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Metabolism of Pyrimidine Deoxyribonucleosides in *Neurospora crassa*

PATRICIA M. SHAFFER,[1] CHIN-AN HSU, AND MITCHEL T. ABBOTT*
Department of Chemistry, San Diego State University, San Diego, California 92115

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The experiments in this report involve the following series of reactions which were previously demonstrated with purified enzyme preparations from *Neurospora crassa*: thymidine $\xrightarrow{a}$ thymine ribonucleoside $\xrightarrow{b}$ thymine $\xrightarrow{c}$ 5-hydroxymethyluracil $\xrightarrow{d}$ 5-formyluracil $\xrightarrow{e}$ uracil-5-carboxylic acid $\xrightarrow{f}$ uracil. The evidence for some of the reactions occurring in vivo has been incomplete and for others totally lacking. In this paper intact cells of *Neurospora* are shown to be capable of converting the substrates of each of the reactions to the corresponding products. Studies are described which were carried out in vivo and in vitro with the pyrimidineless strains *pyr-4,uc-1,uc-2* and *pyr-4,uc-1,uc-3*, developed by Williams and Mitchell. The results reported in the present paper indicate that the *uc-2* mutation affects the pyrimidine deoxyribonucleoside 2'-hydroxylase (reaction a) and the *uc-3* mutation affects thymine 7-hydroxylase (reactions c, d, and e). Evidence is presented for the 2'-hydroxylase reaction being the major, if not only, way by which *Neurospora* can initiate the conversion of thymidine to the pyrimidines of nucleic acids and for the 2'-hydroxylation of thymidine and deoxyuridine being catalyzed by the same enzyme. Deoxycytidine was shown not be be hydroxylated in intact cells but instead deaminated to deoxyuridine, which in turn was converted to uridine. Further studies with the *uc-3*-carrying strain showed that an enzyme other than thymine 7-hydroxylase can also convert 5-formyluracil to uracil-5-carboxylic acid.

Fink and Fink (Fed. Proc. **21**:377, 1962) showed that 5-hydroxymethyluracil and 5-formyluracil accumulated in the mycelia of *Neurospora crassa* during the conversion of thymidine to uracil and cytosine of ribonucleic acid in a process which does not involve fragmentation of the pyrimidine ring. They proposed a pathway for pyrimidine catabolism in which the methyl group of thymidine is removed in a series of oxidative steps (6). Subsequently, purified enzyme preparations were obtained from *Neurospora* which catalyzed the reactions shown in Fig 1 (for reviews see reference 3 and O. Hayaishi, M. Nozaki, and M. T. Abbott, *in* P. Boyer (ed.), The enzymes, in press). Further evidence for 5-hydroxymethyluracil and 5-formyluracil being intermediates in the pathway has come from studies by Williams and Mitchell (18) who have developed several mutants of the pyrimidineless strain *pyr-4*. The *pyr-4* strain can utilize uridine or cytidine as a sole pyrimidine source but not thymidine, thymine, 5-hydroxymethyluracil, or 5-formyluracil, although the latter compounds can be utilized if growth is initiated with uridine or cytidine (5, 18). The *pyr-4,uc-1* strain can utilize thymidine, thymine, 5-hydroxymethyluracil, or 5-formyluracil as a sole pyrimidine source, and Williams and Mitchell have suggested that the *uc-1* mutation affects a gene which controls the activity of one or more enzymes involved in the pathway. The *pyr-4,uc-1,uc-2* strain was thought (18) to be defective in a pyrimidine deoxynucleosidase since this strain could utilize thymine, 5-hydroxymethyluracil, or 5-formyluracil, but not thymidine, deoxyuridine, or deoxycytidine as a pyrimidine source. Moreover, it was suggested (16) that the pyrimidine deoxyribonucleoside 2'-hydroxylation step (Fig. 1, reaction a) or the subsequent hydrolysis step (reaction b) might instead be affected in the *uc-2*-carrying strain. The *uc-3* mutation was thought to affect the conversion of 5-hydroxymethyluracil to 5-formyluracil and possibly of thymine to 5-hydroxymethyluracil but not of 5-formyluracil to uracil-5-carboxylic acid, since the *pyr-4,uc-1,uc-3* strain could grow on medium supplemented with 5-formyluracil or uracil but not when the supplement was thymidine, thymine, or 5-hydroxymethyluracil (18). One aspect of this paper deals with the appar-

[1] Present address: Chemistry Department, University of San Diego, San Diego, Calif. 92110.

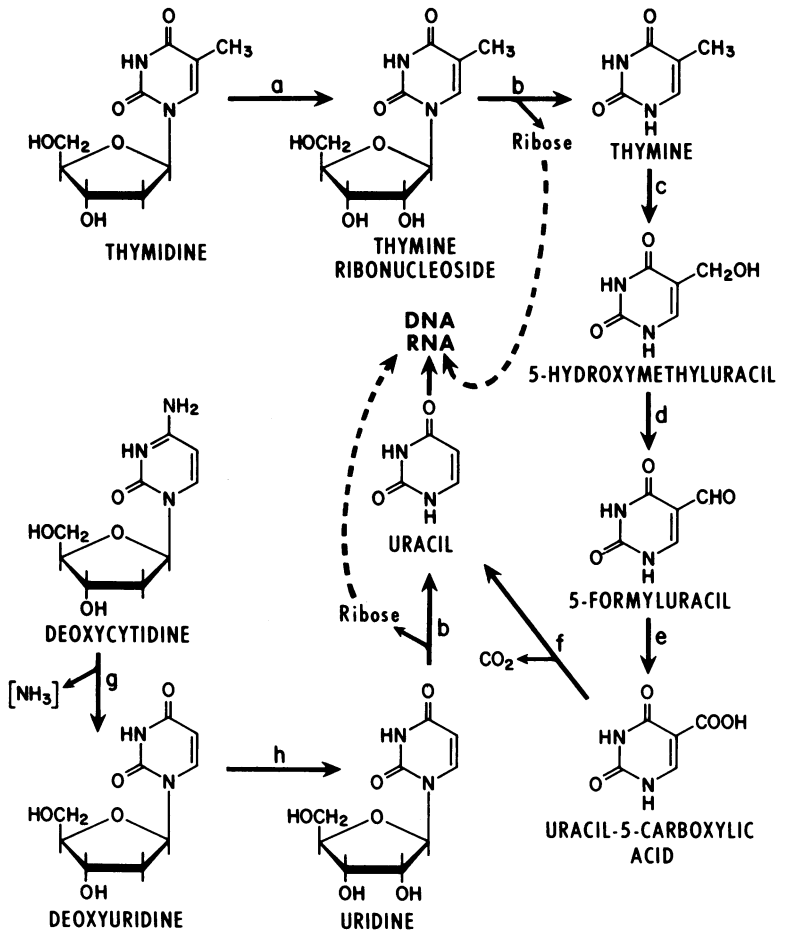

FIG. 1. *Pathway proposed for the utilization of deoxyribonucleosides by Neurospora crassa: a and h, pyrimidine deoxyribonucleoside 2-hydroxylase (15); b, hydrolase reaction (14); c, d, and e, thymine 7-hydroxylase (1, 2, 16); f, uracil-5-carboxylic acid decarboxylase (12); g, deaminase reaction (14).*

ent contradiction of these growth studies with the discovery that the same enzyme, thymine 7-hydroxylase, which catalyzes reactions c and d also catalyzes reaction e (Fig. 1) (9). That uracil-5-carboxylic acid is an intermediate in the pathway was originally suggested by Fink and Fink (Fed. Proc. **21**:377, 1962) when they observed that this compound stimulated the growth of the *pyr-4* strain. However, since Williams and Mitchell (18) were unable to develop a mutant which could effectively utilize or accumulate uracil-5-carboxylic acid, they suggested that uracil-5-carboxylic acid may not be an intermediate and that 5-formyluracil is directly converted to uracil. Evidence is presented in this report for the reactions per se depicted in Fig. 1 occurring in vivo and for the *uc-2* and *uc-3* mutations affecting pyrimidine deoxyribonucleoside 2'-hydroxylase (1.14.11.3) and thymine 7-hydroxylase (1.14.11.6), respectively.

MATERIALS AND METHODS

Strains. *N. crassa* wild-type strain (1A) and two pyrimidineless triple mutants, *pyr-4,uc-1,uc-2* and *pyr-4,uc-1,uc-3*, were used. The triple mutants were obtained from William M. Thwaites (San Diego State University).

Media and chemicals. The cell-free studies were carried out with extracts from *Neurospora* grown on Fries minimal medium (8), supplemented with uridine (0.2 g/liter) for mutants. Westergaard-Mitchell (17) medium was used for growth tests with mutants as described by Williams and Mitchell since it facilitates pyrimidine utilization (18). In experiments in which radioactive substrates were incubated with mycelial pad-sections, Fries medium (supplemented with uridine for mutants) was used in the studies of the 2'-hydroxylase (Fig. 1, reaction a) and hydrolase (reaction b). Repetition of these studies with *Neuro-*

spora grown on Westergaard-Mitchell medium yielded comparable results. However, in studies of the 7-hydroxylase (Fig. 1, reactions c, d, and e) and uracil-5-carboxylic acid decarboxylase (reaction f), the corresponding metabolic intermediates were more easily detected with mycelial pads grown on Westergaard-Mitchell medium supplemented with uridine; hence, this medium was used.

The sources of the radioactive compounds were previously described (10–12, 14, 16). The specific activities (curies per mole) of the pyrimidines and nucleosides used are as follows: 3.0 for [2-^{14}C]thymine ribonucleoside, [2-^{14}C]uridine, [2-^{14}C]thymine, 5-[2-^{14}C]hydroxymethyluracil, 5-[2-^{14}C]formyluracil, [2-^{14}C]uracil-5-carboxylic acid, and [2-^{14}C]deoxycytidine; 2.0 for [2-^{14}C]thymidine and [2-^{14}C]deoxyuridine; 4.0 for ^{14}C and 16.0 for ^{3}H in thymidine which contained ^{3}H in position 6 of the pyrimidine ring and which was uniformly labeled with ^{14}C. All other fine chemicals were obtained from commercial sources as previously described (10, 11).

Preparation of and assay procedures used with pad-sections of Neurospora. The previously unpublished procedures for preparation and assay were developed by R. M. Fink (also described by M. T. Abbott, Ph.D. thesis, University of California, Los Angeles, 1963). Pads of various strains of *Neurospora* were prepared by inoculating 50 ml of medium with a conidial suspension and allowing the culture to grow for 5 days at 28 C with occasional agitation to keep the pad submerged. Each pancake-like pad, along with its incubation medium, was poured out of its Erlenmeyer flask onto a Parafilm disk within a petri dish. After positioning the pad on the Parafilm, a second disk of the latter was added so that the *Neurospora* was sandwiched between the two. The sandwich was cut into pad-sections (7 by 16 mm), and the Parafilm coats were removed. The average dry weight of a pad-section was from 4 to 6 mg. To allow the cut edges to heal, each resultant pad-section was placed in a 5-ml beaker and 50 μliters of medium was added to the section to start a 90-min preincubation period. The incubation period began with the intimate mixing of a solution of 25 μliters, which contained the radioactive substrate, with the medium adhering to the pad-section. Both the preincubation and incubation were carried out in an atmosphere equilibrated with medium. The incubation was concluded after 3 h by adding 1 ml of 80% ethanol. Appropriate chromatographic marker compounds were added and five 1.5-ml portions of 80% ethanol were used to transfer this mixture containing pad-section and media to a chromatographic applicator tube, from which the ethanolic extracts were quantitatively siphoned onto chromatographic paper. Two-dimensional paper chromatography was carried out (4, 14) at 28 C with solvent systems which have been previously described. The separated pyrimidines and nucleosides on the developed chromatograms were outlined under ultraviolet light, and the alignment of radioactive compounds with marker compounds was checked with radioautography. (2). To make radioactivity measurements, portions of the chromatograms which contained the separated compounds were either centered under a thin-window Geiger tube or cut out, added to scintillation fluid and placed in a Packard Tri-Carb scintillation counter at 0 C with the filter paper lying flat on the bottom of the vial (14). Control incubations included those to which 80% ethanol or acetone was added at zero time and others in which the pad-sections were heated at 100 C for 5 min prior to incubation. Typical experiments are shown in Table 1. The standard deviations in this table were calculated from only two or three values, but they indicate the precision observed when pad-sections of a single experiment were compared. Although there was less agreement among values obtained from repetition of experiment, i.e., from pad-sections which were not incubated at the same time, from a qualitative standpoint very similar results were obtained. For example, the results of all experiments are in complete agreement about the following: when thymine or 5-hydroxymethyluracil was used as substrate with the *pyr-4,uc-1,uc-3* strain, no product was detected and all of the substrate was recovered; when a deoxyribonucleoside was used as substrate, more ribonucleoside and pyrimidine were trapped and more substrate was utilized in the wild-type and *pyr-4,uc-1,uc-3* strains than in the *pyr-4,uc-1,uc-2* strain.

In experiments 1, 2, and 3 of Table 1, nonradioactive pyrimidines and ribonucleosides (traps) were added to the pad-sections to slow down the utilization of the corresponding, radioactive transient intermediates. In the pad-section studies of the 2′-hydroxylase reactions (Fig. 1, reactions a and h), the 25-μliter portion of substrate solution which contained [2-^{14}C]thymidine also contained 4 mM thymine ribonucleoside and 2 mM thymine. The substrate solution which contained [2-^{14}C]deoxyuridine contained 20 mM uridine and 2 mM uracil. When [2-^{14}C]thymine ribonucleoside was used as substrate, the substrate solution contained 2 mM thymine. In experiments 4, 5, 6, and 7 of Table 1, no nonradioactive compounds were used as traps. In other experiments, it was shown that nonradioactive 5-hydroxymethyluracil and 5-formyluracil were ineffective in trapping intermediates of the thymine 7-hydroxylase reactions. Presumably this is because each of the pyrimidine substrates of this enzyme inhibits the utilization of the other two (9).

Cell-free preparations and enzymatic assays. The preparation of crude extracts (11) and the purification scheme to which they have been subjected (9, 10) have been previously described. Thymine 7-hydroxylase and pyrimidine deoxyribonucleoside 2′-hydroxylase are dioxygenases which catalyze the following type of reaction:

$$S + O_2 + \alpha\text{-ketoglutarate} \xrightarrow[\text{reducing agent}]{Fe^{2+}} SO + \text{succinate} + CO_2 \quad (1)$$

The standard incubation mixture which was used to assay for these enzymes consisted of 0.1 ml of enzyme preparation and 0.1 ml of 0.02 mM sodium phosphate buffer, pH 7.5, which contained subtrates and cofactors so that the final incubation mixture was 0.75 mM in radioactive pyrimidine or deoxyribonuceloside, 1 mM in α-ketoglutarate, 1 mM in ferrous sulfate, 1 mM in ascorbate, and 0.4 mg/ml in catalase. In the assay for the 7-hydroxylase, the radioactive pyrimi-

TABLE 1. *Detection of reactions in vivo*[a]

Expt	Substrate	Product	Amt (nmol)[b]		
			Wild type[c]	*pyr-4, uc-1 uc-3*[c]	*pyr-4, uc-1, uc-2*[c]
1	Thymidine		35.3 ± 1.3	34.4 ± 3.1	39.6 ± 1.3
		Thymine ribonucleoside	2.9 ± 0.2	3.2 ± 0.6	0.2 ± 0.0
		Thymine	1.4 ± 0.5	2.1 ± 0.4	0.2 ± 0.0
2	Deoxyuridine		33.0 ± 1.4	37.4 ± 0.0	39.6 ± 0.2
		Uridine	2.4 ± 0.8	2.2 ± 0.1	0.4 ± 0.1
		Uracil	0.2 ± 0.1	0.2 ± 0.1	0.0
3	Thymine ribonucleoside		0.3 ± 0.0	1.0 ± 0.1	0.3 ± 0.0
		Thymine	18.4 ± 0.1	19.0 ± 0.7	14.6 ± 2.3
		5-Hydroxymethyluracil	1.3 ± 0.5	0.0	0.9 ± 0.1
4	Thymine		9.8 ± 1.4	20.0 ± 1.1	17.5 ± 1.0
		5-Hydroxymethyluracil	1.6 ± 0.2	0.0	0.4 ± 0.1
		5-Formyluracil	0.0	0.0	0.0
		Uracil-5-carboxylic acid	0.9 ± 0.4	0.0	0.2 ± 0.0
		Uracil	1.1 ± 0.1	0.0	0.5 ± 0.1
5	5-Hydroxymethyluracil		12.4 ± 0.6	20.0 ± 0.2	16.4 ± 2.7
		5-Formyluracil	0.2 ± 0.1	0.0	0.1 ± 0.0
		Uracil-5-carboxylic acid	1.1 ± 0.2	0.0	0.1 ± 0.0
		Uracil	1.5 ± 0.3	0.0	0.2 ± 0.1
6	5-Formyluracil		5.1 ± 1.2	12.1 ± 1.2	10.0 ± 0.7
		Uracil-5-carboxylic acid	3.1 ± 0.3	1.1 ± 0.1	0.2 ± 0.1
		Uracil	2.6 ± 0.5	1.4 ± 0.1	0.3 ± 0.3
7	Uracil-5-carboxylic acid		8.2 ± 2.1	10.7 ± 2.8	9.6 ± 2.5
		Uracil	5.0 ± 0.4	3.4 ± 0.6	4.2 ± 0.5

[a] The values given in this table represent the amount of substrate left over and products produced in mycelial pad-sections after 3 h of incubation. Twenty nanomoles of substrate was added when it was [2-^{14}C]thymine ribonucleoside, [2-^{14}C]thymine, 5-[2-^{14}C]hydroxymethyluracil, 5-[2-^{14}C]formyluracil, and [2-^{14}C]uracil-5-carboxylic acid; 40 nmol was added when the substrate was either [2-^{14}C]thymidine or [2-^{14}C]deoxyuridine. The pad-sections were prepared and incubated as described in Materials and Methods.

[b] The mean and standard deviation of the values obtained from two pad-sections in experiments 1, 2, 3, and 7, and three pad-sections in experiments 4, 5, and 6.

[c] Strain of *N. crassa*.

dine used (S in equation 1) was [2-^{14}C]thymine. In the assay for the 2'-hydroxylase, either [2-^{14}C]thymidine or [2-^{14}C]deoxyuridine was used. The standard incubation mixture, with the appropriate radioactive pyrimidine, was also used to assay for reactions d and e, Fig. 1. The details of how the incubations were carried out, how the radioactive products of the incubation mixtures were chromatographically separated, and how the radioactivity measurements were made have been described (9, 10).

RESULTS

Detection of reactions in vivo. To determine if the reactions depicted in Fig. 1 occur in vivo, radioactive substrates of each of the reactions were incubated with mycelial pad-sections. The data, reported in Table 1, demonstrate that intact cells of the wild-type strain are capable of converting each substrate to the corresponding product. The inability of the *pyr-4,uc-1,uc-3* strain to utilize thymine or 5-hydroxymethyluracil as substrate is consistent with the *uc-3* mutation affecting reactions c and d (Fig. 1). The data in Table 1 also indicate that the *uc-2* mutation affects the conversions of thymidine and deoxyuridine to their respective ribonucleosides. In experiments 1 and 2, in which pad-sections of the wild-type and *pyr-4,uc-1,uc-3* strains were incubated in the presence of nonradioactive nucleoside and pyrimidine traps (see Materials and Methods), thymidine and deoxyuridine were utilized to only small extents, but this utilization of the deoxyribonucleosides was found to be several-fold higher in other experiments (not shown in Table 1) in which the pad-sections were incubated in the absence of the traps. However, the utilization of deoxyribonucleosides by pad-sections of the *uc-2*-carrying strain was never observed to exceed 6%, even when the incubations were carried out in the absence of such traps. Furthermore, the results from experiment 3 in Table 1 indicate that the *uc-2* mutation does not affect

the conversion of thymine ribonucleoside to thymine. This is consistent with growth studies which show that the *pyr-4,uc-1,uc-2* strain can grow on thymine ribonucleoside as a sole pyrimidine source. However, when radioactive deoxycytidine was tested as a substrate for the 2'-hydroxylase reaction with intact cells, no radioactive cytidine was detected even when nonradioactive cytidine was added as a trap to the incubation mixture (Table 2). Instead, deoxyuridine, uridine, and some uracil were formed, except with pad-sections of the *uc-2*-carrying strain in which case more deoxyuridine was formed but little, if any, uridine or uracil.

Direct conversion in vivo of thymidine to thymine ribonucleoside. To determine whether this conversion in whole cells involves free thymine as an intermediate, mycelial pad-sections of the wild-type strain, as well as of the two triple mutants, were incubated with [2-^{14}C]thymine in the presence and absence of nonradioactive thymine ribonucleoside. The formation of radioactive thymine ribonucleoside was not detected. Furthermore, in preliminary kinetic studies the free base could not be detected prior to the accumulation of the ribonucleoside when either radioactive thymidine or deoxyuridine were used as substrates with pad-sections of the wild-type and of the *pyr-4,uc-1,uc-3* strains.

Additional support for this direct transformation of deoxyribose to ribose at the nucleoside level was obtained when pad-sections were incubated with thymidine which was uniformly labeled with ^{14}C and enriched in position 6 of the pyrimidine moiety with respect to ^{3}H. Table 3 shows that the ratio of the specific activities of ^{3}H to ^{14}C was the same for substrate and product. Moreover, this ratio was not changed when incubations were carried out in the presence of nonradioactive, potential intermediates, such as thymine.

Cell-free studies of the effects of the uc-2 and uc-3 mutations. Table 4 shows that the 2'-hydroxylase activity is much reduced in crude extracts of the *uc-2*-carrying strain. The uncentrifuged, homogenized mycelial preparations, from which these extracts were obtained, gave similar results although the assays of these homogenates exhibited less precision than those of the crude extracts. The 2'-hydroxylase activity of the wild-type and *pyr-4,uc-1,uc-3* strains was shown to be relatively constant throughout the growth period by experiments which involved the preparation of extracts from mycelia after 1 to 5 days of growth. Comparable studies of the 7-hydroxylase have shown that this activity does vary during the growth period and is affected by the degree of aeration during growth (11), the composition of the growth medium, and the presence of the *uc-1* mutation (W. R. Griswold and M. T. Abbott, manuscript in preparation). However, under all conditions tested, the extracts prepared from the *uc-3*-carrying strain had no detectable 7-hydroxylase activity. When extracts of the wild-type strain and each of the two triple mutants were combined in various proportions and assayed, no inhibition could be detected, even when incubations were carried out with mixtures in which

TABLE 2. *Testing deoxycytidine as substrate for the pyrimidine deoxyribonucleoside 2'-hydroxylase reaction in vivo*

Substrate[a]	Product	Amt (nmol)[b]			
		Wild type[c]		*pyr-4, uc-1, uc-2*[c]	
		(+)[d]	(−)	(+)	(−)
Deoxycytidine		5.4	1.4	5.8	2.1
	Cytidine	0	0	0	0
	Deoxyuridine	7.6	10.3	14.1	16.4
	Uridine	0.6	0.2	0.1	0
	Uracil	0.8	0.1	0.1	0.4

[a] At the onset of the 3-h incubation, 20 nmol of [2-^{14}C]deoxycytidine was added to each mycelial pad-section.

[b] Each amount is an average of the values obtained from two to four pad-sections.

[c] Strain of *N. crassa*.

[d] The incubations were carried out in the presence (+) and absence (−) of 2 mM cytidine.

TABLE 3. *Retention of ^{3}H and ^{14}C in thymine ribonucleoside produced in vivo*

Substance	Sp act (Ci/mol)		^{3}H/^{14}C[a]
	^{3}H	^{14}C	
Thymidine[b]	15.9	4.0	4.0
Thymine ribonucleoside[b]	15.1	4.0	3.8
Thymidine[c]	16.0	4.0	4.0
Thymine[b]	17.6	2.0	8.8

[a] Thirty-two nanomoles of thymidine, uniformly labeled with ^{14}C and labeled with ^{3}H in the 6 position, was incubated for 3 h with mycelial pad-sections of the *pyr-4, uc-1, uc-3* strain as described in Material and Methods. The specific activities were determined as previously described (15).

[b] Isolated after the incubation. The amounts of thymidine, thymine ribonucleoside, and thymine were 20.8, 5.0 and 7.8 nmol, respec ively.

[c] Isolated at zero time.

TABLE 4. *Comparison of the amounts of pyrimidine deoxyribonucleoside 2'-hydroxylase and thymine 7-hydroxylase in crude extracts of several strains of Neurospora crassa*[a]

Enzyme	Substrate	Units/g (dry wt)[b]		
		Wild type[c]	*pyr-4, uc-1, uc-2*[c]	*pyr-4, uc-1, uc-3*[c]
2'-Hydroxylase	Deoxyuridine	151 ± 68 (11)	22 ± 15 (16)	237 ± 117 (8)
	Thymidine	134 ± 68 (7)	6.7 ± 0.3 (2)	360 (6)
7-Hydroxylase	Thymine	160 ± 77 (16)	368 ± 169 (6)	0 (8)

[a] Assays were carried out in standard incubation mixtures with radioactive pyrimidine and nucleoside substrates.

[b] The mean and standard deviation are given, with number of experiments in parentheses.

[c] Strain of *N. crassa*.

the extract from the mutant was present in an amount three times that of the wild-type strain. In Table 5 data are presented from a typical experiment of this type. Similar results were obtained with the homogenates from which the crude extracts were derived. When the crude extract prepared from the *pyr-4,uc-1,uc-2* strain was subjected to the purification scheme through the diethylaminoethyl-cellulose step as described by Liu et al. (10), so that the 7-hydroxylase activity was purified 70-fold, no evidence was obtained for removal of a component which was inhibiting the 2'-hydroxylase activity. Similar results were obtained with respect to the 7-hydroxylase activity when extracts of the *pyr-4,uc-1,uc-3* strain were subjected to the purification scheme through the ammonium sulfate step, as described by McCroskey et al. (11), yet the purified preparation contained the anticipated 2'-hydroxylase activity.

Hydroxylation of thymidine and deoxyuridine by a single enzyme. That a single enzyme catalyzes the hydroxylation of thymidine and deoxyuridine is suggested by the lower capacity of intact cells of the *uc-2*-carrying strain to hydroxylate either substrate (Table 1) and the corresponding reduced ability of extracts of these cells to catalyze the same reactions (Table 4). In addition, when the 2'-hydroxylase in several extracts from the wild-type strain was subjected to the purification scheme developed by Liu et al. (10) and assayed with both substrates, no separation of the activities was detected, and the ratio of specific activities appeared to be maintained. Likewise, this ratio was maintained during purification of the very low 2-hydroxylase activity of a single extract of the *uc-2*-carrying strain. In addition, preliminary kinetic studies carried out with purified 2'-hydroxylase fractions indicated that thymidine and deoxyuridine inhibit each other competitively.

TABLE 5. *Incubation of various combinations of extracts from Neurospora crassa wild type and the pyr-4, uc-1, uc-2 strains*

Expt	Vol of extracts[a] from strains (ml)		Activity of 2'-hydroxylase in incubation mixtures (units)	
	Wild type	*pyr-4, uc-1, uc-2*	Experimental[b]	Predicted[b]
1	0.10		0.74	
2	0.075	0.025	0.57	0.58
3	0.05	0.05	0.45	0.42
4	0.025	0.075	0.29	0.27
5		0.10	0.10	

[a] The 10,000 × *g* supernatant fraction was prepared from each strain as previously described (11). Incubations were carried out in the standard incubation mixture. It consisted of 0.1 ml of extract or combinations of extracts and 0.1 ml of the buffer that contained the cofactors and substrate.

[b] The units of activity that were experimentally determined are recorded under "Experimental." The values in the "Predicted" column were calculated from the "Experimental" values determined in experiments 1 and 5.

A second enzyme catalyzing the conversion of 5-formyluracil to uracil-5-carboxylic acid. When 5-formyluracil was used as substrate in the assay for 7-hydroxylase activity in crude preparations of the *pyr-4,uc-1,uc-3* strain, no activity was detected, but such negative results are unconvincing since 5-formyluracil is only about one-fifth as effective a substrate as is thymine for the 7-hydroxylase (9), and it had frequently been difficult to demonstrate this activity in similar preparations from the wild type. Therefore, extracts of the 7-hydroxylase from the *uc-3*-carrying strain were purified with a calcium phosphate gel fractionation procedure and concentrated with ammonium sulfate (11). These concentrates had easily demonstrable 2'-hydroxylase activity, but no activity was

detected when thymine, 5-hydroxymethyluracil, or 5-formyluracil was used as substrate. However, the conversion of 5-formyluracil to uracil-5-carboxylic acid could easily be demonstrated in crude extracts of the *uc-3*-carrying strain when oxidized nicotinamide adenine dinucleotide was substituted for the cofactors of the standard incubation mixture (Fig. 2). This suggests the possibility of two enzymes affecting this latter conversion, only one of which is active in the *uc-3*-carrying strain.

DISCUSSION

The utilization of each of the substrates and detection of the respective products (Table 1) of the reactions depicted in Fig. 1 are in accord with their occurring in vivo. The oxidative demethylation of thymine appears to occur primarily at the free base level since the same enzyme, thymine 7-hydrolyase, catalyzes reactions c, d, and e and the hydroxylation of neither thymidine nor thymine deoxyribonucleoside could be detected in vivo or in vitro (3). Since *Neurospora* lacks a thymidine phosphorylating enzyme (6, 7, 18), this pathway provides a means of salvaging the pyrimidine ring of thymidine, but a source of ribose may still be required in order for the uracil end product to be converted to the nucleotide precursors of ribonucleic acid and deoxyribonucleic acid. Thus, the 2'-hydroxylase (Fig. 1, reaction a) allows the deoxyribose moiety also to be salvaged.

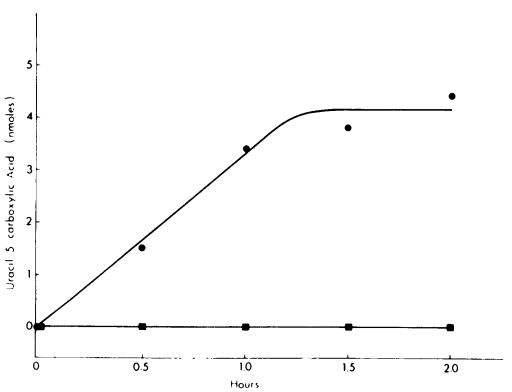

FIG. 2. *Time course of the oxidized nicotinamide adenine dinucleotide stimulated conversion of 5-formyluracil to uracil-5-carboxylic acid by extracts of the strain pyr-4,uc-1,uc-3. An extract (3.8 mg of protein per ml of incubation mixture) was incubated with 0.5 mM 5-[2-^{14}C]formyluracil in 0.02 mM sodium phosphate buffer (pH 7.5) with 1.0 mM NAD$^+$ (●) or with α-ketoglutarate, ascorbate, Fe^{2+}, and catalase (■) as described in Materials and Methods.*

The results obtained in vivo (Table 1) and in vitro (Table 4) indicate that the *uc-2* mutation affects the 2 -hydroxylase. Although the data in Table 1 suggest that the *pyr-4,uc-1,uc-2* strain is much less effective in catalyzing reactions c, d, and e (Fig. 1) than is the wild-type strain, the cell-free studies (Table 4) indicate that this lower activity is not a consequence of less thymine 7-hydroxylase being present. Perhaps the *pyr-4*-carrying mutants (which lack orotidylate decarboxylase [13]) are less effective in the uptake of pyrimidines. Preliminary experiments with the *pyr-4* strain and *pyr-4,uc-1* strain (developed by Williams and Mitchell [18] and recently isolated by W. R. Griswold) have shown that the extent to which these strains metabolize thymine is much less than that of the wild type and similar to that of the *pyr-4,uc-1,uc-2* strain. Although the *pyr-4,uc-1,uc-2* strain has a much reduced 2'-hydroxylase activity, a small amount of this activity did appear to be detected in this strain. It is noteworthy that the *uc-2* mutation blocked not only the utilization of thymine but also of deoxycytidine and deoxyuridine, and thus it would appear that *Neurospora* is deficient in its capacity to phosphorylate all of the common pyrimidine deoxyribonucleosides. This finding is consistent with data of Williams and Mitchell (18) which showed that a small amount of thymidine was incorporated into the nucleic acids of this strain. Studies carried out both in vivo (Table 1) and in vitro (Table 4) showed that the *uc-3*-carrying mutant does not contain an active thymine 7-hydroxylase. However, the data of Table 1 demonstrated that intact cells of the *uc-3*-carrying strain can convert 5-formyluracil to uracil-5-carboxylic acid, an observation which can apparently be explained by the presence in *Neurospora* of an additional enzyme which can convert 5-formyluracil to uracil-5-carboxylic acid (Fig. 2).

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