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THREONINE REQUIRING MUTANTS OF NEUROSPORA

BY

H.J. TEAS

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H. J. Teas

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Acknowledgements

The author is grateful to the following persons for compounds used in this study: Dr. R. T. Major of Merck and Company for D- and L-threonine; Dr. N. H. Horowitz for DL-homocysteine-thiolactone hydrochloride and the keto acid analogue of methionine; Dr. Marguerite Fling for DL-homoserine and alpha-ketobutyric acid; Dr. Marvin Armstrong for D- and L- homoserine; and Dr. David Bonner for the keto acid analogue of isoleucine, alpha-keto-beta-ethylbutyric acid.

THREONINE REQUIRING MUTANTS OF NEUROSPORA

H. J. Teas
Oak Ridge National Laboratory and
California Institute of Technology

A previous study of the nutrition of Neurospora mutant 51504 showed that it requires the amino acids methionine and threonine for growth. Of the 24 amino acids tested, only homoserine could replace threonine and methionine indicating that homoserine is probably a precursor of both threonine and methionine (1). Unpublished work on two other threonine requiring mutants (35423 and 44104) appeared inconsistent. Although neither of them used homoserine, one of them (44104) could utilize either alpha-aminobutyric acid or isoleucine in place of threonine, while mutant 51504 could use neither to replace threonine. Further studies have shown that mutant 51504 cannot utilize alpha-aminobutyric acid or isoleucine in place of threonine even when the inhibitory effect of methionine is overcome, although there is some indication that alpha-aminobutyric acid and isoleucine are stimulated under these conditions. The evidence points toward threonine synthesis occurring by homoserine conversion to threonine by one or several intermediates. This intermediate (or intermediates) can be formed from alpha-aminobutyric acid or isoleucine in mutant 44104 and thus allow threonine synthesis to occur. In mutant 35423, the blocked reaction appears to be between this intermediate and threonine.

A number of inhibitions have been correlated with the mutant characters in these strains.

Materials and Methods

The mutant strains of Neurospora crassa used in this study (35423, 44104 and 51504) were obtained from ultraviolet irradiation of conidia

and kindly made available by Dr. G. W. Beadle. These threonine requiring mutants grow very slightly or not at all on minimal medium in the 72-hour experimental period.

The three mutants have been outcrossed to strains normal with respect to amino acid metabolism and found to give only those types of genetic segregations to be expected if each differed from the normal or wild type by a single gene. The genetic data may be summarized as follows:

35423 outcrossed to normal strains. Forty-two analyzable asci were obtained. The gene for the 35423-threonineless character is not sex linked.

44104 outcrossed to normal strains. One hundred twenty-four analyzable asci were obtained. The gene for the 44104-threonineless character is not sex linked.

35423 crossed with 44104. Thirty-four analyzable asci from the cross between these two mutants showed only mutant spores, indicating that the genes for the threonine requirements of strains 35423 and 44104 are probably located on the same chromosome and closely linked. It is seen that in 92 asci from three different outcrosses of 44104 no crossovers between the threonineless character and the centromere were recovered, whereas in the 22 asci from the cross of 44104A X 12a crossovers were recovered in 11 of the asci. In 35423A crosses the low value (2 units) in the cross with 12a and the higher value (13 units) obtained in the cross with 34508a, in conjunction with the low values for (0 units) the cross of 44104A with 34508 and the high value (25 units) for the cross with 12a suggests a suppression or facilitation of crossing over. McClintock has reported (2) frequent unsynapsed portions of pachytene

chromosomes associated with a heterozygous translocation in Neurospora strain 4637 which had previously been reported to show reduced crossing over in the sex chromosome (3). These observations suggest that a chromosomal aberration which suppresses crossing over by causing a failure of pairing may be responsible for the apparent complete linkage of the mutant genes involved in 35423 and 44104. The anomalous centromere distances and different biochemical requirements will tentatively be taken as evidence for different genes being involved. 51504 outcrossed to normal strains. Eighty-nine analyzable asci showed that the gene conditioning the amino acid requirement of this strain is sex linked (1).

For details of the techniques of crossing and ascospore dissection, and the interpretation of genetic results, reference may be made to Beadle (4) and Beadle and Tatum (5).

Stock cultures of Neurospora were grown on slants of complete medium similar to that used by Beadle and Tatum (5). Cultures were transferred weekly and stored in the refrigerator when they had conidiated, usually after 5-7 days. Unless otherwise stated, conidia for inoculum suspensions were taken from such stored cultures up to an age of approximately two weeks. The quantitative growth responses of the mutant strains to various substances were measured in terms of dry weight of mycelium. For this purpose the mold was cultured in 125 ml Erlenmeyer flasks containing 20 ml of minimal medium (5) and supplement and incubated for 72 hours at 25 C. The flasks were inoculated by a drop of dilute conidial suspension. Mycelial pads were collected on a Buchner funnel, dried in 10 x 75 tubes at 85-95 C, and weighed to the nearest milligram.

The keto acid analogue of threonine, alpha-keto-beta-hydroxybutyric acid, was prepared according to the method of Sprinson and Chargaff (6) from alpha-keto-beta-bromo-n-butyric acid furnished by Dr. David Sprinson. Keto-threonine was identified by its 2, 4-dinitrophenylhydrazone, but was not isolated.

Experimental

Effect of pH, Temperature, and B Vitamins on the Growth of Mutants 35423, 44104, and 51504. The existence of pH (7, 8) and temperature dependent (9, 10) mutants of *Neurospora*, and the fact that pyridoxine can replace threonine for *Lactobacillus arabinosus* (11) suggested testing the above mutants for temperature, pH, and accessory cofactor dependencies. No significant differences were noted for the 3 mutants grown in minimal medium adjusted to pH values 4.5, 5.8 (normal minimal medium), or 7.5, with or without 0.1 mg supplements of threonine for mutants 35423 and 44104, or homoserine for mutant 51504. When grown at temperatures of 25 C, 30 C, or 37 C in the presence or absence of supplements, no differences were found nor were any apparent, due to the addition of a mixture of B vitamins (5).

Response of Mutants 35423 and 44104 to Threonine and its Replacement. Mutants 35423 and 44104 were tested for growth on each of 23 amino acids supplied in 1 mg amounts per flask. None of these amino acids replaced threonine for mutant 35423, but alpha-aminobutyric acid and to a lesser extent isoleucine were found to replace threonine for mutant 44104. The amino acids tested were:

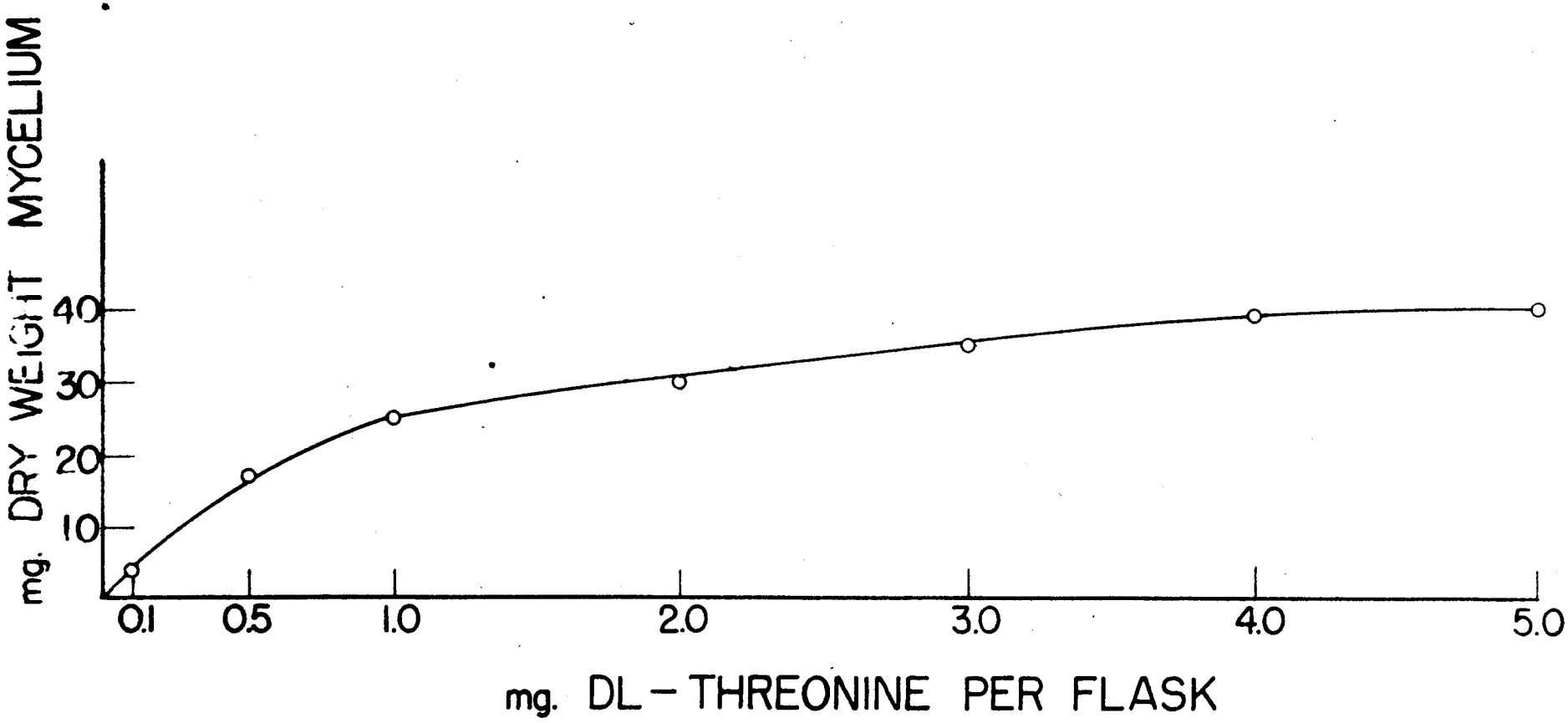
DL-isoleucine, DL-threonine, DL-methionine, L-histidine, DL-valine, L-lysine, L-tryptophane, L-leucine, glycine, DL-alanine, L-aspartic,

L-proline, L-arginine, L-hydroxyproline, L-glutamic, DL-serine, L-tyrosine, L-cystine, DL-norleucine, L-phenylalanine, DL-norvaline, DL-alpha-aminobutyric, and DL-homoserine.

Figures 1 and 2 show the quantitative responses of strain 35423 to threonine, and of strain 44104 to threonine, alpha-aminobutyric acid and isoleucine. In the case of mutant 44104 it is seen that threonine and alpha-aminobutyric acid are almost equally effective, but that isoleucine is only two-thirds as active. Tests in which each of the above mentioned 23 amino acids was tried with these mutants in the presence of 1 mg of DL-threonine ahowed that several of them caused a slight increased growth over the threonine control and that some allowed much less growth. The extent of inhibitions noted will be taken up in a separate section. Tests with single amino acids and mixtures carried out with mutant 51504 have been reported (1).

Optical Specificity of Amino Acids Used by Mutants 35423, 44104, and 51504. The results of tests with DL-, D-, and L-threonine on mutant 35423; with DL-, D-, and L-threonine, DL- and L-isoleucine for mutant 44104; and KL-, D-, and L-homoserine on mutant 51504 are shown in table 1. It may be seen that the D-forms of threonine and homoserine are inactive in initiating growth; however, the racemic mixtures show more growth promoting activity than would be expected from the amount of L-form present. It can be seen in figure 4 that L-homoserine is less active than DL-homoserine 1-2 mg/flask and greater. The D- and L- forms of alpha-aminobutyric acid were not available for testing.

Keto Acid Utilization by Mutants 35423, 44104, and 51504. The results of tests carried out with the alpha-keto acid analogues of threonine,


mg. DRY WEIGHT MYCELIUM
40
30
20
10
0.1
0.5
1.0
2.0
3.0
4.0
5.0
mg. DL-THREONINE PER FLASK

6355

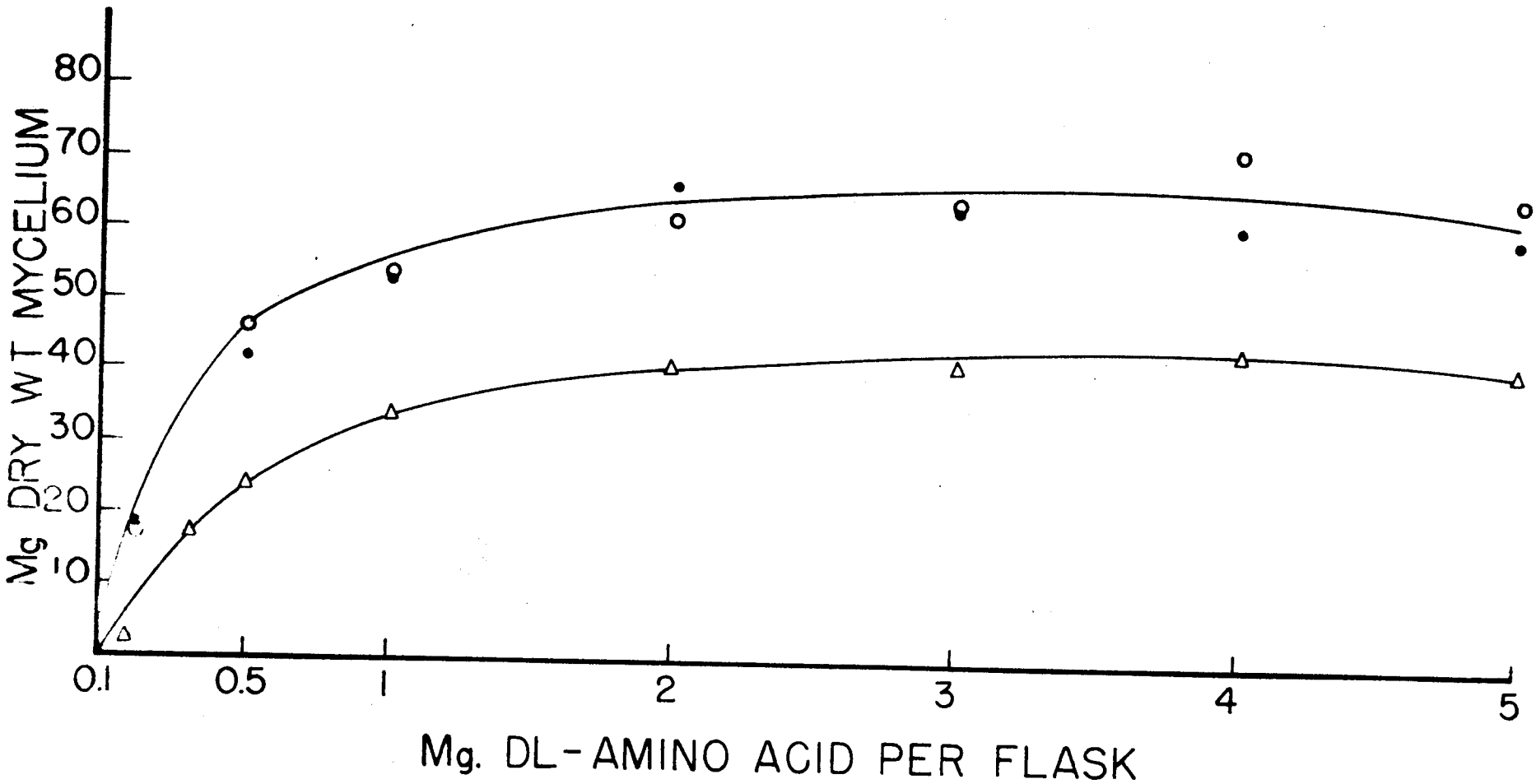

Mg DRY WT MYCELIUM
80
70
60
50
40
30
20
10
0.1
0.5
1
2
3
4
5
Mg. DL-AMINO ACID PER FLASK

Table 1

Centromere Distances Obtained in Crosses* of Mutants 35423 and 44104

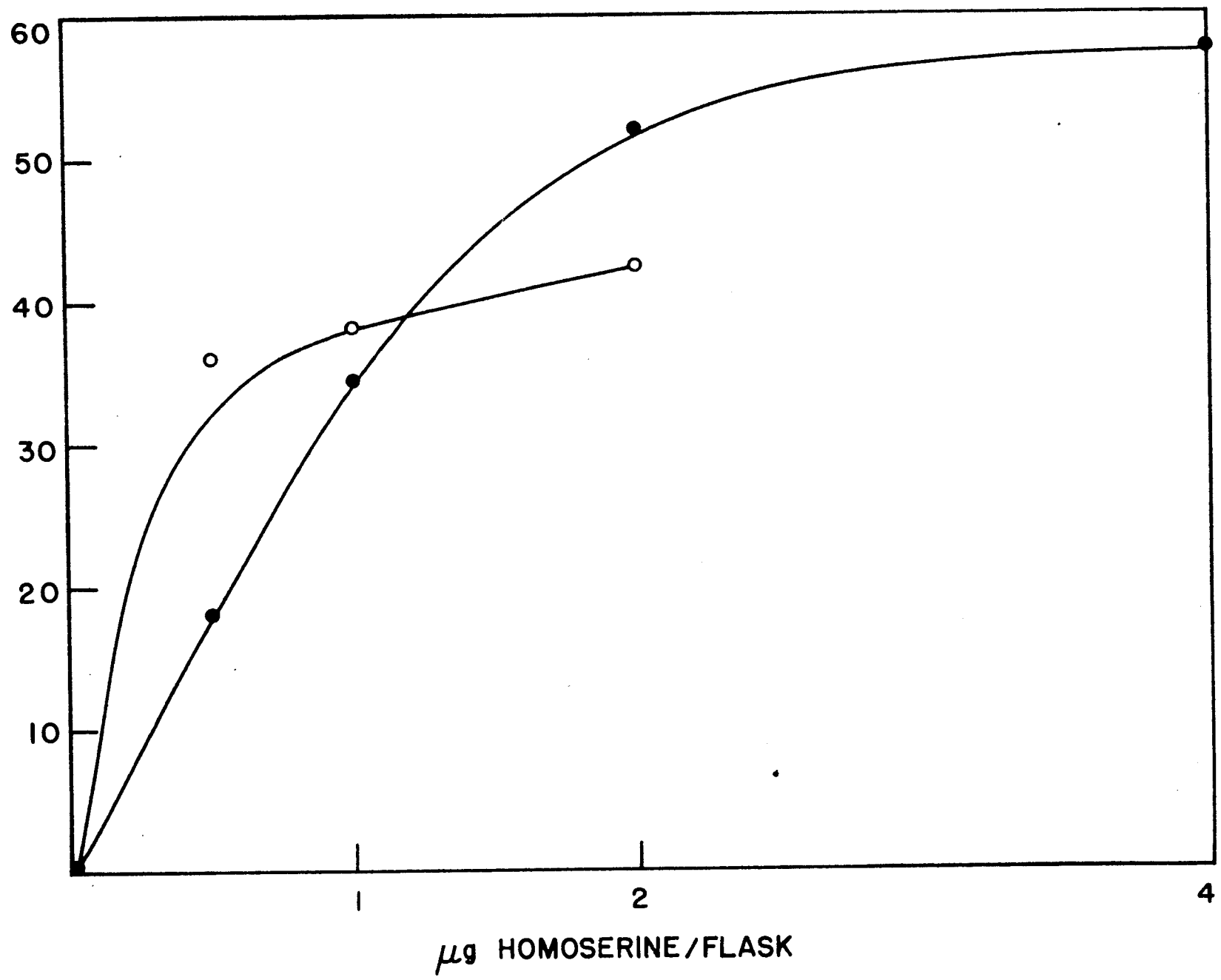
Cross	Number of Analyzable asci+	Centromere Distance
35423A X 34508	20	13
X 12a	22	2
44104A X 34508a	69	0
X 12a	22	25
X 5531	18	0
X 15300, 38701a	15	0
44104A X 35423a**	34	0

*Mutants are as follows:

34508 is aurescent, an albino strain.
12a is a wild type strain (see Beadle and Tatum (5))
5531a is pantothenicless.
15300, 38701a is a double mutant of 15300 an albino and 38701, a purple adenineless strain.

+ Asci are considered analyzable if at least one member of each spore pair germinates.

** Recovered from cross of 35423A X 34508a



GROWTH RESPONSE OF MUTANT 51504 TO L & DL - HOMOSERINE

● = DL-HOMOSERINE ○ = L-HOMOSERINE

FIG. 4

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isoleucine, and alpha-aminobutyric acid showed that only the keto acid analogue of isoleucine, in the case of 44104 is able to replace the corresponding amino acid in the growth of any of the three mutants. The keto acid analogue of methionine, alpha-keto-gamma-methylthiolbutyric acid, was found to replace methionine for mutant 51504, as would be expected from the utilization of D-methionine by this strain (1).

Inhibition Relationships. In view of the inhibitions found with strain 51504 and noted to a lesser extent with strains 35423 and 44104, tests were carried out with the single amino acids plus threonine or homoserine. For mutants 35423 and 44104 growth was measured on 1 mg DL-threonine plus 5 mg of each of the other amino acids, singly, and for mutant 51504 growth was measured on 1 mg DL-homoserine plus 5 mg amounts of the other amino acids singly. The amino acids causing complete or almost complete (90% or greater) inhibition of growth, for the most part, formed a distinct group. The inhibitory amino acids under these conditions were: for mutant 35423, methionine, valine, tryptophane, leucine, glycine, alanine, serine, tyrosine, norleucine, norvaline, alpha-aminobutyric acid, homoserine, and phenylalanine; for mutant 44104, tryptophane, leucine, tyrosine, homoserine, and phenylalanine; and for mutant 51504, methionine, tryptophane, leucine, glycine, serine, tyrosine, norleucine, norvaline, alpha-aminobutyric acid, and to an extent that they constituted borderline cases, also valine and alanine.

In three experiments with wild type *Neurospora* strain 1A the 23 amino acids were tested at the level of 5 mg per flask. The responses were somewhat variable, but tryptophane, glycine, and homoserine gave pad weights indicating 10 to 20% inhibition of growth.

Increasing the threonine for 35423 and 44104 and the homoserine for

51504 was checked for the relief of inhibition with each of the inhibiting amino acids. In all cases increasing the amount of threonine or homoserine at least partially overcame the inhibition. Several ratios of amino acid to threonine were tried with each inhibitor, never using more than 7 mg of total amino acid supplement per flask. Table 2 gives values for the lowest ratio of inhibitor to threonine or homoserine that caused 90% or greater inhibition of growth (column CI) and the highest ratio of inhibitor to threonine or homoserine that allowed approximately normal growth (CO). In several cases it was noted that pad weights at ratios of inhibitor to threonine less than those necessary for normal growth were higher than those of threonine or homoserine controls indicating that a given amino acid can be "inhibitory" or "stimulatory" under different conditions.

The degree of inhibition of mutant 51504 has been observed to vary with the age of the culture used for inoculation. As an illustration of this, the effect of threonine and methionine on growth is shown in figure 3 for cultures 7, 17, and 45 days of age which shows that the optimal ratio of threonine to methionine as well as the amount of growth with a given threonine-methionine supplement is seen to depend upon the age of the culture. The previously reported growth suppression of 51504 by alpha-aminobutyric acid (1) when added to 1 mg DL-threonine plus 1 mg DL-methionine was repeated several times and found to occur only in older cultures and then was not complete. Strains 35423 and 44104 do not appear to be as sensitive to aging as is 51504.

Attempts to Replace the Threonine Portion of the 51504 Requirement. Inasmuch as methionine was inhibitory to strain 51504, it seemed possible that the failure of this mutant to utilize either alpha-aminobutyric acid or isoleucine in place of threonine might be attributable to the inhibitory

Table 2

Growth Responses of Mutants 35423, 44104, and 51504 to Optical Isomers of Nutritionally Active Amino Acids

Mutant Strain	Amino Acid Supplement	Dry Weight of Mycelium in mg.
35423	1 mg. DL-threonine	19.
	2 mg. DL-threonine	28
	1 mg. L-threonine	24
	1 mg. D-threonine	0
	None	0
44104	1 mg. DL-threonine	54
	2 mg. DL-threonine	66
	1 mg. L-threonine	56
	1 mg. D-Threonine	0
	1 mg. DL-isoleucine	34
	2 mg. DL-isoleucine	41
	1 mg. L-isoleucine	37
	None	0
51504	1 mg. DL-homoserine	35
	2 mg. DL-homoserine	53
	1 mg. L-homoserine	37
	1 mg. D-homoserine	0
	None	0

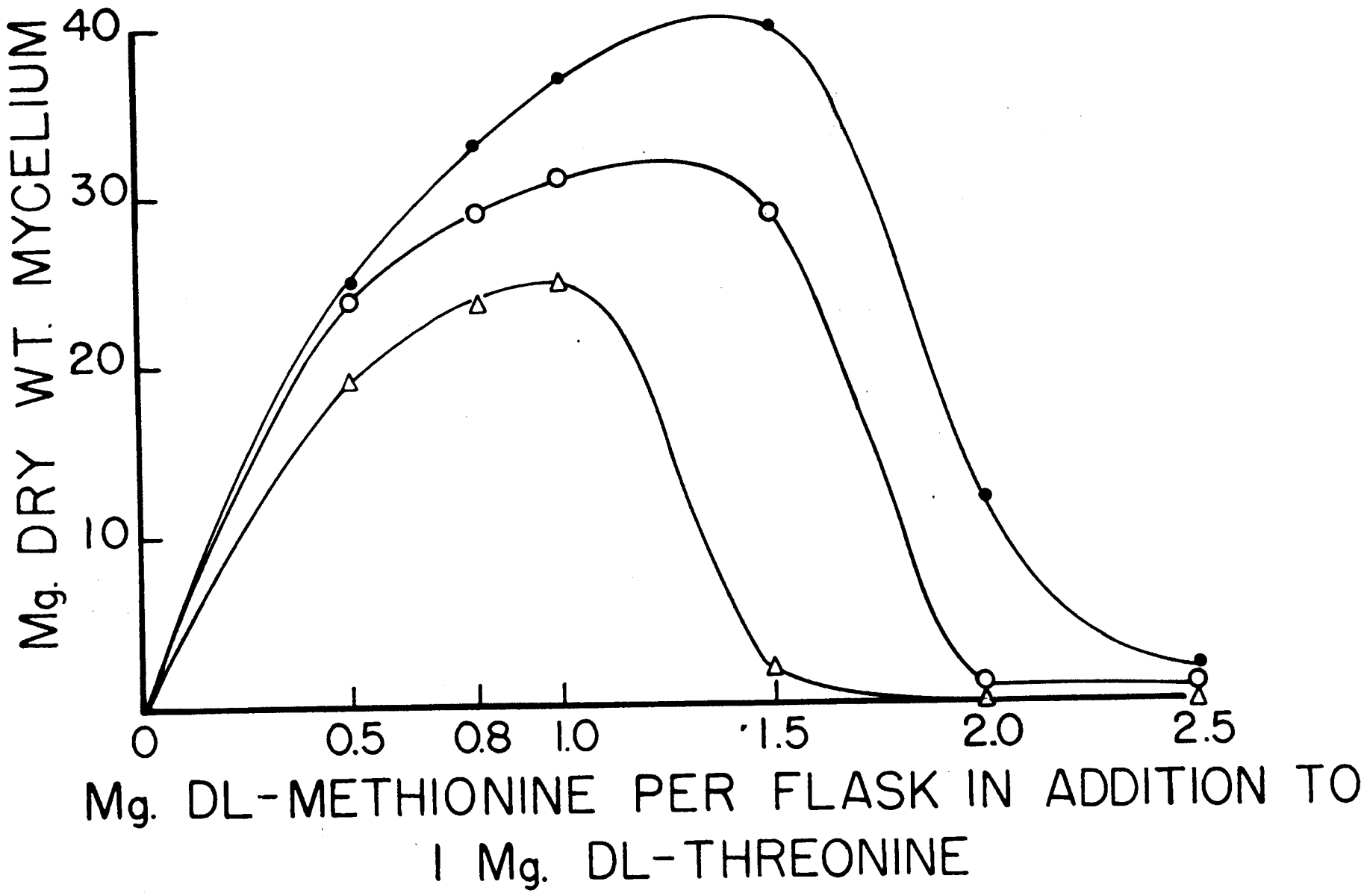

Mg. DRY WT. MYCELIUM
40
30
20
10
0
0.5
0.8
1.0
1.5
2.0
2.5
Mg. DL-METHIONINE PER FLASK IN ADDITION TO
1 Mg. DL-THREONINE

Table 3

Inhibition Ratios for Threonine Requiring Mutants Inhibited 90% or More by 5 mg. Amino Acid and 1 mg. DL-Threonine or DL-Homoserine

Amino Acid	Mutant 35423 Molar Ratio Inhibitory Amino Acid to Threonine		Mutant 44104 Molar Ratio Inhibitory Amino Acid to Threonine		Mutant 51504 Molar Ratio Inhibitory Amino Acid to Threonine	
	C I	C O	C I	C O	C I	C O
DL-Methionine	4.0	1.2	- -	- -	4.0	2.0
DL-Valine	5.0	1.5	- -	- -	5.0*	2.5
L-Tryptophane	2.9	0.9	1.6	0.6	2.9	0.6
L-Leucine	1.0	0.5	2.5	0.9	2.5	0.5
Glycine	7.9	2.6	- -	- -	7.7	4.0
DL-Alanine	6.7	2.0	- -	- -	6.7*	3.3
DL-Serine	5.7	1.7	- -	- -	5.1	2.9
L-Tyrosine	3.3	1.0	2.9	0.7	1.6	0.7
DL-Norleucine	4.6	0.9	- -	- -	4.5	0.9
DL-Norvaline	1.5	0.5	- -	- -	2.6	1.0
DL-alpha-aminobutyric	5.8	1.7	- -	- -	5.9	2.9
DL-Homoserine	1.0	0.3	2.5	0.3	- -	- -
L-Phenylalanine	0.7	0.4	1.8	0.4	1.8	0.4

C I = Complete or almost complete inhibition (90%-100%)

C O = Inhibition completely overcome; growth equal to controls

* Less than 90% inhibition in some experiments

effect of methionine. Single amino acids were tested for relief of methionine inhibition by adding them in 1 mg amounts in the presence of 1 mg DL-threonine and 2 mg DL-methionine (an inhibitory ratio). Five of the 23 amino acids, namely homoserine, threonine, alpha-aminobutyric acid, isoleucine, and lysine allowed approximately as much or more growth as was obtained with 1 mg DL-threonine plus 1 mg DL-methionine. Arginine and proline were effective to a lesser extent. The single amino acids were added in 1 mg amounts to the minimal medium supplemented with 1 mg L-lysine plus 1 mg DL-methionine. Only the expected threonine and homoserine supported growth, indicating that overcoming the methionine inhibition with lysine did not allow replacement of threonine by alpha-aminobutyric acid or isoleucine.

In other experiments the amino acids were tested in 1 mg amounts in the presence of 1 mg DL-methionine plus 1 mg L-lysine (to overcome methionine inhibition), and suboptimal amounts of homoserine (0.2 and 0.5 mg per flask). The results indicated that alpha-aminobutyric acid and isoleucine apparently cause some stimulation of growth but the effectiveness of alpha-aminobutyric acid and isoleucine, when compared to several other amino acids, was of questionable significance. No evidence has therefore been found to show that 51504 can utilize isoleucine or alpha-aminobutyric acid.

Discussion

The study of gene mutations in *Neurospora* that involve blocks in biosynthetic reactions has been a powerful means of elucidating intermediary metabolism (see reviews of Beadle 12, 13, 14). From an analysis of the nutritional relations of threonine requiring mutants it has been possible to shed some light on threonine synthesis. Inasmuch as one mutant (35423) uses only threonine, and another mutant (44104) uses threonine, isoleucine,

or alpha-aminobutyric acid, we can locate the step blocked in the first mutant as involving the conversion of alpha-aminobutyric acid or isoleucine or an intermediate formed from them to threonine. Since a third mutant (51504) used methionine plus threonine or else homoserine, the block in the second mutant must be between homoserine and threonine. Thus it is possible to arrange the steps involved as follows:

51504 threonine and 44104 Isoleucine 35423 threonine
$\longrightarrow$ $\longrightarrow$ Alpha-aminobutyric acid $\longrightarrow$

in which each amino acid subsequent to a block (indicated by the number of the mutant strain) will function in growth. The experimental data obtained to date do not indicate how the two schemes can be made into one. However, the failure of the keto acids corresponding to threonine, or alpha-aminobutyric acid to replace the amino acids in the growth of these mutants indicates that amination of the carbon chain must occur prior to the step controlled by 44104.

It can be argued that a mutant which requires a substance cannot make the substance, otherwise it would not require it. If this is so, threonine and isoleucine must be interconvertible, because either will apparently fulfill all the need of 44104. The dehydration suggested (1) as a step in the conversion of homoserine to threonine would allow the possibility of a C-2 side chain attachment for isoleucine formation from threonine. That threonine-isoleucine interconversion is not a general phenomenon, however, is indicated by the work of various investigators using media or diets in which only single amino acids were omitted at a time. Thus in man (15), dog (16), mouse (17), rat (18), chick (19), and in twenty of the twenty-three lactic acid bacteria studies by Dunn _et al._ (20), the interconversion of isoleucine and threonine has been ruled out by the

demonstration of a requirement for threonine and/or isoleucine.

It is possible that isoleucine and threonine are not interconvertible, but that isoleucine is synthesized by a separate series of reactions and that such a synthesis does not supply enough isoleucine for conversion to threonine. In this event, the beta-ethyl group might irreversibly be removed from exogenous isoleucine and the resulting 4 carbon amino acids be converted into threonine. The less efficient utilization of isoleucine than alpha-aminobutyric acid by mutant 44104 may be due to a relatively slow conversion to threonine as well as the weight difference in threonine that could be formed from a given amount of isoleucine.

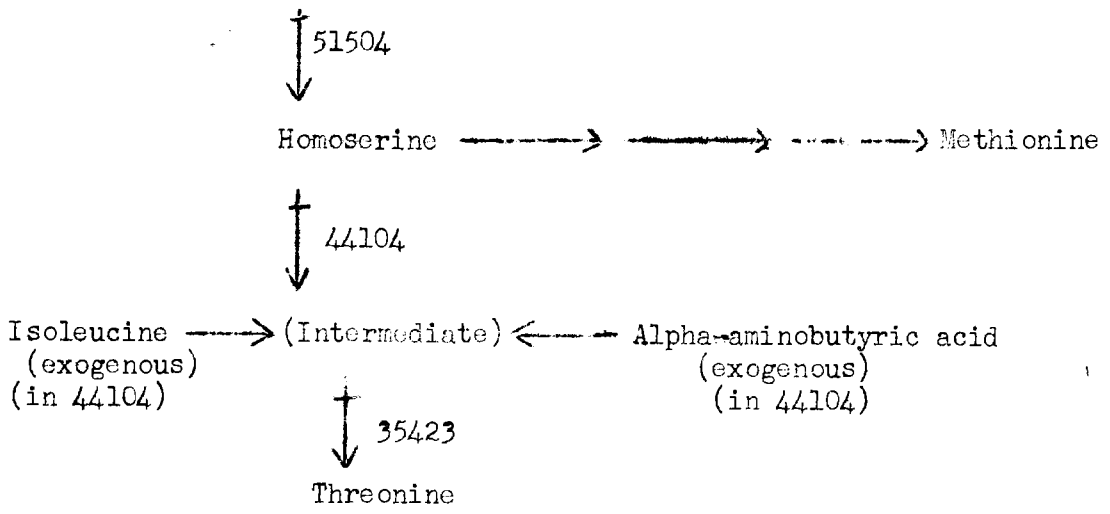
The second difficulty in the relation of precursors and blocks as diagrammed above is the apparent inconsistency of requirements of mutants 44104 and 51504. Irrespective of whether the reactions involving threonine-isoleucine relationships are reversible, these steps should be intact in 51504 as well as 44104 since each of these mutants appear to differ from normal by a single gene. It is possible that this conversion is intact in 51504, but that it is inhibited by some accumulated substance produced by 51504.

The relations of alpha-aminobutyric acid to threonine and isoleucine are uncertain. Dent has shown that alpha-aminobutyric acid occurs in dilute acetic acid extracts of yeast (21), alcoholic extract of potato tuber (22), and in the blood and urine of normal patients after methionine feeding (23), and Fromageot and Clauser have reported that it exerts a sparing action on methionine in the diet of the rat (24). Alpha-aminobutyric acid has not been demonstrated to be a constituent of proteins and, in the absence of evidence establishing the general significance, it has not been extensively tested as a replacement for threonine or isoleucine.

Alpha aminobutyric was found not to be essential for Streptococcus faecalis (25). Of twelve mutants of Bacillus subtilis that required threonine or threonine plus methionine, kindly made available by Dr. N. H. Giles, none utilized either alpha-aminobutyric acid or isoleucine to replace threonine (Teas, unpublished).

The relation of homoserine to threonine in higher forms is likewise not certain. Homoserine did not replace threonine (26) or methionine (27) in the rat, although this finding does not exclude the possibility that it may do so in other higher forms.

A diagrammatic representation of threonine biosynthesis for Neurospora showing what may be the relations is:



In this diagram, alpha-aminobutyric acid and isoleucine are shown as being converted into a threonine precursor. It seems probable that these two amino acids are not normal precursors of threonine.

Glycine has been indicated as a precursor of threonine in yeast by Kogl and Borg (28) and more recently demonstrated by Fanelli (29) and by Rossi and Cinnamo (30); however, no evidence has been obtained that this is the case in Neurospora. If such a pathway were functional in Neurospora,

it would have to be as a precursor of homoserine since the genetic evidence precludes an alternate pathway for the synthesis of threonine.

The observation that the racemic mixtures of threonine, and isoleucine were more than half as effective as the corresponding natural forms indicates that Neurospora can utilize the unnatural forms to some extent even though no activity was found for the corresponding keto acids. With respect to threonine, Neurospora is unlike lactic acid bacteria (31, 32) the mouse (19) and the rat (18), but is similar to Tetrahymena geleii (33) and possibly to man (34) in the utilization of only the L- form by the former or partial use of the D-form in racemic mixtures by the latter.

The inhibitory effects of amino acids have been noted in higher forms (35, 36) and particularly in microorganisms (37, 40). In the case of Neurospora mutants showing inhibitions it is possible to study relationships that are correlated with single gene mutations and compare them with the normal or wild type Neurospora. Since wild type strain 1A was inhibited but 10-20% by 3 of the 23 amino acids in 5 mg amounts, it follows that the inhibitions in threonine requiring mutants are a consequence of the respective mutant characters. Lysineless mutants have been reported to be inhibited by arginine (3); an isoleucine-valine mutant was inhibited by norvaline, norleucine and phenylalanine (41) a threonine-methionine mutant (51504) was inhibited by methionine, alpha-aminobutyric acid, or phenylalanine (1); and in this study 51504 and two other mutants were shown to be inhibited by 5 to 13 of the 23 amino acids tested. The arginine inhibition of the lysine mutant 4545 and methionine inhibition of the threonine-methionine mutant 51504 have been shown to be competitive, the amount of growth depending on the ratio, not the absolute amounts of the amino acids present. However, since mutants of the "less" type are unable to synthesize an essential

metabolite, it is to be expected that growth inhibitions relieved by the metabolite would involve utilization since there is no synthesis to inhibit, and therefore would show competitive inhibition (see review of Ratlin, 42). The structural diversity among amino acids that were found to be inhibitory makes their interpretation on the classical "competition for enzyme" hypothesis difficult.

The complex inhibitions found in threonine requiring mutants suggests that there may be types of amino acid requiring mutants which cannot be obtained with the usual screening procedures, inasmuch as these involve complete media in which the presence of one or more amino acids may inhibit the growth of a mutant requiring another amino acid.

Summary

The amino acid nutrition of three monogenic mutant strains of *Neurospora crassa* that utilize threonine for growth is described. The mutants have requirements as follows: number 51504 uses homoserine, or threonine plus methionine, number 44104 uses threonine, alpha-aminobutyric acid, or to a lesser extent isoleucine; and number 35423 uses only threonine. It is suggested that the biosynthesis of threonine in *Neurospora* involves the conversion of homoserine to threonine by way of one (or several) intermediate steps and in mutant 44104 isoleucine and alpha-aminobutyric acid, can be converted to such intermediate.

Data on the inhibition of threonine or homoserine utilization by amino acids are given. The age of culture used for inoculum is shown to be important in inhibitions with mutant 51504.

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