ALIPHATIC PRECURSORS OF PYRIMIDINES IN *NEUROSPORA CRASSA*

By

Jessie Muriel Boyd

A THESIS

Submitted to the School for Advanced Graduate Studies
of Michigan State University of Agriculture and
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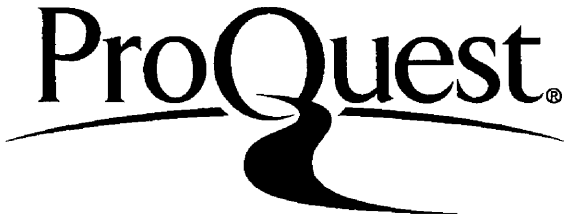

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VITA

The author was born in Billingsley, Alabama and received her secondary and high school education in the public schools of Alabama. She graduated from Sidney Lanier High School, Montgomery, Alabama in June, 1947. The author graduated from Huntingdon College, Montgomery, Alabama, in 1952 with the degree of Bachelor of Arts with majors in Chemistry and Mathematics.

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ABSTRACT

The following investigations were carried out to study the metabolic route by which Neurospora crassa 1298 synthesizes pyrimidine nucleotides from aliphatic acids: 1) a search for compounds which supported growth in a manner similar to certain known precursors, 2) tracer studies with carbon-14 labeled compounds to establish utilization of various compounds as pyrimidine precursors, and 3) attempt to demonstrate enzymatically certain postulated reactions.

Of the numerous compounds studied the following were found to support growth of the mold when added to the basal medium: propionic, acrylic, beta-hydroxypropionic, beta-ureidopropionic, methylmalonic acids, and dihydrouracil. Aspartic and ureidosuccinic acids, compounds found to be precursors to pyrimidines in other organisms by Kornberg, were found to be inactive. Studies of the time-course of growth of the mold on various supplements showed that propionate and aminobutyrate gave similar responses. Beta-ureidopropionate and dihydrouracil required longer periods of adaptation before growth began.

The presence of unlabeled propionic acid or aminobutyric acid in the growth medium depressed significantly the specific activities of the pyrimidines formed by the mold in a medium also containing uracil-2-C^{14}. Propionate-2-C^{14} was found to be incorporated relatively specifically into the pyrimidines of the mold. The purines of the

same experiments contained much lower amounts of carbon-14. The presence of arginine, which inhibited growth of the mold on certain precursors such as propionate, homoserine, aminobutyrate and uracil, was found to lead to an increase in the specific activity of the pyrimidine bases formed by the mold in a medium also containing uracil-2-C^{14}.

Preliminary enzymatic studies show the presence of activities effecting uracil, aminobutyrate and beta-alanyl-pantetheine.

On the basis of the results a new pathway for the synthesis of pyrimidine compounds is suggested, a pathway which leads from propionyl-Coenzyme A through the Coenzyme A derivatives of beta-alanine and beta-ureidopropionic acid to dihydrouridylic acid and finally to uridine-5'-phosphate.

TABLE OF CONTENTS

TABLE OF CONTENTS - Continued

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The discovery of nucleic acids was made in some investigations carried out on the nuclear material of pus cells by Friedrich Miescher in 1897. Later nucleic acids were shown to be normal constituents of all cells and tissues studied. On hydrolysis nucleic acids were found to yield purine and pyrimidine bases, as well as a pentose sugar, and phosphoric acid. Two types of nucleic acid have been found. One of these contained the purine bases, adenine and guanine; the pyrimidine bases, cytosine and thymine; and a sugar D(-)-deoxyribose. The second type contained adenine and guanine; the pyrimidine bases, cytosine and uracil; and the sugar, D(-)-ribose. Both contained phosphoric acid.

Although it is clear that nucleic acids play a fundamental part in cellular metabolism, the exact nature is difficult to determine. Deoxyribonucleic acids have been indicated by many indirect lines of evidence to be the basic genetic material of cells (1). Ribonucleic acid seems to be connected with the process of protein synthesis in the cell.

Most organisms are able to synthesize nucleic acids from simple metabolites. The purine and pyrimidine bases are thought to be incorporated into the nucleic acids in the form of their nucleotides. The pathway of purine biosynthesis has been worked out to a large extent both in mammals and microorganisms. Comparatively little evidence,

however, has been found for a pathway by which pyrimidines are synthesized.

The biosynthesis of pyrimidines from simple precursors was demonstrated in an experiment by Barnes and Schoenheimer (2) in which N^{15} labeled ammonium citrate administered to rats was incorporated into the pyrimidines of nucleic acids. The investigations of Heinrich and Wilson (3) showed that position two of the nucleic acid pyrimidines is derived from CO_2 in the rat. This finding was confirmed by Lagerkvist (4).

In searching for simple metabolic precursors of the pyrimidine nucleus, Mitchell and Houlahan (5) introduced important information in studies of the mutants of Neurospora crassa that require uridine for growth. In these mutants orotic acid was found to accumulate in the medium during growth. For several of these mutants uridine could be replaced by oxaloacetic acid (6). Loring and Pierce (7) found orotic acid would replace pyrimidines as growth factors for certain pyrimidineless mutants of N. crassa. Orotic acid is also a growth factor for Lactobacillus bulgaricus 09; if C^{14} labeled orotic acid is provided in the medium, the isotope appears in uridine-5'-phosphate and cytidine-5'-phosphate but not in adenine or guanine (8). Reichard (9) had obtained similar results earlier in the rat. Orotic acid is used by animal tissues, since the administration of N^{15} (9) or C^{14} (10) labeled orotic acid to rats leads to appearance of the isotopes in the pyrimidines but not in the purines.

Wright et al. (8) demonstrated in L. bulgaricus 09 that DL-ureidosuccinic acid was as effective a precursor for the pyrimidines of the

nucleic acids as was orotic acid. Reichard (11) showed enzymatically with rat liver mitochondria that aspartic acid in the presence of carbamyl phosphate, adenosine triphosphate and magnesium ion could be converted to ureidosuccinic acid by mitochondrial fractions and later he showed labeled aspartic acid to be incorporated into the pyrimidines (12). The synthesis of ureidosuccinic acid required several steps, the first being the production of carbamyl phosphate. The method of formation of this compound was worked out by Jones, Spector and Lipmann (13). In the second part of the reaction, the carbamyl phosphate transferred its carbamyl group to aspartic acid forming carbamyl-aspartic acid, often called ureidosuccinic acid. These reactions were demonstrated to take place in the mitochondrial fraction. Experiments by Woods, Ravel and Shive (14) with L. arabinosus 17-5, which is an aspartic acid requiring mutant, showed that pyrimidines, as well as threonine and lysine, could spare the aspartic acid requirement, indicating the use of aspartic acid for pyrimidine formation in this organism. The precursor relationship of carbamyl aspartic acid to orotic acid was established in nutritional experiments in microorganisms and isotopic experiments in microorganisms and mammals (8, 11, 12). The conversions were shown by Lieberman and Kornberg (15) to require two enzymes, one which effects the ring closure of carbamyl aspartic acid to dihydroorotic acid and the other, a diphosphopyridine nucleotide requiring enzyme, which removed two hydrogens from dihydroorotic acid to produce orotic acid.

The mechanism for the formation of uridine-5'-phosphate from orotate has been clarified by Kornberg and co-workers (16, 17, 18). For the first step of the conversion of orotic acid to uridine-5'-phosphate, Liebermann and Kornberg (16) showed that the formation of 5-phosphoribosyl-1-pyrophosphate from adenosine triphosphate and ribose-5-phosphate was required prior to the formation of orotidine-5'-phosphate from orotate. The enzyme, orotidylic pyrophosphorylase, which catalyzes this reaction, was purified from yeast autolysates and was found to be specific for orotic acid. A second enzyme, orotidylic decarboxylase, catalyzed the decarboxylation of orotidine-5'-phosphate to form uridine-5'-phosphate (17). In the absence of the orotidylic decarboxylase, orotidine-5'-phosphate accumulated. The decarboxylation was found to be irreversible. The conversion of uridine-5'-phosphate to cytidine-5'-phosphate was shown by Kornberg (18) to take place at the triphosphate level and to require adenosine triphosphate and ammonia.

The sequence of reactions suggested by the above experiments is the generally accepted pathway of synthesis of pyrimidine nucleotides. These reactions are shown in schematic fashion in Figure 1.

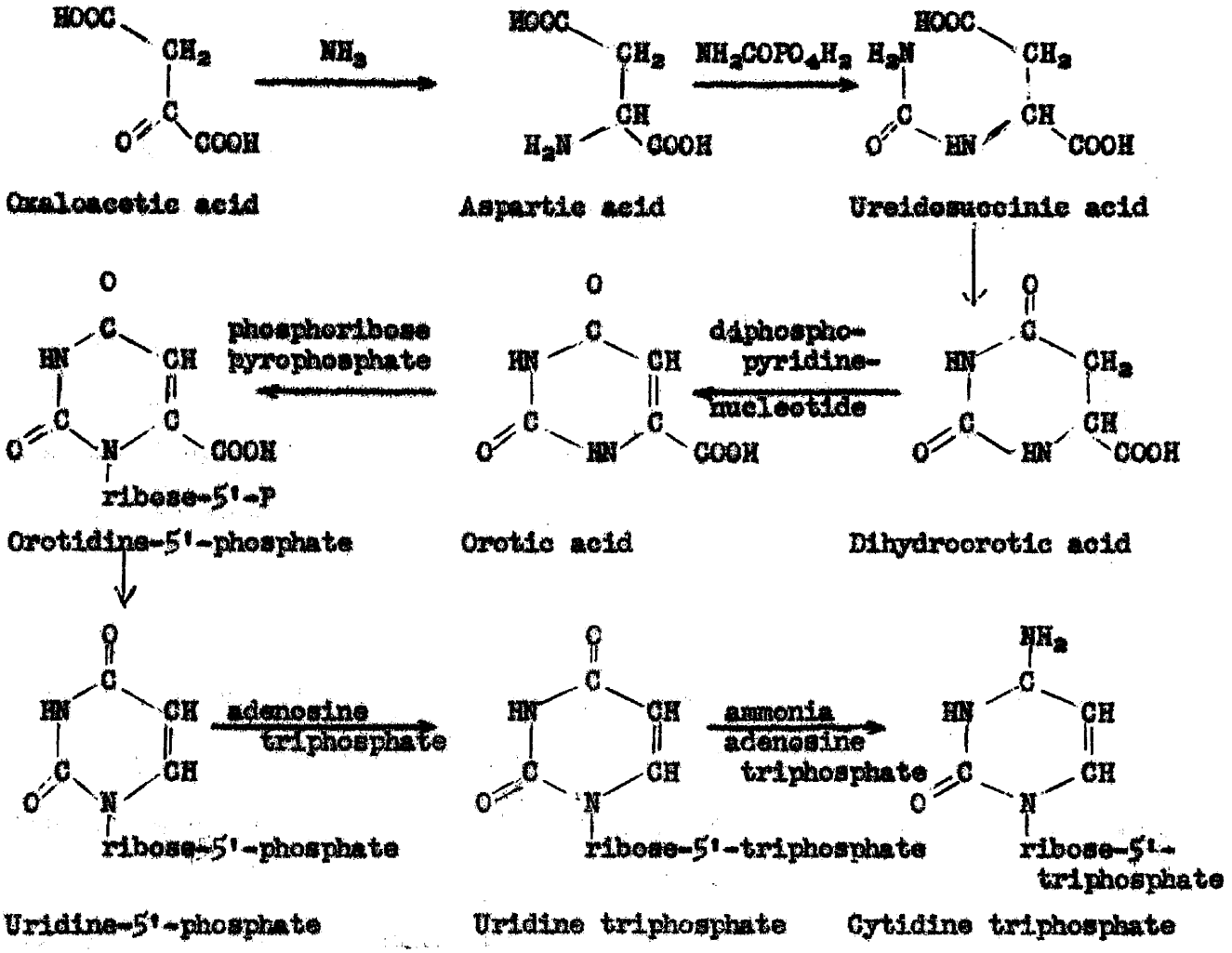


Figure 1. Proposed pathway of pyrimidine biosynthesis (18).

Other pathways of pyrimidine biosynthesis may assume greater importance under different conditions. In mutant organisms, where the normal pathway is blocked, an alternate pathway may be utilized for growth of the organism. Antimetabolites such as azauracil, 6-uracil methyl sulfone and others (19) are known to prevent growth in tumor cells for a period of time, after which the cells start growing again.

This resistance to the antimetabolite may well arise from the adaptive utilization of an alternate pathway of uracil production. Investigations of apparently minor, alternate pathways of biogenesis of necessary compounds may, therefore, have great importance in certain cases.

One possible occurrence of such an alternate route was indicated when Fairley (20) reported the growth of several pyrimidineless N. crassa mutants on the amino acids, alpha-aminobutyrate and threonine. In other experiments Fairley and Herrmann (21) demonstrated the utilization of alpha-aminobutyrate by the mutant 1298 of N. crassa for synthesis of pyrimidines. When aminobutyrate-3-C^{14} was administered to the mold the labeling of the pyrimidines formed ranged from one-tenth to one-fifteenth of the original concentration of radioactivity of the aminobutyrate supplied. The pyrimidines formed were labeled to a considerably greater extent than other mycelial constituents; about four times that of the ribonucleic acid purines and about ten times that of various amino acids. Reisolation of aminobutyrate also showed a dilution to about one-twentieth of the original concentration of radioactivity of aminobutyrate-3-C^{14} administered. Therefore synthesis of the amino acid during growth of the mold could explain the dilution when incorporated into the pyrimidines.

The aminobutyrate was apparently utilized by a route differing from that shown in Figure 1, in that aspartate and ureidosuccinate were incapable of supporting growth.

Another alternate pathway for pyrimidine synthesis are the reactions described by Fink (22), Fritzen (23), Canellakis (24), Griselia (25), and Rutman (26). When uracil was administered to rats, Fink et al. (22) isolated from urine beta-aminopropionic acid and beta-carbamylaminopropionic acid. In similar experiments with labeled thymine, beta-carbamylaminoisobutyric acid and beta-aminoisobutyric acid were isolated. Fritzen (23), Canellakis (24), Griselia (25) and Rutman (26), have shown that uracil-2-C^{14} is rapidly degraded by a enzyme system of rat liver to form the end products, CO_2, NH_3, and beta-alanine. The reduction of uracil to dihydrouracil depends upon triphosphopyridine nucleotide, and is the rate limiting step (24). The components of the carbamyl group are released enzymatically after opening of the ring to form carbamyl-beta-alanine, often called beta-ureidopropionic acid (24). It is quite possible that a reversal of this pathway could afford a pathway of biosynthesis independent of orotic acid. The sequence or reactions as a degradative pathway is shown in Figure 2. The reversal of this pathway has been suggested as a means of synthesizing pyrimidines.

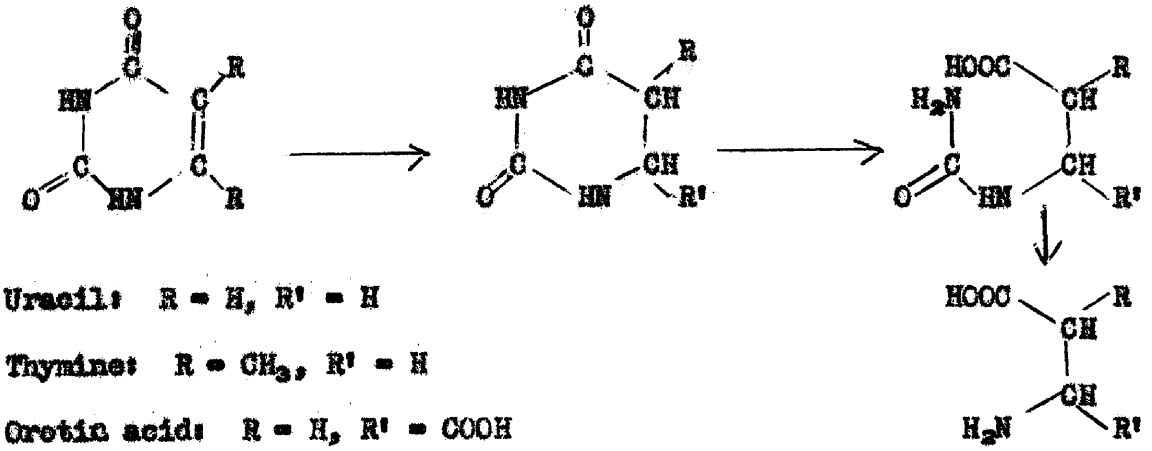


Figure 2. Proposed pathway of pyrimidine degradation (22).

Although the pyrimidine bases have been shown to be extensively degraded to carbon dioxide and the beta-amino acid in mammals, Canellakis (27) has shown that in rat liver slices a considerable portion of uracil-2-C^{14} was converted to nucleotide pyrimidines and nucleic acid pyrimidines and that a smaller proportion was degraded to carbon dioxide. Lagerkvist and Reichard (28) demonstrated that in the mouse, uracil was efficiently utilized for synthesis of nucleic acid pyrimidines in both visceral tissues and Ehrlich ascites cells.

In mammals the pyrimidine bases have been demonstrated to be converted to nucleotides by a nucleoside phosphorylase isolated by Kalckar (29). Nucleoside kinases (30, 31, 32, 33) and phosphotransferases (34) could convert pyrimidine bases, ribose-phosphate and adenosine triphosphate to the nucleotides. Through the use of enzymes isolated from L. delbrueckii and Thermobacterium acidophilus R-26, synthesis of certain deoxribosides were shown by McNutt (35) to take place by the transfer of the deoxyribosyl group from one purine or pyrimidine to another. These are methods by which uracil may be efficiently used for synthesis of nucleic acid pyrimidines.

With the indication that N. crassa 1298 is capable of synthesizing pyrimidine nucleotides by a new metabolic route involving certain aliphatic acids, it became of interest to determine the precise nature of this new mechanism. The present work describes the results of several different approaches to this problem. These approaches involved: 1) a search for compounds which supported growth to a similar extent as

did aminobutyric acid, threonine and homoserine, 2) tracer studies with carbon-14 labelled compounds to establish the utilization of various compounds as pyrimidine precursors, and 3) attempts to demonstrate enzymatically certain postulated reactions.

EXPERIMENTAL AND RESULTS

Effects of Various Compounds on the Growth of Neurospora Crassa 1298

Materials

All compounds, with the exception of beta-ureidopropionic acid, l-amino-l-carboxy-cyclopropane, alpha-hydroxypropionic acid and methylmalonic acid, were obtained from Nutritional Biochemicals Corporation. The beta-ureidopropionic and ureidosuccinic acids were synthesized by the procedure described by Lengfeld and Stieglitz (36). Two grams of beta-alanine and 1.85 g. of potassium cyanate were added to an evaporating dish and enough water added to dissolve both substances. The solution was evaporated on a steam bath to a syrup. The residue was placed in a small beaker and approximately 1 ml. of 0.1 N hydrochloric acid was added. The beta-ureidopropionic acid precipitated out on standing in the refrigerator. The melting point was 169-170° C. The l-amino-l-carboxy-cyclopropane was a gift of Dr. Hans Lau. The beta-hydroxypropionic and methylmalonic acids were gifts of Dr. Minor J. Coon.

Organism

Strain 1298 of Neurospora crassa, which was produced by Beadle and Tatum (37) using X-ray and ultraviolet radiation treatment and later described by Loring and Pierce (7), will not grow on a simple media of salts, sugar and biotin (appendix). This mutant was assumed to be

unable to synthesize pyrimidines since addition of a pyrimidine compound to the basal media permits growth of the organism. The mold was maintained on culture slants consisting of two per cent agar in basal medium with 1 mg. of uracil per ml. (appendix).

Growth Procedure

The mold was grown in 125 ml. Erlenmeyer flasks to which 25 ml. of a basal nutrient having the composition shown in the appendix was added. Each flask was stoppered with a cotton plug and autoclaved at 110° C. for 15 minutes. The heat stable compounds were sterilized along with the media by autoclaving while the heat sensitive compounds were added aseptically after filtration through a Seitz bacteriological filter. After cooling, each flask was inoculated with 0.2 ml. of a spore suspension of the mold made by dispersing two loopfuls of spores in 10 ml. of sterile, distilled water. The cultures, run in triplicate, were incubated at 25° C. for four days. At the end of this time the mycelial pads were removed, rinsed with distilled water, squeezed to remove excess water, and dried overnight in an oven at 50° C. The amount of growth was determined by weighing the dried mycelium. The weights were compared with uracil as a standard. Other controls consisted of basal medium with no supplement.

Results

In Table I a list of the compounds tested for growth is given. They have been placed in three groups indicating their relationship to

TABLE I

GROWTH RESPONSE OF NEUROSPORA CRASSA 1298 TO VARIOUS COMPOUNDS

Supplementary Compounds	Weight of Compound (mg.)	Dry Weight of Mycelium (mg.—4 days)
Uracil Group		
Uracil	5	57.0
Dihydrouracil	5	11.1
β-ureidopropionic acid	10	2
Dihydrothymine	5	2
Thymine	1	2.7
Thymidine	2	0
Malonic acid	5	0
Aspartic Acid Group		
Aspartic acid	5	0
Ureidosuccinic acid	8.55	0
Dihydroorotic acid	5	0
Orotic acid	7	6
Glutamic acid	5	0
Homoserine	6	18
Allothreonine	8	0
α-Aminobutyric Acid Group		
A. α-Aminobutyric Acid	5	43.0
α-Keto butyric acid	8	0
α-Hydroxy butyric acid	8	0
Threonine	10	30
Isoleucine	5	0
Cyclopropane-amino-carboxylic acid	5	0
B. Propionic Acid	6	27
Acrylic acid	6	5
β-hydroxypropionic acid	6.2	15
Methyl-malonic acid	10	14
Succinic acid	5	0
Pyruvic acid	5.1	0
Acetic acid	5	0
Alanine	5	0
n-Propylamine		0
Glycerol	6.3	0
Glycine		0

the previously mentioned pathways of biosynthesis. The uracil group includes the compounds shown by various workers to comprise the pathway suggested in Figure 2. These compounds were beta-ureidopropionic acid, and dihydrouracil, leading to the synthesis of uracil, and dihydrothymine leading to the synthesis of thymine. Uracil had been shown previously to produce growth. In these studies dihydrouracil, beta-ureidopropionic acid, thymine and dihydrothymine were shown to replace the pyrimidine requirement to a lesser degree than uracil itself. The nucleoside, thymidine, was not able to satisfy the pyrimidine requirement for growth. Malonic acid has been shown to be a degradation product of uracil through barbituric acid (38), but it did not replace uracil for growth.

The compounds of the aspartic acid group were those shown in Figure 1. Aspartic acid, ureidosuccinic acid, and dihydroorotic acid did not show any capacity to satisfy the pyrimidine requirement. The growth response to orotic acid indicates its use for pyrimidine biosynthesis. Glutamic acid has been shown to be readily converted to aspartic acid by transamination reactions. Since a mutant of N. crassa which lacks glutamic dehydrogenase has been reported by Fincham (39), the requirement of an alpha amino group was visualized. However, again no growth response resulted. Threonine has been shown previously by Fairlay (20) to be used in place of pyrimidines. The main pathway for the biosynthesis of threonine has been shown to be conversion of aspartic acid to homoserine (40) which in turn gives rise to threonine (41). Homoserine was shown to give a significant amount of growth.

Allothreonine, which differs from the naturally occurring L-threonine by an inversion at the second asymmetric carbon atom, was not suitable for growth.

In the process of looking for compounds which were related to aminobutyric acid and which also gave significant growth responses, propionic acid was shown to give very interesting results. For this reason the aminobutyric acid group was divided into two sections, one which contained compounds related to aminobutyric acid and the other which contained compounds shown by Stumpf (42) and Stadtman (43) to be related more directly to the metabolism of propionic acid itself.

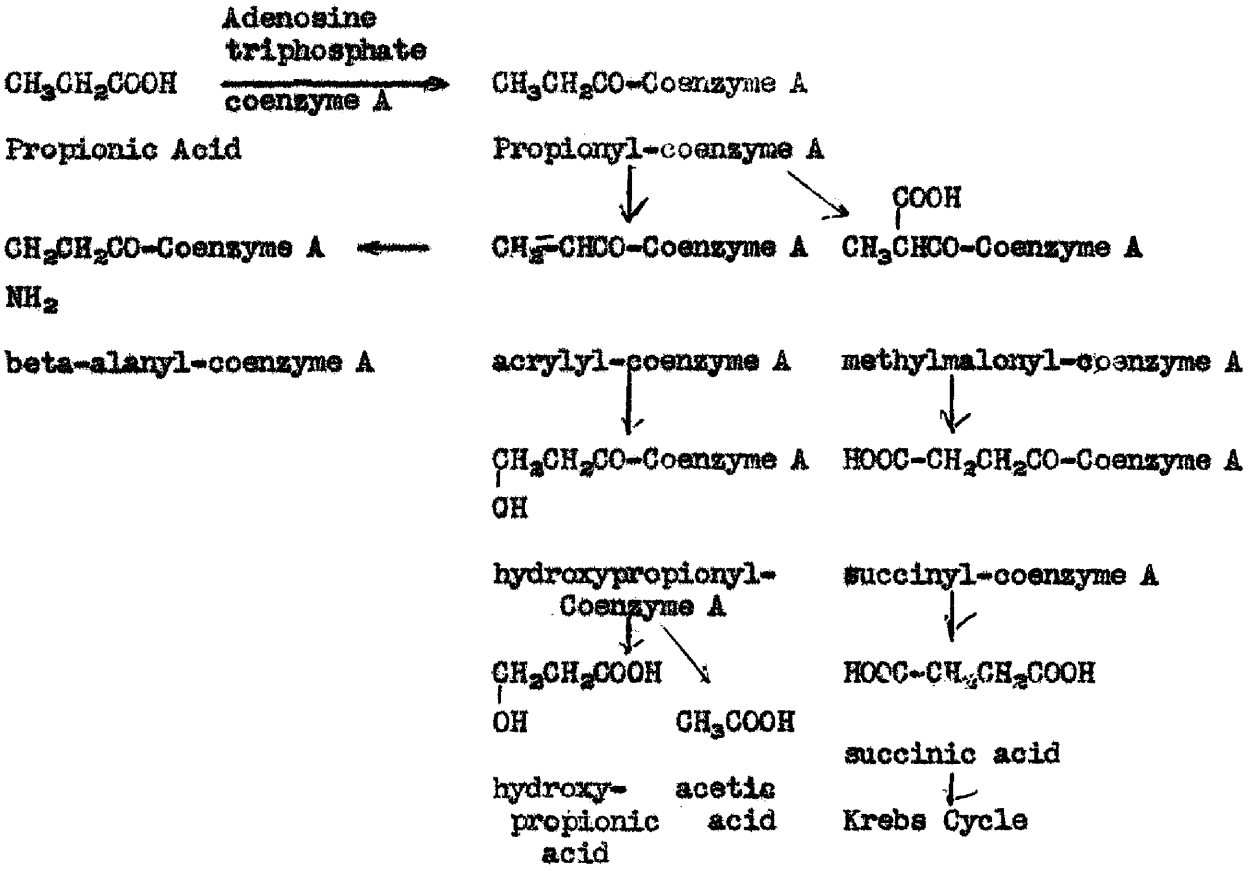


Figure 3. Propionate Metabolism.

Aminobutyrate, which has already been shown to give a high growth response in M. crassa 1298 (20), gives on deamination the keto acid, alpha-ketobutyric acid, in some organisms (44). Although the reaction has been shown to be reversible, the keto acid did not support growth. Neither did the alpha-hydroxybutyric acid. Threonine which is readily converted by rat liver to alpha-aminobutyric acid (45), was indicated by Fairley to be a precursor. Isoleucine has also been demonstrated to yield alpha-ketobutyric acid (46), but evidently did not give rise to aminobutyric acid since no growth resulted.

Of the compounds given in Figure 3, propionic, acrylic, hydroxypropionic and methylmalonic acids gave varying growth responses. Succinic, pyruvic, and acetic acids and alpha-alanine have been shown to be related to the oxidative pathway of propionate metabolism through the Krebs cycle (47). These failed to replace the propionic acid requirement. Other three carbon compounds failing to give growth were n-propyl amine and glycerol. Glycine which has such an important role in purine biosynthesis was found to be inactive here.

Stimulation by Uridine

Table II shows the results of adding uridine to some of the previously reported supplements. Lysine showed a slight stimulation by uridine while uracil and dihydrouracil give a great response. Dihydroorotic acid seems to inhibit growth in the presence of uridine.

TABLE II

STIMULATORY EFFECT OF URIDINE ON VARIOUS SUPPLEMENTS

Supplements	Concentration of Supplement	Weight of Mycelia
A. 0.1 mg. Uridine		6.2
isoleucine	5 mg.	9.5
lysine	5.1	12.6
aspartic acid	5	8.1
dihydroorotic	5	0
Uracil	2	61.7
thymidine	2	6.9
dihydrothymidine	2	10.2
dihydrouracil	2	45.5
B. 0.5 mg. Uridine		27.5
isoleucine	5	30.9
lysine	5.1	31.8
aspartic	5	27.0

Arginine Inhibition

Arginine has been found to have an inhibitory effect on the utilization of certain of the pyrimidine precursors (Fairley, unpublished). Uracil, alpha-aminobutyric acid, propionic acid, and homoserine have been found to be inhibited by very small quantities of arginine. The results are shown in Table III. Ornithine showed the same effect at about 100 times the concentrations used for arginine. Citrulline was not so clear-cut.

TABLE III

ARGININE INHIBITION OF CERTAIN GROWTH PROMOTING COMPOUNDS

Supplements	Concentration of Arginine (mg.)	Weight of Mycelium (mg.--4 days)
A. Uracil (3 mg.)		58
L-arginine	0.01	34
	0.1	17
	1.0	5
	10.0	5
Uracil (5 mg.)		22
L-arginine	10.0	4.7
Uracil (10 mg.)		32
L-arginine	10.0	17
B. Amino butyric acid (5 mg.)		30
L-arginine	0.001	20
	0.01	0
	0.1	0
	0.5	0
C. Propionic acid (6 mg.)		27
L-arginine	0.01	0
	0.1	0
	1.0	0
	10.0	0
D. Homoserine (6 mg.)		18
L-arginine	0.01	0
	0.1	0
	1.0	0

Growth Curves

Growth curves were plotted for the time of incubation response of the mold to selected compounds. Uracil, dihydrouracil, beta-ureidopropionic acid, propionic acid, and alpha-aminobutyric acid were given in equimolar amounts to the mold. Each set was run in triplicate. Five sets of flasks were run for each compound. The results of these experiments are shown in Figure 4. Of the various compounds tested, growth began first on uracil, followed in order by aminobutyric acid, propionic acid, dihydrouracil, and finally beta-ureidopropionic acid. Once growth began the growth rates were essentially the same. Compared with other compounds aminobutyrate and propionate gave lower maximum of growth.

The Utilization of Aminobutyric Acid in the Presence of Uracil-2-C^{14} for the Biosynthesis of Pyrimidines in *Neurospora crassa* 1298

Materials

Uracil-2-C^{14} was obtained from the Isotopes Specialties Company. The specific activity of the sample was given as 0.6 millicuries per millimole. This sample was used in all the experiments involving administration of uracil to the mutant. The aminobutyric acid was obtained from Nutritional Biochemicals. The organism used was the *N. crassa* mutant, 1298, described before.

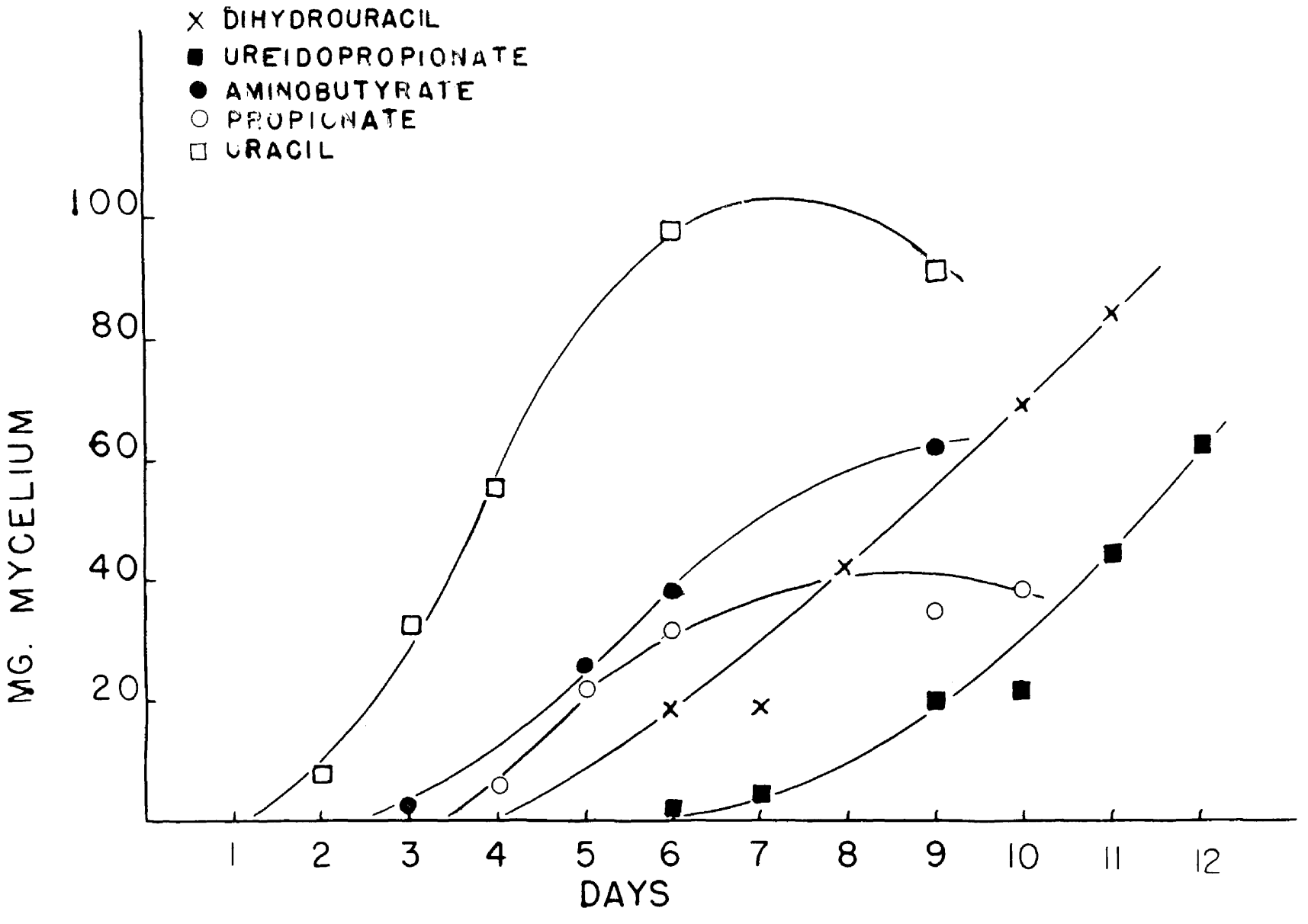


FIGURE 4. RELATIVE RATES OF GROWTH

Growth Procedure

The mold was grown in 125 ml. Erlenmeyer flasks, to each of which was added 25 ml. of basal nutrient medium having the composition shown in the appendix. In each experiment two sets of ten flasks were used. To each of the first ten flasks were added 0.4 mg. of the labeled uracil and 9.6 mg. of unlabeled uracil. In addition to the 10 mg. of uracil the second set of ten flasks contained 10 mg. of aminobutyric acid in each flask. The first set served as a control, giving the incorporation of uracil-2-C^{14} into pyrimidines, while the second set showed the influence of aminobutyric acid on the amount of incorporation of uracil-2-C^{14} into pyrimidines. Ten milligrams of uracil was found from a study of concentration effects on growth to be the most suitable concentration for optimum growth. The autoclaving, inoculation, and incubation were carried out as described in the previous section (page 11). At the end of three days of growth the contents of the flask were filtered on a sintered glass funnel with suction. The mycelial pads were washed with a small amount of water after which they were placed in 100 ml. of acetone. The acetone was removed and the mycelia were placed in the dessicator for 24 hours. At the end of this time the pads were weighed.

Isolation of the Ribonucleotides

The dried, acetone-extracted, mycelial pads were ground to a fine powder using a mortar and pestle with 120 mesh carborundum powder.

This mixture of powders was extracted three times with 5 ml. of ice cold 10 percent trichloroacetic acid (w/v). The residue was washed once with 4:1 ethanol:ether. The first extraction was carried out for thirty minutes and the successive extractions for five minutes. The solid was then washed twice with ether. The mixture of carborundum and extracted mycelial powder was dried. All of the extractions were carried out in a 12 ml. centrifuge tube. By using this procedure, the solution was easily separated from the residue by centrifugation, so that the residue could be reextracted.

Five milliliters of 1 N potassium hydroxide was added to the solid contents of the tube and allowed to stand for 24 hours, stirring occasionally. After centrifuging, the basic solution was adjusted to a pH of 3.0 by using perchloric acid. During this process and a ten minute period of standing the solution was kept cold in an ice bath. The solution was centrifuged and filtered through a sintered glass funnel. The pH was taken up to 11 and the precipitate of potassium perchlorate was filtered off.

This acidic solution was added to a column of Dowex-1-Cl^-, 50-100 mesh, 12X. The column had been previously treated by washing with 200 ml. of 2 N hydrochloric acid, followed by 200 ml. of 5 per cent sodium hydroxide, and again washing with 200 ml. of 2 N hydrochloric acid. The final wash was with water until the eluate was shown to be neutral to pH paper. When all of the solution containing the ribonucleotides had filtered onto the column, the column was developed first with 200

ml. of water. Then the nucleotides were eluted with 2 N hydrochloric acid. The optical density of the eluate was determined at 260 mu. The combined fractions containing the nucleotides was evaporated to dryness on a flask evaporator. Water was added several times to aid in removing the hydrochloric acid.

Hydrolysis of Ribonucleotides

The mixture of ribonucleotides was transferred to a 2 ml. volumetric flask using small amounts of 0.055 N hydrochloric acid. This solution was evaporated to dryness using a charcoal-filtered air stream. The flask stood overnight in a vacuum dessicator containing Drierite. After careful addition of 0.5 ml. concentrated perchloric acid, the flask was heated on a steam bath for forty minutes behind a glass shield. When the flask was cooled, the contents were transferred to a 12 ml. centrifuge tube using small quantities of water. The carbon was centrifuged down, washed with water and the wash combined with the first supernatant. This solution of purines and pyrimidines was allowed to filter into a column of Dowex-50, H^+, 16 X, 50 x 100 mesh. This column had been previously treated in the same manner as the Dowex-1 column. Elution was begun with water (200-300 ml.) and was followed by 0.055 N hydrochloric acid. The uracil came off in the first 150 ml. of water. When the ultraviolet absorption was negligible, the cytosine was eluted with 2 N hydrochloric acid (400-550 ml.). The cytosine was found to come off in a volume of 120 ml. after addition of the first 60 ml. of acid. Guanine was eluted with 3 N hydrochloric

acid (180-200 ml.). The guanine came off in a volume of 45 ml. after addition of the first 40 ml. of acid. The adenine was eluted with 4 N hydrochloric acid (150-200 ml.) in a volume of 75 ml. after 40 ml. of the acid had been added to the column. The elution of the ultraviolet absorbing compounds was detected by absorbance measurements using the Beckmann spectrophotometer Model DU. One hundred lambda of each fraction was plated and counted. There was a direct proportion between absorbance and the amount of radioactivity of each fraction, indicating the radiopurity of each ultraviolet absorbing compound.

The uracil fraction which was eluted from the column in the first 150 ml. of water also contained the perchloric acid used for the hydrolysis. This was removed before further purification of uracil was carried out. First, the fraction was evaporated by means of the flash evaporator to a volume of 2 ml. Then the solution was made neutral to pH paper by addition of 1 N potassium hydroxide. The potassium perchlorate was filtered off and the solution was further evaporated by air to about 0.5 ml. Further purification was carried out by paper chromatography.

One Dimensional Paper Chromatography

The solutions of the substances to be purified were spotted on Whatman #1 paper. Two 100 lambda spots were placed 4 inches from the end on a 7 x 22 inch sheet. The spots were not more than 0.5 cm. in diameter. This could be accomplished by applying small amounts at a time and drying with a hair dryer while the paper was stretched between

two books. After complete application the paper was folded 1.5 inches from the origin and again in the opposite direction 3 inches from the origin. The flaps were placed in glass troughs and the papers passed up and over glass rods, such that the first fold coincided with the glass rod and the paper therefore hung straight down from this point. A bent rod held the papers in the trough. The troughs were held at the top of an all glass rack, and the entire system was contained in a 12 by 24 inch battery jar. The solvents were added to the trough in approximately 75 ml. quantities. The jar was covered with a glass plate and the solvent allowed to flow down the paper until it had almost reached the bottom, or for approximately 24 hours. The papers were then removed and dried in the hood. With an ultraviolet lamp the pyrimidines and adenine appeared as dark blue absorbing spots on a light blue background, and guanine, which fluoresces slightly, appeared as a light blue spot on the paper. The spots were cut out and sliced into small strips, which were then eluted by standing overnight in 0.055 N hydrochloric acid. After centrifuging the concentration was determined from the absorbance at 250 mμ. One hundred lambda of each sample was plated and counted. The radioactivity was found to be associated with the ultraviolet absorbing spots.

Isotope Measurement

One hundred lambda aliquots of the fractions from the column and from the purification procedures were plated on platinum planchets. In all cases the solvent was removed by slow evaporation over an

infrared lamp with a gentle stream of air blowing over the plates. The radioactivity was determined at infinite thinness in a Nuclear proportional Geiger counter, with the Nuclear Ultra Scaler Model 192. Since the concentration of each sample had been determined by finding the absorbance with the Beckmann spectrophotometer, Model DU, the specific activity expressed as counts per minute per micromole could be calculated.

Results

After purification by paper chromatography the cytosine isolated from the mold which was given uracil-2-C^{14} alone, showed a specific activity of 85 and 94 per cent of the specific activity of the original uracil-2-C^{14}. The uracil was labeled to the extent of 77 per cent. There was a negligible amount of radioactivity in both adenine and guanine. When uracil-2-C^{14} and aminobutyrate were given together, the cytosine isolated contained a concentration of radioactivity which was 48 and 73 per cent of the concentration administered. The uracil was labeled to the extent of 56 per cent of the concentration of radioactivity given. Again no significant radioactivity was found in the purines. The results are shown in Table IV.

The Utilization of Propionate for Pyrimidine Biosynthesis in Neurospora crassa

Materials

Sodium propionate-2-C^{14} was obtained from the Volk Radiochemical Company. Two samples were used. The first sample had a specific

activity given as 1.2 millicuries per millimole. The second sample had a specific activity given as 2.52 millicuries per millimole. Two experiments were carried out using the mutant, 1298.

Growth Procedure

The mold was again grown in 125 ml. flasks, to each of which was added 25 ml. of basal nutrient medium. Sixteen flasks were used in each experiment. To each of the flasks was added 0.88 mg. of radioactive propionate (0.65 mg. of propionic acid) and 6.2 mg. of unlabeled propionic acid which had been purified by redistillation. This gave a concentration of 6.85 mg. of propionic acid per 25 ml. of media, a concentration found to be satisfactory for optimum growth. In establishing the specific activity of the sample, it was found that the radioactivity of propionic acid must be determined before dilution with the medium and that two drops of 1 N sodium hydroxide prevented evaporation of the propionic acid from the plates. Observation of these precautions gave the calculated specific activity. Autoclaving, inoculation, incubation for six days, and harvesting of the mycelial pads were carried out in the same manner as described on page 11. The isolation and hydrolysis of the ribonucleotides and the chromatography of the bases on Dowex 50 were made in the same manner as described on pages 21 and 22.

Purification of the Purine and Pyrimidine Bases

The uracil eluted from the column was again removed from the perchlorate and chromatographed on paper as previously described.

Since the radioactivity of the eluted fractions did not agree with the ultraviolet absorbtion data, impurity of the cytosine fraction was indicated. The fact that a high radioactivity was found in those fractions that gave no ultraviolet absorbance indicated that this highly labeled compound was not a purine or pyrimidine. This was shown to be true by chromatographing the highly radioactive fraction that was eluted from the column just before the cytosine fraction. The R_f (ratio of distance that the compound moved to that which the solvent moved) was determined by locating the radioactivity on the paper. This was carried out using the Forro Chromatographic Scanner (Forro Scientific Company, Evanston, Illinois) coupled with a Nuclear-Chicago Model 1620 A ratemeter and a Model AW Esterline-Angus graphic ammeter. The R_f of the activity peak was 0.36.

When the cytosine fraction was chromatographed on paper four spots were located using the ultraviolet lamp. Their R_f values were: (1) 0.55, (2) 0.65, (3) 0.79, and (4) 0.88. Spot (2) was a flourescent spot, while the others were light absorbing. Significant radioactivity was found to be associated with spot (1) which from the spectrum obtained using the Beckmann spectrophotometer, Model DK-2 was shown to be guanine, and with spot (3) which was shown also by spectral studies to be cytosine. Scanning of the paper chromatograph showed a high peak of

radioactivity where the cytosine migrated, a lower peak where the guanine migrated and a much lower peak at a R_f of 0.37 where there was no ultraviolet absorbing spot. The R_f of this latter spot corresponded to that found above in chromatography of the radioactive, non-ultraviolet light absorbing fraction and probably indicates slight contamination of the cytosine fraction with this material. Elution of the spots from the paper chromatograph and counting were carried out as previously described.

This non-ultraviolet light absorbing material was originally eluted from Dowex-50 by 0.055 N hydrochloric acid; whereas cytosine required 2 N hydrochloric acid. However, there was not a complete separation of the two compounds and thus considerable purification was required for the cytosine fraction as was described above. Since this contaminating compound does not absorb light in the ultraviolet region it cannot very well be a pyrimidine or purine. Although the compound did not give a positive test with ninhydrin (0.2 per cent ninhydrin in 1-butanol saturated with water), the concentration may not have been great enough to detect an amino acid. However, calculations based on the amount of radioactivity and the weight of an average amino acid, assuming no dilution of isotope, indicated there should have been sufficient material present to detect had this been of amino acid nature. Neither was a positive test for a ureido acid obtained after spraying with alkali and para-dimethylaminobenzaldehyde, but again there may have been an insufficient concentration.

Adenine was rechromatographed to give an idea of the purity of the purine fraction. Two ultraviolet absorbing spots were found on the paper. The R_f values were (1) 0.57, and (2) 0.77. The adenine was found by the Beckmann DK-2 to be located in spot (2). Elution from the paper and counting of the elutate was carried out in the same manner as the uracil and cytosine.

Radioautography of Paper Chromatograms

Strips to be radioautographed were taped to 8 by 10 inch sheets of Kodak Blue Brand X-ray film. A spot of radioactive solution was placed in the upper right hand corner in order to mark the relation of the chromatogram to the developed film. The films, with attached papers, were placed between plywood boards which were clamped tightly together to insure close contact of chromatograms and films. After a period of 3 weeks the chromatograms were removed and the films were developed for four minutes with Kodak D-19 Developer, then placed in a stop bath of 1 per cent (w/v) acetic acid for 10 seconds, and left in a fixer solution of sodium thiosulfate for at least 10 minutes. The films were then washed in cold tap water for approximately 1 hour, and then hung by clips to dry.

Radioautographs run on the chromatographs of the cytosine fraction showed the radioactivity of spot (3), which corresponds to cytosine itself, to be clearly associated with this spot and not contaminated with other substances.

Results

After purification by chromatography on paper, the specific activity of the cytosine was 31 to 33 per cent of the concentration of radioactivity originally given in the medium. The uracil was 33 to 34 per cent. The guanine was 8 to 14 per cent and the adenine was 9 to 12 per cent. A summary of the data is recorded in Table IV.

Utilization of Propionate in the Presence of Uracil-2-C^{14} for Biosynthesis of Pyrimidine in Neurospora Crassa

Materials

Two samples of uracil-2-C^{14} were used. The first sample with a specific activity given of 0.6 millicuries per millimole was obtained from the Isotopes Specialties Company and was used in experiments with the mutant 1298. One experiment was carried out. The second sample with a specific activity given of 1.2 millicuries per millimole was obtained from Volk Radiochemicals Company and was used with the wild type of N. crassa. One experiment was carried out.

Growth Procedure

Two sets of ten 125 ml. Erlenmeyer flasks were used in the experiment. Twenty-five milliliters of basal medium was added to each flask. In both sets, 0.4 mg. of the labeled uracil and 9.6 mg. of unlabeled uracil were added to each of the flasks. The second set of ten flasks contained 9.92 mg. of propionic acid in addition to the 10 mg. of uracil.

As in the experiment utilizing uracil-2-C^{14} and aminobutyrate, the first set of flasks served as a control and gave the incorporation of uracil into the pyrimidines. The second set showed the influence of propionic acid on the amount of incorporation of uracil-2-C^{14} into pyrimidines of the mutant, 1298. The autoclaving, inoculation, incubation, and harvesting were carried out as described on page 11. The isolation and hydrolysis of the ribonucleotides and the chromatography of the bases were carried out in the manner already described. The fractions from the column were plated and counted. The radioactivity was in direct proportion to the ultraviolet absorbance.

Results

The cytosine from the mold which was fed uracil-2-C^{14} and propionate was found to contain a concentration of radioactivity which was 53 per cent of the initial concentration of the radioactivity furnished the mold as uracil-2-C^{14}. There was essentially no labeling of the purines, adenine and guanine. Again the cytosine from the mold fed only uracil-2-C^{14} and basal constituents showed a dilution of specific activity of 83 per cent of the original specific activity.

The Utilization of Uracil-2-C^{14} in the Presence of Arginine for the Biosynthesis of Pyrimidines in Neurospora Crassa

Materials

Uracil-2-C^{14} was obtained from the Isotopes Specialties Company. The specific activity of the sample was given as 0.6 millicuries per

millimole. The L-arginine was obtained from Pfanstiehl Chemical Company. The organism used was the N. crassa mutant, 1298, described before.

Growth Procedure

The mold was grown in 125 ml. Erlenmeyer flasks, to each of which was added 25 ml. of basal medium. Two sets of ten flasks were used. In both sets, 0.4 mg. of the labeled uracil and 9.6 mg. of unlabeled uracil were added to each of the flasks. The second set of ten flasks contained 10 mg. of arginine in addition to the 10 mg. of uracil per flask. Again the first set served as the control, while the second set showed what effect arginine had on the incorporation of uracil into nucleic acid pyrimidines. The autoclaving, inoculation, incubation, and harvesting were carried out as previously described. The isolation and hydrolysis of the ribonucleotides and the chromatography of the bases were carried out as previously described. The fractions from the column were plated and counted. The radioactivity was in direct proportion to the ultraviolet absorbance.

Results

The cytosine fraction from the mold which was fed uracil-2-C^{14} and arginine was found to contain a concentration of radioactivity which was 100 per cent of the initial concentration of the radioactivity furnished the mold as uracil-2-C^{14}. There was essentially no labeling

TABLE IV

RADIOACTIVITY OF RIBONUCLEIC ACID BASES FROM NEUROSPORA CRASSA 1298 AFTER GROWTH IN THE PRESENCE OF VARIOUS RADIOACTIVE COMPOUNDS

Supplement	Compound Isolated From Mold	Specific Activity of Supplement	Specific Activity of Compound Isolated	Ratio
Experiment 1				
Uracil-2-C^{14}		2.60×10^4		
	cytosine		2.44×10^4	.94
	uracil		--	
	guanine		78	.003
	adenine		125	.006
Uracil-2-C^{14} + aminobutyrate		2.60×10^4		
	cytosine		1.89×10^4	.72
	uracil		--	
	guanine		14	.0004
	adenine		23	.001
Experiment 2				
Uracil-2-C^{14}		2.51×10^4		
	cytosine		2.13×10^4	.85
	uracil		1.94×10^4	.77
	guanine		0	0
	adenine		0	0
Uracil-2-C^{14} + Aminobutyrate		2.51×10^4		
	cytosine		1.21×10^4	.48
	uracil		1.40×10^4	.56
	guanine		8	.0003
	adenine		97	.004
Experiment 3				
Propionate-2-C^{14}		1.44×10^5		
	cytosine		4.43×10^4	.31
	uracil		4.72×10^4	.33
	guanine		2.05×10^4	.11
	adenine		1.80×10^4	.13
Experiment 4				
Propionate-2-C^{14}		1.24×10^5		
	cytosine		4.10×10^4	.33
	uracil		4.19×10^4	.34
	guanine		0.96×10^4	.08
	adenine		1.03×10^4	.09

Continued

TABLE IV - Continued

Supplement	Compound Isolated From Mold	Specific Activity of Supplement	Specific Activity of Compound Isolated	Ratio
Experiment 5				
Uracil-2-C^{14}		2.50×10^4		
	cytosine		2.21×10^4	.88
	uracil	--	--	
	guanine		0.2×10^4	.08
	adenine		0.23×10^4	.09
Uracil-2-C^{14} + propionate		2.50×10^4		
	cytosine		1.45×10^4	.58
	uracil	--	--	
	guanine		0.54×10^4	.21
	adenine		0.63×10^4	.21
Experiment 6				
Uracil-2-C^{14}		1.90×10^4		
	cytosine		1.41×10^4	.74
	uracil		--	
	guanine		0	
	adenine		0	
Uracil-2-C^{14} + Arginine		1.90×10^4		
	cytosine		1.91×10^4	1.01
	uracil		--	
	guanine		0	
	adenine		0	

of the purines, adenine and guanine. Again the cytosine from the mold fed only uracil-2-C^{14} and basal constituents showed a dilution of specific activity of 74 per cent of the original specific activity.

Isolation of Enzymatic Activity Related to Pyrimidine Metabolism

Materials

Sodium propionate-2-C^{14} was obtained from the Volk Radiochemicals Company. The specific activity was given as 1.2 millicuries per millimole. Dihydrouracil and coenzyme A (product of Pabst Laboratories) were obtained from the California Foundation for Biochemical Research. The pyridoxal hydrochloride, adenosine triphosphate, reduced glutathione and the barium salt of ribose-5-phosphate was obtained from Nutritional Biochemicals. The diphosphopyridine nucleotide and triphosphopyridine nucleotide were obtained from Sigma Chemical Corporation. Beta-alanine was a product of Matheson, Coleman and Bell, obtained from the Metro Industries. The beta-alanyl-pantetheine, propionyl-pantetheine, and beta-ureidopropionic acid were synthesized by the author. The preparation of the latter has been described on page 10.

The propionyl-pantetheine and beta-alanyl-pantetheine were prepared by the thiophenol procedure used by Wieland (48) to synthesize thioesters. The propionyl chloride required in this procedure was synthesized according to the procedure of Brown (49). Benzoyl chloride (0.375 moles) and redistilled propionic acid (0.25 moles) were placed in a round bottom flask and were distilled through a Synder-Shriner

reflux condenser in such a manner that the temperature at the top of the column did not go above 78.5° C. The propionyl chloride distillate (0.12 moles) was dissolved in ether. Thiophenol (0.14 moles) was added slowly, and the solution was left standing under nitrogen at 5° C. to form propionyl-thiophenol. Pantethene, obtained from the Mann Research Laboratories, was reduced with a 5 per cent solution of sodium cyanide in 1 N sodium hydroxide. The reduction was followed by the appearance and the depth of the purple color obtained in the presence of a 5 per cent solution of sodium nitroprusside. This is a test for the presence of free sulfhydryl groups. To the pantetheine (0.08 moles) was added approximately 50 mg. of sodium bicarbonate, and nitrogen was bubbled through. A slight excess of the propionyl-thiophenol (0.10 moles) was added. The reaction proceeds for a few minutes during which the solution changed to a bright yellow color and the test reaction with sodium nitroprusside for free sulfhydryl groups disappeared. The mixture now gave a purple color with methanolic hydroxylamine and ferric chloride, a test for an ester group. After adjusting the solution with hydrochloric acid to a blue color with Congo red, it was extracted with ether. This removed excess thiophenol. The aqueous solution was lyophilized, producing a yellow powder. The beta-alanyl-pantetheine was prepared in the same manner with the exception that the acid chloride was prepared using thionyl chloride. These thiophenol procedures were not found to be satisfactory since the investigator developed an extremely painful rash from contact with even very small amounts of thiophenol.

A more satisfactory procedure for making propionyl-pantetheine was found in the use of propionic anhydride (0.1 moles) and pantetheine (0.12 moles). The disappearance of free sulfhydryl groups and the appearance of the ester group took place in about 10 minutes. The chlorides of beta-alanine made with an excess of thionyl chloride (0.14 moles) reacted with the reduced pantethene (0.1 moles) in 3 ml. of triethanolamine buffer (0.1 M, pH 7.4) to give beta-alanyl-pantetheine. Chromatography of these compounds in an 80 per cent 1-propanol solvent by ascending technique for 36 hours showed an ultraviolet absorbing spot which also gave a positive sodium nitroprusside test before and after hydrolysis with 0.1 N sodium hydroxide indicating the presence of a sulphydryl group. Pantetheine itself is reoxidised to the disulfide form during chromatography and does not give the test with nitroprusside unless reduced again.

Methods

Both alpha and beta amino acids were detected after paper chromatography by reaction with ninhydrin. Uracil was determined both in solution from its absorbance using the Beckmann spectrophotometer, Model DU, and after chromatography on paper by locating the spots with an ultraviolet lamp. Attempts at chromatographing propionate were unsuccessful because such small amounts were not detectable by present methods. Dihydrouracil and beta-ureidopropionic acid were detected on chromatograms (with or without previous ninhydrin treatment) by spraying

with 0.5 N sodium hydroxide, followed, after drying for thirty minutes, by spraying with a solution containing 100 ml. of ethanol, 10 ml. of concentrated hydrochloric acid, and 1 gm. of para-dimethylaminobenzaldehyde. This last spray destroyed any color produced by ninhydrin and produced a yellow color with ureide acids (formed by the sodium hydroxide spraying or already present). This procedure is that used by Fink (22). Thioesters were detected on chromatograms by two methods. The first and most satisfactory method was the spraying of chromatograms with a 4 N hydroxylamine solution (pH 6.5-7.0) followed by spraying with ferric chloride (a mixture of equal volumes of 5 per cent ferric chloride, 12 per cent trichloroacetic acid, and 3 N hydrochloric acid). The second procedure involved hydrolysis of the thioester with strong alkali (3 gm. of sodium hydroxide in 100 ml. of 95 per cent methanol), followed by spraying with sodium nitroprusside reagent (1.5 gm. of sodium nitroprusside mixed in 5 ml. of 2 N sulfuric acid, and adding 95 ml. methanol and 10 ml. of 28 per cent ammonia. This solution was filtered to remove salts).

Incubation

The enzymatic reaction was allowed to proceed while kept at constant temperature in a water bath. All experiments were carried out at 35° C. for varying periods of time.

Enzyme Preparations

A number of different procedures were used for preparing solutions of the mycelial constituents to be examined for enzymatic activity. The principle aim in all of the procedures was complete destruction of the mycelial walls to free the inner constituents. In one of the first attempts, mycelia grown for four days were harvested by filtering on a Buchner funnel, and the pads were stored in the deep freeze for two days. After slight thawing the pads were ground with carborundum powder in 0.1 M phosphate buffer. During grinding the mixture was kept cold by dipping in an ethanol-dry ice bath. The suspension was again frozen and upon rethawing and allowing insoluble particles to settle, the supernatant was used in tests for enzymatic activity upon aminobutyric acid. One milliliter of the solution was used with various concentrations of aminobutyric acid in phosphate buffer (pH 7.9). The total volume was 5 ml. and the reaction time was for 30 and 60 minute periods. After addition of 1 ml. of 5 per cent trichloroacetic acid and filtering, 50 lambda spots were placed on Whatman #3 paper. The developing solvent was 1-butanol:glacial acetic acid:distilled water in a ratio of 80:20:20 by volume. After developing for 17 hours, drying, and spraying with ninhydrin, no change in the concentration of aminobutyric acid was observed. When this experiment was repeated the same results were obtained. Even freshly harvested mycelia, ground with carborundum and incubated with aminobutyric acid showed no activity over periods of 30 minutes, 60 minutes, 120 minutes, and 4 hours incubation time.

Another treatment of the mycelial pads in order to study enzyme activity involved preparation of an acetone dried powder. The freshly harvested mycelia were homogenized in the growth medium followed by extraction of the suspension with 6 volumes of acetone at -20° C. The acetone was filtered off and the powder was placed in a dessicator. One hundred milligrams of the powder were allowed to stand in 1 ml. of water for 6 hours. Three substrates were used: propionic acid (3.7 mg.), aminobutyric acid (5.15 mg.), aspartic acid (4.45 mg.) and aminobutyrate plus 1 mg. of pyridoxal-hydrochloride. Incubation was carried out for 50 minutes followed by filtration through a sintered glass filter. Aliquots were placed on Whatman #1 paper and the development of the paper was carried out for 16 hours with a solvent consisting of equal volumes of 0.1 N sodium acetate and ethanol. Again no change was observed except for a small decrease in concentration of the aminobutyrate with the pyridoxal-hydrochloride. This experiment was tried again using acetone dried powder of the residue left after centrifuging the homogenized mycelial pads, but again no activity was observed. All of these preparations showed succinic dehydrogenase activity as determined by the common methylene-blue procedure.

A third preparation was very similar to the first one described in that the freshly harvested mycelia were frozen in the deep freeze for 4.5 hours after which they were ground in phosphate buffer (0.1 N pH 7.9) using a ten Broeck hand grinder. The homogenate was centrifuged in a Servall refrigerated centrifuge for 10 minutes at 11,000 revolutions

per minute at 4° C. One milliliter of the supernatant was used with 1 mg. of uracil. The controls were 1 mg. of uracil to which 1 ml. of enzyme was added at the end of the experiment. One milliliter of the enzyme served as a blank. At the end of 12 hours each of the solutions was diluted to 100 ml. and the concentration of uracil determined on the Beckmann spectrophotometer, Model DU. No change in uracil concentration was observed.

A fourth method of preparation, which was more successful than the previous ones, involved lyophilization of the harvested mycelia. This removes the water under vacuum and at low temperatures, leaving a brittle dried mycelia which can be ground to a powder. One hundred milligrams of this freshly prepared powder, when incubated in phosphate buffer in the presence of uracil showed a slight activity as was detected by a decrease of the uracil concentration determined by the absorbance. This experiment was repeated with similar results for uracil, and at the same time aminobutyrate decreased in concentration as was shown by chromatography on paper. No activity was observed with respect to action on dihydrouracil. No activity could be found in the powder that had been standing at room temperature in a dessicator for several days.

The fifth and most successful preparation consisted of freezing the freshly harvested mycelial pads on blocks of dry ice and homogenizing in tris (tris-hydroxymethylaminomethane) buffer (pH 7.9). A modification of this procedure involved homogenizing the frozen mycelia in a Waring blendor with the dry ice and diluting afterwards with tris buffer. One milliliter of the homogenate was used with 0.05 mg. uracil, 0.07 mg.

of ribose and 0.01 mg. adenosine triphosphate. The total volume was 3 ml. Incubation was carried out for 12 hours. The enzymatic activity was halted by boiling the solutions in a water bath for 15 minutes. Aliquots (100 lambda) were chromatographed on paper and the uracil spots located by use of an ultraviolet lamp. Uracil in the presence of the mycelial preparation showed a great decrease in concentration. With ribose and ATP the uracil concentration decreased but not to the same degree as with uracil alone. This method of preparation was tried using various substrates. Among these were dihydrouracil (0.1 mg.), amino-butyric acid (0.1 mg.), beta-alanine (0.05 mg.), beta-ureidopropionic acid (0.1 mg.), propionic acid (0.1 mg.), uracil (0.1 mg.), propionic acid plus ribose-5-phosphate (2 mg.), beta-ureidopropionic acid plus ribose-5-phosphate (2 mg.), and proiomyl-pantetheine (1 mg.) plus ribose-5-phosphate (2 mg.). The mixtures in a final volume of 5 ml. were incubated for three hours, after which the activity was destroyed by heating. One hundred lambda aliquots were spotted on paper. The paper was developed for 24 hours in a saturated phenol:water solvent. After drying overnight the phenol still prevented detection of any ultraviolet absorbing spots. Spraying with ninhydrin showed a decrease in amino-butyric acid but no change in beta-alanine. Spraying with alkali, followed by spraying with para-dimethylaminobenzaldehyde showed no change in concentration of dihydrouracil or beta-ureidopropionic acid. This latter mycelial preparation was found to give interesting results in a study of its action on propionate and beta-alanyl-pantetheine.

The incubation mixture consisted of the following substituents to which was added 2 ml. of the supernatant from the centrifuged homogenate: propionate-2-C^{14} (0.1 uM, 1.44 x 10^5 counts per minute per micromole), phosphate buffer (10 uM, pH 7.9), potassium chloride (50 uM), adenosine triphosphate (1 uM), coenzyme A (0.3 uM), diphosphopyridine nucleotide (0.2 uM), triphosphopyridine nucleotide (0.1 uM), glutathione (5 uM), ammonium chloride (400 uM), ribose-5-phosphate (0.1 mg.). The total volume was 2.7 ml. This was a combination of the experiments carried out by Stumpf (42) and Stadtman (43). After incubation for 3 hours aliquots of the solutions were placed on paper and developed in an 80 per cent 1-propanol solvent. Each chromatogram was run in triplicate in order that several types of compounds could be located. One chromatograph was used for scanning purposes, however, the propionate evaporated during chromatography. This same chromatograph was used to locate ultraviolet absorbing spots. In the control experiment which contained all components but was heated to boiling at the beginning of the experiment, six ultraviolet absorbing spots were noted, two of which were associated with the components added to the mixture. One of these was an overlapping area of coenzyme A, diphosphopyridine nucleotide and triphosphopyridine nucleotide and the other was adenosine triphosphate. In the case of the active preparations, the ultraviolet light absorbing spot that was associated with Coenzyme A, diphosphopyridine nucleotide and triphosphopyridine nucleotide was not present, and where ribose-5-phosphate and beta-alanyl-pantetheine were added adenosine triphosphate

was not present nor was one of the four spots from the homogenate itself. One of the chromatographs was treated with ninhydrin, then was sprayed with alkali and para-dimethylaminobenzaldehyde. No new ninhydrin-positive spots were found. No beta-ureido acids could be detected. Spraying another chromatograph with alkali and then with the sodium nitroprussaide reagent showed a new thio-ester in the active preparation containing ribose-5-phosphate and beta-alanyl-pantetheine. This spot was not associated with the beta-alanyl-pantetheine itself. No other thio-esters were detectable.

When this experiment was repeated, the results were the same except for the thio-ester spot which may have decomposed, since this experiment was carried out overnight. At the place where the thio-ester spot should have been a new ultraviolet absorbing spot appeared which may be pantetheine resulting from the decomposition of the ester.

Results

The preparation found to be most successful was that using a Waring blendor homogenate of the frozen mold and dry ice diluted with tris buffer. Using this preparation disappearance of aminobutyrate and uracil were observed. Also in the presence of beta-alanyl-pantetheine, ribose-5-phosphate, propionic acid, adenosine triphosphate, and coenzyme A, formation of a new thio-ester was observed.

DISCUSSION

From the results presented in the previous section, various degrees of growth of the mold were observed in the presence of certain compounds in the basal medium. Other compounds when added to the basal media failed to promote growth of the mold. Some of these latter findings were expected since the idea that the mutant utilizes a pathway different from the normal organism has already been introduced. Thus, finding that ureidosuccinic acid and aspartic acid would not support growth of the mold was not surprising, although these compounds have been described by Kornberg (18) as playing an important role in pyrimidine biosynthesis in other organisms. Another compound, orotic acid, shown to be an intermediate in this same sequence of reactions studied by Kornberg, has already been shown by Loring (7) to be used by the mutant for growth. The small amount of growth, in comparison to that found for alpha-aminobutyric acid and some of the other compounds shown in the results in Table I, probably indicates that orotic acid does not give rise to orotidine-5'-phosphate and then to uridine-5'-phosphate as was suggested by Kornberg for the normal pathway. A relatively slow decarboxylation to form uracil followed by conversion of uracil to uridine-5'-phosphate, seems to be another possibility.

Failure of the mutant to grow either on alpha-ketobutyric acid or on alpha-hydroxybutyric acid indicates that these compounds are not intermediates in the use of the amino acids by the mutant.

Both homoserine and threonine have been shown to give rise to alpha-ketobutyric acid (41). Kinnory et al. (44) have traced the catabolism of C^{14}-labeled aminobutyric acid in rat liver homogenates and found that the amino acid is converted to the keto acid followed by oxidative decarboxylation to the next lower monocarboxylic acid, propionic acid. Fairley (20) has demonstrated that aminobutyric acid, homoserine, and threonine were utilized by the mutant to about the same extent for growth. Homoserine and threonine are interconvertible and this interconversion has been suggested to take place through the hydration of a vinylglycine intermediate (41). Hydrogenation of this intermediate has been suggested to occur giving alpha-aminobutyric acid (50). Allothreonine did not replace threonine as an additive to the basal media supporting growth. Threonine racemase, therefore, would not seem to be present in the mutant, although it has been found in Escherichia coli (51). Since isoleucine can be derived from the alpha-ketobutyric acid formed from either threonine or homoserine (52), it was expected to act in a manner similar to these two compounds. However, the failure of isoleucine to support growth seems to further indicate that the keto acid is not the common intermediate between threonine, homoserine and aminobutyric acid. Failure of cyclopropane aminocarboxylic acid to support growth suggests that the mold cannot open the cyclopropane ring to form either homoserine, threonine or aminobutyric acid.

Since threonine is known to give rise to butyric acid and propionic acid (55), both of these compounds were tested as possible intermediates

in the utilization of the aliphatic amino acids. In higher animals threonine can be cleaved to yield glycine and acetate (54). The results show that of the four compounds, propionic acid was the only one used by the mold for growth. These results were comparable to those obtained when aminobutyric acid was added to the medium. This finding is strongly indicative of the use of propionic acid for pyrimidine formation in the mutant by a pathway closely related to that by which the amino acids are utilized.

The metabolism of propionate has received considerable attention. Stadtman (43) has shown that dried cells of Clostridium propionicum metabolize propionic acid in the presence of ammonium salts to form beta-alanine. These reactions take place through Coenzyme A derivatives. Propionyl-Coenzyme A is formed from propionic acid, adenosine triphosphate and Coenzyme A. Propionyl-Coenzyme A or acrylyl-Coenzyme A in the presence of ammonium ions is converted to beta-alanyl-Coenzyme A. An early step in the uitlization of propionate by pig heart involves the addition of carbon dioxide to the three carbon compound (55). This can occur only after the propionic acid is converted to propionyl-Coenzyme A. The product of the reaction is methylmalonyl-Coenzyme A and the reaction requires adenosine triphosphate. This product is then converted to succinic acid by an isomerization reaction.

Other pathways of propionate metabolism exist. In cow udder propionic acid-1-C^{14} is converted to acetic acid-1-C^{14}, a reaction which can not take place by way of the above mentioned mechanism (56).

In peanut mitochondria, propionate appears to be oxidized to beta-hydroxypropionic acid, probably by way of the Coenzyme-A derivatives of propionic acid, acrylic acid, and beta-hydroxypropionic acid (42).

In view of the existence of these pathways, some of the intermediates of these reactions were tested as growth promoters for the mutant. Of the compounds tried acrylic acid, beta-hydroxypropionic acid and methylmalonic acid supported growth, suggesting that some or all of these compounds may be intermediates in the use of propionate for pyrimidine formation.

The finding that propionate and related compounds supported growth of the pyrimidineless strain, led to the suggestion that these compounds were used for pyrimidine formation by conversion to beta-alanine, beta-ureidopropionic acid, and dihydrouracil. When the compounds were tested as growth supplements, beta-alanine was found to be inactive. However, the remaining two compounds did permit growth to occur. Although beta-alanine itself is not utilized for growth, the pathway by which propionic acid is used might require formation of the activated form, beta-alanyl-Coenzyme A. The failure of the mold to use beta-alanine indicates the absence of the enzyme needed for the direct activation.

The results obtained from experiments in which the growth of the mold was determined as a function of time for these various growth-supporting compounds, indicate, however, that neither propionate nor aminobutyrate can be used by a pathway which involves free beta-ureido-propionate or dihydrouracil as intermediates. These conclusions were reached on the basis of the results shown in Figure 4, showing

amino-butyrate and propionate to give a lower maximum of growth as well as beginning growth at a time far before dihydrouracil or ureidopropionate. If propionate and aminobutyrate were utilized by conversion to ureidopropionic acid, dihydrouracil and then to uracil, the lag in initiation of growth, which probably indicates adaptation to the substrate, should have been of the same order or greater than with dihydrouracil and ureidopropionic acid. Since the maximum reached in the curves when the latter substances were used as growth substituents, is similar to that reached by uracil, these substances probably are utilized after conversion to uracil by the reversal of the degradative pathway shown in Figure 2. Once growth began, the growth rates as measured by the slopes of the curves were essentially the same, indicating no great destruction of any of the compounds occurred during the adaptive period. Compared with the other compounds, aminobutyrate and propionate were found to give a lower maximum of growth. This is presumably related to the use during growth of substantial amounts of these compounds for metabolic reactions other than pyrimidine formation.

Since the absence of uridine derivatives for coenzyme reactions has been suspected as the major deficiency in the mold as it begins growth, selected compounds were chosen to see whether the addition of a small amount of uridine would cause any significant growth effect. The results in Table II showed that only dihydrouracil gave a more than additive response in the presence of uridine. A similar effect has been shown (21) for aminobutyric acid and threonine. These results are consistent with the use of an initial supply of a trace of uridine to

provide a coenzyme necessary in the utilization of these compounds; however, this does not rule out the possibility that uridine simply allowed a more rapid adaptation by an unknown process.

The results in Table IV, showing the utilization by the mold of both radioactive uracil and aminobutyrate supplied together in the basal medium, provide further support for the utilization of aminobutyrate in pyrimidine biosynthesis. In the control experiments with uracil-2-C^{14} alone, the nucleic acid pyrimidines were labeled to a very high extent, 85 to 93 per cent of the initial concentration of radioactivity supplied in the uracil. If the pyrimidines formed had as their sole precursor the uracil-2-C^{14} of the medium, they should have been labeled to an identical extent as the precursor. The dilution of the isotope which was actually found indicates that synthesis of the pyrimidines occurred at the same time from the simple non-labeled carbon sources of the medium, such as glucose and tartrate, as well as from the uracil supplied. When aminobutyric acid was added along with the uracil-2-C^{14}, utilization of uracil-2-C^{14} was decreased to an even greater extent, and produced pyrimidines which were labeled to only 55 to 72 per cent of the concentration of radioactivity supplied as uracil-2-C^{14}. These experiments show that when aminobutyrate and uracil were present at the same time the mold used both for synthesis of pyrimidines. These experiments did not indicate whether aminobutyric acid caused dilution of the radioactivity by synthesis of uracil itself or whether it was utilized by another route. However, the data presented previously in the growth

curves (page 19) suggests the two substances were used by different routes.

If propionate and aminobutyrate are used by the mold for pyrimidine biosynthesis by the same pathway, then the concentration of radioactivity found in the pyrimidines isolated from the mold would be expected to be of the same order as that found for aminobutyrate. Indeed, the results shown in Table IV do show considerable dilution, 31 to 38 per cent of the initial concentration of radioactivity, to have taken place in formation of the pyrimidines by the mold when propionate-2-C^{14} was added to the medium. However, aminobutyrate-3-C^{14} was found to be utilized to the extent of 14 per cent of the initial concentration of radioactivity. Thus, although the dilution is to a much greater degree than uracil itself, it is to a lesser degree than aminobutyrate. The relatively low concentration of activity found in purines, 10 per cent of the original radioactivity, indicates that there is a specific incorporation of propionic acid into the pyrimidines.

Further evidence for utilization of propionate for pyrimidine biosynthesis by the mold is found in the results expressed in Table IV. Although in the above experiment the dilution of the initial radioactivity was great enough to question the specificity of propionate utilization for pyrimidine biosynthesis, this experiment shows that propionate is used to a considerable extent. Again in the control experiment using uracil-2-C^{14} alone, a slight dilution of the isotopic carbon was noted as the compound was used for nucleic acid pyrimidine formation. When

propionic acid was added with the uracil-2-C^{14} to the basal medium, the mold used both of the compounds for pyrimidine synthesis. The uracil-2-C^{14} was incorporated to the extent of 53 per cent of the initial concentration of radioactivity into the pyrimidines and practically no utilization for purines. It appears clear, therefore, that propionic acid must be added to the list of known pyrimidine precursors.

With this demonstration of the use of the aliphatic acids, aminobutyric and propionic acids, for pyrimidine biogenesis the problem of the path by which they are utilized presents itself. Because of the similarity of the manner of growth, it seems logical to assume that both compounds are utilized by the same pathway. With the information presented by the experiments described in this thesis, a logical pathway can now be suggested.

The proposed pathway is given in Figure 5. On the basis of propionic acid and aminobutyric acid being closely related, the suggestion is made that they go through a common intermediate which is beta-alanyl-coenzyme A. This latter substance has already been shown to be readily derived from propionyl-Coenzyme A. Note should be made that this is an activated form of beta-alanine which might explain the failure of the latter substance to support growth. The addition of ribose-phosphate is believed to take place at this step with the formation of the beta-alanyl-Coenzyme A-ribotide. The next step would be the formation of the beta-ureidopropionyl-Coenzyme A-ribotide. From this compound, dihydrouracil-ribotide could be formed and then reduced to uridine-5'-phosphate. Actually, the introduction of the ribose-phosphate could

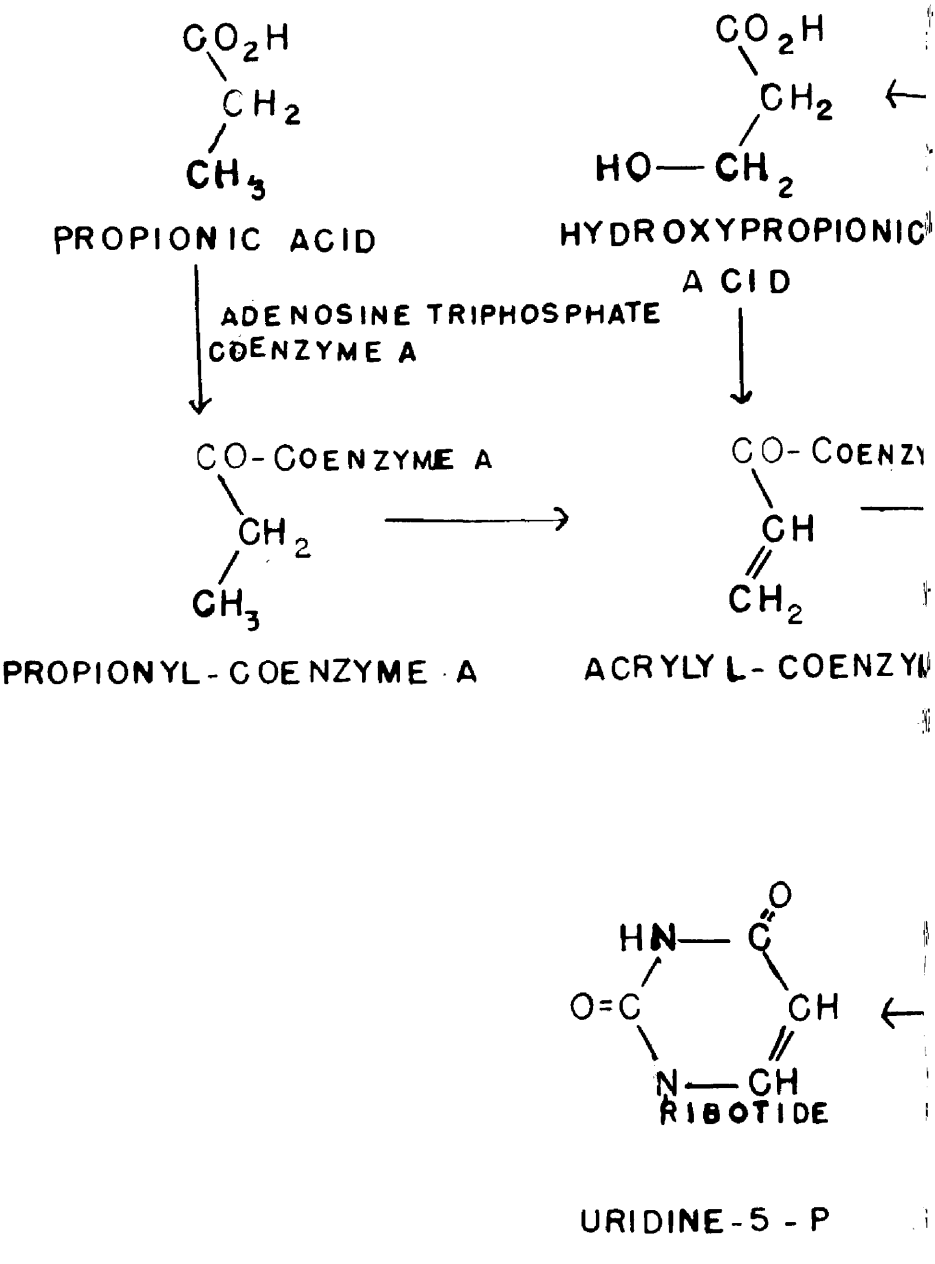
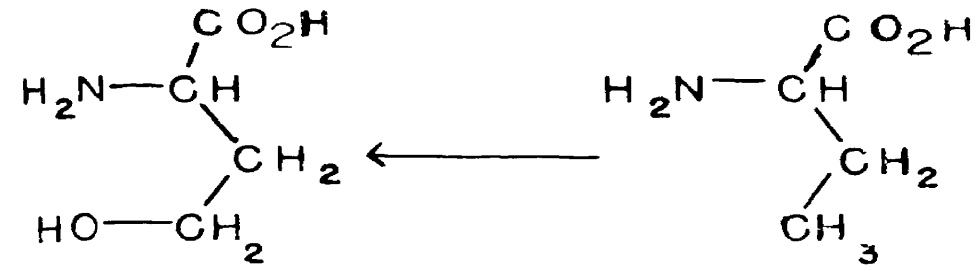
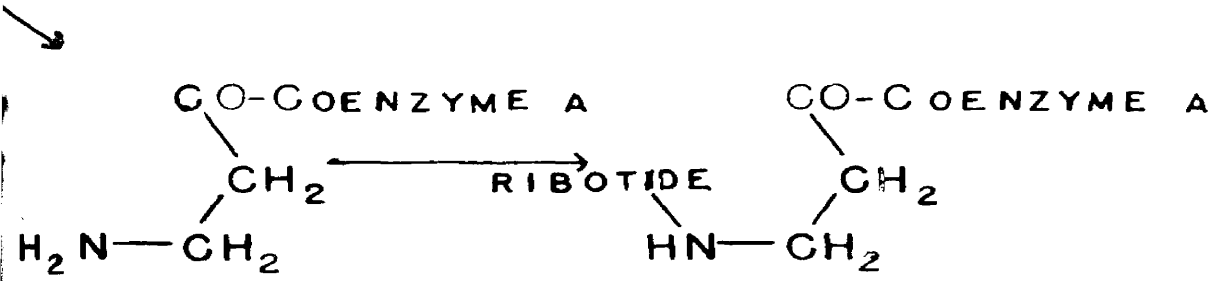
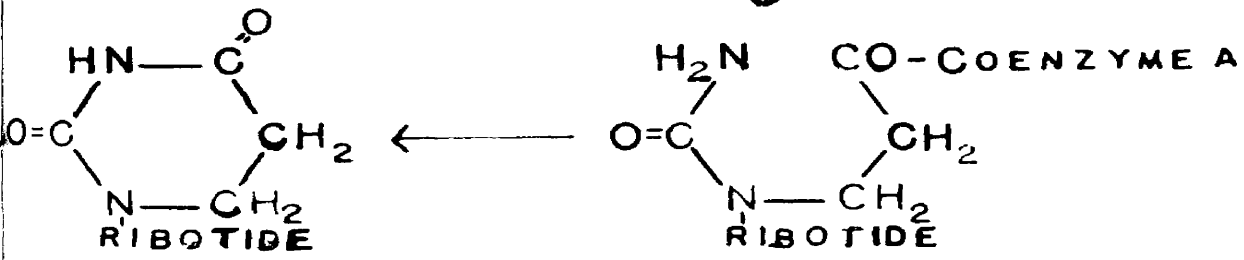


FIGURE 5- SUGGESTED PATHWAY OF PYRIM

CRASSA M







OSYNTHESIS IN THE NEUROSPORA

1298

occur after the formation of beta-ureidopropionyl-Coenzyme A or the release of Coenzyme A might also occur with the formation of beta-alanyl-ribotide. At any rate, it should be noted that this route differs from the simple reversal of the reactions described by Fink (22), which indicate that uridine-5'-phosphate is degraded to beta-alanine by way of free beta-ureidopropionic acid, and dihydrouracil, in that activated compounds in which the glycosidic linkage already exists are used. Preliminary evidence has already been presented by Griscolia (57) showing that beta-ureidopropionyl-ribotide and dihydrouracil-ribotide are enzymatically converted to uridine-5'-phosphate by rat liver.

Little or no evidence is available for the remainder of the proposed pathway, including that part suggesting the route by which aminobutyric acid may be used to enter the pyrimidine structure. As has been stated previously it is known that aminobutyrate can be deaminated to form the keto acid and then decarboxylated to form propionic acid. However, the keto acid does not support growth, making this pathway seem unlikely. Another suggestion is that the formation of homoserine from aminobutyrate by way of the vinylglycine intermediate gives rise to hydroxypropionic acid by a mechanism of deamination and decarboxylation. Hydroxypropionic acid is related to propionic acid, supposedly through acrylic acid or beta-alanine.

Even though little direct evidence is available, this pathway is consistent with the available evidence and is offered as a guide for further study. Fairley (20) has reported the inhibitory action of

arginine on the use of aminobutyric acid and uracil for growth of the mutant. These studies were extended in the present work showing the same inhibitory effect of arginine on propionic acid and homoserine utilization. The experiments demonstrated that only a very small amount of arginine (0.01 mg.) is needed to completely inhibit growth in the presence of 5 mg. of aminobutyric acid, propionic acid, or homoserine. This is a further indication that these compounds are used by the same path. The growth of the mold in the presence of uracil, is inhibited by arginine, but only at much higher concentrations. This indicates that uracil probably does not follow the same pathway as propionate and aminobutyrate. Further evidence of this point was obtained in the experiment in which uracil-2-C^{14} was administered to the mold in the presence of arginine. With the uracil-2-C^{14} alone, a small amount of dilution of the isotopic carbon was again noted. However, with the arginine this dilution was not present. This seems to indicate that the dilution, caused by the formation of propionyl-Coenzyme A from the simple carbon sources once metabolic pathways are underway, is inhibited and all of the pyrimidines are formed from the uracil-2-C^{14}.

The enzymatic experiments were undertaken to obtain evidence as to whether or not some of the reactions postulated for the use of propionate for pyrimidine synthesis actually do occur in the mold. Considerable effort was expended trying methods by which the tough mycelial walls could be ruptured, at the same time giving active enzyme preparations. Best results were obtained with the frozen mycelia

homogenized in dry ice. Activity was shown on uracil, aminobutyrate, and beta-alanyl-pantetheine. The latter compound was tried instead of its Coenzyme A derivative for economical reasons. These were all preliminary experiments and, as such, show that enzymatic activity toward certain substrates is present. However, no conclusions can be drawn on the relation of these activities to the proposed mechanism.

SUMMARY

1. Further evidence for the utilization of aminobutyrate for synthesis of nucleic acid pyrimidines by the mutant N. crassa 1298 was obtained in experiments carried out with the addition of uracil-2-C^{14} and aminobutyrate to the basal medium. The control experiments with uracil-2-C^{14} alone showed a small dilution of the radioactivity of the uracil administered, as it was incorporated into the pyrimidines of the nucleic acids. With uracil-2-C^{14} and aminobutyrate both in the medium the mold showed the ability to use both of the substances at the same time. This was shown by the great dilution of the radioactivity in the isolated pyrimidines.

2. A search for further growth-supporting compounds for N. crassa 1298 added the following compounds: propionic, acrylic, beta-hydroxypropionic, beta-ureidopropionic, methylmalonic acids and dihydrouracil. Various intermediates of the Kornberg scheme were not used for growth. Studies of the time-course of growth of the mold on various supplements showed that propionate and aminobutyrate gave similar responses. Beta-ureidopropionate and dihydrouracil required longer periods of adaptation before growth began.

3. Propinate-2-C^{14} was found to be incorporated, relatively specifically into the pyrimidines of the mold. The purines of the same experiments contained much lower amounts of carbon-14.

4. The presence of unlabeled propionic acid in the growth medium depressed significantly the specific activities of the pyrimidines formed by the mold in a medium also containing uracil-2-C^{14}.

5. Arginine was found to inhibit strongly the growth of the mold with propionate or homoserine as supplements. A weak inhibition of the use of uracil for growth was noted. The presence of arginine in basal medium containing uracil-2-C^{14} led to an increase in the specific activities of the pyrimidines formed.

6. Preliminary experiments showed that enzymes were present in the mold, which effected aminobutyrate, uracil and beta-alanyl-pantetheine.

7. On the basis of the results a new pathway for the synthesis of pyrimidine compounds is suggested, a pathway which leads from propionyl-Coenzyme A through the Coenzyme A derivatives of beta-alanine and beta-ureidopropionic acid to dihydrouridylic acid and finally to uridine-5'-phosphate.

BIBLIOGRAPHY

1. Hotchkiss, R. D.; Brachet, J., The Nucleic Acids, Vol. II, Chargaff, E. and Davidson, J. N., eds., Chapters 27 and 28, Academic Press, New York, 1955, pp. 435, 476.

2. Barnes, F. W., Jr., and Schoenheimer, R., J. Biol. Chem., 151, 123 (1943).

3. Heinrich, M. R. and Wilson, D. W., J. Biol. Chem., 186, 447 (1950).

4. Lagerkvist, U., Acta. Chem. Scand., 4, 1151 (1950).

5. Mitchell, H. K., Houlahan, M. B., and Nyc, J. E., J. Biol. Chem., 172, 525 (1948).

6. Mitchell, H. K., and Houlahan, M. B., Federation Proc., 6, 506 (1947).

7. Loring, H. S., and Pierce, J. G., J. Biol. Chem., 153, 61 (1944).

8. Wright, L. D., Miller, C. S., Skeggs, H. R., Huff, J. W., Weed, L. L., and Wilson, D. W., J. Am. Chem. Soc., 73, 1898 (1951).

9. Bergstrom, S., Arvidson, H., Hammerston, E., Eliasson, N. A., Reichard, P., and von Ubish, H., J. Biol. Chem., 177, 495 (1949); ibid, 179, 169 (1949).

10. Hurlburt, R. B., and Potter, V. R., J. Biol. Chem., 195, 257 (1952).

11. Reichard, P., Acta Chem. Scand., 8, 795, 1102 (1954).

12. Reichard, P., and Hanshoff, G., Acta Chem. Scand., 10, 548 (1956).

13. Jones, M. E., Spector, L., and Lipmann, F., J. Am. Chem. Soc., 77, 819 (1955).

14. Woods, L., Ravel, J. M., and Shive, W., J. Biol. Chem., 209, 559 (1954).

15. Lieberman, I., and Kornberg, A., J. Biol. Chem., 207, 911 (1954).

16. Kornberg, A., Lieberman, I., and Simms, E. S., J. Am. Chem. Soc., 76, 2027, 2844 (1954).

17. Kornberg, A., Lieberman, I., and Simms, E. S., J. Biol. Chem., 215, 389, 403, 417 (1955).

18. Lieberman, I., J. Biol. Chem., 222, 765 (1956).

19. Welch, A. D., Enzymes: Units of Biological Structure and Function. Gaebler, O. H., ed., Academic Press Inc., New York, 1956, p. 547.

20. Fairley, J. L., J. Biol. Chem., 210, 347 (1954).

21. Herrmann, R. L., and Fairley, J. L., J. Biol. Chem., 227, 1109 (1957).

22. Fink, K., Henderson, R. B., and Fink, R. M., J. Biol. Chem., 197 441 (1952); 201, 349 (1953).
Fink, R. M., McGaughey, C., Cline, R. E. and Fink, K., J. Biol. Chem., 218, 1 (1956).

23. Fritzson, P., Acta Chem. Scand., 10, 1674 (1956).

24. Canellakis, E. S., J. Biol. Chem., 221, 315 (1956).

25. Grisolia, S. and Wallach, D. P., Biochim. et Bioph. Acta, 18, 449 (1955).

26. Rutman, R. J., Cantarow, A. and Paschkis, E. S., J. Biol. Chem. 210, 321 (1954).

27. Canellakis, E. S., J. Biol. Chem., 227, 329 (1957).

28. Lagerkvist, U., and Reichard, P., Acta. Chem. Scand., 8, 361 (1954).

29. Kalckar, H. M., J. Biol. Chem., 167, 477 (1947).

30. Ostern, P., and Terszakowec, J., Hoppe-Seyler's Z. physiol. Chem., 250, 155 (1937).

31. Caputto, R., J. Biol. Chem., 189, 801 (1951).

32. Kornberg, A., and Pricer, W. E., J. Biol. Chem., 193, 481 (1951).

33. Greenberg, G. R., Federation Proc., 12, 1 (1953).

34. Brawerman, G., and Chargaff, E., Biochim. et Biophys. Acta, 16, 524 (1955).

35. McNutt, W. S., Biochem. J., 50, 384 (1953).

36. Longfeld, F., and Stieglitz, J., Am. Chem. J., 15, 504 (1893).

37. Beadle, G. W., and Tatum, E. L., Proc. Nat. Acad. Sci., 27, 499 (1941).

38. Hayaishi, O., and Kornberg, A., J. Biol. Chem., 197, 717 (1952).

39. Fincham, J. R. S., J. Gen. Microbiol., 11, 236 (1954).

40. Black, S., and Wright, N., J. Biol. Chem., 213, 27, 39, 51 (1955).

41. Teas, H. J., Horowitz, N. H., and Fling, M., J. Biol. Chem., 172, 651 (1948).

42. Giovanelli, J., and Stumpf, P. K., J. Am. Chem. Soc., 79, 2652 (1957).

43. Stadtman, E. R., J. Am. Chem. Soc., 77, 5765 (1955).

44. Kirmory, D. S., Takeda, Y., and Greenberg, D. M., Biochem. et Biophys. Acta, 17, 561 (1955).

45. Lien, O. G., and Greenberg, D. M., J. Biol. Chem., 200, 367 (1953).

46. Abelson, P. H., and Vogel, H. J., J. Biol. Chem., 213, 355 (1955).

47. Black, A. L., and Klieber, M., J. Biol. Chem., 232, 203 (1958).

48. Wieland, T., and Koppe, H., Justus Liebig Annalen der Chemie, 588, 15 (1954).

49. Brown, H. C., J. Am. Chem. Soc., 60, 1325 (1938).

50. Davis, B. D., Advances in Enzymology, Vol. 16, Nord, F. F. ed., Interscience Publishers, Inc., 1955, p. 247.

51. Ames, H., J. Am. Chem. Soc., 76, 3858 (1954).

52. Umbarger, E., and Adelberg, E. A., J. Biol. Chem., 192, 883 (1951).

53. Cardon, B. P., and Barker, H. A., Arch. Biochem., 12, 165 (1947).

54. Lin, S., and Greenberg, D. M., J. Gen. Physiol., 38, 181 (1954).

55. Flavin, M., Ortiz, P. J., and Ochoa, S., Nature, 176, 823 (1955).

56. James, A. T., Peeters, G., and Lauryssens, M., Biochem. J., 64, 726 (1956).

57. Mokrasch, L. C., Derks, M. A., Caravaca, J., and Grisolia, S., Federation Proc., 17, 278 (1958).

APPENDIX

1. Basal Medium:

Calcium chloride	1 gm.
Ammonium tartrate	50 gm.
Ammonium nitrate	10 gm.
Potassium dihydrogen phosphate	10 gm.
Magnesium sulfate heptahydrate	5 gm.
Sodium chloride	1 gm.
Sucrose	101 gm.
Biotin	26 gamma
Trace element solution	10 ml.
Distilled water	to 10 l.

2. Trace element solution:

Sodium tetraborate	8.8 gm.
Ammonium molybdate	6.4 gm.
Ferric chloride	50.0 gm.
Zinc sulfate heptahydrate	200.0 gm.
Cupric chloride	27.0 gm.
Manganous chloride	4.5 gm.
Distilled water	to 500.0 ml.

3. Culture slants: The mold is maintained on culture slants consisting of basal medium containing 2 per cent agar and 1.0 mg. of uracil per ml. The agar and uracil are dissolved in basal medium by heating and 10 ml. fractions of the resulting culture medium are transferred to Pyrex test tubes. The tubes are stoppered with cotton plugs and are sterilized by autoclaving. The tubes are placed on a slant while still hot to provide maximum surface area and the contents allowed to gel. The mold is transferred from tube to tube at two-week intervals using standard sterile technique.