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Metabolism of Dihydrouracil in *Rhodosporidium toruloides*

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Previous studies, including those done with a similar species, have indicated that dihydrouracil is formed by the breakdown of uracil and is degraded into *N*-carbamyl-beta-alanine. (Fink et al., J. Biol. Chem. **201**:349–355, 1953; S. R. Vilks and M. Y. Vitols, Mikrobiologiya **42**:567–583, 1973; O. A. Milstein and M. L. Bekker, J. Bacteriol. **127**:1–6, 1976). In the present work the conversion of dihydrouracil to uracil is studied in *Rhodosporidium toruloides*, and the growth characteristics of mutants that have lost the ability to use dihydrouracil as a source of nitrogen are examined. It is concluded that dihydrouracil must be converted to uracil before catabolism of the pyrimidine ring can take place.

Studies conducted with *Rhodotorula glutinis* by Vilks and Vitols (22) and by Milstein and Bekker (14) suggested that pyrimidine-ring catabolism proceeds via the following steps: uracil → dihydrouracil → *N*-carbamyl-beta-alanine → NH_4 + beta-alanine. This catabolic route is known as the reductive pathway (5). Vilks and Vitol's implication of the reductive pathway in *R. glutinis* was based on the detection of small amounts of dihydrouracil and *N*-carbamyl-beta-alanine in the medium after supplementation with uracil. Milstein and Bekker (14), also working with a strain designated as *R. glutinis*, reported the induction of dihydrouracil dehydrogenase by uracil and concluded that the reduction of uracil to dihydrouracil was the first step in the catabolism of uracil. Thus, these previous conclusions are in agreement with many findings of the reductive catabolic pathway in a wide variety of organisms (3, 7, 9, 10, 15, 21, 23, 24, 26).

The reductive pathway does not appear to be universal, however. A number of other pathways for pyrimidine-ring catabolism have been suggested for various organisms (see reference 18). Among these only the oxidative pathway, uracil → barbituric acid → urea + malonic acid → NH_4 + CO_2, has been substantiated by independent investigations. This catabolic route seems to be limited to a few genera of procaryotes (8, 9, 11), yet labeled urea is produced from [2-^{14}C]uracil or thymine in both mammalian and fungal systems (2, 4, 16, 17, 20). The mechanism for these conversions has not been established.

The studies reported here were motivated by our observation that all mutants of *Rhodosporidium* sp. selected for an inability to utilize uracil as a sole source of nitrogen were also unable to use dihydrouracil. This observation suggested that dihydrouracil acted as a precursor of uracil and predicted a class of mutants that would be able to use uracil but not dihydrouracil as a nitrogen source. This paper reports the selection of the predicted class of mutants and studies of the wild-type enzymatic activity responsible for the conversion of dihydrouracil to uracil.

Wild-type *Rhodosporidium toruloides*, ATCC 10788, was from the American Type Culture Collection, Rockville, Md. *Rhodosporidium* is the name assigned to *Rhodotorula*-like strains known to have a sexual cycle (1). [2-^{14}C]dihydrouracil was synthesized by condensing [^{14}C]potassium cyanate (New England Nuclear Corp., Boston, Mass.) with a 10-fold molar excess of beta-alanine followed by acidification with HCl and purification by paper chromatography. The procedure was based on that of Fritzson (6). [2-^{14}C]uracil was also from New England Nuclear Corp.

Media, growth tests, etc., used with *R. toruloides* have been described previously (20). Uracil was routinely used as a nitrogen source except as otherwise indicated. Mutagenesis was with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (5 mg/50 ml of culture medium) for ca. 20 min, giving 20 to 50% survival. From each treatment one strain failing to grow with dihydrouracil as a nitrogen source, but growing with uracil, was selected by replica plating (13, 19). Such strains were designated as DHU mutants.

Supplementation of whole cells with [2-^{14}C]dihydrouracil, the determination of $^{14}CO_2$ release, and the detection and identification of other radioactive products of metabolism have been described previously (20). Quantification of chromatogram radioactivity was determined with a Technical Associates radiochromatogram scanner. The scanner was programmed to count strips at 5-mm intervals for periods of 100 s.

Cell-free extracts were prepared from 1-liter cultures grown with agitation for 2 days at 25°C. After being harvested by centrifugation (4,000 × *g* for 1 min), the cells were washed with water and recentrifuged (8,000 × *g* for 10 min). The cells were suspended (1 ml of buffer per 1 g of cells) in 0.1 M sodium phosphate buffer (pH 7.3). The suspension was sonicated at 0 to 5°C. The disrupted cells were centrifuged (30,000 × *g* for 20 min). The supernatant could be frozen with a 35% loss of protein, but no detectable loss of enzyme activity. Protein determination was done with Coomassie brilliant blue (12).

The spectrophotometric assay followed the formation of uracil at 260 nm. The test cuvette contained 50 μl of cell extract and 2.45 ml of 0.1 M sodium phosphate buffer (pH 7.3) with 2.5 μmol of dihydrouracil. Dihydrouracil was omitted from the reference cell. Rates were determined from the initial slope. Anaerobic assays were prepared from solutions that were frozen and subjected to vacuum during thawing. The solutions were added to air-tight cuvettes under an argon atmosphere, and the reactions were started with the injection of dihydrouracil through the cap. Assays with labeled dihydrouracil were essentially the same, except that the reaction was stopped with ethanol and the resulting supernatant was subjected to chromatography before its radioactivity was counted by liquid scintillation. To check

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TABLE 1. Growth of wild-type and mutant strains of *R. toruloides* on various sources of nitrogen[a]

Nitrogen source	Cells per ml ($\times 10^6$)				
	Wild type (ATCC 10788)	DHU-1	DHU-2	DHU-3	DHU-4
Dihydrouracil	625	11	1	2	4
Uracil	768	852	477	537	680
Thymidine	169	92	158	80	196
Thymine	1,356	600	325	205	440
5-Hydroxymethyl-uracil	426	166	285	235	250
Deoxyuridine	130	103	181	147	100
Uridine	360	260	260	280	280
Urea	2,530	1,200	755	910	800
Ammonium sulfate	906	637	680	417	423
None	1	1	1	1	1

[a] Cells were grown for 5 days at 26°C on a rotary shaker from an inoculum of 10^4 cells per ml and were counted with a hemacytometer. Nitrogen sources were added to give a concentration of 1 g/liter.

for the reverse reaction, [2-^{14}C]uracil was used as a substrate.

Table 1 reports cell concentrations reached when various strains were grown with different sources of nitrogen. The principal difference between the wild-type strain and the DHU mutants derived from it is in the inability of the mutant strains to respond to dihydrouracil. Mutants DHU-2, -3, and -4 appear to be absolute mutants, whereas DHU-1 is somewhat leaky. Pyrimidine catabolism is apparently unimpaired in the DHU mutants.

The nutritional results suggest that either the DHU mutants are deficient in the ability to transport dihydrouracil across the cell membrane or they are deficient in the conversion of dihydrouracil to a catabolizable pyrimidine, presumably uracil. To test these ideas, we added labeled dihydrouracil to intact cells of each strain. Table 2 reports the production of $^{14}CO_2$, and Fig. 1 shows the production of other metabolites. With wild type and the leaky mutant DHU-1, there was a rapid conversion of the dihydrouracil substrate to uracil and uridylic acid. These strains also showed a substantial production of labeled CO_2 from the dihydrouracil. On the other hand, the absolute mutants DHU-2, -3, and -4 showed a much slower conversion of the dihydrouracil to other products, and neither the uracil nor the uridylic acid peaks of radioactivity could be discerned. None of the absolute mutants was efficient in the release of labeled CO_2 from dihydrouracil. As an assay for the uracil catabolism in each of the strains, [2-^{14}C]uracil was added to whole cells and the production of $^{14}CO_2$ was observed (Table 2). With uracil all strains gave similar values.

Using dihydrouracil as a substrate with cell extracts prepared from the wild-type strain, we were able to detect the apparent production of uracil by an increase in optical density at 260 nm that was dependent on both enzyme and dihydrouracil. The resulting reaction mixture was subsequently chromatographed, and the UV-absorbing product was seen to have the same R_f as uracil with two solvent systems. The assay was repeated with labeled dihydrouracil, and the resulting product was found to cochromatograph with authentic uracil on both solvents. Using the spectrophotometric assay, we were able to determine that the activity is oxygen dependent, is unaffected by dialysis, and thus apparently requires no cofactors (Table 3). Preliminary attempts to partially purify the enzyme in a manner similar to that reported by Wondrack et al. (25) working with thymine 7-hydroxylase in *R. glutinis* also failed to show any cofactor requirement. When ammonium sulfate was used as a nitrogen source, no activity of the enzyme could be detected. No activity could be detected with extracts prepared from mutants DHU-2, -3, and -4 grown with uracil as an N source, whereas DHU-1, the leaky mutant, was found to have an activity similar to that of wild type.

These results are significant from at least two aspects: the novel enzymatic mechanism for converting dihydrouracil to uracil and the implication that dihydrouracil can act as a precursor of uracil in intact cells.

The characterization of the wild-type enzyme responsible for the oxidation of dihydrouracil to uracil is very preliminary. Nevertheless, it is clear that the activity is oxygen dependent, that the enzyme is soluble, that is has no cofactor requirements, and that it catalyzes an irreversible reaction. These observations suggest that the enzyme is an oxidase

TABLE 2. Production of $^{14}CO_2$ by wild-type and mutant strains of *R. toruloides* supplemented with [2-^{14}C]uracil or [2-^{14}C]dihydrouracil[a]

Strain	$^{14}CO_2$ production with:[b]	
	[2-^{14}C]uracil	[2-^{14}C]dihydrouracil
Wild type (ATCC 10788)	100	100
DHU-1	82	78
DHU-2	89	2
DHU-3	101	2
DHU-4	185	2

[a] Cells were grown with uracil as the nitrogen source. Labled uracil or dihydrouracil was added to the cells resuspended in nitrogen-free medium. The $^{14}CO_2$ produced was trapped and counted by liquid scintillation.

[b] Results are shown as percentage of wild-type control.

TABLE 3. Spectrophotometric assay of uracil production from dihydrouracil in cell extracts of *R. toruloides*[a]

Strain	N source during growth	Assay conditions	Sp act (nmol/min per mg)
Wild type (ATCC 10788)	Ammonium sulfate	Standard	0.0
	Uracil	Standard	11.3[b]
	Uracil	O_2 removed	0.0[b]
	Uracil	O_2 readmitted	7.3[b]
	Uracil	Extract dialyzed overnight	19.0
DHU-1	Uracil	Standard	11.0
DHU-2	Uracil	Standard	0.0
DHU-3	Uracil	Standard	0.0
DHU-4	Uracil	Standard	0.0

[a] Extracts were prepared as described in the text. Activities reported represent the initial slope of the 260-nm absorbtion curve. This slope decreases with time and accumulation of uracil. Activity appears to be a function of growth stage, but no activity has ever been observed in mutants DHU-2, -3, or -4.

[b] These values were obtained sequentially from a single reaction.

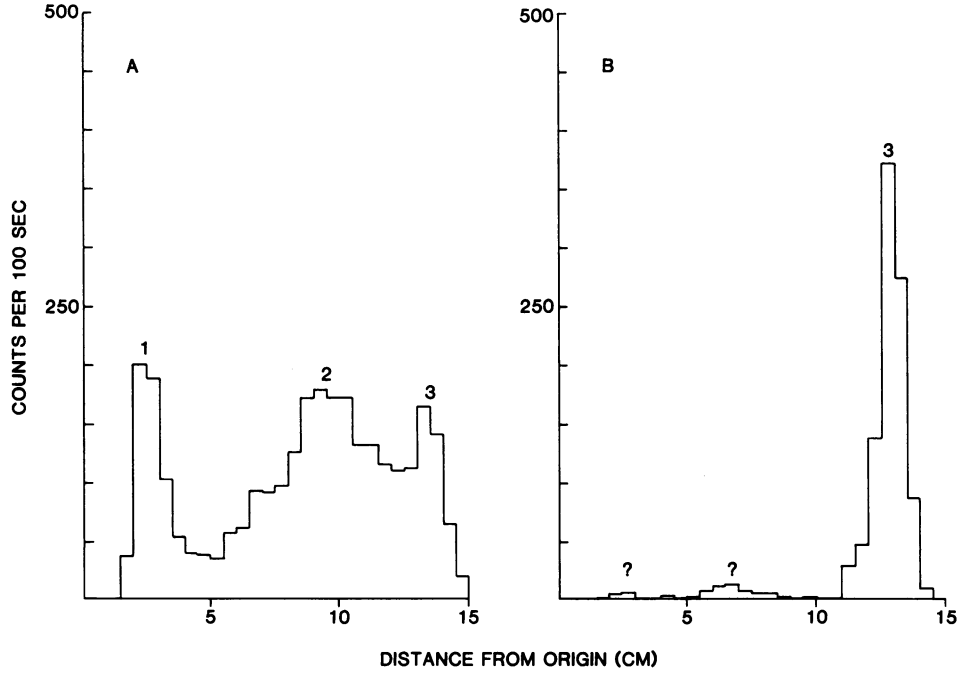

FIG. 1. Conversion of [2-^{14}C]dihydrouracil into uracil and uridylic acid. Supplementation of whole cells with [2-^{14}C]dihydrouracil. Cells were grown with uracil (1 g/liter) as a nitrogen source. Cells suspended (10^{10} cells per ml) in one-tenth strength nitrogen-free medium were supplemented with 0.05 mM (5 Ci/mol) [2-^{14}C]dihydrouracil. After 2 min of incubation, growth was stopped with ethanol. The supernatant was chromatographed and scanned for radioactivity. (A) Wild type (ATCC 10788). (B) Mutant DHU-2. 1, Uridylic acid; 2, uracil; 3, dihydrouracil; 7, unidentified. After 10 min, the mutant continued to show mostly dihydrouracil, whereas the wild type had only a small nucleotide peak remaining.

that has not been described previously. Thus, the enzyme seems to share properties with the biosynthetic form of dihydroorotate oxidase in *Lactobacillus bulgaricus* (18). With this similarity in mind, it has been suggested that the dihydrouracil oxidase activity we have observed is a secondary activity of a biosynthetic dihydroorotate oxidase in *R. toruloides*. This hypothesis seems untenable, however, since none of the four DHU mutants shows a requirement for pyrimidine supplementation.

The nutritional phenotype of the DHU mutants can be explained either through the loss of a specific transport system for dihydrouracil or through an inability to convert dihydrouracil to a catabolizable product. Mutant DHU-1 may be representative of the former category, whereas the absolute mutants DHU-2, -3, and -4 are clearly of the enzyme-deficient type. However, we cannot rule out the possibility that the transport of dihydrouracil is coincident with its oxidation to uracil.

Superficially, these results appear to conflict with those of Milstein and Bekker (14), who reported the induction of the first enzyme of the reductive pathway (dihydrouracil dehydrogenase) in response to uracil supplementation. Although we did employ almost identical conditions of growth and enzyme assay, it is possible that the reductive pathway activity was masked by the oxidase activity seen in our strain. Even if catabolism in *R. toruloides* is initiated by a reduction of uracil to dihydrouracil, previous results (20) have shown that the penultimate step is similar to that of the oxidative pathway. Thus, ambiguity exists regarding pyrimidine-ring catabolism in *R. toruloides*.

Our results show that the assimilation of dihydrouracil and its catabolism to CO_2 and nitrogen are occasioned by the oxygen-dependent conversion of dihydrouracil to uracil. This observation permits some preliminary speculation as to the physiological role of this step. Since the dihydrouracil oxidase activity is not found in cells grown with an ammonium sulfate nitrogen source, it seems possible that dihydrouracil fits into some type of nitrogen salvage system. On the other hand, we cannot draw any strong conclusion from the observation that uracil seems to act as an inducer of the oxidase activity. The uracil effect may be a relaxation of ammonium repression rather than a specific induction by uracil. Further studies will be needed before the actual function of this oxidation can be fully understood.

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