Measurement of Iso-orotate Decarboxylase Activity in *Neurospora Crassa*

By

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Submitted in Fulfillment of the Requirements

for the Degree of

Masters of Science

in the Biology Program

Youngstown State University

August 21, 1999

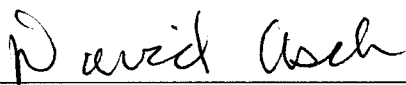Measurement of Iso-orotate Decarboxylase in *Neurospora Crassa*
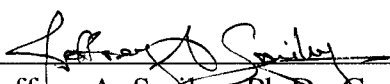
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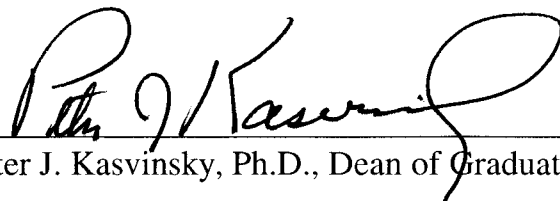
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ABSTRACT

The biosynthesis of uridine-5'-monophosphate (UMP) can occur through two pathways. The first and most common is the *de novo* pathway, while the second and more unique pathway is the thymidine salvage pathway. The fungus *Neurospora crassa,* unlike many organisms, contains both but utilizes the second of these pathways to convert thymidine to uracil.

My research focuses on the fourth enzyme of the thymidine salvage pathway, iso-orotate decarboxylase. Preliminary characterization of IDCase was determined by a radioactivity based assay using ^{14}C to label the substrate IOA. This decarboxylation assay measures the amount of IDCase activity by detecting the amount of radio-labeled CO_2 released from IOA in the reaction at a specified time point.

Wild type *N. crassa* strains had the highest specific enzyme activity of 2.5 nmol/min/mg when grown in a shake culture for 18 hours at 30°C. We have tested iso-orotate decarboxylase levels at various time points in the *N. crassa* life cycle.

We have also examined the rate of induction of IDCase activity in the presence of nitrate as well as in the *N. crassa* strains containing the pyr-4 and nit-2 mutations. As uracil has been shown to be an inducer of the IDCase levels and may therefore act as an inducer for the thymidine salvage pathway.

Acknowledgments

I wish to extend my gratitude to my advisor Dr. David Asch for his time and guidance throughout this thesis project. I would also like to thank Dr. Jeff Smiley for all his patience and understanding when this project got rough for me, and Dr. Gary Walker for helping me whenever I needed the help.

A special thanks to my friends here at YSU and from home. All of your continued encouragement and support was greatly appreciated.

Finally, I would like to dedicate this thesis to my parents, Halina Marshall and Sidney Marshall. Ever since I was a little girl you were there for me. For as long as I can remember you both stressed the importance of education and look where it has taken me. I will always be grateful for everything you have given me and done for me. You are the wings that made me fly.

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Introduction

Neurospora crassa belongs to the class of fungi consisting of about 15,000 different species called Ascomycotina. The defining characteristic of this class of fungi is the existence of spores in a sac called an ascus, that are explosively ejected (Webster 1980). Many ascomycotes also have a perforated transverse septum, which means several different nuclei may occur in a common cytoplasm. These nuclei aren't always identical. Cells which contain nuclei of more than one genotype, meaning that the nuclei are genetically different, are called heterokaryotic. These nuclei are often haploid (Webster 1980).

Like many Ascomycetes, *N. crassa* may undergo both sexual and asexual reproduction. Sexual or "perfect" reproduction involves formation of ascospores and asexual or "imperfect" reproduction undergoes formation of asexual spores called conidia. *N. crassa*, is a heterothallic species meaning that sexual reproduction only occurs following interaction of two different mating types. The female sex organs and the male sex organs can be formed on a single strain but are self-incompatible. During the sexual life cycle, two haploid nuclei fuse resulting in the formation of a diploid cell which immediately undergoes meiosis. This is the only point in the life cycle in which *N. crassa* is diploid. The products eventually differentiate into eight ascospores (Webster 1980).

After ejection the ascospores of *N. crassa* are viable for many years but do not germinate readily unless treated chemically, or are heat shocked. After either of these treatments the spores germinate and produce a coarse rapidly growing mycelium. Within 24 hours after germination, the mycelium can begin asexual reproduction and with time conidia arise. Individual segments of the conidial chain break apart and are dispersed by wind. The *N. crassa* lifecycle is pictured in Figure 1.

N. crassa is widely used in genetical and biochemical studies. This is because *N. crassa* wild type strains are haploid during vegetative growth so no mutations are masked by wild type genes. They also have simple nutritional requirements consisting of a carbohydrate source, simple mineral salts, and biotin. *N. crassa* has only seven chromosomes so it is genetically simple and easy to work with. Though it is a simple organism, it is multicellular with a sexual phase of its lifecycle. This lends to simple genetic analysis.

Nucleotide Metabolism

Nucleotides are substances that are present in virtually all biochemical processes. Nucleotides consist of a nitrogenous base, a 5-carbon sugar, either a deoxyribose or ribose, and a phosphate group. Nucleotide triphosphates are used in the synthesis of nucleic acids. Nitrogenous bases are sorted into two categories.

Figure 1. The *N. crassa* lifecycle. The conidia are used to make an inoculate of that is used in the Iso-orotate decarboxylase experiments in this project.

Ascospore germination←←←←Ascospores ejected from mature ascus

↓   ↑

↓   ←←←←←←

↓   ↓   ↑   ↑

             ↓   asexual   ↑

mycelial growth   cycle   ↑   meiosis and

↓   ↓   ↑   spore formation

          ↓   ↑   sexual   ↑

        conidia→→→→↑   cycle

↓   ↑

                                   Nuclear division

↓   and growth

                                     ↑

                                     ↑

→→→→→→   Fertilization→→→→→→
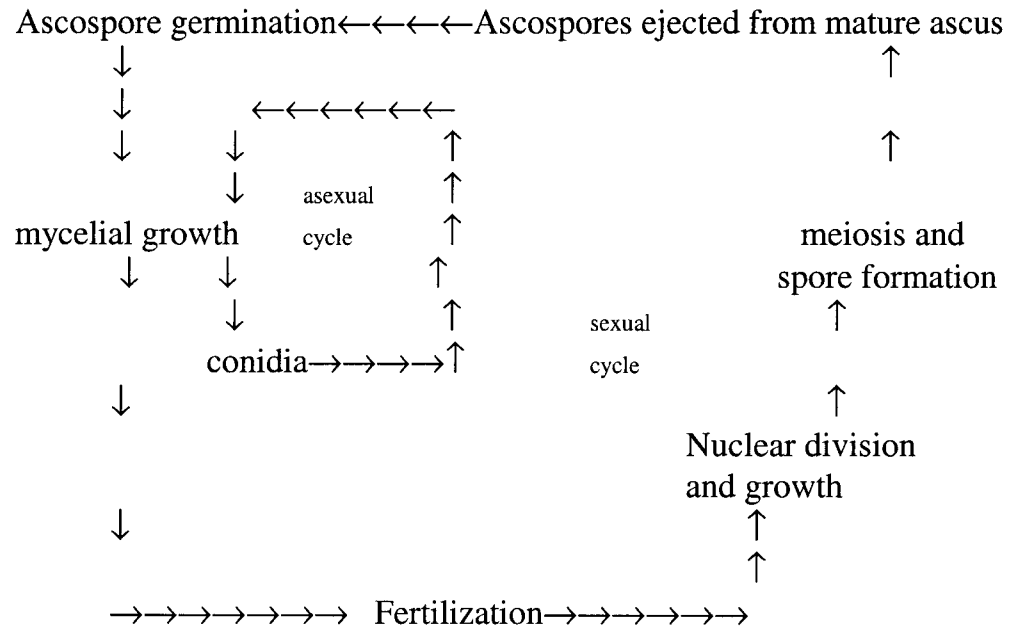
The purine residues are adenine and guanine, while the pyrimidine residues are cytosine, thymine and uracil, which is substituted for thymine in RNA (Voet and Voet 1995).
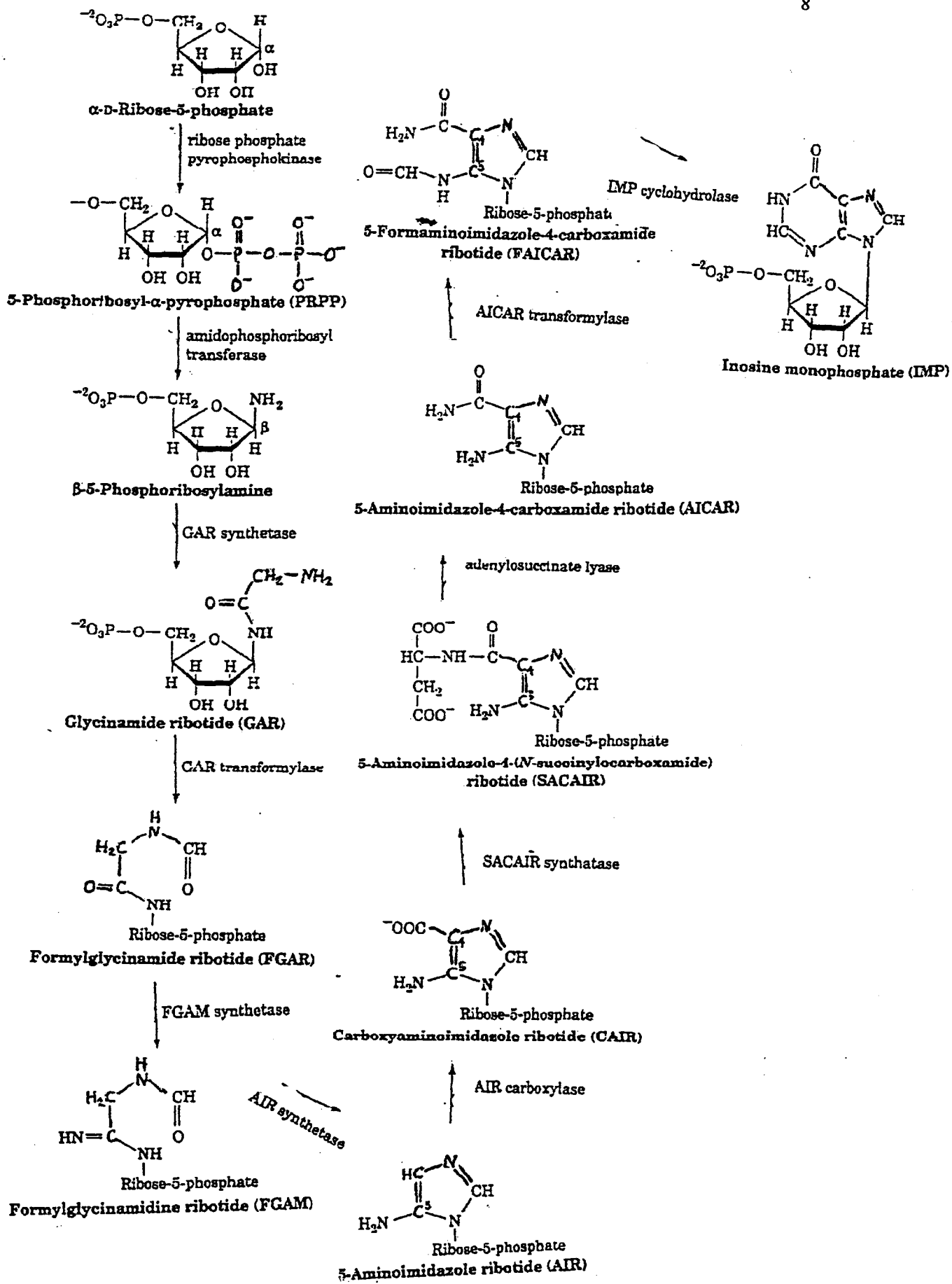
De novo purine pathway

Most organisms, including *N. crassa* contain a pathway for the *de novo* biosynthesis of both purines and pyrimidines. Nucleotides are important in cellular metabolism and nearly all cells can synthesize them through the *de novo* pathways and through degradation products of nucleic acids. Purine nucleotides, from the synthesis or degradation of nucleic acids, are present in every cell in the body. Biosynthesis of a purine nucleotide, Inosine Monophosphate, via the *de novo* purine pathway from alpha D-ribose-s-phosphate occurs using ATP through eleven enzymatic reactions (figure 2). The first step is the activation of ribose-5-phosphate. In this step, ribose phosphate pyrophosphokinase activates ribose-5-phosphate using ATP to form 5-phosphoribosyl-∝-pyrophosphate (PRPP). This reaction is unusual because a pyrophosphoryl group is directly transferred from the ATP to ribose-5-phosphate. PRPP is also a precursor in pyrimidine biosynthesis which will be discussed in the next section. Next amidophosphorybosyl transferase catalyzes the displacement of PRPP's phosphate group by glutamine's amide nitrogen. This is the pathway's flux-generating step. In the third step,

glycerine's carboxyl group forms an amide with the amino group of phosphoriboslyamine, which yields glycinamide ribotide (GAR). This is irreversible due to the hydrolysis of ATP. Then GAR's free α-amino group is formylated to yield formylglycinamide ribotide (FGAR). This is followed by the transfer of an amide amino group from a second glutamine to the growing purine ring in order to form formylglycinamide ribotide (FGAM). This reaction is also driven by the hydrolysis of ATP. The sixth step is the formation of the purine imidizole ring. This condensation reaction yields 5-amino-imidizole ribotide (AIR). Then AIR carbomylase catalyzes a reaction that yields carboxyaminoimidizole ribotide (CAIR). Next, aspartate contributes purine atom N1 in a condensation reaction driven by ATP hydrolysis. Step nine is the elimination of fumerate by cleaving SACAIR. Reaction ten and eleven are the final purine ring atom acquisition of C2 followed by cyclization to form IMP. This is done by the elimination of water rather than the hydrolysis of ATP(Voet and Voet 1995). The reaction is shown in Figure 2.

IMP does not accumulate in the cell, but gets rapidly converted to AMP and GMP. IMO (inosine monophosphate) is synthesized in a two step reaction pathway. First, aspartate's amino group is linked to IMP through the hydrolysis of GTP to yield adenylosuccinate. In the second reaction, adenylosuccinate lyase eliminates fumerate from adenylosuccinate to form ATP(also shown in figure 2).GMP is also synthesized in a two reaction pathway.

Figure 2 Purine Biosynthetic Pathway adapted from Biochemistry Second Edition, Voet and Voet 1995.

α-D-Ribose-5-phosphate

ribose phosphate pyrophosphokinase

5-Phosphoribosyl-α-pyrophosphate (PRPP)

amidophosphoribosyl transferase

β-5-Phosphoribosylamine

GAR synthetase

Glycinamide ribotide (GAR)

GAR transformylase

Formylglycinamide ribotide (FGAR)

FGAM synthetase

Formylglycinamidine ribotide (FGAM)

AIR synthetase

Ribose-5-phosphate
5-Aminoimidazole ribotide (AIR)

AIR carboxylase

Ribose-5-phosphate
Carboxyaminoimidazole ribotide (CAIR)

SACAIR synthatase

Ribose-5-phosphate
5-Aminoimidazole-4-(N-succinylocarboxamide) ribotide (SACAIR)

adenylosuccinate lyase

Ribose-5-phosphate
5-Aminoimidazole-4-carboxamide ribotide (AICAR)

AICAR transformylase

Ribose-5-phosphate
5-Formaminoimidazole-4-carboxamide ribotide (FAICAR)

IMP cyclohydrolase

Inosine monophosphate (IMP)

First, IMP is dehydrogenated to form xanthosinemonophosphate (ZMP) which is then converted to GMP by the transfer of glutamine amide nitrogen. This is also shown in Figure 2.

The de novo pyrimidine pathway

The *de novo* biosynthesis of pyrimidine nucleotides occurs in virtually all organisms through a six-step enzymatic pathway (Jones 1980) and results in uridine-5-monophosphate (UMP). Step one involves the amino acid glutamine, the bicarbonate ion HCO_3^-, and two moles of ATP to form carbomyl phosphate. Step number two involves the synthesis of carbamoyl aspartate catalyzed by aspartate transcarbamylase. The third step is a condensation reaction involving ring closure to form dihydroorotate catalyzed by dihydroorotase. This is followed by the oxidation of dihydroorotate which is an irreversible reaction facilitated by the enzyme dihydroorotate dehydrogenase. In step five, orotate reacts with PRPP to yield orotidine-5'-mono-phosphate (OMP). This reaction is catalyzed by orotate phosphoribosyl transferase. Finally, the sixth enzyme OMP decarboxylase enables the formation of the pyrimidine ribonucleotide UMP. The pyrimidine biosynthetic pathway is pictured in Figure 3. Biosynthesis of thepyrimidine ring requires aspartate, bicarbonate, ammonia, and metabolic energy in the form of ATP.

Figure 3) *de novo* Pyrimidine biosynthetic pathway. Enzyme 1 is carbamyl phosphate synthetase II. Enzyme 2 is transcarbamylase, enzyme 3 is dihydroorotase, enzyme 4 is dihydroorotate dehydrogenase, enzyme 5 is orotate phosphoribosyl transferase, and enzyme 6 is orotidine monophosphate. Adapted from Cannon, 1997.
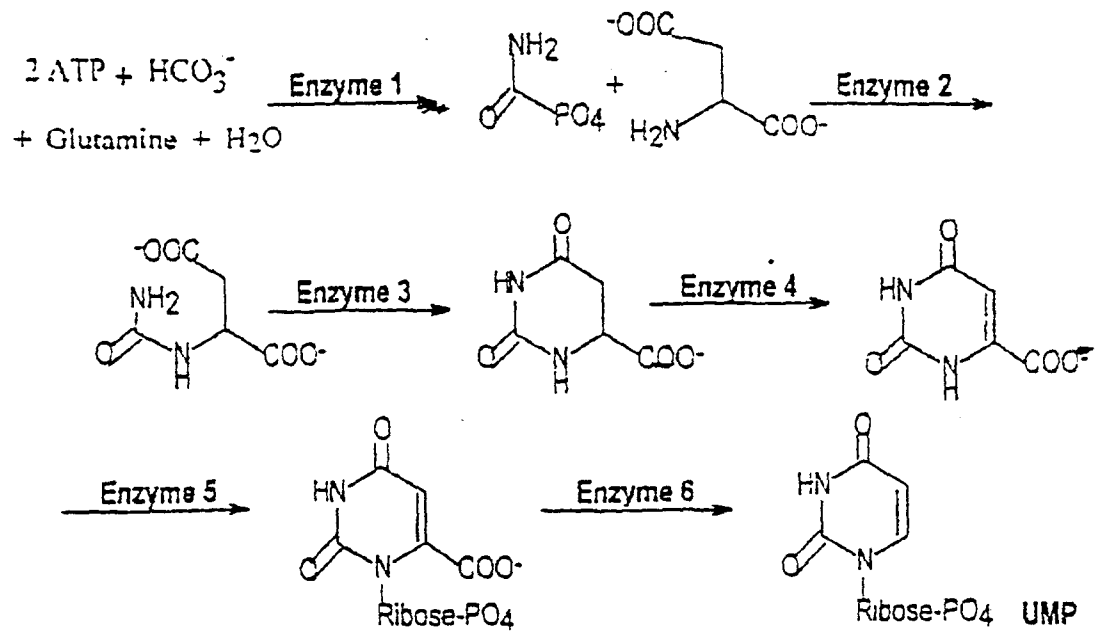
All other pyrimidine nucleotides are synthesized from UMP (Kornberg 1992). In this six-step process, a pyrimidine base if formed before it is converted to a nucleotide, UMP. UTP is then formed by phosphorylation of UMP, and CTP is synthesized by the amination of UTP. Pyrimidine biosynthesis is regulated by feedback inhibition as well as by the concentrations of purine nucleotides.

The biosynthesis of thymine nucleotides from UMP for DNA is carried out by the enzyme thymidylate synthase (Reichard 1988). The formation of thymine nucleotides involves methylation of dUMP to TMP which is an irreversible step. However, in addition to the *de novo* pathway, certain organisms possess a metabolic pathway for the conversion of thymine to uracil (Neuhard 1983). This pathway is referred to as the thymidine salvage pathway and was identified in *N. crassa* with the observation of incorporation of ring-labeled thymidine into RNA (Neuhard 1983). This is indicative of the absence of thymidine kinase and a metabolic route through UMP. The enzymes in this pathway disassemble thymidine into uracil through four enzymatic steps which are shown and described (Figure 4).

The enzymatic steps involved in this conversion from thymidine to uracil are 1) oxidation of thymidine, a deoxyribonucleoside, to thymine ribonucleoside using the enzyme pyrimidine deoxyribonucleoside hydroxylase; 2) hydrolytic cleavage of the glycosidic bond to yield thymine and ribose using the enzyme nucleoside hydrolase; 3) a three-step oxidation of thymine to uracil-5-carboxylate

(iso-orotate, IOA) by a single enzyme, thymine hydroxylase; and 4) decarboxylation of iso-orotate to produce uracil using the enzyme iso-orotate decarboxylase (IDCase). The salvage of uracil is then completed by its conversion to UMP by the enzyme uracil phosphoribosyltransferase, an enzyme common to most organisms. The metabolic role of the thymidine salvage pathway is unknown, although thymine hydroxylase(Thase) has been studied in detail (Thornberg and Stubbel 1993). The existence of the thymidine salvage pathway has been identified by the presence of one or more of the enzymes in *N. crassa, Aspergillus nidulans,* and *Rhodotorula glutinis* (Neuhard 1983).

The only enzyme of the thymidine salvage pathway extensively studied thus far, THase, has been purified 1300-fold from *N. crassa.* Pyrimidines 1-methyluracil, 1-ethyluracil, and 6-azathymine are all chemically related to thymine and are able to serve as substrates for THase.

The enzyme IDCase has also been partially purified. It was also shown that orotic acid from the *de novo* pathway is not able to serve as a substrate for IDCase in the place of IOA. No other related pyrimidines have been found that are able to serve as. Even though there has been no report of the presence of the enzyme in any organism except *N. crassa,* it is presumed that the organisms mentioned above with thymine hydroxylase will complete the pathway to uracil with IDCase.

Figure 4) Thymidine Salvage Pathway adapted from Renita Cannon's Masters Thesis.

DNA
Nucleotides

Enzyme 1 & 2

Thymine →(THase)→ 5-HMU → 5-F*U → IOA →(IDCase)→ Uracil
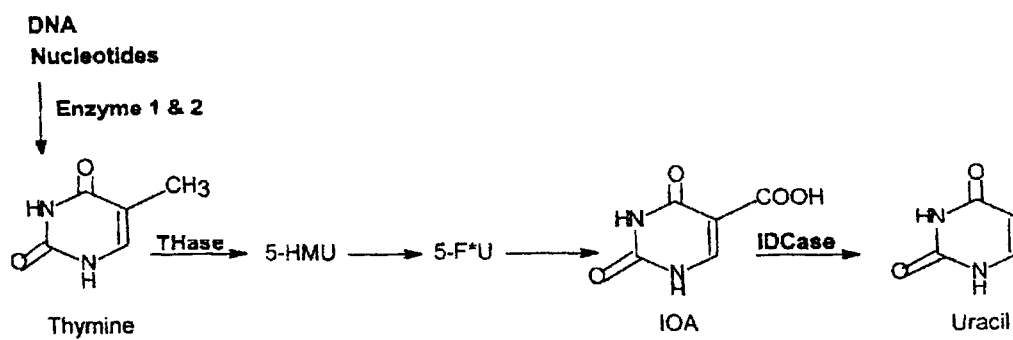
This has been shown to be the case with *R. glutinis* (Smiley et al unpublished data). There is very little information on IDCase itself. It was reported that the enzyme is not sensitive to EDTA, and that addition of common co-factors did not increase the enzyme activity (Palmatier et al 1970). None of the common strategies for decarboxylation are likely to contribute to IDCase catalysis considering the substrate's molecular structure (O'Leary 1992). Also, IDCase probably does not have mechanistic features in common with orotidylate decarboxylase, an enzyme in the *de novo* pyrimidine biosynthetic pathway, since the carboxylate is located at a different position in the ring (Skinner et al 1947).

Most organisms simply salvage thymidine with the enzyme thymidine kinase. The reason why many fungi recycle thymine and thymine nucleosides through UMP is still unclear. It has been suggested the one reason for the pathway is to degrade DNA for use as a total pyrimidine pool, and this pathway is an efficient way to do this. Figure 5 demonstrates this by showing the directionality between fungal metabolism and the metabolism of other organisms.

Nitrogen availability may also be a regulator of the thymidine salvage pathway. When *N. crassa* is grown in Vogels medium, which contains ammonia, it demonstrates a decrease in enzyme activity while when grown in Westergaards medium, which contains nitrate, the enzyme activity is elevated (Cannon 1997). This is due to the differences in nitrogen sources. Westergaards seems to be involved in the observed increase in activity.
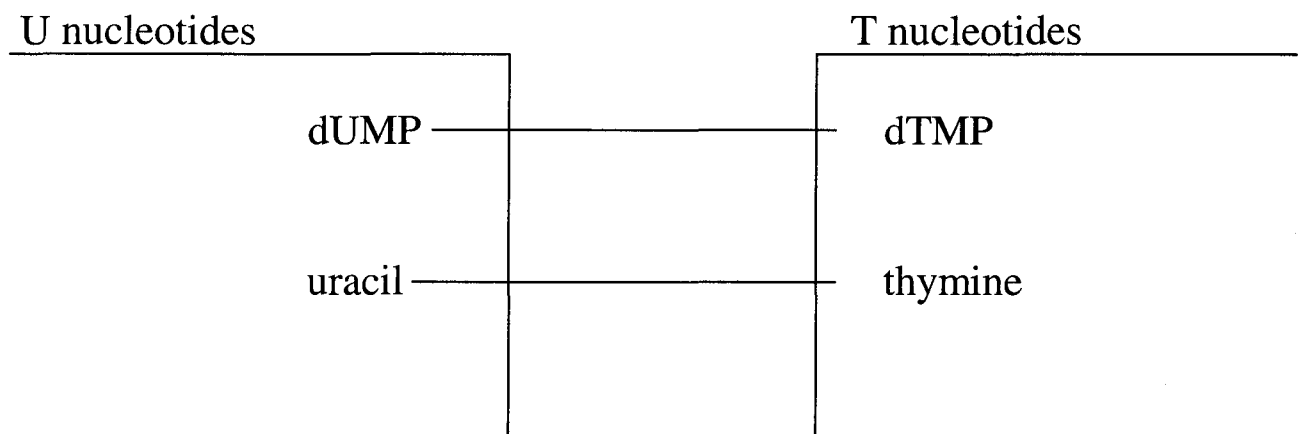
Figure 5. Most organisms metabolism versus fungal Metabolism

Figure 5. Most Organisms Metabolisms (unidirectional)

U nucleotide pool

(RNA)

dUMP ———————————— dTMP

T nucleotide pool

(DNA)

Fungal Metabolism

U nucleotides

dUMP ——————————— dTMP

uracil ——————————— thymine

T nucleotides

The lack of ammonia in Westergaard's may also be the cause for the elevated enzyme expression. The enzyme activity of the thymidine salvage pathway is also affected by the temperature at which the cultures are grown (Williams and Mitchell 1969).

While the significance of this pathway is unclear, fungi may use this pathway because it gives them the ability to conserve metabolic energy that is needed to synthesize the pyrimidine ring in the *de novo* pathway.

Mutants of the Thymidine Salvage Pathway

Two mutations affect the Thymidine Salvage pathway (Williams and Mitchell 1969). These mutations are designated uc-2, uc-3. The uc-2 gene encodes the enzyme that converts thymidine to thymine by forming a defective nucleosidase enzyme. The uc-2 mutant lacks the second step of the thymidine salvage pathway and was isolated using two selection methods.

The first was based on the fact that colonies of the pyrimidine mutant pyr-4, which is defective in the *de novo* pyrimidine pathway, grown on sorbose plates containing limiting uridine and excess thymidine were larger than if thymidine were lacking (Williams and Mitchell 1969). The smaller colonies were selected and tested for their ability to use thymidine as a pyrimidine source.

The second isolation method of the uc-2 mutant, which encodes the enzyme that converts thymidine to thymine and deoxyuridine to uridine, involved a filtration-enrichment procedure. First, irradiated conidia were incubated in filtration-enrichment media containing thymidine. The conidia that did not grow were assumed to be predominantly mutant conidia that were unable to use thymidine. Then these conidia were spread onto sorbose plates that were supplemented with uridine. The resulting colonies were tested to see if they carried a mutation which blocked a step in the thymidine pathway.

The uc-3 mutation was also isolated by the filtration-enrichment method mentioned above (Williams and Mitchell 1969). This mutation encodes the thymidine salvage pathway enzyme THase. It prevents utilization of the first three intermediates of the thymidine degredative pathway. Strains containing the uc-3 mutation are partially able to metabolize thymidine but not thymine.

The uc-1 mutation is a regulatory gene with an unknown mechanism. It was isolated by Williams and Mitchell. The uc-1 mutation was isolated by irradiating pyr-4 conidia, then plating onto sorbose plates containing only thymidine as a pyrimidine source. The uc-1 mutant is able to use thymidine as a sole pyrimidine source, so resulting colonies were presumed to be the mutants and were isolated. Functional mutant strains produce eight to ten times the IDCase activity versus wild type strains (Williams and Mitchell 1969).

Ammonia is the preferred source of nitrogen for *N. crassa*. When this nitrogen source is not available, *N. crassa* is able to use alternative nitrogen sources such as nitrate. Nitrogen control involves the activation of structural genes which are turned on in the presence of these alternate nitrogen sources so they are able to be utilized (Dantzig et al 1978).

N. crassa strains, which contain the nit-2 mutation, lack nitrate and nitrate reductase which in turn means that the nit-2 mutant cannot use nitrate, nitrite, purines, or most amino acids as a nitrogen source but will grow on ammonia, glutamine, or glutamate (Dantzig et al 1978). The nit-2 gene product is a positively acting regulatory protein which mediates global nitrogen control. In the presence of a secondary nitrogen source, nit-2 protein responds by activating a group of genes that enable this secondary nitrogen source to be utilized.

This project focuses on the measurement of IDCase activity in *N. crassa* under various conditions. IDCase is the enzyme which completes the conversion of thymine to uracil. *N. crassa* is the principle organism in which IDCase is readily observable, so it follows that the measurement of IDCase would be most efficient using *N. crassa*.

Materials and Methods

Strains and Media

The different strains of *N. crassa* that were used were purchased from the Fungal Genetics Stock Center in Kansas City, Kansas. Strains used in this study are listed in Table 1.

The ^{14}C that was used in the synthesis of ^{14}C IOA (10 mCi/nmol) was obtained from Dupont-NEN. The ^{14}C labeled IOA was synthesized from uracil and [1-^{14}C] formaldehyde (Cline and Fink 1959). The first step in this condensation reaction was done under basic conditions to produce [7-^{14}C] hydroxymethyluracil (HMU). The second step is an oxidation which involves the addition of Platinum catalyzed-O_2 which completes the synthesis of [5-^{14}C] iso-orotate by oxidizing HMU. ^{14}C-IOA was then diluted to a specific activity of 1mCi/nmol unlabled IOA.

Several kinds of media were used in the following experiments including Westergaards Media(Westergaard and Mitchell 1974), Vogels Media (Vogel 1956), and Horowitz Media and will be described in the next section.

Standing Culture Growth Conditions

Westergaards and Mitchell crossing medium (Table 2) was used to grow the *N. crassa* (Westergaard and Mitchell 1974).

Table 1) Strains of *N. crassa*

FGSC#	Genotype	Reference
2203	uc-2, uc-1, pyr-4	Williams and Mitchell 1969
2204	uc-3, uc-1, pyr-4	Williams and Mitchell 1969
nit-2	nit-2	Dantzig et al 1979
2489	wild type 74A	Authors Stock Collection
pry-4	pyr-4	Caroline 1968

Fifty milliliters of 1X Westergaard's solution with 2% sucrose was aliquoted into

the desired amounts in 250 mL Erlenmeyer flasks and autoclaved. Each flask was

allowed to cool to room temperature and inoculated with Neurospora that was

grown on agar slants containing Horowitz Complete Medium (Table 3). The loop

used to inoculate was sterilized with flame and sterile water, dipped into a flask of

conidia, and then inoculated into the 250 mL of Westergaard's. Each inoculated

flask was incubated at 30°C for a set time and then removed and harvested.

Harvesting involved using a vacuum filtration process that allowed for the *N.*

crassa to be separated from the media which will be explained in a following

section.

Shaking Growth Conditions

The growth conditions for the shaken cultures of *N. crassa* are as follows.

One hundred twenty-five ml of Westergaards or Vogels was sterilized in a 250 mL

flask and inoculated with approximately 3.82×10^6 conidial/ml suspended in 20 ml

of the appropriate media. Conidia were filtered through cloth before inoculation to

remove mycelia. This flask was covered with cotton and shaken at 30°C for the

desired period of time.

Shift Experiments

Shift experiments involved shifting the mycelia from one media to another. The conditions are the same as in the shaking growth conditions as stated in the last section except a shift was added after the culture was shaken for the desired time.

Tissue Homogenization

IDCase lysates were prepared using 1g of mycelia resuspended in 20 mls of GDH buffer (.03 Tris, .01 EDTA, .07% β-mercaptoethanol) (Table 4) along with 13.3µl of pepstatin A (3 mg/ml), 6.8µl of leupeptin (3 mg/ml), 40µl of phenylmethylsulfonylfluoride (PMSF) (3 mg/ml). This mixture was subjected to mechanical agitation using a tissue tearer for 3 minutes at 30 second intervals at 30,000 rpm. After the suspension was centrifuged at 30,000 rpm for 20 min to remove debris, the supernatant was aliquoted in 450µl increments into assay vials.

^{14}C Assay

The levels of IDCase were measured using a decarboxylase assay, a technique that is based on the original assay of the enzymatic conversion of IOA to uracil and CO_2 (Palmatier et al 1970).

Table 2) Westergaard and Mitchell crossing Medium. The following reagents were added to 2 liters of water.

reagents	amount
potassium nitrate	20g
potassium dihrdrogen phosphate	20g
magnesium sulfate	10g
sodium chloride	2g
calsium chloride	2g
trace elements	2mL
biotin	2mL

Table 3) Horowitz Complete Medium. The following reagents were added to 1 liter of water.

reagents	amount
potassium tartrate	5g
sodium nitrate	4g
potassium dihydrogen phosphate	1g
magnesium sulfate	.5g
sodium chloride	.1g
calsium chloride	.1g
glycerol	16mL

This decarboxylase assay measures the amount of $^{14}CO_2$ discharged from the reaction. Following tissue homogenization, vials are placed in a 30°C water bath and 50 µl of ^{14}C iso-orotate, counts per minute: 5000, is added to each vial. Then 450 µl of supernatant is added to each vial and the reaction is run for 10 minutes. Immediately after the supernatant is added a wick soaked in 2 M NaOH is added to the vial in order to collect the radio labeled CO_2. After the reaction (Fig 6) has run for the correct time, it is quenched with 200 µl of 2 M HCL. The reaction being measured is shown in Figure 6.

The wicks absorb CO_2 for 1 hour in the water bath and are then transferred to an oven plate and put in the oven for 40 min to 1 hour. The wicks are removed and placed in previously counted scintillation vials. The CPMs are recorded by the scintillation counter (Packard brand, model number 102992) and used to determine the amount of IDCase activity in the sample by calculating the specific activity in nmol min^{-1} mg^{-1} protein.

Protein Quantitation of IDCase lysates

The Bradford method (Bradford 1976)is used in order to quantify the total protein amount in the sample used. This process involves dispensing duplicate amounts of 0.5 mg/ml BSA (5, 10, 15, and 20 µl) into 8 microcentrifuge tubes and bringing each to 100ul with 0.15 M NaCl.

Table 4) Protein Lysate Solutions, GDH buffer and protease inhibitors

Reagent	amount
distilled water	86.0mL
1M Tris pH 8	3.0mL
Beta-mercaptoethanol	7.0ul
250mM EDTA pH 8	4.0mL

protease inhibitors

pepstatin A	3 mg in 3 ml of EtOH
Leupeptin	3 mg in 3 ml of EtOH
PMSF	3 mg in 3ml of di-water

Into 2 microcentrifuge tubes, aliquot 100 µl of 0.15 M NaCl; these are

blank tubes. 1 ml Coomassie Brilliant Blue solution is added to each and vortexed.

It is allowed to stand 2 minutes at room temperature. The absorbance at 595nm is

determined using a 1-cm pathlength microcuvette (1 ml). A standard curve is

generated by plotting absorbance at 595nm versus protein concentration(Graph 1)

The absorbance for the assayed samples is determined and the standard curve is

used to determine the concentration of protein in the assayed samples.

Figure 6) ^{14}C Assay Reaction

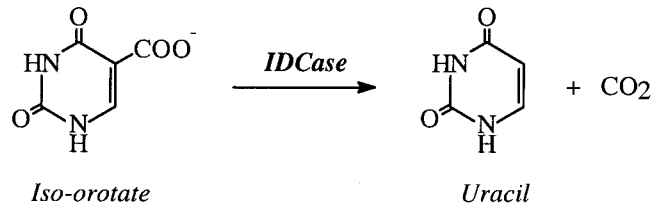*Iso-orotate* → *Uracil* + CO_2 (IDCase)

Table 5) Calculations of Enzyme Activity:
Radioactivity Assay of IDCase Activity

Counts of $^{14}CO_2$ (cpm)	÷	counting efficiency (cpm/dpm)	=	radioactivity of $^{14}CO_2$ (dpm)
Radioactivity of $^{14}CO_2$ (dpm)	÷	Specific activity of $^{14}CO_2$ (dpm/nmol)	=	nmol product
nmoles enzyme activity (nmol product)	÷	time (minutes)	=	(nmol / min) enzyme activity
enzyme activity	÷	total protein (μg)	=	Specific Activity (nmol min μg^{-1})

Results

In order to determine the levels of IDCase activity during different stages of

growth of *N. crassa* cultures; IDCase levels were measured both in standing and

shaking cultures at various time points. Mycelia was grown at 30°C for 24, 48, 72,

and 96 hours, harvested then assayed to determine the levels of IDCase activity.

Table 6 shows that in standing cultures IDCase levels peak at 72 hours and then

drop off as the 96 hour time point is reached. Graph 1 shows that there is almost a

6 fold increase in IDCase activity from 24 to 72 hours. When mycelia was grown

at 30°C while shaking, the IDCase activity was significantly higher than activity in

the standing cultures. The highest IDCase level was observed at 18 hours with

nearly a 7 fold increase over the 12 hour shaking time point. After the peak at 2.5

nmol min μg^{-1} at 18 hours, IDCase activity drops off to 1.611 nmol min μg^{-1} at 24

hours and then to .743 nmol min μg^{-1} after 36 hours (Table 7, Graph 2). Results

from the IDCase assay of standing cultures show a significantly lower enzyme

activity than that of the shaking cultures. The standing cultures were not assayed

at 12 and 18 hours due to the insignificant amount of mycelia that was present.

Table 8 shows the specific activity of both the shaking and standing cultures at

different time intervals. Graph 3 clearly shows that IDCase levels peak at 18 hours

when shaking and at 72 hours when standing.

Table 6) IDCase activity of *N. crassa* standing cultures expressed in nmol min µg^{-1}

Table 6

Time in hours	Media	specific activity nmol min μg^{-1}
24	Westergaards	0.071
48	Westergaards	0.39
72	Westergaards	0.66
96	Westergaards	0.202

Graph 1) Graph of IDCase levels in standing *N. crassa* cultures grown in Westergaards media. The specific activity is in units of nmol min μg^{-1}
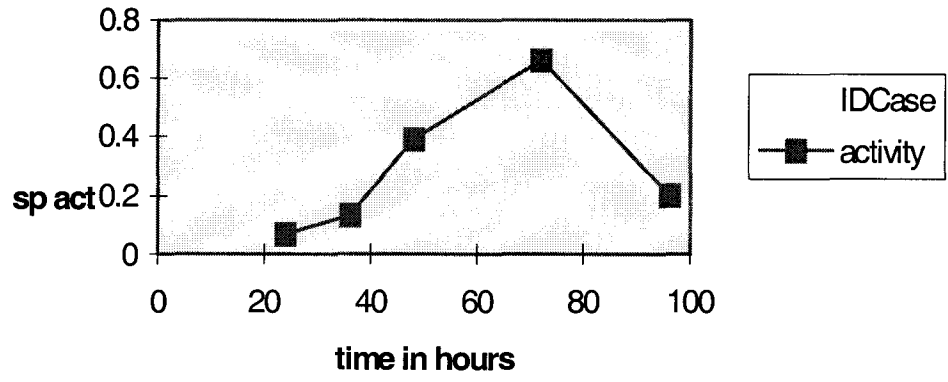
graph of standing cultures

Table 7) Specific activity of IDCase in nmol min μg^{-1} of *N. crassa* shaking cultures grown in Westergaards media.

Table 7

Time in hours	Media	Specific Activity nmol min μg^{-1}
12	Westergaards	0.984
18	Westergaards	2.502
24	Westergaards	1.611
36	Westergaards	0.743

Graph 2) Graph of IDCase activity in shaking *N. crassa* cultures grown in Westergaards media.
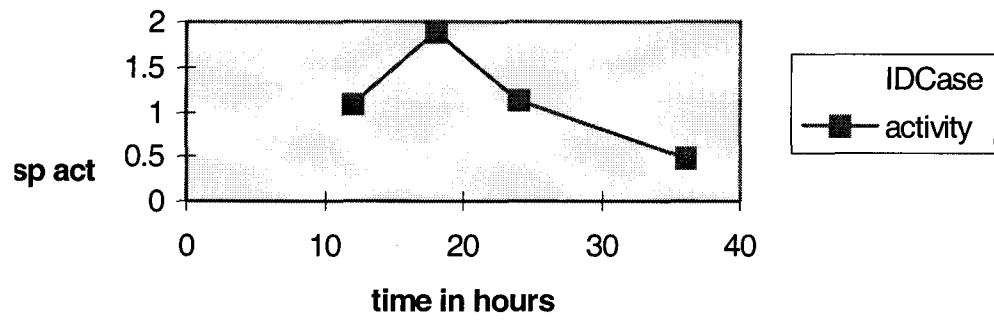
shaking IDCase levels

Table 8) Table of combined standing and shaking IDCase activity in *N. crassa* cultures.

Table 8

Time in hours	Media	Specific Activity of Shaking nmol min μg^{-1}	Specific Activity of Standing nmol min μg^{-1}
12	Westergaards	0.984	
18	Westergaards	2.502	
24	Westergaards	1.611	0.071
36	Westergaards	0.783	0.135
48	Westergaards		0.39
72	Westergaards		0.66
96	Westergaards		0.202

Graph 3) Graph of standing and shaking IDCase activity.

specific activity curve

specific activity

time in hours
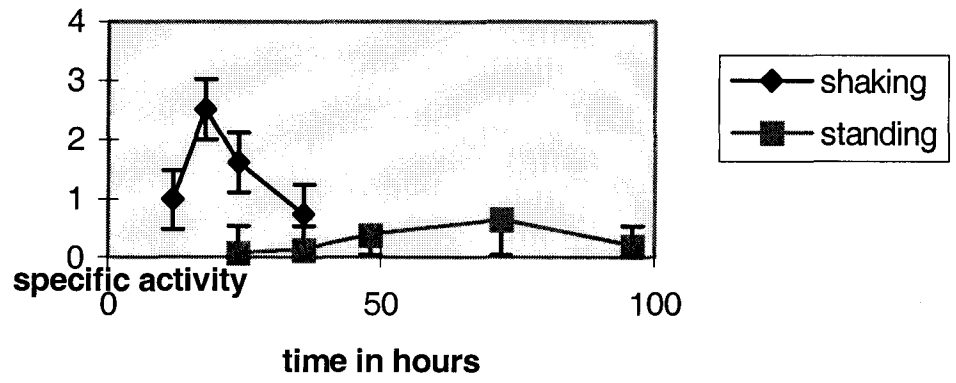
The rapid growth rate of the shaking cultures versus the standing cultures is probably due to the increased amount of oxygen in the shaking cultures.

In order to test the effect of different media on IDCase levels, cultures of *N. crassa* were grown on Vogels media (Vogel 1956) which contains ammonia as a nitrogen source, and on Westergaards media which contains NO_3 as a nitrogen source (Westergaard 1974). Vogels media was previously shown to be the better nitrogen source to produce more growth, but Westergaards worked better to yield a higher IDCase enzyme activity (Cannon 1997).

N. crassa strains were grown in both Vogels and Westergaards media while shaking and harvested at various time points and assayed for IDCase activity. As seen in table 9 and graph 4, IDCase levels were higher when *N crassa* was grown on Westergaards media vs. Vogels media at almost all phases of the growth curve.

To further examine the difference in IDCase levels between *N. crassa* grown on Westergaards versus *N. crassa* grown on Vogels, shift experiments were performed. Conidia were germinated and grown on Vogels media for 12 hours. Then mycelial mats were harvested by filtration. One gram of the harvested tissue was placed in fresh Westergaards and Vogels media. Cultures were then further incubated at 30°C and harvested at various time points. IDCase levels were then determined.

Table 9) Specific activity in nmol min μg^{-1} of *N. crassa* grown in both Vogels and Westergaards media.

Table 9

Time in hours	Specific Activity in Westergaards nmol min μg^{-1}	Specific Activity in Vogels nmol min μg^{-1}
12	1.084	0.336
18	1.8	2.015
24	1.13	0.609
36	0.48	0.565

Graph 4) Graph of IDCase levels expressed in nmol min μg^{-1} of shaking cultures grown in both Vogels and Westergaards media.

vogels vs westergaards shake cultures
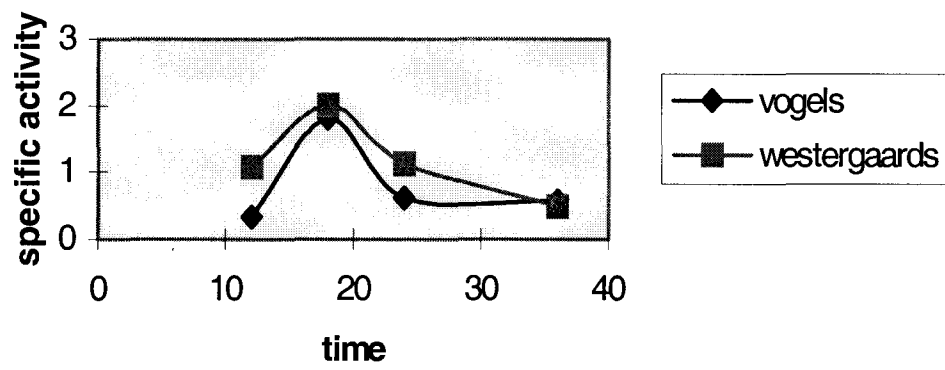
Graph 5 shows that 3 hours after the shift the *N. crassa* that was shifted to Westergaards media had a specific activity of 1.1 nmol min μg^{-1} versus the *N. crassa* still growing in Vogels media at .7 nmol min μg^{-1}. By nine hours, the difference jumps to 3 fold. This verifies that *N. crassa* IDCase seems to be induced when cultures are shifted from Vogels media to Westergaards media, perhaps due to the presence of nitrate in Westergaards media.

When the shift is in the opposite direction, from Westergaards to Vogels it showed that there was practically no change in IDCase activity, but this might be due to the length of the shift. The shift was done after 18 hours for 2 hours followed by the assay. If the shift were longer there is a risk of killing the Neurospora (data not shown).

Thymidine and uracil are the two most likely molecules to effect the levels of IDCase activity. Since the pathway converts thymidine to uracil through several enzymatic steps, it is reasonable to assume that adding uracil would depress IDCase activity. Conversely, thymidine might be able to induce the pathway since there would be an excess of the starting material. To examine the role thymidine would play in regulating IDCase levels, conidia were germinated and grown on Vogels media for twelve hours. Then mycelial mats were harvested by filtration. One gram of harvested tissue was placed in Westergaards media that contained thymidine. Cultures were then further incubated at 30°C and harvested at various time points. IDCase levels were then determined. Table 10 and graph 6 show that

the addition of thymidine did not increase the IDCase activity. The reason for this result is unknown.

In order to examine the effect of uracil on IDCase activity, the same germination and harvesting was done with media containing uracil instead of thymidine. In this case the expected result was that the addition of uracil would depress IDCase levels. The results show that addition of uracil does in fact depress IDCase levels.

As mentioned earlier, *N. crassa* strains that contain the nit-2 mutation lack nitrate reductase. In the presence of a secondary nitrogen source such as nitrate, the nit-2 protein responds by activating a group of genes that enable this nitrogen source to be utilized. In this experiment, the *N. crassa* containing the nit-2 mutation was grown on Vogels media for 12 hours and then shifted to Westergaards for 3, 6, and 9 hours. The control shift was wild type *N. crassa* grown in Vogels media and shifted to Westergaards media and the other was *N. crassa* that contained the nit-2 mutation grown in Vogels media and shifted to Westergaards media. At three hours there is no difference in IDCase activity, and by nine hours the wild type strain is 1.3 times higher than the nit-2 strain (table 12, graph 8).

The next experiment tests to see if IDCase levels are boosted when the *de novo* pathway is blocked. Strains containing the pyr-4 mutation are deficient in the *de novo* pathway and therefore require uracil for growth. If the IDCase levels are

elevated in strains containing the pyr-4 mutation, then the pathway is getting

turned on in order produce uracil in the cell. The *N. crassa* was grown for 18

hours in Vogels media plus uracil and then shifted for one hour to Westergaards.

Table 13 and graph 9 show that there is very little increase in IDCase activity in

the pyr-4 mutant when compared to the wild type.

IDCase is effected by several different inhibitors including 5-nitrouracil, 5-

hydroxyuracil, and 5-fluorouracil. Since the enzyme has not been purified the

inhibition constants have not been determined yet. 5-nitrouracil is found to inhibit

IDCase with an estimated K_i value that is too low for accurate determination

(Smiley et al 1998). One inhibitor, 5-fluorouracil, has demonstrated a 40%

inhibited activity at a 10μM concentration. This inhibitor was used in the

following experiment. The *N. crassa* was grown in vogels media for 12 hours and

then shifted to Westergaards media containing 5-fluorouracil at a 10mM

concentration for 6 and 9 hours. The tissue was then harvested and assayed.

There was virtually no decrease in IDCase activity after six hours. The nine hour

time point demonstrated a slight decrease in IDCase activity. The IDCase levels of

the control was .99 nmol min μg^{-1} compared to the sample at .74 nmol min μg^{-1}.

These results are shown in table 14.

Graph 5) Graph of IDCase levels in *N. crassa* cultures shifted from Vogels media to Westergaards media
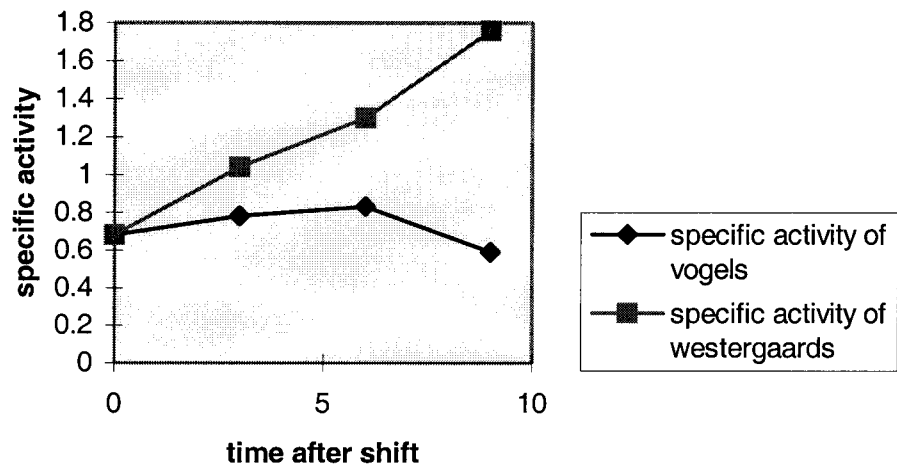
specific activity of shift to westergaards

Table 10) IDCase activity of *N. crassa* cultures shifted from Vogels media to Westergaards media +thymidine (expressed in nmol min μg^{-1}).

Table 10

Time after shift	specific activity after shift to -thymidine	specific activity after shift to +thymidine
0	0.36	0.360.6
3	0.7	0.7
6	1.5	0.9

Graph 6) Graph of IDCase activity of *N. crassa* cultures grown on Vogels media and shifted to Westergaards media +thymidine
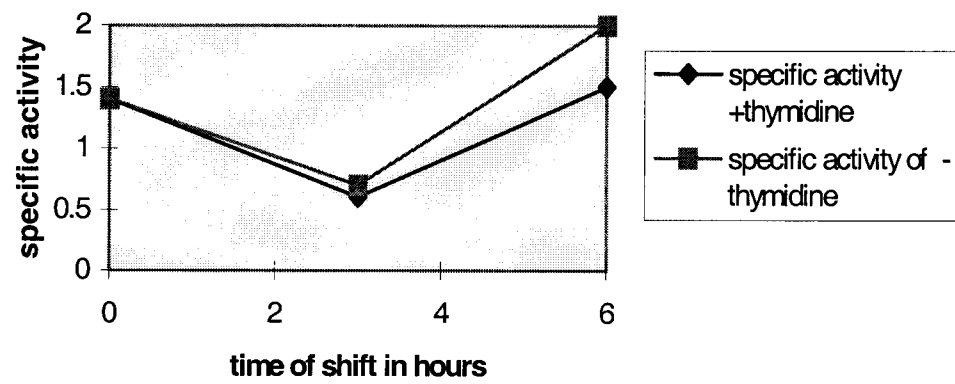
specific activity of shift to thymidine

Table 11) IDCase activity of *N. crassa* shifted to media containing uracil

Table 11

Time after shift	specific activity after shift to - uracil	specific activity after shift to +uracil
0	0.6	0.6
3	0.8	0.6
6	1.2	0.5

Graph 7) Graph of IDCase activity of *N. crassa* cultures shifted to Westergaards media containing uracil
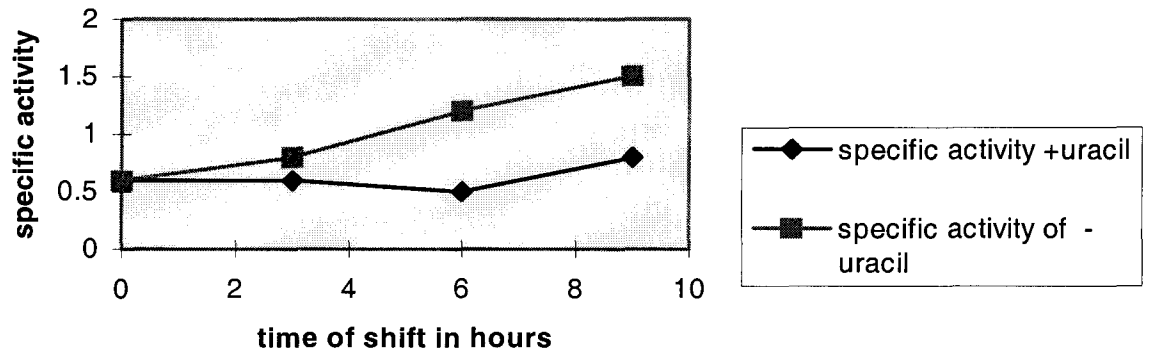
specific activity of shift to uracil

Table 12) IDCase of *N. crassa* cultures containing the <u>nit-2</u> mutation shifted to Westergaards media.

Table 12

Time after shift	specific activity of 74A	specific activity of nit-2
0	0.623	0.623
3	0.46	0.436
6	0.85	0.56
9	1.3	0.11

Graph 8) Graph of *N. crassa* IDCase activity of cultures containing the nit-2 mutation shifted from Vogels media to Westergaards media.

specific activity of nit-2 shift

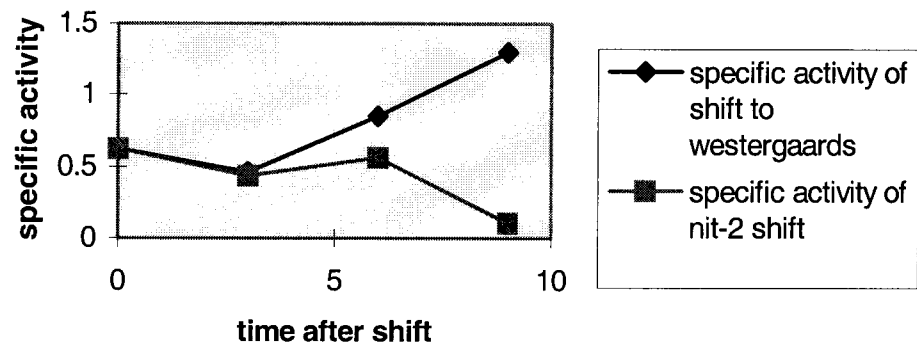Table 13) IDCase activity of *N. crassa* cultures containing the pyr-4 mutation

Table 13

time of shift	specific activity of wild type	specific activity of pyr-4
0	2	2.1
1	0.9	1.1

Graph 9) IDCase activity of *N. crassa* cultures containing the pyr-4 mutation

specific activity of pyr-4 mutant

Chart showing specific activity (y-axis, 0 to 2.5) versus time of shift in hours (x-axis, 0 to 1). Legend: specific activity of wild type; specific activity of pyr-4.
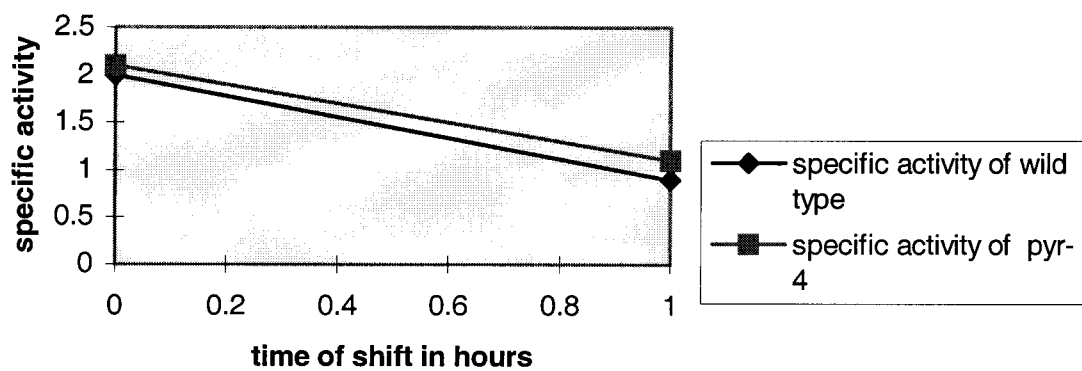
Table 14) IDCase activity of *N. crassa* cultures shifted from Vogels media to Westergaards media containing 5-fluorouracil

Table 14

Time after shift	+ 5-fluorouracil expressed in nmol min μg^{-1}	control expressed in nmol min μg^{-1}
6 hours	1.3	1.3
9 hours	.74	.99

Discussion

Preliminary characterization of the IDCase enzyme of *N. crassa* resulted in

several conclusions. The development of a specific radio-activity based assay

which is capable of accurately measuring IDCase activity was key to these

experiments(Smiley et al 1998). This assay uses a technique that is based on the

original assay of the enzymatic conversion of IOA to uracil and CO_2 (Palmater

1970) as described in the materials and methods section.

The first conclusion is that the *N. crassa* produces significantly higher

IDCase levels and a higher tissue amount when grown in a shaking environment

rather than standing. The standing cultures did not show much activity until 24

hours when they were at .071 nmol/min/mg. At 48 hours, 72 hours, and 96 hours,

the IDCase levels were .39 nmol/min/mg, .66 nmol/min/mg, and .20 nmol/min/mg

respectively. Compared to the shaking cultures, the IDCase activity is very low.

At only 24 hours shaking the *N. crassa* demonstrates a significantly higher IDCase

level of 1.61 nmol/min/mg. The 12 hour, 18 hour, and 36 hour shaking time points

reflect IDCase levels of .98 nmol/min/mg, 2.50 nmol/min/mg, and .74

nmol/min/mg respectively. The optimum growth time for standing cultures was 72

hours and 18 hours for the shaking cultures. This difference in IDCase levels may

be due to the increase in oxygen in the shaking cultures from the constant

agatation.

Nitrogen source was also shown the have an effect on IDCase activity. When *N. crassa* was grown on Westergaards media which utilizes nitrate as a nitrogen source, it was shown to have significantly higher IDCase activity than when grown on Vogels media which uses ammonia as a nitrogen source. The shift experiment involving *N. crassa* shifted from Vogels media to Westergaards media demonstrates induction. Only 3 hours after the shift to Westergaards media there was an IDCase activity of 1.1 nmol/min/mg versus the control at .7 nmol/min/mg. This shows that the thymidine salvage pathway seems to be induced when tissue is shifted from Vogels media to Westergaards media, perhaps due to the presence of nitrate in Westergaards media.

The effect of thymidine and uracil on IDCase levels were tested since they are the most likely controllers of IDCase activity. Since the thymidine salvage pathway converts thymidine to uracil, it is reasonable to assume that the addition of uracil would depress IDCase activity. At 3 hours after the shift to media containing uracil, the specific enzyme activity was at .6 nmol/min/mg versus the control at .8 nmol/min/mg. By 6 hours after the shift the specific enzyme activity was at .5 nmol/min/mg versus the control at 1.2 nmol/min/mg. These results confirm that the addition of uracil does in fact depress IDCase levels.

The addition of thymidine was expected to have the opposite effect of the addition of uracil and be able to induce the thymidine salvage pathway. For reasons that are unknown, this was not the result. At 3 hours after the shift to

media containing thymidine the IDCase levels of both the plus thymidine and the control remained the same. By 6 hours after the shift the IDCase level of the plus thymidine culture was at 1.5 nmol/min/mg versus the control at .9 nmol/min/mg. The reason for this result is unknown.

The nit-2 mutant was tested for its ability to utilize a secondary nitrogen source. As shown in the results section, the nit-2 mutant strain demonstrated a lower IDCase level at .11 nmol/min/mg versus the control at 1.3 nmol/min/mg.

The pyr-4 mutant was used to test if IDCase levels were higher when the *de novo* pathway is blocked. The pyr-4 culture showed a specific enzyme activity of 1.1 nmol/min/mg versus the control at .9 nmol/min/mg. This increase shows that the thymidine salvage pathway is being turned on in order to produce uracil since the *de novo* pathway is dysfunctional.

The chemical 5-fluorouracil was used to see if its addition shut down the thymidine salvage pathway.

REFERENCES CITED

Bradford, M.M. (1976) A Rapid and Sensitive Method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem.* 72:248-254.

Caroline, D.J.F. 1968. Pyrimidine Synthesis in *Neurospora crassa.* Ph.D. thesis, University of Michigan, Ann Arbor. Diss. Abstr. B Sci. Eng.**30**:960

Cannon, R. 1997. Preliminary Characterization of IDCase in *Neurospora crassa.* Masters thesis, Youngstown State University, Youngstown.

Cline, R.E., Fink, R.M., and Kay Fink. (1959) *Journal of American Chemical Society* "Synthesis of 5-Substituted Pyrimidines via Formaldehyde Addition". Vol. 81 pp. 2521-27.

Dantzig, A.H., W.K. Zurowski, T.M. Ball, and A. Nason. (1978) Induction and Repression of Nitrate Reductase in *Neurospora crassa. J. Bacteriol.* **133**: 671-679

Jones, M. E. (1980) Pyrimidine nucleotide biosynthesis in animals: genes, enzymes and regulation of UMP biosynthesis. *Annu. Rev. Biochem.* **49**, 253-279.

Kornberg, A., and Baker, T. A. (1992) Biosynthesis of DNA precursors. In DNA replication, Chapter 2, W. H. Freeman and Co., New York.

Neuhard, J. (1983) Utilization of performed pyrimidine bases and nucleosides, pp. 96-148. In Munch-Petersen, A., ed., Metabolism of Nucleosides, Nucleosides and Nucleobases in Microorganisms. Academic Press, London.

O'Leary, M. H. (1992) Catalytic strategies in enzymatic carboxylation and decarboxylation. *The Enzymes* **20**, 235-69.

Palmatier, R. D., McCroskey, R. P., and Abbott, M. T. (1970) The enzymatic conversion of uracil 5-carboxylic acid to uracil and carbon dioxide. *J. Biol. Chem.*, **245**, 6706-10.

Reichard, P. (1988) Interactions between deoxyribonucleotide and DNA synthesis. *Annu. Rev. Biochem.* **57**, 349-74.

Smiley, J. A., Angelot, J., Cannon, R. C., Marshall, E.M., Asch, D.K. (1998) Radioactivity-Based and Spectrophotometric Assays for Isoorotate Decarboxylase:

Identification of the Thymidine Salvage Pathway in Lower Eukaryotes. *Analytical Biochemistry* **266**, 85-92.

Smiley, J. A. and Jones, M. E. (1992) A Unique Catalytic and Inhibitor-binding Role for Lys93 of Yeast Orotidylate Decarboxylase. *Biochemistry* **31**, 12162-68.

Skinner, C., Emmons, C. and Tsuchiya, H.M. (1947) Molds, Yeasts, and Actinomycetes: A handbook for Students of Bacteriology. 2 Ed. John Wiley and Son, Inc. London

Thornburg, L. D., and Stubbe, J. (1993) Mechanism-based inactivation of thymine hydroxylase, an -ketoglutarate-dependent dioxygenase, by 5-ethynyluracil. *Biochemistry* **32**, 14034-42.

Voet, D., Voet, J.G. (1995) Biochemistry Second Edition, pp. 790-820, Wiley and Sons, Inc., New York.

Vogel, H.J. Microbiol. Gene. Bull. "A Convenient Growth Medium for *Neurospora*" 1956 v. 13 pp. 42-43.

Westergaard, M., and Mitchell, H.K. (1974) *Am. J. of Botany* **34**, 573-77.

Webster, J. (1980) Introduction to Fungi 2nd Edition. Cambridge University Press. pp 248-63.

Williams, L.G., and Mitchell, H. K. (1969) Mutants Affecting Thymidine Metabolism in *Neurospora crassa*. *J. of Bacteriology* **100**, 383-389.