THE EFFECT OF ALPHA-AMINOBUTYRIC ACID AND PROPIONIC ACID ON THE SYNTHESIS OF PYRIMIDINES AND ARGININE IN *NEUROSPORA CRASSA*

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This is to certify that the

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James L. Fairley Jr
Major professor

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ABSTRACT

THE EFFECT OF α-AMINOBUTYRIC ACID AND PROPIONIC ACID ON THE SYNTHESIS OF PYRIMIDINES AND ARGININE IN NEUROSPORA CRASSA

By Donald Eugene Wampler

For several years Dr. Fairley and coworkers have been studying the growth of a pyrimidine-requiring mutant of Neurospora crassa. This mutant, 1298, will grow on a medium containing instead of pyrimidines any one of a number of aliphatic acids including α-aminobutyric acid and propionic acid. This thesis reports work done in an attempt to understand how propionate and α-aminobutyrate promote growth.

The first major finding was that the intracellular arginine concentration fell from about 20 μmoles per gram dry mycelia when the mold was grown on uridine to as little as 1 μmole per gram when grown in the presence of α-aminobutyrate or propionate. Evidence from other laboratories suggested that the biochemical deficiency of this and related mutants is the formation of a pyrimidine-specific supply of carbamyl phosphate. The reduction in arginine caused by propionate or α-aminobutyrate apparently derepresses the arginine-specific supply of carbamyl phosphate to the extent that excess carbamyl phosphate can spill over into the pyrimidine pathway.

On this basis, work was directed toward an explanation of how the growth-promoting compounds reduce the concentration of arginine.

Besides the change in arginine concentration, a change in the concentration of a number of other amino acids was found. These changes are compatible with the idea that arginine production is blocked in the formation of argininosuccinate. Crude extracts of wild type N. crassa displayed about 2.5 units of argininosuccinate synthetase activity per mg of protein. The enzyme was purified approximately 15-fold by treatment with protamine, ammonium sulfate fractionation, and chromatography on DEAE cellulose. Argininosuccinate synthetase was strongly inhibited by inorganic pyrophosphate, L-arginine, AMP, ADP, and ATP. L-Valine, L-α-amino-n-butyric acid, propionate, and L-threonine were also found to be relatively effective inhibitors of the enzyme.

At least part of the reduction in intracellular arginine when cells are grown on propionate or α-aminobutyrate can therefore be explained as a result of the direct inhibition of argininosuccinate synthetase by these growth-promoting compounds. These compounds may also limit argininosuccinate synthetase activity indirectly by increasing the concentration of valine, which also inhibits the reaction. This explanation for the growth of 1298 on propionate and α-aminobutyrate does not exclude the possibility that other metabolic changes may contribute to the reduction in arginine concentration.

The inhibition of argininosuccinate synthetase by arginine found in this work is the first demonstration of end product inhibition in the pathway of arginine synthesis in Neurospora.

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By

Donald Eugene Wampler

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Finally, I would like to express appreciation to the National Institutes of Health for financial assistance during this project.

D.E.W.

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INTRODUCTION

Among the more than 380 Neurospora mutants isolated by Beadle and Tatum (1), some 45 required pyrimidines for growth. On the basis of complementation data, Houlahan and Mitchell (2) arranged a number of these mutants into 3 linkage groups: pyr-1, pyr-2, and pyr-3. All pyr-3 mutants require pyrimidines when grown at 35°, but when grown at 25°, they fall into three phenotypic classes (3): a, b, and c. At 25°, pyr-3a requires the normal supplement of pyrimidines, pyr-3b grows on basal medium, and pyr-3c requires about 1/6 the normal pyrimidine supplement (see Table I).

Table I. Differences Among the Pyr-3 Mutants

Mutant	Number	Growth on αABA (4)	Suppressibility (3,9)	ATC (9,11)	RNA*(2) Requirement
3a	37301	+	+	+	3.3
	1298	+		+	+
3b	37815	+	+	+	0
3c	67602		+		0.38
3d	45502 and several others	0	0	0	3.3

* mg hydrolyzed ribonucleic acid required for one-half wild growth at 25°.

Abbreviations: ATC - Aspartic transcarbamylase
αABA - α-Aminobutyric acid.

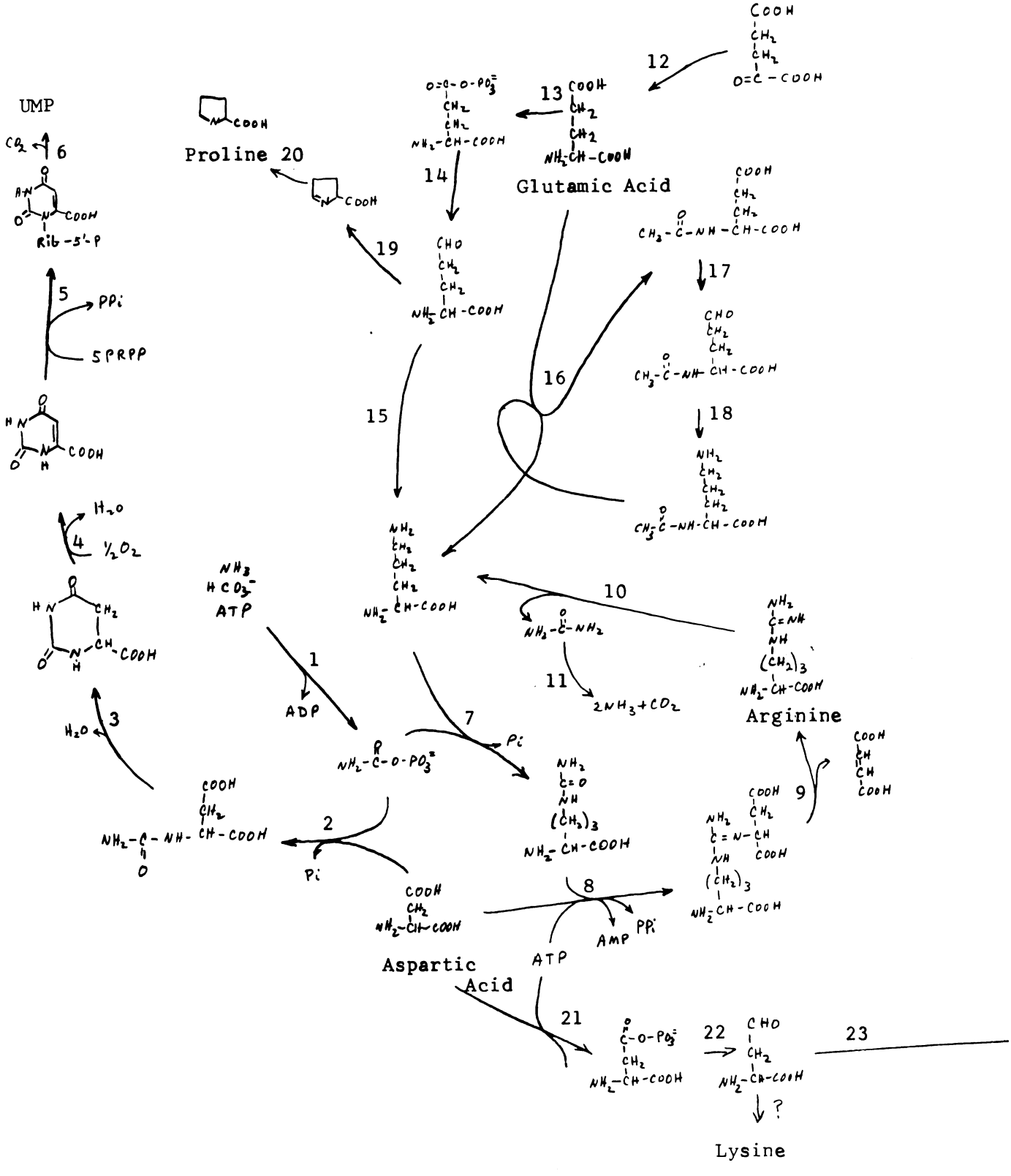
While Houlahan and Mitchell were working on the characteristics of these mutants, one strain of pyr-3a began to grow as if a back mutation had occurred (2). A cross between this apparent reversion and the wild type produced some asci which contained the expected distribution of

mutant and wild spores but also another type of ascus which could not be explained on the basis of a back mutation. This unexpected ascus could be explained, however, if a second, independent mutation had occurred which suppressed the pyr-3a mutation. Further study showed that a second mutation had, indeed occurred. The presence of this suppressor mutation, s, renders the otherwise pyrimidine-deficient mutants phenotypically wild-type and, therefore, the gene can be detected only in conjunction with the mutation which it suppresses. The suppressor phenomenon led to the identification of a fourth member of this allelic group, pyr-3d. Pyr-3d has the same nutritional requirements as pyr-3a but is not suppressible.

There are also two nutritional situations which, like the suppressor mutation, allow pyr-3a mutants to grow in the absence of pyrimidines. Fairley et al. (4,5) have shown that 1298 will grow on basal medium supplemented with any one of several aliphatic acids. These compounds, including α-aminobutyrate, propionate, and threonine, will also support growth of another pyr-3a mutant, 37301, and the pyr-3b mutant, but not the pyr-1 or pyr-2 mutants. Recently, Charles (6,7) has found that strain 1298 can be made to grow on basal medium by increasing the carbon dioxide concentration.

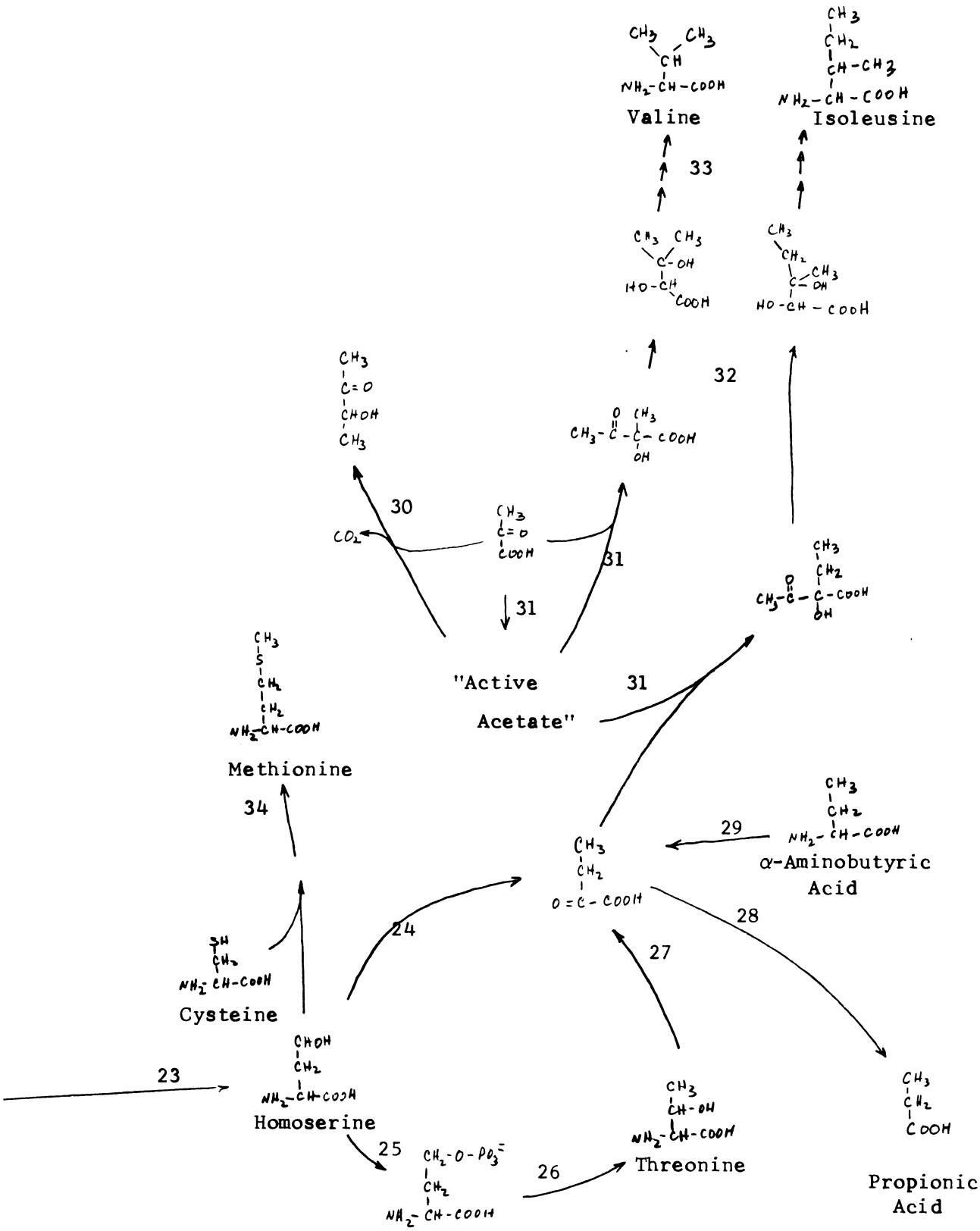
Davis made the first attempts to explain the pyrimidine mutation in terms of enzymatic activity. He found (8) that pyr-1 and pyr-2 are blocked between carbamyl aspartate and uridylic acid (reactions 3, 4, 5, and 6 in Figure 1). Even though the pyr-3 mutants will not grow when carbamyl aspartate is added to the medium, they were shown to contain all the enzymes required for the conversion of carbamyl aspartate

Figure 1. Metabolic Pathways Involved in Pyrimidine and Arginine Synthesis in Neurospora crassa



Legend given on Page 5.

Fig. 1 (Continued)



Footnotes for Figure 1:

Reaction	E. C. No.	Mutant	Ref.	Comments
1	2.7.2.2	pyr-3a*,arg-3	12,41	2 enzymes in N. crassa
2	2.1.3.2	pyr-3d	9,43	same as 2.7.2.2 in N. crassa
3	3.5.2.3	pyr-1*	8	
4	1.3.3.1			
5	2.4.2.10	pyr-2	8	
6		pyr-4	8	
7	2.1.3.3	arg-12	13	
8	6.3.4.5	arg-1	25	
9	4.3.2.1	arg-10	25	
10	3.5.3.1			
11	3.5.1.5			
12	1.4.1.3-4	prol-2 or 3*		
13	2.6.1.1-8	prol-3 or 2*		
14				
15	2.6.1.13			
16		arg-4		
17				
18		arg-6*		
19			12	nonenzymatic
20		prol-1		
21	2.7.2.4		44	3 enzymes in E. coli.
22				
23	1.1.1.3			
24	4.2.1.15			
25	2.7.1.39			
26	4.2.99.2			
27	4.2.1.16		27	inhibited by isoleucine, released by α-aminobutyrate
28				
29				
30				
31		group 4	28,45	stimulated by growth on α-ketobutyrate, threonine, and isoleucine.
32		group I	45	
33		group 2 and 3*	45	
34				

*The enzymes affected by these mutations have not been established.

to pyrimidine nucleotides. Davis, therefore, assumed that the pyr-3 mutants are blocked in or before carbamyl aspartate formation. Pyr-3 mutants are not blocked in aspartic acid formation since they do not require this amino acid. Neither do they seem to be deficient in carbamyl phosphate formation since they do not require arginine. The only enzyme left, then, is aspartic transcarbamylase.

In 1960, Davis showed that the pyr-3d mutants lack aspartic transcarbamylase activity (9) while the pyr-3a and pyr-3b mutants have normal activity. The enzymatic data complement the suppressor data exactly -- those mutants which are suppressible have aspartic transcarbamylase; those mutants which are not suppressible do not have aspartic transcarbamylase. So, the pyr-3a mutants presented a dilemma. Although they seemed to have all of the enzymes necessary for pyrimidine synthesis, they nevertheless required pyrimidines.

Fairley and Adams and Davis simultaneously reported two related observations which provided one of the first clues to solving this dilemma. Fairley and Adams reported (10) that growth of 1298 on α-aminobutyrate, like growth of the suppressed mutant, was strongly inhibited by arginine. Davis reported (11) that the suppressor gene caused a reduction in the intracellular concentration of arginine. In both cases arginine antagonized growth. Since normal cellular concentrations of arginine apparently inhibited pyrimidine formation, Davis proposed that some step in pyrimidine synthesis was abnormally sensitive to arginine.

Shortly thereafter, Davis discovered (12) that suppressed mutants exhibit low ornithine transcarbamylase activity. This production of

an altered, less active form of ornithine transcarbamylase finally allowed the suppressor to be identified independent of the pyrimidine requirement. Further work by Davis and Thwaites (13) established that s is the structural locus for ornithine transcarbamylase.

Work with the suppressor gene, and other data to be discussed later, led to an explanation of the pyr-3a mutation originally put forth by Davis (12,14), and later by Charles (7). This explanation postulates two sources of active carbamyl groups in N. crassa; one specific for arginine production and the other for pyrimidine production. The pyr-3a mutants presumably lack the pyrimidine-specific enzyme. Conditions such as the suppressor mutation which limit arginine production derepress the arginine-specific enzyme to the extent that the extra carbamyl phosphate can be used in pyrimidine synthesis.

The problem of pyrimidine synthesis in pyr-3a mutants thus became a problem in the control of arginine synthesis. This thesis reports studies on the effect of propionate and aminobutyrate on the synthesis of arginine and pyrimidines.

EXPERIMENTAL PROCEDURES

Materials

Neurospora crassa strains used in this work were the wild type, 1A, and a pyr-3a mutant, 1298. These strains are among those in the collection at Dartmouth College. All reagents were obtained from commercial sources.

Growth of Organisms

Organisms were grown in liquid shake culture in 2500-ml Fernbach flasks on a Eberbach reciprocal shaker at 86 strokes per minute. Mycelia used in studies of arginine concentration were grown at room temperature in flasks containing 500 ml of modified Fries medium (15). Mycelia used for enzymatic studies were grown for 22 hours from conidial inocula at 32 - 35° in flasks containing 750 ml Vogel's N medium (16) and 15 g sucrose. In addition, supplemented medium contained 80 mg uracil or 200 mg D,L-α-aminobutyric acid or 200 mg sodium propionate per liter, unless otherwise indicated.

Preparation of Acetone Powders

Mycelia were harvested by filtering with suction over 4 layers of cheesecloth and washing several times with distilled water. The moist pads were torn into chunks and homogenized for about 30 sec. in a Servall Omnimixer containing acetone which had been dried over Na_2SO_4. For free arginine assays and preliminary enzymatic studies, the acetone was at room temperature. For later studies on argininosuccinate synthetase, the acetone was kept at -20° in a dry ice-acetone bath. The homogenized mixture was filtered with suction over Whatman No. 1

filter paper and washed several times with dry, room-temperature acetone.

In those cases when the mycelial cake was not white at this stage or when the initial homogenization was performed at -20°, the cakes were again homogenized in dry, room temperature acetone, filtered, and washed several times with dry acetone. The final white cake was broken up and allowed to air dry at room temperature. The resulting powder was white and very powdery. It could be stored at room temperature for a month or so without change in arginine concentration. Acetone powders used in enzyme studies were stored at 2 - 5° and were always used within a week after harvesting with no noticeable change in enzymatic properties.

Assay for Free Intracellular Arginine

With the use of a Tenbroeck glass tissue grinder, 50 mg of acetone powder was extracted into 5 ml of cold 10 percent trichloracetic acid (TCA). The mixture was centrifuged in a clinical centrifuge and the supernate saved. The residue was resuspended in 3 ml of TCA which had been used to rinse out the tissue grinder and the mixture was again centrifuged. The combined supernates were centrifuged for 10 min. at 4,400 times gravity. This final supernate was poured through a small wad of glass wool and followed by 2 ml of TCA. The resulting solution was adjusted to pH 8.0 (as determined with pH paper) with 5N NaOH and diluted to a volume of 15 ml. Aliquots of this solution were assayed by a modified Sakaguchi procedure (17).

Assays for Amino Acid Pool Sizes

The amino acid concentrations of mycelial extracts were determined by two methods: two-dimensional paper chromatography and analysis on

a Beckman Model 120B amino acid analyzer.

For paper chromatograms, amino acids were extracted from 300 mg of acetone powder with 10 percent TCA as described above. Instead of neutralizing with NaOH, the excess TCA was removed by extracting several times with ether and the solution was then concentrated to about 1 ml with a rotary evaporator at a temperature not greater than 50°. The concentrated solution was filtered and the flask washed with 3 small volumes of 10 percent isopropanol in water.

Two dimensional descending chromatograms were run in a chromatography cabinet. For the first direction, the solvent was a 12:5:3 (v:v:v) mixture of butanol, acetic acid, and water. The second direction was run with a 4:1 (v:v) phenol-water mixture with a petri dish containing 2N NH_4OH in the bottom of the cabinet.

Samples assayed on the amino acid analyzer were extracted from growing mycelia rather than acetone powders. A 350 ml portion of the shake culture (corresponding to about 800 mg of acetone powder) was filtered through Whatman No. 1 filter paper and the mycelial pad washed with several portions of water. The moist pad was homogenized for 20 seconds in 25 ml of cold 1 percent picric acid using a Servall Omni-mixer. This suspension was further homogenized in a glass Tenbroeck tissue grinder until the mycelial fragments passed the walls freely and then was centrifuged for 2 min. in a clinical centrifuge.

Picric acid was removed on a 2.2 x 3 cm column of Dowex 1-X12, 100 - 200 mesh, in the chloride form. Amino acids were washed off of the column with 0.02 M HCl and the combined effluents were reduced to

a small volume (about 1 ml) on a rotary evaporator. The concentrated sample was filtered and the flask washed with small samples of 0.02 M HCl. The final volume of the extract was about 5 ml.

Preparation of Crude Extracts for Enzyme Assays

Extracts used in the initial survey of urea cycle enzymes were prepared by grinding 200 mg of acetone powder in 10 ml of 0.02 M tris-acetate buffer, pH 8.0, with the aid of a Tenbroeck glass homogenizer. The solids were collected by centrifugation and were resuspended in 6 ml of the same buffer. The combined extracts were diluted to two mg protein per ml.

Extracts used in later studies of argininosuccinate synthetase activity were prepared by homogenizing 2 g acetone powder in 18 ml 0.02 M tris-acetate buffer, pH 8.0, for one minute using a Servall Omnimixer. The thick paste was centrifuged and the residue resuspended in 5 ml of the same buffer. The combined extracts were run into a 2.2 x 25 cm column of Sephadex G-25, coarse grade, and eluted with 0.01 M phosphate buffer, pH 7.4. Fractions containing more than 2 mg protein per ml, as measured by the biuret reaction (18), were combined and diluted to 2 mg protein per ml.

Assays for Urea Cycle Enzymes

In all expressions of activity, one unit was defined as the amount of enzyme catalyzing the production of 1 μmole of product (or removal of one μmole of substrate) per hour under the specified conditions. All protein measurements were made by the biuret method (18). Citrulline and urea were measured by the method of Archibald (19), as modified by Gerhard and Pardee (20).

An attempt was made to measure $HC^{14}O_3^-$ incorporation into citrulline but without success. Since a successful assay for the carbamyl phosphokinase reaction has now been reported (21), the unsuccessful methods studied in this laboratory will not be described here.

Ornithine transcarbamylase activity was measured essentially as described by Davis (22). The incubation mixture contained in a volume of 2.6 ml: 20 μmoles L-ornithine; 20 μmoles carbamyl phosphate; 500 μmoles tris-acetate buffer, pH 9.0; and 0.04 ml of mycelial extract. After incubation of the mixture for 15 minutes at 28°C, the reaction was stopped with 0.5 ml of 2N perchloric acid. The precipitate was removed by centrifugation and 1.0 ml portions of the supernatant solutions were analyzed for citrulline.

Argininosuccinase was detected by the formation of argininosuccinate from arginine and fumaric acid (23). The reaction mixture contained in a volume of 1.6 ml: 80 μmoles potassium fumarate; 80 μmoles L-arginine; 100 μmoles phosphate buffer, pH 7.0; and 0.4 ml of the enzyme extract. After incubation for an hour at 28°, the reaction was stopped by boiling for 5 minutes. Coagulated protein was removed by centrifugation, and 25 ml of the supernate was spotted on Whatman No. 4 filter paper. Argininosuccinate was separated from other constituents by ascending chromatography. The liquid phase was water-saturated phenol containing 2 drops of 4N NH_4OH per 25 ml. After the chromatograms were dried, ninhydrin spray was used to locate the amino acids. No quantitative measurements were made other than visual estimation of the size of the spot corresponding to argininosuccinate.

Arginase was measured essentially as described by D. M. Greenberg (24). The reaction mixture contained in a volume of 2 ml: 50 μmoles arginine; 400 μmoles tris-acetate buffer, pH 7.4; and 1 ml of the enzyme extract which had been preincubated with manganese for 1/2 hr at 40°. The reaction was allowed to proceed for 20 min. at 28° and then stopped by adding 0.5 ml 2N perchloric acid. Denatured protein was removed by centrifugation. Arginine was removed by passing a 1 ml sample of the supernate through a 1.5 x 1.5 cm column of Dowex 50-W, 100-200 mesh, hydrogen form. The column was washed with a 2 ml and then a 1 ml portion of water. One ml of the eluate from the column was assayed for urea.

Urease was measured by the disappearance of urea. The reaction mixture contained in a volume of 2 ml: 0.5 μmoles urea; 100 μmoles phosphate buffer; and 1 ml of the enzyme extract. The mixture was incubated for 30 min. at 28° and the reaction was stopped by the addition of 1 ml of 1N perchloric acid. Denatured protein was removed by centrifugation and 1 ml of the supernate was assayed for urea.

Argininosuccinate synthetase activity was measured in two ways. In what will be called the colorimetric method, activity was followed by measuring citrulline disappearance (25). This assay was always used to measure activity in crude extracts and occasionally was used to measure activity in the purified preparation. In what will be called the spectrophotometric method, activity was measured through a series of reactions to the oxidation of NADH*(26). The AMP formed by arginino-

*Abbreviations used in the text are: AMP, ADP, and ATP, adenosine 5'-mono, di, and triphosphate, respectively; DEAE, diethylaminoethyl; NADH, reduced nicotine adenine dinucleotide; tris, tris(hydroxymethyl)-aminomethane.

succinate synthetase was converted to ADP by myokinase; the ADP then accepted the phosphate of phosphoenol pyruvate to give pyruvate and the latter was reduced by NADH to give lactate. The oxidation of NADH was followed by the decrease in absorbance at 340 mμ. Since inorganic pyrophosphate strongly inhibits argininosuccinate synthetase, inorganic pyrophosphatase was also added to the spectrophotometric assay.

For the colorimetric assay, the reaction mixture contained in a volume of 2 ml: 40 μmoles potassium L-aspartate, pH 8.0; 20 μmoles $MgSO_4$; 1.5 μmoles L-citrulline; 2.0 μmoles ATP; 20 μmoles 3-phosphoglyceric acid; 100 μmoles tris-acetate buffer, pH 8.2. Enough crude extract to contain 1 mg protein gave a convenient activity. The mixture was incubated at 37° for 10 minutes and the reaction stopped with 1 ml of 1N $HClO_4$. Three ml of water was added (total volume 6 ml), and the mixture was centrifuged to remove the denatured protein. A 1 ml sample of the supernate was then assayed for citrulline.

For the spectrophotometric assay, the reaction mixture contained in a volume of 1 ml: 0.50 μmoles ATP; 2.08 μmoles phosphoenolpyruvate; 0.47 μmoles NADH; 10 μmoles potassium L-aspartate; 10 μmoles L-citrulline; 2.0 μmoles $MgSO_4$; 100 μmoles tris-acetate buffer, pH 8.2; 150 units myokinase; 125 units inorganic pyrophosphatase; 150 units lactic dehydrogenase containing pyruvate kinase; and 3 - 5 units of argininosuccinate synthetase.

Any assay which measures substrate disappearance has the disadvantage that the substrate concentration must be kept low so that the amount consumed in the reaction will be a significant fraction of the

total. The citrulline concentration in this assay was about 10 percent of the optimal concentration. Aside from this problem, the assay for citrulline requires an hour and a half to run and the reaction cannot be followed continuously.

The spectrophotometric assay has the disadvantage that there are five enzymes involved in the assay. Argininosuccinate synthetase was kept limiting by adding relatively large concentrations of the other four enzymes. An indirect check of their activities was obtained by removing the test cell and following the rate of reaction in the control cell. Because there is a very rapid background reaction in crude extracts, the spectrophotometric assay can only be used on partially purified extracts.

NADH is relatively rapidly removed from the reaction mixture even without the addition of aspartate, citrulline, or ATP. This background reaction was measured in a control cell which contained all of the reagents except citrulline. The removal of NADH in the test cell, which contained all of the reagents, was due to the background reaction plus the series of reactions starting with argininosuccinate synthetase. The difference in the rate of NADH oxidation in these two cells was measured directly on a Beckman DB spectrophotometer. This difference rate is twice the rate of argininosuccinate synthesis since 2 moles of ADP are produced with each mole of argininosuccinate synthesized.

Partial Purification of Argininosuccinate Synthetase

All solutions were kept between 0° and 5°. Using a Servall Omni-mixer, 20 gm of acetone powder was homogenized for 30 sec. in 120 ml

of 0.02 M phosphate buffer, pH 7.4. The thick paste was allowed to soak for 5 min. and then centrifuged 15 min. at 23,000 times gravity. The supernate was saved and the residue reëxtracted in 50 ml of the same buffer and centrifuged for 10 min. The supernates were combined and the protein concentration adjusted to 10 mg per ml.

The solution was adjusted to pH 6.5 and made 0.1 M in ammonium sulfate (0.03 saturation). One-tenth volume of a freshly prepared 4 percent protamine sulfate suspension was added dropwise with constant stirring and the mixture stirred for an additional 5 min. The precipitate was removed by centrifuging for 10 min. at 23,000 times gravity.

The supernate was brought to 0.36 saturation by slowly adding solid ammonium sulfate (200 mg/ml). The mixture was stirred for an additional 15 min. and the precipitate removed by centrifuging for 10 min. This supernate was made 0.71 saturated by adding more solid ammonium sulfate (240 mg/ml), stirred for 15 min. and centrifuged.

The protein precipitated between 0.36 and 0.71 saturation was dissolved in a small volume of 0.01 M phosphate buffer, pH 7.0. This resuspended sample was dialyzed for several hours against four changes of 0.01 M buffer and then dialyzed overnight.

The dialyzed extract was applied to a 2.2 x 15 cm column of DEAE cellulose. Elution, using phosphate buffer, pH 7.0, was carried out by the addition of 15 ml of 0.01 M buffer, 50 ml 0.02 M buffer, and then a linear gradient ranging from 0.02 M to 0.06 M. The elution rate was held at 2 ml per minute (see Figure 2).

A small increase in activity could be achieved by putting this

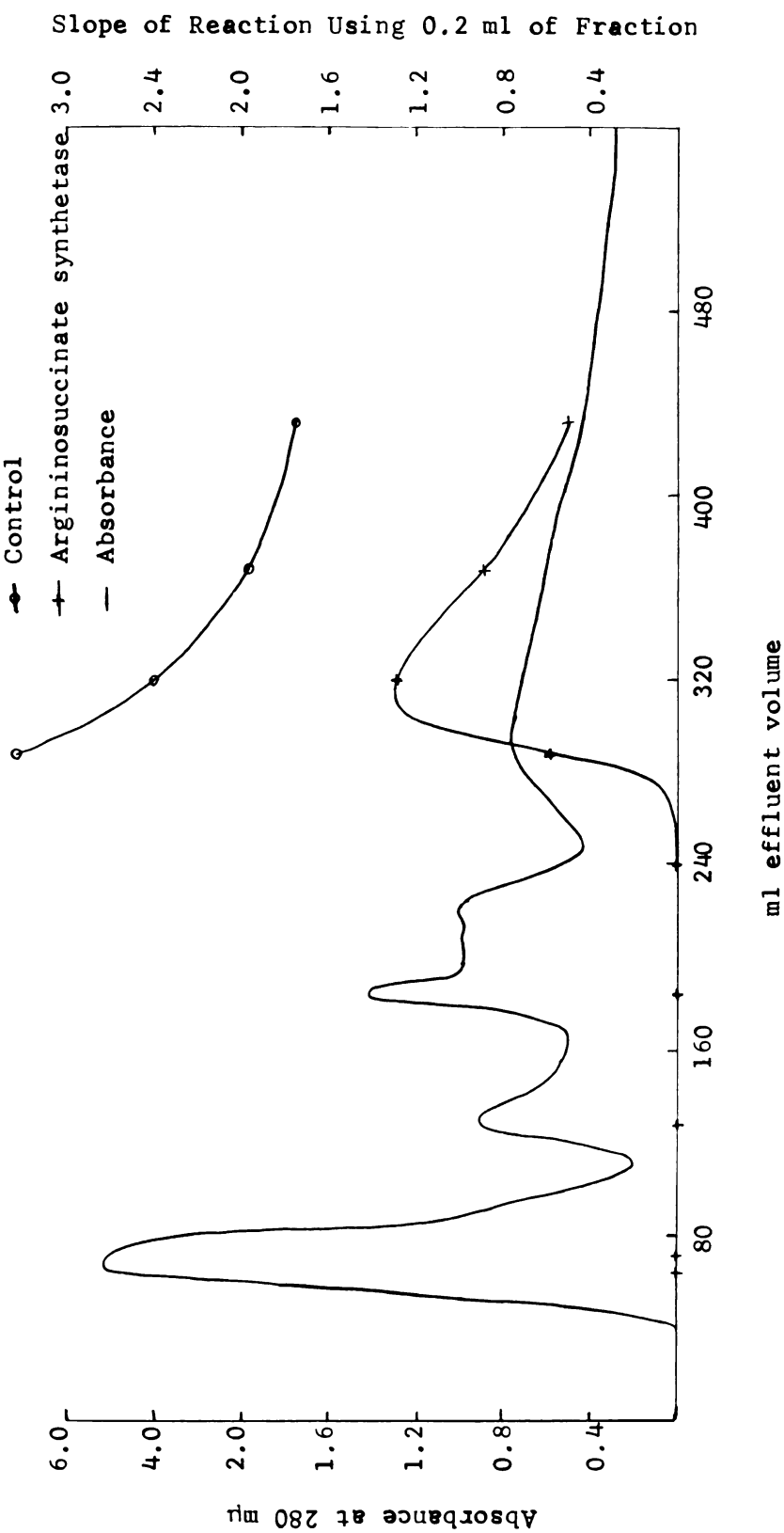


Figure 2. Protein and Argininosuccinate Synthetase Activity Recovered from DEAE Cellulose Column

preparation through a second DEAE cellulose step, using a 1.1 x 10 cm column. Since the only purpose of purification was to get a preparation which could be conveniently measured in the spectrophotometric assay, this second DEAE step was not normally used.

RESULTS

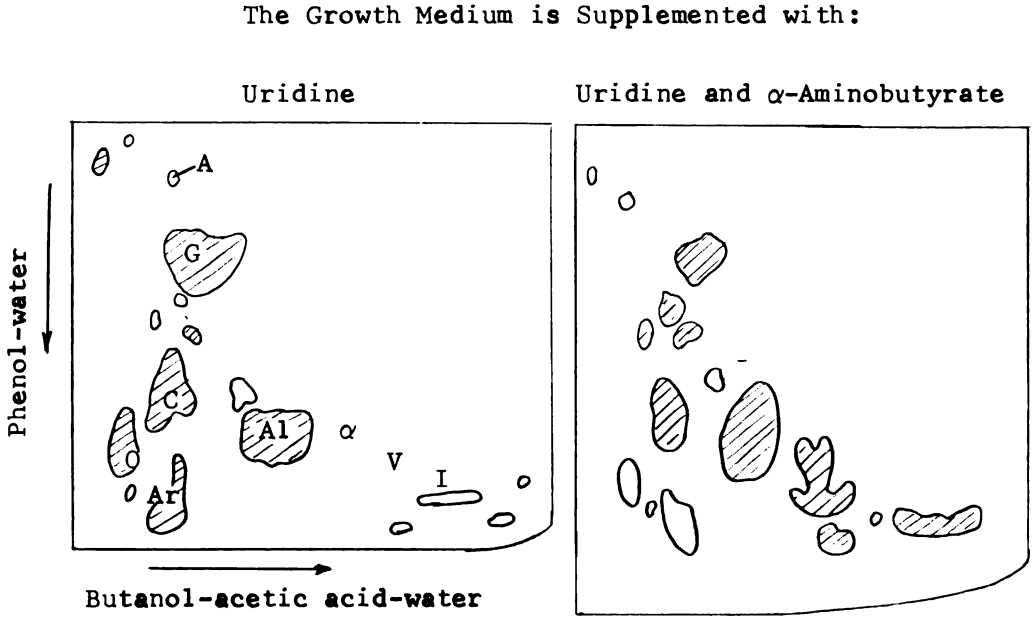
Changes in Free Amino Acid Levels

Growth of both the wild type organism and the mutant 1298 on α-aminobutyrate or propionate caused changes in the intracellular concentrations of several amino acids. Changes in the amino acid composition introduced by growth in the presence of α-aminobutyrate are illustrated in Figure 3. Changes in neutral and acidic amino acids when mycelia were grown with propionate are listed in Table II. The automatic amino acid analyzer gave good separation for most amino acids. The time (measured as elution volume) at which each amino acid came off of the column was the same from one extract to another but extracted amino acids came off progressively slower than amino acids from a calibration mixture. Identification was further complicated by the presence of several unidentified peaks and shoulders.

The amino acid composition of an extract from mycelia grown in the presence of α-aminobutyrate is also presented in Table II. Although there was a substantial increase in isoleucine and β-alanine concentration, this extract shows no increase in valine and a relatively small increase in the peak which should contain α-aminobutyrate. It is likely that this culture was allowed to grow too long and the organism had already removed most of the α-aminobutyrate.

Both the paper chromatograms and the amino acid analyzer show a large increase in the concentration of isoleucine when the mold is grown with α-aminobutyrate. The paper chromatograms

Figure 3. Effect of α-Aminobutyrate on the Free Amino Acid Composition of N. crassa 1298



Letters refer to the position of known amino acids run on a similar chromatogram. Abbreviations are: A, aspartate; Al, alanine; Ar, arginine; α, α-aminobutyrate; C, citrulline; G, glutamate; I, isoleucine; O, ornithine; V, valine.

Table II. Effect of Growth Medium on Free Amino Acid Composition of N. crassa

Amino Acid[2]	Position of Peak[3]	Concentration[1] of Amino Acid when Mycelia were Grown on:		
		Basal	αABA	Propionate[4]
Unknown	55	3.5	8.9	9.2
Aspartate	117	32.4	48.8	24.5
Serine	153	- - - - -Off Scale- - - - - - - -		
Unknown	171	7.0	3.9	13.8
Glutamate	182	- - - - -Off Scale- - - - - - - -		
Glycine	247	31.1	29.0	29.8
Alanine	274	- - - - -Off Scale- - - - - - - -		
Unknown[5]	306	17.0	22.2	4.8
Valine	311	18.4	17.0	33.4
Unknown	316	41.7	40.7	13.4
Methionine	332	1.4	3.8	1.4
Isoleucine	348	4.1	40.5	6.3
β-alanine	518	1.0	9.7	1.5

[1] Concentration was calculated by multiplying the width at one-half the height by the height of the peak. Concentration units are different for each amino acid so comparisons can be made between extracts but not between amino acids.

[2] Leucine, tyrosine, and phenylalanine could also be identified but they were present in small amounts and there was little difference in concentration among the three extracts. There were three peaks which were off the scale. The largest peak was alanine.

[3] Position is measured in ml of effluent from the column.

[4] The extract from mycelia grown with sodium propionate was less concentrated than the other two extracts. Values in this column are the raw data multiplied by 2.5, a factor which brings the glycine value to that of the other extracts.

[5] May contain α-aminobutyrate.

also show an increase in valine and α-aminobutyrate. The reduction in arginine concentration, shown by the paper chromatograms, was studied in detail and will be discussed later. The amino acid analyzer shows an increase in valine and a slight increase in isoleucine when mycelia were grown with propionate. There is, however, no increase in the peak which may contain α-aminobutyrate.

Hayashibi and Uemura (27) have demonstrated that isoleucine exerts feedback control on its own synthesis in Bacillus subtilis by inhibiting threonine deaminase (reaction 27). They further showed that α-aminobutyrate releases this inhibition and cells grown in the presence of α-aminobutyrate accumulate "a large quantity of L-isoleucine." The large increase in isoleucine concentration in cells grown on α-aminobutyrate suggests that a similar situation exists in N. crassa.

Horvath et al. (28) have shown that the presence of threonine, α-ketobutyrate or isoleucine in the growth medium of Pseudomonas aeruginosa or Escherichia coli increases the α-acetolactate forming system (reaction 31) which is the first reaction peculiar to valine and isoleucine synthesis. Since α-ketobutyrate and threonine can readily be formed from α-aminobutyrate (reactions 30 and 27), and isoleucine synthesis is increased with growth on α-aminobutyrate, the logical conclusion is that a similar explanation for the valine increase applies with N. crassa. It is more difficult to explain the increase in valine found when the mold was grown on propionate. Attention must be drawn to the fact that the actual inducer in

the experiments of Horvath et al. is unknown. Conceivably, this could be propionate formed by decarboxylation of α-ketobutyrate.

Intracellular Arginine Concentration

The arginine concentrations in mycelial powders of strains 1A and 1298 grown under various culture conditions appear in Table III. Because the molds grow at considerably different rates under different culture conditions, it was necessary to assay for arginine at comparable stages of growth. Gaps which appear in the data of the Table result chiefly from difficulties in harvesting the mold at the desired stages.

Table III. The Arginine Content of a Wild-Type Strain of Neurospora crassa and the Pyrimidineless Mutant, Strain 1298, under Various Conditions

Supplements to 500 ml. of Fries medium	Strain of the Mold	Arginine Content[1] at Various Growth Stages[2]		
		0.1-0.49	0.5-0.99	1.0-2.5
None	Wild	20	--	20
40 mg. Uridine	Wild	33	23	20
	1298	28	31	30
100 mg. Aminobutyric acid	Wild	10	18	20
	1298	4.5	--	24
40 mg. Uridine + 100 mg. Aminobutyric acid	Wild	10	12	16
	1298	--	7.5	22
100 mg. Sodium Propionate	Wild	1.2	5.0	18
	1298	0.9	--	4
100 mg. Sodium Propionate + 40 mg. Uridine	Wild	--	6.3	--
	1298	1.9	1.2	--

[1] The content of arginine is expressed as μmoles per gram of mycelial acetone powder.

[2] The stage of growth is expressed as grams of mycelium obtained after harvest and extraction with acetone.

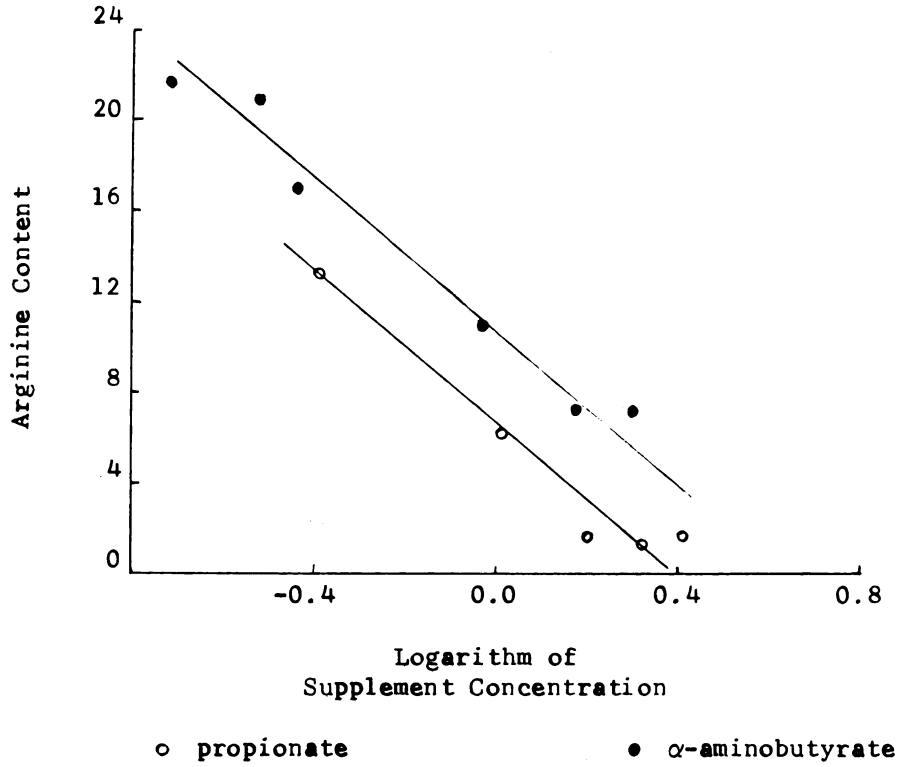
The level of free arginine in wild-type mycelia grown on basal medium was about 20 μmoles per gram of acetone powder. This level was not affected appreciably by the stage of growth or the presence of uridine, except for an apparent increase in the arginine concentration at early stages of growth with uridine. In all cases, when uridine was the supplement, the arginine content of the mutant strain was about 30 μmoles per gram.

For both strains of the mold, the presence of either propionate or aminobutyrate resulted in low arginine concentrations, regardless of the presence or absence of uridine. Levels as low as 1 μmole per gram of acetone powder, 5 percent or less of normal, were obtained with propionate. These effects were most pronounced in early growth, presumably because α-aminobutyrate and propionate are removed by metabolic processes as growth proceeds.

Figure 4 summarizes data which were obtained from experiments designed to determine the relationship between the α-aminobutyrate or propionate concentration in the culture medium and the intramycelial concentration of free arginine. In these experiments, sufficient uridine (80 mg. per liter) was present to provide maximal growth rates regardless of the concentration of propionate or α-aminobutyrate. All mycelia were harvested at essentially the same early stage of growth.

It may be seen that for the concentration range examined, the arginine content proved to be dependent in inverse fashion upon the logarithm of the concentration of either propionate or α-amino-

Figure 4. The Relationship Between the Concentration of α-Aminobutyrate and of Propionate in the Culture Medium and the Arginine Content of the Mycelium.



Arginine content is given in μmoles per gram of mycelial acetone powder. The supplement concentration is given in millimoles per liter.

butyrate. In these experiments, the α-aminobutyrate used was the DL mixture. It is known that only the L-isomer is capable of supporting growth of the mold (5). If the data for α-aminobutyrate given in Figure 4 were replotted under the assumption that only the L-isomer is effective in lowering the arginine concentration, the line for α-aminobutyrate would approach closely that for propionate. This suggests, rather surprisingly, that the two substances may be equally potent on a molar basis in reducing the arginine content of the mycelium.

Enzymatic Studies

General Findings. All of the enzymes involved in the Krebs urea cycle (reactions 7-10) can be demonstrated in extracts of N. crassa (Table IV). During the early stages of this work when the data in Table IV were gathered, crude extracts exhibited only about 0.2 units of activity per mg protein. Later, after making several changes in the preparation of extracts and assay conditions, crude extracts consistently exhibited 2.2 to 2.6 units per mg protein.

The optimal conditions for measuring ornithine transcarbamylase activity had been worked out previously (31). Although adding large amounts of arginine to the growth medium does not repress ornithine transcarbamylase activity (29), conditions which reduce the intracellular arginine concentration do cause derepression, as shown in Table IV.

The last three enzymes listed in Table IV were not studied in detail.

Table IV. Activity of Urea Cycle Enzymes and Urease in Crude Extracts of Neurospora

Enzyme	No.[1]	Activity[2]		
		This Thesis	Other Work	Derepressed[3]
CPK	1	0	0.2 (29)	0.3 Arg 3(29)
OTC	7	20	20 (12)	85 Arg 3(12)
				68 α-ABA
				65 Prop.
ASSase	8	0.2 to 2.6	0.6 (25)	
ASAase	9	+	0.3-0.8 (25)	
Arginase	10	3.0	7 (30)	25 Arginine(30)
Urease	11	0.8		

[1] Reaction numbers in Figure 1.

[2] Activity is μmoles of product formed per mg protein per hour.

[3] These activities are either derepressed or induced. The N. crassa mutant or the supplement used to cause this change is also given.

NOTE: Numbers shown in parentheses refer to references.

Abbreviations used in Table IV:

CPK - Carbamyl Phosphokinase

OTC - Ornithine Transcarbamylase

ASSase - Argininosuccinate Synthetase

ASAase - Argininosuccinase

α-ABA - α-Amino-n-butyric Acid

Prop. - Sodium Propionate

Properties of Argininosuccinate Synthetase in Crude Extracts.

The argininosuccinate synthetase activity found in crude extracts was absolutely dependent upon the presence of aspartate, citrulline, ATP, and magnesium (Table V). Addition of inorganic pyrophosphatase

Table V. Influence of Reaction Components on Argininosuccinate Synthetase Activity from N. crassa

Components of the Reaction Mixture	Percent Control Activity		
	Colorimetric Assay	Spectrophotometric Assay	
		ASSase	Back.
Standard	100	100	100
Standard less aspartate	0	0	100
Standard less citrulline	*	0	100
Standard less ATP	0	23	104
Standard less magnesium	0	0	0
Standard less enzyme	0	0	0
Standard less 3-PGA	60	*	*
Standard plus PPase	104	*	*
Standard with double 3-PGA	100	*	*
Standard less PPase	*	85	100
Standard less PEP	*	0	0
Standard less myokinase	*	0	100
Standard less LDH	*	0	0
Standard, not preincubated with magnesium	*	87	85

Abbreviations used in Table V:

ASSase - Argininosuccinate Synthetase
Back. - Reaction in Control Cell
3-PGA - 3-Phosphoglyceric Acid
PPase - Inorganic Pyrophosphatase
PEP - Phosphoenolpyruvate
LDH - Lactic Dehydrogenase

* Does not apply.

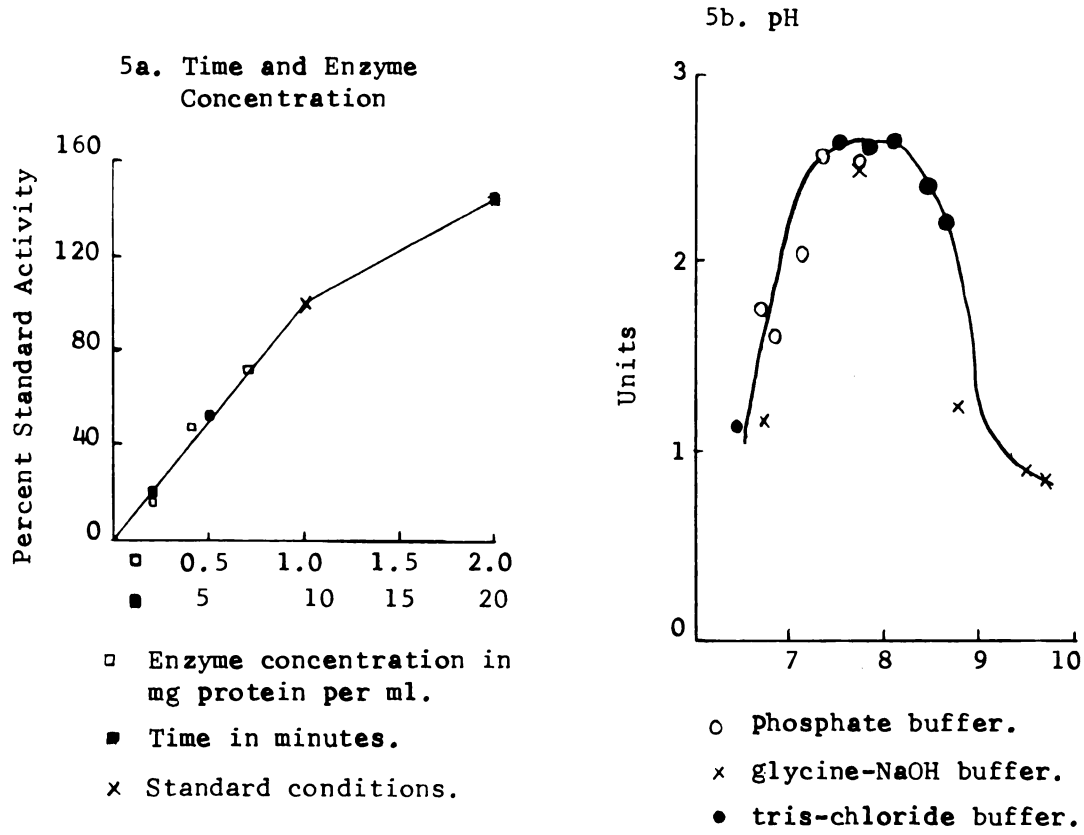
increased the activity slightly, but this enzyme was not normally added. Doubling the concentration of 3-phosphoglyceric acid did not increase the activity. The activity was linear with respect to time for at least 10 minutes and with respect to enzyme concentration within the range used (Figure 5a). The pH optimum was measured using 3 buffers (Figure 5b). There is little difference in activity between pH 7.4 and 8.2.

Figure 6a shows the relationship between activity and ATP concentration. Adenosine triphosphate is both a substrate and inhibitor of argininosuccinate synthetase. Concentrations either above or below 10^{-3} M cause a decrease in activity. Other inhibitors are listed in Table VI.

Of the compounds which allow growth of the pyr-3a mutants -- propionate, α-aminobutyrate, and threonine -- only α-aminobutyrate can be called a good inhibitor (see Figure 6b also). Two products of the reaction -- pyrophosphate and AMP -- also inhibit. Inorganic pyrophosphate is a weak inhibitor when it is added to the reaction with the substrates (upper curve, Figure 6b), but it is a strong inhibitor if it is preincubated with the enzyme for 10 minutes before the substrates are added (lower curve, Figure 6b).

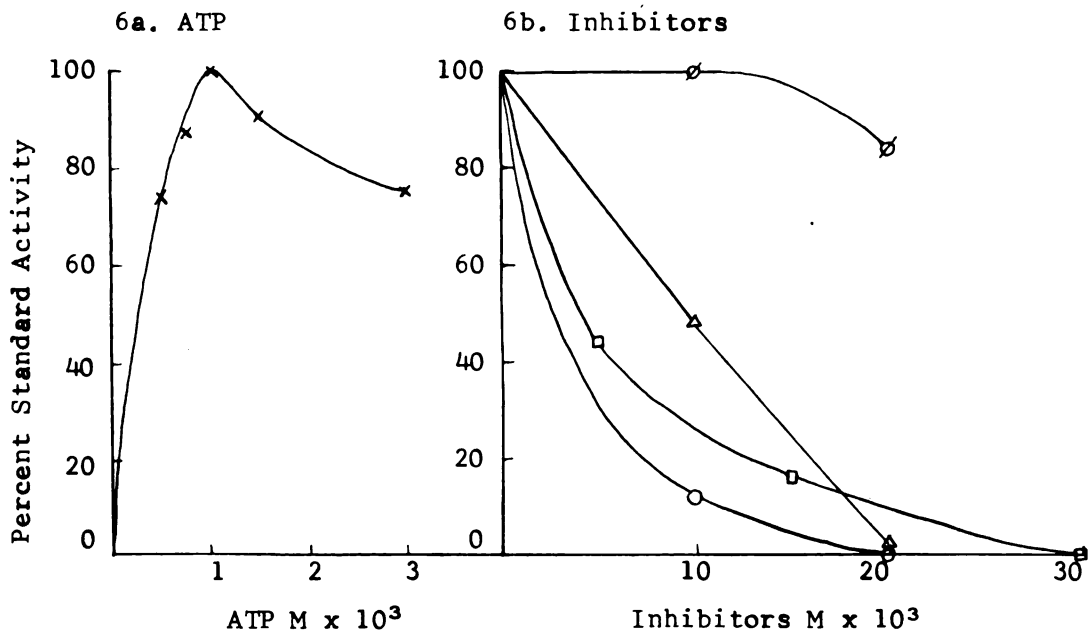
Valine and α-aminobutyrate were the only amino acids which inhibited more than 50 percent. The fact that valine inhibits argininosuccinate synthetase probably explains the observation

Figure 5. Factors Affecting Argininosuccinate Synthetase Measured in the Crude Preparation



For figure 5b, units are μmoles citrulline removed under standard conditions (see Methods).

Figure 6. Factors Affecting Argininosuccinate Synthetase Measured in the Crude Preparation



6b. ø inorganic pyrophosphate, O inorganic pyrophosphate preincubated with enzyme for 10 minutes, □ L-α-aminobutyrate, △ L-valine.

Table VI. Inhibitors of Argininosuccinate Synthetase

Inhibitor	Crude Preparation			Purified Preparation			
	Conc.	Act.	% I	Conc.	Act.	% I	Back.
None		51	0		0.31	0	1.28
PP_i	20	43	16	5	0.00	100	0.37
L-Arginine				10	0.15*	61	0.77*
ATP	5	37	22	10	0.03*	96	0.87*
AMP	10		73				
ADP	10		66				
D,L-Valine				20	0.19	39	1.15
L-Valine	10	24	52				
L-αABA	15	8	84	10	0.21	35	1.28
Propionate	20	49	4	10	0.21	35	1.28
L-Threonine	20	36	29	10	0.23	28	1.28
KGA	20	47	8	10	0.21	35	1.66
KBA	20	56	-10				
L-Canavanine				10	0.25	21	1.28
2-A-3-P				10	0.26	19	1.28
MeAsp				10	0.31	0	1.15
L-Lysine				10	0.33	- 5	1.03
L-Isoleucine				10	0.34	- 6	1.25
L-Leucine				10	0.34	- 6	1.08
L-Glutamate				10	0.36	-13	1.28

* Run with a different enzyme prep. (see Figure 8c). The control reaction is 98% of uninhibited reaction for arginine and 72% for ATP.

Abbreviations used in Table VI:

Conc. - Concentration of the inhibitor M x 10^3.

Act. - For the crude preparation - μmoles citrulline lost out of 250 μmoles (1/6 of assay, see Methods), for purified preparation - slope of the reaction line (see Figure 7). Concentrations of other reagents as in Methods except arginine which was 10^{-2} M.

% I - Percent inhibition.

Back. - Slope of control reaction (see Figure 7).

Abbreviations used in Table VI (Continued):

PP_i - Inorganic pyrophosphate.
AMP - Adenosine 5'-monophosphate.
ADP - Adenosine 5'-diphosphate.
KGA - α-Ketoglutarate.
KBA - α-Ketobutyric Acid.
2-A-3-P - 2-Amino-3-phosphonopropionic acid.
MeAsp - β-Methylaspartic acid.

by Carbonneau and Berlinguet (32) that L-valine inhibits the conversion of citrulline to urea in rat liver homogenates.

Because there is considerable argininosuccinase activity in crude extracts (Table IV) and because argininosuccinate synthetase is probably reversible (26), when argininosuccinate is added to the assay, it is probably converted to both arginine and citrulline. Since arginine interferes with the assay for citrulline, neither argininosuccinate nor arginine were tested for inhibition in the crude extract.

Properties of the Purified Preparation. Fractions from the DEAE cellulose column (Figure 2) exhibited similar argininosuccinate synthetase activities whether measured by either the colorimetric or the spectrophotometric assay. Measured spectrophotometrically, this activity is dependent upon both aspartate and citrulline (Table V). A magnesium requirement could not be demonstrated using this assay method since magnesium is required for myokinase and pyruvate kinase. After a 3 to 5 minute lag, there is some reaction without

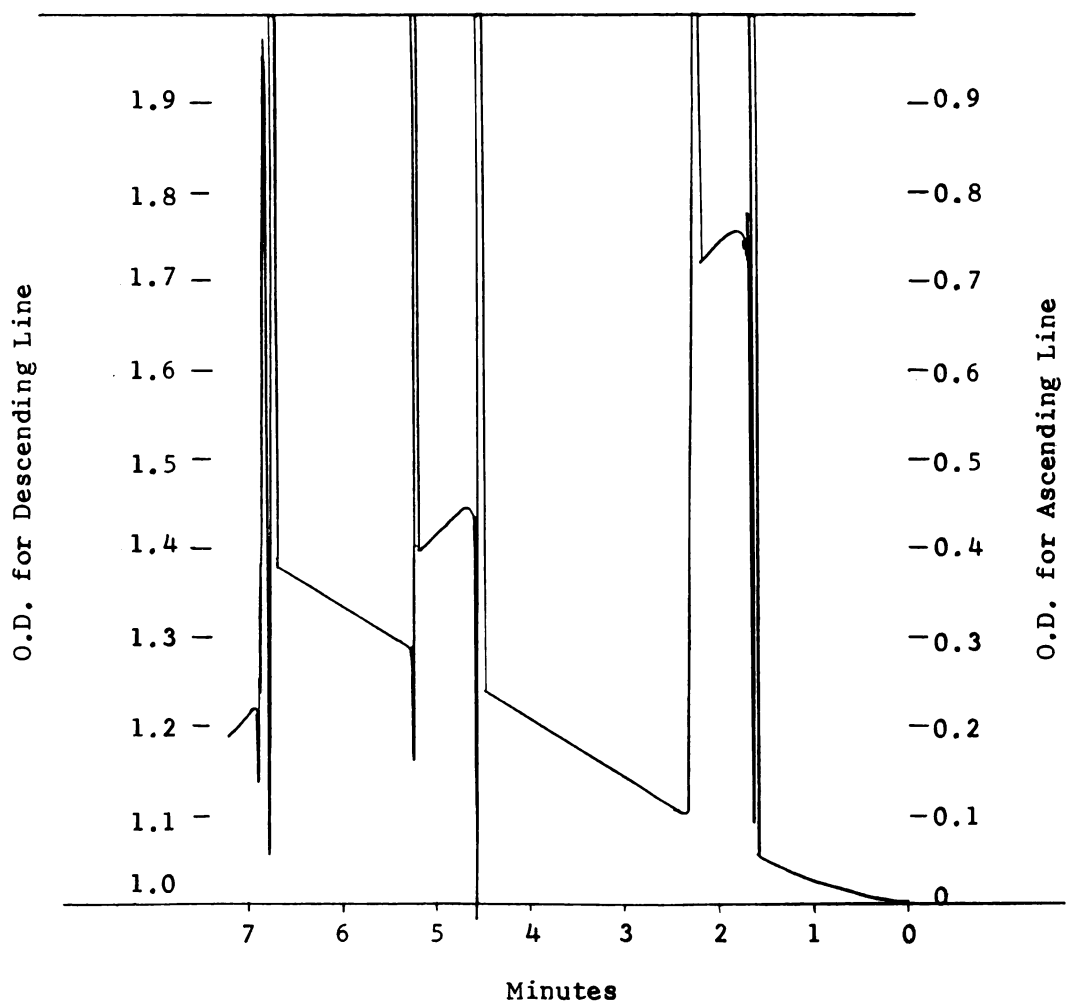
added ATP. This may be due either to bound ATP in the extract or the presence of traces of ATP in the reagents.

The addition of inorganic pyrophosphatase and preincubation with magnesium both cause about a 20 percent increase in activity (Table V). After a slow start (about 30 sec.), the activity is linear with respect to time until 2/3 of the NADH is oxidized (Figure 7). The activity is linear with respect to enzyme concentration up to at least twice the concentration of enzyme used in the studies.

The background reaction does not depend upon the addition of aspartate, citrulline, or ATP (Table V). The rate of this reaction is not linear with respect to either time (Figure 7) or enzyme concentration (Figure 8a). The activity is, however, dependent upon the addition of phosphoenol pyruvate (Table V) so it cannot be explained simply by assuming the presence of an NADH oxidase. A major part of the activity may be ATPase, although the omission of ATP causes a slight increase in activity (Table V). Adenosine triphosphate concentration of 10^{-2} M inhibits the reaction about 30 percent (Figure 6). This background activity is not understood and was not studied further.

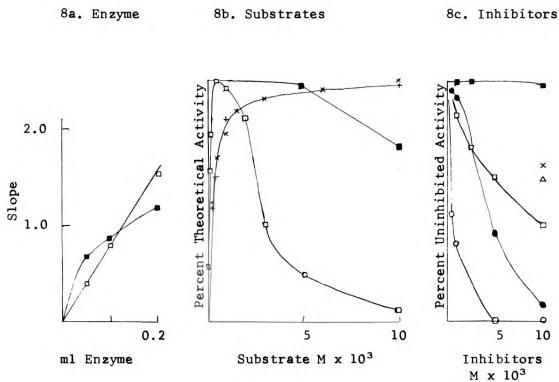
The compounds which inhibit argininosuccinate synthetase in the purified preparation are the same ones which inhibit the reaction in the crude preparation (Table VI). In addition, arginine could be tested in the purified preparation and was found to inhibit strongly (see also Figure 8c). There are some

Figure 7. Raw Data from Spectrophotometric Assay



Ascending line was obtained with the reaction cell in the "Reference" side and the control cell in the "Sample" side of a Beckman DB Spectrophotometer. The descending line was obtained by removing the reaction cell and measuring changes in the control cell only.

Figure 8. Factors Affecting Argininosuccinate Synthetase Measured in the Partially Purified Preparation



Filled-in points refer to activity of the control reaction.

8b. Theoretical activity is the V_{max} obtained from a Lineweaver-Burk plot of the aspartate and citrulline data. For ATP, 100 percent activity is the maximum activity when aspartate and citrulline are 2×10^{-2} M.

▫ ATP, + L-citrulline, × L-aspartate.

8c. ○ inorganic pyrophosphate, ▫ L-arginine,

× L-α-aminobutyrate, △ 20 mM D,L-valine

differences in the degree of inhibition exhibited in the two assays. Pyrophosphate is a much stronger inhibitor in the purified preparation and inhibition by propionate is somewhat more pronounced. It should be remembered that both the specific activity and the citrulline concentration are quite different in these two assays.

DISCUSSION

Carbamyl phosphate was proposed as the carbamyl donor in carbamyl aspartic acid and citrulline synthesis in 1955 (33). Since then, several different enzymes have been found with the ability to form active carbamyl groups (34), although there may be some question of whether or not these enzymes are normally carbamyl, acetyl, or formyl phosphokinases (35,36). Most organisms are thought to derive the carbamyl groups used in both arginine and pyrimidine synthesis from a single source. Escherichia coli is an example of such an organism. Carbamyl phosphate synthesis has been demonstrated in E. coli by several workers (37,38,39). A single gene mutant, P678B1, which lacks this activity requires both arginine and pyrimidine for growth. Reversion of P678B1 to prototrophy is a single genetic event (39).

Neurospora crassa, on the other hand, appears to have two sources of carbamyl groups -- one specific for pyrimidine synthesis and the other specific for the synthesis of arginine (7,12). This conclusion is drawn from attempts to explain the pyr-3a mutation.

Pyr-3a mutants will, of course, grow when pyrimidines are added to the culture medium. But they will also grow under a variety of other conditions which fall into two general groups: (1) conditions which reduce the intracellular concentration of arginine, and (2) conditions which increase the availability of carbon dioxide. In the first case, lowered arginine concentrations presumably derepress the arginine-specific carbamyl source so that

the extra carbamyl groups formed can be used in the pyrimidine pathway. In the second case, an increase in carbon dioxide concentration apparently stimulates the production of carbamyl groups by a mass action effect. In both cases, however, the carbamyl groups which go into pyrimidines are thought to come from the arginine-specific enzyme since the addition of very small amounts of arginine restores the pyrimidine requirement (2,7,10).

The theory provides a plausible explanation for experiments with pyr-3 mutants and is further supported by experiments with other mutants. A situation analogous to pyr-3a suppression has been reported for two unlinked arginine mutants -- arg-2 and arg-3 (40,41). In this case, the arginine requirement is removed by a mutation in the structural gene for aspartic transcarbamylase. The arg-2 and arg-3 loci are thought to be concerned with the formation of arginine-specific carbamyl groups. The reduced pyrimidine production apparently derepresses the pyrimidine-specific carbamyl source.

But the most direct evidence for the existence of two carbamyl sources comes from studies with the carbamyl-forming system itself (21). Extracts from wild type strains incorporate radioactive bicarbonate into the ureido carbon of citrulline when incubated with ammonia, ornithine, ATP, and magnesium. This "carbamyl phosphokinase" activity is not present in the arg-3 mutants. If there are, indeed, two carbamyl sources in N. crassa, one might expect the arg-3 mutants to exhibit activity from the pyrimidine-specific

source. Although this pyrimidine-specific enzyme was not detected by this method, its existence can be inferred from the observation that the double mutant, pyr-1 arg-3, still accumulates carbamyl aspartate. The fact that arg-2 exhibits carbamyl phosphokinase activity may mean that there are two steps in the formation of active carbamyl groups in the arginine pathway.

Even though only one of the two proposed carbamyl sources has been clearly demonstrated, for the rest of the discussion I will assume the theory is correct. It is not even certain that carbamyl phosphate is the carbamyl donor, even though it is an effective substrate in vitro. Nevertheless, I will call the carbamyl donor "carbamyl phosphate" and the enzyme or enzymes involved "carbamyl phosphokinase."

My first contribution toward explaining the action of propionate and α-aminobutyrate was the demonstration that these compounds reduce the intracellular arginine concentration. To this extent, they imitated the suppressor and provided a separate body of evidence for the theory just presented. This reduction in arginine gave a partial answer to the original problem and immediately raised another question -- how do propionate and α-aminobutyrate lower the concentration of arginine?

The arginine concentration might be lowered either by inhibiting arginine synthesis or by increasing its removal. I chose to concentrate on conditions which might inhibit synthesis. Since the suppressor reduces ornithine transcarbamylase activity, a reasonable

starting point was to determine whether or not propionate and α-aminobutyrate inhibit this enzyme.

Although high concentrations of α-aminobutyrate did inhibit ornithine transcarbamylase, this inhibition was not considered a sufficient explanation for the drastic reduction in arginine concentration (42). Not only were propionate and α-aminobutyrate poor inhibitors, but their presence in the growth medium caused derepression of ornithine transcarbamylase to more than three times the normal activity.

It is not likely that the arginine specific carbamyl phosphokinase is inhibited or repressed by the growth-promoting compounds since this enzyme is necessary to supply carbamyl phosphate for both pyrimidine and arginine synthesis.

If we disregard for a moment the possibility that propionate and α-aminobutyrate inhibit the synthesis of aspartate or ornithine, there are only two enzymes which remain to be considered -- argininosuccinate synthetase and argininosuccinase. The finding of Fairley and Adams (10) that citrulline is a poor inhibitor of growth on α-aminobutyrate, while arginine is an extremely potent inhibitor, supports the possibility that the conversion of citrulline to arginine does not readily occur in the presence of α-aminobutyrate.

Fincham and Boylen (23) have shown that the arg-10 mutant, which has lost argininosuccinase activity, accumulates argininosuccinate when grown on arginine. Similarly, the arg-1 arg-10

double mutant, which is also deficient in argininosuccinate synthetase, accumulates citrulline (25). If growth of 1298 on α-aminobutyrate involves the inhibition of one of these enzymes, similar increases in either argininosuccinate or citrulline might be expected.

In the experiments described here, no accumulation of argininosuccinate could be detected. Since there was a considerable concentration of citrulline under normal growth conditions, the results were not sufficiently precise to determine whether or not the citrulline concentration did increase. But the ornithine concentration did go down when the mold was grown on α-aminobutyrate. This relative increase in citrulline concentration with respect to arginine and ornithine may be all that can be expected. It should be remembered that the reduction in arginine synthesis caused by α-aminobutyric acid is considerably different from the complete blocks described by Fincham and Boylen and Newmeyer.

In the first place, growth on α-aminobutyrate does not cause a complete block in arginine synthesis. There is some flux through the pathway from citrulline to arginine. In the second place, and perhaps more important, organisms grown on α-aminobutyrate were not fed arginine. A large part of the citrulline which accumulated in the work described by Newmeyer may have come by way of ornithine from the arginine which was included in the growth medium.

The possible role of ornithine in carbamyl phosphate availability has never been discussed in print. The strong growth

inhibition caused by adding small quantities of arginine may, in part, be due to formation of ornithine which can compete with aspartic acid for the available carbamyl phosphate as well as to repression of carbamyl phosphokinase. This possibility is consistent with the observation of Fairley and Adams that growth on α-aminobutyrate is much more strongly inhibited by ornithine than by citrulline.

A study of argininosuccinate synthetase finally gave clear evidence that at least part of the reduction in arginine concentration is due to inhibition of this reaction. Both α-aminobutyrate and propionate inhibit partially purified argininosuccinate synthetase in vitro. The fact that propionate was a much less effective inhibitor in the crude system was probably due to its rapid removal by other enzymes in the crude extract. Not only do propionate and α-aminobutyrate inhibit but so does L-valine, an amino acid which appears to accumulate in cells grown on either of the other two inhibitors.

So far, evidence has been presented indicating that propionate and α-aminobutyrate inhibit argininosuccinate synthetase, both directly and indirectly. Other unsuspected effects of these compounds may well exist. For example, the possibility that they inhibit the formation of ornithine from glutamic acid, either directly or indirectly, has not been excluded.

The metabolic changes which have been discussed in this thesis emphasize the fact that an organism is a unit and not

just a collection of independent metabolic pathways. The addition of α-aminobutyrate to the growth medium of N. crassa affects the synthesis of valine, isoleucine, arginine, and pyrimidine. I have only considered the most obvious changes in pyrimidine and amino acid metabolism. It is not unlikely that many other changes occur as the cell adjusts to the introduction of a gratuitous metabolite. A thorough study of these interactions is beyond the scope of this study.

In summary, the primary metabolic defect in the pyr-3a mutants of N. crassa is apparently the inability to provide a pyrimidine-linked source of carbamyl phosphate. Derepression and reduced consumption of the arginine-linked carbamyl source allows growth by providing excess carbamyl phosphate, some of which can be used for pyrimidine synthesis. Although some details remain to be examined more fully, the experiments described in this thesis provide a coherent explanation for the growth-promoting properties of propionate and α-aminobutyrate. Growth of N. crassa on either of these compounds is accompanied by a low concentration of intracellular arginine, presumably leading to derepression of carbamyl phosphate synthesis. The low arginine level is due, at least in part, to the direct inhibition of argininosuccinate synthetase by propionate and α-aminobutyrate. These compounds may also reduce argininosuccinate synthetase activity indirectly by stimulating the production of valine which also inhibits the reaction. The discovery that argininosuccinate synthetase is inhibited by arginine may have an important bearing on the control of arginine synthesis in this organism.

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