THYMIDINE METABOLISM IN *NEUROSPORA CRASSA*

Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1968

(Submitted September 19, 1967)

ACKNOWLEDGMENT

I take this opportunity to thank Dr. Herschell K. Mitchell for his guidance and unselfish contribution of time, helpful suggestions and laboratory skills. His interest and understanding have helped to make my stay at Caltech a most pleasant one.

I also wish to thank Professor Mogens Westergaard for acquainting me with the problem of thymidine metabolism in Neurospora and fellow graduate students William Scott and Arthur Riggs for friendly advice and helpful suggestions.

Certainly I will remember with pleasant memories my many lab mates in 287 Alles and those who just stopped by to visit awhile.

I am indebted to the National Science Foundation for the financial support provided by a Graduate Fellowship.

To my wife, Ruth, goes a special vote of appreciation. Her keen eye in spotting mutants, her patience in helping with time consuming laboratory tasks and her steady hand in dissecting Neurospora asci have contributed greatly to this thesis. I am also grateful to her for her patience and excellence in the typing of this thesis.

ABSTRACT

The metabolism of thymidine by Neurospora crassa was investigated after radioactive labeling experiments showed that thymidine is not specifically incorporated into the DNA of Neurospora; a finding in contrast to the results of labeled thymidine experiments with many other organisms. Labeling experiments in which 2-C^{14} deoxyuridine, 2-C^{14} thymidine and 2-C^{14} thymine were administered to Neurospora revealed that in each case ninety per cent of the nucleic acid label was in the RNA fraction. When thymidine methyl-H^3 was administered no label was found in either the RNA or DNA. This and other evidence was taken as proof that Neurospora lacks phosphorylating enzymes for deoxyribose pyrimidine nucleosides but does possess enzymatic reactions by which these same compounds can be converted to RNA precursors.

Three mutants were isolated in which different synthetic steps are blocked in the pathway that converts the pyrimidine ring of thymidine to an RNA precursor. Evidence from genetic studies, nutritional tests and accumulation studies with the three mutant strains 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 prevented is also unable to utilize deoxyuridine and deoxycytidine as pyrimidine sources and suggests a defective deoxyribose pyrimidine nucleosidase enzyme. A second mutation blocks the pathway at the 5-hydroxymethyluracil to 5-formyluracil step and causes the accumulation

of thymine in the growth medium. The third mutation prevents the utilization of uracil and the compounds preceding it in the pathway.

Three mutants were isolated in which the pyrimidine transport system was affected. One of these mutants could utilize the pyrimidine nucleosides (cytidine, uridine, deoxyuridine and thymidine) but could use none of the free bases (thymine, 5-hydroxymethyluracil, 5-formyluracil and uracil). A second mutant could utilize the free bases but not the nucleosides. These results can be most simply interpreted in terms of the first mutation blocking a transport system specific for pyrimidine bases, and the second mutation blocking a system specific for the transport of pyrimidine nucleosides. A third mutation prevented the utilization of both pyrimidine bases and nucleosides in a medium containing ammonium salts but permitted their use in a medium containing nitrate salts.

A strain was isolated carrying a mutation which influences the regulation of the thymidine to RNA precursor pathway. This mutation allows the steps of the pathway that convert thymidine to uracil to function in germinating conidia. A second mutation was found which suppresses the action of the first and restores the normal condition, the absence of the thymidine to uracil conversion in germinating conidia.

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INTRODUCTION

Thymidine metabolism

Since the work of Taylor and associates (1) in 1957 the use of tritiated thymidine for the selective labeling of DNA (deoxyribonucleic acid) and chromosomes in autoradiographic studies has become widespread. The extensive use of such autoradiographic procedures has led to the premise that thymidine is specifically incorporated into DNA and that this is the only pathway of metabolism open to it other than complete degradation. However, scattered throughout the scientific literature are reports of the failure of labeled thymidine to label selectively the DNA of various species.

Brachet (2) reported that the alga, Acetabularia mediterranea, incorporates a large portion of the radioactive label of C^{14}-thymine and H^3-thymidine into RNA (ribonucleic acid), specifically into uridylic and cytidylic acid. Stocking and Gifford (3) administered H^3-thymidine to growing filaments of Spirogyra. Autoradiographs showed little labeling in the cell nucleus, but the chloroplast, which made up one-third of the cell surface in contact with the film, contained ninety per cent of the total radioactivity. Meyer (4) confirmed these experiments with Spirogyra and showed that the cytoplasmic label was not removed by DNase, but that up to fifty per cent of the label was removed by RNase. Berech and van Wagtendonk (5) grew Paramecium aurelia in a medium containing H^3-thymidine. Autoradiographs of organisms grown in the medium revealed marked labeling of the cytoplasm. Chromatography of the Paramecium cellular fractions showed that uridylic acid from the

RNA was radioactive. Baer and St. Lawrence (6) tried to use autoradiographic procedures to correlate DNA synthesis with chromosomal events in Neurospora crassa. They administered H^3-thymine, thymidine-methyl-H^3 and H^3-deoxyuridine in attempts to label specifically Neurospora DNA. The thymine and thymidine compounds yielded light labeling uniformly distributed over both the cytoplasm and nucleus. Deoxyuridine-H^3 gave heavy labeling, but again the distribution of label over the cytoplasm and nucleus was the same. In summary it is clear that at least some organisms have a metabolic pathway capable of converting thymidine to RNA precursors followed by incorporation into RNA.

A metabolic pathway by which thymidine could be converted to RNA precursors was pointed out by Fink and Fink (7, 8, 9). They found that the radioactive labels of thymidine-2-C^{14}, thymidine-5,6-C^{14} and uridine-2-C^{14} were all incorporated to a considerable extent into the nucleic acid fractions of Neurospora crassa. Further, the thymidine compounds gave labeling patterns similar to that of uridine in that there was eight to ten times more label in the RNA fractions than in the DNA fractions. When thymidine-methyl-H^3 was administered no label was found in either the RNA or DNA fractions. These labeling experiments led to three conclusions.

1. Neurospora lacks a thymidine phosphorylating mechanism.
2. The pyrimidine ring of thymidine is incorporated intact into an RNA precursor which can be used to form ribonucleotides that are suitable for both RNA synthesis and for reduction to deoxyribonucleotides useable in DNA synthesis.

3. The conversion of thymidine into an RNA precursor occurs through the stepwise oxidation of the methyl group of thymine followed by elimination of the oxidized methyl group.

Additional proof for the third conclusion was that chromatography of the early metabolic products of radiocarbon labeled thymidine resulted in the identification of labeled thymine, 5-hydroxymethyluracil and 5-formyluracil (10). Crude enzyme preparations from rat liver (11) and Neurospora (12) have been shown to carry out the conversion of thymine to 5-hydroxymethyluracil. Possible intermediates of a pathway involving the stepwise oxidation of the thymine methyl group to yield an RNA precursor are given in the lower half of figure 1.

Pyrimidine metabolism in Neurospora

The general scheme of synthetic reactions involved in the formation of pyrimidines has emerged from research in many laboratories involving a variety of organisms (13). An abbreviated synthetic pathway including the sites of action of the pyrimidine (pyr) mutants of Neurospora is presented in the upper half of figure 1. Research which demonstrates that such a pathway operates in Neurospora includes that of Davis (14), who showed pyr-3a mutants were deficient in carbamyl phosphate synthesizing enzyme and pyr-3d mutants were deficient in aspartate transcarbamylase; Mitchell (15), who discovered the accumulation of orotic acid by pyr-2 and pyr-4 mutants, and Pynadeth and Fink (16), who proved the pyr-4 mutant lacks orotidylic acid decarboxylase activity. McNutt (17) grew a pyr-4 Neurospora mutant in a medium supplemented with uniformly

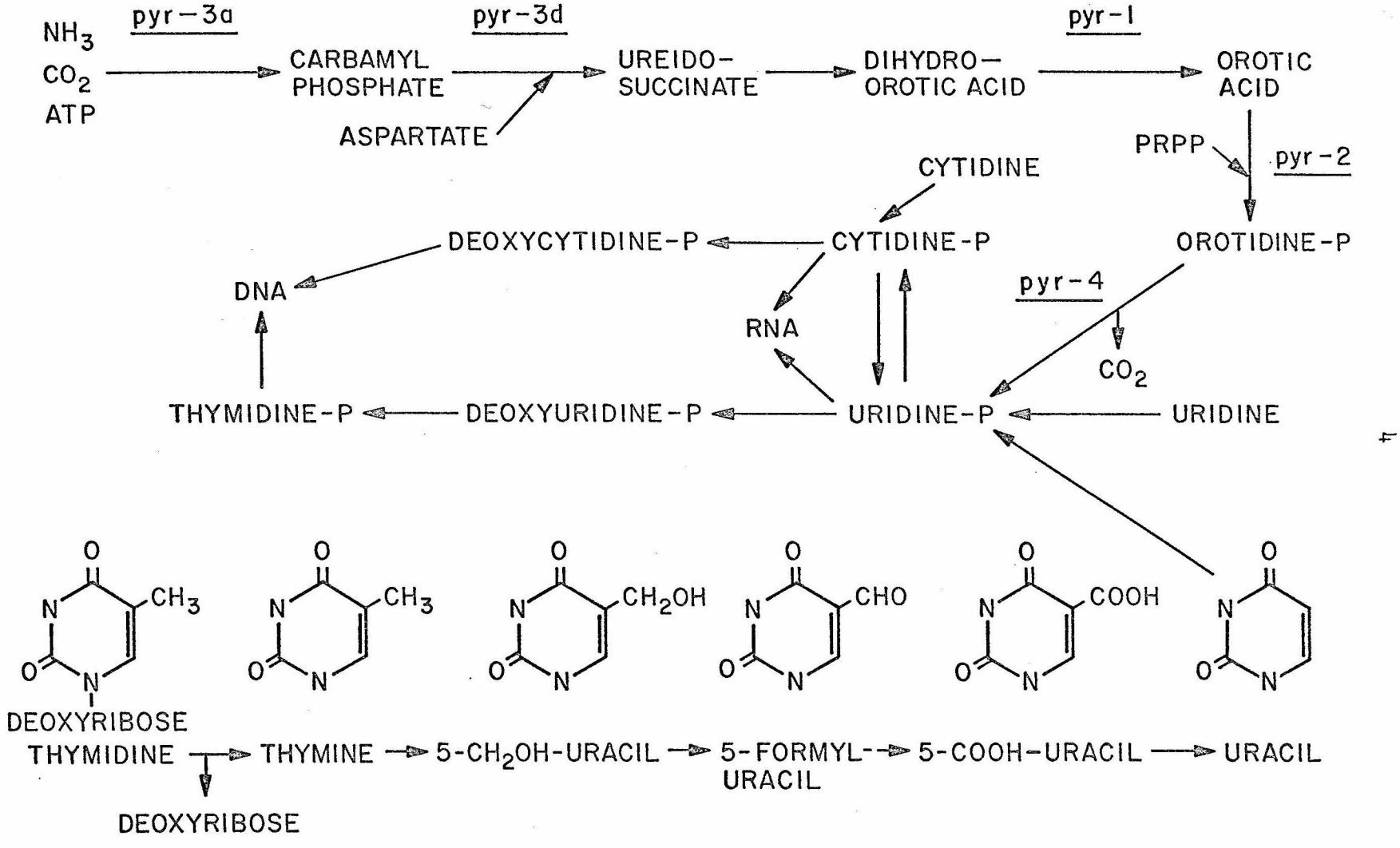


Figure 1. PYRIMIDINE METABOLIC PATHWAYS IN NEUROSPORA.

labeled C^{14}-cytidine and found that the label was incorporated without dilution into the RNA and DNA pyrimidines. In addition the specific activities of the pyrimidine and sugar moieties of both the RNA and DNA pyrimidines were the same as those of the cytosine and ribose components of the initially administered cytidine. These results are consistent with the idea that deoxyribonucleotides are formed from ribonucleotides.

Several interesting facts should be pointed out regarding utilization of pyrimidines by Neurospora pyr mutants. Uridine and cytidine are the commonly used pyrimidine supplements for the mutants as the fungus apparently possesses an efficient phosphorylating enzyme for these ribonucleosides. Conversely, Neurospora is unable to phosphorylate orotidine (18), thymidine, deoxycytidine (9) and deoxyuridine (this thesis). The free pyrimidine bases, orotic acid, cytosine and uracil, are utilized very inefficiently by Neurospora in the standard growth media which contain ammonium salts. In a medium containing nitrate rather than ammonium salts uracil is used just as efficiently as uridine although cytosine and orotic acid are still poorly utilized. Thymidine, thymine and 5-hydroxymethyluracil alone will not support the growth of any pyr mutant but are utilized in the presence of uridine (10). Uptake experiments by Zalokar (19) showed that H^3-uridine was rapidly taken into Neurospora mycelia. Column chromatography of the acid soluble mycelial components revealed all the radioactivity in two peaks corresponding to two uridine diphosphate (UDP) sugar compounds. No activity was detected in free uridine or uridine mono-, di- and triphosphates. The UDP sugar compounds are probably uridine diphosphoglucose and uridine diphosphoacetylglucoseamine (20).

Possible additional pyrimidine mutants of Neurospora

Examples of two classes of reactions for which nutritional mutants are seldom recovered are shown in figure 1. The first class includes those reactions involving phosphorylated intermediates to which the cell membrane is impermeable. Nutritional mutants involving reactions of phosphorylated compounds can be recovered only if the required parent organic compound can enter the cell and be phosphorylated within the cell. For example, the pyr-4 mutant was recovered because uridine could be phosphorylated. A mutant blocking the deoxyuridylic acid to thymidylic acid step cannot be recovered in Neurospora because Neurospora cannot phosphorylate thymidine.

A second and largely untouched class of Neurospora mutants are those which go undetected because they do not affect essential reactions. Reactions involving salvage pathways such as exist for purines, pyrimidines and aromatic compounds are of this class. As an example, a mutation blocking the conversion of thymidine to an RNA precursor would not normally be detected because Neurospora can synthesize de novo sufficient pyrimidines for normal growth. Only when the de novo synthetic pathway is blocked does the salvage pathway become essential and does a means for selecting mutants of the salvage pathway become available. The purpose of this research was to investigate methods by which the specific labeling of Neurospora DNA might be brought about, to devise procedures which would yield mutants of the pyrimidine salvage pathways (specifically the pathway by which thymidine is converted to an RNA precursor) and to use these mutants to elucidate further the steps in the pathway.

MATERIALS AND METHODS

Neurospora strains

Wild type strains 4A and 25a, three pyrimidine (pyr) mutants and one purine (ad-6) mutant were used. The mutants were kindly provided by Mary B. Mitchell from the Neurospora stocks of the Caltech Division of Biology. A brief description of the mutants used, including isolation number, mating type, linkage group and step in the synthetic pathway blocked by the mutation is as follows. Pyr-3 (37301a, linkage group IV R) lacks aspartate transcarbamylase enzyme activity (21, 22). Pyr-1 (H263a, linkage group IV R) is blocked in the pyrimidine synthetic pathway between ureidosuccinic acid and orotic acid (21, 22). Pyr-4 (36601a, linkage group II L) has no orotidylic acid decarboxylase activity (23, 16). The pyr-4 mutant releases large amounts of orotic acid into the growth medium (15). Orotic acid gives strong absorption in the ultraviolet portion of the spectrum and has an absorption maximum at 280 mμ in dilute acid. Neither pyr-1 nor pyr-3 mutants release accumulated compounds into the growth medium. The ad-6 mutation (28610a, linkage group IV R) causes the purine synthetic pathway to be blocked at an early stage, before closure of the imidazole ring (21, 24).

Source of pyrimidines

The four radioactive labeled pyrimidines which were used in experiments with pyrimidine mutants are as follows: 2-C^{14} thymidine (30 mcurie/mmole--New England Nuclear Corp.), 2-C^{14} thymine (45 mcurie/mmole--New England Nuclear Corp.), 2-C^{14} deoxyuridine (23 mcurie/mmole--

California Corporation for Biochemical Research) and thymidine-methyl-H^3 (6100 mcurie/mmole--Schwarz Bioresearch Inc.). The large number of nonlabeled purine and pyrimidine compounds used in the classification of various mutants affecting pyrimidine metabolism came from the following sources: thymidine, 5-hydroxymethyluracil, uridine, cytidine and cytosine (California Corporation for Biochemical Research); thymine, uracil and adenosine (Sigma Chemical Company); uracil-5-carboxylic acid (K & K Laboratories); deoxyuridine, deoxycytidine, deoxyadenosine and deoxyguanosine (Nutritional Biochemicals Corporation). Two compounds, 5-fluorodeoxyuridine and 5-formyluracil could not be obtained commercially. The 5-fluorodeoxyuridine was obtained from Dr. R. Duschinsky of Hoffman-La Roche Inc. while 5-formyluracil was synthesized.

The procedure of Fink et al. (25) was followed for the synthesis of 5-formyluracil from 5-hydroxymethyluracil by using oxygen and a platinum catalyst. Their procedure was an adaptation of the method proposed by Sneeden and Turner (26) for the dehydrogenation of primary alcohols to yield aldehydes.

A suspension of platinum oxide (75 mg in 6 ml of 50% acetic acid) was first reduced to platinum in an atmosphere of hydrogen (in Parr apparatus for 75 minutes at 40 pounds H_2 pressure) after which the hydrogen was removed and replaced with air. Oxygen gas was then slowly bubbled into the suspension for 30 minutes followed by the addition of 12 mg of 5-hydroxymethyluracil which slowly dissolved in the acetic acid. The oxygen was allowed to bubble slowly into the suspension for 18 hours after which the acetic acid solution was removed from the

platinum catalyst and evaporated in an air stream. The products were redissolved in water and separated on a Dowex-50 ion exchange column (separation is discussed in the following section). A yield of 80% 5-formyluracil was attained with the remaining 20% consisting of 5-hydroxymethyluracil (15%) and uracil-5-carboxylic acid (5%).

The criteria used in assuming that the 80% yield product was 5-formyluracil were its chromatographic behavior, its UV absorption spectra in acid and base and the growth response, or lack of response, of mutants in the thymine-methyl oxidative pathway to the product. Using ascending paper chromatography the location of the synthesized 5-formyluracil after development in each of two solvent systems, t-butyl alcohol, methyl ethyl ketone, H_2O and NH_4OH (40:30:20:10) and ethyl acetate, formic acid and H_2O (70:20:10), was the same as reported for 5-formyluracil (27). The maximum and minimum wavelengths of ultraviolet absorption reported for 5-formyluracil (25) and those obtained from the 5-formyluracil synthesized in 80% yield are given below.

Compound	pH	max. (mμ)	min. (mμ)
5-formyluracil	2	275.5	248
80% yield product	2	275	248
5-formyluracil	12	298	267
80% yield product	12	298	268

Ion exchange chromatography of pyrimidines

Wall (28) reported that uracil and thymine could be separated on a column of Dowex-50 resin by eluting with 1.5 N HCl. Further work with

the Dowex-50 column revealed that it could be used to separate several 5-substituted pyrimidines which were not tried by Wall. An additional finding was that water would elute these pyrimidines from the Dowex-50 column and would elute them in a somewhat different order than HCl.

Dowex-50-X8, 200 to 400 mesh resin was cleaned by the following procedure: two washes with hot 6 N HCl, two washes with water, two washes with 1 N NaOH, two washes with 1.5 N HCl and two washes with water. The cleaned resin was suspended in H_2O and poured as a slurry into a glass chromatography tube to form a column 1.3 cm in diameter and 50 cm in height. Water or 1.5 N HCl was washed through the column for 24 hours before its use to reduce to a low level the background of UV absorbing material coming from the resin.

The ion exchange column was used to aid in the identification and purification of pyrimidines accumulated in the growth medium by certain mutants and for separation of 5-formyluracil from a synthetic reaction mixture. A volume of 0.5 to 2.0 ml of used medium or reaction mixture (acidified if 1.5 N HCl was to be eluant) containing two to seven μmoles of pyrimidine was added to the top of the column and washed into the resin with two ml of H_2O or 1.5 N HCl. Elution was then started at a flow rate of about 15 ml per hour. Fractions of volume 1.5 to 2.0 ml were collected and their absorptions at 265 mμ were measured with a model 15 Cary spectrophotometer.

The identity of a pyrimidine compound eluted from the column could generally be determined by the location and characteristic UV absorption spectrum of the fractions containing the compound. The concentration of

pyrimidine in a fraction was calculated from its $O.D._{265}$ above background in relation to the $O.D._{265}$ of a known concentration of that pyrimidine. The amount of each pyrimidine added to the column could be determined from its concentration per fraction and the measured volume of the fractions.

The effluent volumes of 0.35 μmole amounts of various pyrimidines added to a 1.3 X 50 cm Dowex-50-X8 column are shown below. Data with both H_2O and 1.5 N HCl eluant are given as well as wavelength of maximum absorption in 1.5 N HCl.

		Effluent Volume	
Pyrimidine	max. (mμ)	HCl eluant	H_2O eluant
uridine	261	30 - 35 ml	29 - 35 ml
5-CH_2OH uracil	260	35 - 40 ml	38 - 44 ml
thymidine	266	40 - 45 ml	
uracil	258	46 - 50 ml	51 - 57 ml
5-formyluracil	275	46 - 50 ml	51 - 57 ml
5-carboxyuracil	272	46 - 50 ml	29 - 35 ml
thymine	264	62 - 70 ml	67 - 75 ml

RNA and DNA extraction

The method used for extraction of RNA and DNA from Neurospora was developed by Dr. H. K. Mitchell (unpublished data). The method is an adaptation of the perchloric acid extraction procedure described by Ogur and Rosen (29).

Wet mold was placed in a Buchner funnel where the growth medium was removed by rinsing with water and by gentle suction. The pad was then

put in a beaker and alcohol and ether soluble substances were extracted by three changes of absolute ethanol, followed by three changes of a one to one mixture of absolute ethanol and ethyl ether and finally by three changes of ethyl ether. Approximately 20 parts of solvent per gram of mold (wet weight) were used in each solvent change. The ether dry mold was ground and a 250 mg portion was mixed with 5.0 ml of cold 1.0 M $HClO_4$ and let stand 12 hours at 0°C. The mixture was then centrifuged, the supernatant removed and the residue reextracted by cold $HClO_4$ in the same way a total of five times. The cold $HClO_4$ extract contained essentially all the RNA in a partially degraded form. The residue from the RNA extraction was extracted three times at 75°C for fifteen minutes with 5.0 ml portions of 1.0 M $HClO_4$. This treatment partially degraded and made soluble all the DNA.

The RNA concentrations of the cold $HClO_4$ extracts were determined by their optical density at 260 mμ in relation to yeast RNA standards. A second measure of RNA concentration was made by using Bial's reagent (orcinol, $FeCl_3$ and 30% HCl) to test for pentose (29). Conditions for the photocolorimetric estimation of pentose are as follows. Two ml Bial's reagent plus three ml of 1 N $HClO_4$ containing the RNA were mixed, heated 20 minutes in a boiling water bath, allowed to stand eight hours and then centrifuged to remove interfering material. The optical density at 666 mμ was then compared with that of yeast RNA standards that had been treated in a similar manner.

Heating DNA in 1 N $HClO_4$ at 75°C for 15 minutes causes a shift in the UV absorption maximum from 260 to 268 mμ. Therefore, the DNA

concentrations of the hot $HClO_4$ extracts were determined by their optical density at 268 mμ in relation to DNA standards that had also been heated in $HClO_4$. As a second measure of DNA concentration the diphenylamine photocolorimetric test (30) for deoxypentose was used. Two ml of the diphenylamine reagent and one ml of the 1 N $HClO_4$ DNA solution were mixed and allowed to incubate at room temperature for 16 to 20 hours. The optical density of the reaction mixtures were read at 600 mμ.

Radioactive labeling procedures

In experiments where radioactive labeling was used the ether dry pad was placed in cold $HClO_4$ and then ground. Frequently the dry weight of labeled mycelia to be extracted was less than 250 mg. In these cases extraction volumes were reduced proportionately. The perchloric acid RNA and DNA extracts were neutralized at 0°C with KOH. Approximately 80% of the resulting $KClO_4$ salt precipitated from solution and was discarded. In C^{14} labeling experiments 0.01 to 0.05 ml aliquots of the growth medium at the start, used growth medium, alcohol extract, ether extract and neutralized $HClO_4$ extracts were applied directly to aluminum planchets and counted on a Nuclear Chicago low background counter. Each extract or medium was applied to three planchets, and the resulting counts per minute were averaged. The degree of variation involved in the extraction and counting procedure permits an error of $\pm$ 5% in the total counts reported for the various extracts.

In H^3 labeling experiments 0.025 ml aliquots of the extract or medium to be counted were placed in 20 ml glass vials and 10 ml of Bray's

solution (70) was added to each vial. After allowing time for the samples to equilibrate to the temperature of the counter, they were counted in a Nuclear Chicago Scintillation Counter (720 Series). The efficiency of counting in each sample was determined by the channels ratio method.

Growth media and conditions

The growth responses of mutant strains to various pyrimidines were measured in terms of the dry weight of the mycelia. The mold was cultured in 25 ml of medium contained in 125 ml Erlenmeyer flasks for five to six days at 25°C. After two days of growth in standing culture the flasks were placed on a shaker for the remainder of the growth period. The mycelial pads were removed, dried at 65 - 70°C for at least 10 hours and weighed. When this procedure was used and two flasks containing the same mutant inoculum and the same concentration of growth limiting pyrimidine were grown side by side the dry weights of the resulting pads were almost always within 10% of one another. Unless otherwise stated the medium used for growth tests was Westergaard-Mitchell medium (contains nitrate but no ammonium nitrogen) (31) at an unadjusted pH of 4.5. In a few cases the standard Fries medium was used (contains ammonium salts) (32). The trace element solution used was that of Vogel (33). Mutant strains were maintained on agar slants of either Horowitz complete medium (34) or Westergaard-Mitchell medium supplemented with pyrimidines.

Mutant production

All the new mutants to be reported were induced by ultraviolet

radiation. The procedure was the same in all cases, the only variations being the strain and concentration of conidia that received the irradiation and the length of exposure to the ultraviolet light. All the strains used possessed multinucleate conidia.

Conidia from strains grown on complete medium agar slants were suspended in sterile H_2O and the resulting suspension was twice filtered through glass wool. An estimate of conidia concentration was made by determining the optical density of the suspension in a Klett-Summerson colorimeter. With a filter of spectral range 400 to 465 mμ one Klett unit equaled approximately 50,000 conidia per ml. The conidial suspension was then diluted to a concentration of from 1×10^6 to 4×10^6 conidia per ml and 10 ml of the diluted conidial suspension was placed in a 100 X 15 mm Petri dish. The open Petri dish was placed under an ultraviolet lamp with a maximum emission at 254.7 mμ (model UV-30 lamp manufactured by the American Sterilizer Co.). The conidial suspension, which was shaken continually during exposure, was 7.5 cm from the ultraviolet light bulb. The time of ultraviolet exposure ranged from 30 seconds to two minutes depending on the strain irradiated and the degree of killing desired. Samples of conidia were applied to sorbose plates immediately before and after exposure to UV light in order to determine the per cent of killing.

The degree of killing desired was such that the majority of surviving conidia would have only one viable nucleus. Thus, if a mutation occurred in one of these nuclei, the effect of the mutation could not be masked by the presence of a normal nucleus. A commonly used per cent of

kill in mutant hunts involving macroconidial strains is 90 to 99%. In these experiments such a killing range was tried, but was found undesirable for two reasons: (1) a large number of the isolated colonies grew very slowly, often not producing enough mycelia to inoculate a series of flasks; (2) no mutants of the desired type were found. Much better success was achieved when conidia were exposed to UV light until only 40 to 60% were killed.

Filtration enrichment method

The filtration enrichment method is frequently used for isolation of specific nutritional mutants in Neurospora (35, 36, 37). The method works especially well for finding mutants which require a considerable amount of growth factor to give appreciable growth. In the procedure conidia which have been exposed to mutagen are grown in a liquid minimal medium. By repeated filtering through some type of sterile filter, germinated conidia are removed. Conidia which have undergone mutation so that they cannot manufacture some growth factor tend not to germinate and to pass through the filter. Thus, the medium is enriched in mutant conidia. The conidia remaining after several filtrations are inoculated onto agar medium supplemented with the growth factors for which nutritional mutants are sought.

In these experiments the approach was somewhat different in that mutants were desired that block various steps in pyrimidine salvage pathways. In order to observe the presence or absence of the salvage pathways by growth tests the normal pyrimidine synthetic pathway must be blocked. Therefore, instead of wild type a pyrimidine mutant was

used as the source of conidia. In place of minimal medium the filtration enrichment medium was supplemented with a pyrimidine compound. The selection was then for mutants which could not use the pyrimidine supplement in the enrichment medium, but could use whatever pyrimidine supplement was added to the medium onto which surviving conidia were inoculated.

After UV irradiation 2.5 ml of the 4 X 10^6 conidia/ml suspension was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of medium supplemented with two to three μmoles of the pyrimidine for which nutritional mutants were desired. The conidia were kept in suspension by placing the flask on a rotary shaker. Whenever mycelia became visible the contents of the flask were filtered into a second flask through a thistle tube containing a layer of glass wool. The amount of glass wool was such that the flow of conidial suspension through it was only slightly impeded. The time of incubation in the enrichment medium before the final filtration and subsequent inoculation of sorbose plates was 48 to 72 hours. The total number of filtrations required was seven to eight; the first at five to ten hours followed by four or five more filtrations within the next 24 hours.

Sorbose plates

Immediately after UV irradiation or following the filtration enrichment procedure conidia were spread onto agar plates containing sorbose. A combination of a reduction in sucrose concentration to 0.1 - 0.2% and addition of 1.0 to 1.5% L-sorbose to the medium causes each germinating conidium to produce mycelium that is colonial in growth habit (38).

The sterilized sorbose medium was poured into sterile plastic 15 X 100 mm Petri dishes. Each dish contained 25 ml Westergaard-Mitchell medium (normal concentration of salts, trace elements and biotin), 0.2% sucrose, 1.5% sorbose, 2.0% agar and various pyrimidine supplements depending on the type mutant being selected for. For purposes of brevity the above Petri dishes and their contents will be referred to only as sorbose plates containing a given kind and concentration of pyrimidine.

Colonial growth on sorbose plates

Depending on the concentration of viable conidia 0.1 to 1.0 ml of conidial suspension was added to each sorbose plate and spread over the surface with a glass rod. The inoculated sorbose plates were kept at 25°C. At two to three and one-half days after inoculation colonies became easily visible to the naked eye. Beginning at this stage the colonies grew quite rapidly and within an additional 24 to 36 hours would produce aerial hyphae and form conidia. Therefore, with one exception (to be discussed under the section describing the isolation of the pyr-4 uc-2 double mutant), all colonies were removed from the sorbose plates as soon as they became readily visible and were transferred to either complete medium agar tubes or crossing medium agar tubes with a uridine supplement.

A characteristic of UV irradiation is that it slows the germination and initial growth of many conidia. The first colonies appeared on the sorbose plates at two and one-half to three days, but a few colonies were still arising at seven days after inoculation. For this reason the sorbose plates were scanned and newly appearing colonies removed

every 24 hours over a period of four or five days. This repeated scanning and removal of colonies was very important since a large majority of the colonies picked up were not mutant and those mutants that were found were frequently not isolated until the fifth to seventh day after inoculation.

PYR-4 MUTANT

Introduction

Fink and Fink reported that thymidine, thymine, uracil-5-carboxylic acid and 5-hydroxymethyluracil have a sparing action for uridine when uridine is present in amounts suboptimal for the growth of the pyr-4 mutant (7, 10). Further, the radioactive labels of pyrimidine ring labeled thymidine, thymine and 5-hydroxymethyluracil were incorporated into RNA and DNA in such a manner that both nucleic acids had the same specific activity (9, 10). When the radioactive label was in the 5-methyl position of thymine or thymidine no label was found in the RNA and DNA at all. Their conditions for these experiments were a standard Fries medium, a growth limiting concentration of uridine and an equal concentration of some labeled pyrimidine. Experiments were pursued to verify the above results and to determine if feeding a labeled pyrimidine under other growth conditions could result in DNA with a higher specific activity than RNA.

Effect of growth medium

An initial discovery was that thymidine, thymine and 5-hydroxymethyluracil had a much larger sparing action for uridine in Westergaard-Mitchell medium than in the standard Fries medium. For this reason growth experiments involving the pyr mutants and selection procedures for mutants affecting pyrimidine salvage pathways were carried out in Westergaard-Mitchell medium rather than Fries. The sparing actions of thymidine and uracil-5-carboxylic acid for uridine in the two growth media are shown in Table 1. Of the intermediates

Table 1.--Effect of growth medium on utilization of uracil-5-carboxylic acid and thymidine by pyr-4 mutant.

Pyrimidine Supplement µmoles/flask	Dry Wt. (mg) of Pad[1]	
	Fries medium	Westergaard-Mitchell medium
0.5 uridine	13.4	9.1
0.5 uridine + 0.5 thymidine	16.6	21.5
0.5 uridine + 1.5 thymidine	16.7	32.4
2.0 uridine	37.5	30.1
2.0 uridine + 0.5 thymidine	41.0	43.8
2.0 uridine + 1.5 thymidine	42.8	67.3
1.0 uridine	22.6	17.5
1.0 uridine + 1.0 5-COOH uracil[2]	--	18.0
1.0 uridine + 3.0 5-COOH uracil	24.1	18.4
1.0 uridine + 9.0 5-COOH uracil	25.3	19.5
1.5 thymidine	0	0
3.0 5-COOH uracil	0	0

[1] Each figure is the average weight of three pads. Time of growth--5 days.

[2] Uracil-5-carboxylic acid

which could possibly be found in the thymine-5-methyl oxidative pathway (5-CH_3, 5-CH_2OH, 5-CHO and 5-COOH) only uracil-5-carboxylic acid failed to give a large sparing action for uridine on Westergaard-Mitchell medium.

Nucleic acid extraction procedure

Experiments to show the effectiveness of the RNA and DNA extraction procedure and to determine the nucleic acid content of three strains of Neurospora grown for various lengths of time in two different media are given in Table 2. The reliability of both the extraction procedure in separating RNA from DNA and of the calculation of nucleic acid concentration from the UV absorption of the extracts was confirmed by RNA and DNA concentration measurements based on Pentose (Bial's reagent) and deoxypentose (diphenylamine reagent) content of the extracts. An interesting difference noted between mycelial pads grown in the two different media was that the RNA and DNA content of Neurospora grown in Fries medium was always higher than Neurospora grown for the same length of time in Westergaard-Mitchell medium.

2-C^{14} thymine label

The pyr-4 mutant was grown in three types of media containing 2-C^{14} thymine as reported in Table 3. Three observations were made from this experiment. (1) A high proportion of label was found in the nucleic acid extracts--about 85% when the Neurospora was grown in Westergaard-Mitchell medium and about 50% when the Neurospora was grown in Fries medium. (2) A great majority (85 to 90%) of the nucleic acid label was found in the RNA fraction. (3) Changes in the growth

Table 2.--RNA and DNA concentrations of pyr-3, pyr-4 and wild type Neurospora strains in two different growth media.

Strain	Growth Medium	Days Growth	Pad Wt (mg) Per Flask	RNA mg/g[1] measured by		DNA mg/g measured by	
				$O.D._{260}$	Bial's	$O.D._{268}$	Diφamine
pyr-3	W.-M.[2]	6	65	30.0	28.1	2.28	2.50
pyr-4	W.-M.	5	75	27.2	33.3	1.74	2.18
pyr-4	Fries	6	67	48.2	51.9	3.83	4.28
wild	W.-M.	2	31	57.0	--	3.83	--
wild	W.-M.	3	66	31.7	--	3.43	--
wild	W.-M.	4	101	21.2	--	2.56	--
wild	W.-M.	5	97	18.2	--	2.52	--
wild	Fries	1	15	103.3	--	4.57	--
wild	Fries	2	75	75.1	--	4.29	--
wild	Fries	3	109	59.0	--	4.25	--
wild	Fries	4	105	56.0	--	4.42	--
wild	Fries	5	112	50.6	--	4.68	--

[1] Dry, alcohol and ether extracted mycelial pad

[2] Westergaard-Mitchell growth medium

Strains of Neurospora were grown in flasks containing 25 ml of medium at 25°C on a shaker. Pyrimidine supplements were 5.0 μmoles of uracil/flask for the pyr-3 mutant and 5.0 μmoles of uridine/flask for the pyr-4 mutant. RNA and DNA were extracted from 250 mg samples of ground mycelia. Extraction and nucleic acid measuring procedures are described in the materials and methods section.

Table 3.--Utilization and distribution of 2-C^{14} thymine label by pyr-4 mutant grown in three types of media.

Solution Counted	CPM in Growth Medium and Pad Extracts		
	W.-M.[1] limiting ud.	Fries[2] limiting ud.	Fries[3] excess ud.
Growth medium at start	2,479,000	2,479,000	1,240,000
Growth medium at 4 1/2 days	233,000	1,009,000	559,000
Alcohol extracts	38,000	21,000	14,000
Alcohol-ether and ether extracts	500	1,500	1,000
RNA extracts	1,869,000	998,000	595,000
DNA extracts	257,000	113,000	76,000
Per cent of label accounted for after 4 1/2 days growth	97%	86%	100%
Per cent of label at zero time found in RNA and DNA at 4 1/2 days	86%	45%	54%
Per cent of total nucleic acid counts due to DNA	14%	10%	11%

[1] Limiting uridine Westergaard-Mitchell medium--Two flasks each containing 25 ml of Westergaard-Mitchell medium + 1.0 μmole of uridine + 0.045 μmole of 2-C^{14} thymine (1 μcurie) were inoculated with pyr-4 and grown for 4 1/2 days. The resulting two pads were pooled (dry wt. 58 mg) and extracted.

[2] Limiting uridine Fries medium--Two flasks each containing 25 ml of Fries medium + 1.0 μmole of uridine + 0.045 μmole of 2-C^{14} thymine (1 μcurie) were inoculated with pyr-4 and grown for 4 1/2 days. The resulting two pads were pooled (dry wt. 72 mg) and extracted.

[3] Excess uridine Fries medium--One flask containing 25 ml of Fries + 5.0 μmoles of uridine + 0.045 μmole of 2-C^{14} thymine was inoculated with pyr-4 and grown for 4 1/2 days. The resulting pad (dry wt. 79 mg) was then extracted.

medium and alterations of the uridine-thymidine ratio did not cause the ratio of label in RNA to label in DNA to change significantly.

2-C^{14} deoxyuridine label

This experiment, the details of which are given in Table 4, was similar to the one just described except that 2-C^{14} deoxyuridine was the labeled compound and a wild type strain was used in addition to the pyr-4 mutant. One-half or more of the label initially present in the growth medium was found in the nucleic acid extracts of both pyr-4 and wild type strains. In each case approximately 90% of the nucleic acid counts were found in the RNA and only about 10% were in the DNA extract.

Thymidine-methyl-H^3 label

When pyr-3 and pyr-4 mutants were grown in media containing thymidine labeled in the 5-methyl position the amount of label found in the nucleic acid extracts was negligible (Table 5). At the end of the incubation period all the label still remained in the growth media although one-fourth to one-half of the H^3 was in a volatile form, presumably water, indicating that at least this much of the thymidine had been metabolized. If Neurospora had a thymidine phosphorylating enzyme, use of 5-methyl labeled thymidine would provide a way of specifically labeling DNA even in the presence of a pathway converting the pyrimidine ring of thymidine to RNA precursors. The labeling experiments clearly indicate that Neurospora does not have thymidine, deoxyuridine and thymine phosphorylating mechanisms, but does have an efficient system for salvaging the pyrimidine ring of these compounds for use in both RNA and DNA synthesis.

Table 4.--Utilization and distribution of 2-C^{14} deoxyuridine label by *pyr-4* and wild type strains.

Solution Counted	CPM in Growth Medium and Pad Extracts		
	pyr-4 Fries medium[1]	*pyr-4* W.-M. medium[2]	wild W.-M. medium[3]
Growth medium at start	863,000	863,000	762,000
Growth medium at 5 days	196,000	21,000	25,000
Alcohol extracts	10,000	3,000	29,000
Alcohol ether and ether extracts	500	500	2,000
RNA extracts	521,000	334,000	435,000
DNA extracts	52,000	46,000	43,000
Per cent of label accounted for after 5 days growth	90%	47%	70%
Per cent of label at zero time found in RNA and DNA at 5 days	66%	44%	63%
Per cent of total nucleic acid counts due to DNA	9%	12%	9%

[1] Limiting uridine Fries medium--Two flasks each containing 25 ml of Fries medium + 1.0 μmole of uridine + 0.022 μmole of 2-C^{14} deoxyuridine (0.5 μcurie) were inoculated with *pyr-4* and grown for five days. The resulting two pads were pooled (dry wt. 54 mg) and extracted.

[2] Limiting uridine Westergaard-Mitchell medium--Two flasks each containing 25 ml of Westergaard-Mitchell medium + 1.0 μmole of uridine + 0.022 μmole of 2-C^{14} deoxyuridine (0.5 μcurie) were inoculated with *pyr-4* and grown for five days. The resulting two pads were pooled (dry wt. 32 mg) and extracted.

[3] One flask containing 25 ml of Westergaard-Mitchell medium + 0.022 μmole of 2-C^{14} deoxyuridine (0.5 μcurie) was inoculated with wild type and grown for five days. The resulting pad (dry wt. 75 mg) was then extracted.

Table 5.--Utilization and distribution of thymidine-methyl-H^3 by pyr-3 and pyr-4 mutants grown in Fries and Westergaard-Mitchell medium.

Medium or Extract Counted	DPM in Medium or Extract / DPM in Medium at Start	
W.-M. medium at start	100% (47,490,000 DPM)	
Fries medium at start	100% (47,300,000 DPM)	
W.-M. medium (pyr-4) at 92 hours	100%	51%[1/]
W.-M. medium (pyr-3) at 92 hours	105%	54%
Fries medium (pyr-4) at 92 hours	100%	76%
Fries medium (pyr-3) at 92 hours	105%	72%
RNA extract of pyr-4 grown on W.-M.	0.39%	
RNA extract of pyr-3 grown on W.-M.	0.51%	
RNA extract of pyr-4 grown on Fries	0.46%	
RNA extract of pyr-3 grown on Fries	0.46%	
DNA extract of pyr-4 grown on W.-M.	0.10%	
DNA extract of pyr-3 grown on W.-M.	0.16%	
DNA extract of pyr-4 grown on Fries	0.06%	
DNA extract of pyr-3 grown on Fries	0.06%	

[1/] This column represents radioactivity remaining after volatile components of the used medium were evaporated away.

Four flasks were prepared (two with 25 ml of Westergaard-Mitchell medium and two with 25 ml of Fries medium) each containing 5.0 μmoles of uridine and 0.0033 μmole of thymidine-methyl-H^3 (20 μcuries). Pyr-4 and pyr-3 conidia were inoculated into one flask of each type medium and grown for 92 hours on a shaker. Dry weights and nucleic acid contents of nonlabeled controls grown at the same time are as follows.

Mutant	Medium	Dry Wt./Flask	RNA (mg/g)	DNA (mg/g)
pyr-4	W.-M.	35 mg	55.2	3.26
pyr-3	W.-M.	44 mg	47.5	3.21
pyr-4	Fries	59 mg	62.0	4.64
pyr-3	Fries	66 mg	60.0	4.68

PYR-4 UC-1 DOUBLE MUTANT

Selection of mutant

The pyr-4 mutant, like the other pyrimidine mutants will not grow at all when thymidine, thymine or 5-hydroxymethyluracil are the sole pyrimidine sources. However, in Westergaard-Mitchell medium containing limiting amounts of uracil, uridine, or cytidine added thymidine, thymine, or 5-hydroxymethyluracil supplements show a large sparing action. Since a pathway for utilization of these compounds obviously existed an attempt was made to find mutants which would use the compounds as their sole pyrimidine sources. Since such mutants would have activity that the normal pyr-4 mutant did not have large numbers of conidia could be screened.

A 4 X 10^6 conidia/ml suspension of pyr-4 conidia was given UV irradiation for one minute (50% kill) and 400,000 conidia were inoculated onto each of 44 sorbose plates containing 0.75 μmole of thymidine plus 0.75 μmole of thymine per 100 ml. Fiftynine colonies appeared and were transferred to Westergaard-Mitchell medium plus uridine, agar tubes. In testing the isolates 54 were found to grow on minimal medium and were probably pyr-4 revertants or pyr-4 suppressor mutants. Five isolates did not grow on minimal medium, but did grow on crossing medium with only a thymine or thymidine supplement as shown in Table 6. The new mutation present in these isolates was given the designation uracil-1, abbreviated uc-1.

Cross to wild type

Only one isolate, pyr-4 uc-1 #57 was chosen for further work. The

Table 6.--Comparison of growth of pyr-4 parent with five pyr-4 uc-1 isolates in medium supplemented with thymidine, thymine and uridine.

Pyrimidine Supplement µmole/flask	Dry Wt. (mg) of Pad; 5 Days Growth with Following Strains					
	pyr-4 parent	pyr-4 uc-1 no. 36	pyr-4 uc-1 no. 38	pyr-4 uc-1 no. 52	pyr-4 uc-1 no. 57	pyr-4 uc-1 no. 59
None	0	0	0	0	0	0
1.0 thymidine	0	7	32	28	21	20
1.0 thymine	0	13	24	20	21	16
1.5 thymidine + 1.5 thymine	0	20	41	--	62	61
3.0 uridine	61	23	18	44	30	38

mutant was crossed to wild type 4A; asci were dissected; and the genotypes of the spore pairs were determined by growth tests in minimal medium and in medium supplemented with thymine and thymidine. Pyr-4 mutants could not grow in either medium; pyr-4 uc-1 mutants could grow in the supplemented but not the minimal medium. Wild or uc-1 mutants grew profusely in both growth media. By growth tests it was not possible to determine the difference between a wild or uc-1 strain. The data in Table 7 suggest that the pyr-4 and uc-1 loci are linked, although not closely. However, in two later crosses where one parent was a pyr-4 uc-1 mutant reisolated from the above cross, there was no recombination between the pyr-4 and uc-1 loci.

Growth tests

A pyr-4 uc-1 strain selected from those isolated in the cross of pyr-4 uc-1 #57 X 4A was used in the growth response experiment, as well as in labeling experiments and as a source of conidia for UV irradiation in experiments to be discussed later. Figure 2 gives the response of the pyr-4 uc-1 double mutant to a concentration series of four pyrimidines. The mutant utilizes thymidine, thymine and 5-hydroxymethyluracil equally well while uridine is used with less efficiency. It can be seen in Table 6 that all the pyr-4 uc-1 isolates found use uridine only about 50% as well as the parent pyr-4 strain.

2-C^{14} thymidine and 2-C^{14} thymine labeling

The results of administering pyrimidine ring labeled thymine and thymidine to the pyr-4 uc-1 double mutant are given in Table 8. With

Table 7.--Types of asci resulting from cross of pyr-4 uc-1 a (isolate 57) X 4A wild.

Spore Pair		No. Asci	Spore Pair		No. Asci
1	pyr-4 uc-1	5	1	pyr-4 uc-1	2
2	pyr-4 uc-1		2	+ +	
3	+ +		3	pyr-4 uc-1	
4	+ +		4	+ +	
1	pyr-4 +	6	1	pyr-4 uc-1	3
2	pyr-4 uc-1		2	pyr-4 +	
3	+ + or uc-1		3	+ + or uc-1	
4	+ + or uc-1		4	+ + or uc-1	
1	pyr-4 +	2			
2	+ + or uc-1				
3	pyr-4 uc-1				
4	+ + or uc-1				

Parental ditype 7, Nonparental ditype 0, Tetratype 11.

	No. Asci
pyr-4 segregating 1st division	14
pyr-4 segregating 2nd division	4
uc-1 segregation unclassified	2
uc-1 segregating 1st division	5
uc-1 segregating 2nd division	11
Both segregating 1st division of these 5	5
Parental combinations	5
New combinations	0

	Mating Type	
	A	a
17 pyr-4 uc-1 spore pairs	9	8

Conclusion: Uc-1 segregates as a single gene; uc-1 and pyr-4 are linked.

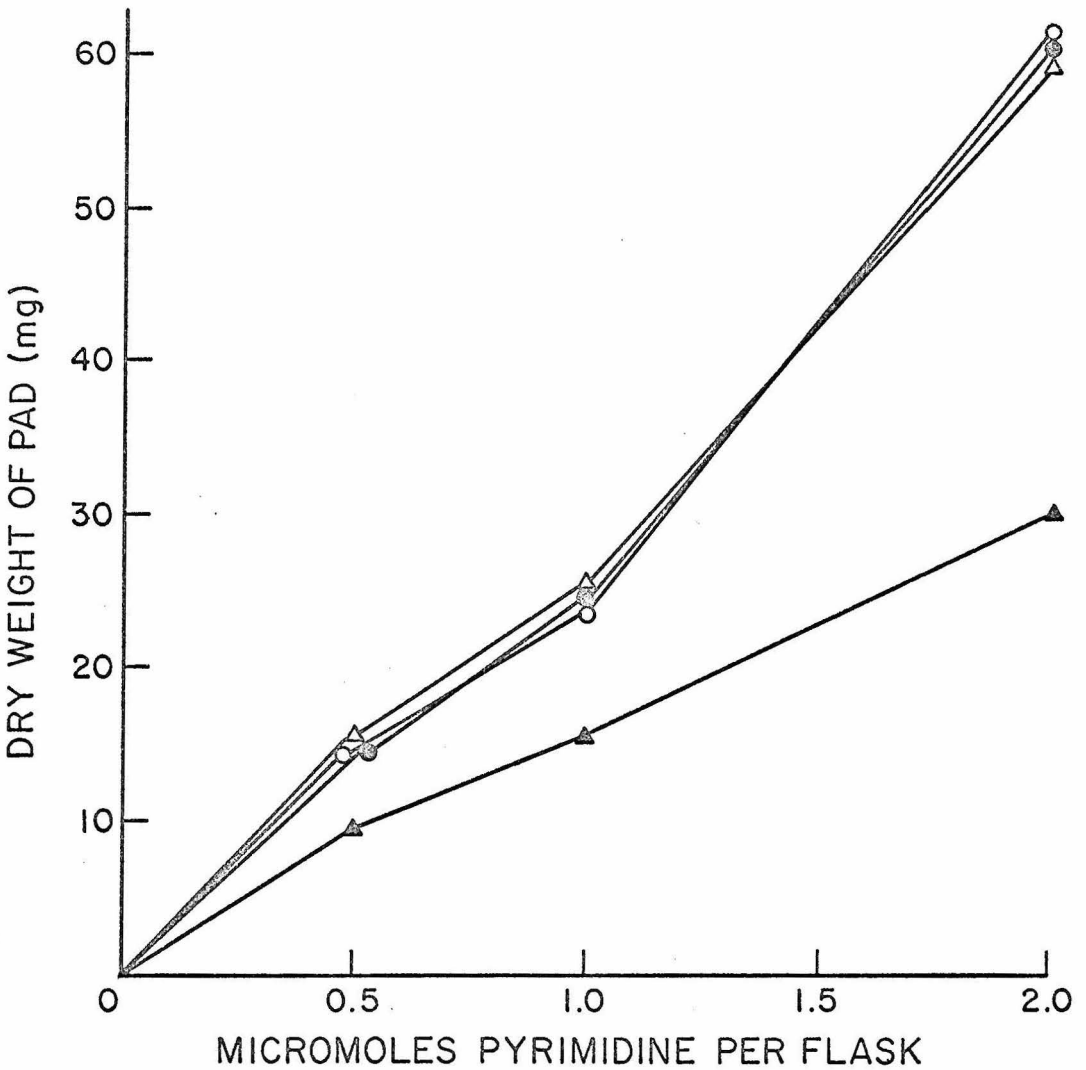


Figure 2 GROWTH RESPONSE OF pyr-4 uc-1 DOUBLE MUTANT TO THYMIDINE △—△, URIDINE ▲—▲, THYMINE ○—○ AND 5-HYDROXYMETHYLURACIL ◉—◉. EACH POINT IS THE AVERAGE DRY WEIGHT OF PADS FROM THREE FLASKS. TIME OF GROWTH WAS 6 DAYS.

Table 8.--Utilization and distribution of 2-C^{14} thymidine and 2-C^{14} thymine label by pyr-4 uc-1 double mutant.

Solution Counted	CPM in Growth Medium and Pad Extracts	
	2-C^{14} thymidine label	2-C^{14} thymine label
Growth medium at start	940,000	1,230,000
Growth medium at 6 days	246,000	198,000
Alcohol extracts	21,000	11,000
Alcohol-ether and ether extracts	500	500
RNA extracts	516,000	696,000
DNA extracts	27,000	50,500
Per cent of label accounted for after 6 days growth	86%	78%
Per cent of label at zero time found in RNA and DNA at 6 days	58%	61%
Per cent of total nucleic acid counts due to DNA	5%	7%
Mg RNA in pad	1.08	1.16
Mg DNA in pad	0.08	0.11
Per cent of total nucleic acid that is DNA	7.4%	9.5%

Two flasks each containing 25 ml of growth medium + 1.0 μmole of 2-C^{14} thymidine (specific activity approximately 0.75 μcurie/μmole) and a second two flasks containing 25 ml of growth medium + 1.0 μmole of 2-C^{14} thymine (specific activity approximately 1.0 μcurie/μmole) were inoculated with pyr-4 uc-1 conidia and grown six days at 25°C on a shaker. RNA and DNA determinations were made from the optical density of the two extracts at 260 and 268 mμ respectively.

both pyrimidines approximately 50% of the label initially present in the medium was recovered in the RNA fraction of the extracted mycelia. The specific activity of the DNA fraction (counts per minute in DNA/mg DNA) was not greater than the specific activity of the RNA fraction. Thus, the uc-1 mutation does not affect the pyrimidine pathways in a way which would permit thymidine or thymine to become incorporated into DNA without passing through an RNA precursor pool.

PYR-4 UC-2 DOUBLE MUTANT

Selection of mutant

Several attempts were made to find mutants in which the pathway converting the pyrimidine ring of thymidine to an RNA precursor would be blocked at different steps. The first method used to select for such mutants was based on the finding that pyr-4 conidia would produce larger colonies on sorbose plates containing very limiting uridine plus thymidine (0.18 μmole of uridine + 2.0 μmoles of thymidine) than on sorbose plates containing only the limiting uridine. Presumably mutants which could not utilize thymidine would produce small colonies on the uridine plus thymidine sorbose plates.

The selection procedure consisted of treating a 1×10^6 pyr-4 conidia/ml suspension with UV irradiation for 90 seconds (50 to 75% kill) and inoculating onto limiting uridine plus thymidine supplemented sorbose plates at a conidial concentration to produce five to twenty colonies per plate. At a time when the largest colonies to appear were almost ready to produce conidia the smallest colonies were transferred to agar tubes of complete medium. Each isolate was tested in two flasks of liquid medium, the first containing only a uridine supplement and the second uridine plus thymidine. Of the 2,100 colonies examined 352 small colonies were isolated and tested further. Of these all but one were either slow growers or demonstrated a large thymidine for uridine sparing action in growth experiments. One isolate showed no sparing action of thymidine for uridine, but thymine and 5-hydroxymethyluracil did have a sparing action. The new mutation present in this isolate was given the designation uracil-2, abbreviated uc-2.

Cross to wild type

The pyr-4 uc-2 double mutant isolate was crossed to wild type 4A, asci were dissected and conidia from the germinated spore pairs were inoculated into liquid medium containing 1.0 μmole of uridine per flask and medium containing 1.0 μmole of uridine plus 1.0 μmole of thymidine per flask. As with the uc-1 mutant, the uc-2 mutant phenotype can only be observed when the pyr-4 mutation is present. The data in Table 9 indicate that uc-2 and pyr-4 are not linked. However, the observation was made that all segregants known to be carrying the uc-2 mutation were a mating type when put into a sexual cross. Therefore, the uc-2 and mating type loci must be rather closely linked.

Growth tests

A pyr-4 uc-2 strain selected from those reisolated in the cross of pyr-4 uc-2 X 4A was used in the growth response and C^{14} labeling experiments. A uc-2 strain taken from the segregants of the above cross was also used in growth experiments and as a parent in later crosses involving the uc-2 mutation. The dry pad weights reported in Table 10 reveal that with both the pyr-4 and pyr-4 uc-2 mutants thymine and 5-hydroxymethyluracil had a large sparing action for uridine while only the pyr-4 mutant gave a large sparing action with thymidine. Furthermore, the pyr-4 mutant can utilize deoxyuridine and deoxycytidine as pyrimidine sources while the pyr-4 uc-2 mutant cannot.

The action of the uc-2 mutation in preventing utilization of the three deoxypyrimidine nucleosides could be caused by an inactive deoxypyrimidine nucleosidase enzyme. Although such enzymes have not been

Table 9.--Types of asci resulting from cross of pyr-4 uc-2 a X 4A wild.

Spore Pair			No. Asci	Spore Pair			No. Asci
1	pyr-4	uc-2	3	1	pyr-4	+	1
2	pyr-4	uc-2		2	pyr-4	uc-2	
3	+	+		3	+	+ or uc-2	
4	+	+		4	+	+ or uc-2	
1	pyr-4	+	1	1	pyr-4	+	1
2	+	+ or uc-2		2	+	+ or uc-2	
3	+	+ or uc-2		3	pyr-4	uc-2	
4	pyr-4	uc-2		4	+	+ or uc-2	
1	+	+ or uc-2	1	1	pyr-4	+	3
2	pyr-4	uc-2		2	pyr-4	+	
3	pyr-4	+		3	+	uc-2	
4	+	+ or uc-2		4	+	uc-2	
1	+	uc-2	1				
2	pyr-4	+					
3	pyr-4	+					
4	+	uc-2					

Parental ditype 3, Nonparental ditype 4, Tetratype 4.

	No. Asci
pyr-4 segregating 1st division	7
pyr-4 segregating 2nd division	4
uc-2 segregation unclassified	3
uc-2 segregating 1st division	6
uc-2 segregating 2nd division	2
Both segregating 1st division of these 6	6
Parental combination	3
New combinations	3

	Mating Type	
	A	a
12 uc-2 carrying spore pairs	0	12

Conclusion: Uc-2 segregates as a single gene; uc-2 and pyr-4 are not linked. Uc-2 and mating type loci are linked.

Table 10.--Growth response of pyr-4 and pyr-4 uc-2 mutants to thymidine, deoxyuridine, deoxycytidine, thymine and 5-hydroxymethyluracil.

Pyrimidine Supplement μmoles/flask	Dry Wt. (mg) of Pad[1/]	
	pyr-4	pyr-4 uc-2
0.5 uridine	11	12
0.5 uridine + 1.0 thymidine	30	13
0.5 uridine + 1.0 deoxyuridine	33	14
0.5 uridine + 1.0 deoxycytidine	25	13
0.5 uridine + 1.0 thymine	29	36
0.5 uridine + 1.0 5-hydroxymethyluracil	35	40
1.0 uridine	18	20
1.0 uridine + 1.0 thymidine	38	20
1.0 uridine + 1.0 deoxyuridine	43	21
1.0 uridine + 1.0 deoxycytidine	31	19
1.0 uridine + 1.0 thymine	39	43
Minimal	0	0
1.0 thymidine	0	0
1.0 deoxyuridine	20	3
1.0 deoxycytidine	6	trace
1.0 thymine	0	0
1.0 5-hydroxymethyluracil	0	0

[1/] Each figure is the average weight of two pads. Time of growth--5 days.

worked with extensively they have been shown to act on a variety of deoxypyrimidine nucleosides while being unable to cleave deoxyribose purine and ribose pyrimidine nucleosides (39, 40). For these reasons growth experiments were undertaken to determine the growth inhibiting effect of the deoxypyrimidine nucleoside, 5-fluorodeoxyuridine (5-FUdR), on strains carrying the uc-2 mutation (Table 11).

In E. coli 5-FUdR acts as a growth inhibitor by specifically blocking the thymidylate synthetase enzyme (41). Attempts were made to use this specific inhibition to select for mutants of Neurospora which could phosphorylate thymidine. Observation of conidia on agar plates containing 5-FUdR showed that the conidia would germinate and grow very slowly for several days until finally overcoming the inhibiting effect, probably because of breakdown of the 5-FUdR by the mold. The fact that strains bearing the uc-2 mutation cannot overcome the inhibition of 5-FUdR as rapidly as strains lacking it (Table 11) could be explained if one assumed that uc-2 carrying strains have much less deoxypyrimidine nucleosidase activity and as a result 5-FUdR is broken down less rapidly. The growth differences between pyr-4 and pyr-4 uc-2 mutants in the presence of 5-FUdR are not so large, probably because uridine is a competitive inhibitor of 5-FUdR.

Labeling experiment

Data reported in Table 12 reveal that both mutants convert 65 to 75% of the label originally present as 2-C^{14} thymine into RNA constituents. The pyr-4 mutant utilizes the label of 2-C^{14} thymidine similarly, but the pyr-4 uc-2 mutant transforms only 11% of this label into RNA

Table 11.--Effect of 5-fluorodeoxyuridine (5-FUdR) on growth of pyr-4, pyr-4 uc-2, uc-2 and 4A strains.

Pyrimidine Supplement μmoles/flask	Dry Wt. (mg) of Pad[1]			
	pyr-4	pyr-4 uc-2	wild 4A	uc-2
Minimal	0	0	94	116
0.05 5-FUdR	--	--	51	4.8
0.25 5-FUdR	--	--	16	1.6
1.25 5-FUdR	--	--	3.6	0.7
4.0 uridine	52	51	--	--
4.0 uridine + 0.05 5-FUdR	57	42	--	--
4.0 uridine + 0.25 5-FUdR	47	33	--	--
4.0 uridine + 1.25 5-FUdR	28	15	--	--

[1] Each figure is the average weight of two pads. Time of growth--72 hours.

Table 12.--Utilization and distribution of 2-C^{14} thymidine and 2-C^{14} thymine label by pyr-4 and pyr-4 uc-2 mutants.

Solution Counted	CPM in Growth Medium and Pad Extracts			
	Pyr-4 Mutant		Pyr-4 Uc-2 Mutant	
	with 2-C^{14} thymidine label	with 2-C^{14} thymine label	with 2-C^{14} thymidine label	with 2-C^{14} thymine label
Growth medium at start	907,000	1,486,000	1,418,000	1,415,000
Growth medium at 90 hours	71,000	119,000	1,104,000	110,000
Alcohol extract	10,000	39,000	17,000	28,000
Alcohol-ether and ether extracts	500	1,000	1,500	500
RNA extracts	660,000	890,000	135,000	966,000
DNA extracts	51,000	81,000	14,000	87,000
Per cent of label accounted for after 90 hours growth	87%	76%	90%	84%
Per cent of label at zero time found in RNA and DNA at 90 hours	78%	65%	11%	74%
Per cent of total nucleic acid counts due to DNA	7%	8%	9%	8%
Mg RNA in pad	1.40	1.33	1.37	1.48
Mg DNA in pad	0.138	0.143	0.132	0.130
Per cent of total nucleic acid that is DNA	9.0%	9.7%	8.8%	8.1%

Five flasks each containing 25 ml of growth medium + 1.0 μmole of uridine were prepared. To each of three flasks was added 1.0 μmole of 2-C^{14} thymidine (specific activity 1.5 μcuries/μmole) while the other two flasks each received 1.0 μmole of 2-C^{14} thymine (specific activity 2.0 μcuries/μmole). Pyr-4 uc-2 conidia were inoculated into two of the thymidine and one of the thymine supplemented flasks while pyr-4 conidia provided the inoculum for the remaining two flasks. The mutants were grown on a shaker for 90 hours at 25°C.

The two pyr-4 uc-2 pads from thymidine supplemented medium were only half the size of pads from the other flasks and were therefore combined for extraction. Each of the four pads extracted had a dry weight of about 35 to 40 mg.

while over 75% remains in the growth medium, presumably as unused thymidine. If a weak thymidine phosphorylating system did exist in Neurospora, it might be expected that the DNA of the pyr-4 uc-2 mutant grown in 2-C^{14} thymidine would have a higher specific activity than the RNA since the conversion of thymidine into an RNA precursor was largely blocked in this mutant. However, no evidence indicating a thymidine phosphorylating enzyme was found. The specific activities of the RNA and DNA extracts (counts in RNA or DNA/mg RNA or DNA) of a pyr-4 uc-2 mutant administered 2-C^{14} thymidine were approximately the same.

Other crosses involving uc-2

A cross of uc-2 a X pyr-4 uc-1 A was made in order to obtain the pyr-4 uc-1 uc-2 triple mutant. The results of this cross and the type growth tests used to distinguish some of the possible mutant combinations which could arise from the cross are shown in Table 13. The action of the uc-2 mutation in the prevention of all but slight utilization of thymidine was also observed in the pyr-4 uc-1 uc-2 triple mutants. In Table 13 and in future references to the triple mutant strain isolated from the uc-2 a X pyr-4 uc-1 A cross the suffix "I" will be added to the mutant designation to distinguish it from triple mutants of similar phenotype which were isolated by other methods.

The same uc-2 a mutant that was used in the above cross was mated to an adenine-6 (ad-6) mutant which is not linked to uc-2. The ad-6 mutant can use deoxyadenosine as its sole purine source, and deoxyguanosine has a large sparing action in the presence of limiting

Table 13.--Types of asci resulting from cross of pyr-4 uc-1 A X uc-2 a.

Spore Pair				No. Asci	Spore Pair				No. Asci
1	pyr-4	uc-1	+	2	1	+	+	uc-2	1
2	pyr-4	uc-1	+		2	pyr-4	uc-1	+	
3	+	+	uc-2		3	pyr-4	uc-1	+	
4	+	+	uc-2		4	+	+	uc-2	
1	pyr-4	uc-1	uc-2	3	1	pyr-4	uc-1	uc-2	1
2	pyr-4	uc-1	uc-2		2	+	+	+ or uc-2	
3	+	+	+		3	pyr-4	uc-1	+	
4	+	+	+		4	+	+	+ or uc-2	

Conclusion: no recombination between pyr-4 and uc-1; uc-1 and uc-2 segregate as unlinked single genes.

Growth Tests to Distinguish Between
Pyr-4, Pyr-4 Uc-1, Pyr-4 Uc-2, and Pyr-4 Uc-1 Uc-2 Mutants

Pyrimidine Supplement µmoles/flask	Dry Wt. (mg) of Pad[1/]					
	pyr-4	pyr-4 uc-2	pyr-4 uc-1 uc-2 I	pyr-4 uc-1	uc-2	wild 4A
Minimal	0	0	0	0	169	166
1.0 thymidine	0	0	0	20	-	-
1.0 thymine	0	0	17	17	-	-
1.0 uridine	16	19	13	13	-	-
1.0 uridine + 1.0 thymidine	43	20	17	38	-	-

[1/] Each figure is the average weight of three pads. Time of growth--5 days.

adenosine. Forty ad-6 carrying ascospores were taken from the cross and conidia from these isolates were inoculated into medium containing deoxyadenosine and medium containing adenosine plus deoxyguanosine. All 40 isolates used the deoxypurines even though half of them should have been carrying the uc-2 mutation. Although uc-2 prevents use of deoxypyrimidines it apparently does not affect deoxypurine utilization.

PYR-4 UC-1 UC-2 AND PYR-4 UC-1 UC-3 TRIPLE MUTANTS

Selection of Mutants

The possession of the pyr-4 uc-1 double mutant made possible a new selection procedure for finding mutants blocked in the pathway between thymidine and RNA. By employing a filtration enrichment procedure and growing UV irradiated pyr-4 uc-1 conidia in a medium containing thymidine the medium should become enriched in mutant conidia which cannot utilize thymidine. The summarized results of several filtration enrichment experiments are reported in the following paragraph.

UV irradiation was applied to 90 X 10^6 pyr-4 uc-1 conidia to give a 30 to 50% kill. The conidia were placed in medium containing 2.5 μmoles of thymidine per 50 ml and incubated for 72 hours, with filtration when growth appeared. The remaining conidial suspension was inoculated onto sorbose plates containing 1.0 μmole of uridine per 25 ml of medium. Over a period of several days 921 colonies were removed from the sorbose plates and transferred to complete medium agar tubes. Of these, 101 were immediately discarded because of no or very little growth on the complete medium. Those that remained were tested in liquid medium plus a thymidine supplement; 784 isolates grew in this medium and were discarded as being the parental double mutants. The remaining 36 isolates were further tested in media containing uridine alone, thymidine alone and uridine plus thymidine. Twenty isolates grew poorly with all pyrimidines and were discarded. Nine isolates would not grow on thymidine alone but did use thymidine to promote growth in the presence of limiting uridine. The possibility that some of these nine isolates

carry uc-1 suppressor mutations will be discussed later. Seven isolates (one of which was lost) met the requirements of the type mutant being searched for by growing well on uridine but not utilizing thymidine either alone or in the presence of uridine. Thus, each of these remaining six isolates must possess a third and new mutation.

Growth tests

The growth response of the six triple mutant isolates to eight different pyrimidines is given in Table 14. The growth tests divide the six triple mutants into two classes. One class, with two members, does not utilize thymidine, deoxyuridine and deoxycytidine while using the other pyrimidines. Since this is the same phenotype given by the pyr-4 uc-1 uc-2 I mutant previously recovered (Table 13) the new mutation in these isolates was also designated uc-2 and the triple mutants were designated pyr-4 uc-1 uc-2 II and III respectively. The second class contained four isolates which could not benefit from thymidine, thymine and 5-hydroxymethyluracil as pyrimidine sources while they grew quite well on 5-formyluracil, uracil, uridine, deoxyuridine and deoxycytidine. The new mutation present in these isolates was designated uracil-3 (uc-3). The four pyr-4 uc-1 uc-3 triple mutants are distinguished by the numerals I, II, III and IV.

Heterocaryons

In order to show possible functional differences among members of the two classes of triple mutants heterocaryons were made in liquid medium. Conidia from the mutants were inoculated into flasks containing 25 ml of medium plus various pyrimidine supplements and grown for

Table 14.--Growth response to several pyrimidines of parent pyr-4 uc-1 double mutant and six triple mutant isolates of filtration enrichment mutant hunt.

Pyrimidine Supplement µmoles/flask	Dry Wt. (mg) of Pad[1/]						
		Triple Mutant Isolates					
	pyr-4 uc-1 parent	pyr-4 uc-1 uc-2 II	pyr-4 uc-1 uc-2 III	pyr-4 uc-1 uc-3 I	pyr-4 uc-1 uc-3 II	pyr-4 uc-1 uc-3 III	pyr-4 uc-1 uc-3 IV
0.5 uridine	9.3	4.5	7.6	8.8	7.9	7.0	7.5
0.5 uridine + 1.0 thymidine	35	5.0	6.9	8.9	7.6	7.2	7.5
0.5 uridine + 1.0 thymine	--	24	32	8.6	7.6	6.9	6.9
0.5 uridine + 1.0 5-CH_2OH uracil	--	27	27	8.7	7.3	6.9	7.3
0.5 uridine + 1.0 uracil	--	15	28	20	21	24	21
0.5 uridine + 1.0 deoxyuridine	--	6.1	10.6	31	52	34	31
0.5 uridine + 1.0 deoxycytidine	--	5.4	9.6	20	22	17	17

Table 14.--Continued

Pyrimidine Supplement µmoles/flask	Dry Wt. (mg) of Pad[1/]						
		Triple Mutant Isolates					
	pyr-4 uc-1 parent	pyr-4 uc-1 uc-2 II	pyr-4 uc-1 uc-2 III	pyr-4 uc-1 uc-3 I	pyr-4 uc-1 uc-3 II	pyr-4 uc-1 uc-3 III	pyr-4 uc-1 uc-3 IV
2.0 uridine	--	17	23	21	19	20	14
2.0 uridine + 1.0 thymidine	--	16	21	23	20	19	17
2.0 uridine + 1.0 thymine	--	38	70	22	19	19	16
2.0 uridine + 1.0 5-CH_2OH uracil	--	36	70	22	20	20	17
2.0 uridine + 1.0 uracil	--	25	59	33	61	58	37
1.0 thymidine	22	0	0	0	0	0	0
1.0 thymine	21	18	19	0	0	0	0
1.0 5-CH_2OH uracil	21	23	22	0	0	0	0
1.0 5-formyluracil	20	19	19	16	17	16	18
1.0 uracil	16	10	17	22	14	16	12
1.0 deoxyuridine	14	2	2	12	47	17	8
1.0 deoxycytidine	--	0	0	15	19	17	5

[1/] Each figure is the average weight of two pads. Time of growth--5 days.

six days. As can be observed from the dry weights of the resulting pads (Table 15) the presumed heterocaryons formed between the pyr-4 uc-1 uc-2 II and III mutants and among the four pyr-4 uc-1 uc-3 mutants gave no utilization of thymidine. However, heterocaryons between pyr-4 uc-1 uc-2 and pyr-4 uc-1 uc-3 triple mutants grew as would pyr-4 uc-1 double mutants. Heterocaryons between pyr-4 uc-1 uc-2 I and each of the two new uc-2 containing triple mutants gave no utilization of thymidine.

Cross feeding experiments

The possibility was investigated that some of the triple mutant isolates, when grown on a medium containing uridine plus excess thymidine, could convert thymidine to a pyrimidine that could be utilized by other triple mutant isolates. The results of the cross feeding experiments (Table 16) demonstrate that the mutants can again be divided into two classes. One class, the three pyr-4 uc-1 uc-2 mutants, excreted nothing into the medium that other uc-2 or uc-3 carrying mutants could use as a pyrimidine source. The second class of four pyr-4 uc-1 uc-3 mutants converted thymidine to a compound or compounds available to all the pyr-4 uc-1 uc-2 mutants.

The amount of growth produced by pyr-4 uc-1 uc-2 mutants growing in pyr-4 uc-1 uc-3 medium indicated that from one-half to almost all of the thymidine originally present had been converted to other pyrimidines by the pyr-4 uc-1 uc-3 mutants. No clue to the identity of the pyrimidine compound or compounds excreted by the uc-3 carrying triple mutants could be gained from the UV spectrum of the used medium since

Table 15.--Heterocaryon formation among six triple mutant isolates.

Inoculum[1/]	Dry Wt. (mg) of Pad with the Following Pyrimidine Supplements (μmoles/flask)		
	1.0 uridine	1.0 uridine + 1.0 thymidine	1.0 thymidine
uc-2 II	9	9	0
uc-2 III	12	12	0
uc-2 II + uc-2 III	12	12	0
uc-3 I	14	14	0
uc-3 II	12	11	0
uc-3 III	11	11	0
uc-3 IV	10	10	0
uc-3 I + uc-3 II	12	13	0
uc-3 I + uc-3 III	13	14	0
uc-3 I + uc-3 IV	13	13	0
uc-3 II + uc-3 III	13	14	0
uc-3 II + uc-3 IV	13	12	0
uc-3 III + uc-3 IV	12	13	0
uc-2 III + uc-3 I	15	57	20
uc-2 III + uc-3 II	16	69	9

[1/] Each of the mutants listed in this table also carry pyr-4 and uc-1 mutations.

Table 16.--Cross feeding experiments with three pyr-4 uc-1 uc-2 and four pyr-4 uc-1 uc-3 triple mutants.

Mutants Receiving Used Medium[1]	Dry Wt. (mg) of Pad with the Following Mutants Producing Used Medium[1]						
	uc-2 I	uc-2 II	uc-2 III	uc-3 I	uc-3 II	uc-3 III	uc-3 IV
uc-2 I	0	0	0	73	61	50	38
uc-2 II	0	0	0	61	47	44	31
uc-2 III	0	0	0	67	53	58	33
uc-3 I	0	0	0	0	0	0	0
uc-3 II	0	0	0	0	0	0	0
uc-3 III	0	0	0	--	--	--	--
uc-3 IV	0	0	0	--	--	--	--

[1] Each figure of the seven mutants listed in this table also carry pyr-4 and uc-1 mutations.

Each of seven pairs of flasks (each flask containing 25 ml of medium, 4 μmoles of uridine and 20 μmoles of thymidine) were inoculated with one of the seven triple mutants listed in the above table. Following five days growth the mycelial pads were removed by filtration (pad of 40 to 80 mg dry wt./flask) and 5 ml portions of each type used medium were placed in series of seven flasks, each flask also containing 20 ml of minimal medium. Then each of the seven triple mutants was inoculated into one flask in each series. Growth was for five days and dry weights of the resulting pads are reported in this table.

the presence of the pyr-4 mutation caused large amounts of orotic acid to be released into the medium as well. Although not conclusively proved, on the basis of growth response tests, heterocaryon formation and cross feeding experiments it was assumed that the uc-2 mutations of pyr-4 uc-1 uc-2 I, II and III were mutations of the same locus and that the uc-3 mutations of pyr-4 uc-1 uc-3 I, II, III and IV were mutations of the same locus.

Crosses

One of the uc-3 carrying triple mutant isolates, pyr-4 uc-1 uc-3 I, was crossed to wild type. Types of asci resulting from this cross are reported in Table 17. Classification of spore pairs was made by determining growth in three pyrimidine supplemented media, 5-hydroxymethyluracil alone, uridine alone and uridine plus 5-hydroxymethyluracil. As with the uc-1 and uc-2 mutations the uc-3 mutation cannot be classified by growth tests unless present with a pyr mutation. Furthermore, uc-1 cannot be classified in the presence of uc-3 since the ability of uc-1 to allow thymidine, thymine and 5-hydroxymethyluracil to be used as sole pyrimidine sources is nullified by the uc-3 mutation which does not allow the use of these compounds under any condition. However, since there was no recombination between pyr-4 and uc-1 in spore pairs that could be tested it was assumed that pyr-4 uc-3 spore pairs also carried uc-1. The asci gave no indication of linkage between uc-3 and pyr-4 loci or uc-3 and mating type loci.

A strain known to be carrying only the uc-3 mutation was selected from spore pairs of the previously discussed cross and was in turn

Table 17.--Types of asci resulting from cross of pyr-4 uc-1 uc-3 A X wild a.

Spore Pair				No. Asci	Spore Pair				No. Asci
1	pyr-4	uc-1	uc-3	8	1	+	+	+ or uc-3	2
2	pyr-4	uc-1	uc-3		2	pyr-4	uc-1		
3	+	+	+		3	pyr-4	uc-1	uc-3	
4	+	+	+		4	+	+	+ or uc-3	
1	pyr-4	uc-1	uc-3	1	1	pyr-4	uc-1	uc-3	2
2	+	+	+		2	+	+	+ or uc-3	
3	pyr-4	uc-1	uc-3		3	pyr-4	uc-1		
4	+	+	+		4	+	+	+ or uc-3	
1	pyr-4	uc-1	+	6	1	pyr-4	uc-1	uc-3	1
2	pyr-4	uc-1	+		2	+	+	+ or uc-3	
3	+	+	uc-3		3	+	+	+ or uc-3	
4	+	+	uc-3		4	pyr-4	uc-1	+	

	No. Asci
pyr-4 segregating 1st division	14
pyr-4 segregating 2nd division	6
uc-3 segregation unclassified	5
uc-3 segregating 1st division	14
uc-3 segregating 2nd division	1
Both segregating 1st division of these 14	14
Parental combinations	8
New combinations	6

	Mating Type	
	A	a
18 uc-3 carrying spore pairs	9	9

Conclusion: Uc-3 segregates as a single gene. There is no evidence of linkage between uc-3 and pyr-4 or between uc-3 and mating type loci.

crossed to a pyr-1 mutant. The data in Table 18 show that pyr-1 and uc-3 loci are unlinked and that uc-3 gives the same phenotype in a pyr-1 background as in pyr-4.

Identification of pyrimidine accumulated by uc-3 mutant

The growth response and cross feeding experiments (Table 14 and 16) indicated that the compound(s) likely to be accumulated by strains carrying the uc-3 mutation would be thymine and/or 5-hydroxymethyl-uracil. In order to gain further information about the accumulated growth factor, wild type strains and strains known to be carrying the uc-3 mutation were grown for a period of five days in flasks containing 25 ml of medium plus 20 μmoles of thymidine. The absorption spectra of wild type and uc-3 used media showed that both had the same wavelength of maximum absorption, 265 mμ. This was taken to indicate that the major pyrimidine(s) present in both media were thymine and/or thymidine. The used media of the uc-3 mutants still had 75 to 100% of the $O.D._{265}$ that was present in the media at the start, while the used media of the wild type strains had only 25 to 50% of the original amount.

In a procedure similar to the one used in the cross feeding experiments (Table 16) uc-3 and wild type used media were used as the source of pyrimidines for growing pyr-4 uc-1 and pyr-4 uc-1 uc-2 (the uc-2 carrying mutant cannot utilize thymidine) mutants. By comparing the weights of pads produced by the two mutants on the used media to standard curves obtained by growing the two mutants on a concentration series of thymine, the amount of thymidine and of pyrimidines other

Table 18.--Types of asci resulting from cross of pyr-1 a X uc-3 A.

Spore Pair			No. Asci	Spore Pair			No. Asci
1	pyr-1	uc-3	6	1	pyr-1	uc-3	1
2	pyr-1	uc-3		2	pyr-1	+	
3	+	+		3	+	+ or uc-3	
4	+	+		4	+	+ or uc-3	
1	pyr-1	+	3	1	pyr-1	+	1
2	pyr-1	+		2	pyr-1	uc-3	
3	+	uc-3		3	+	+ or uc-3	
4	+	uc-3		4	+	+ or uc-3	

Nine random single ascospore isolates carrying the pyr-1 mutation were tested: five were pyr-1 and four were pyr-1 uc-3.

	No. Asci
pyr-1 segregating 1st division	11
pyr-1 segregating 2nd division	0
uc-3 segregating 1st division	9
uc-3 segregating 2nd division	2
Both segregating 1st division of these 9	9
Parental combinations	3
New combinations	6

Conclusion: Uc-3 segregates as a single gene. There is no evidence of linkage between uc-3 and pyr-1.

Growth Tests Used to Distinguish Pyr-1 and Pyr-1 Uc-3 Mutants

Inoculum	Dry Wt. (mg) of Pad[1] with the Following Pyrimidine Supplement (μmoles/flask)			
	minimal	1.0 uridine	1.0 uridine + 1.0 5-CH_2OH uracil	1.0 uridine + 1.0 uracil
pyr-1[2]	0	19	35	34
pyr-1 uc-3[3]	0	18	19	31
uc-3	110	--	--	--
wild	123	--	--	--

[1] Time of growth--5 days.

[2] Average weight of pads from growth tests of three pyr-1 spore pairs.

[3] Average weight of pads from growth tests of three pyr-1 uc-3 spore pairs.

than thymidine in the used media were determined. These biological assays demonstrated that over 70% of the pyrimidine remaining in the used media of wild type strains was thymidine while in the used media of uc-3 strains 70 to 100% of the growth factor was due to pyrimidines other than thymidine. The assays further showed that both wild type and uc-3 used media had about the same level of thymidine, but only the uc-3 medium had an appreciable amount of another pyrimidine. Both strains were capable of degrading a large amount of thymidine, but only uc-3 accumulated one of the early products of degradation.

The used medium of a uc-3 mutant (which had been grown in a medium containing thymidine) was applied to a Dowex-50 column and eluted with 1.5 N HCl as described in the materials and methods section. Two peaks with UV absorption at 265 mμ were eluted from the column. The component of the smaller peak had an absorption maximum at 266 mμ. This component and added thymidine were simultaneously eluted from the column. The component of the major peak had an absorption maximum at 265 mμ. This component and added thymine could not be separated on the Dowex-50 column. The fractions comprising the major peak were pooled, dried, redissolved in a small amount of water and compared with three other pyrimidines by one dimensional paper chromatography. The location of the compounds, after development of the chromatograms, was determined by the appearance of absorbing spots on the Whatman No. 1 paper under a short wavelength UV lamp. The results of Table 19 verify that the major component accumulated in uc-3 used medium is thymine.

Table 19.--Approximate R_F values of pyrimidines.

Pyrimidine	H_2O	ethyl acetate-formic acid-H_2O (70:20:10)	t-butanol-methyl ethyl ketone-H_2O NH_4OH (40:30:20:10)
5-CH_2OH uracil	.77	.37	.34
Thymidine	.81	.59	.63
Thymine	.70	.67	.59
Accumulated by uc-3 mutant	.69	.67	.59

No 5-hydroxymethyluracil or pyrimidine other than thymidine or thymine was detected in the eluate from the ion exchange column. Data in Table 20 show that the growth factor in uc-3 used medium which accounts for essentially all the pyrimidine in the used medium available to the pyr-4 uc-1 uc-2 mutant is thymine. Thus, the uc-3 mutation, shown by growth experiments to block the thymine methyl oxidative pathway between 5-hydroxymethyluracil and 5-formyluracil, causes accumulation of thymine, but not 5-hydroxymethyluracil. A possible explanation of this could be that the steps from thymine to 5-formyluracil are carried out by one enzyme.

Suppressor of uc-1 mutation

During selection of the seven pyr-4 uc-1 uc-2 and pyr-4 uc-1 uc-3 triple mutants, nine isolates were found which behaved in growth tests as pyr-4 mutants. These nine isolates would not grow in medium in which thymidine was the only pyrimidine source, but would utilize thymidine when uridine was present. One of these isolates was crossed to wild type, 10 asci were dissected and the germinated spore pairs were classified as to whether or not they carried the pyr-4 mutation. Conidia arising from the growth of the two pyr-4 carrying spore pairs of each ascus were inoculated into flasks containing 25 ml of medium plus 1.0 μmole of thymidine. The growth results from the 10 asci are as follows: 4 asci--neither pyr-4 segregant produced growth, 5 asci--one pyr-4 carrying segregant produced growth (pyr-4 uc-1 phenotype) the other did not, 1 ascus--both pyr-4 carrying segregants produced growth. This reveals that the parent isolate was still carrying the

Table 20.--Evidence that the pyrimidine growth factor accumulated by uc-3 mutant is thymine.

Pyrimidine Supplement	Dry Weight (mg)		µMoles Pyrimidine in Supplement Calculated by:	
	pyr-4 uc-1 uc-2	pyr-4 uc-1	biological assay	$O.D._{265}$
1.5 ml uc-3 used medium	22	25	1.09	1.30
Thymine fractions from 1.5 ml uc-3 used medium	20	--	0.83	0.84
Thymidine fractions from 1.5 ml uc-3 used medium	0	6	0.26	0.33
1.0 µmole thymidine	24	23	1.00	1.00

A uc-3 mutant was grown five days in flasks containing 25 ml of medium + 20 µmoles of thymidine. A portion of this used medium was used directly in growth experiments and a portion was placed on a Dowex-50 column where the pyrimidine components were eluted with 1.5 N HCl. Estimates of pyrimidine concentration in the used medium as well as the thymine and thymidine fractions from the column were made by comparison of their $O.D._{265}$ to thymine and thymidine standards.

Both thymine and thymidine fractions were pooled, dried to remove HCl and redissolved in water. Portions of these pools and of used medium were placed in flasks containing 25 ml of medium, inoculated with pyr-4 uc-1 or pyr-4 uc-1 uc-2 conidia and grown five days. Each dry weight given is the average of two pads.

uc-1 mutation and must also have had a third mutation, a suppressor of uc-1 (uc-1-su). Since the growth tests do not distinguish among the three genotypes pyr-4, pyr-4 uc-1 uc-1-su and pyr-4 uc-1-su and because a limited number of asci were dissected no definite statement can be made about the possible linkage of uc-1 and uc-1-su.

PYR-1 UC-4 AND PYR-1 UC-5 DOUBLE MUTANTS

Selection of mutants

A filtration enrichment procedure was used to select for mutants which could not utilize uracil, but still could use uridine as a pyrimidine source. UV irradiation was applied to 20 X 10^6 pyr-1 conidia for 35 seconds to give a 33% kill. The conidia were placed in medium containing 0.75 μmole of uracil per 25 ml (2 X 10^5 conidia/ml) and grown for 62 hours using procedures previously described for filtration enrichment. The conidial suspension remaining after 62 hours was inoculated onto sorbose plates containing 0.5 μmole of uridine per 25 ml of medium. From the third to the seventh day following inoculation of the sorbose plates 327 colonies were removed and transferred to agar tubes of complete medium. Sixty of these isolates were discarded because of poor growth on the complete agar medium. The remainder were tested in liquid medium supplemented with uracil and medium supplemented with uridine. All but four isolates were discarded because they behaved as pyr-1 mutants or because they grew poorly on both media. The four isolates kept would not grow in the uracil supplemented medium, but did respond to uridine.

The four presumed double mutants were then tested in media containing limiting uridine further supplemented with one of the following pyrimidines: thymidine, thymine, 5-hydroxymethyluracil or uracil. One of the isolates received no sparing action from any of the four pyrimidine supplements and the new mutation carried by this isolate was designated uracil-4 (uc-4). The other three mutants could not benefit

from thymine, 5-hydroxymethyluracil and uracil, but thymidine did give a sparing action. The new mutation carried by this class of mutants was designated uracil-5 (uc-5).

Crosses

The pyr-1 uc-4 and one of the pyr-1 uc-5 isolates were crossed to wild type 4A, asci were dissected and conidia from growth of the dissected spore pairs were tested in several pyrimidine supplemented media. The results of the crosses and the type of growth tests used in classification of the spore pairs are given in Table 21 for the pyr-1 uc-4 mutant and in Table 22 for the pyr-1 uc-5 mutant. As with the other uc mutations the presence of uc-4 and uc-5 can be determined by growth tests only when they are with a pyr mutation. Uc-4 was found to be linked to the mating type locus while uc-5 was found linked to the pyr-1 locus.

The initial pyr-1 uc-4 isolate gave only about one-third the growth at any given uridine concentration as did the pyr-1 parent. All pyr-1 uc-4 segregants resulting from the backcross of the double mutant to wild type also behaved in this manner. Apparently, the reduced efficiency of uridine utilization and resulting reduced growth are caused by the uc-4 mutation and not a second independent mutation.

Growth tests

Pyr-1 uc-4 and pyr-1 uc-5 double mutants were reisolated from the backcross to wild type. The growth response of these double mutants and the pyr-1 parent to ten different pyrimidines is given in Table 23.

Table 21.--Types of asci resulting from cross of pyr-1 uc-4 a X 4A wild.

Spore Pair			No. Asci	Spore Pair			No. Asci
1	pyr-1	uc-4	4	1	pyr-1	+	4
2	pyr-1	uc-4		2	pyr-1	+	
3	+	+		3	+	uc-4	
4	+	+		4	+	uc-4	

Six random single spore isolates carrying the pyr-1 mutation were tested: three were pyr-1 and three were pyr-1 uc-4.

	No. Asci
pyr-1 segregating 1st division	8
pyr-1 segregating 2nd division	0
uc-4 segregating 1st division	8
uc-4 segregating 2nd division	0
Both segregating 1st division of these	8
Parental combinations	4
New combinations	4

	Mating Type	
	A	a
16 uc-4 carrying spore pairs	1	15

Conclusion: Uc-4 segregates as a single gene; uc-4 and pyr-1 are not linked. Uc-4 and mating type loci are linked.

Growth Tests Used to Distinguish Pyr-1 and Pyr-1 Uc-4 Mutants

Pyrimidine Supplement µmoles/flask	Dry Wt. (mg) of Pad[1]	
	pyr-1[2]	pyr-1 uc-4[3]
1.0 uracil	11.5	0.0
1.0 uridine	14.4	5.9
2.0 uridine	23.7	10.5
1.0 uridine + 1.0 thymidine	28.8	6.3
1.0 uridine + 1.0 thymine	26.2	5.4
1.0 uridine + 1.0 uracil	21.8	5.6

1/ Time of growth--5 days.
2/ Average of pads from growth tests of four pyr-1 spore pairs.
3/ Average of pads from growth tests of eleven pyr-1 uc-4 spore pairs.

Table 22.--Types of asci resulting from cross of pyr-1 uc-5 a X 4A wild.

Spore Pair			No. Asci	Spore Pair			No. Asci
1	pyr-1	uc-5	7	1	pyr-1	+	0
2	pyr-1	uc-5		2	pyr-1	+	
3	+	+		3	+	uc-5	
4	+	+		4	+	uc-5	
1	+	+	1	1	pyr-1	+	1
2	pyr-1	uc-5		2	pyr-1	uc-5	
3	pyr-1	uc-5		3	+	+ or uc-5	
4	+	+		4	+	+ or uc-5	

Seventeen random single spore isolates carrying the pyr-1 mutation were tested: two were pyr-1 and fifteen were pyr-1 uc-5.

	No. Asci		Mating Type	
			A	a
pyr-1 segregating 1st division	8	10 uc-5 carrying spore pairs	6	4
pyr-1 segregating 2nd division	1			
uc-5 segregating 1st division	7			
uc-5 segregating 2nd division	2			
Both segregating 1st division of these	7			
Parental combinations	7			
New combinations	0			

Conclusion: Uc-5 segregates as a single gene; Uc-5 and pyr-1 are linked.

Growth Tests Used to Distinguish Pyr-1 and Pyr-1 Uc-5 Mutants

Pyrimidine Supplement µmoles/flask	Dry Wt. (mg) of Pad[1]	
	pyr-1	pyr-1 uc-5[2]
1.0 uracil	20.1	0.5
1.0 uridine	18.9	15.6
2.0 uridine	29.5	26.0
1.0 uridine + 1.0 thymidine	30.9	27.9
1.0 uridine + 1.0 thymine	30.6	16.3
1.0 uridine + 1.0 uracil	29.8	17.6

[1] Time of growth--5 days.
[2] Average weight of pads from growth tests of ten pyr-1 uc-5 spore pairs.

Table 23.--Growth response to several pyrimidines of parent *pyr-1* mutant and *pyr-1 uc-4* and *pyr-1 uc-5* double mutants.

Pyrimidine Supplement μmoles/flask	Dry Wt. (mg) of Pad[1]		
	pyr-1 uc-4	*pyr-1 uc-5*	*pyr-1*
3.0 uridine	14.9	47	43
2.0 uridine	10.7	36	33
1.0 uridine	5.7	21	18
1.0 uridine + 1.0 thymidine	6.4	38	32
1.0 uridine + 1.0 thymine	6.1	22	31
1.0 uridine + 1.0 5-CH_2OH uracil	5.7	22	32
1.0 uridine + 1.0 5-formyluracil	5.8	21	30
1.0 uridine + 1.0 uracil	5.9	25	31
1.0 uridine + 1.0 deoxyuridine	8.1	41	34
1.0 uridine + 1.0 deoxycytidine	7.8	30	27
1.0 uridine + 1.0 cytidine	10.9	38	30
1.0 uridine + 1.0 cytosine	5.6	21	20
1.0 cytidine	4.7	22	18
1.0 deoxyuridine	0.0	2	14
1.0 deoxycytidine	0.0	4	1
1.0 cytosine	0.0	0	0
1.0 thymidine	0.0	5	0
1.0 uracil	0.0	1	18
2.0 uridine + 1.0 thymidine	11.6	--	--
2.0 uridine + 1.0 thymine	10.2	--	--
2.0 uridine + 1.0 uracil	10.9	--	--

[1] Each figure is the average of two pads. Time of growth--5 days.

The action of the uc-4 mutation seems to be that of blocking the pathway by which the pyrimidine ring of thymidine can be used for incorporation into RNA since thymidine, thymine, 5-hydroxymethyluracil, 5-formyluracil and uracil are not utilized by the pyr-1 uc-4 mutant. The action of the uc-5 mutation is far from clear since it allows thymidine, the first compound in the pathway, to be used but does not allow utilization of the pyrimidines following thymidine in the pathway. The pyr-1 uc-5 mutant can utilize deoxyuridine and deoxycytidine in addition to thymidine. The uc-5 mutation could be looked upon as affecting active transport, that is allowing pyrimidine nucleosides to be carried into the cell, but not the pyrimidine bases. Whether Neurospora is completely dependent on active transport mechanisms to get pyrimidines inside the cell and if it is, whether it has separate systems for pyrimidine bases and nucleosides is not known. The possibility that the uc-5 mutation affects the regulation of the oxidative pathway cannot be ruled out. However, a pyr-1 uc-5 strain grown in medium supplemented with 1.0 μmole amounts of uridine and thymidine and medium containing 1.0 μmole amounts of uridine, thymidine and thymine gave the same amount of growth in both media.

Accumulation products

Pyr-1, pyr-1 uc-4 and pyr-1 uc-5 mutants were grown in medium containing 6.0 μmoles of uridine per flask. At the end of three days the pyr-1 used medium contained no pyrimidine, the pyr-1 uc-5 used medium contained 2.0 μmoles of uracil and the pyr-1 uc-4 used medium had 3.0 μmoles of uracil. At the end of five days growth the pyr-1 uc-5 used

medium had almost no uracil left while the pyr-1 uc-4 used medium still contained almost 3.0 μmoles of uracil. The pyrimidine components of the used medium were eluted from a Dowex-50 column with water. Amounts of pyrimidine in the eluant were determined by the $O.D._{260}$.

As reported in Table 24, the ability of wild type 4A and of uc-4 strains to utilize uridine and uracil was investigated. Wild type rapidly takes up uracil and uridine, and excretes essentially no pyrimidine into the medium. The uc-4 strain rapidly takes up uridine, but releases almost half of it as uracil which it cannot use. This seems to explain why the pyr-1 uc-4 mutant gives less growth from uridine than the pyr-1 mutant.

Table 24.--Accumulation of uracil by uc-4 mutant grown with excess uridine in medium.

Inoculum	Hours Growth	Dry Wt. (mg) of Pad	µMoles Left in Medium of Uridine	Uracil
4A	0	0	9.2	0.0
4A	40	6	7.9	0.0
4A	48	17	4.1	0.5
4A	58	46	0.0	0.0
4A	70	84	0.0	0.0
Uc-4	0	0	9.2	0.0
Uc-4	40	10	6.5	1.2
Uc-4	48	29	0.0	5.5
Uc-4	58	49	0.0	5.1
Uc-4	70	101	0.0	4.5

Wild type 4A and uc-4 strains were inoculated into two series of flasks, each flask containing 25 ml of medium + approximately 10 µmoles of uridine. A flask was incubated for the period of time listed in the above table after which the mycelial pad was removed and a one to two ml aliquot of the used medium was applied to a 1.3 X 50 cm Dowex-50 column. The pyrimidine components were eluted from the column with H_2O and two ml volume fractions were collected. No major 240-290 mµ absorbing materials other than uracil and uridine were eluted from the column. Uridine and uracil were identified by their effluent volumes and their respective UV absorption maximums of 261 and 258 mµ.

Uc-4 and wild type strains were used in identical experiments except instead of uridine the supplement was 10 µmoles of uracil per flask. The wild type strain had removed all the uracil from the medium by 58 hours of growth (49 mg pad). While at 58 hours of growth the uc-4 mutant used medium still contained almost 10 µmoles of uracil (57 mg pad).

PYR-1 UD-1 AND PYR-1 UD-2 DOUBLE MUTANTS

Introduction

In the previous section was discussed the isolation of a group of mutants which could utilize uridine but not uracil. One of these mutants, uc-4, was presumed to be unable to incorporate uracil into nucleic acid. Since the uc-4 mutant could utilize uridine the assumption was made that uridine is phosphorylated directly and is not converted to uracil before incorporation into nucleic acid. Radioactive labeled nucleoside experiments with many organisms (13) including Neurospora (17, 42) have shown that all ribonucleosides are probably directly phosphorylated. The question that remained unanswered was whether uracil could be directly converted to uridine monophosphate such as through a reaction with 5-phosphoribosylpyrophosphate, or whether it was converted to uridine first. Proof for a pathway by which uracil could become incorporated into nucleic acid without passing through a uridine intermediate might be the finding of mutants which could utilize uracil but not uridine.

Selection of mutants

A filtration enrichment procedure was used to select for mutants which could not utilize uridine but still could use uracil as a pyrimidine source. A pyr-1 conidial suspension was given UV irradiation for 40 seconds (55% kill) and inoculated into two flasks each containing 33 ml Fries medium (2×10^5 conidia/ml of medium) supplemented with 1.0 μmole of uridine. The conidial suspension remaining after 44 hours of growth (using previously described filtration enrichment procedures)

was spread onto sorbose plates containing 0.5 μmole of uracil per 25 ml of medium. Of the 643 colonies removed from the sorbose plates and transferred to agar tubes of complete medium all but 45, which were discarded as slow growers, were tested further. Inoculum from each isolated colony was transferred to two flasks; one containing 25 ml of Fries medium plus 1.0 μmole of uridine and the other 25 ml of Westergaard-Mitchell medium plus 1.0 μmole of uracil. The uridine supplement was placed in Fries medium to protect against the possibility that mutants which could not utilize uridine might be discarded because they possessed an adaptive system to convert uridine to uracil. Uracil is used inefficiently by pyr mutants in Fries medium.

Four isolates grew poorly or not at all on the uridine supplemented medium while growing normally on the medium containing uracil. These four presumed double mutants were further tested in Fries and Westergaard-Mitchell media supplemented with one of the following pyrimidines: uridine, cytidine, deoxyuridine and uracil. One isolate behaved like the pyr-1 parent on the uracil supplemented media, but could not utilize the three nucleosides on either medium. The new mutation present in this isolate was designated uridine-1, abbreviated ud-1. An unexpected finding was that the other three isolates utilized the four pyrimidine supplements just as did the pyr-1 parent in Westergaard-Mitchell medium, but could not use any of them in Fries medium. The new mutation present in these three isolates was designated uridine-2 (ud-2).

Crosses

The pyr-1 ud-1 and one of the pyr-1 ud-2 isolates were crossed to wild type 4A, asci were dissected and conidia from the growth of the dissected spore pairs were inoculated into flasks of Fries medium supplemented with 1.0 μmole of uridine. If little or no growth occurred in this medium, indicating the presence of the pyr-1 ud double mutant, the segregant was always further checked in Westergaard-Mitchell medium supplemented with uracil to insure that it could utilize uracil. Segregants known to be carrying only the ud-1 or ud-2 mutation grew in minimal medium like wild type.

The results of the crosses are given in Table 25 for the pyr-1 ud-1 mutant and in Table 26 for the pyr-1 ud-2 mutant. There was no proof for the linkage of the ud mutations either to the pyr-1 or the mating type locus. Of the asci dissected from the backcross of the pyr-1 ud-2 mutant to 4A only three parental ditypes were recovered compared to eight non-parental ditypes. The deficiency of parental ditypes could be due to chance (as small numbers of asci were involved), to poor germination of spore pairs carrying both the pyr-1 and ud-2 mutations (at least one of the four spore pairs failed to germinate in about one-third of the asci dissected) or to more than one gene contributing to the phenotype. However, all the pyr-1 ud-2 segregants were indistinguishable by growth tests from the parent double mutant. The pyr-1 segregants were no different than the original pyr-1 mutant. If two genes are involved in the ud-2 phenotype, they have no visible effect unless present together.

Table 25.--Types of asci resulting from cross of pyr-1 ud-1 a X 4A wild.

Spore Pair			No. Asci	Spore Pair			No. Asci
1	pyr-1	ud-1	8	1	pyr-1	ud-1	1
2	pyr-1	ud-1		2	+	+ or ud-1	
3	+	+		3	+	+ or ud-1	
4	+	+		4	pyr-1	+	
1	pyr-1	ud-1	1	1	pyr-1	+	1
2	pyr-1	+		2	pyr-1	ud-1	
3	+	+ or ud-1		3	+	+ or ud-1	
4	+	+ or ud-1		4	+	+ or ud-1	
1	pyr-1	ud-1	2	1	pyr-1	+	7
2	+	+ or ud-1		2	pyr-1	+	
3	pyr-1	+		3	+	ud-1	
4	+	+ or ud-1		4	+	ud-1	

	No. Asci
pyr-1 segregating 1st division	17
pyr-1 segregating 2nd division	3
ud-1 segregation unclassified	3
ud-1 segregating 1st division	15
ud-1 segregating 2nd division	2
Both segregating 1st division of these 15	15
Parental combinations	8
New combinations	7

	Mating Type	
	A	a
20 ud-1 carrying spore pairs	9	11

Conclusion: Ud-1 segregates as a single gene. There is no indication of linkage between ud-1 and pyr-1 or ud-1 and mating type loci.

Table 26.--Types of asci resulting from cross of pyr-1 ud-2 a X 4A wild.

Spore Pair			No. Asci	Spore Pair			No. Asci
1	pyr-1	ud-2	3	1	pyr-1	+	8
2	pyr-1	ud-2		2	pyr-1	+	
3	+	+		3	+	ud-2	
4	+	+		4	+	ud-2	
1	pyr-1	ud-2	6				
2	pyr-1	+					
3	+	+ or ud-2					
4	+	+ or ud-2					

	No. Asci
pyr-1 segregating 1st division	17
pyr-1 segregating 2nd division	0
ud-2 segregating 1st division	11
ud-2 segregating 2nd division	6
Both segregating 1st division of these 11	11
Parental combinations	3
New combinations	8

	Mating Type	
	A	a
20 ud-2 carrying spore pairs	8	12

Conclusion: There is no indication of linkage between ud-2 and pyr-1 or ud-2 and mating type loci.

Growth Tests Used to Distinguish Pyr-1 and Pyr-1 Ud-2 Mutants

Dry Wt. (mg) of Pad[1] Grown in 25 ml Fries Medium + 1.0 μmole Uridine			
pyr-1 ud-2	pyr-1	ud-2	wild
1.7	24	122	146

[1] Time of growth--5 days. Figures are average weight of pads from testing 10 spore pairs each of pyr-1 ud-2, pyr-1, ud-2 and wild type genotypes.

Growth tests

Pyr-1 ud-1 and pyr-1 ud-2 double mutants which were reisolated from the backcross of the original mutant isolates to wild type were used in growth response tests. The growth response of the pyr-1 ud-1 mutant to ten different pyrimidines is given in Table 27. The results of these growth tests are curious for they show that the ud-1 mutation allows the utilization of thymine, 5-hydroxymethyluracil, 5-formyluracil and uracil, but not of uridine, cytidine, deoxyuridine, deoxycytidine and thymidine. The previously discussed uc-4 mutation (Table 23) gave exactly opposite results; permitted utilization of the nucleosides but not of the free bases. The action of the two mutations, uc-4 and ud-1, suggests that they are affecting specific pyrimidine transport systems.

The growth response of the pyr-1 ud-2 mutant to several pyrimidines in both Fries and Westergaard-Mitchell medium is given in Table 28. An explanation of why the pyr-1 ud-2 mutant is a more efficient user of pyrimidines on one medium (Westergaard-Mitchell) than the pyr-1 parent while utilizing all pyrimidines very inefficiently on another medium (Fries) is difficult. Davis and Zimmerman (43) reported a mutant of Neurospora, UM-300, which in combination with arginine mutants prevented utilization of arginine from the medium. Other double mutant combinations involving UM-300 showed that the uptake of lysine, other amino acids and uridine were affected. The difficulties of assimilation of UM-300 strains were conditional upon the presence of ammonium in the medium. Although it would have been of interest the ud-2 mutation was not transferred into amino acid mutant strains to determine if this mutation prevented the utilization of amino acids.

Table 27.--Growth response to several pyrimidines of parent pyr-1 mutant and pyr-1 ud-1 double mutant.

Pyrimidine Supplement μmoles/flask	Dry Wt. (mg) of Pad[1]	
	pyr-1	pyr-1 ud-1
3.0 uracil	40	37
2.0 uracil	30	28
1.0 uracil	18	18
1.0 uracil + 1.0 thymidine	29	19
1.0 uracil + 1.0 thymine	29	31
1.0 uracil + 1.0 5-CH_2OH uracil	31	25
1.0 uracil + 1.0 5-formyluracil	34	27
1.0 uracil + 1.0 uridine	31	20
1.0 uracil + 1.0 cytidine	29	19
1.0 uracil + 1.0 deoxyuridine	31	19
1.0 uracil + 1.0 deoxycytidine	28	19
1.0 uracil + 1.0 cytosine	19	21
1.0 uridine	18	0
1.0 cytidine	19	0
1.0 deoxyuridine	12	4
1.0 deoxycytidine	0	0
1.0 cytosine	0	0
1.0 thymidine	0	0
1.0 thymine	0	0

[1] Each figure is the average weight of two pads. Time of growth--5 days. Westergaard-Mitchell growth medium.

Table 28.--Growth response of parent pyr-1 mutant and pyr-1 ud-2 double mutant to several pyrimidines in two different growth media.

Pyrimidine Supplement μmoles/flask	Dry Wt. (mg) of Pad[1/]			
	Westergaard-Mitchell Medium		Fries Medium	
	pyr-1	pyr-1 ud-2	pyr-1	pyr-1 ud-2
1.0 uridine	17	21	19	2
1.0 uridine + 1.0 thymidine	25	38	26	2
1.0 uridine + 1.0 thymine	28	40	24	3
1.0 cytidine	21	19	18	0
1.0 deoxyuridine	11	23	6	1
1.0 deoxycytidine	2	3	6	0
1.0 uracil	19	25	5	0
1.0 uracil + 1.0 thymidine	25	41	7	1
1.0 thymidine	0	4	0	0
1.0 thymine	0	8	0	0
1.0 cytosine	0	0	0	0

[1/] Each figure is the average weight of two pads. Time of growth--5 days.

DISCUSSION

Neurospora crassa does not possess a thymidine phosphorylating enzyme and specific labeling of the fungus DNA with thymidine is impossible. However, Neurospora does have a metabolic pathway for the conversion of the pyrimidine ring of thymidine into an RNA precursor. As a means toward clearly defining this pathway mutant isolation procedures were devised and mutants were found in which specific biosynthetic reactions of the thymidine to RNA precursor conversion were blocked. In addition, these isolation procedures yielded mutations which prevent the uptake of pyrimidines from the growth medium and mutations which affect the regulation of one or more steps in the thymidine to RNA precursor pathway. A more detailed discussion of each of these conclusions and findings follows.

Inability of Neurospora to phosphorylate deoxyribose nucleosides

Experiments in which 2-C^{14} thymidine and thymine were administered to Neurospora showed that the majority of the label was incorporated into the RNA of the fungus and supported the hypothesis of Fink (9) that Neurospora lacks a thymidine phosphorylating enzyme. An alternative hypothesis was suggested by the report (44) that in Escherichia coli radioactive labeled thymidine is at first readily incorporated into DNA, but after a short period of time the level of incorporation drops rapidly. This phenomenon is caused by an inducible nucleosidase enzyme which converts thymidine to thymine, the latter compound being very inefficiently incorporated into DNA by E. coli. Therefore,

Neurospora might 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 into DNA.

An answer to the question of whether Neurospora does or does not have a thymidine phosphorylating enzyme was obtained by administering labeled thymidine to a mutant (uc-2) in which the thymidine to thymine conversion step is almost completely blocked. The majority of added thymidine remained in the medium (80%) while ninety per cent of the small amount of label which did become incorporated into the nucleic acid was in the RNA fraction. These results substantiate the hypothesis that Neurospora lacks a thymidine phosphorylating enzyme and gives evidence that the conversion of thymidine to thymine is the only enzymatic reaction thymidine can undergo in this organism.

A conversation with Professor Sterling Emerson provided the information that other researchers had attempted, unsuccessfully, to label specifically the DNA of various fungi species using radioactive thymidine. The presence of a pathway to convert thymidine to an RNA precursor and/or the lack of ability to phosphorylate thymidine are quite likely to be common among the fungi. An inability to phosphorylate thymidine has been reported (45) for the Psittacosis virus while evidence for the presence of a thymidine to RNA precursor pathway exists in Acetabularia (2), Spirogyra (3, 4), Paramecium (5) and rat liver (11) (see introduction section). The pathway for the conversion of thymidine into an RNA component 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 experiments utilizing thymidine for the specific labeling of DNA.

The response of Neurospora to deoxynucleosides other than thymidine was also investigated. Radioactive labeling experiments and/or growth response tests using deoxyuridine, deoxycytidine, deoxyadenosine and deoxyguanosine showed that these nucleosides are rapidly converted to their respective pyrimidine and purine bases which are in turn efficiently utilized as RNA precursors. Although not conclusive this evidence suggests that Neurospora may lack phosphorylating enzymes for all deoxyribose nucleosides. Therefore, a Neurospora strain would require two mutations to permit specific DNA labeling using a radioactive deoxyribose nucleoside. One mutation would be required to prevent the cleavage of the nucleoside to the free base and the second to alter the specificity of a ribose nucleoside phosphorylating enzyme in such a way that the enzyme could phosphorylate a deoxyribose nucleoside. Mutants of the first type were found, but selection procedures for mutants which could phosphorylate thymidine were unsuccessful.

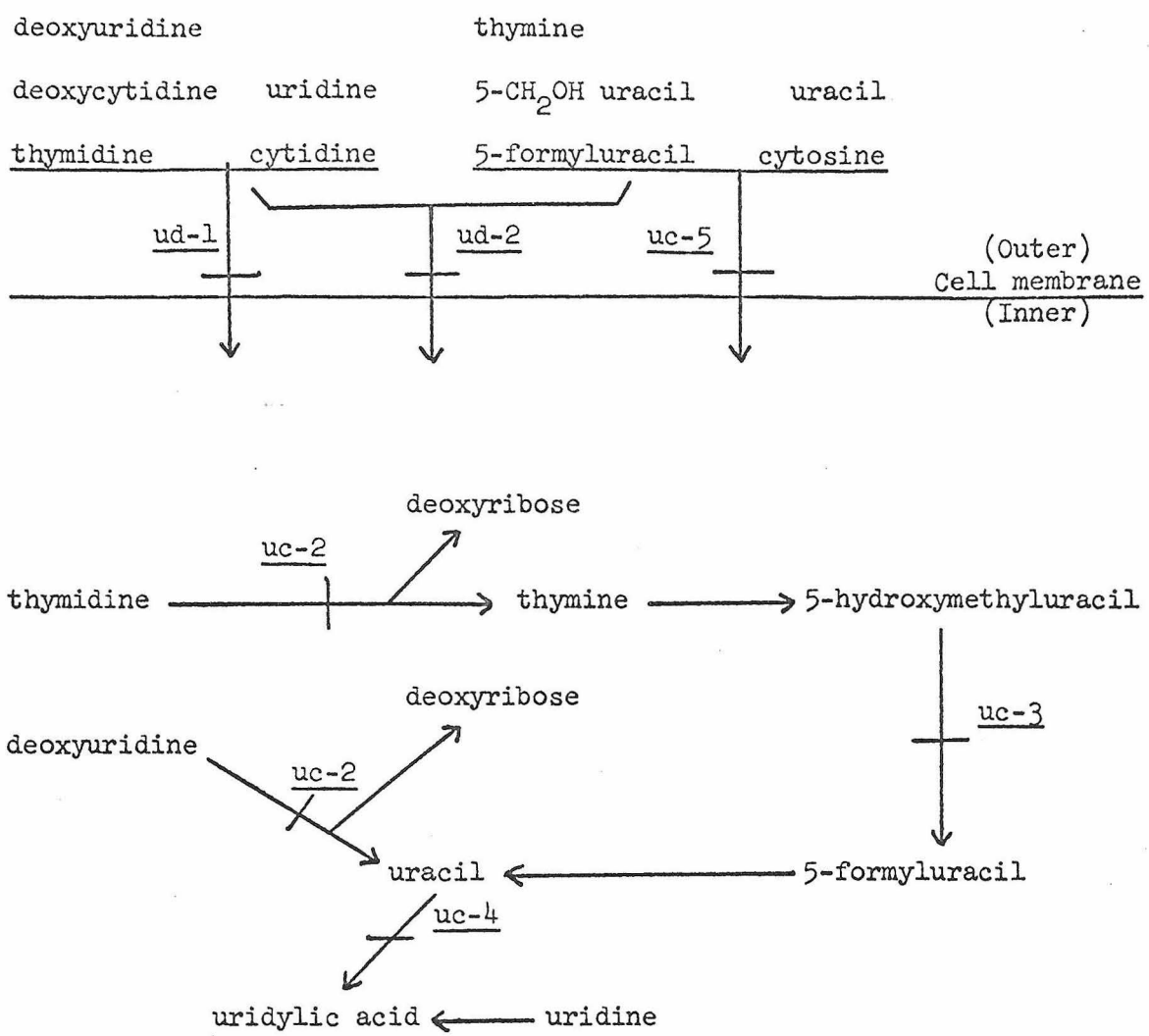
Mutations blocking the thymidine to RNA precursor pathway

One approach for determining the actual reactions involved in the pathway which converts the pyrimidine ring of thymidine to an RNA component was the isolation of mutants in which the pathway was blocked. Evidence gathered from genetic work, nutritional studies and accumulation studies with these mutants, uc-2, uc-3 and uc-4, is

consistent with the conclusion that each of these mutants acts by blocking a biosynthetic reaction in the thymidine to RNA precursor pathway. This pathway, based on the action of the uc-2, uc-3 and uc-4 mutations, is outlined in figure 3.

In addition to blocking the thymidine to thymine conversion step the uc-2 mutation prevents the utilization of deoxyuridine and deoxycytidine as pyrimidine sources. Of interest in this regard is an E. coli mutant (46) which is unable to utilize thymidine, deoxyuridine and deoxycytidine as carbon sources because it lacks the enzymatic activity to cleave the deoxyribose sugar from the three nucleosides. This E. coli mutant was shown to have only two per cent of the normal nucleosidase activity for the thymidine to thymine conversion. The similarity between the Neurospora uc-2 and E. coli mutants in their inability to utilize the three deoxyribose nucleosides led to the conclusion that the uc-2 mutation acts as does the E. coli mutation by causing the production of a defective deoxyribose nucleosidase enzyme.

Further support for the conclusion that the uc-2 mutation causes a lack of deoxyribose pyrimidine nucleosidase activity is provided by work with the deoxyribose pyrimidine, 5-fluorodeoxyuridine (5-FUdR). In E. coli (44) the same inducible enzyme that is responsible for the cleavage of thymidine also splits deoxyribose from 5-FUdR. In Neurospora the inhibitory effect of 5-FUdR is of longer duration in strains carrying the uc-2 mutation than in strains lacking it, indicating that uc-2 strains cannot cleave 5-FUdR. The normal growth response of Neurospora pyrimidine and purine mutants carrying the uc-2 mutation



Two mutations, uc-1 and uc-1-su, not shown above affect the control of the pathway. The uc-1 mutation allows the pathway that converts thymidine to thymine to function in germinating conidia. The uc-1-su mutation suppresses the action of uc-1 and restores the normal condition, the absence of the thymidine to uracil conversion in germinating conidia.

Figure 3.--Proposed sites of action of mutations affecting pyrimidine uptake and pyrimidine salvage pathways.

to pyrimidine ribonucleosides and to purine deoxyribonucleosides and ribonucleosides provides evidence that the specificity of the enzyme altered by uc-2 is restricted to deoxyribose pyrimidine nucleosides, including thymidine, deoxyuridine, deoxycytidine and 5-fluorodeoxyuridine.

The uc-3 mutation when introduced into pyrimidine mutant strains allowed utilization of 5-formyluracil and uracil, but not of thymidine, thymine and 5-hydroxymethyluracil (5-CH_2OH uracil). On the basis of these growth experiments the uc-3 mutation can be classified as blocking the 5-CH_2OH uracil to 5-formyluracil step in the pathway. However, when thymidine was administered to a uc-3 mutant strain thymine was accumulated in the medium while 5-CH_2OH uracil, the expected accumulation product, could not be detected. Two possible interpretations of the thymine accumulation are put forward.

1. The oxidation of the methyl group of thymine to the formyl level (5-formyluracil) is carried out by one enzyme. In this reaction 5-CH_2OH uracil would be an enzyme bound intermediate and presumably would not be formed at all by the mutant enzyme (uc-3).
2. The thymine to 5-CH_2OH uracil to 5-formyluracil conversions are catalyzed by two enzymes. If the equilibrium of the thymine to 5-CH_2OH uracil conversion were to be strongly in favor of thymine, only small amounts of 5-CH_2OH uracil would accumulate even in the presence of a mutation (uc-3) blocking the conversion of 5-CH_2OH uracil to 5-formyluracil.

Although enzymological studies of methyl group oxidation have not as yet produced an example of one enzyme converting a methyl group to the formyl oxidation state the conversion of a primary alcohol group to carboxylic acid by one enzyme is known (47). Consecutive oxidations occur on the surface of a single enzyme without the appearance in the free state of the presumed aldehydic intermediate in the conversions of the primary alcohol groups of histidinol and uridine diphosphate glucose to the carboxylic acid groups of histidine and uridine diphosphate glucuronic acid respectively. Only a very limited amount of work has been reported on the enzymology of the thymine methyl oxidative pathway. Abbott et al. (12) administered C^{14} labeled thymine to the supernatant of a crude Neurospora extract and recovered 5-CH_2OH uracil in high yield (the equilibrium for the reaction is therefore not strongly in favor of thymine), but found no 5-formyluracil or other pyrimidine product. The enzyme carrying out the formation of 5-CH_2OH uracil required NADPH and O_2 and was classified as a hydroxylase. Their data imply that a second enzyme is required for the formation of 5-formyluracil. If the thymine to 5-formyluracil steps are carried out by one enzyme as data presented in this thesis suggest, then the assumption must be made that the enzyme isolated by Abbott et al. (12) had lost the ability to carry the substrate from the alcohol to the formyl oxidation state. The hypothesis of a bifunctional enzyme carrying out two oxidation steps would be strengthened if the uc-3 mutant should be found lacking enzymatic activity for the thymine to 5-CH_2OH uracil conversion. A conclusion which can be reached from the evidence

available is that the thymidine to RNA precursor pathway proceeds thymidine → thymine → 5-CH_2OH uracil → 5-formyluracil →, and that at least three enzymes are required for the first four steps.

Fink (10) proposed that uracil-5-carboxylic acid is an intermediate in the thymine methyl oxidative pathway. This compound would seem a logical intermediate of the pathway since it could be formed in one step from 5-formyluracil and with the release of CO_2 through the action of a decarboxylase would give uracil. However, growth tests with pyr mutants showed that uracil-5-carboxylic acid is the only one of the proposed intermediates of the pathway which is not utilized efficiently. In pyr mutant growth tests involving limiting concentrations of uridine an added supplement of uracil-5-carboxylic acid remained in the medium without loss throughout the growth period. A negative growth response test alone does not rule out a compound as an intermediate of a pathway since, for example, the organism may be unable to transport the compound from the medium to the site in the cell where it is required. Orotic acid (uracil-6-carboxylic acid) although an intermediate of the pyrimidine pathway is utilized inefficiently by Neurospora.

Other evidence obtained in relation to uracil-5-carboxylic acid as a possible intermediate in the oxidative pathway is also of a negative nature. No mutants were found which accumulated uracil-5-carboxylic acid nor were mutant hunts successful in finding strains which could utilize the compound as a pyrimidine source. In view of the lack of proof for a uracil-5-carboxylic acid intermediate, the

possibility that the acid is, in fact, not an intermediate must be considered as likely. A one-step conversion of 5-formyluracil to uracil could be carried out if the formyl group were removed by a tetrahydrofolic acid acceptor.

A third mutation blocking a synthetic reaction necessary for the incorporation of the pyrimidine ring of thymidine into RNA is uc-4. The uc-4 mutation when introduced into a pyrimidine mutant strain allowed utilization of uridine as a pyrimidine source, but not of thymidine, thymine, 5-CH_2OH uracil, 5-formyluracil or uracil. On the basis of the growth experiments the uc-4 mutation can be classified as blocking a reaction required in the incorporation of uracil into uridylic acid.

Two enzymatic mechanisms are known for the conversion of uracil into uridine monophosphate (UMP) (13). The first (reaction a) demonstrated in microorganisms (48, 49) is analogous to the formation of orotidylic acid from orotic acid and 5-phosphoribosylpyrophosphate (PRPP) in the pyrimidine pathway.

(a) uracil + PRPP $\rightleftharpoons$ UMP + pyrophosphate

The second mechanism (reactions b and c) which occurs in mammalian tissue involves the sequential action of pyrimidine nucleoside phosphorylase and pyrimidine nucleoside kinase (49).

(b) uracil + ribose-1-phosphate $\rightleftharpoons$ uridine + Pi

(c) uridine + ATP $\longrightarrow$ UMP + ADP

Nucleoside phosphorylase reactions (reaction b) are now recognized as functioning in vivo for the degradation of nucleosides (44, 50). On

the basis of this evidence it is concluded that in Neurospora uracil is converted to UMP by a pyrophosphorylase enzyme (reaction a) and that the uc-4 mutation blocks this step.

The pyr-1 uc-4 double mutant gave only one-third the growth of a pyr-1 mutant on equivalent amounts of uridine. In addition the double mutant was discovered to accumulate uracil in the growth medium (to a concentration of about one-half the molar concentration of the added uridine). The reduced growth of the pyr-1 uc-4 mutant was apparently caused by nucleosidase action on the uridine with the formation of uracil, which the uc-4 mutation prevents from being utilized. This cleavage of uridine is of interest because of the reports (17, 42, 51, 52) that various organisms including Neurospora (with a notable exception being E. coli) incorporate uniformly labeled ribonucleosides into nucleic acids without separation of the base and ribose moieties.

Two basic differences exist between the experiments of McNutt (17), who showed that pyrimidine ribonucleosides are incorporated into the nucleic acids of Neurospora without breaking the nucleoside bond, and the work reported here, which shows that at least half of the added pyrimidine nucleoside is cleaved. McNutt inoculated a pyr-4 strain of Neurospora into Fries medium (has ammonium salts) containing the nucleoside while in the work reported here a pyr-1 uc-4 double mutant was inoculated into Westergaard-Mitchell medium (no ammonium salts) containing the nucleoside. The possibility exists that a secondary action of the uc-4 mutation could induce the production of a uridine nucleosidase enzyme and cause the cleavage of uridine. It is

believed more likely that the Westergaard-Mitchell medium is responsible (at least indirectly) for the increased nucleosidase action since this medium also has a large effect on the efficiency of utilization of intermediates of the thymidine to RNA precursor pathway.

Mutations affecting pyrimidine transport

It now appears likely that for most physiologically important substrates the fungal cell possesses special transport systems which allow rapid entry into the cell (53). The number of such mechanisms is probably not large since the transport systems thus far discovered have a broad specificity. In yeast, the only fungus in which transport has been studied with any intensity, there are known about fifteen to twenty transport systems; three or four for inorganic ions, three for sugars, four or five for amino acids, three for organic acids and a small number for nucleotides and growth factors. These systems are not normally present, but are induced in the presence of a specific substrate. The mechanisms by which solutes penetrate the membrane are not well understood, although many models have been proposed (53, 54). The kinetics of transport can be explained in most cases by assuming that the substrate must form a chemically specific complex with a receptor site in the membrane in order for transport to occur. It is hypothesized that this complex or a complex formed by transfer of the substrate to a second carrier moves across the membrane and releases the substrate on the other face of the membrane.

Mutants which are defective in the uptake of a given class of compounds (transport negative mutants) have been very useful in the

study of various transport systems. In bacteria two methods have been used to select for transport negative mutants. The first method involves selecting for resistance to various growth inhibitors, for instance some canavanine resistant mutants were found to be defective in the transport of canavanine and the structually related amino acid, arginine (55). The second method takes advantage of the expected absence of growth of specific auxotrophs which have undergone a mutational loss of the transport mechanism specific for their required compound (56, 57). For example, from a histidineless strain (*his*$^-$) of *E. coli* a double mutant was selected (*his*$^-$ histidine transport negative) which required 25 times the concentration of histidine in the external medium to equal the growth rate of the parent strain. The double mutant was shown to lack the ability to accumulate histidine in the cell.

The method described here to select for mutations blocking specific reactions in the pyrimidine salvage pathway is essentially the procedure used in bacteria to isolate transport mutants from an auxotroph. When *pyr* mutant conidia were irradiated and selection was made for isolates which could not utilize a given pyrimidine two types of mutants were possible: those mutants defective in an enzyme necessary for the conversion of the pyrimidine into nucleic acid and those lacking the ability to transport the pyrimidine into the cell. These mutant hunts yielded several *Neurospora* isolates carrying new mutations which prevent the utilization of various pyrimidines. Evidence has been discussed to show that three of these mutations, *uc-2*, *uc-3* and *uc-4*,

cause defective enzymes in the pyrimidine salvage pathways. Data gathered from strains carrying another three mutations, uc-5, ud-1 and ud-2, can be most simply interpreted in terms of these mutations disrupting pyrimidine transport mechanisms.

From a pyr-1 Neurospora strain was isolated a double mutant, pyr-1 uc-5, which was unable to utilize the free pyrimidine bases, thymine, 5-CH_2OH uracil, 5-formyluracil and uracil, although it did utilize in a normal manner the ribose and deoxyribose pyrimidines, uridine, cytidine, deoxyuridine and thymidine. The uc-5 mutation cannot be affecting an enzyme in the synthetic pathways converting deoxyribose pyrimidine nucleosides to RNA pyrimidines since the initial compounds of the pathway, thymidine and deoxyuridine, are utilized while intermediates which normally arise from these two compounds are not metabolized. A hypothesis which explains the growth response of the pyr-1 uc-5 double mutant is that Neurospora possesses one transport system for pyrimidine nucleosides and a second system for pyrimidine bases; the latter system is prevented from functioning by the uc-5 mutation (figure 3).

Support for the hypothesis that Neurospora has two transport systems to carry pyrimidines into the cell was obtained from experiments with a second double mutant, pyr-1 ud-1, isolated because of its inability to utilize uridine. This double mutant behaves in an opposite manner to the pyr-1 uc-5 mutant in that the ud-1 carrying double mutant cannot utilize the ribose and deoxyribose pyrimidines (uridine, cytidine, deoxyuridine and thymidine), although it can use

the pyrimidine bases (uracil, thymine, etc.) in a normal manner. Again it is difficult to propose a single enzyme deficiency in the synthetic pathway which could produce the growth responses given by the pyr-1 ud-1 mutant. The most plausible explanation is that the ud-1 mutation prevents transport of pyrimidine nucleosides into the cell, but does not affect pyrimidine base transport. Verification that uc-5 and ud-1 mutations prevent pyrimidine transport would be the demonstration that strains carrying these mutations possess the normal enzyme complement for utilization of the pyrimidine bases and nucleosides.

Of interest in relation to pyrimidine transport negative mutants in Neurospora was a report of cytidine uptake by E. coli (58). The transport of cytidine into the cell was competitively inhibited by the presence of uridine, thymidine and adenosine, but not by free purine or pyrimidine bases. In a similar study the uptake of adenosine by the E. coli cell was competitively inhibited by pyrimidine nucleosides (uridine, cytidine, deoxyuridine and thymidine) and by several purine nucleosides (59). Guanosine and the purine and pyrimidine bases gave little competition to adenosine uptake. In general the results of E. coli suggest the presence of a transport element common to purine and pyrimidine nucleosides, but which shows little affinity for free nucleic acid bases. Information as to whether the Neurospora uc-5 and ud-1 mutations affect purine transport was not obtained, but the Neurospora and E. coli systems are similar in that they both must possess two pyrimidine transport systems, one for the bases and the other for the nucleosides.

Only a few transport negative Neurospora mutants have been reported. Both Lester (60) and Stadler (61) isolated Neurospora mutants which were resistant to 4-methyl tryptophan (mtr mutants). The germinating conidia of mtr strains had a greatly reduced ability to take up methyl tryptophan, tryptophan, phenylalanine, tyrosine and several other amino acids. The action of the mtr mutant is consistent with the evidence which shows that Neurospora has a small number of amino acid transport systems, each responsible for the transport of several amino acids (62). Crocken and Tatum (63) have investigated sorbose transport in Neurospora and indicate that some sorbose resistant Neurospora mutants may be defective in the ability to take up sorbose and related sugars from the medium.

A limited number of Neurospora mutations have been studied, whose actions affect the permeability of a much wider range of compounds than can be explained by the specificity of most transport systems. St. Lawrence et al. (64) have characterized a mutant, mod-5, in which the germinating conidia initially give enhanced uptake of all the amino acids and of small peptides. Davis and Zimmerman (43) reported a mutant, UM-300, that has deficient uptake of arginine, lysine, unrelated amino acids, and uridine, but at least with regard to arginine the difficulty of uptake is conditional on the presence of ammonium in the medium. The ud-2 mutant discovered here, which cannot utilize any pyrimidine in a medium containing ammonium salts but does use pyrimidines when the medium lacks ammonium salts may well be a mutant of the UM-300 type. The primary action of such mutations as

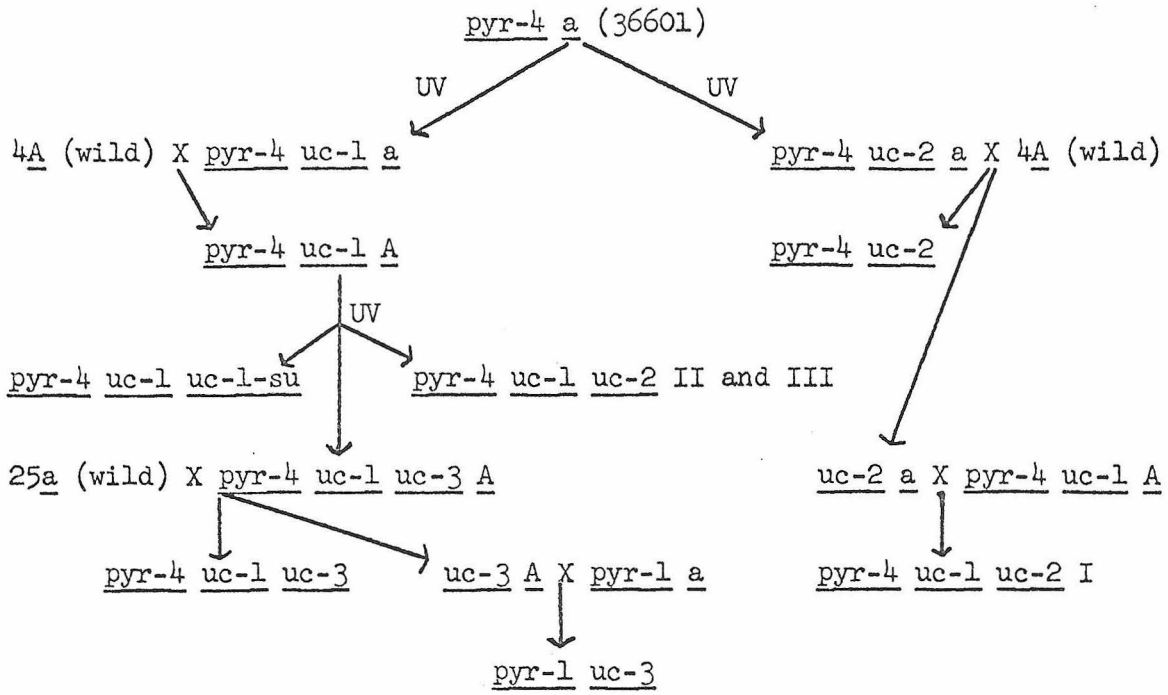
mod-5, UM-300 and ud-2 may not be on a specific transport system itself, but on a cell membrane barrier to permeability or a step in transport common to many specific transport systems.

Mutations affecting the regulation of the thymidine to RNA precursor pathway

Two mutations, uc-1 and uc-1-su influence the regulation of the thymidine to RNA precursor pathway shown in figure 3. Strains carrying the uc-1 mutation were isolated as colonies which appeared on thymidine plus thymine supplemented sorbose plates which had been inoculated with UV irradiated pyr-4 conidia. Conidia of pyr-4 inoculated into medium containing only thymidine, thymine or 5-CH_2OH uracil as a pyrimidine source will not grow. These same compounds are efficiently utilized when a normal pyrimidine source such as uridine is added to the medium to promote some initial mycelial growth. The presence of the uc-1 mutation allows pyrimidine mutants to grow normally when the pyrimidine requirement is met only by thymidine, thymine or 5-CH_2OH uracil.

The uc-1-su mutation suppresses the action of uc-1. (In figure 4 is given the parent strain from which the suppressor mutant was isolated. Also given are the origins of the other isolated mutants.) A pyr-4 uc-1 uc-1-su triple mutant behaves as does a pyr-4 mutant in that neither will grow when thymidine, thymine and 5-CH_2OH uracil are the sole pyrimidine sources.

The pyr-4 uc-1 double mutant was of superficial interest because the two mutations, pyr-4 blocking the pyrimidine synthetic pathway and



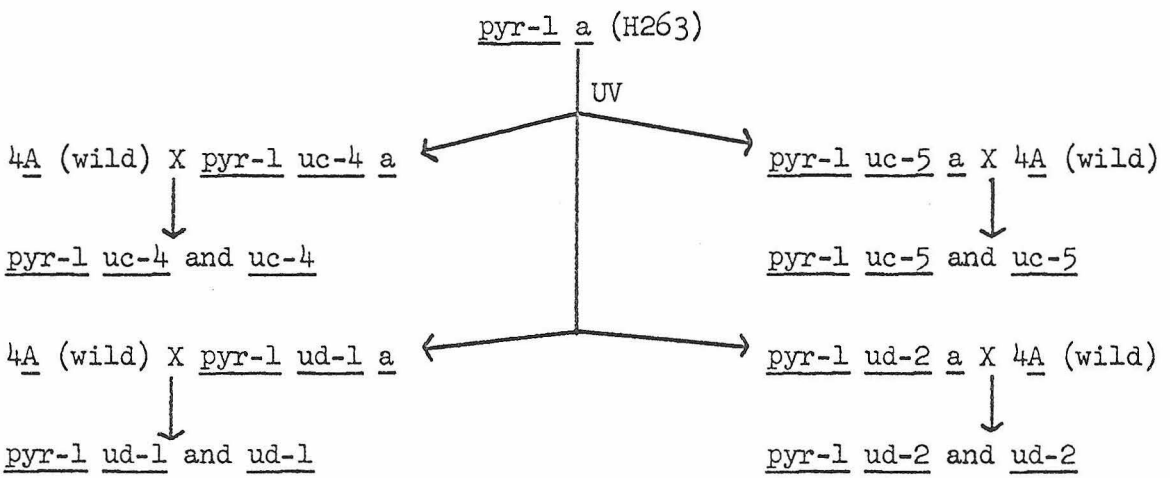


Figure 4.--Origins of uracil (uc) and uridine (ud) mutants. All mutants reported were induced by UV in strains carrying either the pyr-4 or pyr-1 mutation. Strains carrying new mutations were crossed to either wild type 4A or 25a and mutant segregants were reisolated. Crosses were also made between mutant strains to provide segregants carrying the mutations in new combinations.

uc-1 which allows the induction of the thymidine to RNA precursor pathway in germinating conidia, give the same phenotype (no growth on minimal, a response to thymine or thymidine) as would be expected of a thymine mutant. Although looked for, thymine mutants, which have a defective thymidylate synthetase enzyme, have never been recovered in Neurospora. Since Neurospora possesses no alternate means of forming thymidylic acid i.e., a thymidine phosphorylating enzyme, and because thymidylic acid is unable to penetrate the cell wall thymine mutants are irreparable.

The isolation of mutants blocking synthetic reactions involved in the utilization of thymidine (uc-2, uc-3 and uc-4) and of mutants affecting pyrimidine transport systems (uc-5 and ud-1) emphasizes the role of the uc-1 gene in controlling one or more of these reactions. To answer the question of what kind of control the uc-1 gene imposes (feedback inhibition, repression or some less understood control) the reaction or reactions controlled by uc-1 must be known. In answer to the latter question the growth response tests show only that the enzyme(s) necessary for the conversion of 5-CH_2OH uracil and/or 5-formyluracil to uracil are repressed, or possibly are present and subject to an inhibitor, in the germinating conidia of strains not carrying the uc-1 mutation. These 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. Clearly studies of the thymidine to RNA precursor pathway enzymes and of pyrimidine transport systems in uc-1 and normal strains are needed. However, available evidence does permit

at least a superficial comparison of the uc-1 mutant with regulatory mutants in more defined systems.

The presence of a series of mutants affecting both the conversion of thymidine to an RNA precursor and pyrimidine transport systems initially suggests the possibility that these reactions are controlled by a method similar to that described by Jacob and Monod (65) for the lactose operon in E. coli. In such a comparison the uc-1 mutation could be regarded as analogous to the i^- mutation in E. coli permitting the constitutive synthesis of enzymes which normally must be induced. However, two lines of evidence indicate that this new group of Neurospora mutants is not part of an operon. First, neither pyrimidine transport system (one for pyrimidine bases and another for pyrimidine nucleosides) appears to be controlled by the uc-1 gene. Conidia of strains lacking the uc-1 mutation cannot utilize thymine and thymidine, but can use uracil and uridine. Therefore, the pyrimidine transport systems are present under conditions where synthesis of one or more of the enzymes of the thymidine to RNA precursor pathway is prevented. Second, it should be noted that genes governing synthesis and inducibility in the E. coli system are closely linked while those in the Neurospora system are for the most part scattered.

The regulatory uc-1 locus is linked, although not closely to the pyr-4 locus on chromosome II while uc-2 (deoxyribose pyrimidine nucleosidase) is linked to the mating type locus on chromosome I as is uc-4 (uracil to uridylic acid conversion). The uc-5 locus (pyrimidine base transport) is linked to pyr-1 on chromosome IV. The linkages of

uc-3 (thymine to 5-formyluracil conversion), ud-1 (pyrimidine nucleoside transport), ud-2 (all pyrimidine transport in ammonium medium) and uc-1-su (suppressor of uc-1) loci are not known. There was no evidence that uc-3 is linked to a mating type, pyr-4 or pyr-1 locus as were some previously mentioned mutations. Neither was there evidence for the linkage of ud-1 and ud-2 to a pyr-1 or mating type locus. It should be mentioned that a mutant was not found for the enzyme that converts 5-formyluracil to uracil.

The linkage data, in general, fit the pattern in Neurospora and higher organisms where only a limited number of cases are known of genes belonging to the same biosynthetic pathway being adjacent to one another on the genetic map. Several systems have been investigated in Neurospora in which genes producing two or more enzymatic functions of the same biochemical pathway map at the same locus (66, 67). Some of these loci now appear to produce one enzyme with two enzymatic activities, such as trypt-3 (tryptophan synthetase) and pyr-3 (carbamyl synthetase and aspartate transcarbamylase). The hist-3 locus in Neurospora in which three enzymatic activities are located is probably one of the best cases for an operon in an organism other than a bacterium. The possibility exists that the uc-2 and uc-4 loci of the thymidine to RNA precursor pathway might be closely linked, since on the basis of tests with a limited number of ascospores both uc-2 and uc-4 showed close linkage to the mating type gene.

Horowitz et al. (68, 69) have described three unlinked genes, T, ty-1 and ty-2, controlling the synthesis of tyrosinase in Neurospora

crassa. The T gene has a structure determining role in the synthesis of the enzyme while ty-1 and ty-2 influence the synthesis indirectly through their control of an inductive mechanism that operates on the enzyme. Several similarities between the tyrosinase and thymidine to RNA precursor pathway systems bear mentioning.

1. Both the ty genes and the uc-1 gene are not linked to the genes whose products they control.
2. Both tyrosinase and the enzymes of the thymidine to RNA precursor pathway are nonessential enzymes.
3. Both systems respond to a starvation medium. Tyrosinase production is constitutive in a low sulfate or nitrogen medium, and the thymidine to RNA precursor pathway functions most efficiently in a nitrogen starved medium.
4. Tyrosinase production in constitutive strains is greatest in four day old mycelia. This age mycelia is likely the period of greatest thymidine to RNA precursor enzyme synthesis since the degraded nucleic acids released by death of cells could be expected to induce the system.

However, the ty mutations and uc-1 seem to act in an almost opposite manner. The ty mutations prevent the constitutive production of tyrosinase while the uc-1 mutation makes constitutive or at least allows the induction in germinating conidia of the enzymes of the thymidine pathway. The biochemical defect of ty-1 and ty-2 is regarded as either a deficiency in the production of inducer or an excessive production of repressor. If the action of the uc-1 gene is to be regarded as similar

to that of the ty genes then one must assume that the uc-1 mutation causes increased production of inducer or causes a defective repressor.

Preliminary experiments have revealed other evidence relating to control of pyrimidine salvage pathways. When a pyr mutant is grown in a medium containing a growth limiting concentration of uridine plus an added thymine supplement the thymine concentration does not begin to drop until the mycelial pad reaches a weight at which growth would have stopped if only the uridine had been present. A second observation was that a pyr-4 uc-1 double mutant (which utilizes thymidine, thymine and 5-CH_2OH uracil as sole pyrimidine sources just as efficiently as the parent pyr-4 strain uses uridine) utilizes uridine only about one-half as efficiently as a pyr-4 strain. These results show that the reaction(s) by which uridine is utilized and the thymidine to RNA precursor pathway do not function independent of one another.

The control of one pathway by another could be brought about if, for example, uridine monophosphate (a product of the uridine kinase reaction and the end product of the thymidine to RNA precursor pathway) has an end product feedback inhibition action on an enzyme of the thymidine to RNA precursor pathway. The uc-1 mutation might then be regarded as a mutation causing the production of a uridine kinase enzyme of reduced activity. A mutation acting as proposed would directly affect uridine utilization and indirectly affect the thymidine to RNA precursor pathway.

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