EFFECTS OF URACIL AND THYMIDINE ON THE DEVELOPMENT OF RESISTANCE TO 5-FLUOROURACIL IN *PEDIOCOCCUS CEREVISIAE*

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ABSTRACT

WHITE, P. J. (Roswell Park Memorial Institute, Buffalo, N.Y.) AND C. A. NICHOL. Effects of uracil and thymidine on the development of resistance to 5-fluorouracil in *Pediococcus cerevisiae*. J. Bacteriol. **85**:97–105. 1963.—Growth of *Pediococcus cerevisiae* ATCC 8081 in a basal semidefined medium was inhibited by 5-fluorouracil (FU) and 5-fluoro-2′-deoxyuridine (FUDR). Addition of uracil slightly decreased sensitivity to FU, but did not affect inhibition by FUDR. Addition of thymidine alone increased sensitivity to FUDR 500-fold, but did not appreciably affect sensitivity to FU. This organism was able to grow in the presence of high concentrations of either drug when both uracil and thymidine were added to the medium. Substrains resistant to FU were developed in the basal medium, or in basal medium with added uracil, or with thymidine. The substrains developed in the presence of uracil or thymidine were not resistant when these compounds were omitted from the medium. Cross resistance to FUDR was shown only when thymidine was present. The ability to use thymidine as a growth factor in the absence of leucovorin was lost in the substrains developed in the presence of uracil, though not in the other substrains. Washed suspensions of organisms of all strains hydrolyzed FUDR to FU, the parent strain being most active in this respect. The cellular uptake of fluorouracil-2-C^{14} was less in all of the resistant substrains than in the parent strain, whether or not uracil or thymidine was present during the incubation. This uptake of FU-C^{14} was decreased most markedly in the substrains which became resistant to FU in the absence of uracil.

5-Fluorouracil (FU) and 5-fluoro-2′-deoxyuridine (FUDR) were introduced as tumor-inhibiting compounds by Heidelberger and co-workers (Duschinsky, Pleven, and Heidelberger, 1957; Heidelberger et al., 1957). The drugs are also inhibitors of bacterial growth (Scheiner, Kostelak, and Duschinsky, 1957), and the inhibition may be overcome by the addition of uracil, thymine, or thymidine (Cohen et al., 1958; Edinoff, Knoll, and Klein, 1957). Resistance to FU has been observed in tumor cells and in bacteria, and has been associated with loss of enzymes concerned in uracil anabolism. Reichard, Sköld, and Klein (1959) found that Ehrlich ascites tumor cells that became resistant to FU lost uridine phosphorylase activity. Brockman, Davis, and Stutts (1960) showed that enzyme preparations from FU-resistant *Escherichia coli* failed to catalyze the reactions of uracil or FU with 5-phosphoribosyl-1-pyrophosphate to form 5′-ribonucleotides, whereas similar enzyme preparations from FU-sensitive *E. coli* did catalyze these reactions. In both of these cases, it is believed that the administered drug is no longer converted enzymatically to an inhibitory ribonucleotide derivative by the resistant strains. Heidelberger et al. (1960) found that resistance to FU in a line of Ehrlich ascites cells may be due to a decreased sensitivity of the enzyme that converts deoxyuridylic acid to thymidylic acid (thymidylate synthetase) to inhibition by fluorodeoxyuridylic acid, into which administered FU is still converted by the resistant cells.

The present study concerns the effects of uracil and thymidine on the sensitivity of *Pediococcus cerevisiae* 8081 to FU and FUDR, and on the emergence and properties of resistant substrains of this organism. *P. cerevisiae* was used because of its high initial sensitivity to both drugs, and because it can be grown under conditions (in the absence of leucovorin) in which thymidine is an essential growth factor (Sauberlich, 1949). A preliminary report of this work has been presented (White, 1960).

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MATERIALS AND METHODS

Organism. P. cerevisiae (Leuconostoc citrovorum), ATCC 8081, was maintained in Tryptose-glucose-agar stab cultures which had been grown overnight at 37 C. Stock cultures were transferred monthly and stored at 2 C. Substrains resistant to FU and FUDR were similarly maintained.

Medium. A semidefined medium, containing acid-hydrolyzed casein but without added pyrimidines, was used for growth experiments. The basal medium contained (per liter): Vitamin Free Casamino Acids (Difco), 7.5 g; DL-tryptophan, 80 mg; DL-phenylalanine, 50 mg; DL-tyrosine, 100 mg; L-cysteine hydrochloride, 50 mg; D-glucose, 25 g; $CH_3COONa \cdot 3H_2O$, 20 g; NH_4Cl, 3 g; KH_2PO_4, 600 mg; K_2HPO_4, 600 mg; $MgSO_4 \cdot 7H_2O$, 200 mg; $MnSO_4 \cdot 4H_2O$, 20 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; NaCl, 10 mg; adenine sulfate, 10 mg; guanine hydrochloride, 10 mg; xanthine, 10 mg; pyridoxine hydrochloride, 2 mg; pyridoxal hydrochloride, 2 mg; pyridoxamine hydrochloride, 2 mg; riboflavine, 1 mg; thiamine hydrochloride, 1 mg; calcium pantothenate, 1 mg; nicotinic acid, 1 mg; biotin, 15 μg. Then, 10 g of Difco yeast extract or 0.4 μg of leucovorin (calcium salt of 5-formyltetrahydropteroylglutamate) or 100 μg of thymidine had to be added to each liter of medium. The medium was prepared at double strength, adjusted to pH 6.5, filtered through Whatman no. 50 paper, and brought to the desired volume with water (or other experimental additions), before autoclaving at 120 C for 7 min.

Growth conditions. Organisms for inocula were grown overnight in basal medium containing yeast extract, centrifuged, and washed twice in sterile saline of equivalent volume. The washed suspension in saline was diluted to about 2×10^6 organisms per ml, and 0.1 ml of this suspension was used to inoculate 5 ml of medium. Inoculated medium was incubated in air at 37 C in plugged tubes (150 × 19 mm) set up in duplicate. When large quantities of organisms were required for experiments with washed suspensions, medium (500 ml) containing leucovorin, in a 1-liter conical flask, was inoculated with an overnight culture (1 ml) of *P. cerevisiae* grown in basal medium containing yeast extract, and incubated overnight at 37 C.

Assessment of growth. Growth was estimated by measurement of turbidity with a Klett-Summerson photoelectric colorimeter with a red filter, in matched colorimeter tubes (125 × 16 mm); the uninoculated medium was used to give the zero setting. The relation between the colorimeter reading and the dry weight of organisms was linear from 0 to 150 on the colorimeter scale, and a reading of 100 was equivalent to 0.24 mg (dry wt) of organisms per ml. A 1-ml sample of a suspension of cells, which read 100 on the colorimeter scale, gave 2×10^8 colonies on solid medium.

Sensitivity to FU and FUDR. Tubes of medium were prepared, containing the drug at concentrations increasing about threefold between each pair of tubes in a series. Medium was autoclaved after addition of the drug, inoculated, and incubated for 20 hr (or 40 hr when thymidine replaced leucovorin). The concentration of drug inhibiting growth by 50% after this time was determined by plotting the level of growth against concentration of inhibitor in the medium (Fig. 2).

Measurement of uptake of 5-fluorouracil-2-C^{14}. *P. cerevisiae* 8081, and its resistant substrains, were grown in basal medium (50 ml), containing leucovorin (0.9 mμg/ml), in 125-ml Erlenmeyer flasks at 37 C for 14 hr. Organisms were centrifuged and washed twice with saline (20 ml), then resuspended in double-strength basal medium to a concentration of about 1 mg (dry wt) of organisms per ml. The suspension (1.0 ml), double-strength basal medium (1.5 ml), 5-fluorouracil-2-C^{14} (0.5 ml; 1 mg/ml; 100 counts per min per μg), and water or other addition, to a final volume of 5.0 ml, were incubated in duplicate in tubes (130 × 15 mm) at 37 C for 1 hr, along with controls without organisms. After incubation, the mixture was filtered through a Millipore filter (Millipore Filter Corp., Bedford, Mass.) into a Millipore disc, type HA (diameter, 25 mm; pore size, 0.45 μ). Liquid was removed from the disc under slight vacuum for 2 min. Then the disc was transferred to a planchette and dried in a desiccator over calcium chloride. Radioactivity was measured in a gas flow counter (RCL Mark 12, Model 2; Radiation Counter Lab., Inc., Skokie, Ill.) connected to a scaler unit (Model 183A; Nuclear-Chicago Corp., Des Plaines, Ill.).

The difference in counts between discs with and without cells was attributed to fluorouracil-2-C^{14} taken up by the organisms. Washing once with water (2 ml) reduced the readioactivity of discs without organisms almost to the limit of detection. A second wash did not further de-

crease the radioactivity of discs with cells. This residual radioactivity was equivalent to the amount of fluorouracil-2-C^{14} calculated to be present in the organisms by subtraction of the blank without organisms from the counts of unwashed discs. There was a linear relation, after blank correction, between the radioactivity of a disc and the dry weight of organisms collected on the disc, up to at least 5 mg of organisms (dry wt) per disc. Organisms were similarly incubated in basal medium containing polyglucose-C^{14} (1,350 counts per min per ml) to which the organisms were assumed to be impermeable (Werkheiser and Bartley, 1957). The suspension was filtered through a Millipore disc. The radioactivity of this disc was the same as that of a control disc through which medium containing polyglucose-C^{14} without organisms was filtered. The contribution of interstitial liquid to the count of a disc was, therefore, negligible when the weights of organisms collected were small.

Metabolism of fluorodeoxyuridine. Washed suspensions (1 ml) containing about 50 mg (dry wt) of *P. cerevisiae* 8081, or the resistant substrains, in 0.02 M phosphate buffer (pH 6.5) were incubated with FUDR (1 ml, 1 mg/ml) for 1 hr at 37 C. Organisms were centrifuged and the supernatant fluids were diluted 1:50 in 0.1 N KOH. Controls without organisms and without FUDR were included.

Estimation of base mixtures. When present together, FU and FUDR were estimated by measurement of optical density (OD), at 290, 282, 266, and 255 mμ, of solutions in 0.1 N KOH. The concentrations were calculated from the following equations, derived by the method of Loring (1955), after comparison of the absorption spectra of the two compounds in alkaline solution (Fig. 1).

$$\text{FU (moles/liter)} = \frac{7.00\ OD_{290} - 2.42\ OD_{266}}{2.28 \times 10^4}$$

$$\text{or } \frac{5.66\ OD_{266} - 7.00\ OD_{255}}{0.492 \times 10^4}$$

$$\text{FUDR (moles/liter)} = \frac{4.65\ OD_{266} - 4.05\ OD_{290}}{2.28 \times 10^4}$$

$$\text{or } \frac{4.05\ OD_{255} - 2.58\ OD_{266}}{0.492 \times 10^4}$$

$$\text{Total FU plus FUDR (moles/liter)} = \frac{OD_{282}}{0.515 \times 10^4}$$

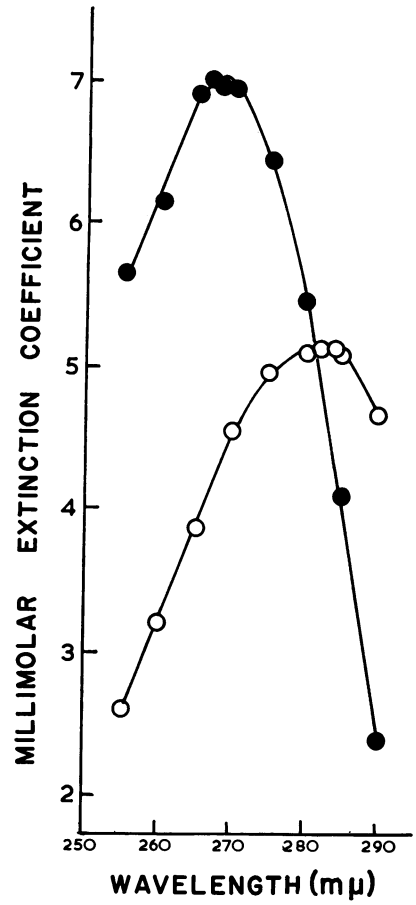

FIG. 1. *Ultraviolet absorption of fluorouracil (FU) and fluorodeoxyuridine (FUDR) in 0.1 N potassium hydroxide.* ○, *FU;* ●, *FUDR.*

Chromatographic procedure. FU and FUDR (about 10 μg per spot) were separated by descending chromatography on Whatman no. 1 paper for 24 hr at room temperature, using as solvent three parts (by volume) of *n*-butanol to one part of 28% (w/v) ammonia (Reichard, 1955). The compounds were detected by examination of the paper in the dark under an ultraviolet lamp. The R_F values for FU and FUDR were 0.20 and 0.15, respectively. After elution for 18 hr with 0.1 N HCl at 37 C, both compounds were estimated by measurement of absorbancies (Beckman DU spectrophotometer) of the solutions in 0.1 N hydrochloric acid at the maxima, 266 and 269 mμ (millimolar extinction coefficients 7.6 and 9.0) for FU and FUDR, respectively. The ratio OD_{230}:OD_{260} was used as

a criterion of purity (0.71 and 0.89 for FU and FUDR, respectively). Determinations after separation agreed with estimations for the mixtures described previously.

Source of chemicals. FU and FUDR were supplied by H. Bond of the Cancer Chemotherapy National Service Center, Bethesda, Md. 5-Fluorouracil-2-C^{14} was purchased from the California Corporation for Biochemical Research, Los Angeles. Orotidylic acid was a gift from R. E. Handschumacher, Department of Pharmacology, Yale University, New Haven, Conn. Deoxyuridylic acid was prepared from deoxycytidylic acid by the method of Flaks and Cohen (1959). A solution of polyglucose-C^{14} was a gift from W. C. Werkheiser of this department. Other materials were commercial samples.

RESULTS

Sensitivity to FU and FUDR. In the basal medium containing leucovorin, growth of *P. cerevisiae* 8081 was inhibited 50% by FU (5×10^{-8} M) and by FUDR (10^{-9} M), after incubation for about 20 hr. The concentration of either drug needed for inhibiton increased about threefold, when incubation was prolonged for 3 days. When the inoculum was increased 1,000-fold, sensitivity to FUDR decreased 30-fold but the sensitivity to FU decreased only 3-fold.

Addition of thymidine to the basal medium containing leucovorin increased by 400-fold the

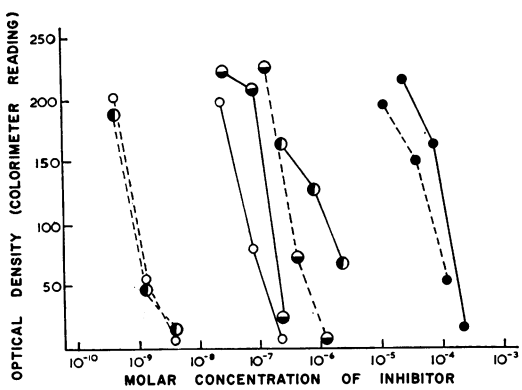

FIG. 2. *Effects of uracil and thymidine on sensitivity of Pediococcus cerevisiae 8081 fluorouracil (FU) and fluorodeoxyuridine (FUDR) after 20 hr of incubation.* ○, *no addition to basal medium;* ◐, *uracil added to basal medium;* ◒, *thymidine added to basal medium;* ●, *uracil and thymidine added to basal medium; solid line, FU; dashed line,* FUDR.

TABLE 1. *Effects of pyrimidines and derivatives on sensitivity of Pediococcus cerevisiae 8081 to fluorouracil (FU) and fluorodeoxyuridine (FUDR)*

Addition (20 μg/ml) to basal medium containing leucovorin	Concn (M) of drug permitting 50% of maximal growth (20 hr of incubation)*	
	FU	FUDR
No addition	4×10^{-8}	8×10^{-10}
Orotic acid	4×10^{-8}	
Orotidylic acid	4×10^{-8}	
Uracil	8×10^{-7}	3×10^{-9}
Uridine	8×10^{-7}	2×10^{-9}
Deoxyuridine	2×10^{-6}	8×10^{-8}
Uridylic acid	4×10^{-7}	
Deoxyuridylic acid	4×10^{-7}	8×10^{-9}
Thymine	4×10^{-8}	
Thymidine	2×10^{-7}	3×10^{-7}
Cytosine	8×10^{-8}	
Cytidine	4×10^{-7}	3×10^{-9}
Uracil + thymidine	8×10^{-5}	4×10^{-5}
Uracil + uridine	2×10^{-6}	
Uracil + deoxyuridine	3×10^{-6}	8×10^{-8}
Uracil + thymine	8×10^{-7}	10^{-9}
Uridine + deoxyuridine	2×10^{-6}	4×10^{-8}
Thymidine + uridine	3×10^{-4}	3×10^{-4}
Thymidine + deoxyuridine	5×10^{-6}	8×10^{-6}
Thymidine + uridylic acid	4×10^{-6}	
Thymidine + deoxyuridylic acid	4×10^{-7}	
Thymidine + orotic acid	2×10^{-7}	
Thymidine + orotidylic acid	2×10^{-7}	
Uracil + uridine + deoxyuridine	2×10^{-6}	4×10^{-8}
Uracil + uridine + thymidine	8×10^{-4}	$>4 \times 10^{-4}$
Uracil + deoxyuridine + thymidine	2×10^{-4}	$>4 \times 10^{-4}$

* Sensitivity to FU or FUDR was determined (see Materials and Methods) in medium to which the various pyrimidine derivatives were added before autoclaving. Although the growth response was measured after incubation for 20 hr, the sensitivity to FU and FUDR did not decrease markedly upon longer incubation.

TABLE 2. *Sensitivity of Pediococcus cerevisiae 8081 and resistant substrains to fluorouracil (FU) and fluorodeoxyuridine (FUDR) in the presence of various additions to the basal medium*

Organism (and addition to basal medium in development of resistance)	Concn of drug (M) for 50% inhibition of growth in basal medium plus:											
	Leucovorin		Leucovorin and uracil		Leucovorin and thymidine		Thymidine*		Leucovorin and uridine	Leucovorin and deoxyuridine	Leucovorin and deoxyuridylic acid	Leucovorin and thymine
	FU	FUDR	FU	FUDR	FU	FUDR	FU	FUDR	FU	FU	FU	FU
P. cerevisiae 8081	4×10^{-8}	8×10^{-10}	8×10^{-7}	3×10^{-9}	2×10^{-7}	3×10^{-7}	2×10^{-6}	—	8×10^{-7}	2×10^{-6}	4×10^{-7}	4×10^{-8}
Substrain 1 (leucovorin† + uracil)	8×10^{-8}	—	8×10^{-4}	10^{-6}	8×10^{-8}	—	NG‡	NG	8×10^{-4}	8×10^{-5}	8×10^{-7}	8×10^{-8}
Substrain 2 (leucovorin + thymidine)	8×10^{-8}	—	2×10^{-6}	—	8×10^{-4}	4×10^{-4}	8×10^{-4}	—	8×10^{-8}	8×10^{-8}	8×10^{-8}	8×10^{-8}
Substrain 3 (thymidine)	8×10^{-8}	—	4×10^{-6}	—	8×10^{-4}	—	8×10^{-4}	4×10^{-4}	8×10^{-7}	8×10^{-7}	—	4×10^{-7}
Substrain 4 (leucovorin)	8×10^{-4}	8×10^{-7}	8×10^{-4}	4×10^{-7}	8×10^{-4}	4×10^{-4}	8×10^{-4}	—	—	—	—	—
Substrain 5 (leucovorin + uracil)	4×10^{-7}	4×10^{-7}	8×10^{-4}	4×10^{-4}	8×10^{-7}	4×10^{-7}	NG	NG	4×10^{-6}	4×10^{-5}	—	4×10^{-6}

* Incubated for 40 hr instead of 20 hr.

† Leucovorin, 0.4 mμg/ml; all other additions, 20 μg/ml.

‡ Did not grow in absence of leucovorin.

concentration of FUDR needed for inhibition, but thymidine had very little effect in overcoming the inhibition due to FU (Fig. 2). When uracil was substituted for thymidine, the concentration of FU needed to cause inhibition was increased 20-fold, but there was very slight effect on inhibition caused by FUDR. When thymidine and uracil were both present, growth occurred in the presence of high levels of either drug (10^{-4} M). Tests were made of the ability of various pyrimidine derivatives to reproduce or to augment these effects of uracil and thymidine (Table 1). Only deoxyuridine was more effective than uracil in overcoming inhibition due to FU, and only deoxyuridine approached the effectiveness of thymidine in overcoming inhibition by FUDR. Uridine plus thymidine was the only binary combination more effective than uracil plus thymidine. In the presence of uracil, uridine, and thymidine, the growth of *P. cerevisiae* 8081 became almost insensitive to FU or FUDR.

Development of resistance to FU and FUDR. Organisms were grown in a series of tubes containing medium and inhibitor. The concentration of inhibitor was trebled at each step in the series. Organisms from the highest level of drug at which growth occurred after 2 to 3 days were used to inoculate a fresh series of tubes. Repetition of this procedure led to a gradual increase of resistance. Resistance to FU was developed by serial passage in four different media (Table 2). Substrain 1 (1,000-fold resistant) was developed in the basal medium containing leucovorin with uracil added; substrain 2 (4,000-fold resistant) was developed in the presence of leucovorin and thymidine; substrain 3 (400-fold resistant) was developed in basal medium containing thymidine, but without added leucovorin; and substrain 4 (20,000-fold resistant) was developed in basal medium containing leucovorin with no other addition. One substrain (5) resistant to FUDR (100,000-fold) was developed by serial passage of the parent organism, in the presence of increasing concentrations of this drug, in basal medium containing leucovorin and uracil. About ten passages at intervals of 2 to 3 days were required in each medium to develop substrains able to grow in the presence of about 5×10^{-4} M drug. In the case of substrain 4, it was necessary to increase the size of the inoculum from 2×10^5 to 2×10^6 organisms per 5 ml of medium in the earlier passages to obtain increased resistance.

Properties of resistant strains. All of the substrains were able to grow in the basal medium in the absence of drug with only leucovorin added; the growth response to leucovorin of the resistant substrains and of the parent strain was over the same range of concentration (0.01 to 0.4 mμg/ml). Substrains 1 and 5, however, were unable to grow in the basal medium when thymidine replaced leucovorin. The parent strain and all of the other substrains could grow, after a lag of about 40 hr, when leucovorin was replaced in the basal medium by thymidine, although the final level of growth (colorimeter reading 80) in the presence of thymidine was lower than in the presence of leucovorin (colorimeter reading 250). This relatively long lag and low level of growth were still shown when organisms were transferred several times into fresh medium containing thymidine without leucovorin. Hence, the growth on thymidine is probably not due to the selection of mutants, but rather to a slow adaptation to use of thymidine as a growth factor.

The resistant strains were able to grow in high concentrations of drug only in the presence of the same additional nutrients that supplemented the basal medium during the development of resistance (Table 2). Thus, substrains 1 and 5 were resistant to FU only if uracil was present; substrains 2 and 3 were resistant only in the presence of thymidine; whereas substrain 4 was resistant in the absence of uracil and thymidine. When incubation in the basal medium plus leucovorin was continued beyond the usual period of 24 hr to 40 to 45 hr, substrains 1 and 5 showed intermediate levels of resistance to FU. and substrains 2 and 3 were able to grow in the highest levels of FU.

When tested in the same medium used for the development of resistant substrains, 2 and 3 were highly cross-resistant to FUDR, whereas substrains 1 and 4 had only slight cross resistance (Table 2). With longer periods of incubation, substrains 1 and 4 were able to grow at increased concentrations of FUDR, but resistance did not reach a very high level. Substrain 4 became highly resistant to FUDR if thymidine was added to the basal medium. Substrain 5 was cross-resistant to FU when uracil was present in the medium.

Uptake of fluorouracil-2-C^{14} from the basal medium by washed suspensions of organisms. The amounts of FU taken up by the various strains of *P. cerevisiae* were compared in the absence and in the presence of uracil and thymidine, singly or together (Table 3). The parent strain took up

more FU from the basal medium than did any of the resistant strains; in substrain 2, no uptake of FU was detected. With the parent strain and with substrain 1, the addition of uracil diminished the uptake of drug; in other strains it had a less marked effect. The addition of thymidine generally had only a slight effect. The uptake of FU per mg (dry wt) of organisms did not change appreciably over a range of FU concentrations in the incubation mixture from 25 to 200 μg per ml. The uptake of FU increased with the time of incubation.

Metabolism of FUDR. Washed suspensions of *P. cerevisiae* 8081 were incubated in the basal medium with FUDR (100 μg/ml) to measure the uptake of the drug. When organisms were centrifuged and the supernatant fluid was examined by chromatography, ultraviolet-absorbing spots corresponding to FU, as well as to FUDR, were found on the paper. The presumed FU had the same OD_{280}:OD_{260} ratio (0.68) as authentic FU. It was inhibitory to *P. cerevisiae* 8081 over the same range of concentrations as was FU, and this inhibition was partly overcome by the addition of uracil to the medium. Washed organisms, incubated in the buffer solution for 1 hr, also hydrolyzed FUDR to FU (Table 4). Since no other ultraviolet-absorbing material was present in the buffer solution, or formed during the incubation, both drugs could be estimated by direct measurement of absorbancy after removal of the cells.

Although the parent strain was the most sensitive to FUDR in growth tests, washed cell suspensions of this strain hydrolyzed FUDR to FU more rapidly than did any of the resistant substrains (Table 4). The two substrains developed in the presence of uracil were considerably less active with respect to such degradation than were any of the other strains.

TABLE 3. *Uptake of fluorouracil (FU)-2-C^{14} from the basal medium by Pediococcus cerevisiae 8081 and resistant substrains*

Organism*	Uptake of FU-2-C^{14} (μg/mg dry wt)			
	No addition	+ Uracil (20 μg/ml)	+ Thymidine (20 μg/ml)	+ Uracil and thymidine
P. cerevisiae 8081	6.9	2.3	7.8	3.7
Substrain 1	4.1	1.1	2.6	1.7
Substrain 2	<0.1	0.6	0.5	0.1
Substrain 3	0.5	0.6	0.4	0.1
Substrain 4	0.8	0.1	<0.1	0.9
Substrain 5	1.1	1.0	1.1	1.1

* Organisms were incubated 1 hr at 37 C in the basal medium containing FU-2-C^{14} (100 μg/ml).

TABLE 4. *Conversion of fluorodeoxyuridine (FUDR) to fluorouracil (FU) by Pediococcus cerevisiae 8081 and substrains*

Strain	Cells (dry wt)	Drug remaining after incubation*	
		FUDR	FU
	mg	*μmoles*	*μmoles*
8081	111	0	3.8
	93	0	3.8
	56	0.7	3.2
	116†	0†	4.2†
Substrain 1	56	2.9	0.9
Substrain 2	92	0.3	3.4
Substrain 3	105	0.4	3.2
Substrain 4	92	0.2	3.8
Substrain 5	80	2.6	1.3

* Cells were incubated in 0.01 M phosphate buffer (pH 6.5) for 1 hr at 37 C with 4.0 μmoles of FUDR.

† Cells incubated in basal medium.

Discussion

The nutrients that are related to metabolic pathways inhibited by FU or its deoxyriboside can influence the characteristics of resistant strains of *P. cerevisiae* 8081 selected in the presence of these metabolites. Both uracil and thymidine must be added to the basal medium to permit growth of the sensitive organism in the presence of high concentrations of either FU or FUDR. Thus, the action of each drug appears to involve at least two separate sites. At one locus, inhibition is overcome by the addition of uracil; at the other, by the addition of thymidine. When organisms become resistant to FU in the absence of uracil and thymidine (substrain 4), mechanisms must be developed to circumvent two blocked reactions or two different functions. In the presence of uracil (substrain 1), only the block in the synthesis of thymidylic acid has to be overcome; in the presence of thymidine (substrains 2 and 3), only the impairment of uracil metabolism needs to be circumvented. In fact, substrain 1 is sensitive to FU if uracil is omitted, and substrains 2 and 3 are sensitive to FU if thymidine is omitted.

Not only do uracil and thymidine render organisms less sensitive to FU, but their presence

may also lead to the selection of substrains of higher levels of resistance. This is in accord with the postulate of Sevag (1946) that organisms may more readily become tolerant of inhibitors if the environment contains substances that can be used for alternative metabolic pathways to bypass the blocked reaction. Several observations are relevant. Kohn and Harris (1942) described a strain of *E. coli* that developed a requirement for methionine during the development of sulfonamide resistance in medium that contained methionine. Harrison and Clapper (1950) produced a strain of *Streptococcus mitis* which used pteroylglutamic acid as a growth factor, by developing sulfonamide resistance in medium containing pteroylglutamic acid. In two instances, a requirement for thymine occurred in strains of *Lactobacillus casei* selected for resistance to two different folic acid antagonists, pyrimethamine (Singer, Elion, and Hitchings, 1958) or amethopterin (Anton and Nichol, 1959). In each of these cases, the new requirement was shown in the absence of drug. With the resistant strains of *P. cerevisiae*, however, uracil or thymidine was required only to permit growth in the presence of high concentrations of inhibitor. Thus, the presence in the medium of metabolites, which can decrease the sensitivity to a growth inhibitor, can condition the selection of resistant substrains with partial or complete requirements for such compounds.

The enzymatic conversion of deoxyuridylic acid to thymidylic acid (thymidylate synthetase) is inhibited by FU, and more powerfully by FUDR, after the administered drug has been converted to fluorodeoxyuridylic acid (Danneberg, Montag, and Heidelberger, 1958; Bosch, Harbers, and Heidelberger, 1958). Thymidine most probably acts by overcoming the inhibition of thymidylate synthetase caused by FU or FUDR. The locus at which uracil acts in preventing inhibition is less definite. FU can be incorporated into the ribonucleic acids (Gordon and Stachelin, 1958; Chaudhuri, Montag, and Heidelberger, 1958; Horowitz and Chargaff, 1959) or into uridine coenzymes (Rogers and Perkins, 1960), and uracil may prevent these incorporations by effective substrate competition. The requirement for uracil to overcome inhibition by FUDR is probably due to conversion of FUDR to FU, a reaction that is carried out rapidly by washed suspensions of these organisms. Furthermore, in the presence of thymidine, approximately equimolar concentrations of FU and FUDR are needed to inhibit growth, suggesting that, when inhibition of thymidylate synthetase is circumvented, organisms can grow in the presence of FUDR until its degradation produces an inhibitory concentration of FU. The decreasing sensitivity to FUDR as the size of the inoculum is increased may be related to the conversion of the drug to a less potent inhibitor. The substrains that are resistant to FU in the presence of thymidine are cross-resistant to FUDR, and substrain 5, developed in the presence of FUDR and uracil, is cross-resistant to FU. Both of these observations are consistent with the degradation of FUDR to FU by the organisms. Substrains 1 and 4, although resistant to FU in the absence of thymidine, are not cross-resistant to FUDR. Possibly these two substrains do not convert FU to deoxyribose derivatives.

Even in the absence of uracil or thymidine, all of the resistant substrains took up less fluorouracil-2-C^{14} than did the parent strain. This decreased uptake may be the result of a decreased permeability of the organisms to the drug, or it may be due to a diminished incorporation of FU into ribonucleic acid (RNA) or uridine coenzymes. Resistance of *S. faecalis* to another uracil analogue, 6-azauracil, was associated with reduced uptake of both uracil and the drug. Also, uridine, but not uracil, was readily incorporated into RNA of the resistant cells (Handschumacher, 1957). The inability of substrains 1 and 5 to use thymidine in place of leucovorin suggests that the loss of enzymes concerned in pyrimidine metabolism may have a part in the resistance of these strains of *P. cerevisiae*. A recent report by Bloch and Hutchison (1962) indicates that thymine-2-C^{14} was incorporated into the deoxyribonucleic acid (DNA) of a FU-sensitive strain of *S. faecalis*, but not into the DNA of a FU-resistant substrain. Any decrease in the ability of the resistant organisms to use exogenous pyrimidines must be compensated for by accelerated *de novo* synthesis. Better understanding of the altered metabolism associated with resistance may indicate means of reducing the alternative ways by which the lethal effect of the drug can be evaded.

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