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Mutants Affecting Thymidine Metabolism in *Neurospora crassa*

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When ^{14}C-thymidine labeled only in the ring is administered to *Neurospora crassa*, the majority of the recovered label is found in the ribonucleic acid (RNA). Three mutants were isolated in which different steps are blocked in the pathway that converts the pyrimidine ring of thymidine to an RNA precursor. Evidence from genetic, nutritional, and accumulation studies with the three mutants shows the pathway to proceed as follows: thymidine → thymine → 5-hydroxymethyluracil → 5-formyluracil → uracil → uridylic acid. A mutant strain in which the thymidine to thymine conversion is blocked is unable to metabolize thymidine appreciably by any route, including entry into nucleic acids. This suggests that *Neurospora* lacks a thymidine phosphorylating enzyme. A second mutation blocks the pathway at the 5-hydroxymethyluracil to 5-formyluracil step, whereas a third prevents utilization of uracil and all compounds preceding it in the pathway. The mutant isolation procedures yielded three other classes of mutations which are proposed to be affecting, respectively, regulation of the thymidine degradative pathway, transport of pyrimidine free bases, and transport of pyrimidine nucleosides.

The use of radioactively labeled thymidine as a means of specifically labeling the deoxyribonucleic acid (DNA) of an organism requires that the organism possess a thymidine phosphorylating enzyme and that it lack enzymes which rapidly degrade thymidine. The latter of these conditions is not met in some organisms, because in autoradiographic studies uniformly labeled ^{3}H-thymidine fails to label specifically the DNA of *Acetabularia* (6), *Spirogyra* (18, 20), *Paramecium aurelia* (5), and *Neurospora crassa* (3). In fact, a majority of the label is incorporated into the ribonucleic acid (RNA) fraction.

A metabolic pathway by which thymidine could be converted to RNA precursors was demonstrated in *N. crassa* (12-14). By administering ^{14}C-thymidine labeled at various positions in the pyrimidine ring, Fink and Fink showed a pattern of labeling in the nucleic acid fraction similar to that obtained with labeled uridine, that is, 8 to 10 times more label in RNA than in DNA. When thymidine ^{3}H-methyl was administered, no label was found in either the RNA or DNA fraction. These data, supplemented by identification of some of the early products of thymidine degradation, including 5-hydroxymethyluracil (5-HMU), indicated a conversion of thymidine to uracil through the stepwise oxidation and ultimate elimination of the methyl group of thymine. A simplified diagram of pyrimidine metabolism, including the thymidine to uracil pathway, is shown in Fig. 1.

The purpose of the experiments reported here was to isolate mutants of the pathway by which thymidine is converted to an RNA precursor. With these mutants, the steps involved in the salvage pathway were better defined. Finally, mutants so blocked were tested to determine whether they could incorporate thymidine specifically into DNA.

MATERIALS AND METHODS

Strains. Wild-type strains 4*A* and 25*a*, three pyrimidine mutants, *pyr-3* (37301*a*), *pyr-1* (H-263*a*), and *pyr-4* (36601*a*), and one purine mutant (*ad-6*) were used. The mutants were kindly provided by Mary B. Mitchell from the *Neurospora* stocks of the California Institute of Technology Division of Biology.

Media and chemicals. All mutant stocks were maintained on the complete medium of Horowitz (16). The medium used for all mutant isolation procedures (with one exception), growth tests, and crosses was that of Westergaard and Mitchell (21), with appropriate modifications of carbon source, agar content, and pyrimidine supplement. The Westergaard-Mitchell medium, which lacks NH_4^+ nitrogen,

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was chosen for these studies because it permits efficient utilization (as pyrimidine sources) of the intermediates of the thymidine degradative pathway. The normal carbon source of 2% sucrose was replaced by 0.2% sucrose and 1.5% sorbose when colonial growth was desired. Radioactive 2-^{14}C-thymidine (30 mc/mmole) and 2-^{14}C-thymine (45 mc/mmole) were purchased from the New England Nuclear Corp., Boston, Mass. Most nonlabeled pyrimidines were purchased from commercial sources. However, 5-formyluracil was synthesized by the method of Cline et al. (9), and 5-fluorodeoxyuridine was obtained from R. Duschinsky of Hoffman-La Roche, Inc., Nutly, N.J.

Mutagenesis and filtration enrichment. A 10-ml conidial suspension (4×10^6/ml) of a *pyr* mutant was added to a petri dish (100 by 15 mm) and placed 7.5 cm beneath an ultraviolet (UV) germicidal lamp for from 30 sec to 2 min. The highest yield of mutants was obtained with a UV exposure giving 50% conidial survival.

For the selection of some mutants, a filtration enrichment procedure was employed (22). A 2.5-ml portion of the irradiated conidial suspension was inoculated into a 250-ml Erlenmeyer flask containing 50 ml of medium, supplemented with 2 to 3 μmoles of the pyrimidine for which nutritional mutants were desired. The conidia were kept in suspension on a rotary shaker, with periodic filtration through a layer of glass wool whenever mycelia became visible. Seven to eight filtrations over a period of 48 to 72 hr at 25 C gave optimal recovery of mutants among surviving conidia.

Immediately after UV irradiation or after the filtration enrichment procedure, 0.1 to 1.0 ml of conidial suspension was spread onto sorbose plates with 0.5 to 2.0 μmoles of pyrimidine supplement. At 25 C, colonies continued to appear on the plates from 3 to 7 days after inoculation. The sorbose plates were scanned each day, and newly appearing colonies were transferred from the agar to complete medium slants.

Mutant selection. In selection of mutants affecting the conversion of thymidine to an RNA precursor, cognizance must be taken of two factors. First, mutations affecting this pathway would not normally be detected since *Neurospora* can synthesize sufficient pyrimidine de novo for growth. However, by employing a *pyr* mutant (Fig. 1) as a parent strain the organism can be made dependent on pyrimidine salvage pathways. Second, *pyr* mutant conidia will not grow when early intermediates of the thymidine degradative pathway, thymidine, thymine, 5-HMU, and 5-formyluracil, are the sole pyrimidine sources, even though these compounds are utilized if growth is initiated by the addition of uridine or cytidine. Levels of uridine as low as 0.1 μmole/25 ml of liquid medium will permit the utilization of some thymidine (0.1 to 0.2 μmole) as a pyrimidine source. Five different methods were devised to select for mutations affecting the thymidine to RNA precursor pathway.

(i) The first selection method was based on the fact that conidial colonies of *pyr-4* on sorbose plates containing limiting uridine plus excess thymidine

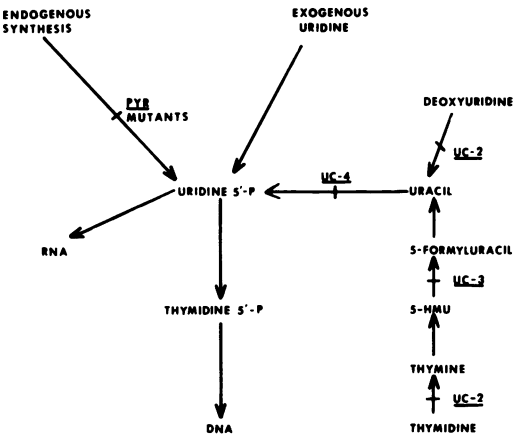

FIG. 1. *Schematic diagram of pyrimidine metabolism in N. crassa and the proposed sites of action of mutations affecting pyrimidine salvage pathways.*

(0.008 and 0.08 mM, respectively) were larger than if thymidine were lacking. Irradiated *pyr-4* conidia were inoculated onto the double-supplemented plates and were incubated until a majority of the colonies had reached a size that indicated they were using thymidine. At this point the smallest colonies were removed and tested for their ability to use thymidine as a pyrimidine source.

(ii) The second procedure designed to yield mutants able to use thymidine as a sole pyrimidine source consisted of inoculating about 4×10^5 irradiated *pyr-4* conidia onto sorbose plates containing only thymidine as a pyrimidine source. The few resulting colonies were isolated.

(iii) The third selection method employed the filtration-enrichment procedure. It was especially useful when derivatives able to use thymidine, thymine, and 5-HMU as sole pyrimidine sources became available by the second method. Irradiated conidia were incubated in filtration-enrichment medium containing thymidine, and the surviving conidia, presumably enriched in mutant conidia unable to use thymidine, were spread onto sorbose plates supplemented with uridine. Resulting colonies were tested to determine whether they carried a mutation which blocked a step in the thymidine to RNA precursor pathway.

(iv) The fourth method is a variation of the above filtration-enrichment procedure, in which mutants which could not utilize uracil were selected specifically. UV-irradiated *pyr-1* conidia were incubated in uracil-supplemented enrichment medium, and surviving conidia were inoculated onto uridine-supplemented sorbose plates.

(v) Finally, the filtration-enrichment procedure was used to select for mutants which could not utilize uridine but which still could use uracil as a pyrimidine source. Irradiated *pyr-1* conidia were incubated in Fries filtration medium (4) supplemented with uridine. Uracil is used very poorly on Fries medium, and the

use of this medium protected against the possibility that mutants which could not utilize uridine as such might be lost because they possessed an adaptive system to convert uridine to uracil. Conidia surviving the filtration step were inoculated onto sorbose plates of Westergaard-Mitchell medium supplemented with uracil.

Growth conditions. Growth responses of mutant strains to various pyrimidines were measured in terms of the dry weight of mycelia. The mold was cultured for 5 to 6 days at 25 C in 25 ml of medium in 125-ml Erlenmeyer flasks. The first 2 days of growth were stationary, after which the flasks were placed on a shaker for the remainder of the growth period. The mycelial pads were removed, dried overnight at 70 C, and weighed. Under these conditions, dry weights were reproducible to 10%.

RNA and DNA extraction. An adaptation of the perchloric acid extraction procedure of Ogur and Rosen (19) was used for extraction of RNA and DNA components. Growth medium was removed from the fresh mycelia by rinsing with water and by gentle suction in a Büchner funnel. Alcohol- and ether-soluble substances were extracted by three changes each of absolute ethyl alcohol, a 1:1 (v/v) mixture of absolute ethyl alcohol and ethyl ether, and ether. Approximately 20 parts of solvent per gram of mold (wet weight) was used in each solvent change. The ether-dry pad was then ground in cold 1.0 M $HClO_4$ (1.0 ml per 50 mg of mycelial dry weight) and allowed to stand for 12 hr at 0 C. The mixture was then centrifuged and the supernatant fluid was removed; the residue was extracted with cold $HClO_4$ in the same way five more times. The cold $HClO_4$ extract contained essentially all of the RNA in a partially degraded form. The residue from the RNA extraction was extracted three times at 75 C for 15 min with 1.0-ml portions of 1.0 M $HClO_4$. This treatment partially degraded and made soluble all of the DNA. The reliability both of the extraction procedure in separating RNA and DNA and of the calculation of nucleic acid concentration from the UV absorption (260 nm) of the extracts was confirmed by RNA and DNA concentration measurements based on pentose (Bial's reagent; 19) and deoxypentose (diphenylamine reagent; 7) content of the extracts.

Radioactive labeling procedure. The $HClO_4$ RNA and DNA extracts were neutralized at 0 C with KOH, and the precipitated $KClO_4$ was discarded. In ^{14}C labeling experiments, samples (0.01 to 0.05 ml) of the growth medium at the start, used growth medium, alcohol extracts, ether extracts, and neutralized $HClO_4$ extracts were applied to aluminum planchets, dried, and counted on a gas flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.).

Chromatography. Nonutilized pyrimidine supplements or pyrimidines excreted into the medium during growth were identified by their elution pattern from a Dowex-50 ion-exchange column, their characteristic UV absorption spectra, and their R_F in comparison with known pyrimidines as determined by paper chromatography. Uridine, 5-HMU, thymidine, uracil, and thymine can be separated on a Dowex-50 column (1.3 by 50 cm) by elution with 1.5 N HCl. Thymidine, thymine, and 5-HMU were also distinguished by one-dimensional paper chromatography (Whatman no. 1) by using the following three solvent systems: water (100), ethyl acetate-formic acid-water (70:20:10), and *t*-butanol-methyl ethyl ketone-water-concentrated NH_4OH (40:30:20:10).

RESULTS

Mutant isolation and genetics. The mutant selection procedures described yielded six classes of mutants which were given the designation uracil (*uc*) or uridine (*ud*). When a selection procedure yielded more than one mutant of a given class, only one was chosen for further work. The six representative mutants and their isolation numbers are *uc-1* (RW-57), *uc-2* (RW-135), *uc-3* (RW-203), *uc-4* (RW-341), *uc-5* (RW-399), and *ud-1* (RW-433). Each mutant isolate was crossed to wild type, and dissection of 10 to 20 ordered asci was carried out for each cross. All asci were consistent with a 2:2 segregation of the new mutations among the spore pairs. Recombinants carrying *uc* or *ud* mutations in an otherwise wild-type background behaved as prototrophs. This is consistent with the hypothesis that pyrimidine salvage pathways are nonessential to growth. Segregants carrying the new mutations were selected from the crosses, and these were used in all further studies.

The genetic map position of the six mutations remains to be determined, but the following linkage relationships are known: *uc-1* to *pyr-4* on chromosome II, *uc-2* and *uc-4* to mating type on chromosome I, and *uc-5* to *pyr-1* on chromosome IV. *Uc-3* and *ud-1* have not been assigned to a linkage group. It is evident that the mutations affecting pyrimidine salvage are not clustered on the genetic map.

Characterization of mutants of the thymidine salvage pathway. The *uc-1* mutation (obtained by the second selection method) when introduced into a *pyr-4* strain allowed the utilization of thymidine, thymine, 5-HMU, and 5-formyluracil as sole pyrimidine sources, whereas in the absence of *uc-1* these compounds could only be used after uridine or cytidine had initiated growth. (Table 1 shows sparing action of thymidine, thymine, and 5-HMU for uridine. Identical results are obtained if cytidine replaces uridine.) Although the regulatory nature of the *uc-1* mutation in no way altered the distribution of administered *2-^{14}C*-thymidine or *2-^{14}C*-thymine (90% of nucleic acid label in RNA, 10% in DNA), the *pyr-4 uc-1* double mutant was very useful as a parent strain for the selection of mutants in which the thymidine degradative pathway is blocked.

The *uc-2* mutation (obtained by the first and

third selection methods) allows all of the proposed intermediates of the thymidine degradative pathway to be used except the initial compound, thymidine (Table 1). It is proposed that the *uc-2* mutation blocks the first step of the pathway, cleavage of the deoxyribose sugar from the thymine moiety, through formation of a defective nucleosidase enzyme. The nucleosidase is thought to be specific for all pyrimidine deoxyribonucleosides since, in contrast to the parent *pyr-4 uc-1* strain, the *pyr-4 uc-1 uc-2* mutant cannot utilize deoxyuridine, deoxycytidine, or thymidine (Table 1). Further, the growth inhibition caused by a fourth pyrimidine deoxyribonucleoside, 5-fluorodeoxyuridine (5-FUdR), is of much longer duration in strains carrying *uc-2* than in those lacking it, indicating that *uc-2* prevents the breakdown of 5-FUdR. The *uc-2* mutation does not affect the utilization of purine deoxyribonucleoside since the growth of an *ad-6 uc-2* mutant was good on deoxyadenosine as a purine source.

When thymidine and uridine are administered to a *pyr-4 uc-2* double mutant, the majority of the thymidine remains in the growth medium (Table 2). This is in contrast to the removal of all thymidine from the medium that a *pyr-4* mutant effects under similar growth conditions.

The *uc-3* mutation (obtained by the third selection method) prevents the utilization of the first three proposed intermediates of the thymidine degradative pathway, thymidine, thymine, and 5-HMU (Table 1). The fact that the use of 5-formyluracil and uracil is unhindered leads to the conclusion that the *uc-3* mutation causes a defective enzyme required in the 5-HMU to 5-formyluracil conversion. That *uc-3*-carrying strains can partially metabolize thymidine was made clear when the used medium of a *uc-3* mutant grown in the presence of thymidine was found to support the growth of a *pyr-4 uc-1 uc-2* triple mutant. This accumulated product was found to be thymine (Table 2). Wild type removes thymidine from the medium as rapidly as does *uc-3*, but excretes very little thymine. Curiously, no 5-HMU was found in the used medium of *uc-3* (nor in the used medium of wild type) incubated with thymidine. It should be reemphasized here that *uc* and *ud* mutants grow well on minimal medium in the absence of a *pyr* mutation. Since the *uc-3* mutant is a prototroph, the addition of uridine or cytidine is not required to initiate the growth necessary

TABLE 1. *Growth response to several pyrimidines by mutants of the pyrmidine salvage pathway*

Pyrimidine supplement[a]	Dry weight of mycelial pad			
	pyr-4	*pyr-4 uc-1*	*pyr-4 uc-1 uc-2*	*pyr-4 uc-1 uc-3*
	mg	mg	mg	mg
Uridine	22	14	13	12
Uridine + thymidine	42	48	14	13
Uridine + thymine	39	45	39	13
Uridine + HMU	45	48	35	13
Thymidine	0	22	0	0
Thymine	0	21	19	0
5-HMU	0	21	22	0
5-Formyluracil	0	20	19	16
Uracil	17	16	17	16
Deoxyuridine	20	14	2	17
Deoxycytidine	6	13	0	17
Thymidine	0	22	0	0

[a] Each supplement was added in the amount of 1.0 μmole per flask.

TABLE 2. *Utilization and excretion of pyrimidines by uc mutants*[a]

Mutant	Medium before growth		Medium after growth		Hours of growth
	Pyrimidine	Amt	Pyrimidine	Amt	
		μmoles		μmoles	
pyr-4	Uridine + ^{14}C-thymidine	1.0 each	None	0	90
pyr-4 uc-2	Uridine + ^{14}C-thymidine	1.0 each	^{14}C-Thymidine	0.80	90
Wild type	Thymidine	20	Thymine	1	120
			Thymidine	5	
uc-3	Thymidine	20	Thymine	11	120
			Thymidine	5	
Wild type	Uridine	10	None	0	70
uc-4	Uridine	10	Uracil	5	70

[a] Pyrimidine supplements were in 25 ml of medium. Dry weights of mycelial pads were: *pyr-4* 38, mg; *pyr-4 uc-2*, 20 mg; wild, *uc-3* and *uc-4*, all 100 to 130 mg.

for the functioning of the thymidine degradative pathway.

The *uc-4* mutation (obtained by the fourth selection method) prevents the utilization of thymidine, thymine, 5-HMU, 5-formyluracil, and uracil as pyrimidine sources for the *pyr-1 uc-4* mutant (Table 3). This is true even if growth is initiated by small amounts of uridine. On the basis of growth data, the *uc-4* mutation can be assigned to blocking a step involved in the conversion of uracil to uridylic acid. The relatively poor growth of the *pyr-1 uc-4* mutant on uridine is explained by the fact that half of the uridine administered is excreted into the medium as uracil, which the mutant cannot use. A *uc-4* mutant given uridine accumulates uracil in the medium, whereas wild type does not (Table 2).

Pyrimidine transport. The procedures used in selection for mutants which could not utilize a given pyrimidine could yield two types of mutants, those defective in enzymes converting the pyrimidine into nucleic acid and those lacking the ability to transport the pyrimidine into the cell. The procedures used here have been used to isolate transport mutants in bacteria (17). Growth data from two double mutants *pyr-1 uc-5* (fourth selection method) and *pyr-1 ud-1* (fifth selection method) suggest the simple interpretation that *uc-5* and *ud-1* disrupt pyrimidine transport mechanisms (Table 4).

The *pyr-1 uc-5* mutant is unable to utilize any free pyrimidine base (thymine, 5-HMU, 5-formyluracil, and uracil), although it can use the ribose and deoxyribose pyrimidine nucleosides (cytidine, uridine, deoxyuridine, and thymidine) in a normal manner. In an exactly reverse manner, the *pyr-1 ud-1* double mutant can use all of the free bases but none of the pyrimidine nucleosides. The hypothesis then is that the *uc-5* locus controls a transport system for the free base pyrimidines

TABLE 3. *Growth response to several pyrimidines by pyr-1 and pyr-1 uc-4 mutants*

Pyrimidine supplement[a]	Dry weight of mycelial pad	
	pyr-1	*pyr-1 uc-4*
	mg	*mg*
Uridine[b]	33	11
Uridine	18	6
Uridine + thymidine	32	6
Uridine + 5-HMU	32	6
Uridine + uracil	31	6
Uracil	18	0

[a] Unless otherwise indicated, each supplement was added in the amount of 1.0 μmole per flask.

[b] Uridine supplement here was 2.0 μmoles/flask.

TABLE 4. *Effect of uc-5 and ud-1 mutations on utilization of pyrimidine nucleosides and free bases*

Pyrimidine supplement[a]	Dry weight (mg) of mycelial pad		
	pyr-1	*pyr-1 ud-1*	*pyr-1 uc-5*
Uridine[b]	33	0	36
Cytidine	18	0	21
Uridine	18	0	21
Uridine + thymidine	32	0	38
Uridine + uracil	31	20	21
Uracil	18	18	1
Uracil + thymidine	29	19	0
Uracil + thymine	29	31	0

[a] Unless otherwise indicated, each supplement was added in the amount of 1.0 μmole per flask.

[b] Uridine supplement here was 2.0 μmoles/flask.

and the *ud-1* locus controls a system for pyrimidine nucleosides. The unlinked *uc-4* and *uc-5* mutations cause similar growth responses to most pyrimidines, but they can easily be distinguished by growth on thymidine. The *uc-4* strains (blocked at the last step of thymidine degradative pathway) cannot utilize thymidine, whereas *uc-5* strains use the thymidine very efficiently.

^{14}C-pyrimidine incorporation. It has been demonstrated that when *Neurospora* is grown in the presence of 2-^{14}C-thymidine and 2-^{14}C-thymine, 90% of the resulting nucleic acid label is recovered in the RNA fraction, and when ^{14}C-methyl-labeled thymidine is administered no label is found in either RNA or DNA (13, 14). These facts alone do not prove that *Neurospora* cannot phosphorylate thymidine and incorporate it directly in DNA. An alternate possibility is that *Neurospora* may have a thymidine phosphorylating system of low activity which would not be detected because of the rapid conversion of its substrate, thymidine, to compounds which are unavailable for specific incorporation in DNA. To test this possibility, 2-^{14}C-thymidine and 2-^{14}C-thymine were separately administered to a *pyr-4 uc-2* double mutant; the *uc-2* mutation, as described previously, blocks the initial step in the degradation of thymidine (Table 5). Both the *pyr-4 uc-1* double mutant and a control *pyr-4* strain convert 60 to 70% of the label originally present as 2-^{14}C-thymine into RNA constituents. The *pyr-4* mutant converts the label of 2-^{14}C-thymidine in a similar manner, but the *pyr-4 uc-2* mutant transforms only 10% of the label to RNA, whereas over 75% of the thymidine remains unused in the growth medium. However, the amount of label found in *pyr-4 uc-2* DNA was proportionately reduced and the distribution of

TABLE 5. *Distribution and utilization of 2-^{14}C-thymidine and 2-^{14}C-thymine label by pyr-4 and pyr-4 uc-2 mutants*[a]

Solution counted	Per cent of initial label after 90 hr of growth			
	pyr-4		*pyr-4 uc-2*	
	2-^{14}C-Thymidine	2-^{14}C-Thymine	2-^{14}C-Thymidine	2-^{14}C-Thymine
Growth medium	8	8	78	8
Alcohol-ether extracts	1	3	2	2
RNA extracts	73	60	10	69
DNA extracts	5	5	1	6
Per cent of total nucleic acid counts due to DNA	7	8	9	8
Per cent of total nucleic acid that is DNA	9	10	9	8

[a] Flasks containing 25 ml of medium, 1.0 μmole of uridine, and either 1.0 μmole of thymidine (S.A., 1.5 μc/μmole) or 1.0 μmole of thymine (S.A., 2.0 μc/μmole) were prepared. *Pyr-4* and *pyr-4 uc-1* conidia were inoculated into one flask of each type, and growth was allowed for 90 hr at 25 C. Extraction of the mycelial pads was accomplished as described in Materials and Methods.

nucleic acid label remained approximately 90% RNA and 10% DNA. Therefore, under conditions in which the conversion of thymidine to RNA precursors was largely blocked, no evidence for the specific incorporation of thymidine into DNA could be shown.

DISCUSSION

Figure 1 shows a pathway by which the pyrimidine ring of thymidine can be converted to uridylic acid and thus made available for incorporation into RNA and DNA pyrimidines. The order of intermediates in the pathway was based on nutritional tests and accumulation studies with strains carrying *uc-2*, *uc-3*, and *uc-4* mutations. The hypothesis that the *uc-2* mutation causes formation of a defective pyrimidine deoxyribonucleosidase enzyme is similar to the explanation of an *Escherichia coli* mutant (11) which had lost the ability to use thymidine, deoxyuridine, and deoxycytidine as carbon sources. This mutant was shown to lack thymidine phosphorylase activity, the enzyme responsible for the cleavage of the deoxy sugar from thymidine.

The *uc-3* mutation prevents the oxidation of 5-HMU to 5-formyluracil because *uc-3*-carrying strains utilize the formyl compound but not 5-HMU as a pyrimidine source. However, since a *uc-3* mutant administered thymidine accumulates large quantities of thymine and none of the expected 5-HMU, the possibility exists that the *uc-3* locus may also control the thymine to 5-HMU conversion. Abbott and co-workers (1, 2) demonstrated the formation of 5-HMU from thymine (thymine-7-hydroxylase) and of 5-formyluracil from 5-HMU by cell-free extracts of *Neurospora*. The two enzyme activities which have been only partially separated by initial purification steps both require O_2 and Fe^{++}, α-ketoglutarate, and ascorbate as cofactors. The *uc-3* mutant strain, whether it is found to lack one or both of the two enzyme activities, should prove quite useful in work on the enzymology of thymine methyl group oxidation. From what is known of uracil salvage enzymes in other microorganisms (8, 10), it is probable that the *uc-4* mutation causes an inactive pyrophosphorylase enzyme for the one-step formation of uridine-5′-phosphate from uracil.

The classification of *uc-1* as a regulatory gene is based on the fact that strains carrying this mutation can use thymidine, thymine, 5-HMU, and 5-formyluracil as sole pyrimidine sources. These growth responses are interpreted to mean that the enzyme(s) necessary for the conversion of 5-formyluracil to uracil is derepressed (or is present at normal levels but not subject to inhibition) in germinating conidia of *uc-1* strains. The growth tests provide no clue as to whether enzymatic steps before 5-formyluracil in the pathway are subject to control by the *uc-1* gene.

Uracil-5-carboxylic acid (5-CU) could be proposed as an intermediate of the thymidine degradative pathway since it might easily be formed from 5-formyluracil and yield uracil upon loss of CO_2. However, growth tests with *pyr* mutants show this compound, in contrast to the other intermediates, to be used very inefficiently as a pyrimidine source. Furthermore, no mutants were found which accumulated 5-CU, nor were mutant hunts successful in finding strains which could utilize the compound as a pyrimidine source. The role of 5-CU as an intermediate awaits the study of enzymes of the pathway.

The pathway for the conversion of thymidine into an RNA precursor is undoubtedly present or capable of being induced in a large number of other organisms. The possibility of the presence of this pathway should be considered in any experiment in which thymidine is to be used for the specific labeling of DNA.

Data were presented showing that 2-^{14}C-thymidine administered to a strain carrying the *uc-2* mutation did not lead to an increased percentage of label incorporated into the DNA. This sub-

stantiates the hypothesis of Fink (13) that *Neurospora* lacks a thymidine-phosphorylating enzyme. Grivell and Jackson (15) have been unable to demonstrate thymidine kinase (E.C. 2.7.1.21) activity in *Neurospora*, *Aspergillus*, and *Saccharomyces*. Since a large portion of the thymidine given to strains carrying *uc-2* remains unused in the medium, it can be assumed that conversion of thymidine to thymine is the only reaction of significance that thymidine can undergo in *Neurospora*. The lack of a thymidine-phosphorylating enzyme makes it quite doubtful that a thymidine mutant (possessing a nonfunctioning thymidylate synthetase enzyme and, therefore, dependent for growth upon a secondary synthesis of thymidylic acid) of *Neurospora* can be isolated.

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