# $^{19}$F Nuclear Magnetic Resonance Analysis of 5-Fluorouracil Metabolism in Four Differently Pigmented Strains of *Nectria haematococca*

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$^{19}$F nuclear magnetic resonance spectroscopy was used to study the metabolism of 5-fluorouracil in four strains of *Nectria haematococca* which displayed similar sensitivities to growth inhibition by this compound but differed in their pigmentation. The major metabolites, 5-fluorouridine and α-fluoro-β-alanine, were excreted into the medium by all four strains. The classical ribofluoronucleotides (5-fluorouridine-5′-monophosphate, -diphosphate, and -triphosphate) and α-fluoro-β-alanine were identified in the acid-soluble fraction of perchloric acid extracts of mycelia. Two hydrolysis products of 5-fluorouracil incorporated into RNA were found in the acid-insoluble pool. They were unambiguously assigned to 5-fluorouridine-2′-monophosphate and 3′-monophosphate with specific hydrolysis reactions on isolated RNA. The lack of fluorodeoxyribonucleotides and the fact that the four strains incorporated similar amounts of fluororibonucleotides into their RNAs strongly suggest an RNA-directed mechanism of cytotoxicity for 5-fluorouracil. The heavily pigmented wild type differed from the three low-pigmented strains in its low uptake of 5-fluorouracil and, consequently, in its reduced biosynthesis of 5-fluorouridine and α-fluoro-β-alanine. At present, it is not clear whether this change in 5-fluorouracil metabolism is a side effect of pigment production or results from another event.

Among the growth inhibitors found active in the production of pigments by the *S1* wild-type strain of the filamentous ascomycete fungus *Nectria haematococca* (Berk. and Br.) Wr, the pyrimidine analog 5-fluorouracil (FU) was shown to stimulate very efficiently this secondary metabolic process (9). In animal cells, FU is metabolized by opposing anabolic and catabolic pathways (6, 12, 17) (Fig. 1). Anabolism forms nucleotides, principally the cytotoxic 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) and 5-fluorouridine-5′-triphosphate (FUTP). Catabolism results mainly in the formation of α-fluoro-β-alanine (FBAL), ammonia, and carbon dioxide. Studies to clarify FU metabolism in *N. haematococca* and to establish the mechanism by which this compound enhances pigmentation were initiated. A preliminary investigation has been carried out by using radiolabeled FU and scintillation counting. To avoid the quenching effects of increasing amounts of various pigments on radioactivity measurements, an FU-sensitive albino mutant (*beiW88*), instead of the *S1* strain (9), was examined. More recently, $^{19}$F nuclear magnetic resonance (NMR) spectroscopy has been used to obtain an overview of FU metabolism in *N. haematococca*. The lack of pigment interference with resonance measurements allowed an analysis of the *S1* strain. Signals corresponding to metabolites of both the anabolic and catabolic pathways were identified in vivo, in cell extracts, and in culture media (10). It was of interest to confirm the similarity of FU metabolism in the *S1* and *beiW88* strains by $^{19}$F NMR spectroscopy. By using this technique, we decided to quantify all FU metabolites produced by both strains. Surprisingly, the first experiments showed that they differed in the amounts of excreted FU

metabolites. The *beiW88* strain was known to carry only one mutation preventing pigment biosynthesis (11). It was supposed that active pigment biosynthesis might result in various disorders in other areas of metabolism. Such an interference could have been detected in FU metabolism because we were looking into this particular metabolic process. In order to clarify the relationship between the pigmentation level and FU metabolism, we further examined two other pigmentation mutants of *N. haematococca* (*beiW877* and *belC505*). We report here that strains differing in their pigmentation abilities also differ in the relative activities of their FU metabolic pathways.

## MATERIALS AND METHODS

**Chemicals.** Media and buffer ingredients were reagent-grade chemicals purchased from Prolabo, Paris, France, or Merck, Darmstadt, Germany. Phosphodiesterase I (from *Crotalus adamanteus* venom) and II (from bovine spleen) were from Boehringer, Mannheim, Germany. [2-$^{14}$C]FU was obtained from CEA, Saclay, France. Aqueous counting scintillant was from Amersham-France, Les Ulis, France. FU, 5-fluorouridine (FUrd), and alkaline phosphatase (from calf intestine) were supplied by Sigma Chemical Co., St. Louis, Mo. 5-Fluorouridine-5′-monophosphate (FUMP), 5-fluorouridine-5′-diphosphate (FUDP), FUTP, and FdUMP were purchased from Sierra Bioresearch, Tucson, Ariz. FBAL was from Koch-Light Laboratories, Colnbrook, United Kingdom. Chromium(III) acetylacetonate [Cr(acac)$_3$] was a product of Spectrométrie Spin Techniques, Paris, France. Sodium fluorobenzoate (FBEN) was prepared by titrating 4-fluorobenzoic acid (Fluka, Buchs, Switzerland) with an NaOH solution.

**Strains and cultivation methods.** The *beiW88*, *beiW877*,
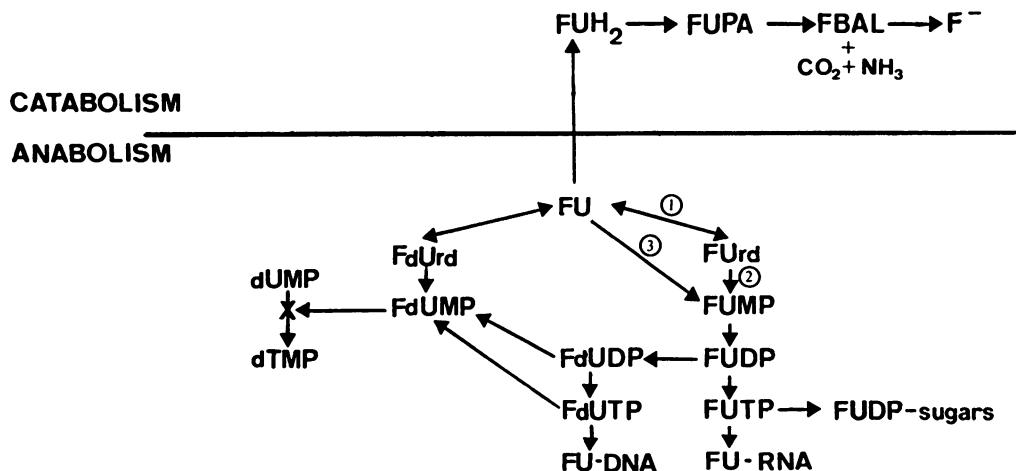
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FIG. 1. Metabolic pathways of fluoropyrimidines. FU, FUrd, and 2'-deoxy-5-fluorouridine (FdUrd) are fluoronucleosides. FUMP, FUDP, FUTP, FdUMP, 2'-deoxy-5-fluorouridine-5'-diphosphate (FdUDP), and 2'-deoxy-5-fluorouridine-5'-triphosphate (FdUTP) are fluoronucleotides. dUMP, 2'-deoxyuridine-5'-monophosphate; dTMP, 2'-deoxythymidine-5'-monophosphate. FUH$_2$, 5,6-dihydro-5-fluorouracil; FUPA, α-fluoro-β-ureidopropionic acid; F$^-$, fluoride ion; ①, uridine phosphorylase; ②, uridine kinase; ③, uridine phosphoribosyltransferase.

and belC505 mutants were derived from the S1 homothallic wild-type strain of N. haematococca (11). Stock cultures were maintained on potato-dextrose-agar medium (8) at 26°C. The basal (MSA) medium contained the following: glucose, 22.5 g; L-asparagine · H$_2$O, 0.95 g; KH$_2$PO$_4$, 1.4 g; Na$_2$HPO$_4$ · 12H$_2$O, 0.35 g; KCl, 0.75 g; MgSO$_4$ · 7H$_2$O, 0.5 g; CaCl$_2$ · 2H$_2$O, 0.15 g; FeSO$_4$ · 7H$_2$O, 0.3 mg; ZnSO$_4$ · 7H$_2$O, 0.3 mg; CuSO$_4$ · 5H$_2$O, 0.125 mg; MnSO$_4$ · H$_2$O, 0.035 mg; Na$_2$MoO$_4$ · 2H$_2$O, 0.025 mg; Bacto-Agar (Difco, Detroit, Mich.), 24 g; and distilled water, 1 liter. For analysis of FU metabolism by $^{19}$F NMR spectroscopy, MSA medium was supplemented with 10 mg of FU per liter. Twenty-five milliliters of this medium were poured into 95-mm (diameter) petri dishes. For studies involving radioactivity measurements, 0.4 ml of [2-$^{14}$C]FU (1 mM; specific activity, 55 mCi/mmol) was diluted with 2 ml of FU (38.5 mM) and 1.6 ml of H$_2$O. One-hundred microliters of this dilution was added to cooled 25-ml portions of MSA medium before the solution was poured into petri dishes. Sterile cellophane disks were layered on both types of media. The inoculation of plates was done by spreading 100-μl aliquots of a microconidium suspension (about 10$^6$ spores per ml of sterile distilled water) on cellophane disks. The dishes were incubated at 26°C for 3 to 7 days.

**Preparation of perchloric acid extracts.** At various times, the mycelia were harvested and sequentially extracted with cold and hot 10% (wt/vol) perchloric acid (PCA), by using the method of Wain and Staatz (19). For each strain, the mycelia from a few petri dishes were scraped off to get about 4 g (fresh weight), rinsed at 4°C with chilled phosphate buffer (10 mM; pH 6.0) on a nylon muslin, squeezed on absorbent paper, and suspended in 8 ml of 10% PCA at 4°C contained in a 50-ml polypropylene centrifuge tube. Mycelia were homogenized for two 15-s periods with an Ultra-Turrax TP18 homogenizer (Jankel and Kunke, IKA Werk, Staufen, Germany) set at maximum speed (20,000 rpm). After 30 min standing in an ice bath, the suspensions were spun at 20,000 × g for 10 min at 4°C. The supernatants were kept on ice. The pellets were washed with 8 ml of cold 10% PCA. After centrifugation as described above, each supernatant was pooled with the previous ones. This fraction (AS) contained the cold acid-soluble materials. The washed pellets were

suspended in 8 ml of 10% PCA and heated for 30 min at 70°C in a water bath to hydrolyze the nucleic acids. The tubes were cooled to 4°C and centrifuged as described above. The supernatants were stored at 4°C. The pellets were washed with cold 10% PCA and centrifuged as described above. Each supernatant was pooled with the previous ones. This fraction (AI) contained the cold acid-insoluble, hot acid-soluble materials. The AS and AI fractions were neutralized with 5 N KOH. The potassium perchlorate precipitates were centrifuged off (10 min at 20,000 × g at 4°C).

**Isolation of RNA.** Total RNA was extracted from the 3-day-old mycelium of the S1 strain by the method of Chomczynski and Sacchi (1) slightly modified for use with fungi. The harvested mycelium was frozen in liquid nitrogen, lyophilized, and powdered with a mortar and pestle under liquid nitrogen. The powder (corresponding to 0.4 to 0.5 g of dry mycelium) was transferred into a 50-ml polypropylene centrifuge tube. Four tubes were run simultaneously (1.9 g of dry mycelium). Five milliliters of denaturing solution was added to each tube, and the contents were mixed. Then, 0.5 ml of sodium acetate (2 M; pH 4), 5 ml of phenol saturated with Tris-HCl buffer (10 mM; pH 7.4), and 1 ml of chloroform-isoamyl alcohol (24:1) were sequentially added, with thorough mixing after the addition of each reagent. After standing on ice for 15 min, the mixtures were spun at 20,000 × g for 20 min at 4°C. The upper aqueous phases were gathered into a fresh 50-ml centrifuge tube. An equal volume (20 ml) of cold isopropanol was added. After mixing, the tube was kept at −20°C for 2 h. After centrifugation at 10,000 × g at 4°C for 10 min, the RNA pellet was dissolved in 6 ml of denaturing solution. This solution was divided in eight 0.75-ml portions, which were transferred into eight 1.5-ml Eppendorf tubes, and precipitated with 0.75 ml of isopropanol at −20°C. After centrifugation in an Eppendorf centrifuge for 10 min at 4°C, the RNA pellets were resuspended in 0.5 ml of 75% ethanol, sedimented, and dried in a vacuum evaporator (Speed Vac; Savant Instruments, Farmingdale, N.Y.). Then, two pellets were dissolved in 1.2 ml of sterile Tris-HCl buffer (0.2 M; pH 9), two others were dissolved in 1.2 ml of sterile sodium citrate buffer (0.2 M; pH 6), and the last four were dissolved in 1.08 ml of sterile distilled water. Ten-microliter aliquots were removed from each tube and

diluted with 990 μl of distilled water, and the $A_{200}$ to $A_{300}$ was measured (Uvikon 810; Kontron AG, Zurich, Switzerland). Other 10-μl aliquots were subjected to agarose gel electrophoresis to check the RNA purity.

**Hydrolysis of RNA.** Fifty microliters of a phosphodiesterase I solution (3 U/ml) was mixed in each tube containing RNA dissolved in Tris-HCl buffer, and the tubes were kept for 3 h at 25°C. Fifty microliters of a phosphodiesterase II solution (4 U/ml) was added to each tube containing RNA dissolved in citrate buffer. One hundred microliters of KOH (5 N) was added to two of the four tubes containing RNA dissolved in water. The tubes with phosphodiesterase II and KOH were incubated for 3 h at 37°C. The two last tubes containing RNA dissolved in water received 200 μl of cold 70% PCA. They were kept on ice for 15 min to precipitate the RNA, then placed for 30 min at 70°C on a heating block, and finally cooled on ice. The enzymatic reactions were stopped by heating the tubes at 90°C for 5 min. The alkaline and acid hydrolysis mixtures were neutralized with PCA and KOH, respectively, and the potassium perchlorate precipitates were centrifuged off. Ten-microliter aliquots of each hydrolyzed sample was subjected to agarose gel electrophoresis to check the disappearance of RNA. The remainders of the samples were lyophilized for ¹⁹F NMR analysis.

**Preparation of samples for ¹⁹F NMR analysis.** After removing the perchlorate precipitates, the AS and AI supernatants were frozen and lyophilized. The lyophilized material (AS and AI fractions and hydrolyzed RNA) was solubilized in 3 ml of distilled water. The pH was adjusted to 5 to 5.5. For quantitative NMR experiments, about 2 mg of the relaxation agent $Cr(acac)_3$ was then added and the solution was transferred to an NMR tube containing a coaxial capillary tube filled with a solution of FBEN in $D_2O$ doped with $Cr(acac)_3$. This solution, previously calibrated, served as the external standard for quantification. A portion of the agar culture medium was melted, transferred into NMR tubes, and analyzed after cooling to room temperature. For quantitative NMR analysis, 100 μl of an internal standard [a solution of FBEN in $H_2O$ saturated with $Cr(acac)_3$, at a known concentration] was added to about 2.5 ml of the melted solution and the exact weight of the mixture was determined after cooling.

**Treatment with $NaIO_4$ and methylamine.** To 2 ml of a neutralized AS (or AI) extract coming from 2 g (fresh weight) of mycelium was added a 300-μl (or 560-μl) portion of $NaIO_4$ (0.5 M) prepared daily; a few minutes later, 375 μl (or 700 μl) of a 4 M solution of methylamine which had been slowly brought to pH 7.5 with $H_3PO_4$ was also added. After being mixed, the sample was incubated for 1 h at 37°C. A 75-μl (or 140-μl) amount of a 1 M solution of rhamnose was then added to destroy the remaining $IO_4^-$. The sample was then acidified (pH, ≈5.2), and the ¹⁹F NMR spectrum was recorded. The ¹⁹F NMR spectrum of a control sample treated under identical conditions but without the reagents was also recorded.

**Incubation with alkaline phosphatase.** An AS or AI extract (2.4 ml) coming from 2 g (fresh weight) of mycelium was adjusted to pH 9. Magnesium acetate was then added to obtain a 0.2 mM concentration in the solution. After the addition of 4 U of alkaline phosphatase, the sample was incubated at 30°C for 3 h and then acidified (pH, 5.0 to 5.5). A control sample without enzyme was treated under identical conditions. The ¹⁹F NMR spectra of both enzymatic and control samples were recorded. The enzymatic sample was then treated with $NaIO_4$ and methylamine as described

above, in order to determine the ribosyl or deoxyribosyl structure of the nucleoside obtained.

**¹⁹F NMR analysis.** Proton-decoupled ¹⁹F NMR spectra were recorded at 282.4 MHz on a Bruker WB-AM 300 spectrometer with 10-mm (diameter) NMR tubes. The magnetic field was shimmed by using the ¹H NMR resonance of water. The chemical shifts (δ) were relative to the resonance peak of the external reference $CF_3COOH$ (5% [wt/vol] aqueous solution). Spectra were recorded under the following conditions: probe temperature, 25°C; sweep width, 29,411 Hz; 32,768 data points zero-filled to 65,536; pulse width, 7 μs (i.e., flip angle, ≈40°); repetition time, 1 or 3 s for qualitative or quantitative analysis, respectively; number of scans, 2,000 for culture media and 4,000 to 8,000 for PCA extracts; line broadening caused by exponential multiplication, 15 Hz for culture media, 5 Hz for the quantitative analysis of PCA extracts, and 1 Hz for the identification of fluoronucleotides (FNUCt). The concentrations of the fluorinated metabolites were measured by comparing the expanded areas of their respective NMR signals with that of FBEN (external or internal). The areas were determined after the different signals were cut out and weighed.

**Radioactivity measurements.** Aliquots from AS and AI fractions (200 μl) and medium (1 ml) were transferred into scintillation vials and mixed with 10 ml of aqueous counting scintillant. In order to estimate label losses, samples from cellophane disks, perchlorate precipitates, and the final mycelium pellet were macerated in known volumes of distilled water. Aliquots of these suspensions were counted. Radioactivity was measured with an LKB Rack Beta 1212 liquid scintillation counter.

## RESULTS

The four selected strains differed by their pigmentation on media containing 77 μM FU as follows: after 7 days, the *beiW88*, *beiW877*, *belC505*, and *S1* cultures were beige, beige, light red, and deep red, respectively. Moreover, compared with unsupplemented medium, the medium supplemented with 77 μM FU led to a growth inhibition of about 40% for the *beiW88* strain and 56% for the three others (11). In order to determine whether there is some correlation between FU metabolism and growth inhibition and pigmentation, we searched for FU metabolites in the culture media and mycelia of each strain.

**Identification of FU metabolites.** The ¹⁹F NMR spectra obtained with PCA extracts of mycelia harvested after increasing incubation times in the presence of 77 μM FU and with corresponding culture media showed that the patterns of internal and external FU metabolites from the four strains were qualitatively similar.

Spectra of media from 3-day-old cultures consisted of a major peak of FU (−93.3 ppm), a signal of FUrd (−89.9 ppm), and, except in the *S1* strain, a small resonance of FBAL (−112.6 ppm) (Fig. 2A). In 4- to 7-day culture media, FUrd and FBAL were always present, but FU was detected only in the *S1* (4 to 7 days), *beiW88* (4 days), and *belC505* (4 and 5 days) strains (Fig. 2B). These signals were assigned by spiking the samples with small amounts of standard compounds and, for FUrd, by an additional treatment with periodate and methylamine, which converted it to FU (10).

Spectra of AS fractions from mycelia showed the signal of FBAL (−112.7 ppm) and sometimes a small FU peak (−93.4 ppm). Moreover, four peaks (−88.95, −89.09, −89.12, and −89.22 ppm) were observed, and, because of their chemical shifts, these might correspond to FNUCt and/or FUDP-
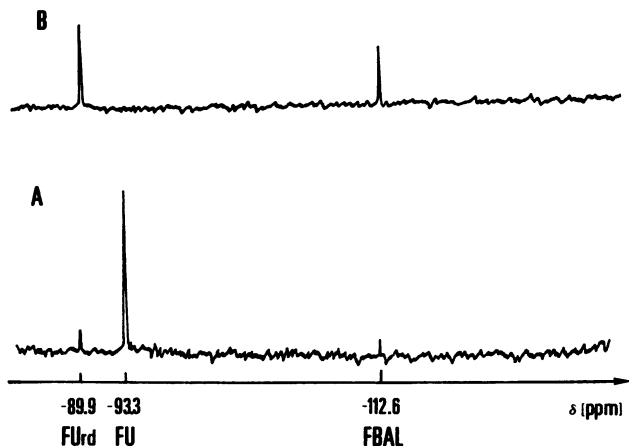
FIG. 2. $^{19}$F NMR spectra of culture media from the beiW88 mutant strain of N. haematococca after 3 (A) and 7 (B) days of culture.



FIG. 4. $^{19}$F NMR spectra of the AI fraction from the S1 wild-type strain of N. haematococca after 5 days of culture. pH, 5.35.

sugars (Fig. 3). The treatment of AS extracts with alkaline phosphatase, which converts nucleotides but not nucleotide sugars into nucleosides (20), led to the disappearance of the four resonances around −89 ppm and the appearance of a signal of fluoronucleosides (FNUCs). This signal was assigned to FUrd with periodate and methylamine treatment (5). These two successive experiments showed that the four resonances corresponded to ribosyl-FNUCt. The direct treatment of AS samples with periodate and methylamine converted these signals into FU, thus confirming that they were ribosyl derivatives. No fluorodeoxyribonucleotide signal was observed in the AS fractions studied. By the addition of authentic FUMP or FUDP, the signals at −89.22 and −89.12 ppm were assigned to FUMP and FUDP, respec-
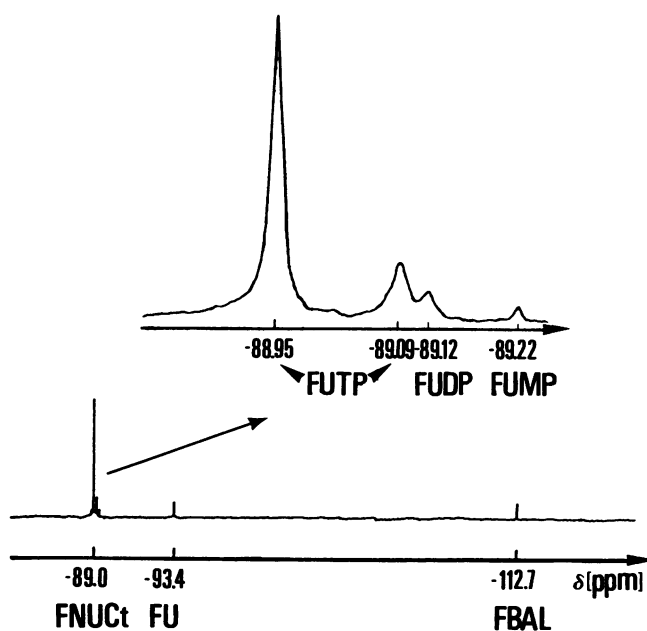


FIG. 3. $^{19}$F NMR spectra of the AS fraction from the S1 wild-type strain of N. haematococca after 3 days of culture. pH, 5.30. See the text for the attribution of signals at −88.95 and −89.09 ppm to FUTP.

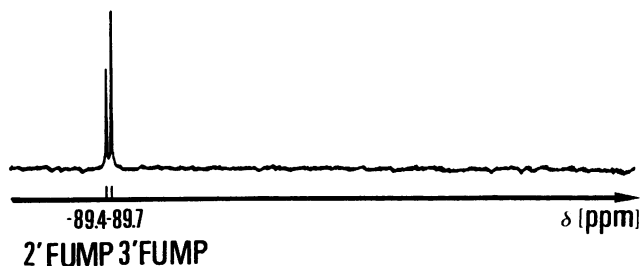tively. The prominent peak at −88.95 ppm and the signal at −89.09 ppm were both enhanced upon the addition of standard FUTP to AS extracts. Indeed, surprisingly, the $^{19}$F NMR spectrum of commercial FUTP showed two signals of equal intensity with very close chemical shifts. One of these signals might correspond to FUTP and the other might correspond to a compound with a similar structure. Work is still in progress to elucidate this problem.

When AI fractions were examined, two signals were found at −89.45 and −89.7 ppm (Fig. 4). In addition, a small resonance corresponding to FUMP appeared occasionally at −89.2 ppm. To identify the two main signals, several experiments were necessary. The treatment of AI extracts with alkaline phosphatase changed these two signals to a single peak of FNUCs that was assigned to FUrd, since a periodate and methylamine treatment yielded FU. The two signals, therefore, corresponded to ribosyl-FNUCt, and, as in the AS extracts, no fluorodeoxyribonucleotide, especially FdUMP, was detected in the AI fractions. The fact that these two compounds were unaffected by the direct treatment of AI extracts with periodate and methylamine implied that they bear a phosphate group on the 2' and/or 3' position of the sugar moiety. For an unambiguous assignment of these signals and to demonstrate that they are fluorinated hydrolysis compounds coming from FU incorporation into RNA, we extracted RNA from S1 mycelium grown for 3 days on MSA medium containing 77 μM FU. The preparation was divided into four equal fractions which were hydrolyzed by various treatments including phosphodiesterase I and II, 0.5 N KOH, and hot 10% PCA. Phosphodiesterase I is known to cut nucleic acids from the free 3' OH terminus, yielding 5'-mononucleoside phosphates (7). Only one signal, at −89.2 ppm, was actually detected in the $^{19}$F NMR spectrum obtained from the RNA fraction treated with phosphodiesterase I. It was attributed to FUMP by spiking with authentic FUMP. Phosphodiesterase II acts on nucleic acids as an exonuclease, yielding nucleoside-3'-phosphates when a 5' OH terminus is available (7). The spectrum resulting from the action of phosphodiesterase II on RNA consisted of a signal at −89.74 ppm corresponding to 5-fluorouridine-3'-monophosphate (3'-FUMP). The alkaline hydrolysis of RNA is known to yield both 5-fluorouridine-2'-monophosphate (2'-FUMP) and 3'-FUMP (7). Two peaks, at −89.45 and −89.7 ppm, were found in the spectrum of the KOH-treated RNA fraction. They were assigned to 2'-FUMP and 3'-FUMP, respectively. These two resonances also appeared in the spectrum obtained from the RNA fraction hydrolyzed with hot PCA. The AI fractions thus contained 2'-FUMP and 3'-FUMP.

**Assessment of the validity of FU and metabolite quantification via NMR.** In order to assess the accuracy of the

TABLE 1. Estimation of error on data obtained from NMR spectra of experiments with *S1* strain

| Day | Amt ($\mu$mol) in[a]: | | | Total compart-ments | Initial $\mu$mol of FU | % Error |
|-----|--------|------|------|------|------|------|
| | Fraction | | | | | |
| | Medium | AS | AI | | | |
| 3 | 26.3 | 0.88 | 1.07 | 28.25 | 28.85 | −2.1 |
| 4 | 25.6 | 0.47 | 0.95 | 27.02 | 26.92 | 0.4 |
| 5 | 21.1 | 0.42 | 0.70 | 22.22 | 25.00 | −11.1 |
| 6 | 26.4 | 0.35 | 0.73 | 27.48 | 25.00 | 9.9 |
| 7 | 25.4 | 0.36 | 0.53 | 26.29 | 25.00 | 5.2 |

[a] Values are expressed in micromoles present in the medium and AS and AI fractions (absolute values).

concentrations determined by NMR spectroscopy, the absolute quantities of each metabolite from each compartment (the medium and AS and AI fractions) were added together and the sum was compared with the initial amount of FU. With the *S1* strain, errors did not generally exceed 10% of the initial FU amount (Table 1). Errors carried out on NMR determinations of fluorinated metabolites produced by the three mutants fell within the same range as that obtained from the *S1* strain (data not shown). These results indicate that the amounts of FU metabolites may be validly deduced from peak areas measured on $^{19}$F NMR spectra.

The percentage of recovery of FU metabolites was determined by following the distribution of the radioactivity in all compartments of the culture, except gases (no attempt was made to trap $^{14}CO_2$). In a preliminary experiment, a *beiW88* culture was labeled with [2-$^{14}$C]FU during the first 3 days of incubation. The results in Table 2 show that the label associated with medium accounted for 80% of the initial radioactivity. About 0.8% remained absorbed on cellophane disks. Radioactivity lost in the final mycelium pellet and in perchlorate precipitates did not exceed 2% of the initial label. The AS and AI fractions contained 7.7 and 4.1% of the initial radioactivity, respectively. The 6.3% loss in radioactivity might result from $^{14}CO_2$ release, adsorption on petri dish plastic, measurement errors, and unknown events.

In order to validate the NMR data, we compared the quantification of FU and metabolites by NMR spectroscopy

TABLE 2. Distribution of $^{14}$C label in different compartments of a 3-day *beiW88* culture

| Compartment | Radioactivity (dpm/U)[a] | % Initial radioactivity |
|-----|------|------|
| Nonseeded | | |
| Medium | 2,079,413 | |
| CD[b] | 17,184 | |
| Total | 2,096,597 | 100.0 |
| Culture | | |
| Medium | 1,663,458 | 79.3 |
| CD | 16,201 | 0.8 |
| AS fraction | 160,979 | 7.7 |
| AS pp[c] | 21,064 | 1.0 |
| AI fraction | 85,595 | 4.1 |
| AI pp[c] | 10,497 | 0.5 |
| Final pellet | 5,642 | 0.3 |

[a] Radioactivity is expressed as disintegrations per minute (dpm) per culture unit (one petri dish). These are absolute values.
[b] Cellophane disk.
[c] Perchlorate precipitate from AS or AI fraction.

TABLE 3. Comparison of data obtained from $^{19}$F NMR and radioactivity measurements in different compartments of a 3-day *beiW88* culture

| Compartment | Mean ± SD % initial FU by $^{19}$F NMR[a] | Mean ± SD % initial radioactivity[b] |
|-----|------|------|
| Medium Fraction | 70.6 ± 3.1 | 73.6 ± 5.7 |
| AS | 7.2 ± 0.2 (9.5 ± 0.2)[b] | 8.1 ± 0.4 |
| AI | 4.4 ± 0.3 | 4.0 ± 0.1 |

[a] Results are expressed as percentages of the initial FU. They are the means of two experiments.
[b] Results are expressed as percentages of the initial radioactivity (before medium inoculation). They are the means of two experiments.
[c] Catabolism of [2-$^{14}$C]FU yields FBAL, $^{14}CO_2$, and $NH_3$. The nonvolatile FBAL is not radiolabeled and cannot be detected by liquid scintillation counting. Since in our experiments $^{14}CO_2$ was not trapped, FBAL was omitted in the sum of AS metabolites obtained from NMR data in order to compare accurately the NMR and radioactivity data. The value in parentheses includes FBAL.

and by radioactivity measurements. Cultures of the *beiW88* strain were simultaneously run for 3 days on MSA medium supplemented with unlabeled or [2-$^{14}$C]FU, harvested and extracted with cold and hot 10% PCA. As shown in Table 3, the relative amounts of fluorinated compounds detected in the medium and AS and AI fractions by $^{19}$F NMR spectroscopy were similar to the amounts of labeled products from the same culture compartments. Moreover, the reproducibility was similar for the two methods and better than 10%.

**Time course of FU metabolism in the *S1* strain.** The results in Fig. 5 show that FU was poorly metabolized, since only 42.5% of initial FU was taken up and metabolized by the *S1* strain within 7 days through two pathways, catabolism to FBAL and anabolism to FNUCt and FNUCs, with the anabolic pathway always being predominant.

Unmetabolized FU was exclusively extracellular, since intracellular FU, when detected, was <0.2% of the total remaining FU. Similarly, FBAL was predominantly present in the culture medium, since intracellular FBAL represented <4% of total FBAL after the third day of culture. FUrd was always extracellular, whereas FNUCt were intracellular (Fig. 6 and 7).

The concentration of extracellular FBAL increased to 13% of the initial FU within the first 6 days and then remained fairly constant. The level of FUrd also increased during the period of pigment synthesis from 8% of initial FU at 3 days to 27% at 7 days (Fig. 6). Inversely, the FNUCt content of the *S1* strain decreased with age from 7% of the initial FU at 3 days to 3% at 7 days. Of this, macromolecule-bound FNUCt (2′-FUMP and 3′-FUMP) represented approximately 60 to 70% of the total FNUCt. Both free and macromolecule-bound FNUCt declined with age (Fig. 7).

**Quantitative comparison of FU metabolism in the mutant and wild-type strains.** The three mutants displayed several significant differences when compared with the wild type. One of the most striking changes was that FU disappeared in culture media in less than 6 days. It was consumed faster by the two beige strains than by the reddish *belC505* mutant (Fig. 5). Changes in the extent of catabolism were also observed. During the first 4 days of incubation, the *beiW88* and *beiW877* mutants converted about 30% of the initial FU to FBAL. At 7 days, FBAL accounted for about 40% of initial FU. Inversely, the percentage of FBAL in the *belC505* culture (19% at 7 days) was lower than in the two beige cultures and slightly higher than in the *S1* strain (Fig. 6).
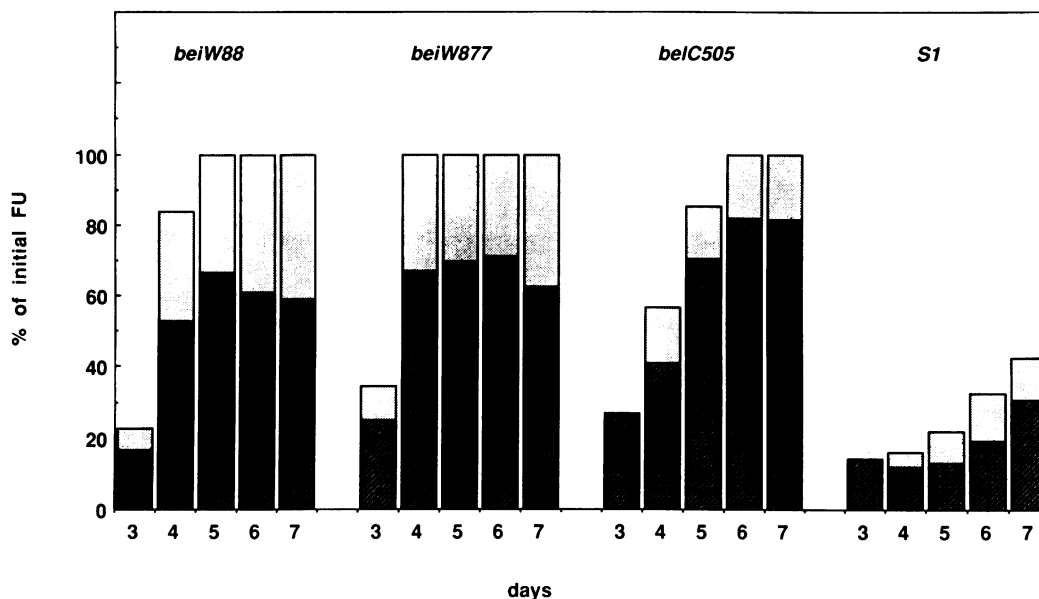
FIG. 5. Proportions of catabolism (□) and anabolism (▨) in the four different strains of *N. haematococca*.

Even if the anabolic pathway always overcame the catabolic pathway as in the *S1* strain (Fig. 5), the relative amounts of FUrd in the cultures of the three mutants were quite different from those observed in wild-type cultures. The *beiW88*, *beiW877*, and *belC505* mutants were found to synthesize FUrd at levels up to 56, 58, and 80% of initial FU, respectively. Anabolism to FNUCt in the *belC505* mutant was as low as in the wild type, but it was higher for the two beige mutants at 3 and 4 days. Free FNUCt from the *beiW88*, *beiW877*, *belC505*, and *S1* strains decreased from 5, 8, 3, and 3% of the initial FU at 3 days to 1, 2, 0.2, and 1%, respectively, at 7 days. The macromolecule-bound FNUCt

contents of the four strains were quite similar, accounting for 3 to 4% of initial FU at 3 to 4 days. In all the strains, they declined slowly, reaching about 2% of initial FU at 7 days (Fig. 7).

## DISCUSSION

The metabolism of FU was examined in four *N. haematococca* strains responding almost similarly to its growth-inhibitory effect but differing in pigmentation potencies (11). The analyses were performed with $^{19}$F NMR spectroscopy, which permits simultaneous determination and quantifica-
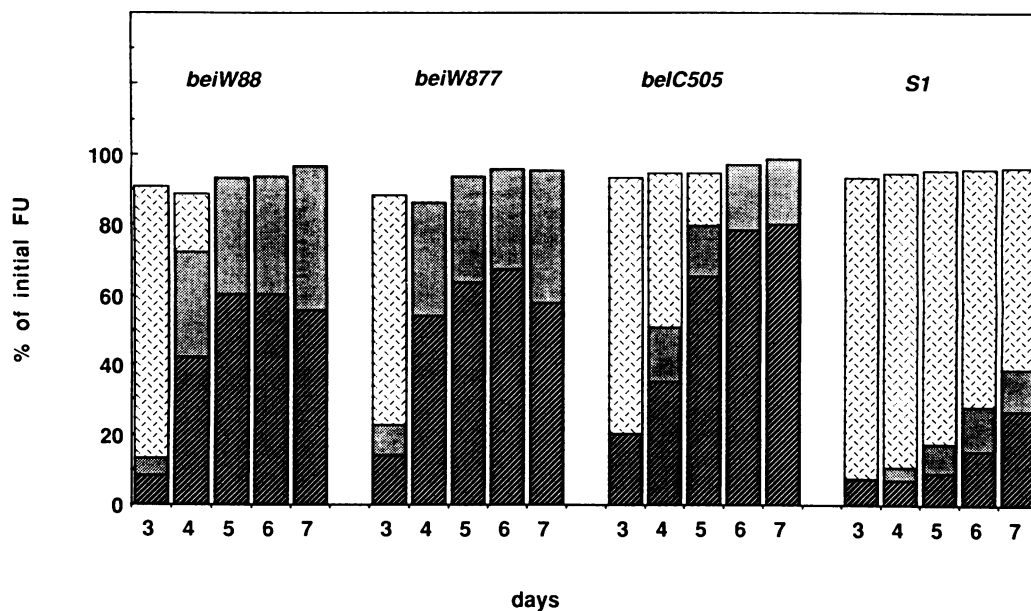


FIG. 6. Changes in the content of the fluorinated compounds FU (▨), FBAL (□), and FUrd (▨) in the culture media of the four strains of *N. haematococca* after different growth periods.
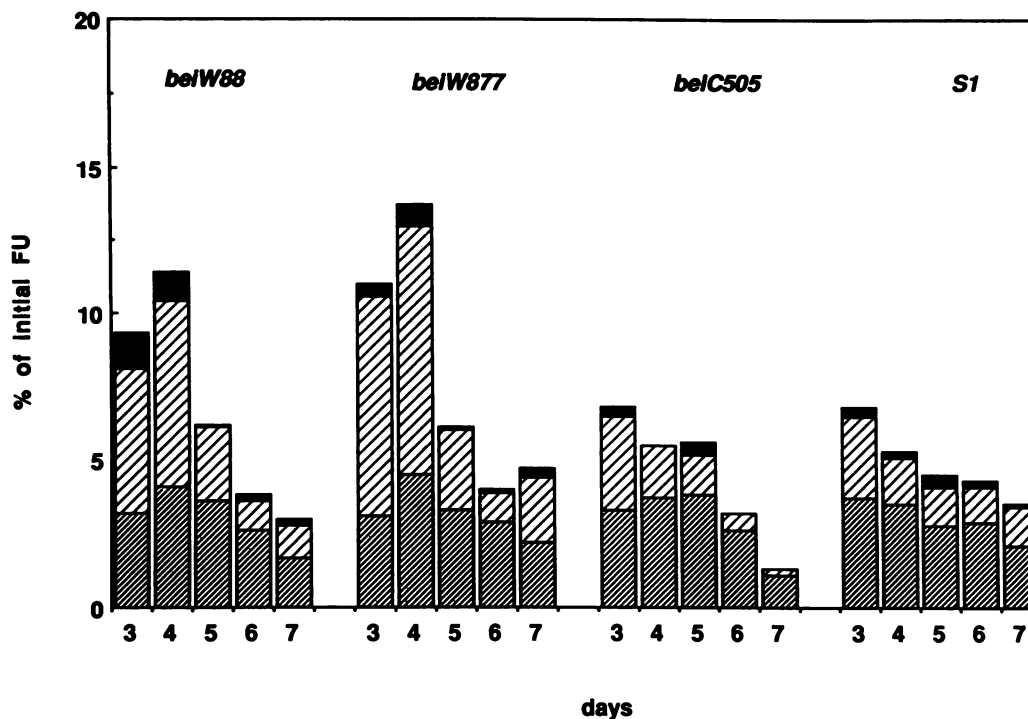
FIG. 7. Changes in the content of the fluorinated compounds free FNUCt (▨), RNA-bound FNUCt (▨), and FBAL (■) in the mycelia of the four strains of *N. haematococca* after different growth periods.

tion of all fluorine-containing compounds in a given sample without the need for radiolabeled drug. For example, it allowed the observation of FU, FBAL, and FUrd in culture media without resorting to any extraction process or chemical treatment (Fig. 2). This technique is also a performing tool for the determination of free or macromolecule-bound FNUCt in PCA extracts. Indeed, the different free FNUCt (FUTP, FUDP, and FUMP) were easily distinguished in the AS fractions (Fig. 3). This can also be gained with reverse-phase, ion-paired high-performance liquid chromatography, but, to our knowledge, only if radiolabeled FU is employed (13, 15). There is also a need for a simple method of quantitating the incorporation of FU into RNA without the use of radioactive precursors. Spears et al. (16) described a method for the determination of levels of incorporation of nonradiolabeled FU into RNA that requires a multistep procedure including the alkaline hydrolysis of PCA precipitates of cell sonicates, the separation of 2'- and 3'-FUMP from the remaining nucleotides by DEAE-cellulose chromatography, the conversion of 2'- and 3'-FUMP to FUrd with alkaline phosphatase, the quantitation of FUrd after periodate oxidation followed by $NaB^3H_4$ reduction of the resulting nucleoside dialdehydes, and isolation of the tritiated FUrd-trialcohol obtained. Recently, another method for identifying and quantitating 2'- and 3'-FUMP from nonradiolabeled FU in alkaline hydrolysates of murine tumor RNA has been developed, by using high-performance liquid chromatography (14). The first drawback of this technique is that 2'- and 3'-monophosphate nucleosides, especially 2'- and 3'-FUMP, are not resolved. Moreover, the method requires the conversion of purine nucleotides to free bases, since the 2' and 3' mixed isomers of GMP cochromatograph with 2'- and 3'-FUMP. ^{19}F NMR is an easier method, since it allows the separate quantification of 2'- and 3'-FUMP coming from

nonradiolabeled FU incorporated into RNA directly in the AI extract.

The ^{19}F NMR spectra of culture media and PCA extracts from all four strains exhibited the same resonance signals (i.e., unmetabolized FU, FUrd, and FBAL in culture media (Fig. 2); free FNUCt (FUTP, FUDP, and FUMP), traces of FU (in some samples), and FBAL in AS extracts (Fig. 3); and 2'- and 3'-FUMP in AI extracts [Fig. 4]). The only observed catabolite was FBAL. Its immediate precursors, 5-fluoro-5,6-dihydrouracil and α-fluoro-β-ureidopropionic acid (Fig. 1), were not detected in cell extracts or in media. The bulk of FBAL was found in culture media (Fig. 6). Thus, the FU catabolic pathway can be regarded as a detoxification mechanism. Another major excreted FU metabolite in *N. haematococca* was FUrd (Fig. 6). Therefore, conversion of FU to FUrd could also contribute to FU detoxification in *N. haematococca*. This is an uncommon FU degradative pathway, since FUrd was not detected in culture media of *Candida* yeasts (3, 4, 18) or filamentous fungi (*Aspergillus* spp. [2]).

The other FU metabolites found in *N. haematococca* cultures were intracellular fluororibonucleotides. No deoxyribosyl FU derivatives could be detected. The lack of fluorinated deoxyribonucleotides in *N. haematococca* extracts suggests that incorporation of FUTP into RNA may be the dominant determinant of growth inhibition by FU. In an earlier study, we found that a FU-resistant mutant with altered uridine phosphoribosyltransferase activity was deficient in macromolecule-bound fluororibonucleotides (10). This result is consistent with an RNA-directed mechanism of cytotoxicity for FU. According to this proposal, the approximately equal percentages of the initial FU incorporated into RNAs in young cultures (before day 5) (Fig. 7) may account

for the similar sensitivity of all four strains to growth inhibition by FU.

The main differences among the four strains were found regarding the mechanism by which the fungi could escape from FU toxicity. This inhibitor was not as much metabolized by the *S1* strain, which became deeply pigmented in its presence, than by the low (*belC505*)- or non (*beiW88* and *beiW877*)-pigmenting mutants. The cause of this difference is not known. From the above results, it seems that the high level of pigment synthesis in the *S1* strain lowers further FU uptake and metabolism. This view is corroborated by the fact that the *belC505* strain, which carries a mutation reducing the production of pigment, has a higher capacity to metabolize FU than the *S1* strain.

## ACKNOWLEDGMENT

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