ARGININE AND PYRIMIDINE BIOSYNTHESIS IN NEUROSPORA CRASSA 1298

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY

A. Birk Adams

1959

ARGININE AND PYRIMIDINE BIOSYNTHESIS IN *NEUROSPORA CRASSA* 1298

By

A. Birk Adams

A THESIS

Submitted to the College of Science and Arts of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Chemistry

1959

ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Dr. James L. Fairley for encouragement and stimulating guidance throughout the course of this work.

The author also wishes to express his appreciation to the various other members of the Chemistry Department for assistance and helpful suggestions.

VITA

The writer was born in Steubenville, Ohio, January 16, 1934 and received his secondary education at North Canton High School, North Canton, Ohio. He entered Bethany College, Bethany, West Virginia in 1951 and received his B.S. degree in 1955 from this institution.

In the fall of 1955 the author entered the Graduate School of Michigan State University. After one year of study and serving as a Graduate Teaching Assistant he was inducted into the United States Army for a period of two years during which time he served as a clerk-typist and medical technician in Stuttgart, Germany. He then returned to Michigan State University in the summer of 1958, again being employed as a Graduate Teaching Assistant in Chemistry while pursuing his studies.

ARGININE AND PYRIMIDINE BIOSYNTHESIS IN *NEUROSPORA CRASSA* 1298

By

A. Birk Adams

AN ABSTRACT

Submitted to the College of Science and Arts of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Chemistry

1959

Approved James L Fairley Jr

ABSTRACT

The purpose of this study was to gain information concerning the mechanisms by which the mutant organism, Neurospora crassa 1298, forms pyrimidine compounds from the precursor, propionic acid. L-arginine had been known to inhibit the growth of this organism in basal medium supplemented with propionate, presumably by blocking a reaction necessary in the conversion of propionate to pyrimidines. Evidence has been obtained in the present work which supports this suggestion and eliminates certain other possibilities.

Use was made of this finding in attempts to identify intermediates of the reaction sequence leading from propionate to pyrimidines. The mold was supplied with propionate and arginine and a search was made, using paper chromatographic techniques, for the accumulation in the growth medium and in the mycelium of the mold of compounds of the synthetic process occurring in the sequence prior to the blocked reaction. No quantitative or qualitative differences were found, in a number of experiments, between compounds formed by the inhibited mold and those formed by the uninhibited control until use was made of radioactive propionic acid. The mold was supplied with propionate-2-C^{14} and the radioactive compounds formed from this by the metabolic activities of the mold and liberated into the medium were separated by two-dimensional paper chromatography and located by radioautography.

A new radioactive compound was detected on the chromatograms of the arginine-inhibited mold, a compound not present in the control. Presumably this compound is an intermediate in propionate utilization. It has not, as yet, been identified.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

In recent years biochemical mutants have been used extensively both for the study of genetics and for the study of biosynthetic pathways of vital cellular components. One of the most useful types of these mutants has been the one produced by Beadle and Tatum (1, 2, 3) in 1940 by the X-radiation of the mold Neurospora crassa. The basic concept behind the use of these mutants is that all biochemical processes are genetically controlled. Only those reactions can occur for which the necessary enzymes are present, and the ability to produce each of the enzymes is a separate inherited characteristic. Destruction of a single genetic unit, a gene, results in the failure to produce a certain enzyme and, in turn, in a block in a particular sequence of biochemical reactions. The organism, however, is capable of essentially normal growth if it is supplied with compounds which occur after the blocked step in this sequence of reactions. Under these conditions the intermediate compounds prior to the blocked reaction will often increase in concentration until one or more is detectable by chemical means.

The generally accepted pathway for pyrimidine biosynthesis in most organisms is the scheme shown in Figure 1 (4). This pathway was proposed by Lieberman and Kornberg and is supported by evidence from several different workers using a variety of different organisms (5-13).

Considerable evidence has been accumulating, however, indicating that the mutant organism, Neurospora crassa 1298, can synthesize pyrimidines by a different, as yet unknown, pathway. This mutant of

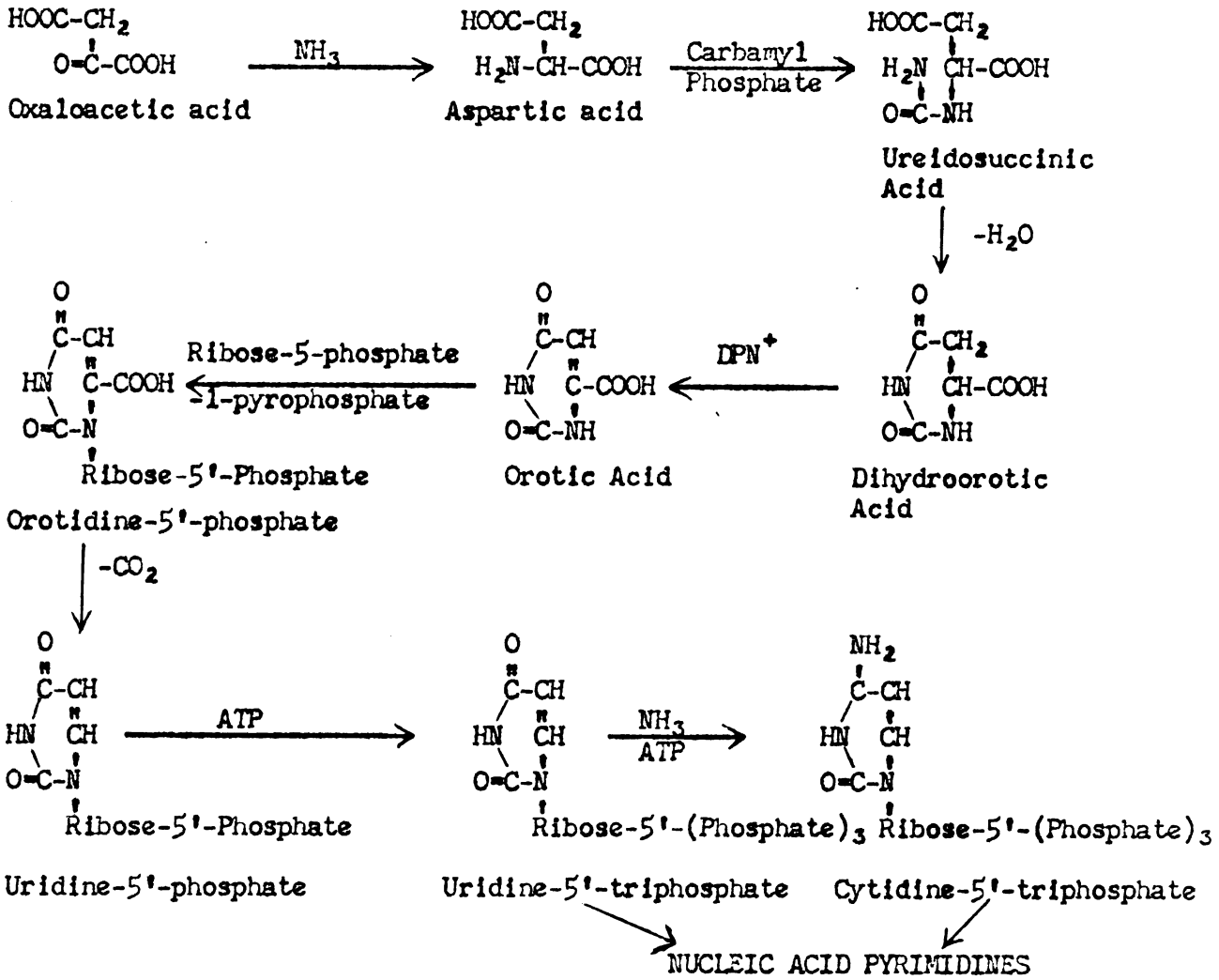


Figure 1. Lieberman-Kornberg pathway of pyrimidine biosynthesis.

a common bread mold was originally characterized as requiring uracil (3) in the basal medium for growth. It was studied further by Loring and Pierce (14) and was found to grow at a much higher rate on uridine or cytidine than it did on uracil. It was also found that orotic acid would support growth only as well as uracil.

Fairley and co-workers (15, 16, 17) using the same organism have shown that α-aminobutyric acid, propionic acid and related compounds

are incorporated into the nucleic acid pyrimidines to a much greater extent than into the purines or the amino acids of the proteins. As a result of these studies it was evident that this mutant did not synthesize pyrimidines by the Lieberman-Kornberg pathway and a new pathway was proposed as outlined in Figure 2 (17).

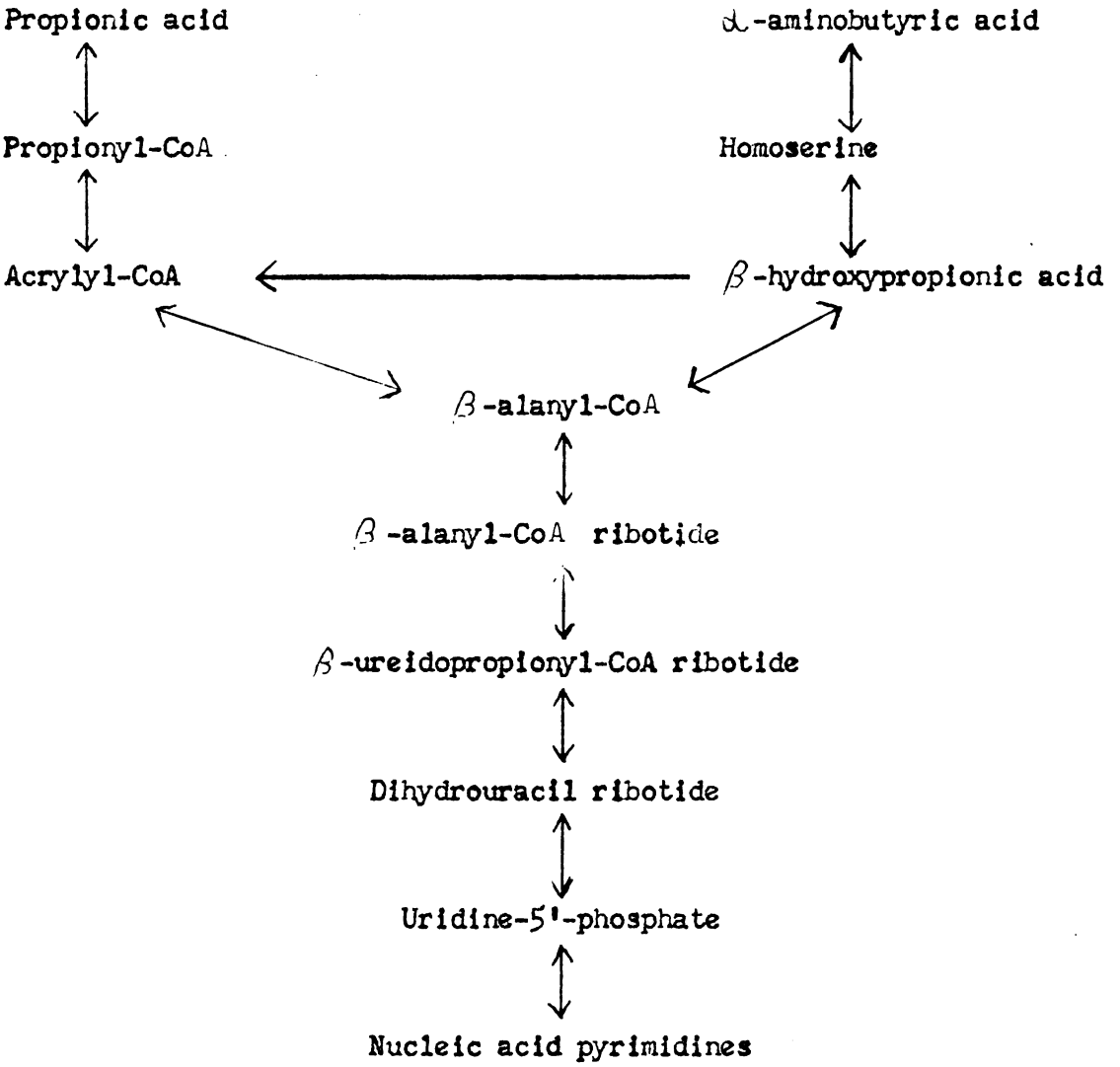


Figure 2. Proposed pathway for pyrimidine biosynthesis in Neurospora crassa 1298.

This scheme is closely related to the degradative path for uracil found in other organisms. When uracil was incubated with rat liver slices, β-alanine was formed (18). If the labeled compounds were incubated with rat liver preparations an interconversion of dihydrouracil and β-ureidopropionate was demonstrated, but the formation of β-alanine from ureidopropionate appeared to be an irreversible process (19). Although these studies seem to involve a purely degradative pathway from uracil to β-alanine, it remains a possibility that the ribotides of the compounds in this sequence could be utilized for the synthesis of pyrimidine nucleotides. Indeed, Mokrasch and Grisolia (20) have reported the incorporation of dihydrouracil ribotide and β-ureidopropionic acid ribotide into ribonucleic acid by rat liver preparations.

Fairley (21) made the interesting observation that very small amounts of L-arginine in medium supplemented with α-aminobutyric acid would completely inhibit the growth of N. crassa 1298, but had less effect when the mold was grown on medium supplemented with uridine. Upon further investigation (18) of this phenomenon it was found that when the mutant was grown on labeled uracil in the presence of arginine, the specific activity of the cytosine isolated from the mycelium was the same as the specific activity of the added uracil. However, when grown in the absence of arginine the specific activity of the cytosine was only about 75% that of the original uracil. This indicated that once growth had started, this organism was capable of synthesizing pyrimidines by an adaptive route from simple compounds and that arginine was able to block this route. It seems quite reasonable to assume from the similarity of the effects of arginine that this

adaptive route is closely related to the synthetic route from propionate and aminobutyrate.

It was also found that the specific activity of the aminobutyric acid isolated from the acid-soluble fraction of the mycelium when the mutant was grown on labeled aminobutyric acid was only about 5 % that of the compound supplied. The nucleic acid pyrimidines isolated had similar, low specific activities (16). Thus it appeared that aminobutyric acid was needed to stimulate adaptation and once this adaptation had occurred the organism was able to synthesize all of its necessary components from the basal medium by the same route used with aminobutyrate.

The present study was undertaken with the aim of gaining further information about the pathway of utilization of propionate and aminobutyrate for pyrimidine synthesis by N. crassa 1298. The approach to this problem was to use L-arginine under different conditions to block, in specific fashion, the sequence of reactions leading from the simple compounds to pyrimidines with the view that intermediates prior to the blocked reaction would accumulate to the point where they could be detected, isolated and identified. This technique has been used successfully, for example, in identifying an intermediate in purine biosynthesis in Escherichia coli inhibited by a sulfa drug (22).

Closely related to this basic problem of the mechanism of pyrimidine formation is the precise nature of the inhibitory action of arginine, and therefore, the arginine effect has also been a subject of the present study. Arginine would be of value to this work only if its inhibitory effect was the result of the blocking of a specific reaction along the biosynthetic route of pyrimidine formation.

It was therefore considered necessary to investigate the possibility that the action of arginine was to affect cell permeability, preventing the absorption of aminobutyrate and propionate, and the chance that arginine simply prevented the adaptive formation of a necessary enzyme in the germinating spores.

EXPERIMENTAL AND RESULTS

Growth Studies on the Arginine Effect

Organism

Neurospora crassa 1293 was the organism used throughout this study and is one of the many mutants produced by Beadle and Tatum in 1940 by X-ray treatment of the wild strain. It differs from the wild strain in that it is unable to grow on modified Fries basal medium (23) containing inorganic salts, sucrose and biotin unless the medium is supplemented with a source of pyrimidines. The organism was maintained on agar slants consisting of 2 % agar and 0.1 % uracil in the basal medium. It was observed that spores obtained from uracil culture slants required one or two days longer to begin growth in medium supplemented with sodium propionate as compared with spores obtained from culture slants containing either sodium propionate or α-aminobutyric acid. Because of this, culture slants containing 1 % DL-α-aminobutyric acid in place of the uracil were also maintained. The mold was transferred to fresh slants about every two weeks.

Materials

The uracil used was a product of the Nutritional Biochemical Corporation and the DL-α-amino-n-butyric acid was a product of the Eastman Organic Chemical Company. L-arginine hydrochloride was from the Pfanstiehl Chemical Company. The sodium propionate was prepared by neutralization of 10N. sodium hydroxide with redistilled propionic acid and recrystallization from a water-ethanol solution.

Growth Procedures

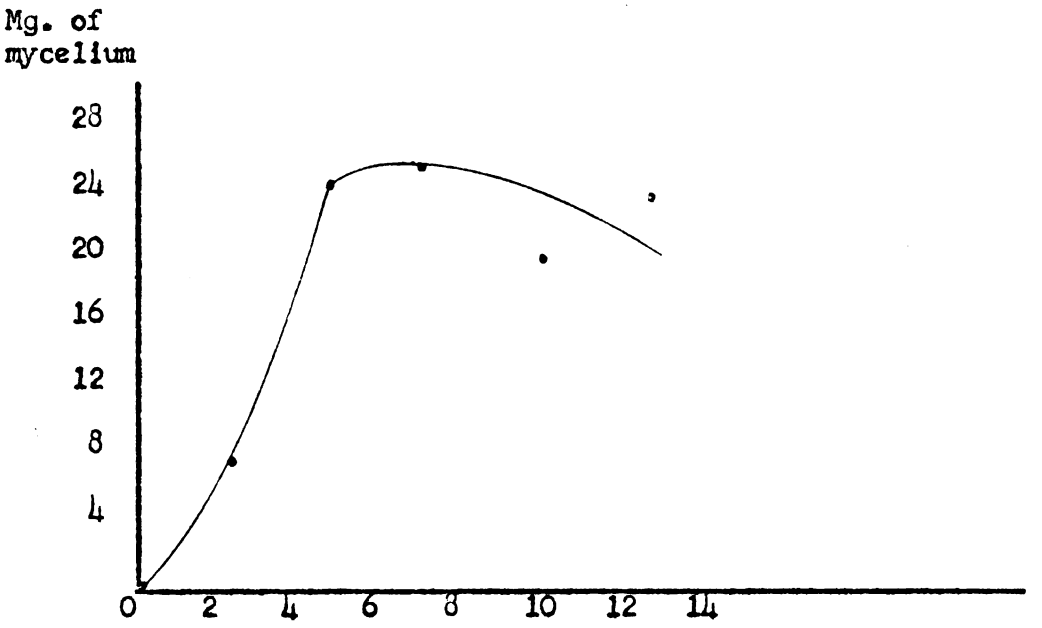
In performing growth studies the mold was grown in 125 ml. Erlenmeyer flasks fitted with cotton plugs. Any heat-stable reagents being used in the experiment and 25 ml. of basal medium were placed in the flasks before sterilization. The flasks were then stoppered with the cotton plugs and sterilized by heating in an autoclave for twenty minutes or more at 12-15 pounds pressure.

The sodium propionate and other similar compounds which were unstable or volatile in the slightly acidic medium were added to previously sterilized flasks after being filtered through a sterile, sintered-glass, bacterial filter using standard aseptic techniques. Finally the flasks were inoculated with 1 ml. of a suspension of spores of N. crassa 1298 in sterile water. Growth was allowed to proceed until a moderately heavy mycelial pad had developed, usually four to six days, and then harvested by filtering the contents of the flasks separately with suction through a sintered glass filter. The mycelia were washed several times with distilled water and then rolled into pellets and dried on a spot plate for a day at 60° C. The dried mycelia were weighed on a torsion balance.

Since higher concentrations of propionate were known to inhibit growth of the mold, it was desirable to learn the concentration of propionate needed to give optimum growth so that this amount could be used throughout the arginine inhibition studies. Six sets of three flasks, each containing 25 ml. of basal medium and concentrations of sodium propionate varying from 2.5 mg. to 12.5 mg. per 25 ml. of medium, were inoculated with 1 ml. of a suspension of spores obtained from a

propionate slant. Growth was allowed to proceed for a period of five days. The mycelia were harvested, dried and weighed and the average weights of the triplicate samples were plotted against the concentration of sodium propionate as shown in Figure 3.

Figure 3



Experiments on Arginine Inhibition of Propionate Utilization

The amount of arginine needed to give one-half inhibition was determined by a growth study. In later experiments aimed at finding an intermediate in pyrimidine biosynthesis this amount of arginine could be used so that growth would continue, but at a highly inhibited rate, allowing intermediates prior to the partially blocked reaction to continually increase in concentration until they were able to be detected.

Eight sets of flasks in triplicate were set up containing 25 ml. of basal medium and varying amounts of arginine. After the flasks were autoclaved, 1 ml. of a sterile solution of sodium propionate was added to each flask and the mycelium harvested after five days. The results are given in Table I and shows that 0.001 mg. to 0.0005 mg. of arginine hydrochloride in 25 ml. of medium is sufficient to give one-half inhibition.

Table I

ARGININE INHIBITION OF N. CRASSA 1298, GROWTH INITIATED BY SPORES

Arg.HCl/25 ml. medium Mg.	Dry weight of mycelium Mg.
1	0
0.1	0
0.005	1.7
0.001	4.5
0.0005	5.5
0.0001	10.5
0.00001	10.5
0.0	10.5
Control (0.0005 mg. Arg. only)	0

In order to eliminate the inhibition of spore germination as a possible effect of arginine on growth of the mutant another experiment was run using a mycelial suspension as the inoculant. One mycelial pad of the mutant grown for six days on propionate medium was placed asceptically into a small, sterile, Waring Blendor containing 50 ml. of basal medium and homogenized for 40 seconds. One milliliter of this suspension was used to inoculate each of five sets of four flasks containing different concentrations of arginine in media containing both

sodium propionate and aminobutyrate. The mycelia were harvested and weighed after six days.

For comparative purposes, three sets of flasks containing sodium propionate in the basal medium were inoculated with 1 ml. of a spore suspension. The results presented in Table II show that mycelial fragments were as effective as spores for initiating growth on medium supplemented with propionate and aminobutyrate and that arginine has essentially the same effect when inoculation is by mycelial fragments as when spores are used for inoculation.

Table II

ARGININE INHIBITION OF NEUROSPORA CRASSA 1298

Substrate	Inoculant	Arginine·HCl	Weight of mycelium
		mg.	mg.
A. Sodium Propionate	Mycelial fragments	0	26
		1	0
		10	0
B. Amino butyric acid	Mycelial fragments	0	40
		1	0
		10	0
A. Sodium Propionate	Spores	0	26
		1	0
		10	0

Because it was not clear if growth in the previous experiment was actually initiated by the mycelial fragments or by the possible presence of spores in the suspension, further evidence was needed that arginine was not merely an inhibitor of germination. For this purpose experiments

on the effect of arginine on the growth of intact mycelia were performed. Eight sets of four flasks containing basal medium supplemented with sodium propionate were inoculated with a spore suspension and allowed to grow for four days, at which time a moderately heavy mycelium had developed. Varying amounts of arginine were added and growth was allowed to continue for two days more before harvesting. The results are given in Table III. This table shows that arginine inhibited growth of the intact mycelium, but a much greater amount was needed for this effect than was needed to inhibit growth from spores or mycelial fragments.

Table III

ARGININE INHIBITION OF INTACT MYCELIUM

Arg.HCl/25 ml. medium mg.	Dry weight of mycelium mg.
0	42.3
1	35.8
0.01	42.9
0.0001	41.7
Control (Wt. of mycelium at start)	25.7

In another experiment the effect of arginine at various stages of growth was studied. This time eight sets of four flasks containing basal medium supplemented with propionate were inoculated with spores. One milligram of L-arginine hydrochloride was added to a different set each day after inoculation. The mycelia from all flasks were harvested at the end of seven days.

An experiment similar to the one just described was run on medium supplemented with 10 mg. of DL-α-aminobutyric acid. In this case 0.01 mg. of arginine hydrochloride was added and the mycelia harvested after

four days. The results of these two experiments are presented in Table IV. Here we see that arginine inhibited growth at all stages on medium supplemented with propionate and apparently also on medium supplemented with aminobutyrate, although the evidence is not as clear.

Table IV

ARGININE INHIBITION OF N. CRASSA 1298 AT DIFFERENT STAGES OF GROWTH

Substrate	Time of addition 1 mg. Arginine.HCl days after inoculation	Dry weight of mycelium mg.
A. Sodium Propionate	1	0
	2	0
	3	Trace
	4	0.5
	5	1.1
	5 1/2	4.8
	6	8.5
Control (No arginine added)		20.8
B. α-amino-butyric acid	0	0
	1	1
	3	32.1
	4	38
Control (No arginine added)		38

Possible Alteration of the Mutant

From time to time during these growth studies, the unsupplemented medium would show heavy growth. This was assumed to be the result of contamination of the culture slant used, perhaps by the wild-type strain, and so the experiment was discarded as being invalid. Later however, the thought occurred that the growth might have been the result of alteration of the mutant allowing it to synthesize pyrimidines from

simple constituents in a more efficient manner. The result of one experiment in which growth appeared on the blanks is given in Table V. It is seen that sodium propionate slightly inhibited growth of this organism and that arginine plus sodium propionate completely inhibited the mold when present at the start of growth.

Table V

EFFECT OF ARGININE ON "ALTERED" N. CRASSA 1298

Supplement	Time of arg. addition	Dry weight of mycelium*
	days after inoculation	mg.
6 mg. Na prop.	1	0
6 mg. Na prop.	2	Trace
6 mg. Na prop.	3	4.3
6 mg. Na prop.	4	7.2
6 mg. Na prop.	4.5	32.1
6 mg. Na prop.	5	35.0
6 mg. Na prop.	No arginine added	37.6
None	No arginine added	47.4

*The mold was harvested after six days total growth.

To see if the wild strain would behave in a similar fashion, flasks containing sodium propionate and arginine, separately and in combination, were inoculated with spores of the wild-type strain. Sodium propionate had no effect at all at the concentration used (6 mg./25 ml.) and arginine at a 1 mg. per 25 ml. concentration had a slightly stimulatory rather than inhibitory effect.

Experiments On The Arginine Effect With Unlabeled Propionate

Materials

The compounds used were the same as those described for the growth studies. The organism was again mutant 1298 of Neurospora crassa. The solvents and color reagents used for chromatography were those described by Block, et al. (24) in a laboratory manual of paper chromatography.

Chromatographic Procedures and Results

In various experiments thirty or more flasks containing basal medium supplemented with sodium propionate were inoculated with 1 ml. of a suspension of spores of the mutant and allowed to grow until mycelia developed. At this time each of twelve or more mycelia of approximately equal size was lifted from its flask, rinsed in three successive beakers of sterile, distilled water and finally placed in sterile Petri dishes containing 10 ml. of an aqueous propionate solution and 1 mg. of arginine hydrochloride. As a control, twelve or more additional mycelia of equivalent size were transferred in the same manner into Petri dishes containing propionate only. Aseptic techniques were followed during the complete transfer procedures.

The mycelia were allowed to remain in the aqueous solutions at room temperature for periods of from five to ten hours. At the end of the incubation period the contents of the dishes containing propionate and arginine were filtered collectively by suction through a sintered filter, rinsed with water and the mycelial pad placed in acetone for 30 minutes and then dried in a desiccator.

To remove the propionate the filtered solution was first acidified and then extracted once with one-half its volume of ethyl ether and twice more with one-quarter quantities. The extracts were combined. The aqueous solutions and the ether extracts were both evaporated nearly to dryness on a flash evaporator and taken up in a small amount of water.

The mycelia were allowed to dry for at least a day and then ground to a fine powder in a mortar with 120 mesh carborundum. The powder was placed in a 12 ml. centrifuge tube and 5 ml. of ice-cold, 10 % trichloroacetic acid was added. The solution was kept cold in a refrigerator for thirty minutes with occasional stirring. The tube was then centrifuged and the supernatant solution collected. The residue was extracted twice more with 5 ml. of the trichloroacetic acid solution for periods of five minutes each. The extracts were combined and extracted three times with 5 ml. of ether to remove the acid and then were evaporated to dryness and taken up in 3 ml. of water.

The mycelia and the solutions of the controls, for which the incubation solutions originally contained only propionate, were treated simultaneously in the same manner as the arginine-inhibited samples described earlier.

Paper chromatographs of the aqueous incubation solutions, the ether extracts of these solutions and the trichloroacetic acid extracts of the mycelia were prepared for the arginine-inhibited samples and the controls to determine if any differences existed between them which might be the result of an increase in the concentration of an intermediate compound in the biosynthetic route of pyrimidine formation. One-dimensional chromatograms were first prepared for screening the different solvents and color reagents to be used.

The prepared solutions were applied to 18 inch long strips of Whatman No. 1 filter paper in dropwise fashion, drying between drops with a hair-dryer until the desired volume had been spotted. Volumes of 25-100 µl. were applied with micropipettes attached to a 1 cc. syringe. The spotted papers were then developed in the solvent by a descending technique in which the entire system was contained in a 12 X 24 inch battery jar covered with a glass plate. After developing, the chromatograms were allowed to dry in a hood and then examined for various types of compounds.

If any differences were noted on the one-dimensional chromatograms or if better resolution of the components observed was desired a two-dimensional chromatogram was prepared. In all cases the solvents used were phenol-water in a ratio of four to one and n-butanol-acetic acid-water in a ratio of four to one to five. The order of solvents was changed after several runs so that the phenol-water was the last solvent making the front easier to see in the chromatographic cabinet.

Sheets of Whatman No. 1 filter paper, 18 1/4 X 22 1/2 inches were spotted near one corner in the same way as used for the one-dimensional chromatograms. Development of the sheets took place by a descending technique in closed chromatocabs in which the paper and atmosphere had been allowed to equilibrate with the solvent vapors for 30 minutes before the solvent was added to the trays holding the paper. The developed chromatograms were thoroughly dried and examined in the same manner as the one-dimensional chromatograms.

Both types of chromatograms were sprayed with the reagents necessary to produce colored spots with certain types of compounds. The

reagents used are listed below.

The Sakaguchi spray for the guanidine group.

Ammonical silver nitrate for reducing sugars.

p-Dimethylaminobenzaldehyde for indoles and arylamines.

Aniline-phthalic acid for simple sugars.

o-Phenylenediamine for α-keto acids.

Diazosulfanilic acid for imidazoles.

Ninhydrin for amino acids.

In addition the chromatograms were examined under ultra-violet light for the presence of any absorbing or flourescent compounds.

Two different solvents were used to develop the one-dimensional chromatograms. Ethanol-ammonia-water (95:1:5) was used because of its usefulness in the chromatography of sugar phosphates. However, when chromatograms developed in this solvent were sprayed with the various color reagents, the only color produced was at the origin. This was true even when a molybdate spray for phosphates was used. Therefore, no results were obtained with this solvent.

The other solvent used, n-butanol-acetic acid-water (4:1:1), is a general solvent capable of resolving almost any type of compound to some extent. The results which follow were observed with this as the developing solvent.

Nearly all of the reagents produced colored spots or streaks with the aqueous incubation solutions but these were impossible to interpret without better resolution. The α-keto acid spray gave spots of about equal size on chromatograms of both the arginine-inhibited sample and the control at a Rf value of .28. Since the spots were of equal size

no further characterization of it was attempted. The ether extracts of the aqueous solutions gave no color with any of these sprays nor did they show any spots under ultra-violet light. The chromatograms of the trichloroacetic acid extracts gave colored streaks when sprayed with ninhydrin, Sakaguchi, diazosulfanilic acid and the p-dimethylaminobenzaldehyde sprays and also fluoresced at the origin under ultra-violet light.

Two-dimensional chromatograms were then prepared of the aqueous solutions and the trichloroacetic acid extracts of the mycelium. The aniline-phthalic acid spray showed two spots which were more intense on chromatograms of the aqueous incubation solution containing arginine than on chromatograms of the control, however, those turned out to be due to fructose and glucose present as the result of inadequate rinsing of the mycelia during the transfer from the medium to the incubation solutions. In addition, a bromophenol blue reagent for detecting acids and a molybdic acid reagent for detecting phosphates were sprayed on the two-dimensional chromatograms but again the spots produced were of equal size and intensity and so no further investigations were made along these lines. The other reagents which produced colored spots or streaks on the one-dimensional chromatograms were also sprayed on the two-dimensional chromatograms, but with the exception of ninhydrin, either no spots appeared or those spots which did appear were of equal size on both the control and sample chromatograms.

Many spots appeared when the chromatograms of both the acid extracts of the mycelium and the incubation solutions were sprayed with ninhydrin. The largest spot which appeared was one with Rf values corresponding to known values for alanine. Another prominent spot was

in the area expected for the dicarboxylic amino acids. The "alanine" spot was particularly interesting because of its relation to the proposed pathway of pyrimidine biosynthesis and also because of the relatively large amount present.

In order to determine if the spot assumed to be alanine was due to the α-or β-isomer, the area of the spot was cut out of an unsprayed chromatogram and eluted with water. The resulting solution was rechromatographed on one-dimensional strips and again sprayed with ninhydrin. The results as given in Table VI indicate the compound to be α-alanine.

Table VI

IDENTIFICATION OF A NINHYDRIN-POSITIVE COMPOUND

Compound	Color with Ninhydrin	Rf in BuOH-HAc-H_2O	Rf in Phenol-H_2O
Unknown	pink	0.28	0.58
α-alanine	pink	0.29	0.59
β-alanine	blue-pink	0.33	0.60
α-aminobutyric acid	pink	0.40	0.69

Alanine was by far the largest amino acid present in the incubation solution but the amounts were the same in both the arginine-inhibited sample and the control. It was also a major component of the trichloroacetic acid extract of the medium, but of a concentration about equal to the concentration of the dicarboxylic amino acid present.

In one case a ninhydrin-positive spot having an Rf value of 0.42 in butanol-acetic acid-water and 0.07 in phenol-water appeared on chromatograms of the incubation solution containing arginine and not on chromatograms of the control. However, when an attempt to duplicate

the results was made, the spot did not appear. It was tentatively concluded to be due to the presence of ornithine, which has similar Rf values, resulting from arginine degradation.

Experiments on the Arginine Effect with Sodium Propionate-2-C-^{14}

Materials

The sodium propionate-2-C^{14} was obtained from the Volk Radiochemicals Company having a stated activity of 0.5 millicuries per 21.5 mg. The other compounds used were the same as those used in the previous experiments. The X-ray film was from the Eastman Kodak Company.

Paper Chromatography

Two mycelial pads growing on medium supplemented with sodium propionate were transferred aseptically with rinsing to two sterile Petri dishes containing 10 ml. of a solution of 2.6 mg. of sodium propionate-2-C^{14} and unlabeled sodium propionate so that the total activity of the solution was about 0.01 mc. To one Petri dish was also added 1 mg. of L-arginine hydrochloride. The pads were incubated for four hours and then filtered by suction directly into 50 ml. beakers by use of a small bell jar. The mycelia were rinsed and placed in 25 ml. of acetone for one hour, filtered and placed in a desiccator to dry.

The total volume of the medium and washings was about 15 ml. in both cases. This was divided into equal parts. One part was first acidified to pH 1 with 1N. hydrochloric acid, extracted three times

with 5 ml. of ether and finally evaporated to dryness and taken up with 1 ml. of water.

One milliliter of the other half of the media was poured into a 5 ml. beaker, covered and placed in the refrigerator. The remaining portion was evaporated to dryness with a stream of dry air, and taken up with 1 ml. of water.

The acid extracts of the mycelia were prepared in the same manner as in the previous experiment. In an attempt to recover any volatile constituents that might be present in the incubation solution, the untreated sample and control and the ether-extracts were applied to one-dimensional chromatograms in 50 µl and 100 µl quantities and immediately placed in the chromatography jars before the paper was dry. The papers were developed in phenol-water (4:1), then cut into 1 1/2 inch-wide strips and the radioactivity counted on a Forro chromatograph scanner coupled to a Nuclear-Chicago Model 1620 A ratemeter and an Easterline-Angus graphic recorder. It was necessary to dry the chromatograms before counting so that any volatile compounds were possibly lost in spite of the original precautions.

Strip chromatograms were also prepared from the ether-extracted incubation solutions and the trichloroacetic acid extracts and a graphic record made of the radioactive spots present.

Two-dimensional chromatograms were prepared of the incubation solutions and of the acid extracts of the mycelia. The papers were again developed in phenol-water and butanol-acetic acid-water and sprayed with ninhydrin. The ninhydrin-positive spots for the arginine-inhibited sample and the control were again equivalent, but a few of

these spots were counted to determine the amount of activity being incorporated into the mold components. A Geiger tube attached to a Nuclear-Chicago Model 190 ultrascaler was used for counting the spots directly from the paper. The results are given in Table VII.

Table VII

ACTIVITY OF SOME NINHYDRIN-POSITIVE COMPONENTS OF N. CRASSA 1298 INCUBATED WITH SODIUM PROPIONATE-2-C^{14}

Rf value of spot		Probable Component	Activity in counts /min.	
Phenol-H_2O	BuOH-HAc H_2O		sample	control
Trichloroacetic acid extract of mycelium				
.59	.23	alanine	130	156
.26	.17	(dicarboxylic amino acid)	154	173
Origin		--	346	345
Background		--	106	106
Medium after ether extraction				
.59	.23	alanine	172	186
.79	182	leucine, isoleucine	123	122
Background		--	104	104
.67	.13	arginine	108	---

The strip chromatograms showed high activity at the origin and at a few other areas, but no significant differences were seen between the controls and the samples inhibited with arginine. The activity present was for the most part of a very low order, especially with the acid extracts of the mycelia.

Because of the high amount of activity at the origin, another solvent frequently used to chromatograph sugar phosphates was used in an attempt to find a radioactive, phosphorylated intermediate. For this purpose one-dimensional strip chromatograms were developed in methanol-

formic acid-water (80:15:5 v/v) and the activity determined as before. again a very low order of activity was recorded except at the origin and no positive conclusions could be drawn from the record produced.

Radioautography

Chromatograms of the trichloroacetic acid extracts of the mycelia were placed against X-ray film and placed in Kodak X-ray film holders in the dark. Exposure was allowed to proceed for a period of 10 weeks. At the end of this period the films were developed in the darkroom of the Food Science Laboratory, Michigan State University.

Radioautographs were also made of the samples incubated for 10 hours in radioactive sodium propionate-plus-L-arginine hydrochloride (0.001 mg.) and of the control containing only the labeled propionate. The longer incubation period was used to increase the activity of the mold constituents and the smaller amount of arginine was used to prevent smearing of the chromatograms and also as an attempt to get only partial inhibition. The incubation solutions of the sample and the control were acidified, ether extracted and concentrated as before and spotted on the chromatograms.

A new solvent was used since several chromatographic charts of plant constituents chromatographed in n-butanol-propionic acid-water and phenol-water were kindly furnished by Dr. Kieth Richardson of the Agricultural Chemistry Department, Michigan State University.

The new solvent was prepared by mixing 1246 ml. of n-butanol with 84 ml. of water as solution "A". Next a solution, "B", was made by mixing 620 ml. of propionic acid with 790 ml. water. Equal amounts of "A"

and "B" were stirred together until only one phase remained. More of solution "B" was added before the two phases merged.

The developed chromatograms were trimmed to fit a 14 X 17 inch sheet of Kodak X-ray film and placed together with the film in film-holders. Exposure was for five weeks. The films of the sample and the control were then developed and compared.

At first glance, no significant difference was noted, however, on closer inspection a large spot having an R-alanine value of approximately 1.5 in phenol-water, 1.8 in butanol-propionic acid-water and 2.8 in butanol-acetic acid water was observed to consist of two contiguous spots on the radioautograph of the incubation solution inhibited by arginine and not on the radioautograph of the control. The complete areas were cut out of both chromatograms, eluted with water and rechromatographed on 1 1/2 inch strips of Whatman No. 1 filter paper using phenol-water as the solvent. The strips were counted with the Forro strip scanner and showed two distinct peaks with the strip of the arginine-inhibited sample and only one peak with the control strip. The compounds have not been identified as yet. The radioautographs of the trichloroacetic acid extracts did not show the same large spot just mentioned, but did show many other spots in common with the incubation solutions including a spot for alanine. However, no significant difference was seen between the sample and the control.

The nature of the significant component is not known since it was never seen when the chromatograms were sprayed with the various color-producing reagents. These reagents are relatively insensitive compared with the radioautograph process, so the fact that no colored spot was observed with these reagents does not definitely mean that the specific type of compounds tested for was absent.

DISCUSSION

To determine the practicality of the use of arginine as a tool for the isolation of an intermediate in the biosynthetic route from propionate to pyrimidines in N. crassa 1298, the nature of its inhibitory action was investigated. The possibility that the effect of arginine was due solely to the inhibition of spore germination was studied since in all previous observations of this inhibitory effect arginine had been present in the medium at the time of inoculation with spores. The results showed that arginine had the same inhibitory effect when growth of the mutant was initiated from mycelial suspensions as when spores were used as the inoculant. This does not provide conclusive evidence that spore germination was not inhibited since the lag phase and growth response observed with both types of inoculation were the same, indicating that growth might actually have started from spores present in the mycelial suspension rather than from the fragments of mycelia themselves. However, the fact that arginine definitely inhibited growth of intact mycelia as shown in Table III shows that inhibition is not solely an effect on sporulation nor an inhibition of adaptation at the spore stage.

The results of the growth studies show that much larger concentrations of arginine are needed for inhibition of growing, intact mycelia than are needed to inhibit initial growth. Probably the most plausable explanation of this would be that the larger intact mycelium has the enzyme systems available for destroying the arginine or of otherwise removing it from the medium through synthesis of protein and other mycelial components. This explanation would in turn help to explain

the presence of the ninhydrin-positive spot assumed to be ornithine found on chromatograms of the incubation solution containing arginine.

Another possible action of arginine was indicated by the work of others (25) investigating the inhibitory action of arginine toward certain histidineless mutants of N. crassa. In this case arginine, in combination with other amino acids, blocks the transport of histidine across the mycelial membrane. Evidence that this type of action is not the reason for the inhibition of N. crassa 1298 by arginine is given in Table VII. The activity of the amino acids extracted with trichloroacetic from the mycelium of the mold incubated with labeled propionate in the presence of arginine demonstrates clearly that the propionate was being metabolized. Also, the approximately equal density of the major spots seen on radioautographs of the trichloroacetic acid extract of mycelium incubated with labeled propionate in the presence of arginine and in the absence of arginine shows that propionate is metabolized to about the same extent in both cases. Thus the assumption that the inhibition by arginine is due to the blocking of a specific, vital, metabolic reaction occurring in the mutant organism seems valid.

Further investigation of the nature of the inhibition of the growth of the mutant by arginine leads to a consideration of the growth characteristics of the mutant on different types of media. The mutant grows very rapidly on uridine (14), closely paralleling growth of the wild strain on basal medium. Growth of the mutant on aminobutyrate and propionate shows a lag period of several days followed by a shorter period of rapid growth. The lag period is shortened by one to two days by inoculation with spores from an agar slant containing aminobutyrate as

opposed to uracil. These observations led Fairley (28) to conclude that the new pathway which exists in the mutant grown on propionate or aminobutyrate is of an adaptive nature and that the process of adaptation is stimulated by the presence of increased concentrations of these simple compounds. This conclusion is further supported by the fact that arginine has no effect on the growth of the mutant on medium containing uridine or of the wild strain on basal medium. The interesting observation in the present study of the growth of the mutant on basal medium may be of importance in confirming the adaptive nature of this organism. It was very clearly shown that the organism growing in the blanks was not the wild-type strain since arginine inhibited its growth in the same manner as it inhibited the mutant when present in the medium supplemented with propionate. Propionate also produced slight inhibition of this new organism while neither arginine nor propionate had any inhibitory effect toward growth of the wild-type strain. Therefore, it seems logical to assume that this new organism is the same mutant 1298 of N. crassa, but has undergone further alteration so that it is now able to utilize the simple carbon sources of the medium, sucrose and tartrate, for the synthesis of pyrimidines.

After a study of all these observations the conclusion was reached that N. crassa 1298, because of a genetic block, is no longer able to synthesize pyrimidines in the manner utilized by the wild-type strain, but, through adaptation, is able to synthesize pyrimidines by an alternate route from simple compounds such as propionate. Furthermore, the inhibitory action of arginine is caused by the blocking of a specific reaction in this biosynthetic sequence, and as such, may be properly utilized in the present study to increase the concentration of the intermediate compounds, which

occur prior to the inhibited reaction, in an attempt to gain more information about this new biosynthetic route.

Many attempts were made to find a compound present in the mold inhibited by arginine which was not present in the control by varying such conditions as the concentration of arginine or the time of incubation with arginine. The concentrations of any intermediates under these conditions evidently did not increase enough to be detected by the various reagents employed or the nature of these intermediates was quite different from what was expected. The pathway suggested by Boyd (17) and presented on page five of the Introduction was used as a guide to the nature of these possible intermediates. And, although no direct evidence was available that arginine acted as a competitive inhibitor, it seemed very likely because of its specific nature. If this was the case, arginine probably would structurally resemble the intermediate immediately prior to the blocked reaction. Therefore the most reasonable compound fitting these specifications would be a derivative of β-ureidopropionate. Another possibility would be that the arginine competes for the enzyme with the carbamyl donor which supposedly combines with the β-alanyl-CoA derivative. Because of the high acidity attained by the samples during the extractions and chromatography, hydrolysis of glycosidic, thioester and other similar bonds was likely and so all combinations of the basic compounds and their derivatives were looked for as possible spots on chromatograms. No success was achieved until radioautographs of the incubation solutions were developed. Many dark spots, corresponding to radioactive compounds on the chromatograms, were found on the radioautographs. However, only one

distinct difference was noted between the radioautograph of the incubation solution which contained arginine in addition to propionate as compared with the radioautograph of the solution originally containing only propionate. This spot was a close neighbor of another major spot found on both radioautographs and might conceivably have been due to a streak of that compound. However, rechromatography showed that the spot was indeed a distinct compound. Having definitely established the presence of another compound by rechromatography there is still some slight doubt as to its significance because of, among other things, the small amount of arginine used. However, it is hard to imagine any other explanation for the presence of the spot except that it is an intermediate in propionate utilization since aseptic techniques were carefully employed and no contamination was evident when the mold was harvested. Even if some small amount of bacteria had been introduced into the incubating sample it seems unlikely that this much activity could be incorporated into just one compound without producing other compounds which would be evident on the radioautographs. Further work must now be done to identify the compound and to relate it to the new route of pyrimidine biosynthesis.

An interesting sidelight to this problem was the finding of large amounts of alanine which the mold excreted into the propionate solutions during incubation periods. This proved to be the major radioactive compound present in the incubation solutions, but not in the trichloroacetic acid extracts of the mycelia. This would seem to indicate that the mutant is capable of converting propionate very quickly into alanine outside the mycelium. When paper chromatography was used to identify

alanine it indicated the compound to be α-alanine, however, when co-chromatography was employed in which the unknown was spotted together with α-and β-alanine, the mixture moved together in both cases, even in two dimensions. So it is not known with certainity which isomer is present or if it is a mixture of the two, but the experimental data favors α-alanine.

The conversion of propionate to β-alanine through the coenzyme A derivatives of propionic acid and acrylic acid has already been demonstrated in some microorganisms (26) and has been postulated as being part of the biosynthetic pathway under study in this thesis. However, β-alanine did not support growth of this mutant, so that if the compound was β-alanine, its presence in the medium would not seem to be of significance as a utilizable pyrimidine precursor.

If the alanine is indeed the α-isomer as indicated or a mixture of the two isomers, then it is interesting to speculate on the mechanism of its production from propionate. Alanine is known to arise from pyruvic acid by a transamination reaction or directly by the addition of ammonia by the enzyme "L-alanine dehydrogenase" (27). In support of this mechanism was the presence of a spot on the radioautographs with Rf values corresponding to those of pyruvic acid.

The extremely rapid conversion to alanine and the possibility of the presence of both isomers makes one consider the possibility of a dehydrogenation of propionate and then immediate recombination with ammonia, either specifically or randomly to form equal amounts of both isomers. No other evidence to support this possibility is available and so the conversion to pyruvate and subsequent transamination seems to be the most plausible mechanism.

Labeling was also found very high in an amino acid thought to be either glutamic or aspartic acid. This spot appeared to be the densest of all the spots on the radioautograph of the trichloroacetic acid extract of the mold mycelium. The presence of both of these amino acids would be expected through operation of the citric acid cycle. The high amount of labeling of this amino acid in the trichloroacetic acid extracts as compared to alanine may be significant when more is known about the metabolism of the mutant.

SUMMARY

1. The action of arginine as a specific inhibitor of pyrimidine biosynthesis in N. crassa 1298 was examined with a series of growth and radioactive tracer experiments.

2. The preparation of solutions of the constituents of the mold and their separation by paper chromatography are described. Samples in which the mold was inhibited by arginine were compared with samples from the uninhibited mold, but no significant differences were seen when the chromatograms were sprayed with color-producing reagents. When labeled propionate was used as the substrate subsequent radioautograms of the incubation solution showed the presence of a new compound in the arginine-inhibited sample. The compound has not yet been identified.

3. The possible significance of this new compound and of other mold components found is discussed.

BIBLIOGRAPHY

1. Tatum, E. L., Science, 129, 1711 (1959).

2. Beadle, G. W., Science, 129, 1715 (1959).

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

4. Lieberman, I., and Kornberg, A., J. Biol. Chem., 212, 909 (1955).

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

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

7. Yates, R. A., and Pardee, A. B., J. Biol. Chem., 221, 793 (1956).

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

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

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

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

12. Bergström, S., Arvidson, H., Hammersten, E., Eliasson, N. A., Reichard, P., and van Ubisch, H., J. Biol. Chem., 177, 495 (1949).

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

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

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

16. Hermann, R. L. and Fairley, J. L., J. Biol. Chem., 227, 1109 (1957).

17. Boyd, J. M., Unpublished Thesis, Mich. State Univ. (1958).

18. Fink, K., Henderson, R. B., and Fink, R. M., J. Biol. Chem., 197, 349, 441 (1952).

19. Fritzsen, P., J. Biol. Chem., 226, 223 (1957).

20. Mokrasch, L. C. and Grisolia, S., Biochim. et Biophys. Acta, 27, 226 (1958).

21. Fairley, J. L., Paper No. 102, Division of Biological Chemistry, American Chemical Society, New York City Meeting, 1954.

22. Slotnick, I. J. and Sevag, M. G., Arch. Biochem. and Biophys., 57, 491 (1955).

23. Horowitz, N. H., and Beadle, G. W., J. Biol. Chem., 150, 325 (1943).

24. Block, R. J., LeStrange, R., and Zweig, G., Paper Chromatography, A Laboratory Manual, Academic Press Inc., New York, 1952.

25. Mathieson, M. J., and Catcheside, D. G., J. Gen. Microbiol., 13, 72 (1955).

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

27. Waimee, J. M., and Pierard, A., Nature, 176, 1073 (1955).

28. Boyd, J. M., and Fairley, J. L., "Tracer Studies of Pyrimidine Biosynthesis by Neurospora Crassa 1298", Submitted for publication.