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A ^{19}F Nuclear Magnetic Resonance Study of Uptake and Metabolism of 5-Fluorocytosine in Susceptible and Resistant Strains of *Candida albicans*

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The metabolism of the antifungal drug 5-fluorocytosine (5-FC) was studied in intact viable cells of *Candida albicans* by ^{19}F nuclear magnetic resonance (NMR). The uptake of the drug and its conversion to the deaminated product 5-fluorouracil (5-FU) were easily observed by NMR analysis of both the cells and the supernatants of the incubation mixture. In the 5-FC-resistant mutant D14 of *C. albicans*, which lacked cytosine deaminase activity, the resonance peak of 5-FU was not observed. In intact cells of all 5-FC-susceptible strains the metabolism of 5-FU progressed to the formation of other fluorinated derivatives which were visualized as a single, broad resonance band at a lower field with respect to 5-FC and 5-FU. This band was resolved into three distinct peaks in the acid extract of treated cells, one of these peaks being attributable to 5-fluoro-dUMP (5-FdUMP). In strain 72R of *C. albicans*, which is 5-FC resistant because of a low level of UMP-pyrophosphorylase activity, the broad, low-field resonance band was detected later and with much less intensity than in the 5-FC-sensitive strains. This suggests that, besides 5-FdUMP, this band is also contributed to by 5-FUMP and possibly other phosphorylated derivatives. ^{19}F NMR analysis also revealed that a significant amount of 5-FU is secreted into the external medium, the rate of secretion being higher in 5-FC-resistant strain 72R than in 5-FC-sensitive strain 72S. Although not all resonances were definitely identified, this study shows that ^{19}F NMR spectroscopy may be an important tool for noninvasive analysis of the metabolism of fluorinated drugs in yeasts.

Nuclear magnetic resonance (NMR) spectroscopy provides a potent noninvasive approach for studies of intermediate metabolism in intact cells and tissues (3) and also in microorganisms, including yeasts (1, 8). Little work, however, has been addressed to investigating the mechanisms of action of antibiotics by these techniques. ^{19}F NMR seems particularly suitable in this view thanks to the natural abundance and relatively high sensitivity of the fluorine nucleus. In particular, we focused our attention on 5-fluorocytosine (5-FC), which is a powerful antimycotic agent especially active against *Candida* and *Cryptococcus* pathogenic fungi (10), owing to the presence of the fluorine label intrinsic to the molecule and essential for its antibiotic action. The usefulness of this molecule in the clinical situation is, however, lowered by the relative ease by which strains of pathogenic yeasts resistant to 5-FC emerge during treatment, and this especially applies to infections caused by *Candida albicans*. Another point of interest is that the biochemical studies performed so far have not yet clarified whether incorporation of fluorine into RNA or inhibition of DNA synthesis by fluorinated UMP derivative(s) (or both) is the main determinant of antibiotic action (12). In principle, ^{19}F NMR may contribute to the elucidation of these fundamental aspects of 5-FC action. In the present study, we addressed the biochemical mechanisms of action of 5-FC in *C. albicans* and evaluated the feasibility of using ^{19}F NMR technology for this problem. To this aim, we used a panel of genetically characterized 5-FC-sensitive and -resistant strains of the microorganism and established the conditions under which ^{19}F NMR may be used to obtain significant information on 5-FC metabolism in intact and viable cells of *C. albicans*.

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MATERIALS AND METHODS

Organisms and growth conditions. Five *C. albicans* isolates were used in this study. 5-FC-sensitive strain BP was from the established culture collection of the Istituto Superiore di Sanità (Rome, Italy); strain MEN was a heterozygous, partially 5-FC-resistant, clinical isolate which gave rise by mitotic segregation to a susceptible (72S) and a resistant (72R) homozygous segregant (13). MEN, 72S, and 72R were biochemically characterized with respect to their UMP-pyrophosphorylase activity, and a partial or total defect in this enzymatic activity was shown to correlate well with 5-FC resistance (14). Strain D14 was a 5-FC-resistant variant from a clinical isolate of *C. albicans* devoid of cytosine deaminase activity (W. L. Whelan et al., manuscript in preparation). A classification of the above mentioned strains on the basis of their genetic, biochemical, and serological characteristics and 5-FC susceptibility is given in Table 1.

All strains were routinely maintained on malt-extract agar slopes at 28°C. Samples for NMR measurements were obtained by following two distinct procedures.

(i) Yeast-form cells of *C. albicans* were pregrown at 28°C in Winge medium (5) under moderate agitation for 24 h. Cells were washed, suspended in glucose-salt-biotin medium [glucose, 20 g liter^{-1}; $(NH_4)_2SO_4$, 5 g liter^{-1}; $MgSO_4$, 0.2 g liter^{-1}; biotin, 0.01 g liter^{-1}] containing 20% (vol/vol) D_2O, and packed in the NMR tube. The final volume of the samples, which contained about 10^{11} yeast cells, was about 4

TABLE 1. General characteristics of *C. albicans* strains

Strain	Serotype	Genotype	5-FC resistance class	Biochemical basis of resistance
BP	A	Unknown	Susceptible	
MEN	A	Heterozygous	Partially resistant	Intermediate level of UMP-pyrophosphorylase
72S	A	Homozygous	Susceptible	
72R	A	Homozygous	Resistant	Low level of UMP-pyrophosphorylase activity
D14	Not determined	Homozygous	Resistant	Cytosine deaminase absent

ml; 5-FC (5 mg) was added directly to the NMR tube at the beginning of measurements.

(ii) Washed yeast-form cells, obtained as in procedure i, were resuspended (at a density of about 2×10^9 cells ml^{-1}) in glucose-salt-biotin medium containing 5-FC (0.1 mg ml^{-1}) and incubated at 28°C under slight agitation; at different time intervals 50-ml samples of the suspension were removed, cells were harvested and washed by centrifugation (10 min, 2,500 × *g*) and resuspended in fresh medium containing D_2O (20%, vol/vol) at a final volume of about 4 ml in the NMR tube.

Acid extracts of 5-FC-treated cells were prepared by stirring packed cells for 10 min in 0.5 N perchloric acid (PCA) at 4°C (ratio, 100 g of wet cells/100 ml of PCA). The mixture was centrifuged, and then the deposit was extracted with a further portion of 0.2 N PCA; the latter procedure was repeated twice. The three supernatants were pooled and neutralized by the addition of 3 N KOH. The volume was reduced under slight pressure at low temperature and made up to about 2 ml with D_2O.

NMR measurements. ^{19}F NMR spectra were recorded at 28°C with a Varian XL 100 15-in. (38-cm) spectrometer working at 94.1 MHz, interfaced to a 620L computer. The field/frequency ratio of the spectrometer was stabilized by "locking" on the D_2O resonance.

Sample tubes (12-mm outside diameter) were used. Each spectrum represented the Fourier transform of accumulated free induction decays, obtained with a sequence of 60° pulses and acquisition time of 1.00 or 0.50 s. The frequency of resonance signal (chemical shift) was measured in parts per million relative to 25 mM NaF (external reference). All spectra from intact cells were generally taken within 30 to 60 min, while extracts required accumulation periods of up to 9 h.

Peak assignment was performed by adding standard compounds to PCA extracts of 5-FC-treated cells. Moreover, the presence in PCA extracts of fluorinated derivatives of 5-FC identified by ^{19}F NMR analysis was confirmed by high-pressure liquid chromatography of the same extracts, performed by the method of Pogolotti et al. (6). The following compounds were obtained from Sigma Chemical Co. (St. Louis, Mo.): 5-FC, 5- fluorouracil (5-FU), 5-fluoro-2-dUMP (5-FdUMP).

RESULTS

In a series of preliminary experiments, we examined the ^{19}F NMR spectra of 5-FC-susceptible strains BP and 72S during incubation of packed yeast-form cells in the NMR tube (as described in procedure i above) in glucose-salt-biotin medium, at 28°C, in the presence of 5-FC (5 mg/10^{11} cells). Figure 1 shows the kinetics of 5-FC uptake and metabolism over a 24-h period of incubation with the BP strain. It is easily observed that 5-FC (−45.6 ppm, peak A) is progressively but not completely metabolized to 5-FU (−46.7 ppm, peak B) (Fig. 1a through d) and that 5-FU formed during this incubation is intracellular (Fig. 1e). Under these experimental conditions, no further metabolism of the compound was observed. Similar results were obtained with other 5-FC-sensitive isolates. Under the same experimental conditions, the ^{19}F NMR spectrum of strain D14 did not show any signal attributable to 5-FU over the experimental period, only the 5-FC signal being detectable (data not shown).

In the above experiments the NMR spectra did not show a complete metabolism of 5-FC, as expected from its mode of action (2, 7). One possible explanation would relate to the experimental conditions of procedure i, in which uptake and metabolism of 5-FC occurred at very high cell density (approximately 1 g [dry weight] per ml) in a minimal volume (4 ml).

For this reason, in subsequent experiments the cell density was reduced to approximately 1 mg (dry weight) per ml, in keeping with the conditions used for biochemical studies of 5-FC action (14), and uptake and metabolism of the drug were allowed to occur before packing cells for NMR examination. Under these conditions the cells maintained full viability throughout the incubation period with 5-FC and the subsequent NMR observation time (as measured by CFU counts). The results of these experiments are reported in the sections below.

^{19}F NMR spectra of 5-FC metabolism in 5-FC-sensitive strains of *C. albicans*. Figure 2 shows the NMR spectra taken at different time intervals during 24 h of incubation of the homozygous, 5-FC-susceptible strain 72S. 5-FC was rapidly taken up and metabolized so that, after 90 min of incubation, two intracellular signals were detected, one arising from 5-FU (peak B) and another signal at a much lower field (−42.2 ppm) (Fig. 2a, peak C). Metabolism of 5-FU progressed (Fig. 2b through c) until the latter resonance and another signal (−43.1 ppm, peak D) were the major signals detected at 24 h (Fig. 2d). The compounds giving rise to signals C and D were tentatively assigned to phosphorylated derivatives of 5-FU (see Discussion).

No 5-FC signal from the cellular suspension could be detected, indicating that if 5-FC were present within the cells, its concentration would be less than the instrumental sensitivity (0.5 mM). The spectral analysis of the incubation medium of the cellular samples (Fig. 2) showed that some 5-FU was also secreted into the medium as incubation progressed. However, no other 5-FC metabolite was se-

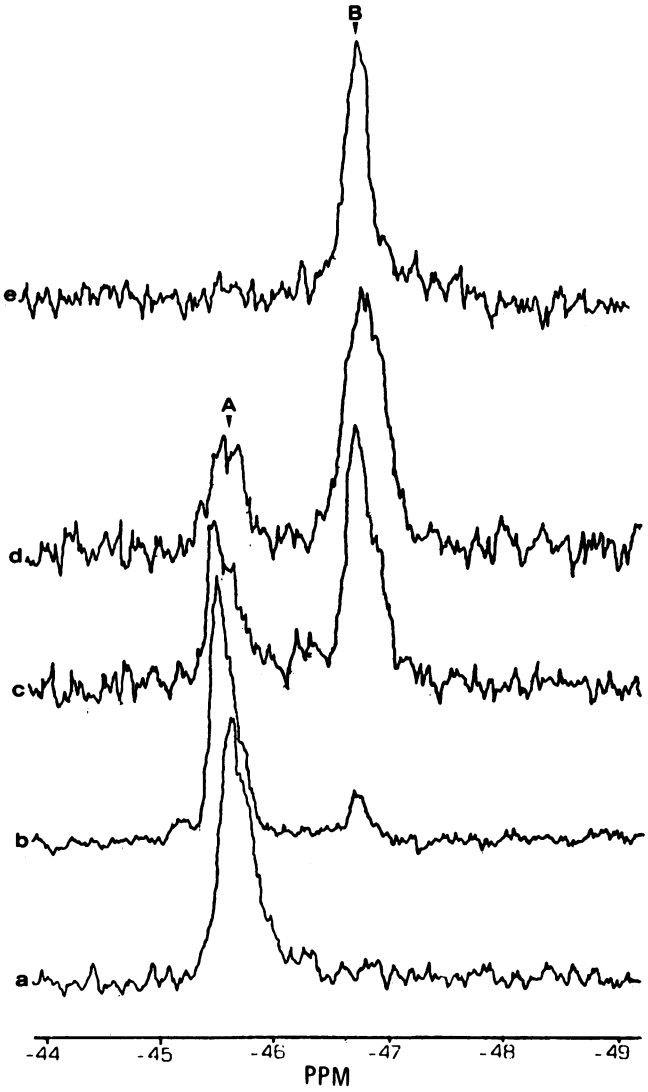

FIG. 1. ^{19}F NMR spectra were taken at various time intervals after addition of 5-FC to the cells (strain BP) in the NMR tube (procedure i of Materials and Methods): (a) 5 min, 287 scans (1 s each); (b) 2 h, 12 min, 700 scans; (c) 19 h, 15 min, 190 scans; (d) 24 h, 30 min, 190 scans. After experiment d, cells were washed and analyzed (spectrum e), 675 scans. Peak A, 5-FC; peak B, 5-FU. For any other details, see Materials and Methods.

creted from the cells, as judged by the absence of detectable signals other than those arising from 5-FC and 5-FU (Fig. 3) up to 24 h of incubation. In particular, Fig. 3 shows that secretion of 5-FU had already occurred by the first 30 min of observation when a major fraction of 5-FC had not yet been taken up (Fig. 3a). Figure 3 also shows the progressive uptake of 5-FC and the parallel progressive secretion of 5-FU. The heterozygous MEN strain examined by ^{19}F NMR under the same experimental conditions as those reported for strain 72S exhibited an essentially similar pattern of 5-FC uptake and 5-FU metabolism (data not shown).

^{19}F NMR spectra of 5-FC-resistant strains of *C. albicans*. Our experiments included two strains resistant to 5-FC, either because of decreased UMP-pyrophosphorylase activity (strain 72R) or because of the absence of cytosine deaminase activity (strain D14) (Whelan et al., in preparation). Some of the experiments performed with strain 72R

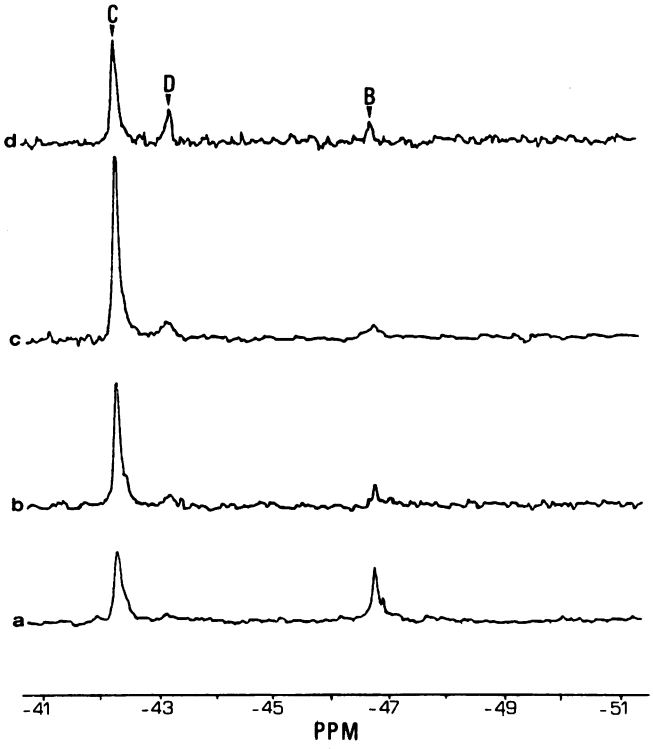

FIG. 2. ^{19}F NMR spectra of 5-FC-susceptible strain 72S incubated as described in procedure ii of Materials and Methods. (a) 90 min; (b) 240 min; (c) 360 min; (d) 24 h of incubation. Peak B, 5-FU. Peaks C and D, 5-FU phosphorylated derivatives. For any other details, see the text.

are detailed in Fig. 4. It was observed that, while capable of converting 5-FC into 5-FU (Fig. 4a), this strain was much less efficient than 5-FC-sensitive strain 72S in further metabolizing 5-FU, as shown by the absence of peak C at 90 min and by the low intensity of peaks C and D at later times of incubation (Fig. 4b and c). Table 2 reports the ratios between the peak area of signal C and that of 5-FU in 72S and 72R. It is easily observed that at 240 min, a time when signal C is also present in the 5-FC-resistant strain, the ratio is about 1 order of magnitude higher in 72S than in 72R. The capacity of 72R cells to take up 5-FC and convert it to 5-FU was confirmed by the spectral analysis of the supernatants of incubation medium (not shown here), which also documented that much 5-FU was secreted by the cells. Spectral analysis of strain D14, carried out as reported above, did not show any ^{19}F NMR signal with the exception of 5-FC.

^{19}F NMR spectra of acid extract of cells incubated with 5-FC. To get further insight into the metabolism of 5-FC in susceptible and resistant strains, we analyzed the acid extracts of cells incubated with 5-FC (procedure ii of Materials and Methods) by ^{19}F NMR spectroscopy at different times of treatment. In strain 72S, the acid extract taken at 3 h of incubation showed the presence of 5-FU and at least three other well-resolved fluorinated metabolic intermediates (C1, at −42.1 ppm; C2, at −42.4 ppm; C3, at −42.5 ppm; Fig. 5a). The resonance band at the higher field (C3) was assigned to 5-FdUMP by the use of a standard compound. This band was not detected in 72R cells. The other two signals are at present unknown but they are likely to be phosphorylated 5-FU derivatives, since they were not detected (Fig. 5b) in the extract of strain 72R which is deficient in UMP-pyrophosphorylase activity (14).

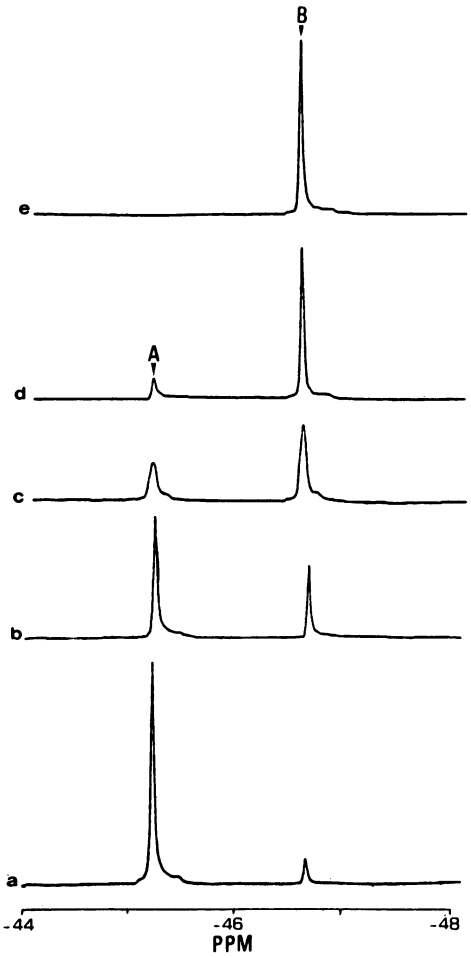

FIG. 3. ^{19}F NMR spectra of supernatants of samples of strain 72S incubated with 5-FC as reported in Materials and Methods and in the legend to Fig. 2. (a) 30 min; (b) 90 min; (c) 240 min; (d) 360 min; (e) 24 h of incubation.

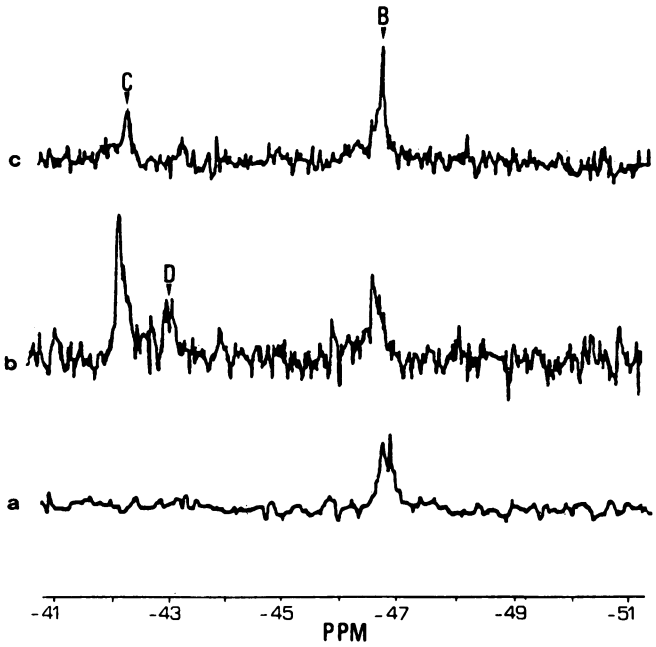

FIG. 4. ^{19}F NMR spectra of 5-FC-resistant strain 72R incubated as described in procedure ii of Materials and Methods. (a) 90 min; (b) 360 min; (c) 24 h of incubation. Note that the vertical scale of this figure is twice that of Fig. 2. For any other details, see Materials and Methods.

DISCUSSION

In this study, we evaluated the feasibility of the NMR approach for a noninvasive investigation of the uptake and metabolism of 5-FC, an important antifungal chemotherapeutic drug. Other workers had already shown that by ^{19}F NMR it is possible to follow the metabolic pattern of fluorinated compounds, in particular the specific pathways of 5-FU metabolism, in bacteria and tumors (4, 11). However, no ^{19}F NMR studies have been performed on the metabolic pathways of 5-FC in fungal organisms, in which this drug is metabolized in a characteristic and specific way (12).

Different methodological approaches were followed in this study to assess the potential information that could be obtained in these systems, and a number of *C. albicans* strains, either 5-FC sensitive or 5-FC resistant, were employed. Under all experimental conditions tested, even in the susceptible strains, a full cellular viability was maintained, thanks to the relatively low ratio between the amount of the drug and the number of cells and possibly also to the use of cells pregrown to the stationary phase. In this paper, three distinct resonance signals (−45.6, −46.7, and −42.5 ppm) were attributed to 5-FC, 5-FU, and 5-FdUMP, respectively. This attribution were based on the following observations. (i) Chromatographically pure standards of the above compounds had chemical shifts identical to those of the resonance signals detected both in the cells and in the PCA extracts. The intensity of the resonance signals tentatively attributed to 5-FC, 5-FU, and 5-FdUMP specifically increased upon the addition of the respective standard to PCA extracts. (ii) Each compound identified by ^{19}F NMR was indeed present in the PCA extract as shown by high-pressure liquid chromatography performed as described by Pogolotti et al. (6) (data not shown). The results of NMR examination of well-characterized, 5-FC-resistant, biochemical mutants of *C. albicans* can also be taken as indirect, biochemical evidence for the consistency of the attribution of the resonance signals to 5-FC and 5-FU. In particular, mutant D14, which does not possess cytosine deaminase activity (Whelan et al., in preparation), did not show the resonance attributed to 5-FU, as expected. Overall, the attributions assumed in

TABLE 2. Relative amount (R) of compound(s) in peak C and 5-FU (peak B) in 5-FC-sensitive and -resistant strains of *C. albicans* (72S and 72R, respectively)

Incubation time	R[a]	
	72S	72R
90 min	1.21	0[b]
240 min	6.22	0.86
360 min	8.83	ND[c]
24 h	7.08	0.61

[a] Calculated as ratio between peak area of signal C and peak area of signal B. Note that R is proportional to the real ratio of absolute concentration of the compounds giving rise to the two resonances.
[b] Peak C not detected at the indicated time.
[c] ND, Not determined.

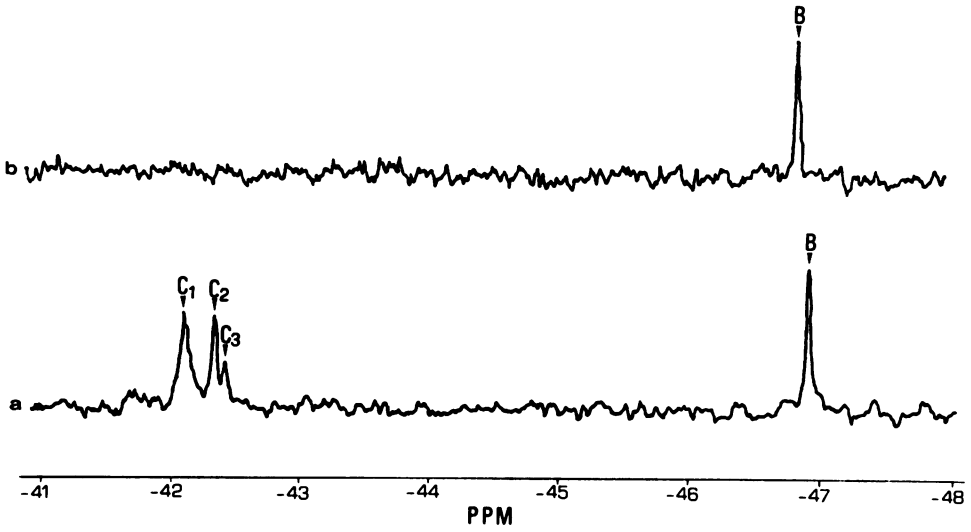

FIG. 5. ^{19}F NMR spectra of acid extracts of *C. albicans* cells incubated in 5-FC medium for 3 h (procedure ii). (a) Strain 72S; (b) strain 72R. For any other details, see Materials and Methods.

this paper are in substantial accord with those resulting from previous ^{19}F NMR studies of bacteria and animal tissues (4, 11). From biochemical studies (2, 7) it is known that 5-FU is phosphorylated to 5-fluoro-UMP and then to 5-fluoro-UDP. This is a key compound in the mechanism of action of the drug since it gives rise to either 5-fluoro-UTP or to 5-FdUMP. These latter compounds, together or independently, determine the antifungal activity of the drug by faulty RNA production or inhibition of DNA synthesis, respectively (12). A scheme showing the structure of the above compounds and their metabolic conversion is depicted in Fig. 6. When three 5-FC-sensitive strains were examined by ^{19}F NMR through the parallel examination of both the intact cells and their supernatants, consistent kinetics of drug uptake and conversion to the deaminated product 5-FU were observed.

In the packed cells (see procedure i in Materials and Methods), no further metabolism of 5-FC beyond the production of 5-FU could be detected. It should be stressed here that a high cell density was required to obtain significant

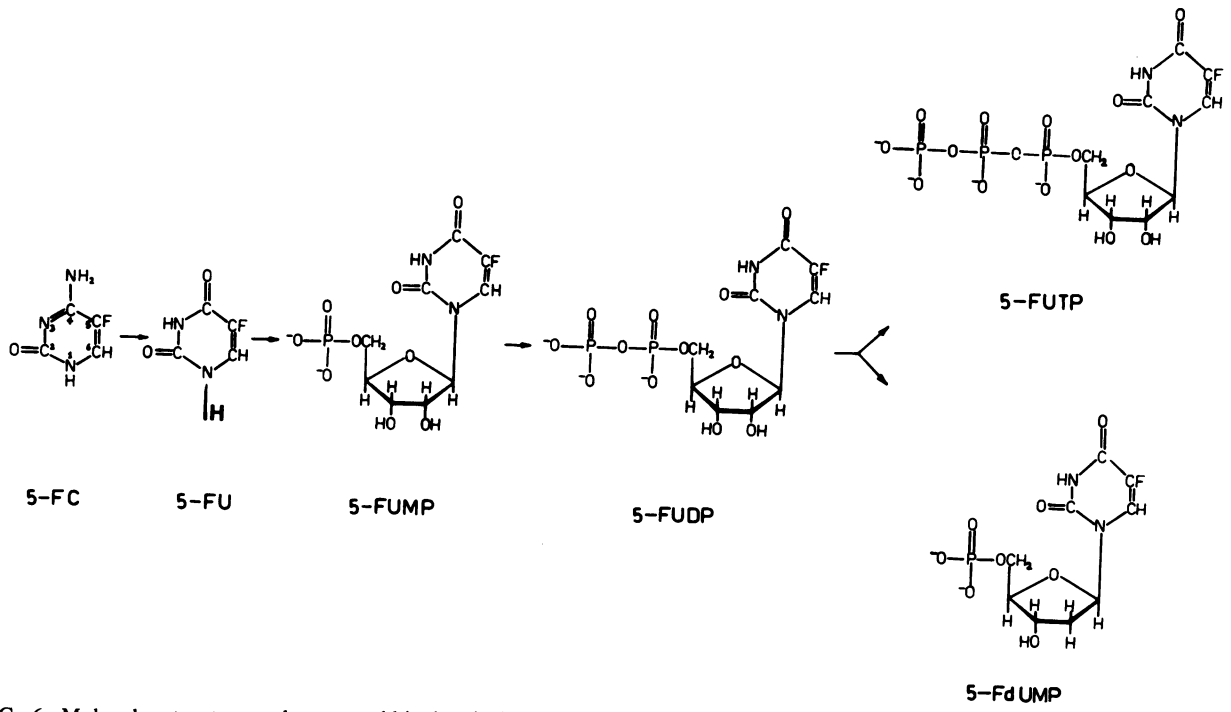

FIG. 6. Molecular structure and presumed biochemical pathways of 5-FC metabolic derivatives in *C. albicans*. 5-FUMP, 5-Fluoro-UMP; 5-FUDP, 5-fluoro-UDP; 5-FUTP, 5-fluoro-UTP.

information from these experiments and that these conditions likely resulted in a metabolic block. The absence of signals originating from putative phosphorylated metabolites of the drug may be a consequence of this block. However, by incubating cells under more physiological conditions (procedure ii), thus allowing metabolic conversion of 5-FU before concentrating the cells to the density required for NMR analyses, a metabolic pathway consistent with previous biochemical reports was observed. Although the low NMR sensitivity requires a large number of cells, procedure ii is comparable to the experimental conditions of conventional biochemical methods used to study uptake and metabolism of 5-FC. 5-FU conversion into phosphorylated derivatives was visualized by ^{19}F NMR in the form of an early, major resonance centered at −42.2 ppm and a later, minor one at −43.1 ppm. Although still unidentified, it seems likely that both resonances represent phosphorylated derivatives of 5-FU for the following reasons.

(i) In the UMP-pyrophosphorylase-deficient mutant 72R, the conversion from 5-FC to 5-FU was identical to that occurring in the sensitive parental strain, but the resonance at −42.2 ppm was detected only at later times of incubation (as seen both in intact cells and in the acid extract) and was much less intense compared with that in the parental, sensitive strain. After 24 h, the ratio between the area of this resonance and that of 5-FU was more than 1 order of magnitude lower in 72R than in 72S. This difference is in keeping with the differences in UMP-pyrophosphorylase activity in the two strains, as determined biochemically (14).

(ii) When an acid extract of the sensitive strain was examined, three peaks in the spectral region between −42.0 and −42.5 ppm were visible, one of them being attributed to 5-FdUMP. The fact that these three peaks were discernible only in the extracts could be due to either the better spectral resolution achievable in cell extract conditions or the possible intracellular immobilization of some fluorinated derivatives through binding to macromolecules, for instance, enzymes, as observed by others (11). The direct detection of 5-FdUMP in intact cells by ^{19}F NMR is of special relevance since biochemical assay of this metabolic product is rather difficult and it is known that 5-FdUMP forms a covalent complex at the active site of thymidylate synthetase (6, 9). Another point of interest in this investigation is the demonstration that a significant amount of 5-FU is secreted into the external medium by all strains, although the release itself is certainly greater in the 5-FC-resistant strain, a finding which would agree with the data of Polak and Scholer (7). Moreover, recent studies on the uptake of 5-fluoro-[^{14}C]cytosine by *C. albicans* have shown that release of radioactively labeled material from the cells does indeed occur (D. Kerridge et al., unpublished observations).

Although within the limitations already indicated, which mostly concern the direct identification of the signals attributable to phosphorylated metabolites of 5-FU in the drug-sensitive strains, the results reported in this study show that ^{19}F NMR is a valuable and feasible approach for investigations concerning the mechanism of action of the important clinical drug 5-FC in intact and viable cells. This approach can represent a powerful integration or even an alternative to the more conventional biochemical methods. In particular, ^{19}F NMR, if extended to a sufficiently high number of strains, could be a rapid and convenient way to assess the nature (sensitive versus resistant) of isolates and, more important, the type of the resistance (uptake, deamination, phosphorylation) which characterizes the isolate itself. This could be of a particular advantage with respect to classical biochemical investigations requiring enzyme isolation and the use of radioactive compounds.

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