conversion to 5-FUMP (Fig. 1).

In the present study, we addressed the biochemical mechanisms of action of 5-FC in a related pathogenic yeast, *C. glabrata*. The choice of organism was dictated by the fact that *C. glabrata* is haploid (30) and therefore gives rise to a wider range of mutants resistant to 5-FC (and other fluorinated pyrimidines) than does the diploid *C. albicans* when subjected to UV mutagenesis and selection (5). For these studies, a range of mutant strains of *C. glabrata* resistant to the inhibitory effects of 5-FC and 5-FUrd because of specific lesions in enzymes of the pyrimidine salvage pathway was employed (Table 1). This study was extended to include an analysis, in both *C. albicans* and *C. glabrata*, of the fate of the fluorinated pyrimidine nucleosides 5-FUrd and 5-fluoro-

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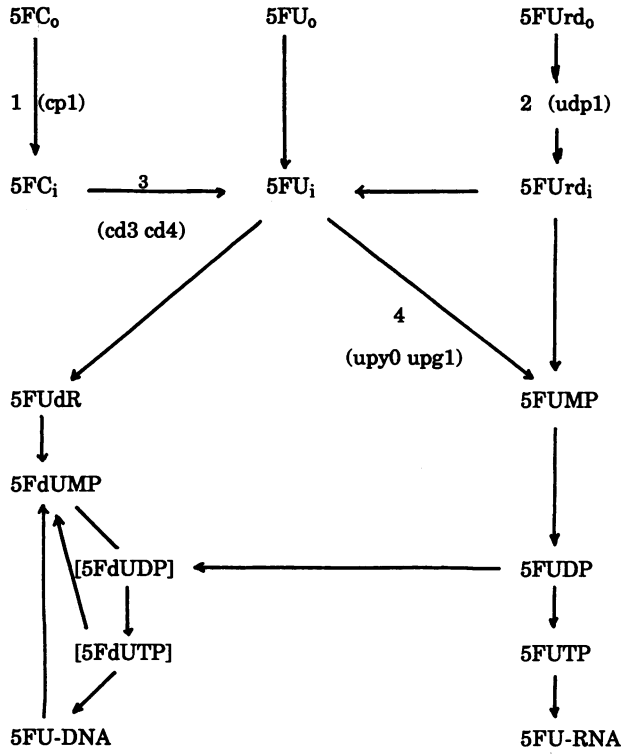

FIG. 1. Presumed biochemical pathways of 5-FC and 5-FU metabolism in *Candida* spp. Pathways: 1, cytosine permease; 2, uridine permease; 3, cytosine permease; 4, pyrimidine phosphoribosyl transferase.

2'-deoxyuridine (5-FdUrd), which are widely used in the treatment of disseminated human cancers (17).

MATERIALS AND METHODS

Chemicals. All fluorinated pyrimidines were products of the Sigma Chemical Co., St. Louis, Mo., except for 5-FdUMP, which was obtained from Behring Diagnostics, La Jolla, Calif. [2-^{14}C]cytosine was obtained from CEA, Cedex, France; [2-^{14}C]uracil and [2-^{14}C]uridine were obtained from Amersham International plc, Amersham, United Kingdom. D_2O was obtained from Norsk Hydro. All other chemicals used were of analytical grade.

Organisms. *C. glabrata* 4 (30) and *C. albicans* 72S (29) were used as the parental (wild-type) strains in this study. Mutant isolates were obtained from these by UV mutagenesis and fluoropyrimidine selection, as previously described (5). These mutants were defective in specific functions of (fluoro)pyrimidine transport or metabolism. The biochemical characteristics of these strains are given in Table 1.

All strains were routinely maintained on Yeast Morphology Agar (Difco Laboratories, Detroit, Mich.) plates at 37°C. Cells for NMR experiments were grown in Yeast Nitrogen Base (Difco Laboratories, Detroit, Mich.) made up in 0.1 M phosphate buffer (pH 7.0) containing 1% (wt/vol) glucose and 0.15% (wt/vol) asparagine (B-YNB-GA).

Samples for NMR measurements. Yeast-form cells of *C. albicans* and *C. glabrata* were pregrown in B-YNB-GA medium for 24 h with agitation (190 rpm) at 37°C. Cells were washed in 0.9% sterile saline and suspended in fresh, prewarmed (37°C) B-YNB-GA containing 100 μg of 5-FC or 5-FUrd per ml at a final cell concentration of 10^9 cells per ml.

TABLE 1. Characteristics of strains of *C. glabrata* analyzed by NMR spectroscopy

Phenotype	Strain(s)	Pyrimidines that can act as N source	IC_{50}[a] (μg/ml) of:			Uptake[b]			Activity[c] of:				Major pyrimidine in soluble pool
			5-FC	5-FU	5-FUrd	Cytosine	Uracil	Uridine	Cytosine deaminase	UMP pyrophosphorylase	Uridine ribohydrolase	Uridine kinase	
Wild type	4	Uracil, cytosine, uridine	0.1–1.0	0.1–1.0	0.1–1.0	+++ sat	+++ sat	+++ sat	22	3.6	8	0.034	Uracil
Cytosine permease deficient	*cp1*	Uracil, cytosine, uridine	1.0–10	0.1–1.0	0.1–1.0	++ lin	+++ sat	+++ sat	20	3.9	7	ND	Uracil
Cytosine deaminase deficient	*cd3, cd4*	Uracil, uridine	>1,000	0.1–1.0	0.1–1.0	–	+++ sat	+++ sat	0, 3	3.7, 3.8	8, 9	ND	Cytosine
UMP pyrophosphorylase deficient	*upy0, upy1*	Uridine	100–1,000	100–1,000	1.0–10	–	–	++ sat	22, 23	0, 0.4	9, 7	ND	Uracil
Uridine permease deficient	*udp1*	Uracil, cytosine	0.1–1.0	0.1–1.0	>1,000	+++ sat	+++ sat	+ lin	20	3.4	7	0.031	Uracil

[a] Defined in the text.
[b] Uptake of radioactively labeled pyrimidines into macromolecules. sat, Uptake occurs in a saturable, concentrative manner; lin, uptake is linearly dependent on external pyrimidine concentration. Symbols indicate relative rates of uptake, ranging from rapid uptake (+++) to no uptake (–).
[c] Activities of enzymes expressed in nanomoles of product formed per minute per milligram of protein. ND, Activity not determined.

The cells were then incubated with moderate agitation (110 rpm), and 20-ml samples were removed after 1, 3, or 5 h or as described below.

For each time point of incubation, cells were harvested by centrifugation and the supernatant fluids were stored at −20°C. The pelleted cells were washed with 0.9% sterile saline containing $MgSO_4$ (12 mM), suspended in B-YNB-GA, and packed into a 5-mm NMR tube to a final volume of 0.6 to 0.8 ml which contained approximately 2×10^{10} cells. Supernatants were thawed prior to NMR analysis and concentrated by rotoevaporation at 30°C. The residual deposit was then dissolved in 0.075 M citrate buffer, pH 6.0, containing D_2O (20%, vol/vol) and transferred into a 5-mm diameter NMR tube in a final volume of 0.7 ml.

In some experiments, perchloric acid (PCA) extracts of highly susceptible *C. glabrata* were prepared. The cell pellet was stirred in 1.3 M PCA at 0°C for 10 min. The mixture was centrifuged, and the deposit was extracted twice with 1.3 M PCA; the three supernatant extracts were collected and carefully neutralized with 3 M KOH to a pH of 5 to 6. After a 24-h interval to allow precipitation of $KClO_4$, the extracts were concentrated by rotoevaporation at 30°C and further prepared for NMR analysis in the same way as the supernatants. The number of viable cells did not change during the 5-fluoropyrimidine treatment, as assessed by comparing the number of CFU in suspensions at the beginning and end of drug treatment.

NMR measurements. ^{19}F NMR spectra were recorded without proton decoupling in 5-mm NMR tubes at 20°C by using a Brucker AM 400 wide-bore NMR spectrometer operating at 376 MHz. The field-frequency ratio of the spectrometer was stabilized by locking on the D_2O resonance for spectra of supernatants and acid extracts. Spectra for cell suspensions were recorded unlocked, but no NMR-detectable drift was observed over the duration of the experiments. The ^{19}F spectra were obtained by Fourier transformation of 200 to 20,000 accumulated free-induction decays (FIDs) with an exponential line broadening of 2 Hz for supernatants and extracts and of either 15 or 25 Hz for cell suspensions (see figure legends for details). In the case of cell suspensions, FIDs were accumulated in blocks of 500 to check for time-dependent changes; none was observed, and so blocks were co-added prior to transformation. FIDs consisting of 16,000 datum points were acquired by using a sweep width of 10 kHz, a nominal pulse width of 45°, and interpulse intervals of 2 s for supernatants and extracts and 0.82 s for cell suspensions.

Peak assignments were performed by spiking the samples with standard fluoropyrimidines (when available) when only one signal was present or by correlating the chemical shifts of the signal observed with those of the known standards at the same pH value (see below) if two or more signals were detected. The frequency of the resonance signal (chemical shift) was measured in parts per million relative to 5-FU, whose chemical shift at pH 4.86 was taken as 0 ppm. Coupling constants for ^{19}F nucleoside derivatives were determined from the spectra reported in Fig. 13. These were 5.5, 6.0, and 8.0 Hz for the mono-, di-, and triphosphate nucleosides respectively. Values were not determined for 5-FC and 5-FU.

Because there was variation in pH between the sample categories analyzed (intact cells, supernatants, and acid extracts) and also between different samples of the same sample category, it was important to determine whether the chemical shifts of the fluorinated species detected were affected by changes in the pH value. This was performed by taking spectra of 0.1 M phosphate solutions containing 5-FU, 5-FUrd, 5-FC, and 5-FdUMP at a series of pH values within the approximate pH range of 5 to 9. The observed pH variation of the samples was well within this range. Enough hydrochloric acid was added to the solution to bring the pH to 4.86. The solution was then titrated with NaOH, and its pH value was measured by a pH meter fitted with a 4-mm Russell electrode to permit measurement in 5-mm NMR tubes.

Ability to utilize pyrimidines as nitrogen sources. Cells were grown by using Difco Yeast Nitrogen Base without amino acids and $(NH_4)_2SO_4$ containing 1% (wt/vol) glucose; 1.0 mg of cytosine, uracil, or uridine per ml as nitrogen source; and 2% (wt/vol) agar. Approximately 10^6 cells were spotted onto the agar, and the plates were inspected for growth of the yeast after 1 week of incubation at 37°C.

Fluoropyrimidine susceptibility testing. Broth dilution tests were performed in duplicate with Difco Yeast Nitrogen Base containing 1% (wt/vol) glucose (YNBG). Drugs were dissolved in the same medium and diluted in tubes to concentrations ranging from 1,000 to 0.01 μg/ml. Yeast inocula were obtained from overnight cultures grown in YNBG at 37°C with shaking at 150 rpm; ~10^5 CFU, as determined by plate counts, was used to inoculate 5 ml of medium plus drug. After 24 h of incubation at 37°C with shaking at 150 rpm, the growth of the cultures was determined turbidimetrically at 600 nm by using drug-free medium as a blank. The readings were used to calculate IC_{50}, a value for the concentration at which the fluoropyrimidine had a standard effect on growth yield, defined as the lowest concentration at which $\%T > \%T_{control} + 0.5(100 - \%T_{control})$, where $\%T$ is the percent transmission of the culture grown in the presence of the drug and $\%T_{control}$ is the percentage transmission of the control culture grown in the absence of the drug. A 50-μl portion from each culture at each concentration tested was plated onto Yeast Morphology Agar after a brief sonication to disperse clumps of cells, and the plates were incubated for 48 h at 37°C. The concentration of drug resulting in complete loss of viability was termed the fungicidal value.

Uptake of nucleosides. Cells were grown from a small inoculum (~10^4 CFU/ml) in 5 ml of YNBG broth in the presence of radiolabeled pyrimidines ([2-^{14}C]cytosine, [2-^{14}C]uracil, or [2-^{14}C]uridine) for 18 h at 37°C with shaking at 150 rpm. After this period, the suspension density was determined turbidimetrically at 600 nm, PCA was added to a final concentration of 5% (wt/vol), and the resulting mixtures were placed on ice for 1 h. They were then filtered through presoaked (water) Whatman GF/C filter disks, washed twice with 10 ml of ice-cold 5% (wt/vol) PCA, and dried, and the radioactivity was measured in the presence of Optiscint HiSafe (LKB) scintillant in a Beckman LS3801 scintillation counter. The specific activity of the [^{14}C]pyrimidines was held constant at 0.01 μCi/μmol, and the pyrimidine concentration was varied from 100 to 2,000 μM. Uptake is expressed in counts per minute per unit of optical density and indicates the ability of a particular strain to salvage pyrimidines from the extracellular medium into its nucleic acids.

Measurement of enzyme activities and preparation of cell extracts. Cells grown at 37°C in YNBG with shaking at 190 rpm were harvested in the exponential phase to yield approximately 200 mg dry weight, washed once in distilled water, and suspended in 3 ml of Tris or phosphate buffer (0.05 M, pH 7.4) in a large boiling tube. Tube and contents were cooled on ice for 10 min. Glass beads (0.45 to 0.50 mm, B. Braun Melsungen AG) were added to just below the meniscus, and the cells were broken by vigorous agitation on

a vortex mixer for periods of 30 s with cooling on ice in between. After checking for at least 95% breakage by microscopic examination, the homogenate was centrifuged for 5 min at 800 × *g* to remove glass beads and broken cells, and the supernatant was stored on ice and used within 1 h as enzyme source.

Enzyme assays. Cytosine deaminase activity was determined spectrophotometrically as previously described (9). Measurements of the activities of UMP pyrophosphorylase and uridine kinase have also been described previously (10). The methodology involves the assay of radioactivity adsorbed to cationic disks after incubation with radiolabeled precursor. In both cases, the product (UMP) but not the labeled substrate (uracil or uridine) remains fixed to the disks, whereas the substrate is eluted by washing. Uridine ribohydrolase activity was measured spectrophotometrically (2). Protein determinations were carried out as previously described (16).

Composition of soluble pyrimidine pool. Cells grown overnight on Yeast Morphology Agar were inoculated into 5 ml of YNBG medium containing radiolabeled cytosine, uracil, or uridine (50 μM; 1 μCi/μmol). The culture was incubated overnight at 37°C with vigorous shaking (190 rpm). After 15 h of incubation, the stationary-phase cells were harvested by centrifugation, washed twice with fresh medium, and suspended in 200 μl of ice-cold PCA (5% wt/vol). After 1 h, the PCA insoluble material was removed by centrifugation and the supernatant was chromatographed. Samples were applied to Whatman 1-mm paper and chromatographed by descending chromatography for 18 h with isopropanol–water–11.6 M HCl (130:37:33, vol/vol). Radioactively labeled uracil, cytosine, and uridine were used as standard markers. After drying, radioactivity was located on the developed chromatogram by cutting out paper strips 1 cm wide progressively from the base line to the solvent front and measured in a 2,5-diphenyloxazole (PPO)-toluene mixture (5 mg/ml) with a Packard Tri-Carb liquid scintillation spectrophotometer.

RESULTS

pH titration and peak assignments. The products of fluoropyrimidine metabolism in fungi may include the free base and nucleoside (5-FU, 5-FUrd), the nucleotide products (5-FdUMP, 5-FUMP, 5-FUDP, 5-FUTP), and the catabolites (dihydrofluorouracil, fluoroureidopropionic acid, fluoro-β-alanine, fluorocitrate, and fluoroacetate), as well as 5-FC.

Fig. 2 illustrates how the chemical shifts of 5-FU, 5-FUrd, 5-FC, and 5-FdUMP vary with pH. The chemical shift of 5-FU at the lowest pH tested (4.86) was taken as reference and was arbitrarily set at 0.00. The chemical shifts of 5-FU and 5-FC show little variation with pH within the range tested, though 5-FU certainly titrates over this pH range, whereas those of 5-FUrd and 5-FdUMP vary considerably. The values obtained were fitted to the Henderson-Hasselbach equation by using the simplex method for function minimization to fit values to constant terms of a given equation using experimentally determined results (15). From the values obtained, pK_a values of 7.72 ± 0.16, 7.54 ± 0.17, and 7.40 ± 0.13 were determined for 5-FU, 5-FUrd, and 5-FdUMP, respectively. The pK_a values for 5-FU and 5-FUrd are in agreement with previously published values (15, 28).

^{19}F NMR spectra of 5-FC metabolites in highly susceptible *C. glabrata* 4 (wild type). Fig. 3 shows the ^{19}F NMR spectra

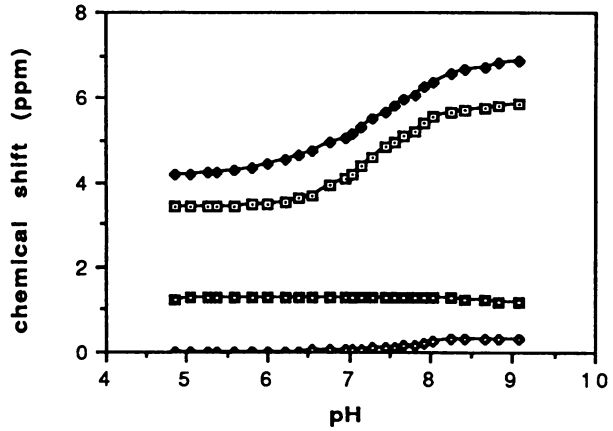

FIG. 2. Fluoropyrimidine chemical shifts as a function of pH. ⊡, 5-FUrd; ◆, 5-FdUMP; ■, 5-FC; ◆, 5-FU.

recorded after 1, 3, and 5 h of incubation of the 5-FC-sensitive, wild-type *C. glabrata* 4 with 5-FC. After 1 h of treatment, two intracellular ^{19}F signals were detected: a signal at 0 ppm attributable to 5-FU and a second signal approximately 4.5 ppm downfield, attributable to fluoronucleotides (5-FNTs). From 1 to 5 h, metabolism in intact cells progressed, as indicated by the increase in the ratio between the peak area of the signal corresponding to 5-FNTs to that corresponding to 5-FU.

The fact that no intracellular 5-FC signal was detected, even at the earliest time point of incubation of 1 h, indicates that 5-FC is rapidly taken up and deaminated to 5-FU and that these two steps occur at a faster rate than do subsequent (5-FU→5-FNTs) metabolic steps. The 5-FNTs signal after 1 h of incubation appears composite, suggesting the presence of more than one fluorinated species. This suggestion was confirmed in the spectrum of acid extracts of cells taken after 2 h incubation with 5-FC, which shows two peaks at ~4.3 and ~4.4 ppm (Fig. 4): the former is tentatively assigned to a monophosphate 5-FNT species, and the latter signal possibly to a di- or triphosphate 5-FNT species.

Fig. 5 shows the spectra of the supernatants of intact cells treated with 5-FC. After 1 h of incubation, two extracellular signals could be detected: a large signal at 0 ppm (5-FU) and a much smaller signal ~1.4 ppm downfield attributable to 5-FC. The presence of a 5-FU signal in the supernatant indicates that efflux of intracellular 5-FU occurred. Extracellular deaminase activity was ruled out as an explanation for the above results because no such activity could be detected in the cell supernatants, despite an average level of intracellular cytosine deaminase activity of 20 nmol uracil formed per min per mg of protein in *C. glabrata* (Table 1). Cell damage and subsequent release of intracellular contents was also dismissed as an explanation on the basis that cell viability did not change during 5-fluoropyrimidine treatments.

Measurement of the relative peak areas of the resonance signals in Fig. 5 after 1 h of incubation indicated that the amount of 5-FU in the extracellular medium was ca. 20 times the amount of 5-FC. This means that the deamination of 5-FC and subsequent efflux of 5-FU from the cell occur very rapidly relative to the transport of 5-FC into the cell. The small amount of residual, unmetabolized 5-FC was subsequently metabolized, as indicated by the absence of 5-FC in spectra taken after 3 and 5 h of incubation. From an overall consideration of the spectra of intact cells and their super-

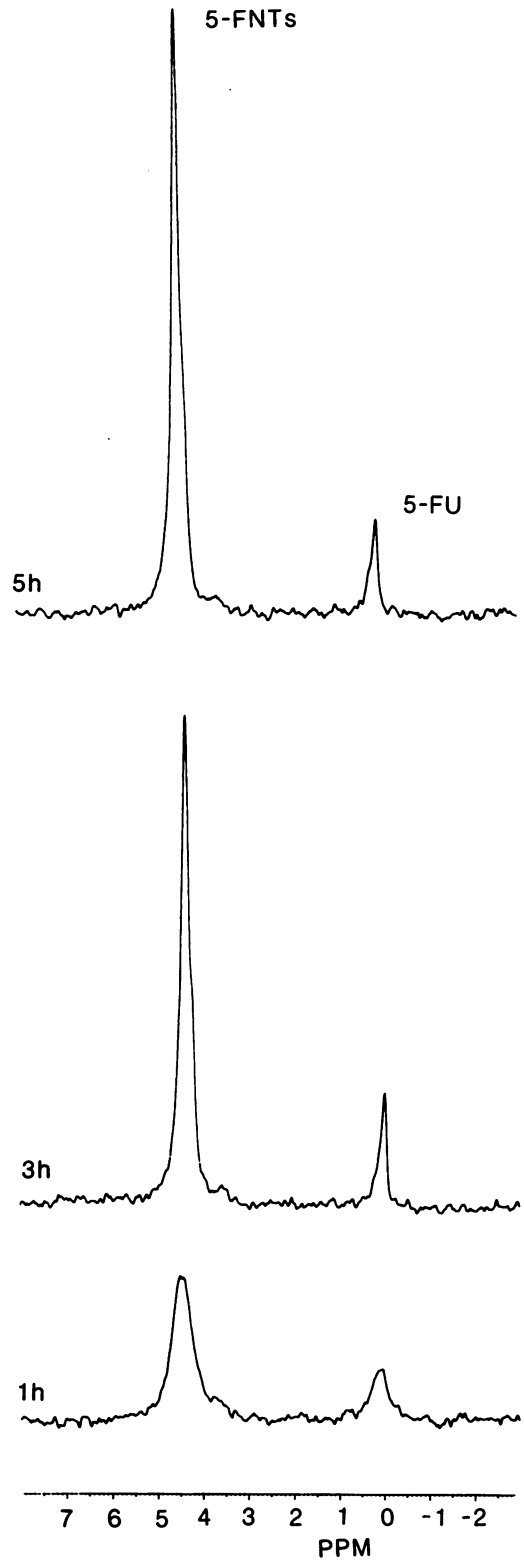

FIG. 3. ^{19}F NMR spectra of intact cells of 5-FC-susceptible *C. glabrata* 4 incubated with 5-FC for 1, 3, and 5 h. For each sample, 3,500 FIDs were accumulated and a line broadening of 2 Hz was used. The pH of the samples when the spectra were recorded was 6.9 to 7.1.

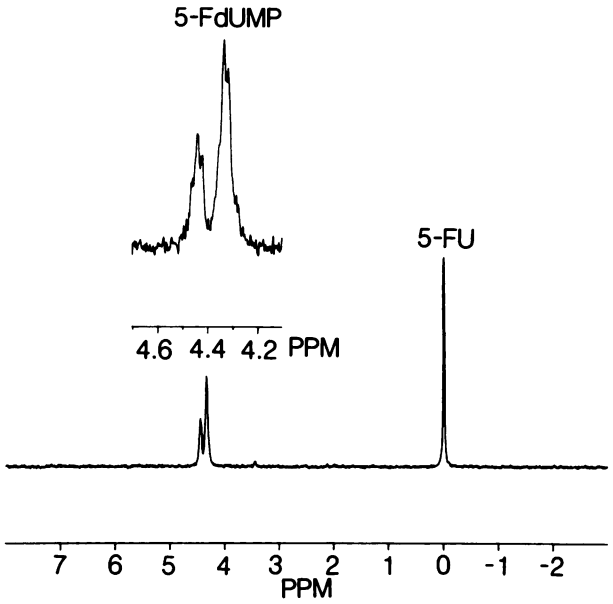

FIG. 4. ^{19}F NMR spectrum of an acid extract of *C. glabrata* 4 incubated with 5-FC for 2 h. 20,000 FIDs were accumulated, and a line broadening of 2 Hz was used. The pH of the sample when the spectrum was recorded was 5.55.

natants, it is clear that transport of 5-FC into the cell, its subsequent deamination to 5-FU, and 5-FU efflux from the cell all occur very rapidly in the context of overall 5-FC metabolism.

^{19}F NMR spectra of 5-FC metabolites in cytosine deaminase-deficient strains of *C. glabrata*. Fig. 6 and 7 show the NMR spectra taken after 1, 3, and 5 h of incubation of *C. glabrata cd3* and *cd4*, which are resistant to 5-FC because they have a deficiency in their cytosine deaminase function (Table 1). In strain *cd3* only one intracellular signal was detected after 1 h of incubation with 5-FC (Fig. 6). This signal is attributable to 5-FC, and its size increases between 1 and 3 h of incubation, after which time it remains constant. As expected, the only signal present in the culture medium is that corresponding to 5-FC; this signal shows a decrease between 1 and 3 h which is consistent with the intracellular increase during this time (not shown).

These results are in contrast with those for the parental strain (Fig. 3) but are consistent with the phenotype of strain *cd3*, which is unable to deaminate 5-FC. After 1 h of incubation with 5-FC, *C. glabrata cd4* showed three intracellular signals at 0, ca. 1.2, and ca. 4.3 ppm, corresponding to 5-FU, 5-FC, and 5-FNTs, respectively. These signals can be explained by considering that in this strain, the lesion at the cytosine deaminase locus is not absolute (as it is in strain *cd3*) and there is some residual cytosine deaminase activity. This accounts for the presence of both some unmetabolized intracellular 5-FC and the same products of 5-FC metabolism which were detected in the wild type (Fig. 3), i.e., 5-FU and the 5-FNTs. This explanation would predict efflux from strain *cd4* of some of the 5-FU formed, as occurs in the wild type (Fig. 5). The spectrum of the supernatant fluid of strain *cd4* (Fig. 8) shows that efflux did occur, albeit at a much lower level than in the wild type, as indicated by the relative peak area ratios (5-FC/5-FU) in the two strains.

After 3 and 5 h of incubation, 5-FU was barely visible in the intracellular pool, and the increase in the ratio of 5-FU metabolites (5-FC and 5-FNTs) to 5-FU indicates the pro-

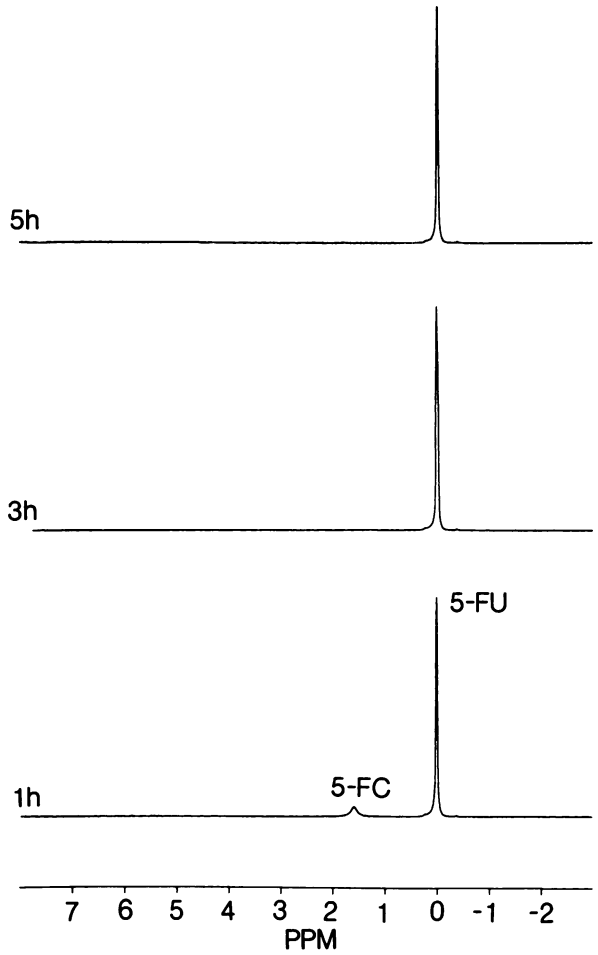

FIG. 5. ^{19}F NMR spectra of the supernatants from suspensions of *C. glabrata* 4 incubated with 5-FC for 1, 3, and 5 h. For each sample, 200 FIDs were accumulated and a line broadening of 2 Hz was used. The pH of the samples when the spectra were recorded was 5.3 to 5.5.

gression of 5-FU metabolism, particularly between 1 and 3 h (Fig. 7); there is a corresponding increase in the levels of 5-FC and 5-FU in the supernatants; the ratio of these two signals remains roughly constant for up to 3 h of incubation, after which time it decreases as the extracellular concentration of 5-FC increases (Fig. 8). This behavior can be rationalized in terms of altered intracellular (fluoro)pyrimidine pool levels caused by a disturbance of the normal flux rate of cytosine deaminase. The results also suggest that under these anomalous conditions, 5-FC may be transported out of the cell, presumably by being driven in reverse through the cytosine transporter, although this result was not observed in strain *cd3*.

Strains *cd3* and *cd4* have the same high level of resistance to 5-FC (Table 1), and there is a direct correlation between the levels of cytosine deaminase activity measured in these strains (Table 1) and their ability to metabolize 5-FC (Fig. 6 and 7). These results indicate that high levels of resistance to 5-FC can be achieved even with reduced cytosine deaminase activity. Presumably, as long as the overall flux of 5-FC metabolism is slowed down to below a certain critical level, the cells can respond to the inhibitory effects of 5-FC, e.g., by increasing the rate of de novo nucleotide biosynthesis.

^{19}F-NMR spectra of 5-FC metabolites in UMP pyrophosphorylase-deficient strains of *C. glabrata*. Fig. 9 and 10 show the NMR spectra taken after incubation of two strains of *C. glabrata*, *upy0* and *upy1*, which are resistant to 5-FC because they have a defective UMP pyrophosphorylase (Table 1). In both strains, the total intracellular concentration of 5-FC metabolites is much lower than in the wild type (Fig. 3). In strain *upy0*, only one intracellular signal attributable to 5-FU was detected after up to 5 h of incubation with 5-FC (Fig. 9). This signal is clearly of much smaller magnitude than the corresponding signal in the wild-type strain (Fig. 3). Lack of a fluoronucleotide signal is indicative of a complete blockage of the UMP pyrophosphorylase enzymic step.

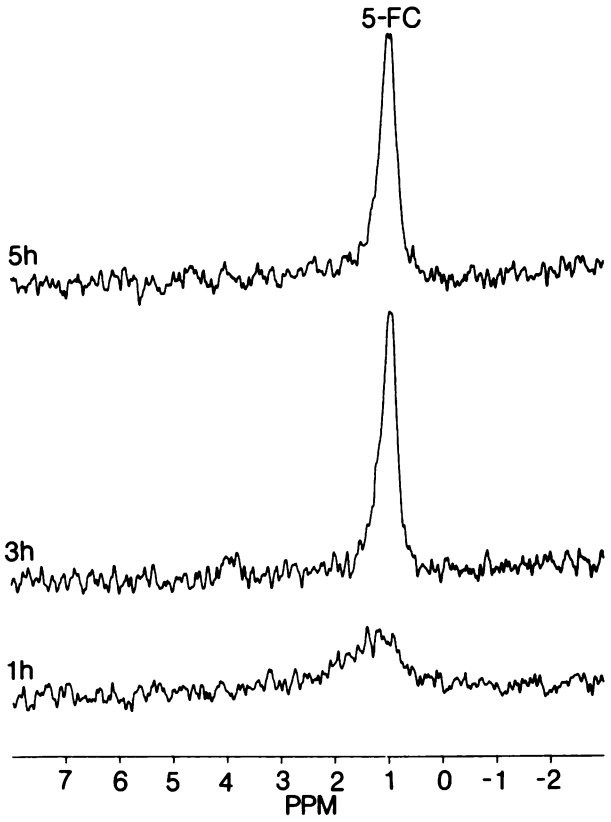

FIG. 6. ^{19}F NMR spectra of intact cells of *C. glabrata cd3* (cytosine deaminase deficient) incubated with 5-FC for 1, 3, and 5 h. For each sample, 3,500 FIDs were accumulated and a line broadening of 15 Hz was used. The pH of the samples when the spectra were recorded was 5.9 to 6.1.

By contrast, strain *upy1* had more than one intracellular 5-FC metabolite after incubation with 5-FC (Fig. 10). Both 5-FU and 5-FNTs were present intracellularly after 1, 3, and 5 h of incubation. The total concentration of fluorinated metabolites in strain *upy1* (and *upy0*), however, was lower than in the wild type (Fig. 3). After 5 h, only 5-FU was present in the supernatant of both strains (not shown), indicating that 5-FU efflux is taking place normally as in the wild type. Therefore, it appears that strain *upy1*, unlike strain *upy0*, still retains some detectable UMP pyrophosphorylase activity and that the lesion at this locus is not absolute, even though both strains are highly resistant to the inhibitory effects of 5-FC (Table 1).

These observations are consistent with the measured levels of UMP pyrophosphorylase activity in these strains (Table 1). This situation is therefore analogous to that in the cytosine deaminase-deficient mutants, in which high levels

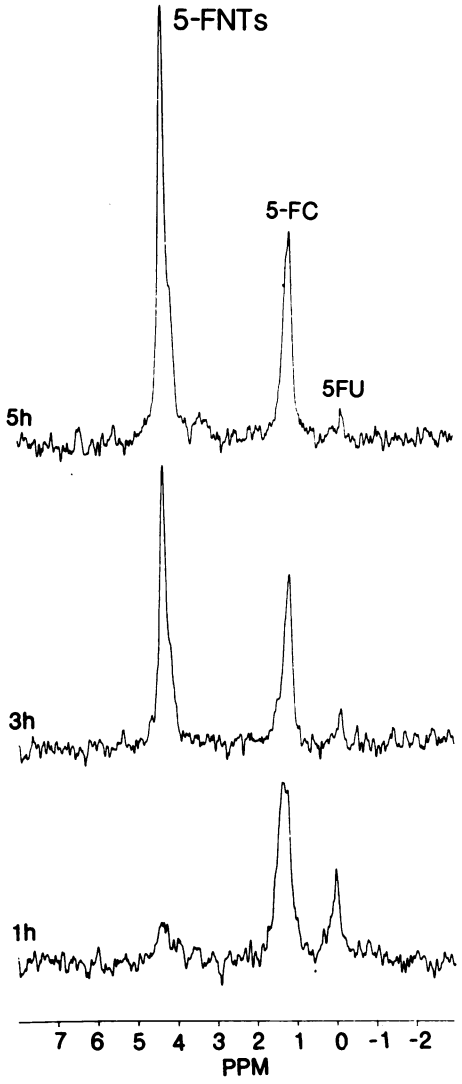

FIG. 7. ^{19}F NMR spectra of intact cells of *C. glabrata cd4* (cytosine deaminase deficient) incubated with 5-FC for 1, 3, and 5 h. For each sample, 1,000 FIDs were accumulated and a line broadening of 15 Hz was used. The pH of the samples when the spectra were recorded was 6.2 to 6.4.

of resistance to 5-FC can be achieved even with a partial loss of enzyme activity.

^{19}F NMR spectra of 5-FC metabolites in a cytosine permease-deficient strain of *C. glabrata*. *C. glabrata cp1* was isolated as mildly resistant to the inhibitory effects of 5-FC (Table 1). It was unable to transport 5-FC in a concentrative, saturable manner because it had lost a specific permease for cytosine, although 5-FC could still enter the cell by passive diffusion. NMR spectra of intact cells incubated with 5-FC show the same pattern of metabolites as that of the wild type. An analysis of the supernatants after 1 h of incubation (Fig. 11), however, indicates a higher concentration of unmetabolized 5-FC in strain *cp1* than in the wild type. After 1 h, 5-FC was utilized more slowly in strain *cp1* than in the wild type, and some 5-FC remained unmetabolized after 5 h of incubation in the former strain (Fig. 11) but not in the wild type (Fig. 5). These results are consistent with the assigned phenotype of strain *cp1* (Table 1).

^{19}F NMR spectra of 5-FUrd metabolites in wild-type and uridine permease-deficient strains of *C. glabrata* and *C. albicans*. The precise metabolic fate of 5-FUrd in *C. glabrata* and *C. albicans* is unclear (Fig. 1). Mutant strains *C. glabrata udp1* and *C. albicans* 6F1 were isolated which were highly resistant to 5-FUrd by virtue of a loss of a specific uridine

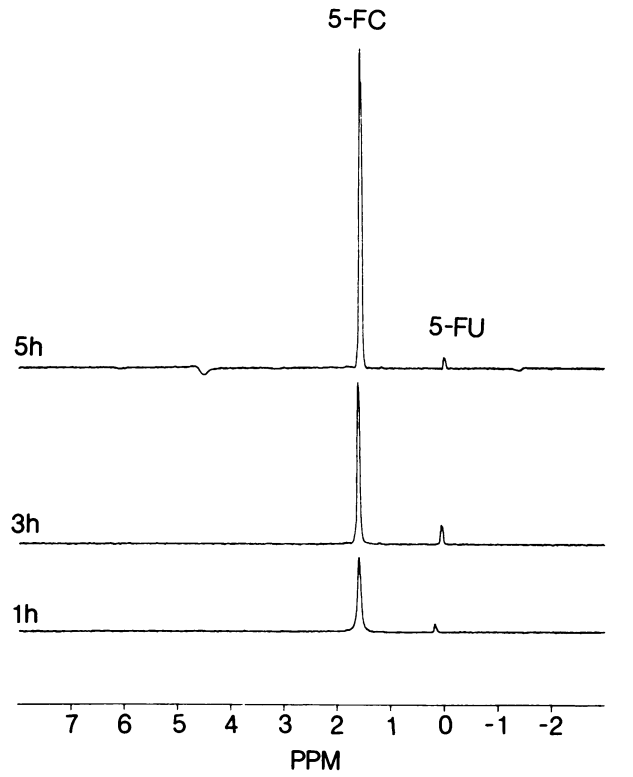

FIG. 8. ^{19}F NMR spectra of supernatants from suspensions of *C. glabrata cd4* (cytosine deaminase deficient) incubated with 5-FC for 1, 3, and 5 h. For each sample, 2,500 FIDs were accumulated and a line broadening of 2 Hz was used. The pH of the samples when the spectra were recorded was 5.3 to 5.5.

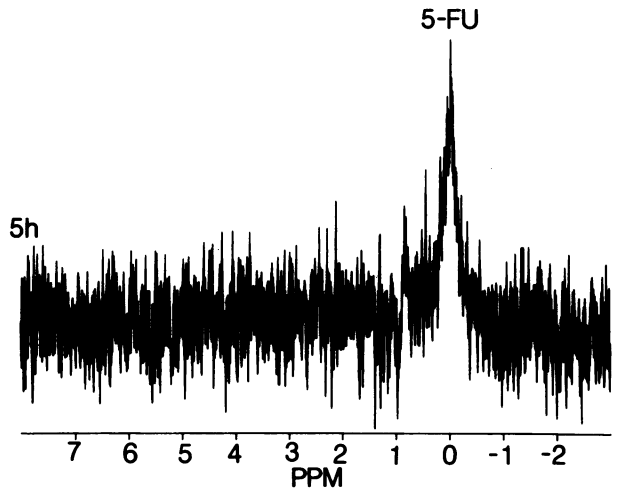

FIG. 9. ^{19}F NMR spectrum of intact cells of *C. glabrata upy0* (UMP pyrophosphorylase deficient) incubated with 5-FC for 5 h. A total of 2,500 FIDs were accumulated, and a line broadening of 15 Hz was used. The pH of the sample when the spectrum was recorded was 6.78.

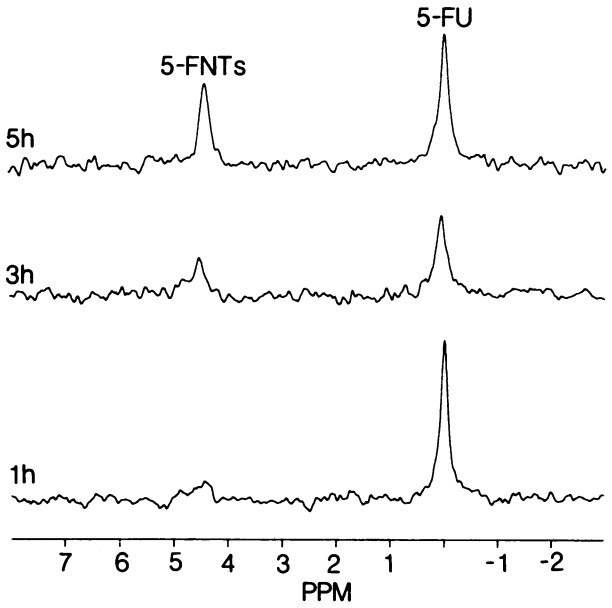

FIG. 10. ^{19}F NMR spectra of intact cells of *C. glabrata upy1* (UMP pyrophosphorylase deficient) incubated with 5-FC for 1, 3, and 5 h. For each sample, 3,500 FIDs were accumulated and a line broadening of 25 Hz was used. The pH of the samples when the spectra were recorded was 6.5 to 6.7.

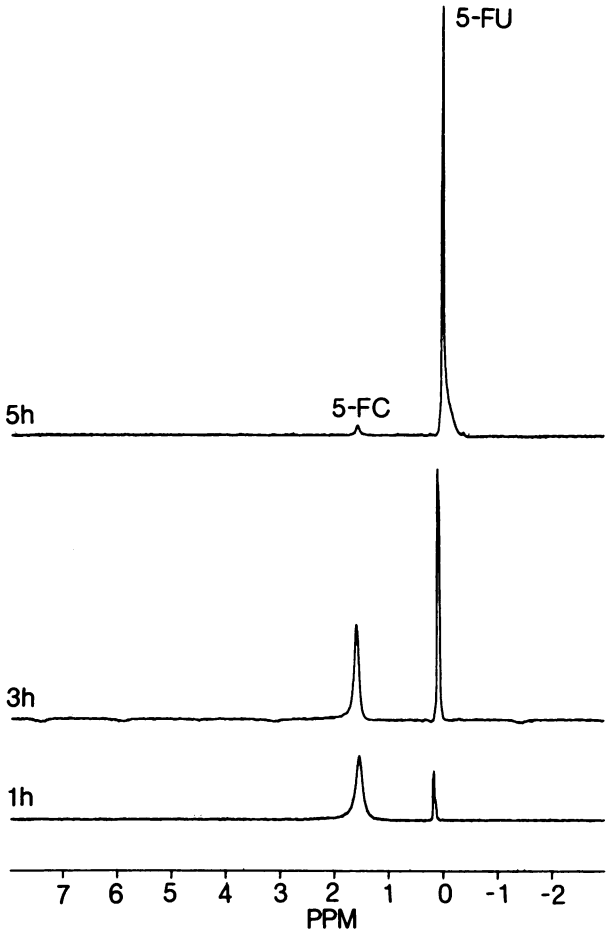

FIG. 11. ^{19}F NMR spectra of supernatants from suspensions of *C. glabrata cp1* (cytosine permease deficient) incubated with 5-FC for 1, 3, and 5 h. For each sample, 256 FIDs were accumulated and a line broadening of 2 Hz was used. The pH of the samples when the spectra were recorded was 5.3 to 5.5.

permease (Table 1). Analysis of NMR spectra of these mutants incubated with 5-FUrd confirmed the assigned phenotype of these strains.

No 5-FUrd was detected and there were only traces of 5-FUrd metabolites in *C. glabrata udp1* after 5 h of incubation with 5-FUrd. These were identified as 5-FNTs. This indicated a failure of 5-FUrd to enter these cells (Fig. 12). This was in marked contrast to the wild type, where large quantities of 5-FNTs were detectable after the same time (Fig. 12). A large 5-FNT signal was also detected in cells of *C. albicans* 72S following 5 h of incubation with 5-FUrd (not shown). There was no detectable intracellular ^{19}F signal after 5 h of incubation in *C. albicans* 6F1.

In the wild-type strains, the signal of the 5-FNTs was the only intracellular signal detected. This indicates that, once inside the cell, 5-FUrd is rapidly metabolized to its nucleotide derivatives and that there are no sizable pools of metabolic intermediates. Analysis of the spectra of the acid extracts of these strains reveals three 5-FNT species at 4.16, 4.31, and 4.41 (Fig. 13). The first and largest of these signals is attributable to a monophosphate derivative. The two signals downfield in all likelihood correspond to the di- and triphosphate derivatives (e.g., 5-FUDP and 5-FUTP). When wild-type cells were preincubated with 5-FC, two 5-FNT signals were detected (Fig. 4), whereas preincubation with 5-FUrd resulted in three signals (Fig. 13). As 5-FC and 5-FUrd share the same pathway of metabolism once 5-FUMP is formed (Fig. 1), it is difficult to account for this finding since the only difference in the experimental conditions is the duration of the incubation, with 5FC-treated cells being incubated for 2 h and the 5-FUrd-treated cells being incubated for 5 h. The only signal in the supernatants was that corresponding to 5-FUrd (not shown), which suggests that 5-FU efflux did not occur.

^{19}F NMR spectra of wild-type *C. glabrata* and *C. albicans* in the presence of 5-FdUrd. The drug 5-FdUrd, widely used as an anticancer agent (17), is not toxic to *C. glabrata* and *C. albicans*, although both yeasts are highly susceptible to 5-FUrd. This was somewhat surprising since the two drugs are structurally very similar, differing only in the presence of a hydroxyl moiety at position 2 of the sugar ring. It was thought that one of two explanations could account for these differences in toxicity. Either 5-FdUrd is unable to enter the cells or, once transported across the plasma membrane, it cannot be metabolized to any toxic nucleotide derivative. To resolve these two possibilities, the fate of 5-FdUrd in intact cells of wild-type *C. glabrata* and *C. albicans* was followed by NMR spectroscopy. For both strains, there was no detectable intracellular ^{19}F signal, even after 5 h of incubation, while the supernatants showed a very large signal attributable to 5-FdUrd. This clearly indicates that 5-FdUrd, unlike 5-FUrd, is not transported into these yeasts (Fig. 12). The implication of this observation for the specificity requirements of (fluoro)uridine transport are discussed elsewhere (M. O. F. Fasoli and D. Kerridge, unpublished data).

DISCUSSION

Under the experimental conditions tested, cellular viability remained constant even in the susceptible strains, as determined by CFU measurements at the beginning and end

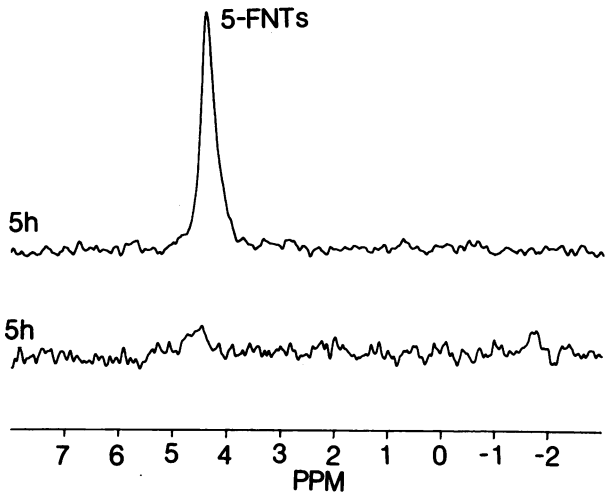

FIG. 12. ^{19}F NMR spectra of intact cells of 5-FUrd-susceptible *C. glabrata* 4 (top spectrum) and of 5-FUrd-resistant (uridine permease-deficient) strain *udp1* (bottom spectrum) incubated with 5-FUrd for 5 h. For each sample, 3,500 FIDs were accumulated and a line broadening of 25 Hz was used. The pH of the samples when the spectra were recorded was 6.2 to 6.4.

of the incubation period. This was possibly because of the low ratio between the amount of drug and the number of cells and because the cells used in the incubations had been pregrown to the stationary phase.

Assignments of NMRs were made either by correlating the chemical shifts observed with those of chromatographically pure standards recorded at the same pH or by spiking the spectra with suitable standards. A control experiment indicated that the chemical shifts of two of the 5-FC metabolites (5-FUrd and 5-FdUMP) were affected by the pH of the sample. pK_a values for these compounds were obtained. The previous ^{19}F NMR studies on intracellular fluoropyrimidine metabolism in *C. albicans* (2, 25) did not take into account the possibility of pH variations affecting the chemical shifts

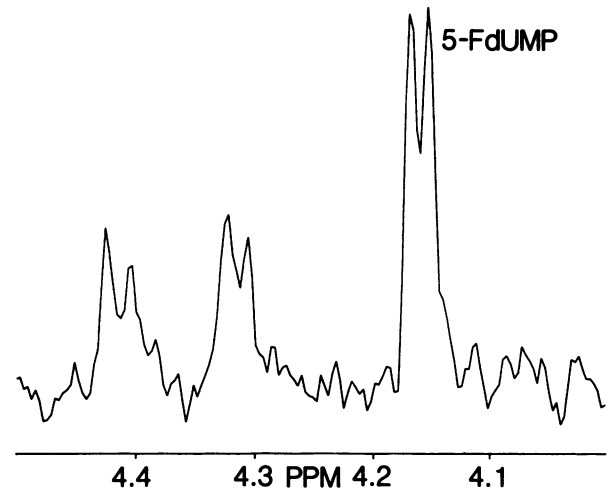

FIG. 13. ^{19}F NMR spectrum of extracts of *C. glabrata* 4 incubated with 5-FUrd for 5 h. For each sample, 805 FIDs were accumulated and a line broadening of 2 Hz was used. The pH of the sample when the spectrum was recorded was 5.32. No other signals were detected.

of fluorinated compounds, which could therefore have led to misinterpretations of data. Such misinterpretations can be avoided by addition of authentic compounds to the samples being examined. Although 5-FUrd and 5FdUMP titrate over the physiological pH range (6.8 to 8.0), there are difficulties in using these compounds to estimate the internal pH of *Candida* species. For example the high cell density required may affect the internal pH value. Also, 5-FUrd was not detected in any quantity within the cells and therefore cannot be used in wild-type strains, and finally it is possible that, in vivo, the ^{19}F NMRs of different 5-FNTs may not be resolved.

The absence of signals corresponding to products of fluoropyrimidine catabolism indicates that degradative metabolism of fluoropyrimidines does not occur in these yeasts under the experimental conditions used. But it is of interest to note that in *Aspergillus* strains (3) the catabolic pathway becomes operative when the cells are packed in the NMR tube. In the filamentous *Nectria* fungus, the catabolic pathway is operative under normal conditions of culture (20). The metabolism of 5-FC in this fungus is similar to that occurring in mice (23) and bacteria (14).

The results of NMR examination of a 5-FC-susceptible wild-type strain of *C. glabrata* gave some interesting information on 5-FC and 5-FUrd metabolism. It is clear, for example, that transport of 5-FC into the cell and its subsequent deamination to 5-FU occurs rapidly in the context of overall 5-FC metabolism and that the transport step is slower than deamination and uracil efflux. The metabolism of 5-FU progresses to the formation of phosphorylated derivatives which were visualized as distinct peaks in the acid extracts of treated cells. The largest of these 5-FNT signals was tentatively identified as a monophosphate derivative.

Another clear result is the confirmation of the existence of a mechanism for 5-FU efflux in *C. glabrata*. These observations are similar to those determined with *C. albicans* (2, 25). The issue of 5-FU efflux is particularly interesting because it could provide an alternative explanation for the mechanism of toxicity of 5-FC in humans. Results of a recent study (7) suggested that toxicity of 5-FC could arise from conversion to 5-FU by human intestinal microflora. The results obtained in this and other studies (2, 25) suggest that the pathogens themselves (*C. glabrata* and *C. albicans*) excrete large quantities of 5-FU into the surrounding medium and may contribute to any 5-FU-induced toxicity in the human host.

The possible role of uracil efflux in yeast cells is unclear. However, since it is the major free pyrimidine in the intracellular pool (Table 1) and may have some regulatory role in pyrimidine salvage metabolism, it is possible that an efflux mechanism has evolved to regulate the size of the uracil pool and maintain it at an optimal value.

The results of NMR examination of 5-FC-resistant strains of *C. glabrata* treated with 5-FC were consistent with their assigned phenotype, and it was possible to correlate the ^{19}F NMR profile of a given strain with its levels of specific enzyme activities. In particular, in one strain which lacked cytosine deaminase, the resonance peak of 5-FU was not observed, and in one strain which lacked UMP pyrophosphorylase, the broad, low-field resonance corresponding to the 5-FNTs was not detected. These "tight" mutants contrasted with more "leaky" mutants in which some metabolism did occur. The fact that both tight and leaky strains showed the same level of resistance to 5-FC indicated that even a partial loss of enzyme activity is sufficient to confer resistance. This may have some important implications in the context of 5-FC chemotherapy.

Analysis of NMR spectra of cells of *C. glabrata* and *C. albicans* preincubated with 5-FUrd indicated that the transport of this drug into the cell and its subsequent metabolism to the nucleotide derivatives occurs very rapidly, although the details for this pathway could not be resolved. Analysis of acid extracts of cells after 5 h of incubation with 5-FUrd revealed the presence of three phosphorylated species. The largest of these 5-FNT signals was identified as a monophosphate derivative. In mutant strains 1,000-fold more resistant to the toxic effects of 5-FUrd than the wild type, there was no intracellular ^{19}F signal after incubation with 5-FUrd. This observation confirmed the uridine permease-deficient phenotype of these strains. Analysis of NMR spectra of cells of *C. albicans* and *C. glabrata* preincubated with 5-FdUrd indicated that this drug is not transported into these yeasts and that this probably explains their inherent resistance to this compound.

Overall, the NMR analyses that have been carried out indicate metabolic pathways in *Candida* species which are consistent with previous biochemical reports (21, 27). The difficulty of identifying precisely those signals attributable to the phosphorylated metabolites was at least partly overcome by correlating these signals in the acid extracts of cells with the signals of suitable standards at the same pH. The low intrinsic NMR sensitivity requires a large number of cells, and this clearly differs from the situation in vivo. Nevertheless, results reported in this study show conclusively that ^{19}F NMR is a valuable and feasible approach for studies on the mode of action of clinically important fluorinated drugs in intact, viable cells of pathogenic fungi. It may also find important applications in the rapid determination of the precise mechanism(s) of resistance to fluorinated pyrimidines in antifungal or anticancer chemotherapy.

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LITERATURE CITED

1. **Bachelard, H. S., D. W. G. Cox, and P. G. Morris.** 1987. NMR as a tool to study brain metabolism. Gerontology **33**:235–246.
2. **Carter, C. E.** 1955. Partial purification of a nonphosphorolytic uridine nucleosidase from yeast. J. Am. Chem. Soc. **73**:1508–1510.
3. **Chouini-Lalanne, N., M. C. Malet-Martino, R. Martino, and G. Michael.** 1989. Study of the metabolism of flucytosine in *Aspergillus* species by ^{19}F nuclear magnetic resonance spectroscopy. Antimicrob. Agents Chemother. **33**:1939–1945.
4. **Di Vito, M., F. Podo, A. Torosantucci, G. Carpinelli, W. L. Whelan, D. Kerridge, and A. Cassone.** 1986. A ^{19}F nuclear magnetic resonance study of uptake and metabolism of 5-fluorocytosine in susceptible and resistant strains of *Candida albicans*. Antimicrob. Agents Chemother. **29**:303–308.
5. **Fasoli, M. O. F., and D. Kerridge.** 1988. Isolation and characterisation of fluoropyrimidine-resistant mutants in two Candida species. Ann. N.Y. Acad. Sci. **544**:260–263.
6. **Gochin, M., T. L. James, and R. H. Shafer.** 1984. *In vivo* ^{19}F-NMR of 5-fluorouracil incorporation into RNA and metabolites in *Escherichia coli* cells. Biochim. Biophys. Acta **804**:118–124.
7. **Harris, B. E., B. W. Manning, T. W. Federle, and L. B. Diasio.** 1986. Conversion of 5-fluorocytosine into 5-fluorouracil by human intestinal microflora. Antimicrob. Agents Chemother. **29**:44–48.
8. **Hull, W. E., R. E. Port, R. Herrmann, B. Britsch, and W. Kunz.** 1988. Metabolites of 5-fluorouracil in plasma and urine as monitored by ^{19}F nuclear magnetic resonance spectroscopy for patients receiving chemotherapy with and without methotrexate treatment. Cancer Res. **48**:1680–1688.
9. **Ipata, P. L., and G. Cercignani.** 1978. Cytosine and cytidine deaminase from yeast. Methods Enzymol. **51**:394–401.
10. **Jund, R., M. R. Chevallier, and F. Lacroute.** 1977. Uracil transport in *Saccharomyces cerevisiae*. J. Membr. Biol. **36**:233–251.
11. **Keniry, M., C. Benz, R. H. Shafer, and T. L. James.** 1986. Noninvasive spectrophotometric analysis of fluoropyrimidine metabolism in cultured tumour cells. Cancer Res. **46**:1754–1758.
12. **Kirschenlohr, H. L., J. C. Metcalfe, P. G. Morris, G. C. Rodrigo, and G. A. Smith.** 1988. Ca^{2+}, transient Mg^{2+} and pH measurements in the cardiac cycle by ^{19}F NMR. Proc. Natl. Acad. Sci. USA **85**:9017–9021.
13. **Krugh, T. R., and M. E. Nuss.** 1979. Nuclear magnetic resonance studies of drug-nucleic acid complexes, p. 113–175. *In* R. G. Schulman (ed.), Biological applications of magnetic resonance. Academic Press, Inc., New York.
14. **Lee, K. R., and S. Y. Sheu.** 1989. Evidence of a new metabolic pathway of 5-fluorouracil metabolism in *Escherichia coli* from *in vivo* ^{19}F-NMR spectroscopy. Biochim. Biophys. Acta **1011**:12–17.
15. **Loomis, R. E., and J. L. Alderfer.** 1986. Halogenated nucleic acids: effects of 5-fluorouracil on the conformation and properties of a polyribonucleotide and its constituents. Biopolymers **25**:571–600.
16. **Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87**:206–210.
17. **Martindale, W.** 1982. *In* J. E. F. Reynolds (ed.), The general pharmacopoeia, 28th ed. Pharmaceutical Press, London.
18. **Morris, P. G.** 1988. NMR spectroscopy in living systems. Annu. Rep. NMR Spectroscopy **20**:1–60.
19. **Nelder, J. A., and J. A. Mead.** 1965. A simplex method for function minimization. Computer J. **7**:308–313.
20. **Parisot, D., M. C. Malet-Martino, P. Casnier, and R. Martino.** 1989. ^{19}F nuclear magnetic resonance analysis of 5-fluorouracil metabolism in wild-type and 5-fluorouracil-resistant *Nectria haematococca*. Appl. Environ. Microbiol. **55**:2474–2479.
21. **Scholer, H. J.** 1980. Flucytosine, p. 35–106. *In* D. C. E. Speller (ed.), Antifungal chemotherapy. John Wiley & Sons, Inc., Chichester, England.
22. **Semmler, U., P. Bachert-Baumann, F. Guickel, F. Ermark, P. Schlag, W. J. Loran, and G. Van Konick.** 1990. Real time follow up of 5-fluorouracil metabolism in the liver of tumor patients by means of F-19 NMR spectroscopy. Radiology **174**:141–145.
23. **Stevens, A. N., P. G. Morris, R. A. Iles, P. W. Sheldon, and J. R. Griffith.** 1984. 5-Fluorouracil metabolism monitored *in vivo* by ^{19}F NMR. Br. J. Cancer **50**:113–117.
24. **Stewart, E., N. A. Gow, and D. V. Bowes.** 1988. Cytoplasmic alkalinization during germ tube formation in *Candida albicans*. J. Gen. Microbiol. **134**:1079–1087.
25. **Vialaneix, J. P., N. Chouini, M. C. Malet-Martino, R. Martino, G. Michel, and J. P. Lepargneur.** 1986. Noninvasive and quantitative ^{19}F nuclear resonance study of fluorocytosine metabolism in *Candida* strains. Antimicrob. Agents Chemother. **30**:756–762.
26. **Wain, W. H., and W. D. Staatz.** 1973. Rates of synthesis of ribosomal protein and total ribonucleic acid through the cell cycle of the fission yeast Schizosaccharomyces pombe. Exp. Cell Res. **81**:269–278.
27. **Waldorf, A. R., and A.-M. Polak.** 1983. Mechanisms of action of 5-fluorocytosine. Antimicrob. Agents Chemother. **23**:79–85.
28. **Wempen, I., R. Duschinsky, L. Kaplan, and J. J. Fox.** 1961. Thiation of nucleosides. IV. The synthesis of 5-fluoro-2′-deoxycytidine and related compounds. J. Am. Chem. Soc. **83**:4755–4766.
29. **Whelan, W. L., and D. Kerridge.** 1984. Decreased activity of UMP pyrophosphorylase associated with resistance to 5-fluorocytosine in *Candida albicans*. Antimicrob. Agents Chemother. **26**:570–574.

30. **Whelan, W. L., S. Simon, E. J. Beneke, and A. L. Rogers.** 1984. Auxotrophic mutants of *Torulopsis glabrata*. FEMS Microbiol. Lett. **24**:1–4.
31. **Wolf, W. M., J. Albright, M. S. Silver, H. Weber, V. Reichardt, and R. Sauer.** 1987. Fluorine-19 NMR spectroscopic studies of the metabolism of 5-fluorouracil in the liver of patients undergoing chemotherapy. Magn. Reson. Imaging **5**:165–169.
32. **Wolf, W., C. A. Presant, K. I. Servis, A. El-Tahtawy, M. J. Albright, P. B. Barner, R. Ring, R. Atkinson, R. Ong, M. King, M. Singh, M. Ray, C. Wiseman, D. Blayney, and J. Shan.** 1990. Tumor trapping of 5-fluorouracil in vivo ^{19}F NMR spectroscopic pharmacokinetics in tumor-bearing humans and rabbits. Proc. Natl. Acad. Sci. USA **87**:492–496.
33. **Zon, G.** 1980. NMR studies of drug metabolism and mechanism of action, p. 110–170. *In* J. S. Cohen (ed.), Magnetic resonance in biology. John Wiley & Sons, Inc., New York.