THE BIOCHEMISTRY AND GENETICS OF THREONINE-REQUIRING MUTANTS OF *NEUROSPORA CRASSA*

A Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1947

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. G. W. Beadle for advice and encouragement, to Dr. N. H. Horowitz for assistance in the biochemical aspects of the problem, to Dr. M. Fling for several compounds that she prepared, to Dr. B. O. Phinney and Mrs. Mary Houlahan for permission to report asci that they dissected, to Dr. A. J. Haagen-Smit and to Dr. Gertrud Oppenheimer for the micro-analyses, as well as to other members of the genetics laboratories at Stanford University and at California Institute of Technology, who helped in many ways.

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INTRODUCTION

That genetic differences in organisms may be correlated with chemical ones was appreciated by Garrod in his 1923 edition of "Inborn Errors of Metabolism" where he described a number of human metabolic abnormalities that behave as genetic characters (1). More recently, evidence has accumulated to show that genes are concerned with particular steps in the metabolism of organisms, as in Drosophila eye color (2), flower anthocyanins (3), guinea pig coat color (4), and growth factors for the fungus Neurospora (5). Such studies, particularly those on Neurospora, have suggested a one-to-one relationship between genes and particular biochemical reactions (5); more specifically, between genes and the enzymes mediating such biochemical reactions.

Beadle and Tatum, and Horowitz et al. have shown that x-ray, ultra violet, neutron and chemical treatments may increase the mutation rate in the ascomycete Neurospora crassa (6, 7). A number of mutant strains resulting from treatments have been investigated genetically and have been shown to differ from normal strains by single gene changes. Some of these mutant strains are characterized by the inability to synthesize essential growth substances, as amino acids or vitamins, and therefore cannot grow on a "minimal" medium containing only the salts, sugar and biotin required by the

normal wild type strain (8). A mutant strain unable to synthesize a substance which can be supplied exogenously may be expected to grow if the minimal medium is supplemented with the appropriate compound. Biochemical mutant strains are designated by adding the suffix "-less" to the name of the compound that they are unable to synthesize (9). A mutant strain unable to synthesize pyridoxine is referred to as "pyridoxineless"; one unable to synthesize threonine, as "threonineless."

In Neurospora, nuclei of vegetative mycelia are haploid. The species is heterothallic, the two sexes or mating types being designated arbitrarily as A and a. When nuclei of opposite mating type fuse, a diploid zygote is formed which immediately undergoes meiosis (10). As Dodge has shown, the first meiotic spindle is oriented parallel to the length of the ascus; the second meiotic and post meiotic spindles are arranged oblique to the long axis (11). In Neurospora crassa, eight spores, arranged as four pairs in linear order, are formed per ascus. The ascus is sufficiently narrow to prevent rearrangement of the spores, so that by the dissection and germination techniques developed by Shear and Dodge, by Lindegren, and by Beadle and Tatum the four meiotic products can be separated and recovered in order (12, 13, 6). The fact that the spores occur in pairs allows for genetic analysis of some asci in which not all eight of the spores have germinated.

It is known that heterozygous genes at the centromere segregate in the first meiotic division. As a result, spore order will show whether an effective crossover has occurred between a gene and its centromere. Thus, the frequency of chiasmata between a given gene and the centromere can be measured as the percentage of asci showing second division segregation for the character. Since only one half of the chromatids in an ascus showing a second division segregation are crossover types, the distance of a gene from its centrmere in crossover or map units is one half the frequency of second division segregations.

A cross of strains which differ with respect to only a single gene will give rise to asci containing four spores of each parental type. In the cross of a mutant strain with wild type the absence of asci showing other than four mutant and four wild type spores has been taken to indicate the single genic nature of the mutant difference (14).

If two genetically different biochemical mutant strains are crossed, asci of several types should be obtained: those in which all four spore pairs are mutants; those in which three pairs are mutant and one pair wild type; and those in which two pairs are mutant and two pairs wild type. In the last case the two pairs of spores that are mutant presumably carry both mutant genes and are thus double mutants. The recovery of both single mutant strains by outcrossing a double mutant to wild type confirms the genetic difference of the original strains.

Beadle and Coonradt have shown that hyphae of genetically different biochemical mutant strains of Neurospora may fuse producing a heterocaryotic mycelium in which the rate of progression of the mycelial frontier on solid minimal medium exceeds that of either single strain (15). This "heterocaryotic vigor" was attributed by them to an intracellular symbiosis of the two types of nuclei. The heterocaryon test for allelism that they developed is based on this phenomenon. In pure biochemical mutant stock cultures all the nuclei are genetically alike. When an inoculum from such a culture is transferred to minimal medium, growth is slow or does not occur at all. If two biochemical mutants inoculated together on minimal medium give rise to a vigorous heterocaryon, the strains may be assumed to be different. This technique has been applied to the separation of a large number of mutants into genetical classes (16). However, as pointed out by Beadle and Coonradt, negative results do not necessarily prove the genetic identity or allelic nature of two mutants.

The study of intermediary metabolism by means of genetic blocks has been notably successful with Neurospora. If a particular gene controlled step is blocked, the mold cannot synthesize the end products of the blocked reaction and if the end product or products are essential, it cannot grow. However, if a compound normally produced subsequent to the block is supplied from outside, the deficiency can be overcome. But supplying a substance which is normally an inter-

mediate produced before the block should not allow growth of the organism. By testing a series of genetically different mutants on possible intermediates, Tatum and Bonner were able to elucidate several steps in the biosynthesis of the amino acid tryptophane (17); Srb and Horowitz were able to confirm the Krebs ornithine cycle (18); and Horowitz was able to discover steps in the biosynthesis of choline (19).

Another fruitful approach has been that of "cross feeding." In the case of a genetic block in the synthesis of an essential metabolite, the immediate precursor may accumulate in the mycelium and diffuse into the medium. If this occurs, another mutant strain blocked at some prior step in the same series of synthetic reactions may be stimulated by the sterile filtrate of the medium in which the first strain has been growing. This approach has lead to the isolation and identification of accumulated precursors such as monomethylaminoethanol, a choline precursor, by Horowitz (20); cystathionine, a methionine precursor, by Horowitz (21); and anthranilic acid, a tryptophane precursor, by Tatum, et al. (17).

Among the mutations concerned with nutritional deficiencies that were obtained in Neurospora crassa, five strains initially classified as threonineless were selected for this investigation. These strains were all obtained by ultra violet irradiation of asexual spores (6). The purpose of

this study was twofold: first, to establish whether these mutant strains resulted from different single gene mutations; and second, to investigate the biochemical requirements of these strains with a view toward elucidating the biosynthesis and biochemical relations of threonine.

The amino acid threonine,

$$CH_3\text{-}CHOH\text{-}CH\cdot NH_2\text{-}COOH,$$

is one of the stereoisomeric alpha-amino-beta-hydroxy-n-butyric acids. Its first isolation was from hydrolyzed oat protein by Schryver and Buston in 1926 (22). The isolation was reported from other sources, (23, 24), but the structural configuration was not determined until 1936 when Rose and his co-workers at the University of Illinois obtained it in pure form in relatively large amounts (25, 26). They were able to replace the amino group with an hydroxyl group and obtain an alpha,beta-dihydroxybutyric acid structurally related to d(-)threose (25). It was on the basis of this relationship that the name threonine was selected. The configuration of natural threonine corresponds to that of an amino acid of the "l" series. The designation of the natural form of threonine as d(-)threonine by Meyer and Rose because of its relation to d(-)threose has not been generally accepted and will not be used in this thesis. The more acceptable name for the natural form is l(-)threonine.

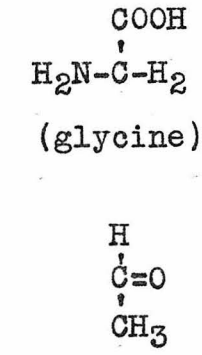
The chemical synthesis of the four isomeric alpha-amino-beta-hydroxy-n-butyric acids was accomplished in the Illinois

laboratory, as was the resolution of the racemic mixture into d- and l-threonine and d- and l-allothreonine. The Rose group also demonstrated that l(-)threonine is an essential dietary component for the dog, rat and man (27). Threonine has since been shown to be required by the mouse (28), chick (29) and a number of microorganisms (30, 31,32). Only the l(-) form has been reported to show growth promoting activity.

The biological synthesis of threonine has not been definitely established. However, it was shown by Stokes and Gunness that three lactobacilli, Lactobacillus delbruckii, L. casei, and L. arabinosus, require threonine only when grown under conditions of pyridoxamine or pyridoxal deficiency (33). This finding may indicate that pyridoxamine or pyridoxal is essential for threonine synthesis, perhaps as a coenzyme. Such an hypothesis is suggested by the role of pyridoxal in bacterial transaminases and decarboxylases (34, 35).

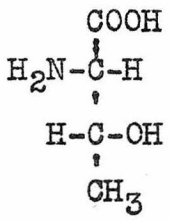
In 1941 Kögl and Borg established that threonine is identical with the "factor Z" of Euler which stimulates the respiration of Saccharomyces cerevisiae in short experimental periods without cell multiplication (36). They also found that glycine will replace threonine after a lag period. Moreover, once maximum stimulation had occurred from glycine, no further effect was produced by adding threonine. This indicated to them that glycine is converted into threonine by

yeast, a synthesis which they visualize as occurring through a condensation of glycine and acetaldehyde. They were able to demonstrate that quantities of acetaldehyde ample for such a synthesis were present in the yeast suspension. They suggested two possible schemes for this reaction: either directly by the condensation of acetaldehyde and glycine,



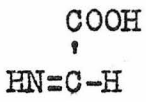
(glycine)

(acetaldehyde)

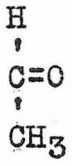


(threonine)

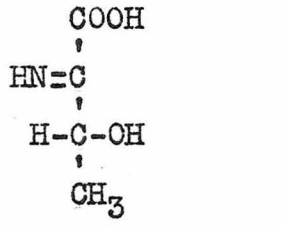
or indirectly by the condensation of iminoacetic acid and acetaldehyde, with the subsequent addition of two hydrogens.



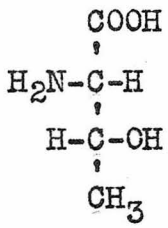
(iminoacetic acid)



(acetaldehyde)



(alpha-imino-beta-hydroxy-n-butyric acid)



(threonine)

The authors found that glycine produced factor Z activity that could be interpreted as a 60-70% conversion of glycine to l(-)threonine. However, they could not isolate threonine or obtain any evidence for the existence of a substance showing factor Z activity in the medium after glycine addition. They explained that this failure might be attributed to synthesis and retention of threonine within the cells.

More recently, in 1946, Fanelli showed that glycine is actually converted into l(-)threonine by yeast (37). He demonstrated this synthesis by incubating yeast suspensions for two hours with and without glycine and determining the threonine content chemically. He also reported that pantothenic acid stimulated the synthesis of threonine. This is in agreement with the observation of Kögl and Borg that pantothenic acid was one of several vitamins that exerts a synergistic effect on factor Z stimulation by threonine or glycine.

GENETIC EXPERIMENTS AND RESULTS

A. Heterocaryon Tests

In order to classify the threonineless mutants genetically, heterocaryons of the same sex were made on minimal agar in growth tubes as described by Beadle and Coonradt. Most of the threonineless mutant strains do not appear to form vigorous heterocaryons. Attempts to show genetic differences by this means were unsuccessful except in the case of 44104 and 35423 and then only in one of six attempts. The results of the successful heterocaryon formation are shown in Table 1. Heterocaryons in liquid culture medium as described by Doermann were tried without success (38).

TABLE 1

Heterocaryon Formation Between 44104 and 35423 on Solid Minimal Medium

Inoculum	Growth Rate mm./hr.
44104A	1.1
35423A	1.5
44104A + 35423A	2.7

B. Outcross to Wild Type

The five threonineless mutants were crossed with wild type or with a morphological marker strain. Ascospore derived

cultures from these crosses were tested for the threonineless character by the transfer of a small amount of conidia to minimal medium. Those cultures which grew on minimal medium were considered to be wild type; those which did not grow on minimal medium were considered to be threonineless. Cultures from several asci of each mutant outcross were tested quantitatively by growth in flasks containing relevant supplements. The similarity of their biochemical characteristics to those of the parental threonineless and wild type strains were thereby checked.

The results obtained in crosses of threonineless mutants, shown in Table 2, indicate that each of the five mutants differs in its threonineless character from the wild type by a single gene.

C. Crosses Between Threonineless Mutants

The ten possible intercrosses between the five mutants were made to test for genetic differences with respect to their threonine requirements. The results are shown in Table 3. The crosses of mutants 35423 x 44104 and 44105 x 46003 gave only asci in which all spores were mutant. This result indicates that the genes involved are either the same or closely linked.

D. Proof of Genetic Difference by Outcrossing Double Mutants

Proof of genetic difference by recovery of the two original mutants from a double mutant was obtained for 35423-46003,

TABLE 2

Types of Asci Resulting from Outcrosses

of Threonineless Mutant Strains

35423A x 34508a

thr = threonineless
aur = aurescent, a sex-linked morphological character
\+ = wild type allele of aur or thr

Spore Pairs								Number
1st		2nd		3rd		4th		of Asci
+	aur	+	aur	thr	+	thr	+	1
thr	+	thr	+	+	aur	+	aur	3
+	+	+	+	thr	aur	thr	aur	1
thr	aur	thr	aur	+	+	+	+	3
+	+	+	aur	thr	+	thr	aur	1
thr	aur	thr	+	+	aur	+	+	3
thr	aur	thr	+	+	+	+	aur	1
thr	+	thr	aur	+	+	+	aur	1
thr	+	thr	aur	+	aur	+	+	1
+	+	thr	+	+	aur	thr	aur	1
thr	aur	+	aur	+	+	thr	+	1
+	aur	thr	+	+	aur	thr	+	1
thr	+	+	aur	thr	+	+	aur	1
+	+	thr	aur	+	+	thr	aur	1
								20

	No. Asci
thr segregated 1st division	15
thr segregated 2nd division	5
aur segregated 1st division	10
aur segregated 2nd division	10
both segregated 1st division	8
of these eight,	
parental combinations	4
new combinations	4

Conclusion: thr-35423 segregates as a single gene; it and aur are not linked; therefore, thr-35423 is not on sex chromosome.

Table 2 (Continued)

35423A x 12a

thr = threonineless
\+ = wild type allele of threonineless

Spore Pairs

1st	2nd	3rd	4th	Number of Asci
+	+	thr	thr	8
thr	thr	+	+	13
+	thr		thr	1
				22

	No. Asci
thr segregated 1st division	21
thr segregated 2nd division	1

Conclusion: thr-35423 segregates as a single gene

44104A x 34508a

thr = threonineless
aur = aurescent
\+ = wild type allele of aur or thr

Spore Pairs

1st		2nd		3rd		4th		Number of Asci
+	aur	+	aur	thr	+	thr	+	4
thr	+	thr	+	+	aur	+	aur	5
+	+	+	+	thr	aur	thr	aur	4
thr	aur	thr	aur	+	+	+	+	5
+	+	+	aur	thr	+	thr	aur	10
+	aur	+	+	thr	+	thr	aur	7
+	aur	+	+	thr	aur	thr	+	7
+	+	+	aur	thr	aur	thr	+	3
thr	aur	thr	+	+	aur	+	+	4
thr	aur	thr	+	+	+	+	aur	8
thr	+	thr	aur	+	+	+	aur	3
thr	+	thr	aur	+	aur	+	+	9
								69

	No. Asci
thr segregated 1st division	69
thr segregated 2nd division	0
aur segregated 1st division	18
aur segregated 2nd division	51
both segregated 1st division	18
of these eighteen,	
parental combinations	9
new combinations	9

Conclusion: thr-44104 segregates as a single gene; it and aur are not linked.

Table 2 (Continued)

44104A x 5531a*

thr = threonineless
pnt = pantothenicless-5531
\+ = wild type allele of thr or pnt

Spore Pairs

1st		2nd		3rd		4th		Number of Asci
pnt	+	pnt	+	+	thr	+	thr	7
+	thr	+	thr	pnt	+	pnt	+	2
pnt	thr	pnt	thr	+	+	+	+	1
+	+	+	+	pnt	thr	pnt	thr	4
pnt	thr	+	thr	+	+	pnt	+	2
pnt	+	+	+	+	thr	pnt	thr	1
pnt	thr	+	thr	pnt	+	+	+	1
								18

	No. Asci
thr segregated 1st division	18
thr segregated 2nd division	0
pnt segregated 1st division	14
pnt segregated 2nd division	4
both segregated 1st division	14
of these fourteen,	
parental combinations	9
new combinations	5

Conclusion: thr-44104 and pnt are not linked; therefore, thr-44104 is not in the 4th chromosome.

* Mary Houlahan dissected all of these asci.

TABLE 2 (Continued)

44104A x 38701, 15300a*

thr = threonineless
alb = albino-2 (15300)
\+ = wild type allele of thr or alb

Spore Pairs								Number of Asci
1st		2nd		3rd		4th		
alb	thr	alb	thr	+	+	+	+	1
+	thr	alb	thr	alb	+	+	+	4
alb	+	+	+	alb	thr	+	thr	3
alb	thr	+	thr	+	+	alb	+	1
alb	thr	+	thr	alb	+	+	+	1
alb	+	alb	+	+	thr	+	thr	1
alb	+	+	+	+	thr	alb	thr	2
+	+	alb	+	+	thr	alb	thr	1
								14

	No. Asci
thr segregated 1st division	14
thr segregated 2nd division	0
alb segregated 1st division	2
alb segregated 2nd division	12
both segregated 1st division	2
of these two,	
parental combinations	1
new combinations	1

Conclusion: thr-44104 is not linked with alb-15300.

*Segregations for 38701, adenineless, not reported.

Table 2 (Continued)

44104A x 12a*

thr = threonineless
\+ = wild type allele of thr

Spore Pairs

1st	2nd	3rd	4th	Number of Asci
+	+	thr	thr	7
thr	thr	+	+	4
thr	+	thr	+	5
thr	+	+	thr	4
+	thr	thr	+	2
				22

	No. Asci
thr segregated 1st division	11
thr segregated 2nd division	11

Conclusion: thr-44104 segregates as a single gene.

* Mary Houlahan dissected 14 of these asci.

Table 2 (Continued)

44105a x 34508A

thr = threonineless
aur = aurescent
\+ = wild type allele of thr or aur

Spore Pairs

1st		2nd		3rd		4th		Number of Asci
+	aur	+	aur	thr	+	thr	+	1
thr	+	thr	+	+	aur	+	aur	1
+	+	+	+	thr	aur	thr	aur	2
thr	aur	thr	aur	+	+	+	+	1
+	+	+	aur	thr	+	thr	aur	1
+	aur	+	+	thr	+	thr	aur	1
+	aur	+	+	thr	aur	thr	+	1
+	+	+	aur	thr	aur	thr	+	2
thr	aur	thr	+	+	aur	+	+	1
thr	aur	thr	+	+	+	+	aur	4
thr	+	thr	aur	+	+	+	aur	3
thr	+	thr	aur	+	aur	+	+	4
thr	aur	+	+	thr	aur	+	+	1
								23

	No. Asci
thr segregated 1st division	22
thr segregated 2nd division	1
aur segregated 1st division	5
aur segregated 2nd division	18
both segregated 1st division	5
of these five,	
parental combinations	2
new combinations	3

Conclusion: thr-44105 segregates as a single gene; it and aur are not linked.

Table 2 (Continued)

46003A x 34508a

thr = threonineless
aur = aurescent
\+ = wild type allele of thr or aur

Spore Pairs

1st		2nd		3rd		4th		Number of Asci
+	aur	+	aur	thr	+	thr	+	2
thr	+	thr	+	+	aur	+	aur	6
+	+	+	+	thr	aur	thr	aur	8
thr	aur	thr	aur	+	+	+	+	6
+	+	+	aur	thr	+	thr	aur	5
+	aur	+	+	thr	+	thr	aur	5
+	+	+	aur	thr	aur	thr	+	3
thr	aur	thr	+	+	aur	+	+	1
thr	aur	thr	+	+	+	+	aur	2
thr	+	thr	aur	+	+	+	aur	2
thr	+	thr	aur	+	aur	+	+	2
+	+	thr	aur	thr	+	+	aur	1
thr	+	+	+	+	aur	thr	aur	1
+	aur	+	+	thr	aur	thr	+	1
								45

	No. Asci
thr segregated 1st division	43
thr segregated 2nd division	2
aur segregated 1st division	23
aur segregated 2nd division	22
both segregated 1st division	22
of these twenty-two,	
parental combinations	8
new combinations	14

Conclusion: thr-46003 segregated like a single gene; it and aur are not linked.

Table 2 (Concluded)

51504A x 34508a

thr = threonineless
aur = aurescent
\+ = wild type allele of thr or aur

Spore Pairs

1st		2nd		3rd		4th		Number of Asci
+	aur	+	aur	thr	+	thr	+	9
thr	+	thr	+	+	aur	+	aur	20
+	aur	thr	+	+	aur	thr	+	10
+	aur	thr	+	thr	+	+	aur	7
thr	+	+	aur	+	aur	thr	+	13
thr	+	+	aur	thr	+	+	aur	8
								67

	No. Asci
thr segregated 1st division	29
thr segregated 2nd division	38
aur segregated 1st division	29
aur segregated 2nd division	38
both segregated 1st division	29
of these twenty-nine,	
parental combinations	29
new combinations	0

Conclusion: thr-51504 segregated as a single gene; it and aur are closely linked; therefore, thr-51504 is on the sex chromosome.

TABLE 3

Crosses Between Threonineless Strains

Types of Asci

Ascus type	Spore Pairs 1st	2nd	3rd	4th
1.	+	+	thr	thr
2.	thr	thr	+	+
3.	+	thr	+	thr
4.	+	thr	thr	+
5.	thr	+	+	thr
6.	thr	+	thr	+
7.	+	thr	thr	thr
8.	thr	+	thr	thr
9.	thr	thr	+	thr
10.	thr	thr	thr	+
11.	thr	thr	thr	thr

Frequency of Ascus Types

Cross	1	2	3	4	5	6	7	8	9	10	11	Total No. Asci
35423 x 44104											34	34
35423 x 44105	4	7					1		1		12	25
35423 x 46003	4	4	1			1	1				5	16
35423 x 51504	2	2	1	1	4	1				1	4	16
44104 x 44105	5	7							1		4	17
44104 x 46003	10	9						3			5	27
44104 x 51504	1	2				1	4	6		2	5	21
44105 x 46003											24	24
44105 x 51504	1						5	2	2	2	3	15
46003 x 51504		2			1		5	2	3	4	3	20

44104-46003, 44104-51504, and 46003-51504.

E. Evidence of Linkages

The linkages indicated by the genetic crosses are shown in Table 4.

Table 4

Summary of Linkages from Crosses of Threonineless Mutants

Mutant	Linkages
35423	Not sex linked. Linked to 44104.
44104	Not sex linked. Linked to 35423.
44105	Not sex linked. Genetically the same as 46003.
46003	Not sex linked. Not in same chromosome as 35423 and 44104.
51504	Shows sex linkage. Close to aurescent.

F. Special Considerations

1) Evidence for genetic difference between 35423 and 44104.--The biochemical difference in the responses of strains 44104 and 35423 as well as the vigorous heterocaryon formation indicate that the two are not mutations of the same gene. The centromere distances calculated from the outcrosses of each of these strains are shown in Table 5. The recovery of only asci showing eight mutant spores among 34 asci from the cross of 35423 x 44104 would indicate that the two are genetically the

TABLE 5

Centromere Distances from Outcrosses of 35423 and 44104

	No. Asci	Cm. Distance
Mutant 35423		
35423A x 34508a	20	13
35423A x 12a	22	2
Mutant 44104		
44104A x 34508a	69	0
44104A x 12a	22	25
44104A x 5531a	18	0
44104A x 15300a	15	0

same. However, the fact that 44104 gave a centromere distance of 25 units in one cross and 0 in three others suggests that strain 44104 carries a crossover suppressor, perhaps a chromosomal aberration. An alternative to this is that strains 34508, 5531 and 15300, and also 35423, carry crossover suppressors.

When crossed with 12a, strain 35423 showed a centromere distance of 2 units. This appears to be a reduction or incomplete suppression of crossing over when compared to the value of 13 units obtained from a comparable sample of asci in the cross of 35423 with 34508.

2) Evidence for the cnr gene and the genetic identity of strains 46003 and 44105.--Strains 46003 and 44105, although apparently identical by genetic test, differed in their growth response to the amino acid canavanine. Since the growth of several wild type strains was shown to be inhibited by canavanine (Horowitz, unpublished), the utilization of this compound as a substitute for threonine was surprising. In asci resulting from the outcross of 46003, the ability to resist canavanine and the threonineless character segregated independently as single gene differences. Thus, the following new types were derived:

canavanine resistant strains able to synthesize threonine

and

threonineless strains unable to utilize canavanine

Ascus types for the cross are shown in Table 6. The genetic character which seems to determine resistance to the amino acid canavanine has been named "canavanine resistant", abbreviated "cnr." The discovery of this gene for canavanine resistance prompted a check of the two wild type strains 4A and 25a from which mutant 46003 had been derived. Strain 25a was inhibited by canavanine. Strain 4A was found to be resistant to canavanine and was thus very probably the source of the cnr gene in strain 46003.

Table 6

Ascus Types Obtained from a Cross of 46003

Analyzed for Canavanine Resistance

46003A x 34508a

cnr = canavanine resistant
aur = aurescent
thr = threonineless
\+ = wild type allele of cnr, aur or thr

Spore Pairs												Number of Asci
1st			2nd			3rd			4th			
cnr	+	+	+	aur	+	+	aur	thr	cnr	+	thr	2
cnr	+	+	cnr	+	+	+	aur	thr	+	aur	thr	2
+	aur	thr	+	aur	thr	cnr	+	+	cnr	+	+	2
cnr	+	+	+	aur	+	cnr	+	thr	+	aur	thr	3
cnr	+	+	+	aur	thr	cnr	+	thr	+	aur	+	1
+	aur	thr	cnr	+	thr	cnr	+	+	+	aur	+	1
cnr	+	thr	cnr	+	+	+	aur	+	+	aur	thr	1
+	aur	+	cnr	+	+	cnr	+	thr	+	aur	thr	1
+	aur	+	cnr	+	+	+	aur	thr	cnr	+	thr	1
cnr	+	thr	+	aur	thr	+	aur	+	cnr	+	+	1
cnr	+	thr	cnr	+	thr	+	aur	+	+	aur	+	1
cnr	+	thr	+	aur	thr	cnr	+	+	+	aur	+	1
												17

	No. Asci
cnr segregated 1st division	6
cnr segregated 2nd division	11
aur segregated 1st division	6
aur segregated 2nd division	11
thr segregated 1st division	15
thr segregated 2nd division	2
both cnr and thr segregated 1st division	5
of these five, parental combinations	1
of these five, new combinations	4
both cnr and aur segregated 1st division	6
of these six, parental combinations	6
of these six, new combinations	0

Conclusion: thr-46003 and cnr are not linked; cnr and aur are closely linked.

BIOCHEMICAL EXPERIMENTS AND RESULTS

A. Methods

The growth responses of threonineless mutant strains to various substances were measured in terms of the dry weight of mycelium. The mold was cultured in 20 ml. of medium contained in 125 ml. Erlenmeyer flasks at 25° for 72 hours. Mycelial pads were collected on a buchner funnel, dried at 90-100° for eight hours, and weighed. The medium and procedure are the same as those described by Bonner and Beadle (39).

B. Growth Curves

The responses of the five threonineless mutant strains to added threonine are plotted in Figs. 1-5. These figures show that four strains, 35423, 44104, 44105 and 46003, but not strain 51504, can be made to grow almost normally (i.e., from 70-100% of wild type growth on minimal medium) if supplied with sufficient threonine.

C. Responses to Single Amino Acids

The five mutant strains were tested for growth on each of twenty-six alpha-amino acids to determine the specificity of their response. The results of these experiments are shown in Table 7. On the basis of growth responses to single amino acids, the mutants can be characterized as follows:

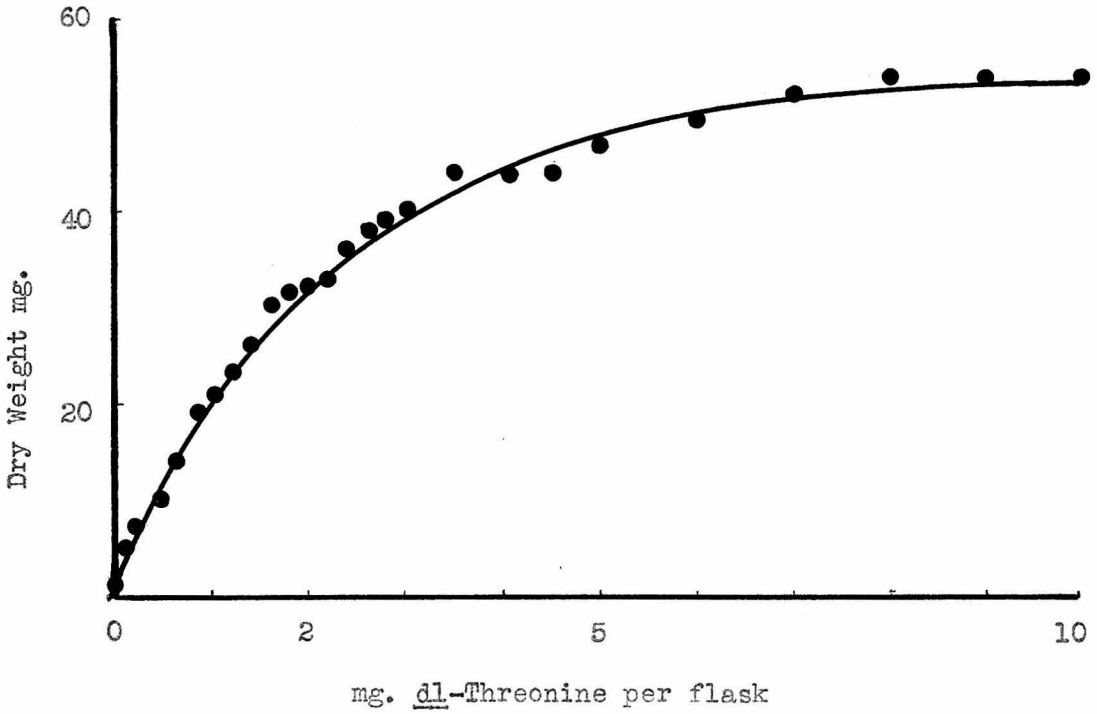


Fig. 1 Growth Response of Mutant 35423 to Threonine

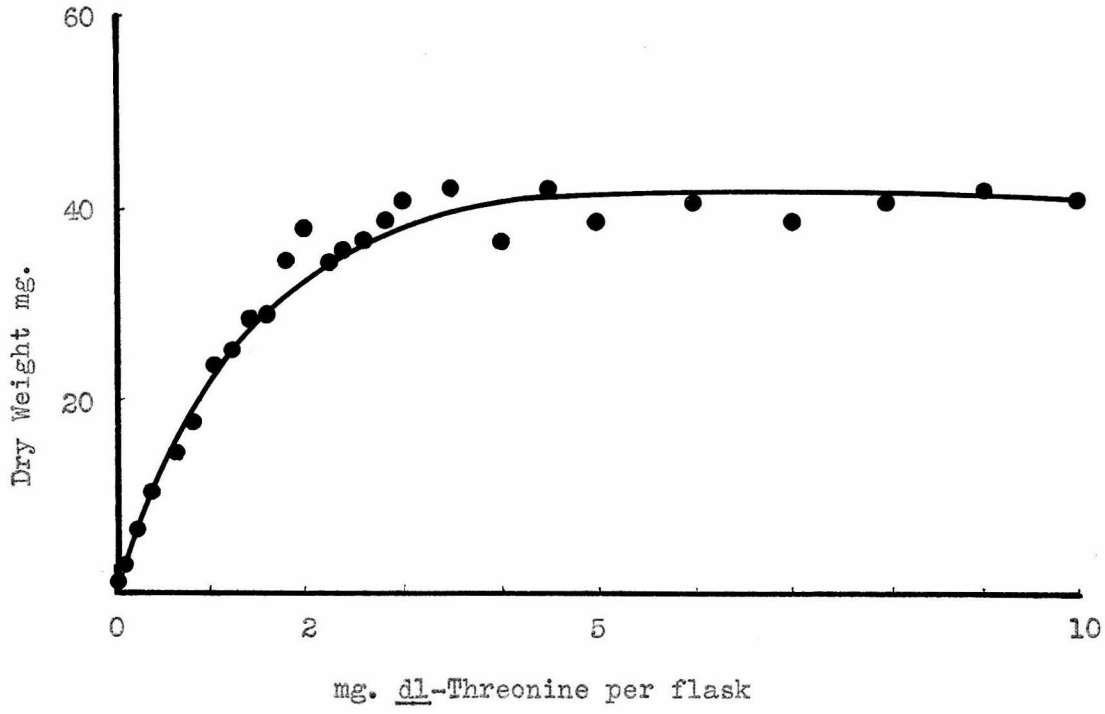


Fig. 1 Growth Response of Mutant 44104 to Threonine

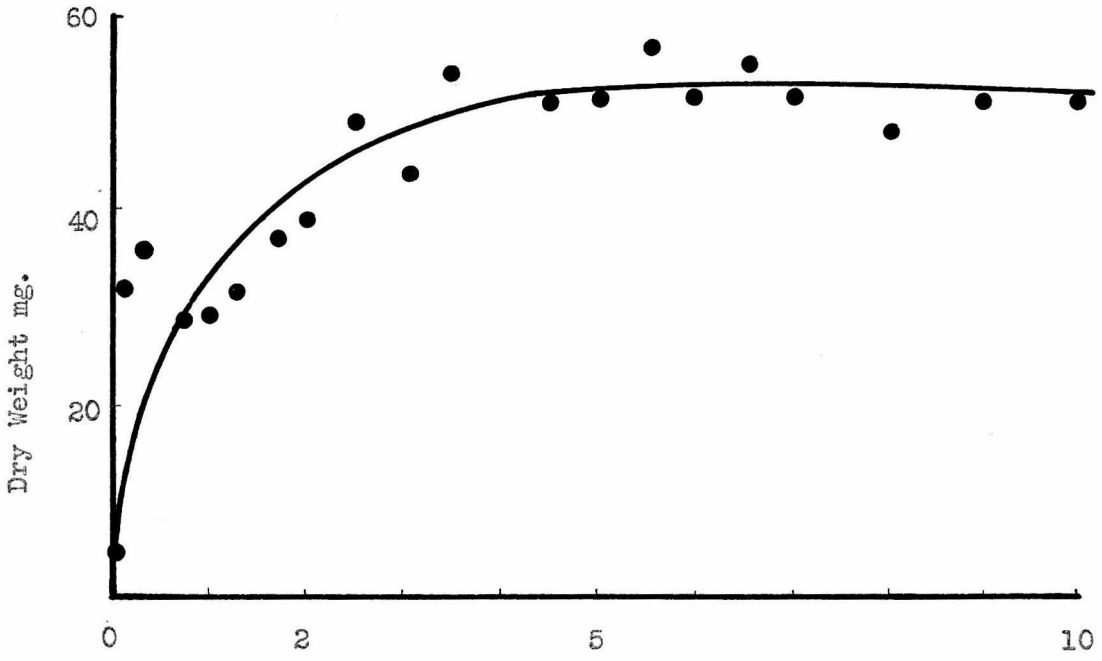


Fig. 3 Growth Response of Mutant 44105 to Threonine

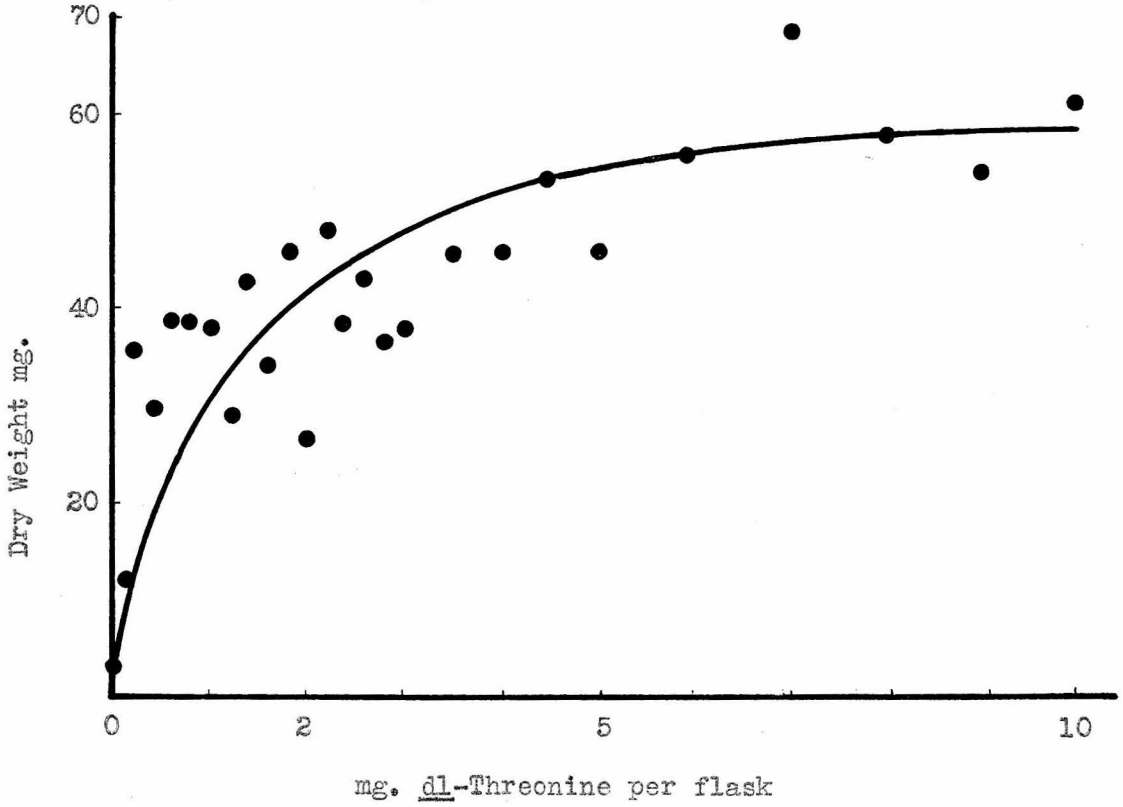


Fig. 4 Growth Response of Mutant 46003 to Threonine

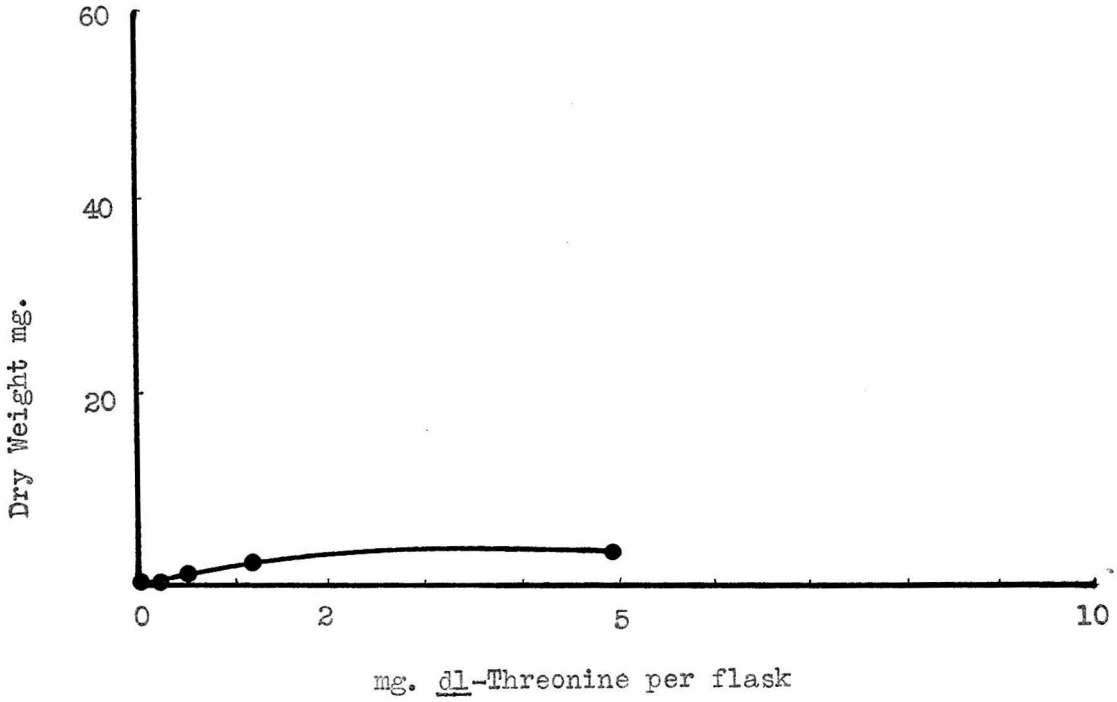


Fig. 5 Growth Response of Mutant 51504 to Threonine

TABLE 7

Growth Responses of Threonineless Mutant Strains to Single Amino Acids

72 Hour Pad Weights in mg. on 1 mg. Amino Acid*

Amino Acid	Mutant Strains 35423	44104	44105	46003	51504
none	0-1	1-2	9-15	14-17	0
dl-isoleucine	1	12	29	36	0
dl-threonine	28	26	26	36	1
dl-methionine	1	3	12	10	0
l-histidine·HCl	1	3	6	6	0
dl-valine	1	1	17	20	0
dl-lysine·HCl	1	2	7	5	0
l-tryptophane	1	0	8	17	0
dl-leucine	1	0	4	10	0
glycine	1	1	15	16	0
dl-alanine	1	3	14	14	0
dl-aspartic acid	1	2	17	13	0
l-proline	1	2	9	13	0
l-arginine·HCl	1	3	17	4	0
l-hydroxyproline	1	1	2	15	0
l-glutamic acid	1	2	18	11	0
dl-serine	1	2	9	12	0
l-tyrosine	1	1	11	11	0
l-cystine	1	1	9	15	0
dl-norleucine	1	1	5	14	0
dl-phenylalanine	1	1	12	15	0
dl-norvaline	1	1	14	14	0
dl-alpha-amino-n-butyric acid	1	18	43	42	0
dl-citrulline	0	1	3	10	0
dl-ornithine·HCl	0	1	1	10	0
l-canavanine·SO_4	0	0	0	35	0
dl-alpha-amino-gamma-hydroxybutyric acid	0	1	35	36	48

*Significant variations from minimal control are underlined.

35423 requires threonine.

44104 utilizes threonine, alpha-aminobutyric acid, or isoleucine.

44105 utilizes threonine, alpha-aminobutyric acid, isoleucine, or alpha-amino-gamma-hydroxybutyric acid; it shows some growth on minimal medium.

46003 utilizes threonine, alpha-aminobutyric acid, isoleucine, alpha-amino-gamma-hydroxybutyric acid, or canavanine; some growth is obtained on minimal medium.

51504 requires alpha-amino-gamma-hydroxybutyric acid, but grows to a slight extent on threonine.

D. Growth of Strains on Minimal Medium

Table 7 showed that strains 35423, 44104 and 51504 grew very slightly or not at all on unsupplemented minimal medium. However, strain 46003 gave considerable growth on minimal medium. Since growth on minimal medium was not visible before approximately sixty hours had elapsed, it is suggested that 46003 adapted to growth in the absence of threonine. An indication of the extent of this adaptation is shown by a comparison of the growth on minimal medium for the four genetically different strains, as follows:

Pad Weight in Mg.

Mutant	After 3 days	After 7 days
35423	1	11
44104	1	21
46003	8	94
51504	0	1

Mutant 46003 appeared to have a greater capacity for growth on minimal medium than did the other strains, but it did not reach the amount of growth shown by wild type.

In most experiments mutant 35423 gave low minimal control pad weights. However, several times 35423 became altered so that it grew on minimal medium. No type 35423 ascospores were recovered from asci of crosses made with a stock of 35423 that grew on minimal medium. This finding indicates that strain 35423 must have mutated or become contaminated. A similar change occurred in a 35423 stock carrying a morphological marker gene; therefore, it seems more likely that the change represented a mutation than a contamination.

E. Responses to Threonine Plus Single Amino Acids

With the view toward finding possible stimulating or inhibiting effects of other amino acids, experiments were carried out in which the mutants were tested on threonine plus each of the twenty-six amino acids singly. These results are shown in Table 8; they can be summarized as follows: strain 35423 responds to many amino acids once it is supplied with threonine. Strain 35423 is strongly inhibited by phenylalanine. The addition of threonine does not allow strains 44104, 44105 or 46003 to utilize any amino acids that they do not grow on in the absence of threonine. Strain 51504 utilizes threonine plus alpha-amino-gamma-hydroxybutyric acid, as expected, and utilizes methionine when present with threonine.

TABLE 8

Growth Responses of Threonineless Mutant Strains to Amino Acids plus Threonine

72 Hour Pad Weight in mg. on 1 mg. Amino Acid plus 1 mg. dl-threonine

Amino Acid in addition to 1 mg. dl-threonine	Mutant Strain 35423	44104	44105	46005	51504
none	28	26	26	36	1
dl-isoleucine	33	42	51	48	1
dl-threonine	40	47	40	38	1
dl-methionine	41	47	30	25	48
l-histidine·HCl	39	27	35	35	1
dl-valine	38	31	36	40	1
dl-lysine·HCl	26	26	32	42	1
l-tryptophane	28	20	31	26	1
dl-leucine	27	27	26	23	1
glycine	31	44	31	35	1
dl-alanine	30	33	31	38	1
dl-aspartic acid	24	26	35	37	1
l-proline	30	26	35	37	1
l-arginine·HCl	30	26	28	45	1
l-hydroxyproline	25	30	33	38	1
l-glutamic acid	26	29	29	39	1
dl-serine	26	30	30	39	1
l-tyrosine	37	42	25	32	1
l-cystine	26	26	33	45	1
dl-norleucine	29	38	26	26	1
dl-phenylalanine	15	33	23	23	1
dl-norvaline	30	38	28	22	1
dl-alpha-amino-n-butyric acid	41	44	50	49	1
dl-citrulline	22	21	13	23	0
dl-ornithine·HCl	25	31	17	31	1
l-canavanine·SO_4	20	25	0	34	0
dl-alpha-amino-gamma-hydroxybutyric acid	1	9	36	42	41

Mutant 35423 responded to so many other amino acids when they were supplied in addition to threonine that an experiment was carried out to determine the threshold of this stimulation. The results (not reported in detail) showed that none of the other amino acids added to a basal supplement of 0.01 or 0.1 mg. of dl-threonine per flask gave over 2 mg. of mycelium. Thus, it appears that mutant 35423 is stimulated by a number of other amino acids when sufficient threonine is present to allow for at least moderate growth.

F. Responses to Threonine Stereoisomers

The growth responses of threonineless mutants to threonine stereoisomers are shown in Table 9. These experiments indicate that only the natural or l(-) form of threonine has appreciable growth-promoting activity.

G. Responses to Keto-Acid Analogs

The responses of threonineless mutants were determined for several keto-acid analogs of growth-promoting amino acids. The results are shown in Table 10. None of the strains utilized alpha-keto-beta-hydroxy-n-butyric acid, the keto-acid analog of threonine; only strain 46003 utilized alpha-keto-n-butyric acid, the keto acid analog of alpha-amino-butyric acid or alpha-keto-beta-ethyl-n-butyric acid, the keto-acid analog of isoleucine.

H. Amino Acid Inhibitions

As Table 8 showed, phenylalanine was inhibitory to mutant 35423.

TABLE 9

Response to dl-Threonine and the Four Stereoisomeric Alpha-amino-beta-hydroxy-n-butyric acids in Amounts of 1 Mg. per Flask

Mutant	72 Hr. Pad Wt. Mg. Minimal Control	dl-Thr.	l(-) Thr.	d(+) Thr.	d-Allo-Thr.	dl-Allo-Thr.
35423	0	32	50	0	0	0
44104	3	18	21	4	4	8
46003	3	18	25	3	1	7
51504	1	2	1	2	3	2
51504*	2	35	35	2	2	1

*With 1 mg. dl-methionine per flask

TABLE 10

Response of Threonineless Mutants to Keto-acid Analogs of Threonine, Isoleucine and Alpha-aminobutyric Acid*

Mutant	72 Hr. Pad Wt. Mg. 1 mg. amounts per flask			
	Minimal Control	Keto-thr.**	Keto-isol.	Keto-aminobut.**
35423	0	0	0	0
44104	0	0	0	0
46003	17	23	41	47
51504	0	0	0	0
51504/	0	0	0	0

*The keto-acid analogs referred to are: alpha-keto-beta-hydroxy-n-butyric acid, the analog of threonine; alpha-keto-beta-ethyl-n-butyric acid, the analog of isoleucine; and alpha-keto-n-butyric acid, the analog of alpha-amino butyric acid.

**Sterilized by filtration.

/With 1 mg. dl-methionine added per flask.

High ratios of methionine to threonine were found to be inhibitory to mutants 35423, 46003 and 51504, but not to 44104, as shown in Table 11. Growth of mutant 51504, which requires methionine plus threonine or else alpha-amino-gamma-hydroxybutyric acid alone, is inhibited when the ratio of the molar concentration of methionine to that of threonine exceeds about 0.8 to 1. This inhibition is shown for several concentrations of each amino acid in Table 12. No explanation is offered for this inhibition. Many others have been reported in microbiological literature and two somewhat similar inhibitions have been found in other Neurospora mutants; the inhibition of lysineless strains by arginine has been reported by Doermann (40) and the inhibition of a serineless strain by asparagine, by Hungate (41).

TABLE 11

The Effect of High Ratios of Methionine to Threonine on the Growth of Threonineless Mutants

	72 Hr. Pad Weights in Mg.		
Mutant	Minimal Control	1 mg. dl-threonine	1 mg. dl-threonine plus 5 mg. dl-methionine
35423	1	24	3
44104	1	22	32
46003	6	45	15
51504	0	0	0
51504*	0	47	0

*With 1 mg. dl-methionine added per flask.

TABLE 12

The Effect on Mutant 51504 of Varying Threonine and Methionine Concentrations

Mg. 72 Hour Pad Weight

dl-Threonine per flask	Mg. dl-Methionine/flask 0	.1	1	5	10
0	0	0	0	0	0
.1	0	0	0	0	0
.5	0	32	1	0	0
1	1	40	47	0	0
2	2	47	56	1	0
5	2	58	62	64	2
10	3	59	59	53	50

I. Growth Requirements of Strain 51504

Experiments were designed to test for possible substitution of the threonine and methionine portions of the requirement of 51504. The same series of twenty-six amino acids was tried with a basal supplement of 1 mg. of methionine to test for threonine substitution. Only one of the twenty-six amino acids substituted for threonine, that being the expected alpha-amino-gamma-hydroxybutyric acid. A number of compounds, in addition to the twenty-six amino acids shown on page 29, were tested for possible methionine substitution. The results, shown in Table 13, indicate that homocysteine, cystathionine, methionine sulfoxide and the keto-acid analog of methionine are able to replace methionine. In addition, several compounds have a slight activity in methionine replacement. This is probably due to the sparing of methionine as a methyl donor, since choline, methylaminoethanol, and dimethylaminoethanol have slight activity.

J. Biochemical Differences Arising from the cnr Gene

As noted in the presentation of genetic data, strain 46003 was found to carry a gene modifying its biochemical behavior. The gene allowed canavanine to substitute for threonine in supporting growth of the mutant. The outcrosses of 46003 made three stocks available:

46003-thr-cnr - threonineless, canavanine resistant

46003-thr-non-cnr - threonineless, canavanine sensitive

TABLE 13

Tests of Compounds for Methionine Replacement in Growth of Mutant 51504

Compound in 1 mg. Amount	In the Presence of 1 mg. of dl-threonine 72 Hr. Pad Wt. Mg.
none	0
dl-methionine	47
dl-homocysteine (as thiolactone·HCl)	42
dl-homocystine	3
taurine	5
l-cysteine	6
choline	15
aminoethanol	3
methylaminoethanol	15
dimethylaminoethanol	13
dl-methionine-sulfone	0
dl-methionine-sulfoxide	44
keto-methionine (alpha-keto-gamma-methiolbutyric acid)	30
l-cystathionine*	15

*0.5 mg. dl-threonine and 0.25 mg. l-cystathionine used.

T-3-cnr - derived wild type, canavanine resistant (genetic analysis of the ascus in which T-3 occurred proves that it must have the cnr gene derived from the original 46003-thr-cnr strain)

Also, since one parent of the original 46003-thr-cnr was canavanine sensitive and the other canavanine resistant, two strains in addition to the above three were available:

25a - wild type, canavanine sensitive

4A - wild type, canavanine resistant (25a and 4A probably differ by 4A having the cnr gene and 25a lacking it)

TABLE 14

Growth Responses of Five Strains to Canavanine, Canavanine Plus Arginine, and Threonine

Strain	Mg. Supplement to Medium l-canavanine	l-arginine	dl-threonine	72 Hr. Pad Wt. Mg.
46003-thr-cnr	0	0	0	18
	1	0	0	43
	0	0	1	32
	1	5	0	18
46003-thr-non-cnr	0	0	0	21
	1	0	0	0
	0	0	1	38
	1	5	0	27
T-3-cnr	0	0	0	62
	1	0	0	46
	1	5	0	59
	0	5	0	57

TABLE 14 (Continued)

Strain	Mg. Supplement to Medium l-canavanine	l-arginine	dl-threonine	72 Hr. Pad Wt. Mg.
25a	0	0	0	59
	1	0	0	0
	1	5	0	53
	0	5	0	59
4A	0	0	0	74
	1	0	0	64
	1	5	0	63
	0	5	0	65

Table 14 showed that strain 46003-thr-cnr grows on canavanine, but is inhibited when grown on canavanine plus arginine. Strain 46003-thr does not grow on canavanine, but is slightly stimulated by canavanine plus arginine. Strain T-3-cnr grows almost normally in the presence of canavanine. Of the wild type strains, 25a is inhibited by canavanine and strain 4A is resistant to canavanine.

Experiments were carried out with 46003-thr-cnr and 25a in which the amount of arginine was varied but the canavanine held constant. The results shown in Table 15 indicate that the inhibition of canavanine utilization as a threonine substitute by arginine in one case, and the inhibition of growth by canavanine in the other case, varies with the amounts of the two substances present. The growth of 46003-thr-cnr was inhibited by increasing the amount of arginine; the growth inhibition of 25a was relieved by adding arginine.

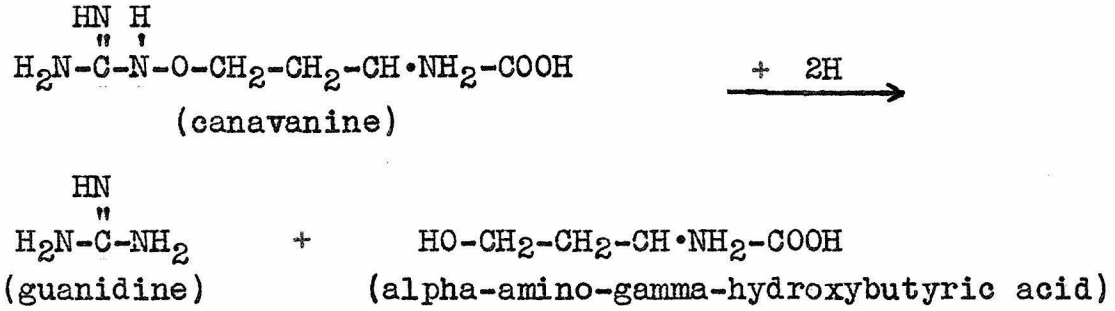
TABLE 15

Growth of Strains 46003-thr-cnr and 25a on Arginine and Canavanine

Strain	mg. l-arginine	mg. l-canavanine	72 Hr. Pad Wt. Mg.
46003-thr-cnr	0	0	18
	0	1	43
	1	1	35
	2	1	30
	3	1	24
	4	1	23
	5	1	18
25a	0	0	65
	0	1	0
	1	1	2
	2	1	34
	3	1	46
	4	1	53
	5	1	58

It can be seen from the growth responses shown in Table 14 that 46003-thr-cnr is not inhibited by canavanine. On the contrary, it utilizes canavanine as a threonine substitute. This utilization of canavanine as a threonine substitute is inhibited by arginine. Strain 46003-thr-non-cnr is inhibited by canavanine; arginine reverses this inhibition to the extent of allowing it to attain the growth that it normally achieves on minimal medium. Strain T-3 is slightly inhibited by canavanine and not affected by canavanine plus arginine. Strain 25a is inhibited by canavanine, but the inhibition is relieved by arginine as previously noted (Horowitz, Teas and Srb, unpublished). This inhibition by canavanine and relief of inhibition by arginine suggests that

canavanine competes with an arginine metabolizing enzyme and that only strains carrying the cnr gene are able to overcome the inhibiting effect of canavanine. The possibility exists that an enzyme is present that splits canavanine to form alpha-amino-gamma-hydroxybutyric acid and guanidine as follows:



The difference between canavanine sensitive and resistant strains might be that one is inhibited by an end product of the reaction. In order to test this possibility, two canavanine resistant and two sensitive strains were tested on guanadine and alpha-amino-hydroxybutyric acid, as shown in Table 16. Neither compound was found to be differentially inhibitory.

K. Tests of Glycine and Acetaldehyde

Because of the suggestion of Kogl and Borg (36) that threonine is synthesized from glycine and acetaldehyde, an experiment was carried out to test the threonineless mutants for growth response to glycine and/or acetaldehyde. These results, all negative, are shown in Table 17.

TABLE 16

Growth of Canavanine Sensitive and Resistant Strains
In the Presence of Guanidine and
Alpha-amino-gamma-hydroxybutyric Acid

Strain	Supplement to Minimal Medium: dl-threonine	Guanidine Carbonate	Alpha-amino-gamma-hydroxybutyric acid	72 Hr. Pad Wt. Mg.
46003-thr-cnr	0	0	0	8
	1	0	0	25
	1	1	0	28
	0	0	1	29
46003-thr-non-cnr	0	0	0	18
	1	0	0	34
	1	1	0	33
	0	0	1	23
4A	0	0	0	58
	0	1	0	55
	0	0	1	57
25a	0	0	0	56
	0	1	0	52
	0	0	1	52

TABLE 17

Growth Responses of Threonineless Mutants to Glycine and/or Acetaldehyde*

Mutant	Mg. Supplement Minimal Medium		72 Hr. Pad Wt. Mg.
	Glycine	Acetaldehyde*	
35423	0	0	1
	1	0	0
	0	1	1
	1	1	1
44104	0	0	2
	1	0	1
	0	1	1
	1	1	2
46003	0	0	5
	1	0	5
	0	1	2
	1	1	2
51504	0	0	0
	1	0	0
	0	1	0
	1	1	0
51504**	0	0	0
	1	0	0
	0	1	0
	1	1	0

*Sterilized by filtration.

**With 1 mg. dl-methionine added per flask.

DISCUSSION

By way of review, the characteristics of the mutant strains under consideration are as follows:

35423 requires threonine.

44104 utilizes threonine, alpha-aminobutyric acid, and isoleucine; is linked with 35423.

44105 appears to be genetically the same as 46003 in its threonineless character; is biochemically the same as 46003 without the cnr modifier.

46003 utilizes threonine, alpha-aminobutyric acid, isoleucine, alpha-amino-gamma-hydroxybutyric acid, and the keto-acid analogs of alpha-aminobutyric acid and isoleucine; grows some on minimal medium.

51504 utilizes alpha-amino-gamma-hydroxybutyric acid or requires both threonine and either methionine, cystathionine, homocysteine or methionine sulfoxide; is closely linked to aurescent on the sex chromosome.

A. Genetics

Genetic evidence as well as biochemical tests on the segregating products indicates that the threonineless characters of the above mutant strains are each differentiated from wild type by a single gene. Conclusive evidence was obtained that 35423 and 46003 differ genetically, as do 44104 and

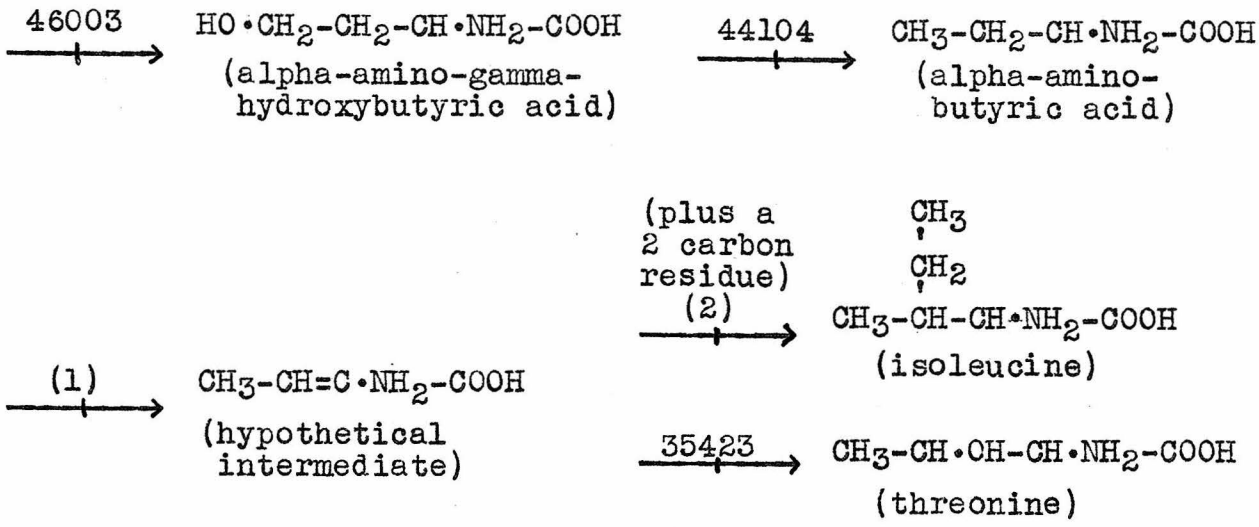
46003; 44104 and 51504; and 46003 and 51504. In addition, heterocaryon tests indicate that strains 35423 and 44104 are genetically different.

Strains 35423 and 44104, although different biochemically and different by heterocaryon test, did not give rise to any double mutant types. It is suggested that in the crosses of mutant 44104 to 34508, to 5531, and to 15300 as well as to 35423, crossing over is suppressed by the same mechanism as in the cross of 35423 to 12. In the cross of 44104 to 12, the zygote would be homozygous for the suppressor. This suppressor might be a chromosomal aberration. In the cross of 35423 to 34508, the zygote would be homozygous normal. The two crosses in which there is no suppression of crossing over, therefore, give approximately true values of 25 units and 13 units for the centromere distances of 44104 and 35423 respectively. The suggestion that 44104 and 12 contain an aberration which suppresses crossing over receives some support from the fact that one parent of 44104 was a sister isolate of 12.

By way of precedent for this hypothesis, it is to be noted that McClintock, working with Neurospora, found that strain 4637 carries a reciprocal translocation. In this strain unsynapsed portions of the involved chromosomes were frequently observed at pachytene (10). Srb obtained genetic evidence that strain 4637 acts as a crossover suppressor when crossed with a sex linked arginineless strain 29997 (16).

B. Threonine Biosynthesis

The growth responses of mutants 35423, 44104, and 46003 give some suggestion of the means of threonine biosynthesis. Strain 35423 is able to utilize only threonine. This indicates that the genetic block in 35423 is close to threonine. Strain 44104 utilizes alpha-aminobutyric acid and isoleucine in place of threonine, which indicates that the block in 44104 involves a step prior to the one involved in the block of 35423. Strain 46003 utilizes threonine, alpha-aminobutyric acid, isoleucine, alpha-amino-gamma-hydroxybutyric acid, and the alpha-keto-acid analogs of isoleucine and alpha-aminobutyric acid, so that the genetic block in 46003 must involve a step prior to that of either 44104 or 35423. The utilization of the alpha-keto-acid analogs of isoleucine and alpha-aminobutyric acid by 46003 and not by 44104 suggests that the amination of a four carbon alpha-keto-acid occurs before the block in 44104 and after the block of 46003. The failure of the alpha-keto-acid analog of threonine to stimulate growth in any mutant probably indicates that it is not an intermediate. The utilization of isoleucine, alpha-aminobutyric acid, or threonine by 44104 indicates that these three compounds have a common precursor. If 44104 could make any one of the three compounds, the mutant would presumably not require threonine. A scheme into which these observations have been incorporated but which does not take into consideration mutant 51504 is as follows:



Steps (1) and (2) are ones for which no mutant has been found in Neurospora. Inasmuch as strain 46003 will utilize four carbon alpha-keto-acids that 44104 will not use, and 46003 will grow on alpha-amino-gamma-hydroxybutyric acids which will not support the growth of 44104, it seems probable that at least one more step belongs between the blocks 46003 and 44104.

The unsaturated four carbon amino acid postulated as an intermediate appears to have basis in fact. Chargaff and Sprinson reported that several bacterial enzyme preparations showed hydroxy-amino acid deaminase activity (42). They found that four bacteria, as well as mouse, rat or rabbit liver, would convert serine,

$HO{\cdot}CH_2{-}CH{\cdot}NH_2{-}COOH$, to pyruvic acid, $CH_3{-}C{:}O{-}COOH$. The reaction was inhibited by O-methyl serine. They suggested, therefore, that the steps in the deamination are as follows:

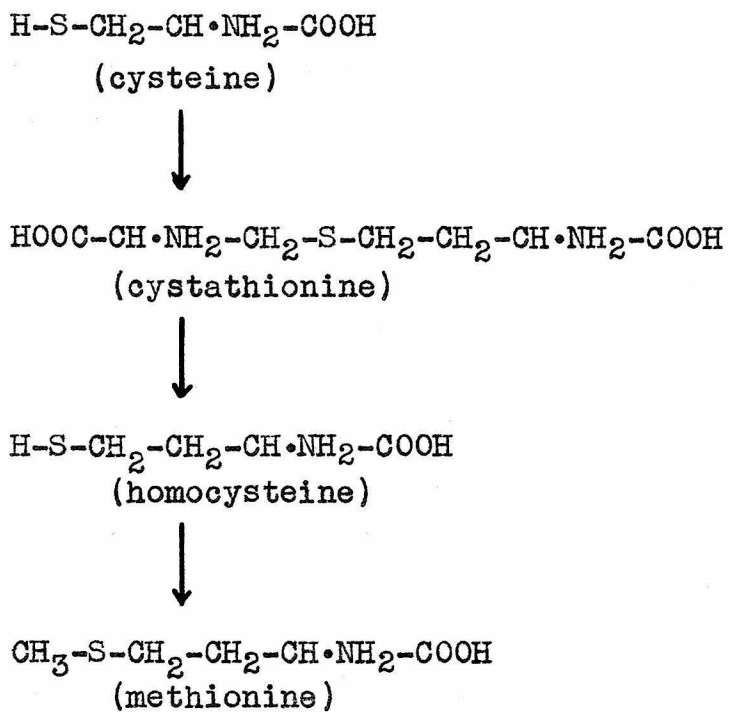
$HO \cdot CH_2\text{-}CH \cdot NH_2\text{-}COOH \xrightarrow{-HOH} CH_2{=}C \cdot NH_2\text{-}COOH \xrightarrow{\text{(rearranges)}}$

$CH_3\text{-}C{:}NH\text{-}COOH \xrightarrow{+HOH} CH_3\text{-}C{:}O\text{-}COOH + NH_3$; and that probably the deamination of threonine proceeds in a similar manner. Either of the intermediates they postulate would be possible threonine precursors by assuming that threonine is synthesized by a reversal of their deamination. This would involve starting with alpha-ketobutyric acid (utilized by 46003) and obtaining threonine as a product.

The possibility that Neurospora synthesizes threonine from glycine and acetaldehyde as suggested by Kögl and Borg (36) does not seem likely. The evidence from threonineless mutants suggests that alpha-ketobutyric acid is a precursor of threonine. If the glycine condensation product were an alternate scheme of threonine synthesis, the mutants would not have been detected because they would have been able to make threonine by another path. If both means of threonine synthesis had been blocked, the strains would have been double mutants and been detected genetically. This was not the case because each was shown to differ from wild type by a single gene. If a glycine condensation product is involved as a precursor of threonine, it would have to be a precursor of alpha-amino-gamma-hydroxybutyric acid.

C. Interrelation of Threonine and Methionine

Horowitz (21) has shown that methionine synthesis involves the following steps:



In 1942 Binkley and du Vigneaud found that rat liver slices would convert homocysteine and serine to cysteine (determined as cystine) (43). This suggested that cysteine was formed by transferring the S of homocysteine to serine via a condensation product, cystathionine. Alpha-amino-gamma-hydroxybutyric acid and cysteine would be the two products.

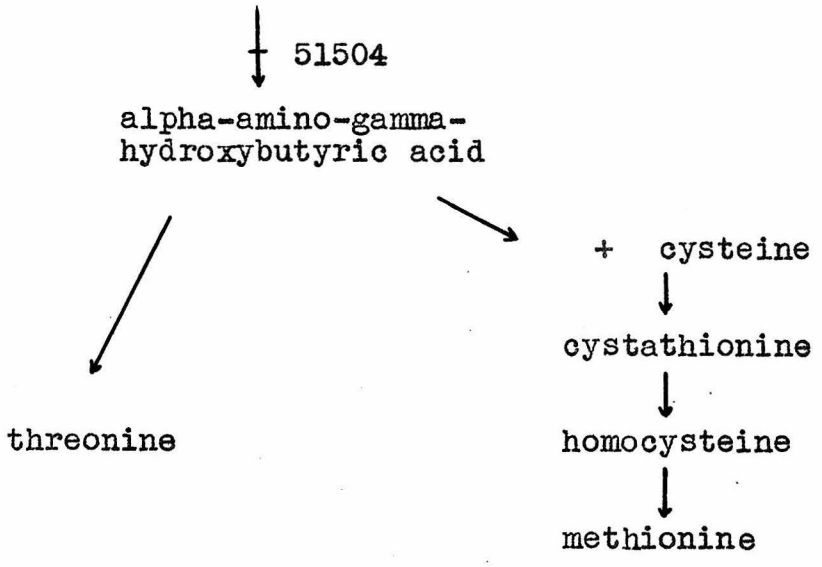
The finding that a single compound, alpha-amino-gamma-hydroxybutyric acid, will substitute for threonine and methionine in mutant 51504, indicates that alpha-amino-gamma-hydroxybutyric acid is a precursor of both threonine and methionine. This would fit into the scheme for methionine synthesis, since alpha-amino-gamma-hydroxybutyric acid might condense with cysteine to form cystathionine by analogy with the reaction of Binkley and du Vigneaud, as follows:

$HOOC-CH\cdot NH_2-CH_2-S-H$ (cysteine)

$HO\cdot CH_2-CH_2-CH\cdot NH_2-COOH$ (alpha-amino-gamma-hydroxybutyric acid) $\longrightarrow$

$HOOC-CH\cdot NH_2-CH_2-S-CH_2-CH_2-CH\cdot NH_2-COOH$
(cystathionine)

The block in mutant 51504 would then be prior to the formation of alpha-amino-gamma-hydroxybutyric acid,



No scheme has been devised that will explain the growth requirements of all four genetically different mutants.

D. Action of the cnr Gene

Any explanation for the action of the cnr gene must take into account the following observations:

1) a wild type strain with the cnr gene is not inhibited by canavanine.

2) a threonineless-46003 strain with the cnr gene is able to use canavanine as a substitute for threonine.
3) the utilization of canavanine by strain 46003-thr-cnr is inhibited by arginine.

The observations seem to be best explained by assuming that both canavanine sensitive and canavanine resistant strains have an essential arginine metabolizing enzyme (or enzymes) that is inhibited by canavanine. It is known that liver arginase converts canavanine much more slowly than it hydrolyzes arginine (44). The enzyme common to both sensitive and resistant strains might be arginase. The canavanine resistant strains would have a second enzyme, the presence of which is conditioned by the cnr gene. The second enzyme would be absent in the canavanine sensitive strains; it would relieve the canavanine inhibition of the arginine metabolizing enzyme by splitting canavanine and thereby removing it. In this conversion of canavanine it would be inhibited by an excess of arginine. In the special case, 46003-thr-cnr, where a product of canavanine conversion is necessary for the growth of the strain, arginine would inhibit growth by competing for the canavanine converting enzyme.

The following table shows the experimental results and explanations according to this hypothesis:

Strain	Supplement to Minimal Medium: None	Canavanine	Canavanine + Arginine
25a Canavanine sensitive wild type	Growth	No Growth arginine metabolizing enzyme is competitively inhibited	Growth inhibition by canavanine relieved
4A Canavanine resistant wild type	Growth	Growth arginine metabolizing enzyme not inhibited because a canavanine splitting enzyme removes the canavanine	Growth no inhibition expected
46003 Canavanine sensitive threonine-less	No Growth (or slight growth) no source of threonine supplied, so only slight adaptive growth	No Growth no enzyme for converting canavanine to a threonine substitute	No Growth no enzyme for converting canavanine to a threonine substitute
46003 Canavanine resistant threonine-less	No Growth no source of threonine supplied, so only slight adaptive growth	Growth canavanine is split to form a substance that will substitute for threonine	No Growth canavanine splitting enzyme inhibited by arginine, so that threonine substitute is not formed. Slight adaptive growth

The structural similarity of part of the canavanine molecule to alpha-amino-gamma-hydroxybutyric acid, which will function as a substitute for threonine, has suggested that canavanine is converted to alpha-amino-gamma-hydroxybutyric acid in strains having the cnr gene. The conversion of canavanine to alpha-amino-gamma-hydroxybutyric acid has been accomplished chemically through an enzymatically produced intermediate, canaline (45). A test of this hypothesis would be to introduce the cnr gene into mutant 51504. Inasmuch as 51504 is able to utilize alpha-amino-gamma-hydroxybutyric acid for growth, strain 51504-cnr should utilize canavanine in place of threonine and methionine if alpha-amino-gamma-hydroxybutyric acid actually were formed from canavanine. However, the close linkage (no crossovers in 67 asci) between thr-51504 and aurescent on the one hand, and the close linkage (no crossovers in 17 asci) between aurescent and the cnr gene on the other hand, has precluded such a direct test.

It is possible that both canavanine resistant and canavanine sensitive strains have the canavanine splitting enzyme as well as the arginine metabolizing enzyme (enzymes). If this were the case, the inhibition of canavanine might be due, not to the absence of an enzyme to remove the canavanine, but to an end product of canavanine conversion being inhibitory to sensitive strains but not resistant ones. This cannot be the case if the products of canavanine split-

ting are alpha-amino-gamma-hydroxybutyric acid and guanidine, since both compounds were shown to be without inhibitory effect on canavanine sensitive or resistant strains (Table 16). The possibility that other products of canavanine splitting are differentially inhibitory to sensitive and resistant strains cannot be excluded.

Summary

Five mutant strains of Neurospora crassa that require the amino acid threonine were studied. Of these, four strains, each differing from wild type by a single gene, were found to be genetically different from each other. These strains are of four biochemical types:

35423 requires threonine.

44104 utilizes threonine, alpha-aminobutyric acid, or isoleucine.

46003 utilizes threonine, alpha-aminobutyric acid, isoleucine, alpha-amino-gamma-hydroxbutyric acid, or the alpha-keto acid analogs of alpha-aminobutyric acid or isoleucine.

51504 utilizes alpha-amino-gamma-hydroxybutyric acid or else threonine plus either methionine, homocysteine, cystathionine or methionine sulfoxide.

From the requirements of the first three mutants it is suggested that threonine is synthesized from a four carbon keto-acid, and that alpha-aminobutyric acid and isoleucine are interrelated through a common precursor, an unsaturated four carbon alpha-amino acid. Evidence from mutant 51504 indicates that threonine and methionine have a common precursor, alpha-amino-gamma-hydroxybutyric acid. No satisfactory scheme has been developed to relate all four mutants.

Evidence is presented that strain 44104 carries a crossover suppressor, possibly a chromosomal aberration.

A gene modifying the expression of the threonineless character in strain 46003 is reported. An hypothesis is proposed to account for the gene which determines resistance to the amino acid canavanine. This is done by assuming that a canavanine-splitting enzyme is present in canavanine resistant strains, but not in canavanine sensitive strains.

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APPENDIX

A. Source of compounds

The author is grateful to the following persons for supplying compounds used in this study:

Compound	Donor
d- and l-threonine and d- and dl-allothreonine	Dr. R. T. Major, Merck Co.
l-canavanine·SO_4	Dr. N. H. Horowitz, Calif. Inst. of Tech.
alpha-keto-gamma-methiol butyric acid	Dr. N. H. Horowitz
l-cystathionine	Dr. N. H. Horowitz
dl-homocysteine·thiolactone	Dr. N. H. Horowitz
monomethylaminoethanol	Dr. N. H. Horowitz
dimethylaminoethanol	Dr. N. H. Horowitz
dl-methionine-sulfoxide	Dr. J. W. Dubnoff, Calif. Inst. of Tech.
dl-methionine-sulfone	Dr. J. W. Dubnoff
dl-alpha-amino-gamma-hydroxybutyric acid	Dr. M. Fling, Calif. Inst. of Tech.
alpha-ketobutyric acid	Dr. M. Fling
alpha-keto-beta-ethyl-n-butyric acid	Dr. D. Bonner, Yale Univ.
dl-alpha-keto-beta-bromo-butyric acid	Dr. D. Sprinson, Columbia Univ.

All other compounds were of commercial origin except the ones prepared by the author (see following page).

B. Preparation of compounds

Alpha-keto-beta-hydroxy-n-butyric acid (keto-threonine): Prepared according to the method of Sprinson and Chargaff (46), from alpha-keto-beta-bromo-n-butyric acid. The acid was not isolated because of its reported instability, but was identified by its 2,4-dinitrophenylhydrazone:

$C_{10}H_{10}O_7N_4$	calculated	N 18.8
	found	N 18.75

Melting point of 2,4-dinitrophenylhydrazone

literature	157-8° d.
observed	157° d.

Canavanine:

Prepared according to the method of Cadden (47).

Canavanine, free amino acid:

$C_5H_{12}O_3N_4$	Calculated	N 31.8
	found	N 30.0

Analyses reported in literature: N 30.6, N 30.7

Melting point of free acid	literature	184°
	observed	184°

Canaline:

Prepared according to the method of Kitagawa (48). The canaline obtained by hydrolysis of the picrate was not characterized.

Canaline picrate:

$C_{10}H_{11}O_{10}N_5$	calculated	N 19.3
	found	N 18.5

Melting point of picrate	literature	193-4° d.
	observed	189° d.