GENETICS AND BIOCHEMISTRY OF THE CYSTEINE-TYROSINE RELATIONSHIP IN *NEUROSPORA CRASSA*

Thesis by

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ABSTRACT

Mutant 84605 which was obtained from wild type Neurospora crassa following X-ray treatment differs from wild type by a single gene located 4.8 units from the centromere of the second chromosome.

At 25°, the mutant requires both cysteine and tyrosine for normal growth. At 35° only cysteine is required. The block in cysteine synthesis is in the step sulfite ⟶ thiosulfate.

High tyrosinase activity was found in the mutant grown at 25°, but not when grown at 35°. Under the same conditions, wild type shows little or no tyrosinase activity.

The addition of cysteine to the medium causes an inhibition of the growth of wild type, and at the same time a marked increase in the tyrosinase activity occurs. The inhibition can be overcome by adding tyrosine to the medium, or by culturing at 35°.

It is suggested that the tyrosine requirement is caused by the high tyrosinase activity and that the latter, in turn, is caused by the defect in sulfur metabolism.

Two natural inhibitors of tyrosinase have been found in Neurospora.

A powerful inhibitor of the growth of wild type accumulates in cultures of the mutant.

Experiments designed to test whether sulfide can serve as a sulfur source for Neurospora indicate that sulfide is utilized slightly, if at all.

Experiments with a double mutant have indicated that the production of cysteine from methionine by Neurospora does not involve simple reversal of the step homocysteine ⟶ methionine.

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INTRODUCTION

The biochemical mutants of Neurospora crassa which are induced by physical or chemical agents have been shown mostly to differ from the wild type by the change of a single gene. They are unable to carry on the normal biological syntheses and hence they fail to grow on the minimal medium unless the appropriate compounds which the mutant strains can no longer synthesize are added (3). Evidence has accumulated to show that many of the chemical reactions by which the organism can function and live are under immediate control of the gene. Each gene is concerned with a specific step in a chemical reaction (17). In a mutant, a defective gene results in a block of a certain chemical reaction. Therefore, by use of a number of biochemical mutants blocked in different steps in a series of chemical reactions, the whole metabolic process can be clearly demonstrated. It has already been pointed out that a biochemical mutant gene serves just as reliably as the radioactive tracers in the study of intermediate metabolism of organisms.(19)

The methionine synthesis in Neurospora has been studied by Horowitz and his colleagues. In higher animals, sulfur is usually supplied in the form of organic compounds, inorganic sulfate being the end product of much of the metabolized sulfur. However the normal strain of Neurospora is able to synthesize cysteine and methionine by using inorganic sulfate. Cysteine is a normal intermediate in the synthesis of methionine from sulfate. To facilitate reference to these sulfur mutants which have lost the ability of synthesizing

sulfur amino acids, they have been divided into two groups: Those mutants with a genetic block before cysteine are arbitrarily referred to as cysteineless mutants, and those mutants which are blocked at any step between cysteine and methionine are referred to as methionine-less mutants. In studying cysteine metabolism in Neurospora, Horowitz found that among the cysteineless mutants, some strains cannot reduce inorganic sulfate to sulfite, others cannot reduce sulfite to thiosulfate, and still others can use only cysteine or the intermediates between cysteine and methionine. The unpublished experiments of Phinney, Fling and Horowitz show that those mutants which can use sulfite can also use cysteic acid. The pathway of cysteine synthesis was suggested in two possible ways. It proceeds either through the reduction of sulfate to sulfite and then to thiosulfate which combines with the three carbon compound or through the reduction of sulfate after it combines with the three carbon compound to form cysteic acid and then follows a series of reductions to give rise to cysteine.

Lampen, Roepke and Jones (21) in their studies of sulfur metabolism of Escherichia coli found some biochemical mutants in which sulfur metabolism is interfered with in one way or another. On the basis of growth experiments on the sulfur mutants in this category, they suggested that sulfate can be reduced through sulfite to sulfide which combines with a three carbon compound to form cysteine, and that the reactions of cysteine synthesis in E. coli are reversible.

The process of methionine synthesis from cysteine is better known than that of cysteine synthesis from inorganic sulfate in Neurospora. There are a number of specific genes which control the synthetic reactions.

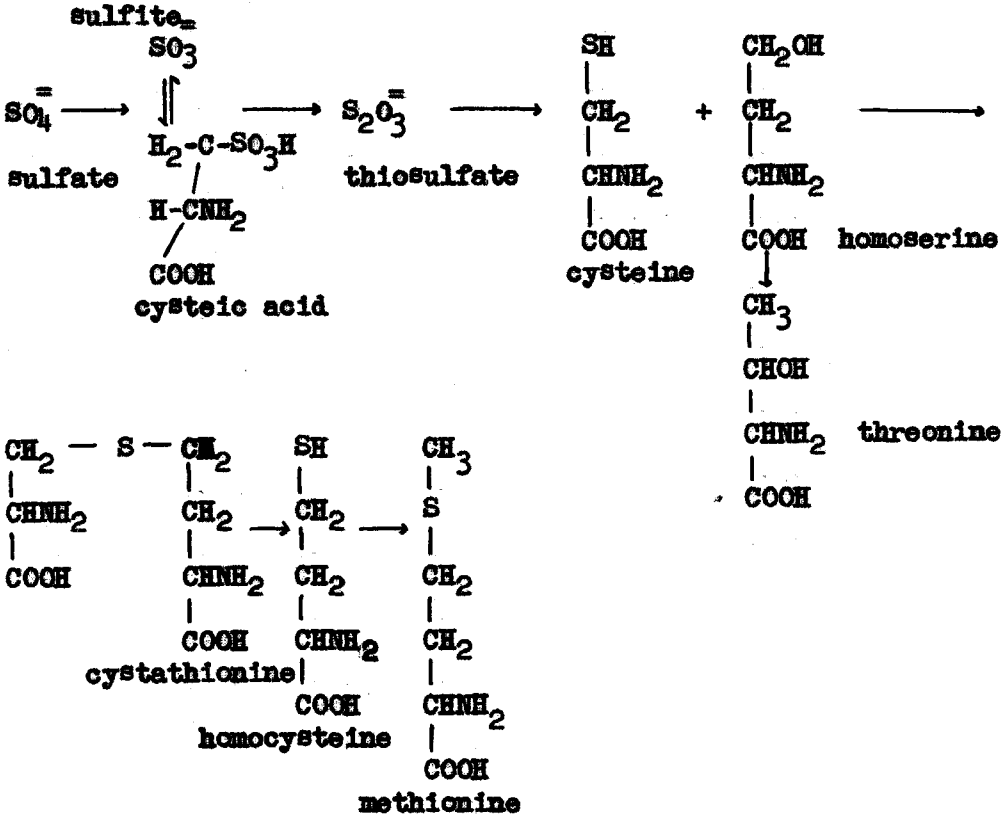
Some methionineless mutants can use homocysteine, but others can use only methionine, even if the medium is supplemented with homocysteine plus choline or betaine. The latter case suggests that the methylation of homocysteine is blocked in these mutants. Cystathionine has been found accumulated in cultures of one strain with a genetic block between cysteine and homocysteine (18). This finding confirms the experiments of Binkley and Du Vigneaud that cysteine is formed from serine and homocysteine through the intermediate formation of cystathionine (6). In Neurospora serine is not produced from the direct cleavage of cystathionine, as shown by the fact that the serineless mutant does not necessarily require exogenous methionine for growth.

Lampen et al present evidence consistent with the idea that homocysteine is the precursor of methionine, and that the conversion of homocysteine to cysteine is through cystathionine in E. coli (21). The biological activity of the four possible optically active isomers of cystathionine were investigated and found different in different organisms. Anslow and Du Vigneaud found that both L-cystathionine and L-allocystathionine can support the growth of the animal on a diet free of cysteine, while D-cystathionine and D-allocystathionine were ineffective (1, 2). In Neurospora, Horowitz (18) found that only L-cystathionine is active for the mutant strain. Simmonds (32) reported on her work on E. coli K-12 that L-cystathionine and D-allocystathionine are as active as methionine.

In 1947 Teas, Horowitz (36) showed that a mutant of Neurospora requires both threonine and methionine for its optimal growth. Genetic tests indicate that the mutant is induced by the change of a single

gene. Their further experiments have proved the assumption that the gene responsible for the synthesis of the common precursor of both threonine and methionine is defective in the mutant. Homoserine was found to promote the growth of the mold in place of threonine and methionine. This clearly indicates that in Neurospora the four carbon fragment of cystathionine originates from homoserine (γ -hydroxy- α - amino butyric acid), and the latter is also surprisingly the precursor of threonine. Recently Teas (37) found a similar case in the mutants of Bacillus subtilis. Two mutants of the bacteria require both threonine and methionine but can utilize homoserine instead. This result suggests that in Bacillus subtilis, as in Neurospora, homoserine is the precursor of both methionine and threonine.

The results of investigations so far obtained in Neurospora crassa indicate that the pathway of methionine synthesis controlled by a series of specific genes proceeds as follows:



The original purpose of the present investigation was to discover the source of the carbon skeleton of cysteine. In the course of this study, however, a problem of basic importance in connection with the concept of the gene-enzyme relationship was encountered. A mutant was found which requires cysteine (or certain other sulfur compounds) plus the unrelated amino acid tyrosine for growth. The further investigation of this mutant forms the main part of this thesis.

PART I

A Mutant Requiring Both Cysteine and Tyrosine

Mutant No. 84605 was obtained by X-irradiation of the asexual spores of the wild type 4A and isolated from a cross with the wild type 25a. It was originally classified as a cysteineless mutant#, but it grows very slowly on this amino acid. If, in addition to cysteine tyrosine is supplied, its growth rate approaches that of wild type.

GENETIC STUDY AND RESULTS

1. Evidence of one gene difference between the mutant and the wild type.

Since every chemical reaction in the organism is controlled by certain specific genes, it is important to know whether the given strain is differentiated from the wild strain by a single gene, before going to the biochemical investigation. A cross was made between the mutant and the wild type 4A, using the media described in Westagard and Mitchell (38). 400 ascospores were isolated at random and 20 asci in order. The result obtained (Table 1) indicated that the mutant differs from the wild type by a single gene.

2. Evidence of linkage group.

Crosses were made between the given mutant and four other mutants in four different linkage groups. The results are summarized in table 2. The data in the first cross, between mutant 84605 and the sex-linked adenineless purple mutant, show that the frequency of segregation at first meiotic division of both mutant genes is 25 among which 10 types of asci are parental type and the other 15 new

Table 1

Segregation types resulting from the cross between the cysteine-tyrosineless mutant 84605a and wild type 4A isolated at random

+ = wild type

cys-t = cysteine-tyrosineless

o = not germinated

Segregants	Number
+	164
cys-t	158
o	78
Total	400

Type of asci resulting from the cross between 84605a and 4A

Spore pairs				No. of asci
1st	2nd	3rd	4th	
+	+	cys-t	cys-t	16
cys-t	cys-t	+	+	3
+	cys-t	cys-t	+	1
				20

Table 2

Type of segregation resulting from a cross between mutant 84605a and the sex-linked adenineless purple mutant 35203-pa5-5A.

cys-t= cysteine-tyrosineless

ad-p = adenineless purple

+ = wild type allele of cys-t or ad-p

Spore pairs				No. of asci
1st	2nd	3rd	4th	
cys-t, ad-p	cys-t, ad-p	++	++	9
++	++	cys-t, ad-p	cys-t, ad-p	6
cys-t +	cys-t +	+ ad-p	+ ad-p	7
+ ad-p	+ ad-p	cys-t +	cys-t +	3
++	+ ad-p	cys-t, ad-p	cys-t +	2
cys-t +	++	cys-t, ad-p	+ ad-p	1
+ ad-p	cys-t +	cys-t +	+ ad-p	2
				30

Indicated centromere distance of cys-t = 5 units

Table 2 (Continued)

Type of segregation resulting from a cross between mutant 84605a and the pyrimidineless mutant 37815-12945-1A.

cys-t = cysteine-tyrosineless

pyr = pyrimidineless

+ = wild type allele of cys-t or pyr.

Spore pairs				No. of asci
1st	2nd	3rd	4th	
cys-t +	cys-t +	+ pyr	+ pyr	6
+ pyr	+ pyr	cys-t +	cys-t +	3
++	++	cys-t, pyr	cys-t, pyr	5
cys-t, pyr	cys-t, pyr	++	++	3
++	cys-t, pyr	+ pyr	cys-t, pyr	1
++	+ pyr	cys-t, pyr	cys-t +	1
cys-t, pyr	cys-t +	++	+ pyr	2
cys-t, pyr	cys-t +	+ pyr	++	2
cys-t, pyr	++	++	cys-t, pyr	1
cys-t +	++	+ pyr	cys-t, pyr	1
cys-t +	cys-t, pyr	++	+ pyr	1
+ pyr	++	cys-t +	cys-t, pyr	2
cys-t +	cys-t, pyr	+ pyr	++	1
				30

Indicated centromere distance = 5.2 units

Table 2 (Continued)

Type of segregation resulting from a cross between mutant 84605a and the adenineless mutant 44206a.

cys-t = cysteine-tyrosineless

Ad = adenineless

+ = wild type allele of cys-t or Ad

Spore pairs				No. of asci
1st	2nd	3rd	4th	
cys-t +	cys-t +	+ Ad	+ Ad	2
+ Ad	+ Ad	cys-t +	cys-t +	1
++	++	cys-t, Ad	cys-t, Ad	6
cys-t, Ad	cys-t, Ad	++	++	6
cys-t +	++	+ Ad	cys-t, Ad	1
cys-t +	cys-t, Ad	+ Ad	++	1
++	cys-t +	cys-t, Ad	+ Ad	1
++	+ Ad	cys-t, Ad	cys-t +	1
++	+ Ad	cys-t +	cys-t, Ad	2
++	cys-t, Ad	+ Ad	cys-t +	1
++	cys-t +	+ Ad	cys-t, Ad	1
cys-t +	+ Ad	cys-t, Ad	++	1
+ Ad	++	cys-t, Ad	cys-t +	2
cys-t, Ad	cys-t +	++	+ Ad	1
				27

Indicated centromere distance = 9.3 units

Table 2 (Continued)

Type of segregation resulting from a cross between mutant 84605a and the riboflavineless mutant 51602a.

cys-t = cysteine-tyrosineless

rib = riboflavineless

\+ = wild type allele of cys-t or rib

Spore pairs				No. of asci
1st	2nd	3rd	4th	
cys-t +	cys-t +	+ rib	+ rib	24
+ rib	+ rib	cys-t +	cys-t +	14
+ rib	cys-t +	cys-t +	+ rib	1
cys-t +	++	+ rib	cys-t, rib	1
				40

Indicated centromere distance of cys-t = 2.5 units

Average centromere distance (all data) = 4.8

recombinations. Therefore mutant 84605 is not linked with mutant Ad-p 35203-5A, and hence is not on the sex chromosome.

The second cross, between mutant 84605 and the pyrimidineless mutant 37815-12945 1a, shows that there are 17 asci of both mutant genes segregated at the first division of meiosis and that among the 17 asci, 9 show the parental types and the others new recombinations. Therefore the cysteineless mutant is not linked with the pyrimidineless mutant and it does not belong to the fourth linkage group.

The third cross, between the cysteineless mutant and the adenineless mutant 44206a, shows that both mutant genes segregate at first meiotic division in 15 asci of the total 27, and that of the 15 asci three are parental type and the other 12 types of new recombination. These results again indicate that these two mutant genes are independently located on different chromosomes.

In the fourth cross, between mutant 84605 and the riboflavineless mutant 51602a, 36 asci of the total 40 show first division segregation of both mutant genes, and none of them are new recombination types. Therefore it indicates that the gene of strain 84605 is linked with the gene of riboflavineless mutant 51602 on the second chromosome.

The data from the five crosses given in tables 1 and 2 show that the cysteineless mutant gene segregates from its wild type allele in the second division in 14 cases out 146, giving a cross-over value between the gene and thecentromere of 4.8%. Since the centromere distance of the gene of the riboflavineless mutant as shown by Houlahan et al is about 1.7 (20), and the recombination value between the cysteineless and theriboflavineless mutant is about 1.25%, it is

probable that the sequence is as follows: centromere-51602-84605.

BIOCHEMICAL STUDY AND RESULTS

Growth

a. On the series of known intermediate substances of methionine synthesis

The growth was measured as usual by dry weight of the mycelium produced by the mold after 72 hours at 25° C in 20 ml. of medium (the medium and technique used are those described in Horowitz and Beadle, 16). The mutant was originally classified as a cysteineless mutant, since cysteine or methionine were the only substances tested which gave growth. When quantitative growth experiments were carried out, however, it was found that the mutant actually grows very poorly on cysteine. The known sulfur-containing intermediates in the synthesis of methionine were therefore tested, with the results shown in table 3. It is seen that none of them produces normal growth of the strain.

b. On the series of known intermediate substances of methionine synthesis plus single amino acids.

Since the mutant grows to only a slight extent on those sulfur compounds as shown in table 3, it was suggested that, in addition, another substance could be indispensable for the optimal growth of this mutant. Further study of the growth requirement of the mutant showed that another factor effective for its growth was present in casein hydrolysate. Experiments were then carried out with twenty single amino acids plus cysteine. Tyrosine was found as the only effective substance in addition to cysteine (Table 4). Tested with other indispensable sulfur compounds plus tyrosine, the growth of the mutant is shown in table 5. These results confirm those of table 3, namely that the block is between sulfite and thiosulfate.

Table 3

Growth of mutant 84605 on 20 ml. minimal medium plus sulfur compounds.

Sulfur compound	in mg.	Dry weight in mg.
None		0
$SO_3^=$	2	0
DL-cysteic acid	1	0
$S_2O_3^=$	1	1.8
L-cysteine Hcl	1	3.2
L-cystathionine	1.5	1.0
Dl-homocysteine thiolactone Hcl	2	1.6
Dl-methionine	1	2.0

Table 4

Effect of single amino acids in addition to cysteine on the growth of strain 84605

Amino acid 1 mg. in amount in addition to 1 mg. l-cysteine Hcl	Dry weight in mg.
None	1.8
Glycine	0.8
Alanine	1.4
Dl-valine	2.0
L-leucine	1.8
Dl-isoleucine	2.8
L-aspartic acid	1.0
D-glutamic acid	1.0
Dl-serine	0.6
Dl-threonine	1.8
L-proline	1.4
L-hydroxyproline	1.8
Dl-phenyl alanine	1.4
L-tyrosine	26.4
Dl-tryptophane	3.0
D-arginine	1.0
Dl-lysine	1.0
L-histidine	0.6

Table 5

Effect of l-tyrosine in addition to the sulfur compounds on the growth of strain 84605.

Supplements in addition to 1 mg. l-tyrosine	in mg.	Dry weight in mg.
None		0
$SO_3^=$	2	0
$S_2O_3^=$	1	29.6
l-cysteine Hcl	1	29.8
l-cystathionine	1.5	24.6
Dl-homocysteine thiolactone HCl	2	26.4
Dl-methionine	1	25.0

c. Responses to tyrosine and its analogs.

Several compounds were tested for their ability to replace tyrosine. Among them only tyramine and to a lesser extent phenol are effective for promoting the growth of the mutant (Table 6). Trans-cinnamic acid, quinic acid and shikimic acid found recently by Haskins and Mitchell (15), Gordon (unpublished data) and Tatum (unpublished data) respectively to replace tyrosine in Neurospora are not effective. Contrarily trans-cinnamic acid seems inhibitory. Because of being easily oxidized in the medium, the effect of Dopa (L-3, 4-dihydroxyphenylalanine) is still not exactly known. Dopa has been found to replace tyrosine for the growth of a tyrosineless mutant of E. coli (31).

In testing for the optimal ratio of cysteine to tyrosine for maximum growth of the mutant, it was found that high concentrations of cysteine are inhibitory unless the tyrosine concentration is also proportionally increased. Table 7 indicates the result of different proportions of cysteine and tyrosine on the growth of mutant. The effect of equal amounts of tyrosine and sulfur compounds on the growth of the mutant are shown in Figure 1.

d. Temperature effect.

An experiment was carried out testing the growth of the mutant in different temperature conditions. At 35° C good growth of the mutant was obtained with thiosulfate or any of the sulfur compounds following it without the necessity of adding tyrosine (Table 8). This suggests that the mutant is only a cysteineless strain and proves that its requirement for both amino acids at 25° C is not due to a block in the chemical reaction of a common precursor. Figure 2 shows

Table 6

Effect of tyrosine analogs on the growth of strain 84605 in presence of cysteine.

Compounds in mg. in addition to 1 mg. of cysteine HCl	Dry weight in mg.
None	2.8
L-tyrosine	35.0
L-tyramine	22.8
hydroxybenzoic acid	5.0
gallic acid	2.6
phenol	10.8
p-coesol	0.8
catechol	1.0
Dopa	1.0
hydroquinone	1.0
trans-cinnamic acid	0
shikimic acid	3.8
quinic acid	4.4

Table 7

Effect of various concentrations of cysteine and tyrosine on the growth of strain 84605.

L-cysteine mg.	L-tyrosine mg. 0	.5	1	5	10
0	0	0	0	0	0
.5	1.4	23.2	23.4	22.0	21.0
1	3.2	30.0	28.4	27.4	28.0
5	7.1	44.2	42.6	33.6	37.0
10	1.8	13.6	14.0	42.0	54.0

Table 8

Growth of mutant 84605 on sulfur compound at 35° C.

Compound	mg.	Dry weight mg.
None		0
l-tyrosine	1	0
Na_2SO_3	2	0
$Na_2S_2O_3$	1	60.0
l-cysteine HCl	1	51.0
L-cystathionine	1.5	36.0
L-homocysteine thiolactone HCl	1	49.0
dL-methionine	1	48.8

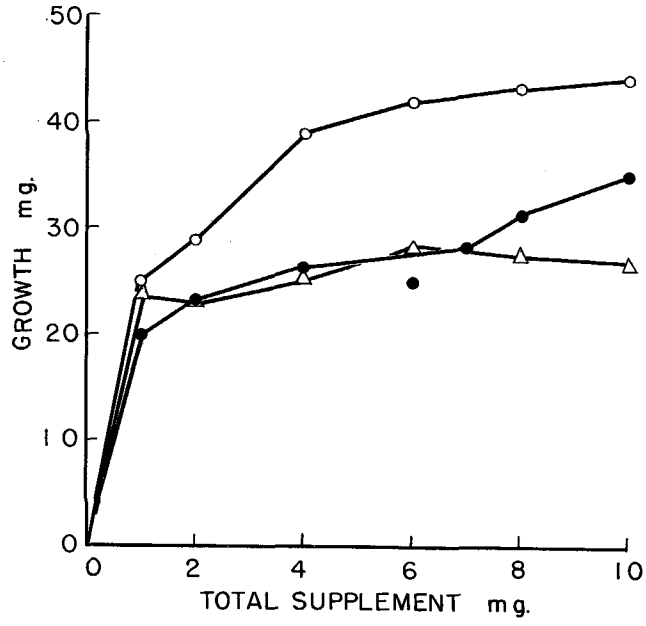


Figure 1. Growth response of mutant 84605 to equal amounts of tyrosine and sulfur compounds. Circles represent growth with $Na_2S_2O_3$ plus L-tyrosine; dots represent growth with L-cysteine plus L-tyrosine; and triangles growth with DL-methionine plus L-tyrosine.

the growth response of the mutant to thiosulfate, cysteine and methionine at 35° C.

e. Inhibition by choline of the utilization of methionine at 25° C.

A mixture of B-vitamins was found to exert a significant inhibition of the growth of the mutant in the medium with methionine plus tyrosine at 25° C, but no appreciable effect was detected when it was added to the medium in presence of sodium thiosulfate or cysteine plus tyrosine. Nine vitamins have been tested for their inhibitory effect upon this mutant. Choline was found to be the only substance which inhibits the growth of the mutant at 25° C in the medium supplemented with methionine plus tyrosine (Table 9). It is of interest for comparison to note here that the mutant reported by Teas et al which is blocked in homoserine synthesis and which requires both threonine and methionine, can grow to a certain extent on choline plus threonine instead of methionine plus threonine (36). Because of the role of choline and methionine in the transmethylation system in animals, it was suggested that the inhibition caused by choline could be due to the result of a competitive action between choline and methionine. Experiments were done, as shown in Table 10, increasing the amount of either methionine or tyrosine, but this had no effect at all on the inhibition by choline.

f. Accumulation in the mutant medium of a substance which can inhibit the growth of wild type.

An inhibitory substance has been detected in medium containing 2 mg. or more of cysteine or methionine per 20 cc in which the mutant has grown. The substance becomes detectable at

Table 9

Effect of choline on the growth of mutant 84605.

Compounds				Dry weight 25° C	Dry weight 35° C
Minimal				0	0
methionine			1 mg.	1.4	56.4
"	+ L-tyrosine		1 mg.	28.0	67.0
"	"	+ inositol	250 γ	21.0	60.0
"	"	+ pantothenic acid	250 γ	22.6	72.0
"	"	+ folic acid	250 γ	28.4	66.4
"	"	+ PAB	250 γ	25.0	81.4
"	"	+ thiamine	250 γ	30.0	67.4
"	"	+ riboflavin	250 γ	34.0	44.4
"	"	+ pyridoxine	250 γ	16.0	52.4
"	"	+ nicotinic acid	250 γ	20.0	53.9
"	"	+ choline	250 γ	1.4	53.6

Table 10

Growth on various concentrations of methionine and tyrosine of mutant 84605 in presence of choline at 25° C.

Compounds								Dry weight mg.
Minimal								0
Dl-methionine	1 mg.							1.4
"	1 mg. +	L-tyrosine	1 mg.					24.6
"	1 mg. +	"	1 mg. +	choline	100	γ		4.5
"	2 mg. +	"	1 mg. +	"	100	γ		4.6
"	3 mg. +	"	1 mg. +	"	100	γ		4.4
"	5 mg. +	"	1 mg. +	"	100	γ		4.4
"	10 mg. +	"	1 mg. +	"	100	γ		3.4
"	1 mg. +	"	1 mg. +	"	100	γ		4.6
"	1 mg. +	"	2 mg. +	"	100	γ		4.1
"	1 mg. +	"	3 mg. +	"	100	γ		4.4
"	1 mg. +	"	5 mg. +	"	100	γ		3.4
"	1 mg. +	"	10 mg. +	"	100	γ		3.0

about 144 hours after inoculation at 25° C. From 168 to 192 hours its inhibitory effect on the growth of wild type is so intense that 4 ml. of the medium containing the inhibition substance can completely stop the growth of the mold (Figure 3). The composition of the medium affects the amount of inhibitory substance produced by the mutant. If the concentration of cysteine or methionine is less than 2 mg. per 20 cc of medium, no appreciable inhibition can be detected. If tyrosine is supplemented in addition to cysteine or methionine, inhibition is also decreased. The inhibitory agent is not soluble in ether and is precipitated by alcohol. Its inhibitory effect cannot be counteracted by methionine, cysteine or tyrosine.

g. Inhibition of wild type by cysteine.

As was shown before in the growth experiment of the cysteineless mutant 84605 (Table 6), if the concentration of cysteine is increased to an extent which far offsets the proper ratio of the amount of cysteine to that of tyrosine necessary for the optimal growth of the mold, an inhibition results. Cysteine at high concentrations also inhibits the growth of the wild type 25a (unpublished data of Fling). By using the wild types 4A and 1A for experiment, the inhibition due to cysteine becomes obvious, when the latter is added in excess of 3 mg. in 20 cc of minimal medium. About 80% inhibition is produced when the cysteine concentration reaches 10 mgs. per 20 cc. (Figure 4).

h. Effect of tyrosine on cysteine inhibition.

The result of the competitive effect of cysteine and tyrosine on the optimal growth of the cysteine mutant 84605 suggested that

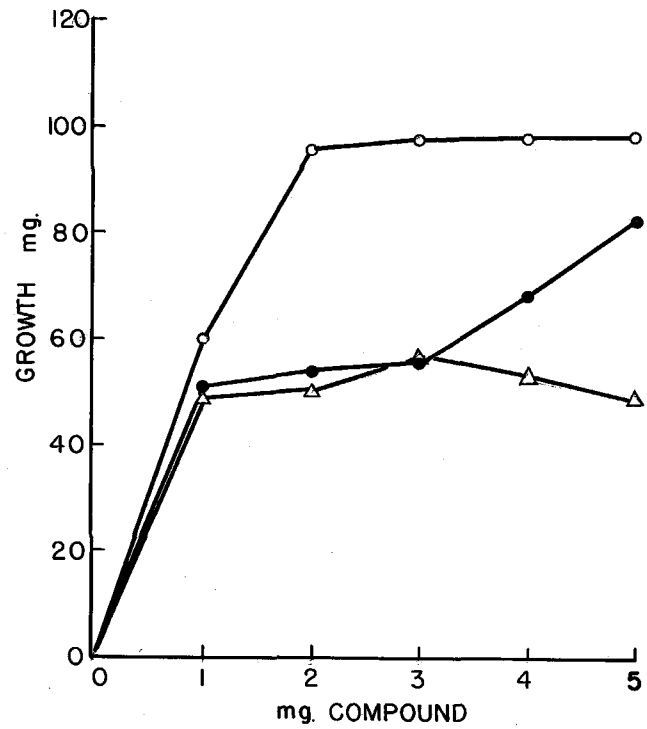


Figure 2. The growth response of mutant 84605 to sulfur compounds at 35° C. Circles represent growth with $Na_2S_2O_3$; dots growth with L-cysteine; and triangles growth with DL-methionine.

cysteine inhibition of the growth of the wild type could be released by adding appropriate amounts of tyrosine. The suggestion was confirmed with the experiment in which the wild types 1A and 4A were grown with increasing concentrations of both cysteine and tyrosine. As shown in Figure 4 tyrosine exerts an effect which overcomes the inhibition by cysteine.

1. Effect of temperature on cysteine inhibition.

The effect of increasing the temperature which takes the place of adding tyrosine for promoting the growth of the cysteine-tyrosineless mutant has been pointed out in the previous experiments. Similarly, the inhibition by excess of cysteine, which can be released by adding tyrosine can also be diminished by increasing the temperature. The result of the experiments in which the wild type was grown in high concentration of cysteine at 35° C indicates that the inhibitory effect caused by cysteine is decreased to a certain extent by raising the temperature. Interestingly enough, temperature plays a similar role in accelerating the growth of the mutant blocked in cysteine synthesis and that of the wild type when inhibited by cysteine. A comparison between the effect of increasing tyrosine concentration and the effect of high temperature treatment on the growth of wild type inhibited by cysteine is shown in Table 11.

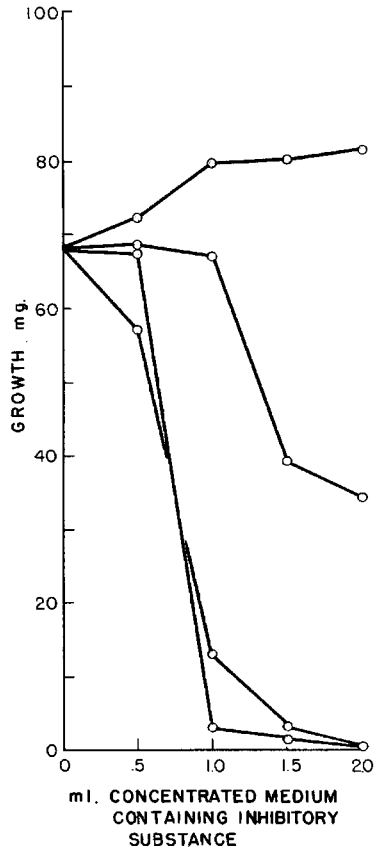


Figure 3. The inhibitory effect on growth of wild type 4A of medium in which strain 84605 has grown for (A) 120 hours, (B) 144 hours, (C) 168 hours, and (D) 192 hours. Medium concentrated twofold before testing.

Table 11

Effect of tyrosine and high temperature on the growth of wild type 4A inhibited by cysteine.

25° C				35° C	
l-cysteine HCl mg.	Dry weight mg.	l-cysteine HCl + L-tyrosine mg. of each	Dry weight mg.	l-cysteine HCl mg.	dry weight mg.
None	59.0	None	59.0	None	57.0
1	60.0	1	56.4	1	66.4
2	61.2	2	57.0	2	66.6
3	61.4	3	61.0	3	67.0
5	40.1	5	62.2	5	78.6
6	23.8	6	62.0	6	62.2
8	11.0	8	62.0	8	36.0
10	4.0	10	56.6	10	28.0

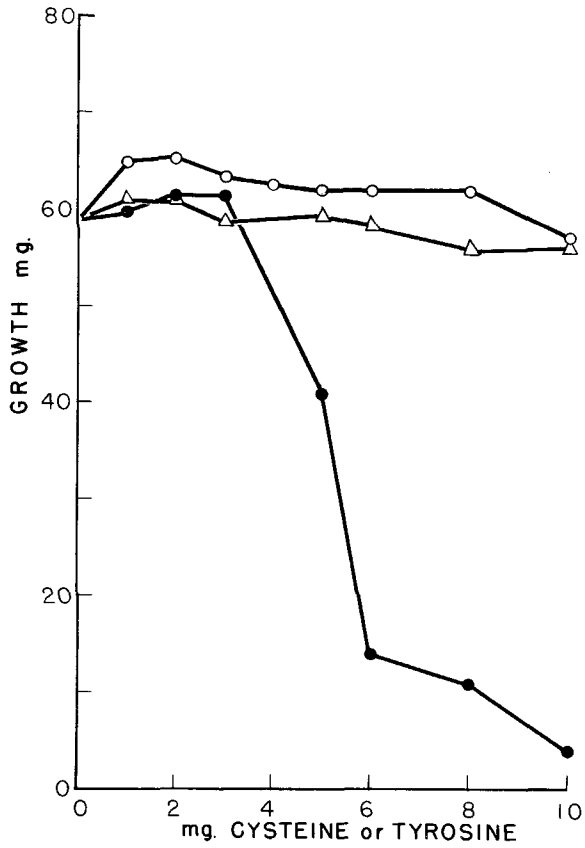


Figure 4. Effect of L-tyrosine on cysteine inhibition of wild type 4A. Circles represent growth with L-cysteine plus L-tyrosine; dots growth with L-cysteine; and triangles growth with L-tyrosine.

2. Enzyme

a. Tyrosinase activity of the mutant.

As previously pointed out, the mold requires tyrosine in addition to its unsynthesizable sulfur compounds at 25° C, but at 35° C it grows normally just like the ordinary cysteineless mutants without any necessity of adding tyrosine. As a working hypothesis, an assumption was made that the tyrosine requirement of the cysteine-tyrosineless mutant could be due either to an interference with tyrosine synthesis or to a destruction of tyrosine. In his investigation on tyrosinase in Glomerella, Markert found that environmental and nutritional conditions exert a significant influence on the tyrosinase activity (27). The interesting thing that arouses the attention is due to Markert's finding (personal communication) that the high activity of tyrosinase in certain Glomerella strains was found to disappear when the cultures of the mold were grown at 35° C. It was therefore decided to investigate the tyrosinase activity in the cysteine-tyrosineless mutant of Neurospora under different conditions.

The crude enzyme preparation was prepared from mycelium which had grown about 7 to 10 days in flask culture at 25° C in 20 cc of medium containing 1 mg. of cysteine or methionine plus a trace of tyrosine. The mycelial tissue was collected and macerated by grinding with sand. Five cc of distilled H_2O was added for each gram of wet weight of mycelial tissue. The pH was maintained at 7.0 by adding one-fourth volume of .25 molar sodium phosphate buffer. Throughout the process of preparation the material was kept ice cold. Enzymatic activity was measured by the manometric method on the basis of the rate of oxygen consumed during the aerobic oxidation

of the phenolic substrate as catalyzed by the enzyme. Two ml. of enzyme solution were added to the main part of the Warburg flask, .2 ml. of 20% KOH to the central well, and .25 ml. of the phenolic substrate was placed in the side arm of the flask. All experiments were run at 30° C. When the contents of the flask reached temperature equilibrium the substrate was tipped from the side arm to the enzyme solution. The rate of reaction was measured every 10 minutes. Figure 5 shows the tyrosinase activity of the mutant when grown for 7 days in flask culture at 25° C. The tyrosinase activity is higher when the mold is grown in agar medium for 2 weeks (Figure 5). About 2.9 atoms of oxygen were consumed per molecule of dopa and 4.3 atoms of oxygen per molecule of tyrosine. The total oxygen consumption is close to that reported by Mason and Wright (26) for mushroom tyrosinase.

In order to study the properties of Neurospora tyrosinase, the following experiments were done:

(1) The specificity of tyrosinase. Tyrosinase obtained from different sources possess different specific properties. Generally the enzyme isolated from plants and lower animals is less specific than that from higher animals (24). The enzyme prepared from the strain of Neurospora has a higher affinity toward the substrates tyrosine or dopa than the other phenolic compounds which have been tested (Figure 6). In this respect, it is similar to mammalian tyrosinase which oxidizes tyrosine and dopa much more rapidly than other substances (22). Hydroquinone is not supposed to be oxidized by tyrosinase unless a small amount of catechol is present (12). However, tyrosinase from

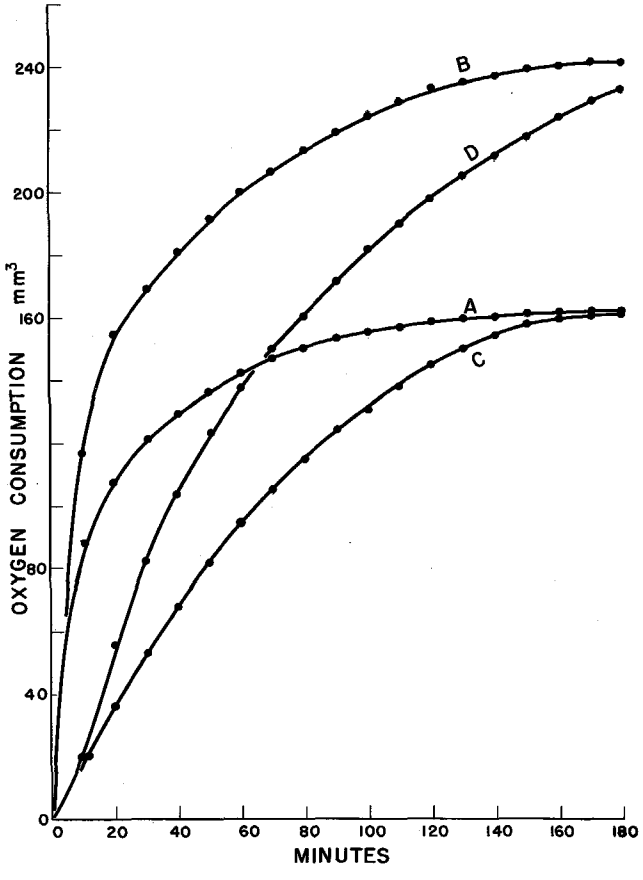


Figure 5. The oxygen consumed in the oxidation of L-tyrosine and 3, 4-dihydroxyl-L-phenylalanine catalyzed by the enzyme from mutant 84605.

Curve A: substrate dopa, enzyme prepared from the mycelium grown in agar medium.
Curve B: substrate L-tyrosine, enzyme prepared from the mycelium grown in agar medium.
Curve C: substrate dopa, enzyme prepared from the mycelium grown in liquid medium.
Curve D: substrate L-tyrosine, enzyme prepared from the mycelium grown in liquid medium.

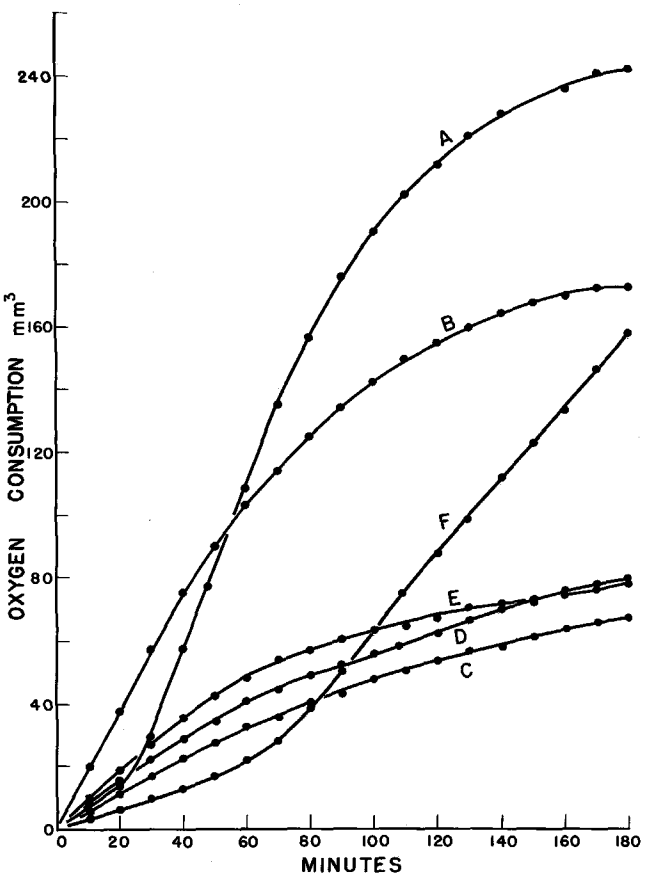


Figure 6. Activity of tyrosinase from strain 84605 on different phenolic compounds: (A) L-tyrosine, (B) dopa, (C) catechol, (D) gallic acid, (E) hydroquinone, (F) tyrosylamide.

Neurospora can oxidize hydroquinone to a slight extent; on the other hand, catechol is not easily oxidized by the enzyme. This is possibly due to the presence of laccase which can oxidize only hydroquinone instead of other orthoquinoid compounds in the crude enzyme preparation.

(ii) Effect of pH on reaction rate.

Because dopa is easily autooxidized when the reaction mixture is brought to above pH 7.0, l-tyrosine was used as the substrate to evaluate the effect of pH on the tyrosinase activity. The optimal pH for the oxidation of tyrosinase by the enzyme is 6 to 7. At pH 5 the oxidation rate is decreased; when the pH is increased above 7.0, the induction period is prolonged (Figure 7). This is in agreement with the observation of Bordner and Nelson (9), and Gaubard and Nelson (12) on the enzymatic oxidation of p-cresol that the length of the induction is a function of the pH of the reaction mixture.

(iii) Effect of enzyme concentration on total oxygen uptake.

In the oxidation of a fixed weight of dopa or tyrosine an increase in the amount of tyrosinase leads to an increase in the total amount of oxygen consumed. Figure 8 shows the results obtained when .916 mg. of dopa and .985 mg. of tyrosine were oxidized in the presence of increasing amounts of enzyme at pH 7.0. Two ml. of enzyme solution catalyzed the consumption of oxygen to 152 ml. in the oxidation of dopa and to 223 ml. in the oxidation of tyrosine within 180 minutes; with the half volume of the enzyme solution the oxygen consumption dropped to 92ml. and 155 ml. respectively. Change of the total amount of oxygen consumption by the variation of mushroom tyrosinase concentration has been clearly shown by Mason and Wright (26).

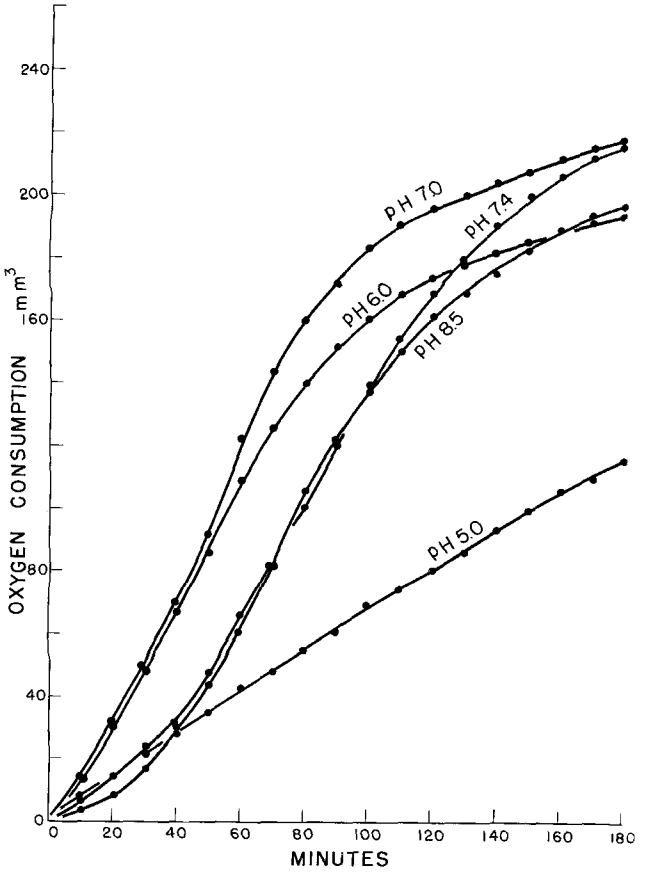


Figure 7. pH effect on tyrosinase activity. 1.2 ml. enzyme plus 0.8 ml. 0.2 M acetate (pH 5), phosphate (pH 6.0, 7.0 and 7.4), or pyrophosphate (pH 8.5) buffer were used in each Warburg vessel.

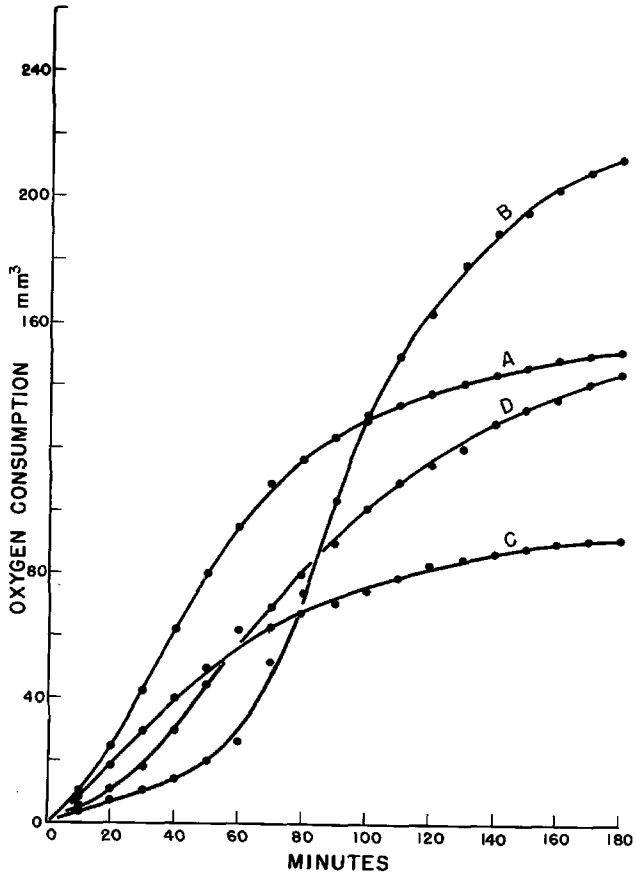


Figure 8. Effect of enzyme concentration on the oxygen uptake.

A. 2.0 ml. enzyme plus dopa.
B. 2.0 ml. enzyme plus L-tyrosine.
C. 1.0 ml. enzyme plus dopa.
D. 1.0 ml. enzyme plus L-tyrosine.

(iv) Michaelis and Menten dissociation constant.

The dissociation constant Km was evaluated according to the equation, $1/v = Km/V\ max \cdot s + 1/V\ max$ (25, 28), where v = initial rate, s= substrate concentration and V max = maximum rate. When 1/v is plotted against 1/s, the ordinate intercept is 1/V max, and the slope of the straight line is Km/V max. In order to eliminate complications due to the induction period in the oxidation of tyrosine, the initial velocity, v, was measured after 20 minutes from the beginning of the reaction by the term of oxygen uptake within 20 minutes. The range of substrate concentration, s, varied from .4 mg. to 1.4 mg. for 2.25 ml. Figure 9 indicates:

$1/V\ max = .66$

$Km/V\ max = .216$

Thus Km = .216 x 1.5 = .324 mg./2.25 ml. =.0079 M.

(v) Temperature effect on tyrosinase activity in the mycelium.

The tyrosinase activity fluctuates to a certain extent in different preparations grown under the same conditions. Temperature apparently exerts the most significant influence upon the activity of tyrosinase in the present mutant. High activity of tyrosinase has only been obtained from the culture grown at 25° C. The mold shows no enzyme activity at all when it is grown at 35° C. Figure 10 shows the decrease in tyrosinase activity when the culture was transferred on the fourth day from 25° C to 35° C for an additional 72 hours. It should be added here that temperature does not produce such a noticeable effect on the crude mycelial extract. The enzyme seems stable toward the change of temperature in vitro.

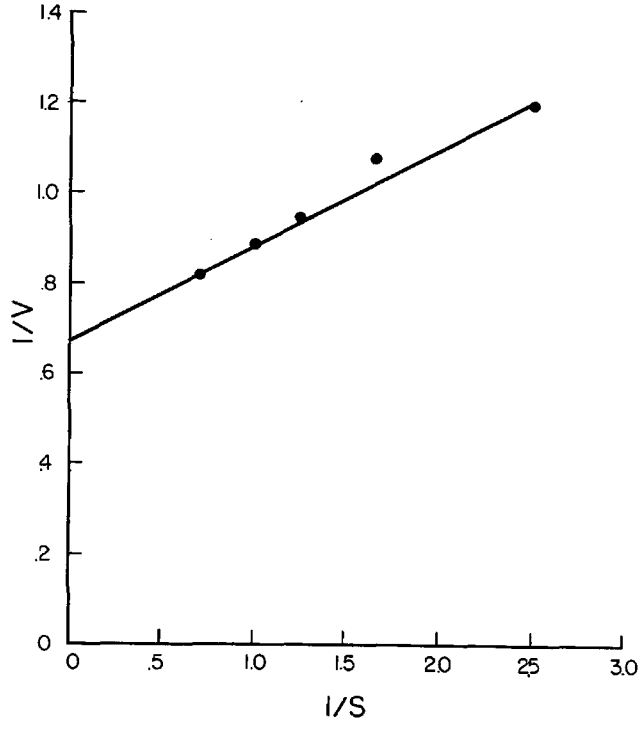


Figure 9. Evaluation of Km and V max in the oxidation of L-tyrosine by tyrosinase.

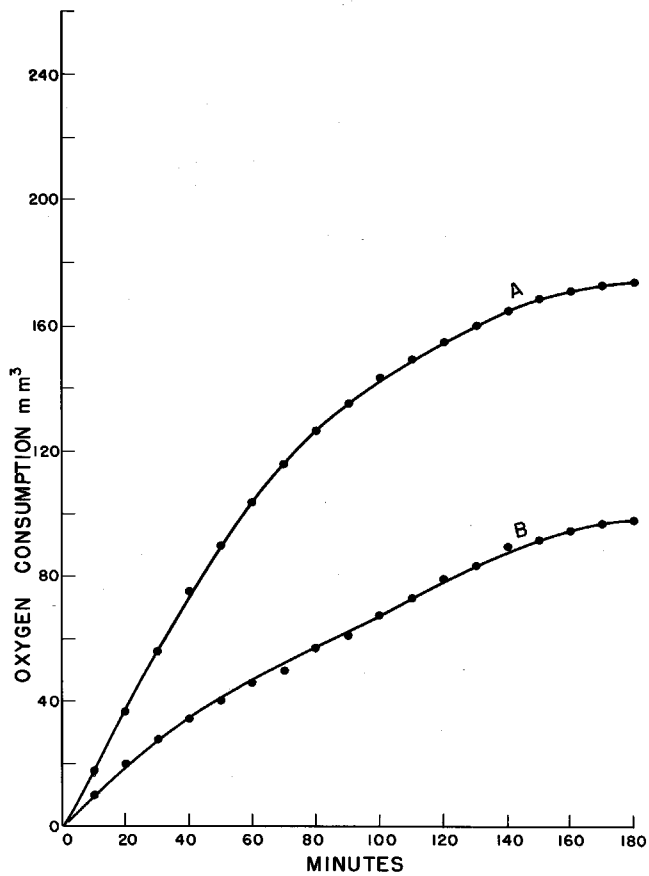


Figure 10. Oxygen consumed in the oxidation of 0.98 mg. of dopa catalyzed by tyrosinase:
A. prepared from the mycelium grown at 25° C for 7 days.
B. prepared from the mycelium grown at 25° C for 4 days and another 3 days at 35° C.

(vi) The natural inhibitor of tyrosinase.

Tyrosinase activity cannot be demonstrated in the wild type under the same conditions in which the mutant shows strong enzyme activity. Likewise, in the submerged culture of the mutant, tyrosinase activity is also greatly decreased as compared with cultures grown on agar or on the surface of liquid medium. The same activity of tyrosinase under both conditions might be due to the absence of certain substances which are necessary for the conversion of the enzyme from an inactive to an active form. An experiment was carried out to find out whether tyrosinase is present, but in its inactive form, in the wild type or in the mutant of a submerged culture. A small amount of crude enzyme preparation with strong tyrosinase activity was added to the mycelial extract of the wild type or that prepared from the mycelia of a young submerged culture of the mutant. The result of the experiment was entirely different from expectation. Such a mixture of mycelial extract not only fails to show any sign of increased tyrosinase activity but the small amount of tyrosinase added becomes inhibited. Results of further experiments to confirm the presence of such an inhibitory substance in extracts of the wild type as well as in extracts of the young mycelial tissue from submerged culture of the mutant are shown in Figure 11. The inhibitory substance present in extracts of the wild type or of the submerged culture of the mutant strain is more effective in inhibiting the oxidation of tyrosine than of dopa by the enzyme. The inhibitory substance is thermostable and not dialyzable. The mycelial extract of the wild type which contains as usual the inhibitory agent was boiled until the proteins in the extract became coagulated. After

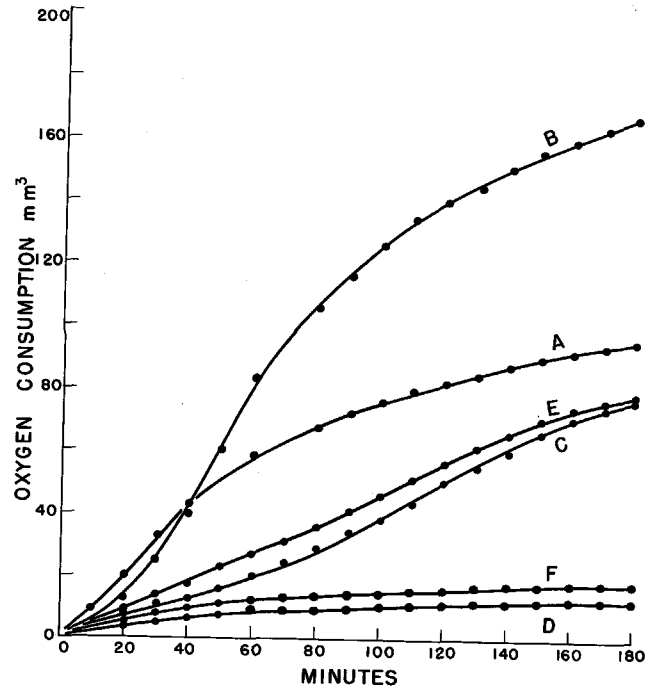


Figure 11. Inhibition of tyrosinase activity by mycelial extract.

- **A.** Dopa plus enzyme.
- **B.** L-tyrosine plus enzyme.
- **C.** Dopa plus enzyme plus extract of wild type 25a.
- **D.** L-tyrosine plus enzyme plus extract of wild type 25a.
- **E.** Dopa plus enzyme plus extract of mutant 84605 grown in submerged culture.
- **F.** L-tyrosine plus enzyme plus extract of mutant 84605 grown in submerged culture.

centrifuging, the coagulated protein precipitate and the supernatant solution were separately tested for their inhibitory function. As shown in Figure 12, only the protein part of the mycelial extract possesses the inhibitory principle.

(vii) Induction period in the oxidation of tyrosinase.

When the tyrosinase reacts on tyrosine in the presence of oxygen, a lag period is observed. If dopa is used as the substrate instead of tyrosine, there is no induction period (22). The induction period of mouse melanoma tyrosinase can be shortened by adding a small amount of dopa or compounds related to dopa to the tyrosinase-tyrosine system (22, 23). In Neurospora the induction period of the tyrosinase-tyrosine reaction can be effectively shortened by adding a small amount of a powerful sulhydryl inhibitor, sodium-p-chloromercuribenzoate (Figure 13). Rothman et al (24) demonstrated that the action of the inhibitory substance on plant tyrosinase found in human skin can be counteracted by adding p-chloromercuribenzoic acid. Probably the lag period of tyrosine oxidation in Neurospora is due to the presence of some sulfhydryl compound which inhibits the tyrosinase activity specifically in its oxidation of tyrosine. Frequently tyrosinase prepared from the mold under standard conditions showed high activity on dopa, but none on tyrosine at all, even when the mixture of the enzyme and tyrosine was incubated for more than 12 hours. In such a case p-chloromercuribenzoate effectively assumes its function of restoring the tyrosinase activity on tyrosine.

To summarize, two naturally occurring inhibitors of tyrosinase have been found in Neurospora extracts. The first of

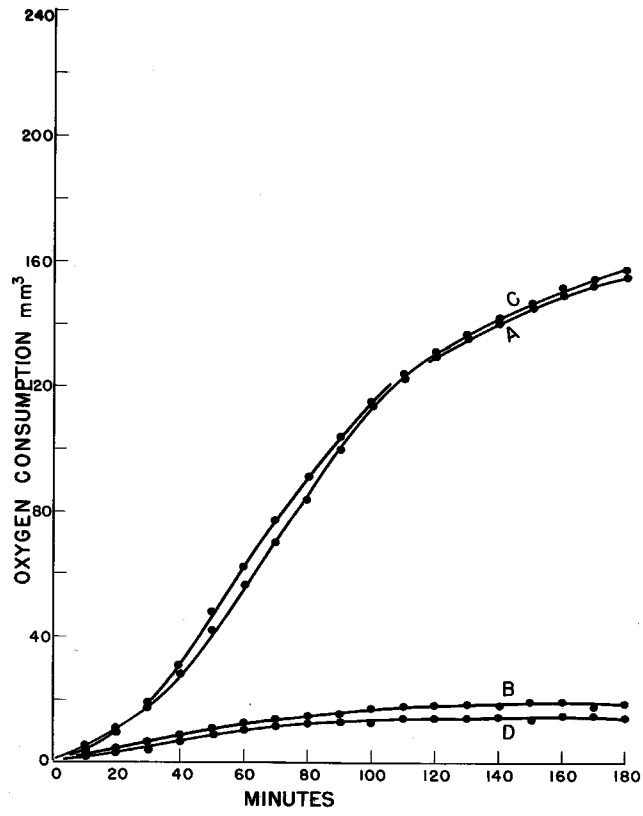


Figure 12. Effect of boiling on the inhibitor of tyrosinase.

A. L-tyrosine plus 1 ml. enzyme.
B. L-tyrosine plus 1 ml. enzyme plus 1 ml. boiled extract of wild type 25a.
C. L-tyrosine plus 1 ml. enzyme plus supernatant of boiled extract.
D. L-tyrosine plus 1 ml. enzyme plus precipitate of boiled extract.

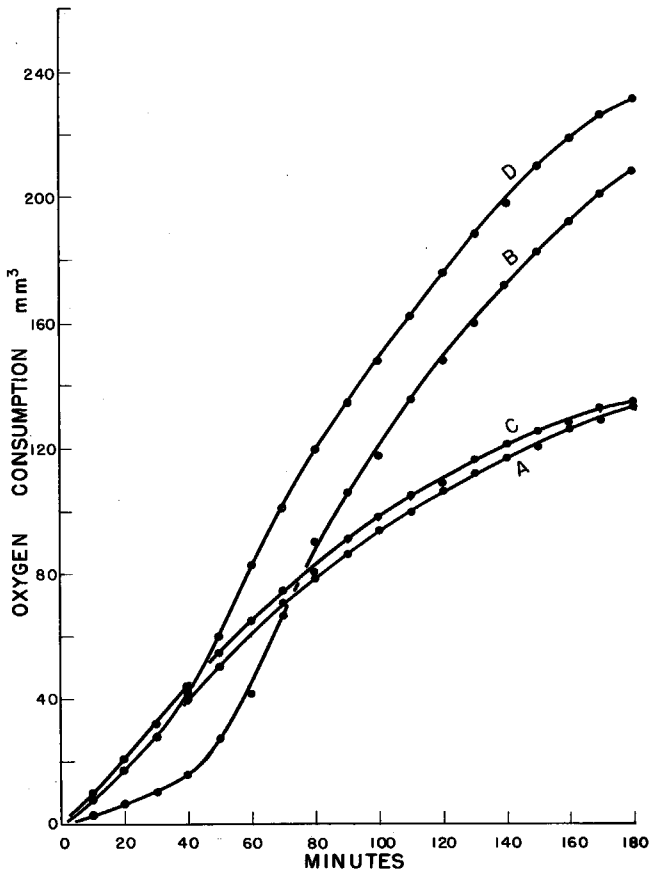


Figure 13. Effect of Na-p-chloromercuribenzoate on tyrosinase activity.

A. Dopa plus enzyme.
B. L-tyrosine plus enzyme.
C. Dopa plus enzyme plus 8.8×10^{-5} M Na-p-chloromercuribenzoate.
D. L-tyrosine plus enzyme plus 8.8×10^{-5} M Na-p-chloromercuribenzoate.

these inhibits the oxidation of both tyrosine and dopa. It is found in wild type cultures, but not in cultures of the mutant except when the latter is grown under conditions of limiting O_2 supply. The second inhibitor has no effect on dopa oxidation but inhibits tyrosine oxidation. It is found both in wild type and mutant extracts and is presumed to contain an -SH group, since it is overcome by the reagent p-chloromercuribenzoate. The second inhibitor is usually present in extracts of the mutant grown in liquid medium, but is usually absent from mutant cultures grown on solid medium.

b. Tyrosinase activity of wild type.

No tyrosinase activity has been detected in wild types 25a and 7A under the same conditions in which the mutant exhibits its active tyrosinase. Wild type 4A occasionally shows some slight tyrosinase activity. In their investigation of L-amino acid oxidase of Neurospora, Bender and Krebs (5) using an old culture of 25a, found that with tyrosine as the substrate more oxygen was consumed than is expected for oxidative deamination and the final solution became dark. This suggests that old cultures of 25a may also produce small amounts of tyrosinase.

In the growth experiments with wild type, when the concentration of cysteine added to the culture medium exceeds 3 mg. per 30 cc, the growth of the mold is inhibited. The inhibition can only be overcome by adding appropriate amounts of l-tyrosine at 25° C. The role of tyrosine and high temperature on cysteine inhibition of wild type growth seems similar to the case for optimal growth of the cysteineless mutant 84605, hence they are

possibly related to tyrosinase activity induced directly or indirectly by an excess of cysteine. The assumption that cysteine inhibition in the growth of wild type is due to its induction of tyrosinase activity in the wild type was tested by experiments. When the wild type 4A was grown in minimal media at 25° C for 7 days, only minimal tyrosinase activity was detected. If cysteine was added up to 5 mg. per 20 cc, which concentration is sufficient to inhibit the growth, a high tyrosinase activity was found as expected, but when the cysteine concentration was increased to 10 mg. per 20 cc, the tyrosinase activity was decreased again (Figure 14). To avoid the possible interference of excess cysteine on tyrosinase activity, the enzyme solutions were dialyzed against distilled water over night in the cold and then treated with sodium-p-chloromercuribenzoate. For comparison of the tyrosinase activity of 4A grown in media with different concentrations of cysteine with that of mutant 84605, the activity of the enzyme was expressed as Q_{O_2}, using the amount of oxygen absorbed by the system within a half hour (Table 12).

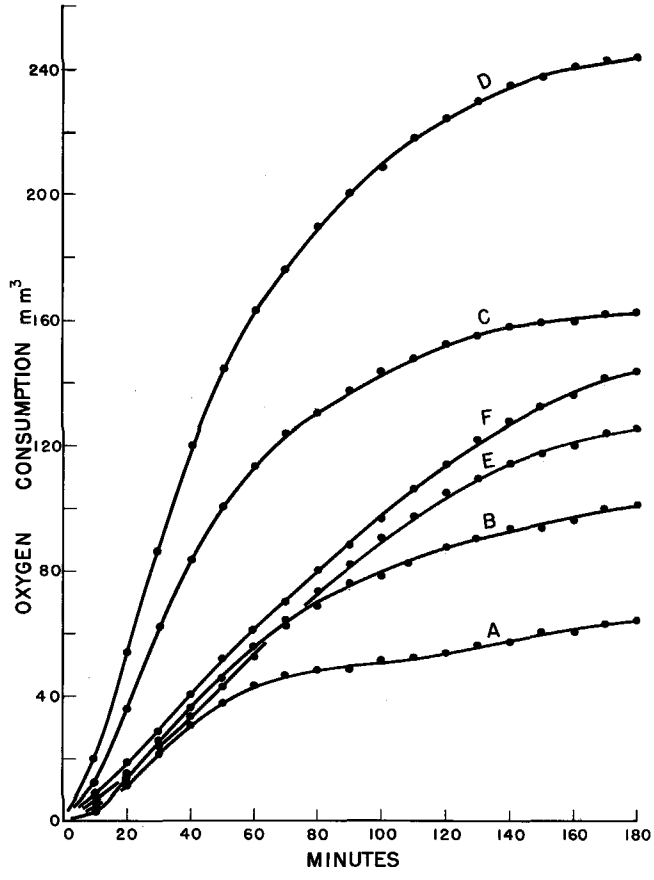


Figure 14. Effect of cysteine on tyrosinase production by wild type 4A.

A. Oxidation of dopa by extract of culture grown on minimal medium.
B. Oxidation of tyrosine by extract of culture grown on minimal medium.
C. Oxidation of dopa by extract of culture grown on minimal medium containing 5 mg. L-cysteine per 20 ml.
D. Oxidation of tyrosine by extract of culture grown on minimal medium containing 5 mg. L-cysteine per 20 ml.
E. Oxidation of dopa by extract of culture grown on minimal medium containing 10 mg. L-cysteine per 20 ml.
F. Oxidation of tyrosine by extract of culture grown on minimal medium containing 10 mg. L-cysteine per 20 ml.

Table 12

Strain	Media	Qo_2/.916 mg. of tyrosine/30 min.
84605	l-cysteine plus trace of tyrosine	202.6
4A	minimal	14.02
4A	l-cysteine 5 mg./20 ml.	48.29
4A	l-cysteine 10 mg./20 ml.	16.40

$Qo_2 = \frac{O_2 mm^3}{d \times hrs}$ d= dry weight of the total heat precipitated protein of the enzyme solution.

DISCUSSION

To review the above investigations, the characteristics of the cysteine-tyrosineless strain 84605 can be summarized in the following table:

	25° C	35° C
Growth requirement (amino acids)		
Wild type	none	none
Mutant	$S_2O_3^=$ or cysteine etc. + L-tyrosine	$S_2O_3^=$, or cysteine etc.
Tyrosinase activity		
Wild type	inactive or slightly active	inactive
Mutant	very active	inactive

In addition to the indispensable sulfur compound, the given mutant requires l-tyrosine for growth. At 35° C it grows normally like the ordinary cysteineless mutants without the need of tyrosine. Investigations on the tyrosinase activity shows the opposite effect. High tyrosinase activity of the mutant has been demonstrated in the mycelium grown at 25° C. No tyrosinase activity can be detected from the culture grown at 35° C. Furthermore, the wild type Neurospora so far investigated shows almost no tyrosinase activity at all. These contrary but compatible facts offer a possible interpretation of the metabolism of the double requirement of the cysteineless mutant; that is, the requirement for tyrosine by the mutant could be due to the high activity of tyrosinase.

It is generally assumed that a block occurring in the biochemical reaction is due to the absence or inactivity of a specific enzyme which is controlled by the gene responsible for its function. Many examples in Neurospora as well as in other plants have given reliable evidence for the concept of the gene and enzyme relationship. The mutant of the present investigation was genetically different by the change of a single gene which is essential for the reduction of sulfite to thiosulfate in the chain of cysteine synthesis. How can the gene which controls the cysteine synthesis also function in directing the activity of tyrosinase in the mutant? This is the question important to those who are interested in the gene-enzyme hypothesis. The following hypotheses are an attempt to answer this question briefly on the basis of the relationship of gene and enzyme.

(1) The gene responsible for the reduction of sulfite to thiosulfate at the same time controls directly the activity of tyrosinase or the synthesis of it. This indicates that a single gene performs two different chemical reactions. According to Horowitz (19) in his recent discussion on the number of functions per gene, this explanation seems unlikely.

(2) The strain 84605 could be a double mutant. The position of the gene controlling a stp in cysteine synthesis to that of the gene responsible for the activity or synthesis of tyrosinase in the chromosome might be so close that separation of one from the other becomes almost impossible. Therefore phenotypically two enzymatic reactions would appear to be controlled by a single gene.

In the present study, no recombinations of the cysteineless and tyrosineless characters were observed in 146 ascospores dissected in order and 400 spores taken at random. If these characters are assumed

to be determined by two genes one unit apart, the probability that no recombinations between them would be observed in 146 asci and 400 random ascospores is as follows:

$$P_1 = 0.98^{146} \times 0.99^{400} = 0.000902$$

If it is assumed that the genes are 0.1 unit apart, the probability of no recombination is as follows:

$$P_{0.1} = .998^{146} \times .999^{400} = 0.512$$

It is concluded that the data effectively excluded the possibility that the two characters are determined by genes more than one map unit apart, although they do not exclude the possibility that they are closer than one unit. The simplest hypothesis, especially in view of the biochemical evidence, is that the characters are determined by a single locus.

(3) The high activity of tyrosinase in the strain 84605 is due to the secondary effect of the gene which is primarily concerned with cysteine synthesis. Similar suggestions have been given to other single gene mutants requiring two entirely different substances. Bonner (7, 8) found a mutant strain 16117, differing from the normal by a single gene which requires the two amino acids, isoleucine and valine for growth. This strain cannot use the keto acid analog of isoleucine, but it can use the keto acid analog of valine. On the other hand, the keto acid analog of isoleucine inhibits the conversion of α-keto-isovaleric acid to valine. Bonner suggests that the strain 16117 is actually an isoleucineless mutant and that the accumulation of α-keto-B-methyl-n-valeric acid, resulting from a genetic block before isoleucine, inhibits the biological conversion of α-ketovaleric to valine. Tatum and Bell also found two double requirement thiamineless mutants; both

strains require thiazole and pyrimidine and produce a biologically thiamine-like compound (35). It was suggested that the formation of the thiamine-like compound at the expense of the normal precursors of thiamine results in the need for exogenous pyrimidine and thiazole for growth.

The present mutant has been shown to be evidently a cysteine-less mutant. The genetic block between sulfite and thiosulfate presumably causes the accumulation of a certain compound which initiates the activity of tyrosinase.

This view is supported by the growth experiments with wild type Neurospora on high concentrations of cysteine. These experiments indicate that cysteine inhibition of the growth of the mold can be released by either adding tyrosine or raising the temperature. The results of the investigation on tyrosinase activity in wild type show that cysteine, when it reaches certain high concentrations, can accelerate the activity or synthesis of tyrosinase. It appears, therefore, that high tyrosinase activity and an accompanying tyrosine requirement may result from derangement of the sulfur metabolism. This derangement can result either from a genetic block in cysteine synthesis, or from the presence of excess cysteine in the medium. The simplest view would appear to be that in both cases the increased tyrosinase activity is induced by the presence of abnormally high concentrations of certain sulfur compounds. In the case of the mutant, this accumulation is assumed to be the result of the genetic block. Numerous precedents have already shown the accumulation of intermediates in Neurospora mutants.

The mechanism of the increase in tyrosinase activity may be related to the inhibitor of tyrosinase which has been found in wild type. This inhibitor has not been found in the mutant, except in young submerged cultures. If the inhibitor is active *in vitro*, then its intracellular concentration may determine the rate of tyrosine oxidation in the living cell, and in its turn may be determined by the intracellular concentration of certain sulfur compounds. On this hypothesis one would expect by fractionation procedures to be able to demonstrate tyrosinase in extracts of wild type cultures grown on minimal medium. One might also expect a decrease in the tyrosine requirement of the mutant grown in submerged culture. Several attempts to fractionate wild type extracts, using acetone and ammonium sulfate, have failed to increase the enzyme activity. Experiments on the tyrosine requirement of submerged cultures of the mutant have not been done.

Other hypothetical possibilities relating to the possible effect of sulfur compounds on the rate of tyrosinase synthesis or destruction can be suggested, but in view of the lack of any conclusive evidence, they will not be discussed here.

The experiments have also indicated a strong temperature effect on the tyrosinase activity of the mutant and of the wild type grown on cysteine medium. Enzyme activity *in vivo* and *in vitro* is demonstrable in cultures grown at 25°, but not at 35°. Extracts of cultures grown at 25° show no loss of tyrosinase activity when incubated at 35°, while extracts of cultures grown at 35° develop no tyrosinase activity when held at room temperature. It would appear, therefore, that tyrosinase synthesis is inhibited at 35°.

The temperature effect on tyrosinase activity is of considerable interest, especially in view of the fact that a similar phenomenon is well known in connection with the development of melanin in mammals. Melanic pigmentation and tyrosinase activity in the guinea pig, in the Himalayan rabbit and in the Siamese cat, are also inhibited at skin temperatures above 33° (4, 40). It appears very likely that in all of these cases the same fundamental phenomenon is involved. Cysteine has also been implicated in melanin production in mammals. Pavcek and Baum (29) found that the effect of pantothenic acid in restoring hair pigmentation in deficient rats is augmented by cysteine.

PART II

Experiments on Cysteine Synthesis

1. Utilization of hydrogen sulfide.

It has been shown in higher animals that cysteine sulfur can be split off the organic molecule in the form of hydrogen sulfide. This desulfuration reaction is due to the presence of an enzyme, cysteine desulfhydrase (11, 33). Using radioactive sulfur, Smythe (34) was able to demonstrate that sulfur of hydrogen sulfide was incorporated into cysteine under the catalysis of cysteine desulfhydrase *in vitro*. Lampen *et al* suggest that in *E. coli* cysteine could by synthesized from hydrogen sulfide and the requisite organic compound by desulfhydrase (21). The enzyme has also been found by Horowitz in Neurospora (unpublished experiments). To test for the utilization of hydrogen sulfide in Neurospora, wild type 5297 was used. It was grown in a sulfur free medium to which hydrogen sulfide was added as the only sulfur source. The amount of hydrogen sulfide was determined by iodometric titration. Because of its being easily oxidized hydrogen sulfide was added to the autoclaved sulfur free medium as a sterilely filtered solution. 250 cc Erlenmeyer flasks with glass stoppers were used for the experiments. The utilization of hydrogen sulfide in comparison with that of sulfate by the mold is shown in Table 13. The growth effect of hydrogen sulfide in sulfur free medium and in medium containing sulfate is shown in Table 14. In both cases hydrogen sulfide exerts its inhibitory effect when its concentration reaches 100 γ per 20 ml.

Table 13

Growth response of wild type 5297 to H_2S S in comparison with its response to sulfate S in sulfur free minimal media.

[S] γ /20 ml.	Dry weight in mg.	
	H_2S S	$SO_4^=$ S
None	6.6	6.6
10	8.4	11.4
20	8.6	15.8
30	9.4	17.4
40	11.6	18.4
60	11.8	19.0
80	11.2	20.0
100	0	21.0

Table 14

Utilization of H_2S S by wild type 5297 grown in the minimal medium with and without sulfate.

added S γ/20 ml.	Dry weight in mg. H_2S S	$SO_4^=$ S
0	6.2	22.2
2	8.6	24.0
4	9.2	30.6
6	9.1	24.6
8	9.1	24.6
10	10.1	26.0
50	10.4	26.0
100	0	0

Other experiments have been carried out to test the possibility that the small amount of H_2S used by the mold might have first been oxidized by the air. Two kinds of minimal medium were prepared. One contained sulfate and the other was supposedly free from sulfur. Both were devoid of the trace elements except for a small amount of iron. After the introduction of H_2S the media were kept at 25° C for three days in the glass stoppered flasks. On the fourth day, H_2S was eliminated by bubbling nitrogen through the media. The glass stoppers were then replaced by cotton plugs, and the media were inoculated with the mold. The growth was determined as usual after three days' incubation. The results obtained (Table 15) fail to answer conclusively the question of whether H_2S is oxidized spontaneously in the media during the three day incubation. Since the mold is inhibited when grown in the sulfate containing minimal media previously treated with H_2S, the results obtained in the case of the sulfur free media in the previous experiments can be explained either by saying that the H_2S is not oxidized in the media and is slightly utilized by the mold or that the H_2S is oxidized but the growth effect of the oxidized form of H_2S is counteracted by the inhibitory effect of the H_2S which remains in the media.

2. The pathway of the conversion of methionine to cysteine.

All the known cysteineless mutants of Neurospora can grow if methionine, homocysteine, or cystathionine is supplied instead of cysteine. Since in higher animals methionine is converted to cysteine through the intermediates homocysteine and cystathionine (2, 6), it seems possible in Neurospora that the conversion of methionine to cysteine is the reverse of the reactions of methionine synthesis from

Table 15

Growth of wild type 5297 in the minimal media pretreated with H_2S.

	dry weight mg.	
$(S^=)$* mg.	medium with $SO_4^=$	medium without $SO_4^=$
none	75.6	8.1
0.5	66.0	8.0
0.8	57.0	7.4
1.0	55.8	7.2

cysteine. In order to study the path of the conversion of methionine to cysteine in Neurospora the double mutants with one block before cysteine and another before methionine were sought. The possibility of converting methionine to cysteine by reversal of the reaction homocysteine ⟶ methionine, or by oxidation of methionine sulfur to sulfate which in turn is converted to cysteine, is eliminated in the double mutant.

A cross was made between the cysteineless mutant 36106 which has a block just before cysteine and the methionineless mutant 29627 which has a block before methionine. Only 11 mature asci were found from this cross, two being identified as the asci of the recombination type. Table 16 shows the growth of ascospores of the two asci resulting from the cross. The double mutants, 4-1, 7-3 and 9-3 (abbreviation for the first spore pair of the fourth ascus and third spore pair of the seventh ascus, etc.) grow on methionine only about half as well as they do on methionine plus cysteine. However the double mutant 9-7 segregated from the same ascus as 9-3 can grow on methionine just as well as on methionine plus cysteine. Further experiments to test the growth of the double mutants 9-3 and 9-7 with different proportions of methionine and cysteine are shown in Table 17. Methionine is necessary for the growth of double mutant 9-3, but in addition cysteine is indispensable for its optimal growth, whereas in the case of 9-7, the growth of which depends solely upon the exogenous methionine, cysteine does not show any effect on growth. It is interesting to note that cysteine inhibition in these double mutants can be slightly overcome by increasing methionine.

Table 16

Growth of ascospores resulting from the cross 36106 x 29627.

Amino acid mg./20 ml.	Dry weight in mg.													
	4-1	4-3	4-5	4-8	7-1	7-3	7-5	7-7	9-1	9-3	9-5	9-7	36106	29672
None	0	52.0	0	0	58.8	0	0	0	48.0	0	56.2	0	0	0
L-cysteine HCl 2 mg.	0	52.2	30.8	0	58.9	0	30.8	0	43.2	0	59.8	0	36.0	0
DL-methionine 2 mg.	13.2	50.0	59.8	48.2	57.8	15.6	22.4	42.0	49.0	12.6	58.8	26.8	19.8	49.8
DL-methionine 1 mg. plus L-cysteine HCl 1 mg.	25.0	54.4	44.4	36.2	60.0	26.0	30.2	46.4	51.4	28.4	60.0	26.4	36.4	45.0

Table 17

Growth of double mutants 9-3 and 9-7 on the various concentrations of cysteine and methionine.

Compounds mg./20 ml.					Dry weight in mg. 9-3	9-7
None					0	0
l-cysteine HCl	1				0	0
dl-methionine	.4				7.4	6.8
"	1.0				9.0	22.0
"	2.0				10.4	22.8
"	4.0				11.0	26.4
"	8.0				11.0	23.0
"	.4	plus	l-cysteine	.4	10.0	10.0
"	"	"	"	1.0	12.8	11.0
"	"	"	"	2.0	16.4	11.0
"	"	"	"	4.0	19.8	3.0
"	"	"	"	8.0	0	0
"	1.0	"	"	.4	14.6	22.6
"	"	"	"	1.0	19.6	21.0
"	"	"	"	2.0	25.0	21.6
"	"	"	"	4.0	33.4	22.2
"	"	"	"	8.0	0	0
"	2.0	"	"	.4	14.6	25.8
"	"	"	"	1.0	18.0	24.8
"	"	"	"	2.0	31.0	25.0
"	"	"	"	4.0	33.8	25.6
"	"	"	"	8.0	0	6.4

Table 17 (Continued)

Compounds mg./20 ml.					Dry weight in mg. 9-3	Dry weight in mg. 9-7
dl-methionine	4.0	plus	l-cysteine	.4	13.8	26.4
"	"	"	"	1.0	19.0	25.6
"	"	"	"	2.0	28.0	25.8
"	"	"	"	4.0	36.0	24.8
"	"	"	"	8.0	12.8	11.6
"	8.0	"	"	.4	22.8	27.4
"	"	"	"	1.0	29.8	25.6
"	"	"	"	2.0	30.0	26.0
"	"	"	"	4.0	35.6	23.8
"	"	"	"	8.0	14.0	13.0

Homocysteine and cystathionine were tested for their effect in promoting the growth of double mutants 9-3 and 4-1. Neither is active by itself, but both can replace cysteine for these strains. Homocysteine seems more effective than cystathionine in replacing cysteine (Table 18).

Of the four optical isomers of cystathionine tested, L-cystathionine has some effect in replacing cysteine in the growth of the two double mutants; D-cystathionine and L-cystathionine are slightly effective, whereas D-allocystathionine inhibits growth. The results are shown in Table 19.

Temperature exerts an effect on the conversion of methionine to cysteine. The double mutants, 7-3, 9-3 and 9-7 were inoculated in the media supplemented only with methionine at 35° C. The double mutants 7-3 and 9-3 which usually require cysteine in addition to methionine for optimal growth at 25° C, can grow as well as the double mutant 9-7 which grows regularly in methionine at both ranges of temperature (Table 20). The experiments lead to the conclusion that the cysteine-methionineless double mutants are able to convert methionine to cysteine, but the two strains of the double mutants differ in the rate of the conversion.

Table 18

Effect of L-homocysteine and L-cystathionine on the growth of double mutants 7-3 and 9-3

Amino acids	Dry weight in mg. 7-3	9-3
None	0	0
L-cysteine HCl 1 mg.	0	0
L-cystathionine 1.4 mg.	0	0
L-homocysteine 0.9 mg.	0	0
DL-methionine 1 mg.	8.9	9.0
DL-methionine 2 mg.	15.4	10.6
DL-methionine .5 mg. plus L-cysteine HCl .5 mg.	21.0	20.4
DL-methionine .5 mg. plus L-cystathionine .74 mg.	15.4	10.6
DL-methionine .5 mg. plus L-homocysteine .45 mg.	30.0	21.0
DL-methionine 1 mg. plus L-cysteine HCl 1 mg.	35.0	25.7
DL-methionine 1 mg. plus L-cystathionine 1.4 mg.	24.6	19.0
DL-methionine 1 mg. plus L-homocysteine .9 mg.	34.6	25.6

Table 19

Activity of cystathionine on cysteine-methionineless double mutants.

Amino acids	Dry weight in mg. 7-3	9-3
None	0	0
DL-methionine	12.0	9.8
DL-methionine	13.8	11.4
DL-methionine 1 mg. plus L-cystathionine 1.4 mg.	19.8	15.8
DL-methionine 1 mg. plus D-cystathionine 1.4 mg.	13.8	12.8
DL-methionine 1 mg. plus L-allocystathionine 1.4 mg.	12.8	13.8
DL-methionine 1 mg. plus D-allocystathionine 1.4 mg.	7.6	8.4
DL-methionine 1 mg. plus L-cysteine HCl 1 mg.	31.4	25.0

Table 20

Growth of cysteine-methionineless double mutants at 35° C.

Amino acid mg/20 ml.	Dry weight in mg. 7-3	9-3	9-7
None	0	0	0
L-cysteine 1	0	0	0
DL-methionine 1	30.0	34.4	24.0
DL-methionine 2	30.0	34.5	34.6
DL-methionine 3	44.6	36.4	36.8

DISCUSSION

The knowledge about how a sulfur compound combines with a C_3 compound to form cysteine in the lower organisms which are able to synthesize cysteine from inorganic sulfur compounds is very rudimentary. However, the results of the above experiments indicate that hydrogen sulfide is not, as had been believed for some time, the form of sulfur which represents the intermediate product in the reduction of thiosulfate to cysteine.

As regards the question of the conversion of methionine to cysteine in Neurospora, it has been established by the growth experiments of the double mutants that methionine S can be converted to cysteine S without prior oxidation to $SO_4^=$ and without simple reversal of the reaction, homocysteine $\longrightarrow$ methionine. A number of possibilities for the mechanism of the conversion exist. The double mutant 9-7 can convert methionine to cysteine normally, but the other two do so rather slowly except at high temperature. The following possibilities for the pathway of the conversion of methionine to cysteine are suggested: (1) Methionine is converted through a different path back to homocysteine which then goes reversibly to cysteine through cystathionine; (2) Methionine is converted to cystathionine which gives rise to cysteine by cleavage; (3) Methionine is directly converted to cysteine or to an organic precursor of cysteine through an entirely different pathway.

Because either homocysteine or cystathionine can take the place of cysteine in promoting the growth of those double mutants for which cysteine is necessary in addition to methionine for optimal

growth, the reactions from homocysteine through cystathionine to cysteine (the reversal of the synthetic path) seems to occur. The conversion of methionine to cysteine in Neurospora possibly can take place by both means, namely by the reverse of the reactions of Methionine synthesis or by any one of the three possible new paths as suggested above.

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APPENDIX

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Compound	Donor
L-homocysteine	Dr. J. W. Dubnoff
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L-cystathionine	Dr. N. H. Horowitz
D-cystathionine	Dr. N. H. Horowitz
L-allocystathionine	Dr. N. H. Horowitz
D-allocystathionine	Dr. N. H. Horowitz
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